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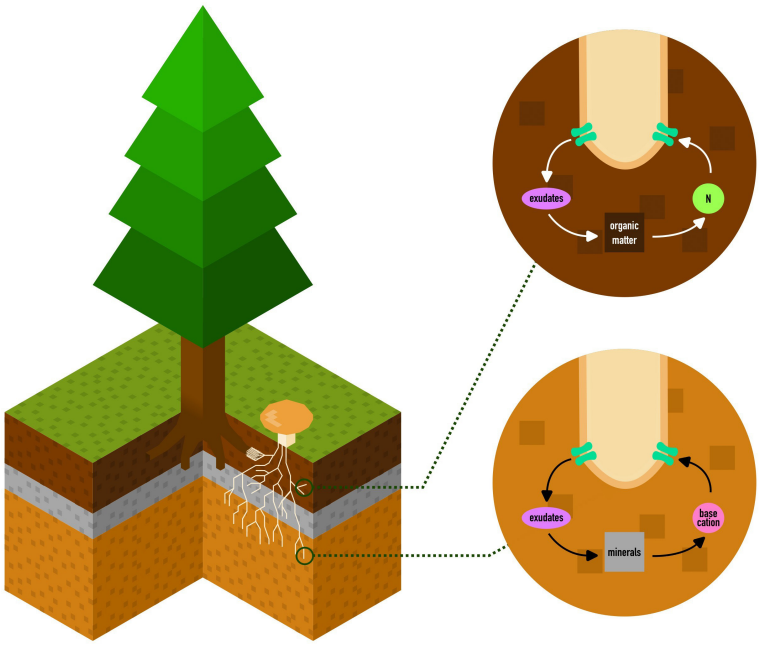
Their role in mineral weathering and nitrogen mobilisation

Katharine King



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Ectomycorrhizal fungi in boreal forests: their role in mineral weathering and nitrogen mobilisation

Abstract

Ectomycorrhizal (ECM) fungi play key roles in mineral weathering and nitrogen (N) mobilisation in boreal forests, yet knowledge gaps remain regarding how these processes interact and mechanistic explanations of mineral weathering are lacking. While progress has been made in understanding N mobilisation and transport gene regulation, the regulation of base cation transporter genes, uptake, and low molecular weight organic acid (LMWOA) production remains largely unexplored. This thesis investigates the relationships between mineral weathering, N mobilisation, and plant C allocation, focusing on ECM fungal communities, the evolution of base cation transporter gene families, and the regulation of base cation transport and LMWOA production. The results show that mineral weathering and base cation mobilisation are up-regulated in plants with greater organic matter availability, driven by increased nutrient demand and C allocation to the O and B soil horizons, where the ECM fungi *Piloderma sphaerosporum* and *Suillus bovinus* were most abundant. Base cation transporter gene families in *Suillus* evolved rapidly, with expansions linked to base cation uptake, especially in the MgtE gene family, which was significantly positively correlated with Mg uptake. Significant expansions were also observed in *Piloderma*. Transcriptional analysis of *S. bovinus* revealed up-regulation of several base cation transporter genes in response to mineral treatment, with regulation occurring at both gene copy number and transcription levels. Additionally, *Piloderma fallax* in symbiosis with *Pinus sylvestris* showed significantly higher LMWOA production and exudation in the organic N treatment compared to the inorganic N treatment, with gene expression influenced by the N source. These findings highlight the interconnectedness of mineral weathering and N mobilisation and underscore the need for integrated approaches to understand these processes and their regulation, with implications for sustainable forestry and climate change mitigation.

Keywords: *Suillus*, *Piloderma*, community analysis, stable isotope probing, phylogenomics, evolution, transporter proteins, base cations, organic acids, transcriptomics

Ektomykorrhizasvampar i boreala skogar: deras roll i mineralvittring och kvävemobilisering

Abstract

Ektomykorrhizasvampar spelar nyckelroller i mineralvittring och mobilisering av kväve (N) i boreala skogar, men kunskapsluckor kvarstår om hur dessa processer samverkar och mekanistiska förklaringar till vittring saknas. Medan framsteg har gjorts för att förstå mobilisering av N och reglering av upptag via N transportörer, så har reglering av gener för baskatjontransportörer, liksom för produktion av organiska syror med låg molekylvikt förblivit i stort sett outforskad. Denna avhandling undersöker sambanden mellan mineralvittring, N-mobilisering och kolallokering. Fokus är på mykorrhizasvampsamhällen, evolutionen av genfamiljer för baskatjontransportörer, samt regleringen av baskatjontransport och produktion av lågmolekylära organiska syror. Resultaten visar att mineralvittring och upptag av baskatjoner uppregleras i växter med större tillgång på organiskt material, drivet av en ökad efterfrågan på näringsämnen samt ökad kolallokering till det organiska jordskiktet samt anrikningsskiktet (B-horisonten), där mykorrhizasvamparna *Piloderma sphaerosporum* och *Suillus bovinus* främst förekommer. Genfamiljer för baskatjontransportörer i släktet *Suillus* utvecklades snabbt, med expansioner kopplat till baskatjonupptag, särskilt i MgtE-genfamiljen, vilket även var signifikant positivt korrelerat med magnesiumupptag. Det förekom även betydande expansioner i släktet *Piloderma*. Transkriptionsanalys av *S. bovinus* avslöjade uppreglering av flera gener för baskatjontransportörer som svar på mineralbehandling, och regleringen sker både genom antalet genkopior och transkriptionsnivåerna. Dessutom visade *Piloderma fallax* i symbios med *Pinus sylvestris* plantor signifikant högre produktion av lågmolekylära organiska syror i den organiska N-behandlingen jämfört med den oorganiska N-behandlingen, med genuttryck påverkat av N-källan. Resultaten visar på en sammankoppling mellan mineralvittring och N-mobilisering, och understryker behovet av integrerade metoder för att förstå dessa processer och deras reglering, vilka har konsekvenser för hållbart och klimatanpassat skogsbruk.

Nyckelord: *Suillus*, *Piloderma*, samhällsanalys, stabil isotopsondering, fylogenomik, evolution, transportörproteiner, baskatjoner, organiska syror, transkriptomik

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I. Mahmood, S., Fahad, Z., Bolou-Bi, E., King, K., Köhler, S., Bishop, K., Ekblad, A. and Finlay, R. (2024). Ectomycorrhizal fungi integrate nitrogen mobilisation and mineral weathering in boreal forest soil. New Phytologist, vol 242 issue 4, pp. 1545-1560, DOI: 10.1111/nph.19260

II. King, K., Fransson, P., Finlay, R. and Sanchez-Garcia, M. (0000). Transporter gene family evolution in ectomycorrhizal fungi in relation to mineral weathering. (submitted manuscript)

III. King, K., Sanchez-Garcia, M. and Fransson, P. (0000). Expression of base cation transporter genes during early mineral weathering in the ectomycorrhizal fungus *Suillus.* (manuscript)

IV. Fransson, P., King, K., Bent, E., Norström, S., Bylund, D. and Elfstrand, M. (0000). Organic acid exudation and transcriptome responses to organic and inorganic nitrogen sources in the ectomycorrhizal fungus *Piloderma.* (manuscript)

The contribution of Katharine King to the papers included in this thesis was as follows:

I. Contributed to data analysis, visualisation and writing.

II. Jointly planned with other authors, conducted mineral weathering experiments, performed bioinformatic and statistical analysis with MSG, performed visualisation and writing.

III. Planned and performed microcosm experiments, lead author in bioinformatic and statistical analysis, visualisation and writing.

IV. Contributed substantially to data analysis, writing, and visualisation.

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Abbreviations

$\delta^{15}N$	Isotopic signature of nitrogen
$\delta^{26}Mg$	Isotopic signature of magnesium
^{13}C	Heavy isotope of carbon
ANOSIM	Analysis of Similarity
ANOVA	Analysis of Variance
B horizon	Illuvial soil horizon
C	Carbon
Ca	Calcium
CAZymes	Carbohydrate-active enzymes
CO_2	Carbon dioxide
DEGs	Differentially expressed genes
E horizon	Eluvial soil horizon
ECM	Ectomycorrhizal
Fe	Iron
FPKM	Fragments per kilobase million
GO	Gene Ontology
HOM	High organic matter

ICP-AES	Inductively Coupled Plasma Mass Spectroscopy
ICP-EOS	Inductively Coupled Plasma Optical Emission Spectroscopy
K	Potassium
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC-MS-MS	Liquid Chromatography Tandem Mass Spectrometry
LMWOAs	Low Molecular Weight Organic Acids
LOM	Low organic matter
MC-ICP MS	Multicollector-inductively Coupled Plasma Mass Spectroscopy
Mg	Magnesium
MOM	Medium organic matter
MRPP	Multi-Response Permutation Procedure
N	Nitrogen
Na	Sodium
NH_4Cl	Ammonium chloride
NMDS	Nonmetric Multi-Dimensional Scaling
NOL	No organic layer
O horizon	Organic soil horizon
O_2	Oxygen dimer

P	Phosphorus
PCA	Principal Component Analysis
ROS	Reactive oxygen species
TCA	Tricarboxylic acid
TCDB	transporter classification database

1. Forests, fungi and biogeochemical cycles

Forests are complex ecosystems, rich in biodiversity. They commonly comprise trees, shrubs, grasses, mosses, lichens, fungi, bacteria and archaea, as well as macro- and microfauna. Globally, forests cover 31 % of terrestrial land (FAO 2020) and perform a multitude of vital functions. Not only are forests an important source of essential resources, such as clean air and water, they also play pivotal roles in global nutrient cycles, such as the carbon (C) and nitrogen (N) cycle, and the sequestration of atmospheric carbon dioxide (CO_2) belowground (Hamelin 2024).

Plants, together with their symbiotic partners (commonly fungi), take up essential nutrients for growth like N, phosphorous (P) and base cations – calcium (Ca), potassium (K), iron (Fe), magnesium (Mg), sodium (Na) – from organic and mineral soil (Lindahl et al. 2007; Clemmensen et al. 2015; Finlay et al. 2020). This process requires the allocation of photosynthetically derived C from plant hosts to their symbiotic partners, resulting in C sequestration belowground (Finlay et al. 2020).

Forests are estimated to store 662 gigatonnes (Gt) of C globally, in living and dead biomass, above- and belowground (FAO 2020) – about 5.81 Gt of which is stored in soil fungi (He et al. 2020). This is particularly important in the context of climate change as the rate of climate change and global warming accelerates with elevating atmospheric CO_2. It is therefore vital to maintain and, where possible, increase C sequestration and C stocks in forests. To do this, our knowledge of forest ecosystems must be expanded and incorporated into the development and implementation of sustainable forestry practices.

Basic and applied research into key processes like N mobilisation and mineral weathering, and how these two processes are interconnected, are essential to help make informed decisions towards sustainable forestry, particularly in light of the impact of climate change. Nitrogen mobilisation and mineral weathering, two separate processes performed by interacting fungi, and in particular ectomycorrhizal (ECM) fungi, have been often studied separately (Finlay et al. 2020; Pérez et al. 2022; Tunlid et al. 2022), but rarely simultaneously. Several studies have examined the genomic

evolution of ECM fungi to perform organic matter decomposition and mobilise N (Kohler et al. 2015; Martin et al. 2016; Miyauchi et al. 2020; Lebreton et al. 2021; Martin & van der Heijden 2024), and the molecular and regulatory mechanisms of N mobilisation from organic substrates (Nicolás et al. 2019; Pérez et al. 2022; Plett et al. 2024), however, more research to further elucidate these mechanisms is warranted. To date, there are no studies on the genomic evolution of ECM fungi to perform mineral weathering and base cation uptake, and very few studies focusing on related molecular and regulatory mechanisms (Sun et al. 2019; Pinzari et al. 2022, 2024).

The broader aim of this thesis is to contribute to basic research that can support informed decision-making in sustainable forestry. Here, I focus on the role of symbiotic ECM fungi in N mobilisation and mineral weathering in boreal forests. The following studies investigate how the separate processes of N mobilisation and mineral weathering influence each other and which fungal communities are involved; base cation uptake by ECM fungi and how genes involved have evolved; and which genes are actively transcribed during N mobilisation and mineral weathering.

2. Boreal forests: their belowground fungal community and processes

Boreal forests account for 27% of all forested area globally (FAO 2020) and are a vital component of global biogeochemical processes and, in particular, nutrient cycles. They are predominantly located between latitudes 50 °N and 60 °N, forming a near-continuous circumpolar belt across the Northern Hemisphere (Bonan & Shugart 1989). The climate in the boreal zone is characterised by short, cool summers and long, harsh winters, resulting in a short growing season (Taggart & Cross 2009).

Coniferous trees dominate in boreal forests, particularly pine (*Pinus*) and spruce (*Picea*) (Aerts 1995). Broad-leaved trees, such as birch (*Betula*), alder (*Alnus*) and aspen (*Populus*), are also common. The understory is commonly composed of ericaceous shrubs, such as blueberry (*Vaccinium myrtillus*) and lingonberry (*Vaccinium vitis-idaea*), and mosses and lichens (Esseen et al. 1997; Nilsson & Wardle 2005). Boreal forests are also rich in diverse fungal communities with varied lifestyles, including saprotrophic, pathogenic and symbiotic fungi (Sterkenburg et al. 2015).

Boreal forests are limited in N (Vitousek & Howarth 1991), and typically form stratified podzols with a distinct organic (O) horizon, overlying a mineral eluvial (E) horizon, and mineral illuvial (B) horizon (Rosling et al. 2003). This results from low temperatures and pH, recalcitrant plant litter, slow decomposition rates, and the absence of earthworms resulting in a lack of mixing (Lundström et al. 2000; van Breemen et al. 2000). Distinct fungal communities inhabit each of these soil horizons, and play diverse roles in biogeochemical processes like mineral weathering – which primarily occurs in the E and B horizons (Finlay et al. 2009, 2020), N mobilisation – which primarily occurs in the O horizon (Lindahl et al. 2007; Lindahl & Tunlid 2015), and C sequestration (Rosling et al. 2003; Clemmensen et al. 2013; Kyaschenko et al. 2017).

Biogeochemical processes in boreal forests contribute to global C, N and other nutrient dynamics, with direct implications for climate change outcomes, particularly through their influence on C sequestration and

atmospheric CO_2 levels (Law et al. 2018; López-Blanco et al. 2019). A number of climate change mitigation strategies have been proposed, including reforestation and increasing C density in forests (Law et al. 2018). However, forestry industries are adopting increasingly intense practices, including whole-tree harvesting, which can deplete forest soils of essential nutrients critical for tree growth and result in a reduction of C storage (Akselsson et al. 2007, 2019; Ameray et al. 2021; Hertog et al. 2022; Kastner et al. 2022). A deeper understanding of biogeochemical processes – and the role of fungi in mediating them, is therefore critical for informing sustainable forestry practices and climate change mitigation strategies. The following sections provide an overview of the diversity and ecological roles of fungi in boreal forest ecosystems and the biogeochemical processes they partake in.

2.1 Fungi of the boreal forest

Boreal forests are home to complex and diverse fungal communities that are intimately involved in a multitude of ecological processes. A large proportion of this diversity is belowground, with total fungal biomass in boreal forest soils estimated at 8 Gt across the entire biome (He et al. 2020). Fungi are heterotrophic, meaning they must acquire energy from other organisms, either by consuming C from dead organic matter – as done by saprotrophic fungi – or by consuming C from living organisms (Smith et al. 2017; Genre et al. 2020). Consumption of C from living organisms can be achieved by either weakening or killing the host organism – as done by pathogenic fungi – or by forming a symbiotic relationship with host plants – as done by ericoid mycorrhizal and ectomycorrhizal (ECM) fungi (Oliver 2024). These constitute the main fungal guilds in boreal forests, and their abundances vary across the soil horizons (Rosling et al. 2003; Lindahl et al. 2007).

Ectomycorrhizal fungi

Ectomycorrhizal fungi form the most prevalent symbiotic relationships in boreal forests, most commonly with trees of the Pinaceae family, and exchange N, P, and base cations for photosynthetically derived C (Smith & Read 2008). They are the dominant fungal guild in boreal forest soils, accounting for as much as 70 % of fungal amplicons (Sterkenburg et al. 2015). Conservative estimates of ECM mycelium in boreal forest soils propose a biomass range of 700-900 kg ha^{-1} (Wallander et al. 2001), while more generous estimates propose a biomass range of 4000 – 6000 kg ha^{-1} (Wallander et al. 2004). Annually, ECM fungi are estimated to produce 219 kg ha^{-1} of mycelial biomass on average, and ECM necromass is estimated to account for one-third of total biomass turnover in boreal forests (Hagenbo et al. 2024).

Fungal hyphae of ECM fungi colonise the fine roots of their host plant ectotrophically and form a thick sheath around the root tips called the mantle,

functioning as a nutrient store and a barrier to other microorganisms (Fig. 1). Between the epidermal and cortical cells of the root tip, a Hartig net forms, facilitating nutrient and signal exchange between the host plant and the ECM fungus. Ectomycorrhizal extraradical mycelia extend into the surrounding soil and colonise nutrient-rich microsites, mobilising nutrients and transporting them to their host plant (Smith & Read 2008). To do this, ECM fungi exhibit a range of exploration strategies, ranging from contact and short-distance types, to more extensive medium- and long-distance types, often adapted to specific ecological niches (Agerer 2001).

Figure 1. *Pinus sylvestris* roots in a peat and vermiculite substrate colonised by the ECM fungus *Suillus bovinus*. ECM root tips are circled. (Photo: Petra Fransson)

Ectomycorrhizal fungi play key roles in biogeochemical cycles, such as mineral weathering, N mobilisation, and C cycling (Clemmensen et al. 2013; Van Der Heijden et al. 2015; Genre et al. 2020). Participation in these processes is energetically expensive and requires the allocation of plant photoassimilates, contributing to the sequestration of atmospheric CO_2 into long-term C storage belowground (Fig. 2) (Clemmensen et al. 2013, 2015, 2021; Finlay et al. 2020). Boreal forest trees have been shown to allocate approximately 60 % of photoassimilated C belowground to roots and mycorrhizal symbionts (Litton et al. 2007; Gill & Finzi 2016), with ECM fungi estimated to receive approximately 20 % (Hobbie 2006; Hagenbo et al. 2017).

Ectomycorrhizal fungi have the highest abundance of all fungal guilds in the E and B soil horizons (Rosling et al. 2003), where they are able to weather minerals and mobilise and acquire base cations and P in low C environments, whilst receiving C from their host plants. As much as two-thirds of ECM root tips are found in the E and B horizons, along with almost half of the ECM taxa across the boreal forest podzol, many of which are exclusive to these horizons (Rosling et al. 2003). Ectomycorrhizal fungi are also dominant in the lower O horizon (Rosling et al. 2003; Lindahl et al. 2007) where they decompose organic matter to mobilise and acquire N and other nutrients for their plant hosts (Read & Perez-Moreno 2003; Simard et al. 2003; Hobbie & Horton 2007; Van Der Heijden et al. 2015), contributing an estimated 60-86 % of total plant N (Hobbie & Hobbie 2006; Kohler et al. 2015). The role of ECM fungi in plant nutrient acquisition, resulting from mineral weathering and N mobilisation, combined with their expansive colonisation of boreal forest soils and their role in biogeochemical processes and nutrient cycles, makes them one of the most important guilds of fungi in boreal forests.

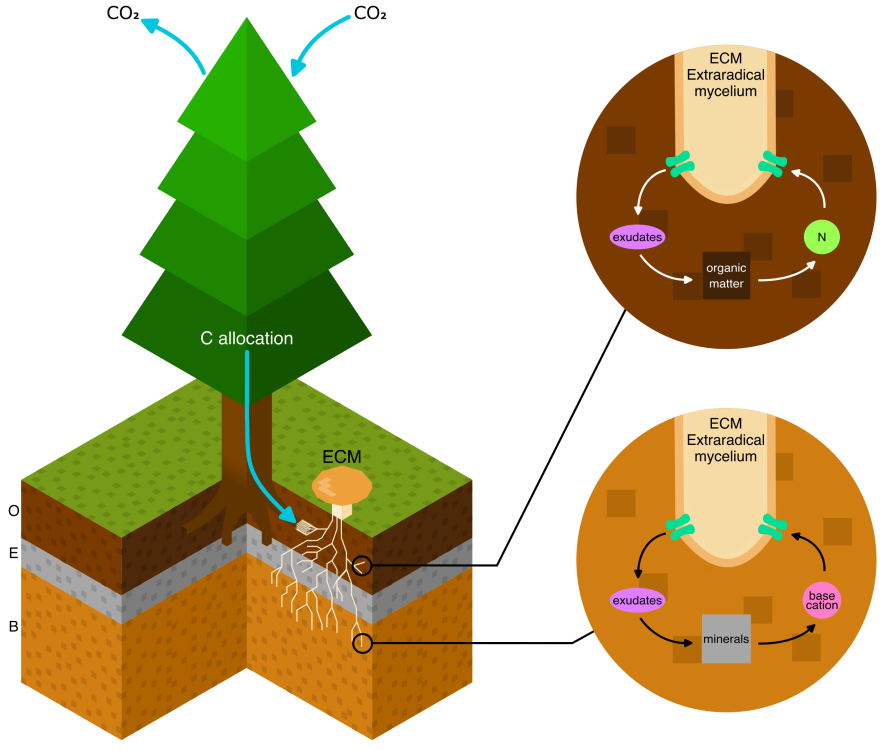

Figure 2. Schematic diagram of the ECM symbiosis between host trees and ECM fungi, and the biogeochemical processes they perform in boreal forest podzols. A host tree and an ECM fungus fruiting body, including an ECM root tip and radiating exploratory mycelia, are illustrated, as well as soil horizons, which are indicated with letters (O, E, B) adjacent to each horizon (left). Blue arrows indicate C flow. Host trees both photosynthesise (CO_2 entering the tree) and respire (CO_2 exiting the tree). Photosynthetically derived C is allocated belowground through ECM root tips to ECM fungi, which can then utilise C to decompose organic matter and mobilise N (upper right), or weather minerals and mobilise base cations and P (bottom right). Decomposing agents are exuded from extraradical mycelia, which then degrade organic matter and mobilise N for uptake. Weathering agents are also exuded from extraradical mycelia, which then weather minerals and mobilise base cations and P for uptake. Both the exudation of decomposing and weathering agents, and the uptake of N, P and base cations typically occur through transporter proteins.

Key ECM genera in this thesis

The *Suillus* genus

The ECM genus *Suillus* belongs in the order Boletales (Wu et al. 2020), has widespread distribution and primarily forms species-specific ECM symbioses with Pinaceae, with a long-distance exploration type growth pattern by extraradical mycelia (Agerer 2001). *Suillus* has been suggested as a new model genus for ECM ecology and evolution (Lofgren et al. 2024). Many species grow well in culture and are commonly used in experiments (Fransson & Johansson 2010; Lofgren et al. 2024). *Suillus* species have been frequently reported to perform mineral weathering, exude organic acids (Olsson & Wallander 1998; Wallander & Wickman 1999; Adeleke et al. 2012), take up base cations from mineral sources (Balogh-Brunstad *et al.*, 2008; Fahad *et al.*, 2016), and are found primarily in the B horizon in boreal forests (Marupakula et al. 2021; Lofgren et al. 2024). *Suillus bovinus* occurs in forests of all successional stages, but the number of genets has been shown to decrease over time while individual genets increase in size, and *S. bovinus* is commonly replaced by *S. variegatus* in older forests (Dahlberg & Stenlid 1990; Dahlberg 1997).

The *Piloderma* genus

Piloderma species are phylogenetically nested within the ecologically diverse Atheliales clade (Sulistyo, Larsson et al. 2021) and constitutes a small genus of soft, corticoid, and widely distributed ECM species (Svantesson et al. 2025) that display short- to medium-distance fringe exploration types (Agerer 2001). *Piloderma* has been considered a species complex since delimitation based on morphology is difficult, but in recent years several new species have been described (Larsson et al. 2024; Svantesson et al. 2025). Some *Piloderma* species, especially *P. fallax* and *P. byssinum*, have been commonly used in various experiments, including pure culture work and synthesised with pine seedlings (Rosling et al. 2004; Fransson & Johansson 2010; Weigt et al. 2011). They are predominantly found in the Northern Hemisphere (Larsson et al. 2024), and are especially common in boreal forests (Varenius et al. 2016), (Soil fungi in Swedish woodland, 2024, https://svamparisverige.se). *Piloderma* dominate in younger forest stands, and are partly replaced in older successional stages (Kyaschenko et al. 2017). Different *Piloderma* species grow predominantly in the organic or mineral soil, or are found throughout the soil profile (Rosling, Landeweert et al. 2003).

Ericoid mycorrhizal fungi

Ericoid mycorrhizal fungi form symbiotic relationships with Ericaceous shrubs, and also acquire soil nutrients in exchange for photosynthetically derived C (Smith & Read 2008; Ward et al. 2022; Rains et al. 2024). Ericaceous shrubs have fine, delicate roots which lack root hairs. The epidermal cells of these roots are colonised by ericoid mycorrhizal fungi, which form coil-like hyphal structures and extend extraradical mycelia that act as root hairs into the surrounding soil (Read & Perez-Moreno 2003). Growth of extraradical mycelia into the soil is limited, suggesting that their primary role lies in the conversion of nutrients to usable forms, rather than the mass acquisition of nutrients across an extensive soil volume (Read & Perez-Moreno 2003). Ericoid mycorrhizal plants are present in 96 % of boreal forests, and have been found to increase organic matter accumulation, slow organic matter decomposition, and have a strong influence on nutrient cycling in boreal forests (Clemmensen et al. 2015, 2021; Ward et al. 2022).

Saprotrophic fungi

Saprotrophic fungi encompass free-living moulds, litter-decaying and wood-decaying fungi that acquire C from dead organic matter (Smith et al. 2017), and they have served as the ancestral origin from which ECM and ericoid mycorrhizal fungi have evolved on multiple separate occasions (Matheny et al. 2006; Tedersoo et al. 2010; Hess et al. 2018; Martino et al. 2018; Miyauchi et al. 2020). Saprotrophic fungi are the dominant decomposers of organic material in boreal forest soils (Hobbie & Horton 2007; Lindahl et al. 2007), have been shown to have higher abundance in the O horizon (Rosling et al. 2003; Lindahl et al. 2007), and are estimated to contribute between 2000 and 4000 kg ha^{-1} of biomass in boreal forest soils (Wallander et al. 2004)

Many saprotrophic fungi, termed brown-rot fungi, rely on Fenton chemistry, by oxidising ferrous iron (Fe^{2+}) and releasing reactive oxygen species (ROS) which degrade cellulose, hemicellulose and pectin (Smith et

al. 2017; Finlay & Thorn 2019). Other saprotrophic fungi employ oxidative enzymes such as cellulases, laccases and peroxidases to degrade cellulose and lignin (Baldrian 2004). These are termed white-rot fungi (Finlay & Thorn 2019), and together with brown-rot fungi and other saprotrophic fungi, are a driver of organic matter decomposition and nutrient cycling in boreal forests (Bödeker et al. 2016).

Pathogenic fungi

Pathogenic fungi are disease-causing fungi that colonise their hosts and exploit their resources, often resulting in the reduction of growth, damage or fatality of the host (Oliver 2024). Pathogenic fungi are commonly categorised as necrotrophic, biotrophic, or hemibiotrophic (Ferreira et al. 2006; Oliver 2024). Necrotrophic pathogenic fungi obtain nutrients from dead host tissue, which is killed either prior to or during colonisation (Wang et al. 2014), by the secretion of ROS, cell wall degrading enzymes, or toxins (Daub & Chung 2009; Tudzynski & Kokkelink 2009; Horbach et al. 2011). Biotrophic pathogenic fungi obtain nutrients from live host tissue by supressing host immunity with high-specificity effector molecules (Horbach et al. 2011; Yi & Valent 2013). Hemibiotrophic pathogenic fungi initially exhibit biotrophic behaviour, before switching to a necrotrophic lifestyle (Ferreira et al. 2006; Horbach et al. 2011; Oliver 2024). Pathogenic fungi are a significant source of disturbance in boreal forests, contributing to substantial economic losses and ecological damage – impacts that are expected to intensify with climate change (Sturrock et al. 2011).

2.2 Biogeochemical processes of the boreal forest

Biogeochemical processes play essential roles in boreal forest ecosystem functioning, with implications for nutrient availability, plant productivity, plant and fungal community composition and activity, and C sequestration and belowground C storage (Lindahl et al. 2007; Quirk et al. 2014; Clemmensen et al. 2015; Finlay et al. 2020). These processes are in turn influenced by above- and belowground abiotic and biotic factors (Wardle et al. 2004; Quideau et al. 2013). In particular, plant nutrient acquisition, primarily mediated by associated ECM and ericoid mycorrhizal fungi, is a strong driver of biogeochemical processes (Gill & Finzi 2016), as plants not only allocate photosynthetically derived C to their symbiotic partners to perform these processes, but also act as nutrient sinks. Ectomycorrhizal fungi contribute to mineral weathering by exuding organic acids and other compounds that mobilise base cations and P from mineral sources (Leake & Read 2017; Smits & Wallander 2017; Finlay et al. 2020). Similarly, some ECM fungi have been shown to have a significant capacity to mobilise and assimilate organic N associated with both SOM and mineral surfaces (Tunlid et al. 2022), and they, and ericoid mycorrhizal fungi, exude organic acids and enzymes to perform this process (Lindahl & Tunlid 2015; Ward et al. 2022). Meanwhile, saprotrophic fungi employ different strategies to decompose organic matter (Sterkenburg et al. 2015; Finlay & Thorn 2019). Through these processes, ectomycorrhizal, ericoid mycorrhizal and saprotrophic fungi drive nutrient and C dynamics in boreal forests.

Mineral weathering and base cation uptake

The process of mineral weathering entails the breakdown of rock into smaller particles, the dissolution of minerals, and the precipitation of secondary minerals (Finlay et al. 2020). Mineral weathering is a key process in the carbon cycle (Pagani et al. 2009) and while the consumption of CO_2 by weathering is relatively small on a short-term scale when compared with fluxes associated with photosynthesis and respiration, it is proposed to be the

dominant sink of global C and exert significant influence on atmospheric CO_2 and climate patterns over geological time-scales, typically spanning millennia or longer (Goudie & Viles 2012). It is driven by physical, chemical and biological agents and is further influenced by environmental conditions, such as temperature, soil moisture, and pH (Uroz et al. 2009; Leake & Read 2017; Smits & Wallander 2017; Finlay et al. 2020).

Physical weathering agents such as thermal stresses and mechanical forces (Bonneville et al. 2009, 2016) contribute to the fragmentation of rock material, increasing the surface area available to be weathered. Chemical weathering agents, such as protons, hydroxides and organic acids, can alter and dissolve minerals by interfering with ionic bonds in mineral structures, liberating weathering products such as base cations and P (Smits & Wallander 2017). The concentration of weathering products also influences mineral weathering, as these products can interact with chemical weathering agents and, at high concentrations, slow the process (Finlay et al. 2020).

Biological mineral weathering shares similarities with physical and chemical weathering; however, it is facilitated by plants, fungi, and other organisms (Smits & Wallander 2017). Plants and fungi can apply mechanical force through the growth of roots and hyphae (Howard et al. 1991; Bonneville et al. 2016). They can also influence the chemical composition of soils through water and nutrient uptake, acting as a nutrient sink and preventing nutrient saturation (van Scholl et al. 2006; Van Scholl et al. 2006; Finlay et al. 2020). Additionally, plant necromass increases soil acidity, contributing to more favourable conditions for mineral weathering (Leake & Read 2017).

Plants also allocate photosynthetically derived C to fungal symbiotic partners, which can then be utilised by fungi to weather minerals and mobilise base cations and P (Fomina et al. 2010; Schmalenberger et al. 2015). Ectomycorrhizal fungi in particular are strong drivers of biological mineral weathering in boreal forest soils (Finlay et al. 2020) and dominate the mineral soil horizons (Rosling et al. 2003; Marupakula et al. 2021). To weather minerals, ECM fungi produce and exude low molecular weight organic acids (LMWOAs), free radicals, and protons, which can lower soil pH and disrupt ionic bonds in mineral structures (Fomina et al. 2010; Smits

& Wallander 2017). Additionally, they can produce and exude siderophores, which chelate metal ions, further facilitating mineral weathering (Smits & Wallander 2017; Finlay et al. 2020). Furthermore, ECM fungi facilitate nutrient uptake and transfer to plant hosts, reducing the concentration of weathering products such as base cations and P in the soil and preventing their accumulation, which can lead to a reduction in mineral weathering (Finlay et al. 2020).

Decomposition and nitrogen mobilisation

In boreal forest soils, decomposition of organic matter is primarily carried out by saprotrophic and ECM fungi in the O soil horizon. Whilst saprotrophic fungi decompose organic matter to obtain C, ECM fungi do so to obtain N (Hobbie & Horton 2007; Clemmensen et al. 2015; Leake & Read 2017), which is present in soil organic matter and bound to mineral particles (Cotrufo et al. 2022). Saprotrophic and ECM fungi have been shown to inhabit spatially separate zones of the soil, with saprotrophic fungi dominating in the relatively undecomposed, recently shed litter of the upper O soil horizon, while ECM fungi are more prevalent in the more decomposed lower O soil horizon (Hobbie & Horton 2007; Lindahl et al. 2007).

Saprotrophic fungi have evolved several strategies to degrade organic matter and access C from different substrates (Lebreton et al. 2021) and typically degrade organic matter from least to most recalcitrant (Boer et al. 2005). Two key chemical reactions used by saprotrophic fungi are the Fenton reaction and peroxidase reactions. The Fenton reaction, employed by brown-rot saprotrophic fungi, requires Fe ions (Op De Beeck et al. 2020) and generates free radicals that break bonds in cellulose, hemicellulose, and lignin, promoting their degradation (Boer et al. 2005). This facilitates access for enzymes produced by brown-rot fungi and other microbes (Rineau et al. 2013; Op De Beeck et al. 2018, 2020). Peroxidase reactions, employed by white-rot saprotrophic fungi in aerobic conditions, involve enzymes with high oxidation potential, such as lignin peroxidases and manganese peroxidases, which work together to degrade woody substrates (Boer et al. 2005; Op De Beeck et al. 2020). Peroxidases catalyse the incorporation of

oxygen dimers (O_2) to C-centred radicals, facilitating polymer cleavage (Bödeker et al. 2014, 2016).

Ectomycorrhizal fungi play a critical role in mobilising and assimilating organic N from SOM and mineral surfaces (Tunlid et al. 2022), an important process in N-limited boreal forests, where N is the primary constraint on plant growth (Vitousek & Howarth 1991; Gill & Finzi 2016). This N is then transferred to plant hosts and exchanged for photosynthetically derived C. Most of the N required to meet plant demand is derived from organic litter and soil (Sponseller et al. 2016). Ectomycorrhizal fungi have retained some decomposition capabilities from their saprotrophic ancestors (Kohler et al. 2015; Bödeker et al. 2016; Miyauchi et al. 2020; Lebreton et al. 2021; Ward et al. 2022) and, like saprotrophic fungi, can utilise the Fenton reaction, exude extracellular enzymes such as carbohydrate-active enzymes (CAZymes) and peroxidases, and LMWOAs and other compounds to modify SOM and mobilise N (Lindahl & Tunlid 2015; Wang et al. 2017; Tunlid et al. 2022). Similarly, ericoid mycorrhizal fungi also decompose organic matter to acquire N and have retained some decomposition capabilities from their saprotrophic ancestors, including the ability to exude CAZymes and peroxidases, and LMWOAs and other compounds (Lebreton et al. 2021).

Carbon dynamics

Boreal forests are a large and persistent sink of C and play a key role in C dynamics globally, accounting for 32 % of forest C stocks, of which 60 % is stored in boreal forest soils (Pan et al. 2011). Plants, which use solar energy and atmospheric CO_2 to form C-rich photoassimilates, are the main contributors of C to forest soils (Finlay & Clemmensen 2017). Carbon can enter the soil through aboveground plant litter and deadwood deposition, and via belowground roots, contributing to SOM accumulation and long-term C storage (Godbold et al. 2006). Belowground allocation of photosynthetically derived C by plants to symbiotic partners is another major source of C input into the soil and strongly influences the C cycle (Litton et al. 2007; Sterkenburg et al. 2015). Plant C allocation not only drives mycorrhizal fungi-mediated processes like mineral weathering and organic matter

decomposition, but also mycorrhizal fungal growth. Mycorrhizal hyphal turnover has been shown to be a major source of C flux (Godbold et al. 2006), and ECM hyphal turnover, in particular, has been shown to account for one third of biomass turnover in boreal forests (Hagenbo et al. 2024).

Biological mineral weathering by ECM fungi is an energy-intensive process that requires significant plant allocations of C, thereby driving both C sequestration of atmospheric CO_2 and long-term C storage (Goudie and Viles, 2012; Finlay *et al.*, 2020). Carbon is a fundamental prerequisite for the production of weathering agents such as LMWOAs and siderophores. While weathering agents are typically labile and undergo rapid turnover, their contribution to nutrient mobilisation from mineral substrates can result in the long-term sequestration of C in both organic (Clemmensen et al. 2013) and inorganic (Sun et al. 2019) recalcitrant substrates. Weathering agents can also form relatively stable C-rich secondary minerals, further contributing to long-term C storage (Fomina et al. 2010).

Ectomycorrhizal fungi also influence the C cycle through the decomposition of organic matter to attain N (Tunlid et al. 2022). Not only does the energy-intensive production of metabolites, such as peroxidase enzymes and free radicals generated during the Fenton reaction, result in C sequestration via C allocation by plants, but these metabolites can also be amalgamated into recalcitrant molecular aggregates, contributing to C storage (Lehmann & Kleber 2015; Tunlid et al. 2022). Although ECM fungi contribute to C flow through organic matter decomposition, they compete for organic substrates with the more efficiently decomposing saprotrophic fungi. This has been observed to increase SOM and C accumulation in boreal forest soils by limiting saprotrophic fungi activity and is commonly referred to as the Gadgil effect (Gadgil & Gadgil 1971; Averill et al. 2014; Sterkenburg et al. 2018).

Whilst plants and fungi, and other microorganisms, contribute to C sequestration and C storage, they must also respire – utilising C for energy and releasing CO_2 into the atmosphere (Lindahl et al. 2002). Fungi are the most significant contributors to CO_2 efflux in boreal forest soils, with ECM fungi alone shown to emit more than 50 % of total CO_2 emissions (Högberg et al. 2001), meanwhile estimates of plant roots CO_2 emissions range from

40-50 % (Hanson et al. 2000). The balance of C sequestration, storage, and emissions is complex, fluctuating with community composition, growth, interactions and the processes they perform, as well as with soil matter type and quality (Lindahl et al. 2002).

3. Objectives and Aims

The pivotal role of ECM fungi in mineral weathering and N mobilisation is well-documented. Whilst these processes have been studied separately, information on how different ECM fungi drive mineral weathering and N mobilisation, and by extension C sequestration in different soils, as well as how N supply impacts these processes, is lacking. In addition, the evolutionary and regulatory mechanisms involved in these processes at the genomic and gene level remain understudied.

This thesis aims to improve understanding of ECM-driven biogeochemical processes in boreal forests, which underlie the mobilisation of base cations from mineral sources and N mobilisation from organic substrates necessary for sustainable forest production. This work can potentially support informed decision-making in sustainable forestry and aid the development of new CO_2 mitigation strategies. The following studies investigate how N mobilisation and mineral weathering influence each other, the fungal communities involved, the evolution of base cation uptake genes in ECM fungi, and the transcriptional activity of ECM fungal genes during these processes.

Paper I considers the relationship between organic substrate decomposition and mineral weathering using reconstructed podzol microcosms with non-sterile forest soils and *P. sylvestris* seedlings. **Paper II** investigates transporter gene family evolution in ECM fungi in relation to mineral weathering capability, with a focus on base cation transporters and the *Suillus* genus. **Paper III** further examines base cation transporter genes, assessing the gene expression profile of *S. bovinus* when undergoing mineral weathering both alone and whilst in symbiosis with *P. sylvestris* seedlings. Finally, **Paper IV** explores the impact of organic and inorganic N sources on organic acid production and exudation and C release, and gene expression profiles of *Piloderma* mycelia and ECM root tips when in symbiosis with *P. sylvestris* seedlings.

4. Nutrient mobilisation

In boreal forests, plants rely heavily on associated ECM fungi to acquire N via N mobilisation from organic matter, and P and base cations via mineral weathering from minerals. In turn, ECM fungi depend on photosynthetically derived C supplied by their plant hosts to perform these processes (Smith & Read 2008). Nitrogen – the main limiting factor of plant productivity in boreal forests (Vitousek & Howarth 1991; Gill & Finzi 2016), and P and base cations are essential for growth (Finlay et al. 2020). Larger plants produce more photoassimilates, enabling them to allocate more C to their symbiotic partners, which in turn can be utilised to perform more organic matter decomposition and mineral weathering. In stratified boreal forest soils, ECM-mediated organic matter decomposition and mineral weathering occur in spatially separate soil horizons, each with distinct fungal communities (Rosling et al. 2003; Lindahl et al. 2007; Finlay et al. 2020; Marupakula et al. 2021).

4.1 The microcosm experiment: nitrogen mobilisation and mineral weathering

The contributions of N mobilised from organic matter, and P and base cations mobilised from minerals to plant nutrition are well-documented. The majority of studies have focused on N mobilisation in the upper O horizon, and have shown that many ECM fungi retain decomposing capabilities from their saprotrophic ancestors (Kohler et al. 2015; Lindahl & Tunlid 2015; Bödeker et al. 2016; Miyauchi et al. 2020; Lebreton et al. 2021; Ward et al. 2022). A handful of studies have focused on the deeper mineral horizons, where two-thirds of mycorrhizal root tips and more than half of ECM taxa were reported to be found (Rosling et al. 2003). Additionally, a number of laboratory studies of individual ECM species have demonstrated their proficiency in biological weathering and base cation uptake from minerals.

These studies, however, have considered the processes of N mobilisation and mineral weathering separately.

This chapter examines N mobilisation and mineral weathering, and their influence on each other, in the same system. Carbon and N dynamics in plant tissue, soil and soil solution of different soil horizons were assessed. Special focus was placed on the base cation Mg, which exists as three abundant and stable isotopes in nature (^{24}Mg, 78.99 %; ^{25}Mg, 10.00 %; ^{26}Mg, 11.01 %), making it highly suitable in the identification of biogeochemical processes that fractionate isotopes (Wallander & Wickman 1999; Wallander 2000; Balogh-Brunstad et al. 2008; Black et al. 2008; Adeleke et al. 2012; Schmalenberger et al. 2015; Fahad et al. 2016). Furthermore, ECM fungi have been shown to discriminate against heavier Mg isotopes during mineral weathering and base cation uptake, leading to enrichment of heavier Mg in soils (Fahad et al. 2016). Additionally, Mg plays a central role in plant nutrition, including photosynthesis (Black et al. 2007), enzyme function and cation transport and homeostasis (Bose et al. 2011).

In **Paper I,** the integration of the spatially separate processes N mobilisation and mineral weathering was investigated. For 14 months, *Pinus sylvestris* seedlings were grown in microcosms containing reconstructed podzols using natural forest soils with differing proportions of O horizon soil. These microcosms were used to elucidate the effect of organic matter availability on N mobilisation and mineral weathering and, more specifically, on biomass of plant and fungal mycelia; spatial allocation of plant-derived C; spatial distribution, abundance and composition of fungal communities and guilds; and N, P and base cation mobilisation and uptake. Elemental and stable isotopic analyses of C (^{12}C, 98.92 %; ^{13}C, 1.08 % (Delikatny & Poptani 2005)), N (^{14}N, 0.36 %; ^{15}N 99.64 % (Bovey & Mirau 1996)), and Mg, as well as $^{13}CO_2$ pulse-labelling, and high-throughput DNA sequencing of fungal communities in different soil horizons, were employed to support these investigations.

4.2 Hypotheses

In **Paper I**, we hypothesised that increased organic matter availability would enhance N mobilisation by ECM fungi and the subsequent supply of N to their plant hosts. This, in turn, would promote plant growth and increase the allocation of photosynthetically derived C to ECM fungi. We further hypothesised that greater C allocation to ECM fungi would increase mobilisation of base cations and P from mineral substrates. Additionally, we hypothesised that enhanced Mg uptake would lead to an enrichment of $\delta^{26}Mg$ signatures in soil solution, particularly in areas of high ECM abundance.

4.3 Methodology

Natural soil microcosms and in-growth mesh bags

To test our hypotheses in a controlled system that closely mimics natural soil conditions, microcosms with reconstructed podzols using natural forest soils with differing proportions of O horizon were used. Four treatments were designed to simulate a gradient of organic matter availability, with O horizons accounting for 150 % (high organic matter), 100 % (medium organic matter), 50 % (low organic matter), and 0 % (no organic layer) of total microcosm volume (Fig. 3c). Four replicates of each treatment were prepared, each with six *P. sylvestris* seedlings, along with four controls with no plants.

Forest soils were acquired from a well-documented forest, Jädraås, located in central Sweden (60°490′ N, 16°300′ E, altitude 185 m) (Persson & Bockheim 1981; Mielke et al. 2022). Soils were sieved and placed into microcosms in layers, with B horizon soil at the base, followed by E horizon soil, and finally O horizon soil. Whilst E horizon soil remained constant at 800 g, B horizon soil varied with the O horizon volume, resulting in a B horizon of 250 g and O horizon of 600 g in the high organic matter (HOM) treatment, a B horizon of 650 g and O horizon of 400 g in the medium organic matter (MOM) treatment, a B horizon of 1050 g and O horizon of 200 g in the low organic matter (LOM) treatment, and a B horizon of 1450 g and O horizon of 0 g in the no organic layer (NOL) treatment (Fig. 3c). Each layer was separated by a 1 mm thick, 2 mm pore size nylon mesh, and a lysimeter and two mesh bags containing soil and glass beads were embedded in each horizon, to extract soil solution and obtain clean fungal mycelium, respectively (Fig. 3a).

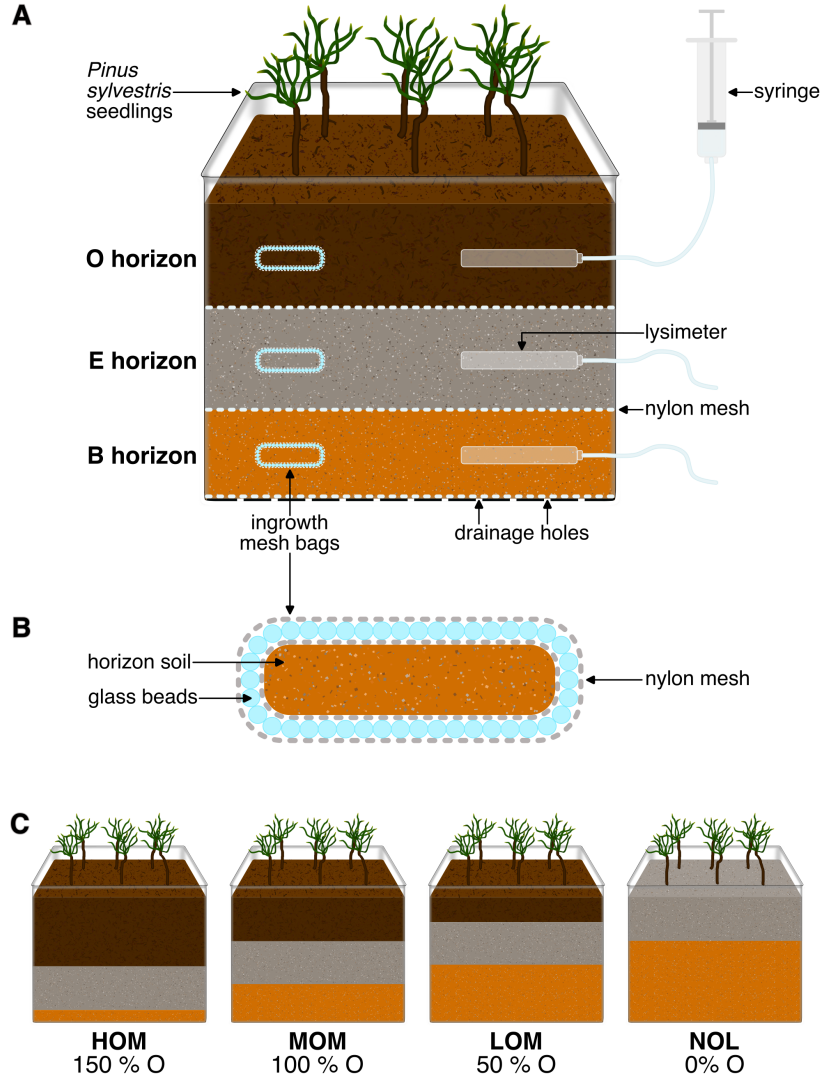

Figure 3. Schematic illustration of microcosm design (A), mesh bag design (B), and treatments (C). (A) shows all components comprising each microcosm. In acrylic containers, podzols using natural forest soils were reconstructed, with variable O horizon volumes according to treatment (as indicated in (C)). Nylon mesh was placed at the base and between each horizon to aid harvesting, and ingrowth mesh bags and lysimeters were embedded into each horizon. A syringe was used to extract soil solution. Ingrowth mesh bags comprised two compartments: the inner, filled with soil corresponding to the horizon the bag was to be embedded in, and the outer, with acid-washed glass beads to aid harvesting of clean fungal mycelium.

In-growth mesh bags were constructed of 50 μm pore size nylon mesh and consisted of an inner compartment, filled with O, E or B horizon soil from the corresponding horizon, and an outer compartment of acid washed 1 mm glass beads (Fig. 3b). Ectomycorrhizal fungi were expected to grow through the outer compartment to access soil in the inner compartment during their exploration and colonisation of the bulk soil. Elemental composition and concentration of mycelium in the outer compartment were expected to mirror that of mycelium in the bulk soil, whilst avoiding root and soil contamination, thus facilitating the harvest of clean, representative fungal mycelium for elemental and isotopic analyses, and DNA extraction.

Stable isotope and elemental analyses

To address the hypotheses that increased organic matter availability would enhance ECM-mediated N mobilisation and transfer to plant hosts, and that this would result in larger plants and increase C allocation to ECM fungi, stable isotope analysis of ^{15}N and ^{13}C natural abundances was conducted. This analysis was performed on plant shoots and roots, as well as soils from each horizon, using an elemental analyser and a continuous flow Isoprime isotope ratio mass spectrometer.

To assess whether greater C allocation to ECM fungi, resulting from increased plant growth, would lead to greater mobilisation of base cations and P from mineral substrates, elemental analysis was performed. Concentrations of base cations and P in plant shoots and roots, as well as in soil and soil solution, were determined. Fungal mycelium from each soil horizon was also analysed using inductively coupled plasma mass spectroscopy (ICP-AES).

To test the hypothesis that enhanced Mg uptake, resulting from increased mineral weathering due to greater N mobilisation and transfer by ECM fungi in treatments with higher organic matter availability, would lead to enriched $\delta^{26}Mg$ signatures in soil solution, and particularly in areas of high ECM abundance, isotope analysis of Mg was conducted. This analysis was

performed using ion chromatography and multicollector-inductively coupled plasma mass spectroscopy (MC-ICP MS).

Community analysis

To further test the hypothesis that δ^{26}Mg would be enriched in areas of high ECM abundance, and to ascertain the role of ECM fungal species and different fungal guilds in N mobilisation and mineral weathering, community analysis was performed. DNA was extracted from soil and clean mycelium from the mesh bags of each horizon and in each treatment, to identify guild and fungal community composition and abundance. Following PCR amplification, sequencing, library preparation and adaptor ligation were performed at SciLifeLab, NGI-Uppsala, Sweden. Combining community and guild analyses with stable isotope and elemental analyses of spatially separate samples from different soil horizons and treatments facilitated interpretations of the roles different fungi and guilds are likely to play in N mobilisation, mineral weathering, and plant nutrient acquisition.

Statistics

The effects of treatments on each parameter were assessed using Analysis of Variance (ANOVA). Linear regression was used to evaluate relationships between pairs of parameters, while Principal Component Analysis (PCA) assessed correlations among multiple parameters in the statistical software JMP PRO v15.0.0. Fungal beta diversity patterns were analysed using Nonmetric Multi-Dimensional Scaling (NMDS) with Bray-Curtis dissimilarity based on rarefied abundance data. Fungal community composition differences across O, E, and B horizon soils (or mycelial samples) in the reconstructed podzol microcosms or treatments were tested using nonparametric Analysis of Similarity (ANOSIM) and PERMANOVA with 9999 permutations (PAST v.4.03). Significant differences in fungal taxa abundance across treatments were identified using ANOVA in QIIME (Caporaso et al. 2010).

4.4 Results and Discussion

Nitrogen, P and base cations are essential to plant growth and productivity and, in the case of boreal forest trees, are most commonly acquired through symbiotic ECM fungi. By utilising plant allocated C, ECM fungi can decompose organic matter to mobilise N – the primary limitation on plant productivity in boreal forests (Vitousek & Howarth 1991; Gill & Finzi 2016), and transport it to their plant hosts. Plants with higher N availability can grow larger, and in turn allocate more C to their symbiotic partners. However, forestry practices are moving beyond the removal of only tree trunks, to include other key inputs of organic matter, such as branches, roots, stumps and foliage, to keep up with demands for sustainable fuel sources like bioenergy (Daioglou et al. 2019; Kastner et al. 2022). Carbon allocations to ECM fungi are not only used to mobilise N, but also to weather minerals and mobilise base cations and P (Finlay et al. 2020). The processes of N mobilisation, mineral weathering and C allocation are therefore intimately intertwined, and are likely to be affected by organic matter availability.

Plant and mycelial biomass increase with organic matter availability

The impact of organic matter availability on plant productivity has long been well established, with additions shown to increase forest productivity (Brockway 1983; Couillard & Grenier 1989; Henry et al. 1994), and removals shown to reduce it (Ginter et al. 1979; McLeod et al. 1979; Jurgensen et al. 1997). In **Paper I**, increased organic matter availability resulted in significantly greater plant biomass (Fig. 4a), likely due to greater N supply. Mycelial biomass also increased significantly with increasing organic matter availability, with an increase of 121 % from the MOM treatment to the HOM treatment (Fig. 4b). Furthermore, there was 3.64 times the density of mycelia in the B horizon soil of the HOM treatment compared to the MOM treatment, likely reflecting increased C allocation to ECM fungi by larger plants, which supports fungal proliferation. Taken together, these findings provide evidence for our first and second hypotheses that greater

organic matter availability would lead to greater ECM-mediated N mobilisation and supply to host plants, and the subsequent increase in plant growth would increase C allocation to ECM fungi.

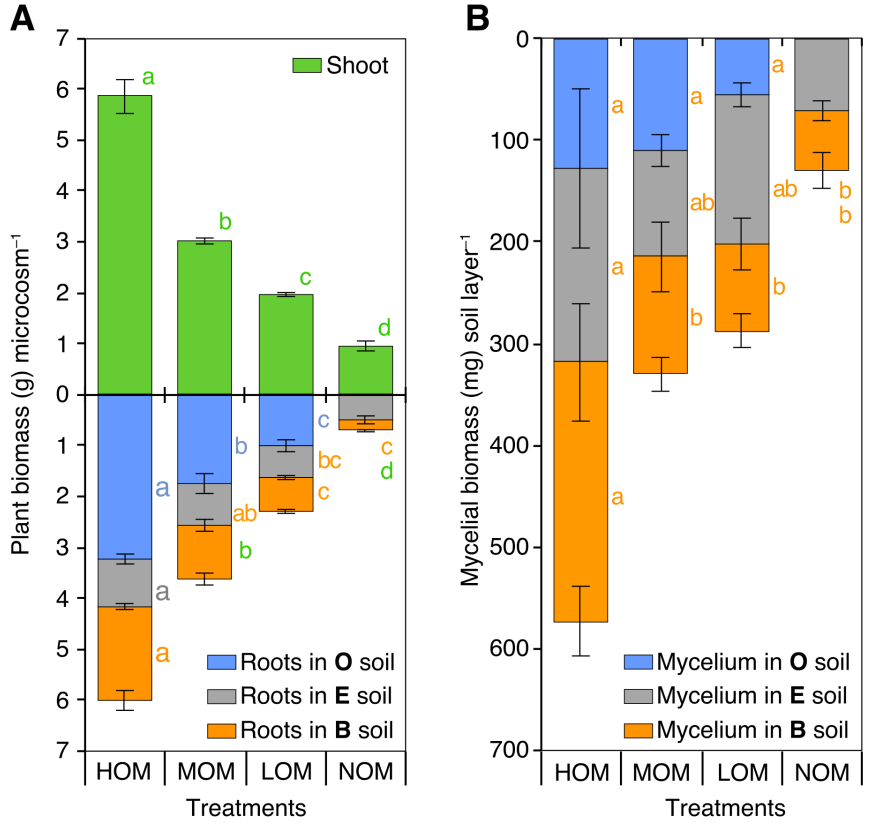

Figure 4. Plant (A) and mycelial (B) biomass in HOM, MOM, LOM and NOL treatments. (A) shows plant biomass (g) with standard errors for shoots (green) and roots in the O (blue), E (grey), and B horizons (orange). (B) shows mycelial biomass (mg) in the O (blue), E (grey), and B horizons (orange). A compact letter display (CLD) indicates significant differences between corresponding values of different treatments. (*Adapted from* ***Paper 1***).

Plant N content increased significantly with increasing organic matter availability and plant biomass (***Paper I*** *Fig. 2d*). Additionally, plants in HOM treatments were significantly depleted in $\delta^{15}N$, indicating the fractionation of isotopes and preferential uptake of lighter isotopes by associated ECM fungi. Discrimination against heavier isotopes by ECM fungi has been shown previously, and leads to the enrichment of heavier isotopes in substrates (Fahad et al. 2016). The significant increase in plant N content and greater depletion of $\delta^{15}N$ in plants in HOM treatments compared to plants in other treatments strongly suggests enhanced N mobilisation by ECM fungi as a result of greater organic matter availability, and further supports our first hypothesis.

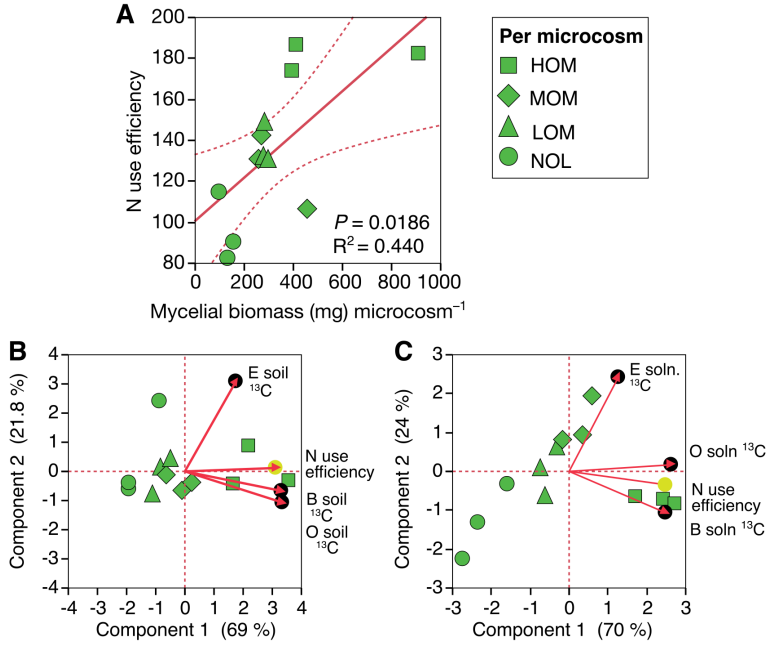

Figure 5. Plant N use efficiency in each treatment. (A) shows a significant positive correlation between plant N use efficiency and mycelial biomass per microcosm. PCAs show the ordination of treatments explained by plant N use efficiency and ^{13}C allocation to soil (B) and soil solution (C) in each horizon. Treatments are indicated by shape. (*Adapted from* ***Paper 1***).

Plant N use efficiency, which is the biomass production of plant shoots per unit of N taken up, was shown to be significantly positively correlated with mycelial biomass (Fig. 5a), indicating that plants with access to organic matter not only received more N, but also other key nutrients required for growth, such as base cations and P. This is likely related to the vast increase in mycelial biomass with increasing organic matter availability.

Plant ^{13}C assimilation and allocation increase with organic matter availability

Plant ^{13}C assimilation increased significantly with plant biomass, which increased with organic matter availability. In the HOM treatment, where plants were largest and mycelial biomass in the B horizon was 3.64 times greater than in the MOM treatment, allocation of ^{13}C by plants to the B horizon soil and soil solution increased significantly. Moreover, ^{13}C allocation was shown to be significantly positively correlated with mycelial biomass in the B horizon (Fig. 6), supporting our second hypothesis that larger plants allocate greater quantities of C to ECM fungi. The increased allocation of C to the B horizon suggests a plant-driven demand for base cations and P – nutrients most commonly derived from minerals via ECM-mediated mineral weathering, providing preliminary evidence for our third hypothesis that greater C allocation to ECM fungi would lead to an increase in base cation and P mobilisation from minerals. The observed C allocation to ECM fungi in the mineral B horizon is consistent with previous studies, where experimental microcosms inoculated with single fungal species demonstrated selective allocation of photosynthetically derived C through ECM fungal networks to mineral-dense areas (Rosling et al. 2004; Smits et al. 2012). This allocation was shown to enhance the weathering of apatite and promote subsequent P uptake by plants (Smits et al. 2012). Plant N use efficiency was also shown to be significantly positively correlated with ^{13}C allocation to O and B horizon soils and soil solutions (Fig. 5b and c, respectively), indicating a plant-driven demand for not only base cations and P, but also N.

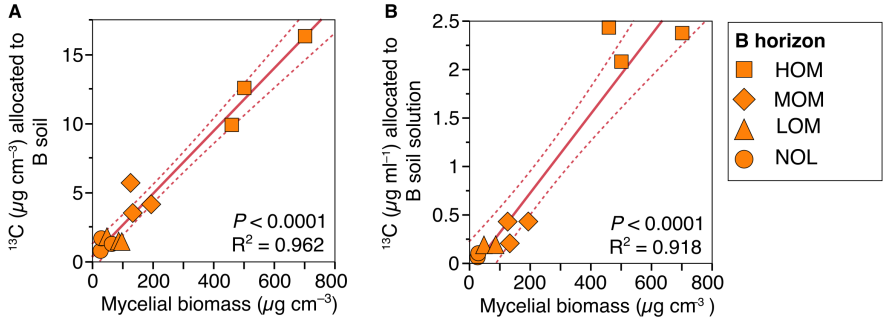

Figure 6. Correlations between mycelial biomass and ^{13}C allocation to B horizon soils (A) and soil solutions (B). Treatment is indicated by shape. (*Adapted from* ***Paper I***).

Plant base cation and P content correlate with mycelial biomass

Total base cation and P content in plants were significantly positively correlated with E (Fig. 7a and c, respectively) and B horizon mycelial biomass (Fig. 7b and d, respectively). Plant content of the individual base cations Ca, K and Mg was also shown to be significantly positively correlated with E and B horizon mycelial biomass (***Paper I***, Fig. S2), indicating that ECM fungi are weathering minerals and supplying plants with base cations and P, as seen in previous studies (Jentschke et al. 2000, 2001; Andrews et al. 2011; Adeleke et al. 2012). Furthermore, these results suggest that greater mycelial biomass in B horizon soils – proposed to result from greater C allocation by larger plants due to increased organic matter availability, leads to greater mineral weathering and subsequent mobilisation, uptake, and transfer of base cations and P to plants. The observed correlations, in conjunction with the earlier discussed significant correlation between ^{13}C allocation and mycelial biomass in the B horizon, offer robust evidence for our third hypothesis that increased C allocation to ECM fungi would enhance the mobilisation of base cations and P from mineral substrates.

Furthermore, the signature of δ^{26}Mg in B horizon soil solution became increasingly enriched with increasing organic matter, and this was significantly positively correlated with plant biomass (Fig. 7e), root biomass

(Fig. 7f), plant Mg content (Fig. 7g), and ^{13}C allocation by plants (Fig. 7h). The enrichment of $\delta^{26}Mg$ was also positively correlated with mycelial biomass in B horizon soils, though insignificantly (***Paper I****, Fig. 5c*). These results provide support for our fourth hypothesis that enhanced Mg uptake would lead to an enrichment of $\delta^{26}Mg$ signatures in soil solution, particularly in areas of high ECM abundance. These finding aligns with pure culture studies showing that ECM fungi, in contrast to saprotrophic fungi, are more efficient at mobilising Mg from granite particles (Fahad et al., 2016). The improved access to base cations and P, facilitated by greater mycelial colonisation of B horizon soil, may account for the improved N use efficiency observed in plants grown in treatments with greater organic matter availability.

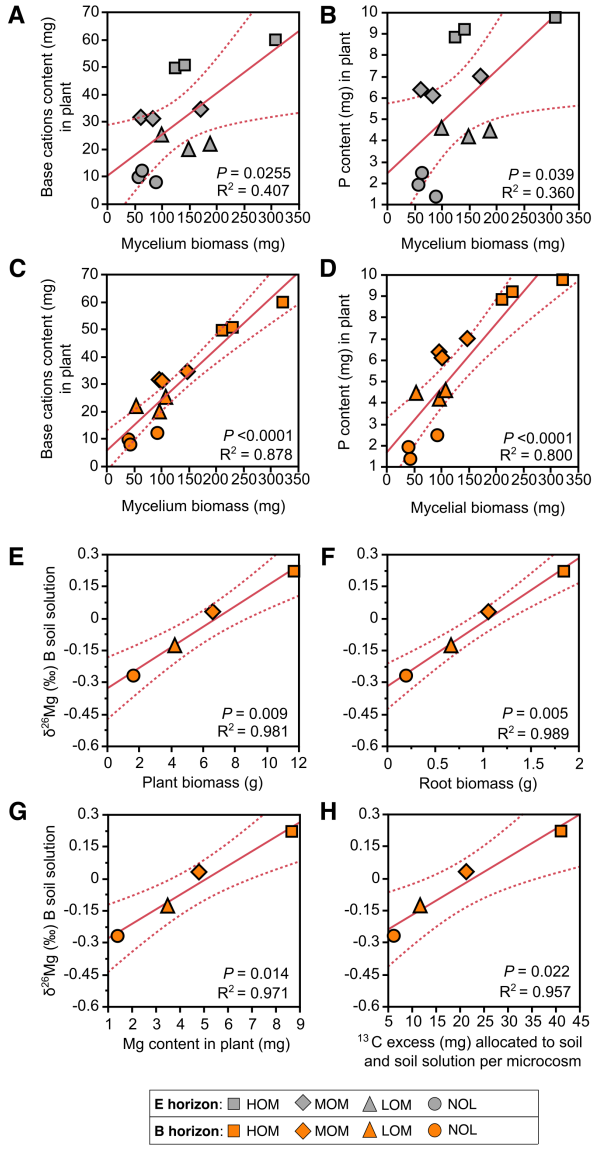

Figure 7. Correlations between: base cation content in plants and mycelial biomass in E horizon soils (A) and B horizon soils (C); P content in plants and mycelial biomass in E horizon soils (B) and B horizon soils (D); and δ^{26}Mg in B horizon soil solution and plant biomass (E), root biomass (F), plant Mg content (G), and ^{13}C allocation to soil and soil solutions. Treatment is indicated by shape, with the E horizon in grey and the B horizon in orange. (*Adapted from* ***Paper 1***).

Ectomycorrhizal abundance increases with plant biomass

Organic matter availability had a significant effect on fungal community composition and relative abundance in O and B horizon soils (***Paper I****, Fig. 6a*). The fungal community of the B horizon soil in particular, was strongly associated with plant content of Ca, K, Mg, N and P, likely due to the role of ECM fungi in mineral weathering in this horizon. Soil horizon also had a significant effect on fungal community composition (***Paper I****, Fig. S4a*), which has been demonstrated previously (Rosling et al. 2003; Marupakula et al. 2021). Fungal guilds were strongly influenced by organic matter availability. The ECM fungal guild was significantly positively correlated with greater plant biomass, which resulted from increased organic matter availability (***Paper I****, Fig. 6b*), and dominated all ingrowth mesh bags regardless of soil horizon or treatment, making up more than 80 % of total amplicons in almost all cases (***Paper I****, Fig. S6d-f*). The saprotrophic guild showed the opposite trend to ECM fungi, with a significant negative correlation with increasing plant biomass (***Paper I****, Fig. 6c*). This is likely due to the increased competitiveness of ECM fungi with increasing organic matter, and the subsequent increase in plant growth and C allocation, allowing them to colonise substrates more effectively. This interpretation is in keeping with the well-documented "Gadgil effect", which posits that ECM fungi compete with and suppress saprotrophic fungi in N-limited conditions, such as those found in boreal forests, leading to the accumulation of organic matter and increased C storage (Gadgil & Gadgil 1971).

This dominance of ECM fungi supports the assumption in all our hypotheses that ECM fungi play a central role in plant nutrition, contributing to N mobilisation, mineral weathering, and the transfer of essential nutrients to host plants. This assumption is further supported by previous findings, showing the significant capacity of ECM fungi to mobilise and take up N from organic matter (Wang et al. 2017; Tunlid et al. 2022), and weather minerals and mobilise and take up base cations (Hoffland et al. 2004; Finlay et al. 2009, 2020; Fomina et al. 2010). Moreover, a negative correlation was found between soil solution pH and ECM amplicon abundance in B horizon soils (***Paper I****, Fig. S8*), indicating an association between base cations and P uptake and acidification, which could result from LMWOA and proton

exudation by ECM fungi during mineral weathering, as shown in previous studies (Olsson & Wallander 1998; Wallander & Wickman 1999; van Hees et al. 2005; Adeleke et al. 2012).

The ECM fungi *Suillus bovinus* and *Piloderma sphaerosporum* dominated the soil horizons (Fig. 8) and ingrowth mesh bags, showcasing the prevalence of these two species. *Suillus bovinus*, dominating the mineral soils, and *P. sphaerosporum*, dominating the organic soil, are common in boreal forest soils (Rosling et al. 2003; Marupakula et al. 2021). A number of *Suillus* species have been shown to perform mineral weathering and have been observed to produce and exude greater quantities of LMWOAs in the presence of minerals (Olsson & Wallander 1998; Wallander & Wickman 1999; Adeleke et al. 2012), and take up base cations from mineral sources (Balogh-Brunstad *et al.*, 2008; Fahad *et al.*, 2016). *Piloderma* species have been shown to produce and exude proteases which can break down proteins and aid N mobilisation from organic sources, and transfer N to host plants (Heinonsalo et al. 2015). They have also been shown to mobilise base cation and P from mineral sources (Fahad et al. 2016). The high abundance of each of these taxa in their respective horizons suggests they may play a significant role in mineral weathering and base cation and P mobilisation, as well as organic matter degradation and N mobilisation, followed by the subsequent transfer of nutrients to host plants, although this requires experimental validation.

Though insignificant in all horizons apart from the E horizon, there was a trend of increasing *S. bovinus* abundance with increasing organic matter availability (Fig. 8), which may indicate increased C allocation to *S. bovinus* with increasing plant biomass, driven by plant nutrient demands for additional base cations and P. The opposite trend was seen in *P. sphaerosporum*, with abundance decreasing with increasing organic matter availability, perhaps indicating that *P. sphaerosporum* is highly competitive and can suppress other species when organic matter availability becomes sparse. Alternatively, this trend may suggest that plant demand for N is reduced with increasing organic matter availability, having already received sufficient N for growth, and in turn reduced C allocation to *P. sphaerosporum,* resulting in lower abundance. Under this interpretation,

the energetic expense of sustaining *P. sphaerosporum* may also have outweighed the benefits of N acquisition.

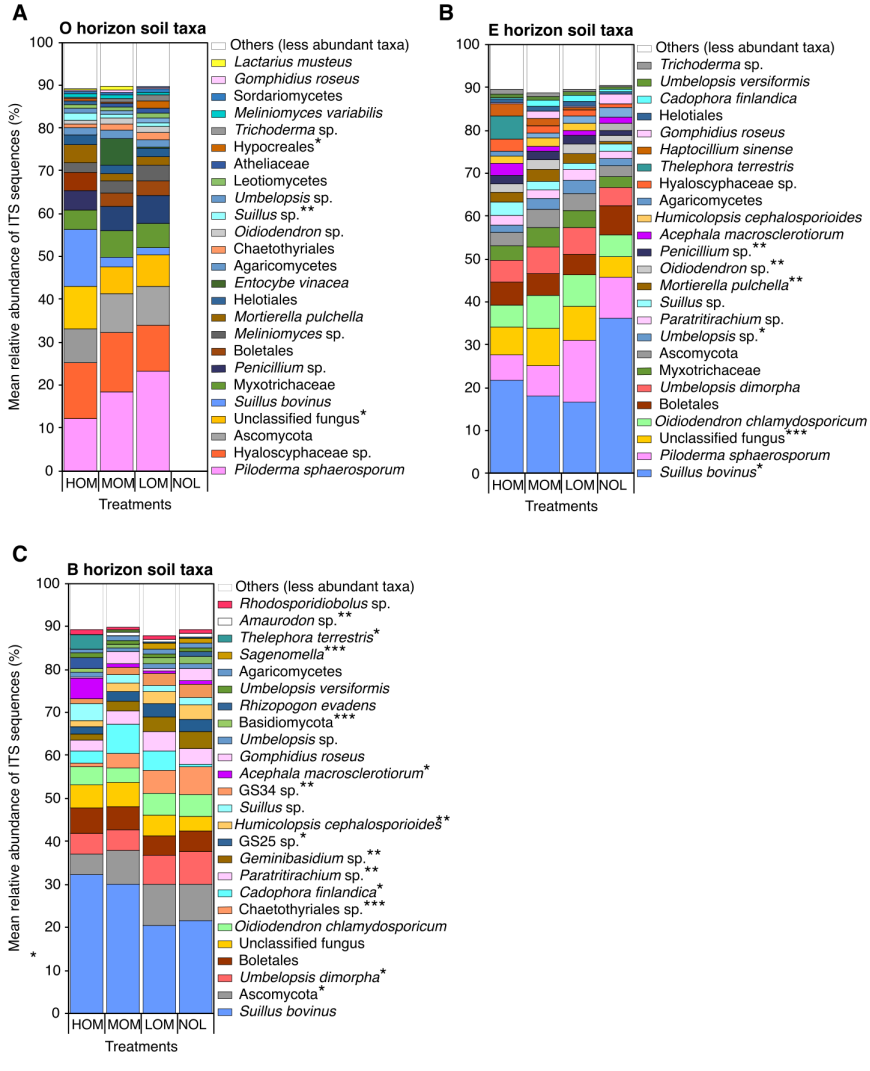

Figure 8. Histograms of the relative abundance of the 25 most abundant fungal taxa in O (A), E (B), and B (C), horizon soils. Asterisks indicate the significance of one-way analysis of variance of treatment differences for each fungal taxon (* = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$). (*Adapted from* ***Paper 1***).

4.5 Concluding remarks

Nitrogen mobilisation and mineral weathering are two key processes in boreal forest soils that are tightly interconnected and have strong implications for plant productivity, soil C dynamics including C sequestration and storage (Godbold et al. 2006), and climate change mitigation (Vicca et al. 2022). C sequestration and storage. In **Paper I**, we investigated how these two processes were integrated and how they were affected by differing organic matter availability. We found that increased organic matter availability led to increased N supply to plants, increasing their biomass and enabling them to allocate greater quantities of C belowground to fungal mycelia, soil and soil solution, particularly in the O and B horizons. Greater C allocation to fungal mycelia led to greater N mobilisation in the O horizon, and greater base cation and P mobilisation in the B horizon, followed by the subsequent transfer of nutrients to plants. The dominance of ECM fungi in fungal amplicons is an indicator of the fundamental role they play in boreal forest soil ecosystems, and strongly implicates them as the recipient of plant allocated C. Though in this study, it is not possible to confirm the roles of *P. sphaerosporum* in N mobilisation and *S. bovinus* in mineral weathering, their high abundance in the O and B horizons, respectively, is a strong indicator of their involvement. Further research is required to explore both the mechanisms and regulation of these processes, and the potential roles of *Suillus* and *Piloderma* species.

5. Genome evolution of nutrient uptake

Ectomycorrhizal fungi-mediated mobilisation of nutrients from soils requires the production and exudation of LMWOAs, protons, free radicals, siderophores, and enzymes, which contribute to the decomposition of organic matter and weathering of minerals. Once mobilised, these nutrients must be taken up by ECM fungi through specific transporter proteins (Fig. 9). The capacity for nutrient mobilisation and uptake is governed by the genetic composition of fungal genomes, as well as the regulation of transcriptional, post-transcriptional, translational and post-translational processes (Mukhopadhyay et al. 2024). Genetic composition and regulatory processes have evolved through random mutations and natural selection of traits that enhance the degradation of organic substrates, mineral weathering, and the uptake of key nutrients.

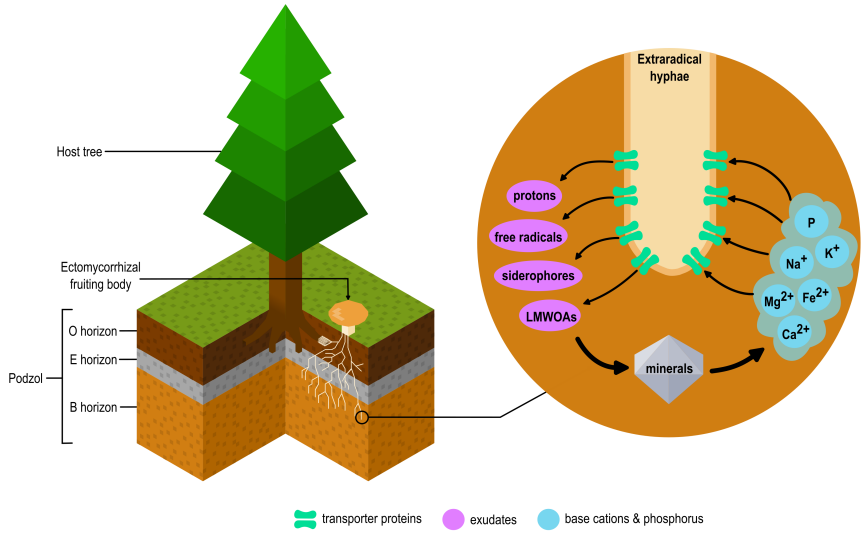

Figure 9. Overview of ECM-mediated mineral weathering in boreal forest podzols. ECM fungi, in symbiosis with host trees (left), exude LMWOAs, siderophores, free radicals and protons, through transporter proteins in their extraradical hyphae, to weather minerals and mobilise base cations and P (right), which are taken up through transporter proteins. (*Taken from* ***Paper II***).

5.1 The genomic evolution study: transporter gene family evolution and mineral weathering

One driver of genome evolution and environmental adaptation is changes to the number of copies of a gene (Steenwyk & Rokas 2017; Tralamazza et al. 2024). Genes involved in environmental adaptation, such as those involved in nutrient mobilisation and uptake, undergo frequent duplications and losses (Wapinski et al. 2007). Changes in gene copy numbers have been demonstrated to be instrumental in the emergence of the ECM lifestyle from saprotrophic ancestors, with extensive losses of plant cell wall-degrading enzymes and increases in symbiosis-induced secreted proteins (Kohler et al. 2015; Miyauchi et al. 2020). A similar pattern of gene duplication and loss can be expected in the evolution of nutrient mobilisation by ECM fungi.

This chapter explores the emergence of strong mineral weathering capabilities in ECM fungi and the evolution of base cation transporter gene families, which are involved in the uptake of nutrients mobilised from minerals. Particular emphasis is placed on the ECM genus *Suillus*, highlighted in **Paper I**, which has previously been shown to exude weathering agents such as LMWOAs (Olsson & Wallander 1998; Wallander & Wickman 1999; Adeleke et al. 2012), and take up base cations (Balogh-Brunstad *et al.*, 2008; Fahad *et al.*, 2016).

In **Paper II**, the evolution of mineral weathering capabilities in ECM fungi was investigated by analysing expansions (gene gain) and contractions (gene loss) of gene copy numbers in transporter gene families across a large phylogeny of Agaricomycetes species, with a particular focus on base cation transporter families and the ECM genus *Suillus*. Base cation transporter gene families were selected as a proxy for mineral weathering activity, as many mechanisms, such as LMWOA exudation, are ubiquitous across multiple processes and not specific to mineral weathering. To complement genome analyses, mycelial base cation uptake from minerals was quantified in the ECM genera *Suillus* and *Piloderma*, alongside two saprotrophic species. Correlations between base cation uptake and base cation transporter gene family copy numbers were then assessed to explore potential mechanistic links between genomic evolution and mineral weathering capacity.

5.2 Hypotheses

In **Paper II**, we hypothesised that greater base cation uptake is dependent on base cation transporter gene family copy numbers that result from evolutionary expansions. Furthermore, we hypothesised that mineral weathering results in base cation uptake by members of the genus *Suillus* and that base cation uptake from minerals by *Suillus* species will be greater compared to other fungal species.

5.3 Methodology

Gene family evolution

To test the hypothesis that greater base cation uptake is linked to evolutionary expansions in base cation transporter gene families, the evolution of transporter gene families was first examined across a large phylogeny of 108 Agaricomycotina taxa. First, a phylogenomic tree was constructed using OrthoMCL v2.0.9 (Chen et al. 2006) and IQ-TREE v2 (Nguyen et al. 2015), and then time-calibrated with r8s v1.8.1 (Sanderson 2003). The resulting ultrametric tree was used as input for CAFE5 v5.1 (Mendes et al. 2020) – a tool which analyses copy number changes in gene families while accounting for phylogenetic relationships and enabling statistically robust inference of gene family evolution. Copy number data of 173 transporter gene families present in the 108 Agaricomycotina taxa, annotated with the transporter classification database (TCDB) system (Saier 2006; Saier et al. 2009, 2014, 2016, 2021) and taken from JGI MycoCosm (https://mycocosm.jgi.doe.gov/mycocosm/annotations/browser/tcdb/summary;FdfSB_?p=agaricomycotina), was also used as input for CAFE5.

Three datasets were established for CAFE5 analyses. The first, termed the "full dataset", used the 108 taxa phylogenomic tree and scaled copy number values (0-80) of all 173 transporter gene families, to accommodate limitations of CAFE5. The second, termed the "partial family dataset", was run with a subset of 28 taxa from the full phylogeny and used actual (unscaled) copy numbers of the 126 transporter gene families present within these taxa. This subset included members of the genera *Suillus* and *Piloderma*, and the saprotrophic species *Coniophora puteana* and *Serpula lacrymans*, facilitating observations in key taxa of interest and accounting for any error introduced by scaling. The third, termed the "partial subfamily dataset", again used the 28 taxa phylogeny and used actual copy numbers of 51 transporter gene subfamilies, enabling a finer-scale analysis within particularly large transporter gene families. The resulting trees from each of these datasets were visualised with CafePlotter (https://github.com/moshi4/CafePlotter), and expansions and contractions

were visualised by heatmaps using the 'Tidyverse' suite v2.0.0 (Wickham et al. 2019) in R v4.3.2 (R Core Team 2023).

The evolution of the Mg^{2+} transporter-E (MgtE) gene family was investigated in greater detail to elucidate whether there had been an expansion of one homologous gene or several of different origins contributing to the observed high copy numbers. A phylogenetic tree of protein sequences of all MgtE family genes present in the 28 taxa of the partial family dataset was constructed. Sequences were extracted from proteomes using SAMTOOLS (Danecek et al. 2021), and the tree was reconstructed using the methods described above and visualised in R using ggtree (Yu et al. 2017; Wang et al. 2020; Yu 2020).

Base cation uptake from minerals

To test the hypothesis that base cation uptake from minerals by *Suillus* species will be greater compared to other fungal species, twenty-three isolates – comprising 14 *Suillus*, six *Piloderma* and two saprotrophic isolates – were grown in pure culture in the presence and absence of minerals. Gabbro and granite sands were used as mineral sources. Four treatments were established as shown in figure 10: a limited treatment (providing only C and N), a rich treatment (providing all essential nutrients in readily available forms), and two mineral treatments – gabbro (limited medium supplemented with 20 g/L of gabbro sand) and granite (limited medium supplemented with 20 g/L of granite sand) (*see* ***Paper II****, section 2.2.2 for details*). Isolates were incubated in continuous darkness for nine, five or three weeks, depending on the species (*Suillus* and *Piloderma*, and *C. puteana* and *S. lacrymans*, respectively).

Following incubation, biomass was harvested, washed, dried, pooled to obtain sufficient material for analyses, and milled into a fine powder. Pooled samples were subjected to C and N analysis (Mettler MT5 Microbalance, and Thermo Finnigan FlashEA 1112 Elemental Analyser), nitric acid microwave digestion (PerkinElmer Titan MPS) and Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-EOS) analysis (PerkinElmer AVIO 500 ICP-

OES) to measure C, N, Ca, Fe, K, Mg, Na and P concentrations in isolate biomass.

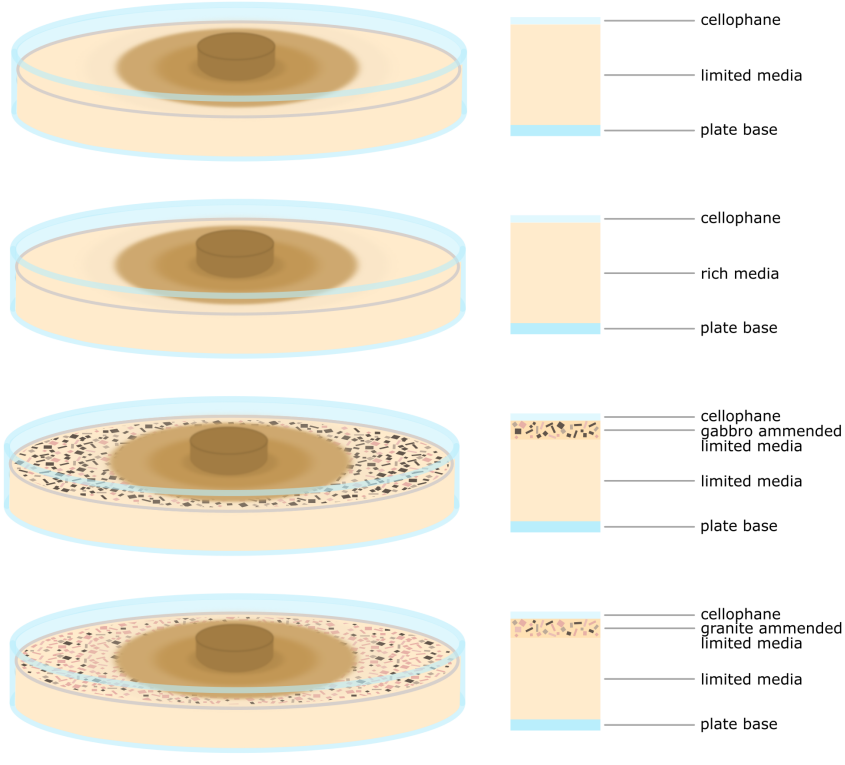

Figure 10. Schematic illustration of plate design for the base cation uptake experiment. From top to bottom: limited treatment – with one layer of limited media, rich treatment – with one layer of rich media, gabbro treatment – with a base layer of limited media and a thin upper layer of limited media amended with gabbro sand, and granite treatment – with a base layer of limited media and a thin upper layer of limited media amended with gabbro sand. All plates were covered with a layer of sterile cellophane to aid harvesting of fungal mycelium. Fungal plugs were placed on top of cellophane and allowed to grow.

Statistical analyses were performed in R v4.3.2 (R Core Team 2023). Linear models were fitted for Ca, Fe, K, Mg, Na and P concentrations, to evaluate the effects of isolate, treatment, and their interaction on elemental uptake. Post hoc comparisons were carried out using the 'emmeans' v1.10.4 package (Lenth 2024), with Tukey's HSD tests applied to identify significant pairwise differences. Compact letter displays were generated using 'multcomp' v1.4-26 (Hothorn et al. 2008) to summarise groupings. Mineral weathering was defined as a significantly higher element concentration in biomass grown in gabbro and/or granite treatments compared to the limited treatment.

To aid isolate-to-isolate comparisons, linear models were also fitted to estimate the ratios of Ca, Fe, K, Mg, Na and P concentrations in the gabbro and granite treatments relative to the limited treatment, with isolate and treatment as explanatory variables. Ratios were calculated with 'emmeans' v1.10.4 (Lenth 2024), and based on the mean concentration of each element, per isolate per treatment. Differences were considered significant when 95 % confidence intervals did not overlap.

Base cation uptake and gene family copy numbers

To further test the hypothesis that greater base cation uptake is dependent on higher copy numbers of base cation transporter gene families, correlations were performed between base cation uptake by isolates and gene copy numbers of relevant base cation transporter gene families. Only gene families identified as significantly expanding or contracting in the CAFE5 analysis and functionally linked to the transport of Ca, Fe, K, Mg, Na, or P (based on TCDB annotations) were included. Correlations were conducted on a per-element basis, meaning gene families were only tested against the corresponding element. Species lacking copy number data were excluded from the analysis.

5.4 Results and Discussion

The co-evolution of fungi and plants, and the emergence of the plant-fungal symbiosis, was essential in the colonisation of terrestrial habitats (Pirozynski & Malloch 1975; Humphreys et al. 2010; Bidartondo et al. 2011). Ectomycorrhizal fungi have evolved from saprotrophic ancestors across multiple lineages, frequently losing genes associated with plant decay mechanisms as they transitioned to a symbiotic lifestyle (Kohler et al. 2015; Miyauchi et al. 2020). In exchange for plant-allocated photosynthetically derived C, ECM fungi supply N, P, and base cations in intimate symbiotic relationships. Ectomycorrhizal-mediated mineral weathering and P and base cation uptake are, in part, driven by plant nutrient requirements (Finlay et al. 2020). Adaptation to these requirements is likely the result of evolutionary changes in ECM genomes, which support mineral weathering and nutrient uptake. Genes associated with ecological adaptations in variable environments and stress have been shown to exhibit greater copy number variation than those associated with cellular maintenance and growth (Wapinski et al. 2007). Transporter genes, which are critical in abiotic and biotic environmental interactions, are likely to undergo frequent expansions and contractions, facilitating swift adaptation to environmental conditions and plant nutrient requirements.

Rapid base cation transporter gene family evolution in Suillus *species*

A Principal Component Analysis (PCA) based on raw copy numbers of individual transporter gene families in 108 Agaricomycotina genomes revealed distinct clustering of the Suillaceae and Russulaceae families (Fig. 11a). Base cation transporter gene families comprise 18 of the 30 most influential transporter gene families (Fig. 11b), indicating that these base cation transporter gene families are integral to the ecological strategies and environmental adaptations of these two groups.

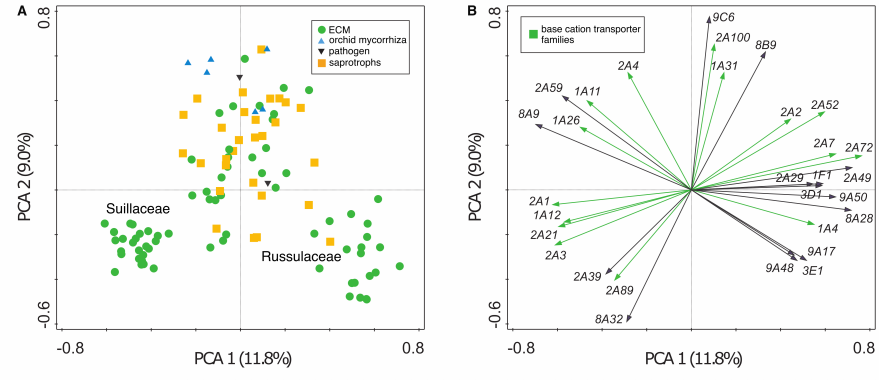

Figure 11. Variation in transporter gene family composition in 108 Agaricomycotina taxa, based on Principal Component Analysis of copy numbers of 173 transporter gene families and visualised by (a) sample and (b) species plots showing the 30 transporter gene families (with TCDB ID) with the largest contribution to the ordination of taxa. Lifestyle (a) is coded by shape and colour of sample points, and in (b) base cation transporter families are indicated in green. The first two axes together explained 20.8 % of the total variation. (*Taken from* ***Paper II***).

Our analyses confirmed the presence of significant expansions and contractions in base cation transporter gene families. Significant expansions and contractions were observed in 13, 22, and 15 transporter gene families in the full, partial, and partial subfamily datasets, respectively. Despite differences in the number of significant transporter gene families, general trends across all datasets were consistent. Differences are likely due to the scaling of gene copy numbers in the full dataset, and the difference in the number of taxa between the full and the partial and partial subfamily datasets. Notably, all of these transporter gene families exhibited significant changes in the genus *Suillus*, highlighting their rapid evolution within this genus.

Of these transporter gene families, approximately half are involved in base cation transport (Fig. 12). The observed expansions and contractions may be an indicator of the potential importance of base cation transport in adaptative strategies to stress and the environment, as genes with varying copy number are more likely to have roles in adaptation (Wapinski et al. 2007). Furthermore, expansions and contractions in base cation transporter gene families may be associated with base cation uptake demand, and their prevalence in taxa such as the genus *Suillus* could indicate mineral

weathering proficiency. In addition to the significant expansions and contractions identified in base cation transporter gene families, we found 28 significant correlations between copy numbers of some of these families and uptake of the base cations or P that they transport. These results support our first hypothesis that greater base cation uptake is driven by evolutionary expansions of copy numbers in base cation transporter gene families.

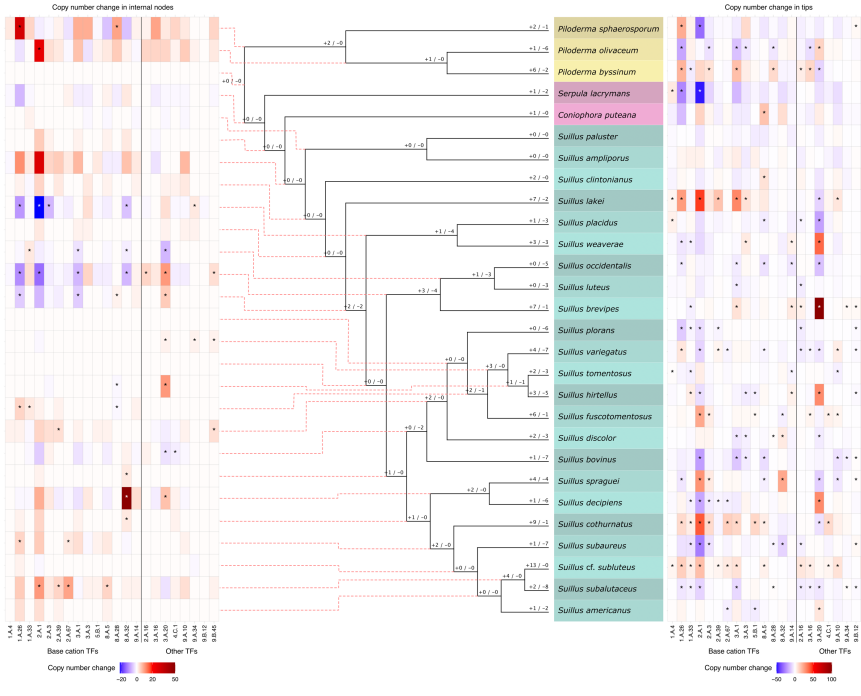

Figure 12. Summary of copy number changes in significantly expanding and contracting transporter families (TFs) across the partial family dataset. At each node of the phylogenomic tree (not to scale), the number of expanding (+) and contracting (-) TFs is indicated. Heatmaps show gene copy number changes of significantly expanding and contracting TFs, with contractions in blue and expansions in red at internal nodes (left heatmap) and tips (right heatmap). Significant expansions and contractions are indicated with an asterisk (*). Copy number changes range from -22 to +49 at internal nodes and -48 to +101 at tips. For both heatmaps, base cation TFs are positioned to the left and other TFs are positioned to the right. (*Taken from* ***Paper II***).

Mg^{2+} transporter-E gene family evolution

Magnesium is a crucial component in photosynthesis (Black et al. 2007) and enzyme function (Bose et al. 2011), and can be mobilised and taken up by ECM fungi from mineral substrates (Fahad *et al.*, 2016; ***Paper I*** - Mahmood *et al.*, 2024). The Mg^{2+} transporter-E (MgtE) gene family (TCDB ID: 1.A.26), which had significantly higher copy numbers in the Agaricomycotina compared to most other transporter gene families (*see* ***Paper II****, section 3.1.2*), was found to have a high number significant expansions and contractions in the full and partial family datasets, compared to the majority of other transporter gene families (Fig. 12 & ***Paper II****, Fig. S3*). MgtE genes have been shown to facilitate the influx of Mg^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, Fe and Cu (Smith et al. 1995; Goytain & Quamme 2005), and are involved in Mg^{2+} homeostasis (Hattori et al. 2009; Conn et al. 2011; Hermans et al. 2013; Franken et al. 2022). Many organisms possess multiple copies of a single type of Mg^{2+} transporter gene family, which may account for the low abundance of other Mg^{2+} transporter gene families in our datasets (Franken et al. 2022). In plants, multiple copies of the Mg^{2+} transporting CorA/Mrs2 metal ion transporter (MIT) gene family have been identified, with no functional redundancy identified (Gebert et al. 2009; Schmitz et al. 2013).

The phylogeny of protein sequences of MgtE family genes showed that several clades of MgtE genes were present in each taxon, indicating that duplications occurred across multiple different transporter genes of origin (Fig. 13). This, compared to the expansion of one homologous gene, suggests more rapid evolution. Although in some cases, gene duplications can result in dysfunctional or redundant genes, duplications can also result in greater genetic diversity between genes (Fujita et al. 2007). Diversification of gene function resulting from gene duplications may lead to certain genes exhibiting greater functionality in particular fungal tissues, at particular life stages, and under particular environmental conditions (Copley 2020). This may explain the high copy numbers of MgtE transporter family genes found. To make definitive conclusions, however, functional validation of MgtE genes under different conditions is required.

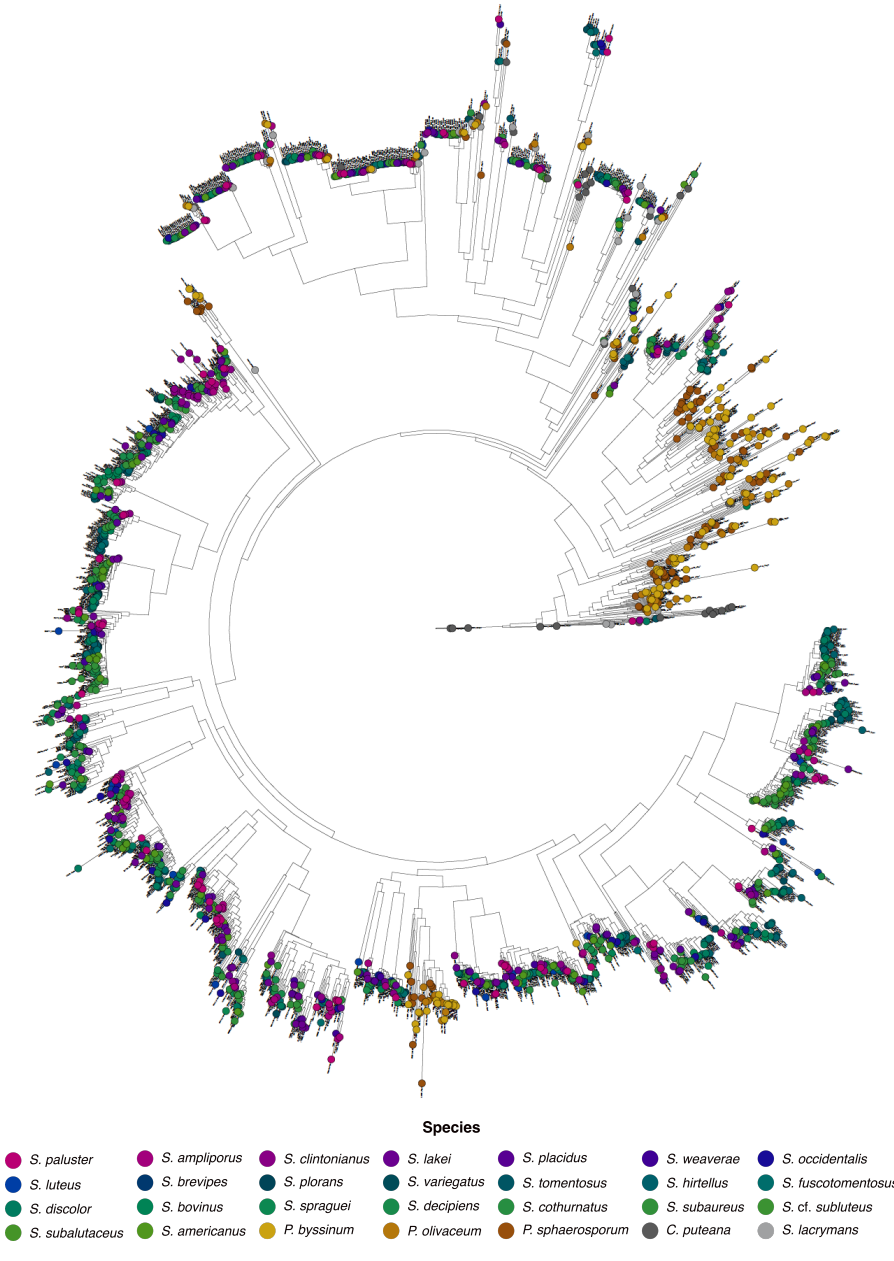

Figure 13. Phylogenomic tree based on protein sequences of Mg^{2+} transporter-E family genes from 28 taxa from the partial family dataset (*Suillus* and *Piloderma* species, and the saprotrophic fungi *Coniophora puteana* and *Serpula lacrymans*). Genes from each taxon are marked with a coloured dot. (*Adapted from* ***Paper II***).

Mg^{2+} transporter-E gene family copy numbers correlate with Mg uptake

Within the genus *Suillus*, significant expansions in the MgtE gene family were found in four taxa and two ancestral nodes. The MgtE family was also found to be significantly expanding in the genus *Piloderma*, in two taxa and the ancestral node for the *Piloderma* clade. Magnesium uptake by fungal mycelia, including 14 *Suillus* and six *Piloderma* isolates, was significantly positively correlated with copy numbers of the MgtE transporter gene family in the gabbro, granite and limited treatments, and a non-significant positive correlation was found in the rich treatment (Fig. 14), further supporting our first hypothesis, and our second hypothesis that mineral weathering results in base cation uptake by members of the genus *Suillus*.

One isolate of *S. variegatus*, in which the MgtE gene family was significantly expanding, was found to have significantly higher mycelial Mg concentrations in gabbro treatments compared to granite and limited treatments (*see* ***Paper II****, Fig. S6d*). The weathering capabilities of *S. variegatus* have been demonstrated in a number of studies, including its ability to take up K^+ from granite particles (Fahad et al. 2016), produce high levels of organic acids in the presence of minerals (Olsson & Wallander 1998; Wallander & Wickman 1999), and display enhanced weathering activity in symbiosis with *P. sylvestris* compared to non-mycorrhizal plants (Wallander & Wickman 1999). Taken together, these findings further support our first and second hypotheses that greater base cation uptake is dependent on expansions in base cation transporter gene families, and that base cation uptake results from mineral weathering by *Suillus* species, respectively.

Although no significant expansions of the MgtE gene family were found in *S. bovinus*, all four isolates were shown to have significantly higher mycelial Mg concentrations in gabbro treatments compared to granite and limited treatments (*see* ***Paper II****, Fig. S6d*), and two of these isolates were found to have significantly higher ratios of Mg in the gabbro relative to the limited treatment, compared to almost all other fungal isolates (*see* ***Paper II****, Fig. S7c*). This finding offers additional support to our second hypothesis, and supports our third hypothesis that base cation uptake from minerals by

Suillus species will be greater compared to other fungal species. It also aligns with our previous findings in **Paper I**, where significant δ^{26}Mg enrichment (indicating Mg^{2+} uptake) was identified in the soil solution of the B horizon – where *S. bovinus* was the most abundant species.

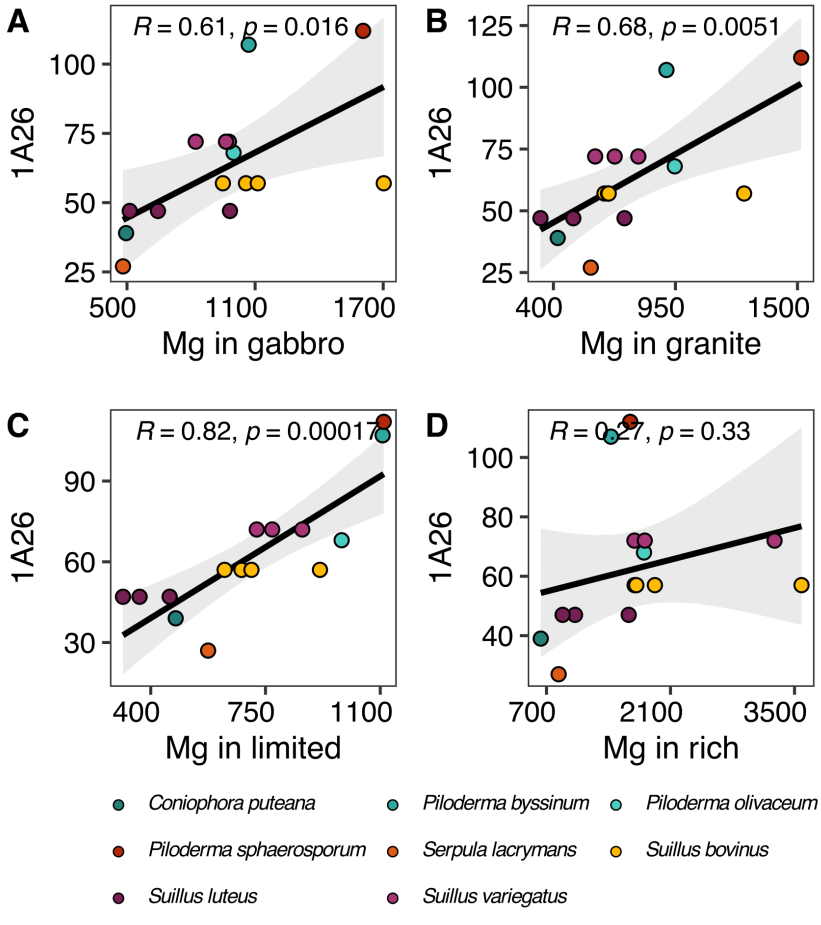

Figure 14. Significant positive correlations between estimated mean Mg mycelial content in species and gene copy numbers of the Mg^{2+} transporter-E gene family in gabbro, granite, and limited treatments. A non-significant positive correlation is present in the rich treatment. Species are indicated with coloured dots. (*Adapted from* ***Paper II***).

One *S. luteus* isolate, which similarly had no significant expansions of the MgtE gene family, was also found to have significantly higher mycelial Mg concentrations in gabbro and granite treatments compared to limited treatments (*see* ***Paper II****, Fig. S6d*). The same *S. luteus* isolate was found to have significantly higher ratios of Mg in the gabbro relative to the limited treatment, compared to almost all other fungal isolates (*see* ***Paper II****, Fig. S7c*), supporting our second and third hypotheses. Certain populations of *S. luteus* exhibit tolerance to the heavy metals zinc and cadmium, both of which are cations (Colpaert et al. 2011; Ruytinx et al. 2011, 2017, 2019; Bazzicalupo et al. 2020). These adaptations were attributed to single-nucleotide polymorphisms and gene copy number variation in genes involved in heavy metal homeostasis, including metal ion transporter genes (Bazzicalupo et al. 2020). These findings are in line with our first hypothesis and indicate that environmental adaptations, such as greater base cation uptake, can occur through gene family expansions (Bazzicalupo *et al.*, 2020).

Interestingly, *P. sphaerosporum* was found to have significantly higher mycelial Mg concentrations in gabbro and granite treatments compared to limited treatments, and *P.* aff. *fallax* was found to have significantly higher mycelial Mg concentrations in the gabbro treatment compared to the granite and limited treatments (*see* ***Paper II****, Fig. S6d*). Two of the three *Piloderma* species included in our analysis of evolutionary expansions and contractions of gene family copy numbers, *P. sphaerosporum* and *P. byssinum* (which had poor growth in the base cation uptake experiment), were found to have expansions in the MgtE gene family (Fig. 12). The similar performance of *Piloderma* in comparison to *Suillus* species suggests, in slight contrast to our third hypothesis that *Suillus* species would take up more base cations than other species, that *Piloderma* species may also exhibit mineral weathering capabilities resulting in base cation uptake.

Higher Ca and Fe uptake in Suillus *species compared to other fungi*

Suillus bovinus possessed no expansions in any base cation transporter families; however, all isolates had significantly higher mycelial concentrations of not only Mg, but also Ca and Fe in the gabbro treatment compared to the limited treatment. Among the isolates, Sb1 stood out with significantly higher mycelial concentrations of all measured base cations in the gabbro treatment compared to the limited treatment. *Suillus luteus*, which also had no significant expansions in any base cation transporter families, and *S. variegatus* both had significantly higher Ca and Fe mycelial concentrations in the gabbro treatment compared to the limited treatment (*see* ***Paper II****, Fig. 4*). Taken together, these findings provide additional support to our second hypothesis that mineral weathering results in base cation uptake in *Suillus* species.

When comparing ratios of Ca mycelial concentrations in gabbro treatments compared to limited treatments, *S. bovinus*, *S. variegatus*, and *S. luteus* took up significantly more Ca than almost all *Piloderma* species, excluding *P. sphaerosporum* (*see* ***Paper II****, Fig. S6*), lending support to our third hypothesis that base cation uptake resulting from weathering of minerals by *Suillus* species will be greater than that of other fungal species. However, when considering other base cations, including Mg, results were mixed, with *Piloderma* species sometimes performing equally well as *Suillus* species. This inconsistency with our third hypothesis adds to the suggestion that *Piloderma* species are capable of weathering minerals and mobilising base cations for uptake.

5.5 Concluding remarks

Suillus species, which typically inhabit B horizon soil in boreal forests, are proficient at mineral weathering and base cation mobilisation and uptake from mineral substrates. In **Paper II**, we investigated evolutionary expansions in base cation transporter gene families and whether these expansions were a driver of greater base cation uptake in some ECM species, like *Suillus*. These gene families were among the most abundant significant families and were shown to be rapidly evolving in the *Suillus* genus. This suggests that the observed expansions and contractions in these transporter gene families are likely an adaptation to the requirement of base cation uptake and plant nutrient demand, supported by our finding that mycelial uptake of some base cations in the presence of gabbro and granite – two base cation rich rocks, increased with the copy number of some base cation transporter gene families, in particular, the MgtE gene family. In slight contrast to our *Suillus*-centric hypotheses, notable expansions and enhanced mycelial uptake of base cations were also observed in several *Piloderma* species. This highlights the need for future controlled experiments that examine mineral weathering across a broader range of species. Moreover, further research into other mechanisms of base cation uptake regulation, such as transcriptional regulation of transporter proteins, in *Suillus* and other ECM fungi could shed light on the underlying mechanisms driving mineral weathering and base cation mobilisation.

6. Genes involved in nutrient mobilisation

Plant and ECM fungal nutrient demands are strong drivers of mineral weathering and N mobilisation in boreal forests, as demonstrated in **Paper I**. The production and exudation of LMWOAs, protons, free radicals, siderophores, and enzymes, as well as the uptake of N, P, and base cations, must be tightly regulated in accordance with ECM fungal and plant nutritional requirements, recalcitrance of substrates and other biotic and abiotic factors. Although the mechanistic regulation of these processes is poorly understood, they are likely to be regulated by not only genetic composition and gene copy number, as was indicated in **Paper II**, but also at the transcriptional level (Mukhopadhyay et al. 2024).

6.1 The transcriptomic studies: gene expression in mineral weathering and LMWOA production

Transcriptional regulation is essential for maintaining the functionality of living organisms and their ability to adapt to environmental conditions (Mukhopadhyay et al. 2024). The capacity of an ECM fungal species to perform mineral weathering and N mobilisation is crucial for its competition with other ECM fungi in securing photosynthetically derived C from its plant host. As such, genes involved in mineral weathering and N mobilisation are expected to be up- or down-regulated to meet the nutritional demands of both plants and ECM fungi, as well as to respond to changing environmental and nutritional conditions.

Studies on the transcriptional regulation of mineral weathering in ECM fungi remain limited; however, upregulation of K^+ transporter genes during mineral weathering has been observed in *Hebeloma cylindrosporum* (Corratgé et al. 2007; Garcia & Zimmermann 2014), *Amanita pantherina* (Sun et al. 2019), and *Paxillus involutus* (Pinzari et al. 2022, 2024). Similar studies of transcriptional regulation of LMWOA production in response to differing N sources by ECM fungi are also scarce, however transcription of

a range of N transporter proteins and enzymes has been shown in an ECM metatranscriptome study (Pérez et al. 2022), and a substantial number of genes encoding enzymes, transporters, and relevant secondary metabolites have also been identified (Casieri et al. 2013; Nehls & Plassard 2018). To improve our understanding of C and nutrient dynamics in boreal forest soils, further research is needed to elucidate which genes are involved in the production of key components of mineral weathering and nutrient mobilisation and how they are regulated – including those involved in LMWOA production and exudation, and genes encoding enzymes, siderophores, and nutrient transporter proteins that have yet to be investigated.

This chapter explores the transcriptional regulation of two key components of mineral weathering and nutrient mobilisation: base cation transporter genes during mineral weathering and genes involved in the production of LMWOAs in response to differing N sources. Based on the findings of **Paper I** and **Paper II**, as well as those of previous studies (Shah et al. 2016; Finlay et al. 2020), the ECM fungi *Suillus bovinus* and *Piloderma fallax* were utilised in the mineral weathering and LMWOA production experiments, respectively.

In **Paper III**, the transcriptional response of fungal base cation transporter genes to growth with and without minerals was assessed to better understand the regulation of base cation uptake. Two *S. bovinus* isolates, selected based on their base cation uptake capacity in **Paper II**, were grown in pure culture and in symbiosis with *P. sylvestris* seedlings in Petri dish microcosms. Differentially expressed genes (DEGs) were related to expanding (genes gained) base cation transporter gene families identified in **Paper II**, to elucidate mechanisms of regulation during mineral weathering.

In **Paper IV**, the transcriptional response of fungal genes in extraradical mycelia and ECM root tips related to LMWOA exudation to growth with organic and inorganic N was investigated to better understand how C release in the ECM symbiosis is affected by different N sources. To achieve this, *P. fallax* was grown in symbiosis with *P. sylvestris* seedlings in a Petri dish microcosm. Additionally, exuded LMWOAs were characterised and related to DEGs associated with LMWOA exudation by network analysis.

6.2 Hypotheses

In **Paper III**, we hypothesised that base cation transporter genes would be up-regulated in treatments with minerals compared to those without minerals, and that these genes would exhibit greater up-regulation in symbiotic plant-fungal systems compared to pure culture systems, as plants act as a sink for base cations. We further hypothesised that there would be intraspecific variation among isolates, and that the transcriptional regulation of base cation transporter genes would either work together or independently of observed evolutionary expansions of gene copy numbers of base cation transporter families.

Paper IV was an exploratory study, with the aim of gathering information about the response of C release to organic and inorganic N sources. The transcriptional regulation of genes and pathways putatively relevant to the production of LMWOAs, including those involved in the Tricarboxylic Acid (TCA) cycle and the glyoxylate pathway, was related to exudation, and we expected to identify differential expression between the organic and inorganic N treatments.

6.3 Methodology

Petri dish microcosms

In **Paper III**, to test the hypothesis that base cation transporter genes would be up-regulated in the presence of minerals, two treatments – gabbro and limited – were prepared, similar to the base cation uptake experiment in **Paper II**. To assess the hypothesis that base cation transporter genes would be up-regulated in *S. bovinus* in symbiosis with *P. sylvestris* seedlings compared to *S. bovinus* grown in pure culture, two experimental systems incorporating the two treatments were established, as shown in Figure 15a and b. For the pure culture systems (termed plant–), *S. bovinus* was pre-grown for six weeks before being transferred to 9 cm gabbro or limited treatment plates. For symbiotic systems (termed plant+), synthesised ECM seedlings were pre-grown in a peat-vermiculite substrate (*see* ***Paper III****, materials and methods for details*) for three weeks before transfer to 15 cm gabbro or limited treatment plates. In both experimental systems, mycelial biomass was destructively harvested and snap-frozen in liquid N for RNA extraction at three timepoints – 10, 15, and 20 days.

To test the hypothesis that there would be intraspecific variation in base cation transporter gene response to mineral treatment, the *S. bovinus* isolates Sb16 (*isolate Sb2 in* ***Paper II***) and Sb4 (*isolate Sb1 in* ***Paper II***) were used. Isolate Sb16 was used for all timepoints in both the pure culture and the symbiotic systems, and isolate Sb4 was used for timepoint two in the symbiotic system only.

In **Paper IV**, to explore the response of C release, patterns of LMWOA exudation, and genes related to these processes to different N sources, an organic and an inorganic treatment were established. The amino acid L-alanine was used for the organic treatment, and ammonium chloride (NH_4Cl) was used for the inorganic treatment. To facilitate the harvesting of ECM exudates, glass beads and liquid nutrient solution were used, as shown in Figure 15a and c. *Piloderma fallax* synthesised *P. sylvestris* seedlings were pre-grown for five weeks, after which nutrient solutions were removed and replaced with nutrient solutions containing either L-alanine or NH_4Cl (*see*

***Paper IV**, materials and methods for details*). Ectomycorrhizal seedlings were grown in treatments for seven days, after which mycelial biomass and ECM root tips were harvested and snap-frozen in liquid N, and nutrient solutions containing exudates were collected and frozen at -20 °C.

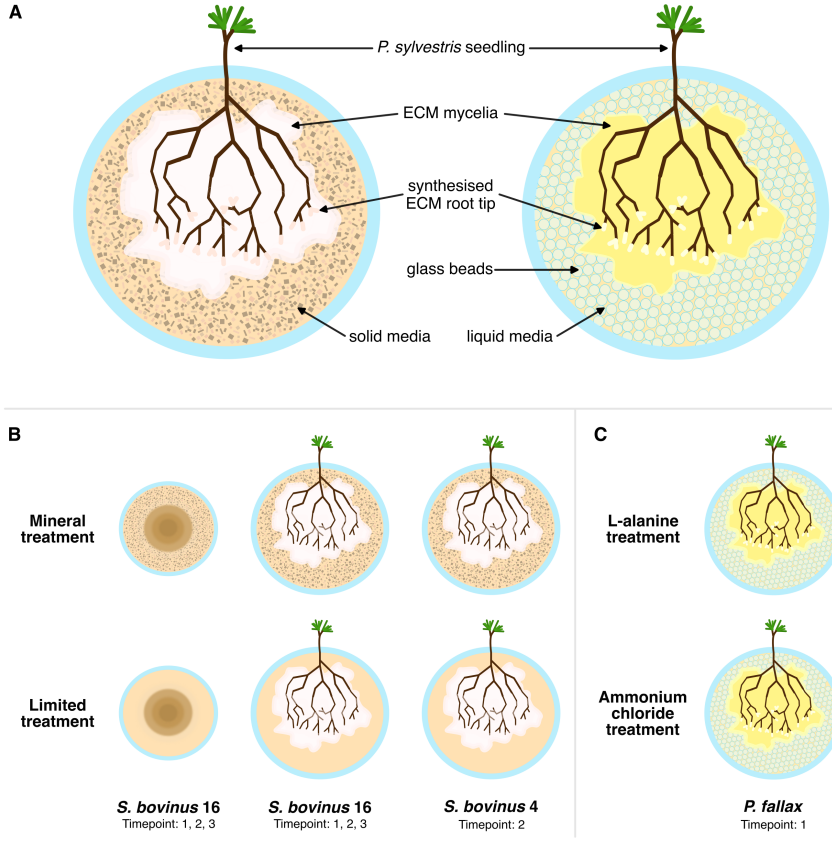

Figure 15. Schematic illustration of plate design for the mineral weathering (left) and LMWOA production (right) experiments. (A) shows the overall design of plant-fungus plates for each experiment, highlighting the common components: seedling, ECM mycelia, synthesised ECM root tips; and the separate components of solid media in the mineral weathering experiment (left) and glass beads and liquid media in the LMWOA production experiment (right). (B) provides details of the treatments, timepoints and isolates used in the mineral weathering experiment, and (C) shows the same details for the LMWOA production experiment.

Differential gene expression and enrichment analysis

In **Paper III**, differential gene expression analyses were performed to test the hypotheses that base cation transporter genes would be up-regulated in response to mineral treatment, that these genes would be more up-regulated in *S. bovinus* in symbiosis with *P. sylvestris*, and to assess intraspecific transcriptional response between isolates. Following RNA extraction and sequencing (*see **Paper III**, materials and methods for details*), raw RNA-seq data processing, quality control, alignment, and mapping were carried out using the nfcore/rnaseq v3.18.0 pipeline (Patel et al. 2025) within the nf-core framework (Ewels et al. 2020), and implemented using Nextflow v24.10.4 (Di Tommaso et al. 2017). Reads were aligned to the *S. bovinus* reference genome (Suibov1) (Lofgren et al. 2021). Further quality control and normalisation were performed in R using the DESeq2 v1.44.0 package (Love et al. 2014). Differential gene expression was performed using DESeq2 by contrasting each gabbro treatment with a specific plant+/– treatment, isolate and timepoint with its corresponding limited treatment, with mineral treatment as an explanatory factor. Resulting DEGs would therefore be considered up- or down-regulated in the gabbro treatment. Differentially expressed genes (DEGs) were identified with an absolute log2 fold change (logFC) > 1 and an FDR-adjusted p-value (FDR) < 0.05. Global gene expression was visualised by PCA, and significant treatment differences were tested using the Multi-Response Permutation Procedure (MRPP) with Vegan v2.7-1 (Oksanen et al. 2025). To aid interpretation, DEGs were annotated with Gene Ontology (GO) terms and analysis of enriched GO terms was performed using clusterProfiler v4.14.6 (Xu et al. 2024). All visualisations were created using the 'Tidyverse' suite v2.0.0 (Wickham et al. 2019).

To explore the transcriptional response of genes related to LMWOA production and exudation in *P. fallax*, differential gene expression analysis was also performed in **Paper IV**. After RNA extraction and sequencing, raw RNA-seq data were processed using Nesoni and mapped to *Piloderma croceum* F 1598 v1.0 genome using Bowtie 2 (Langmead & Salzberg 2012). Differential expression analysis was conducted using edgeR in R by contrasting each tissue type within each treatment, and each treatment within

each tissue type. DEGs were identified by normalising transcripts of all treatments against ECM root tips in L-alanine. Genes with an absolute log2 fold change (logFC) > 1 and an FDR-adjusted p-value (FDR) < 0.05 were considered differentially expressed. DEGs were annotated with GO terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, and analysis of enriched GO terms and KEGG pathways was performed using clusterProfiler v4.14.6 (Xu et al. 2024). The majority of visualisations were carried out using the 'Tidyverse' suite v2.0.0 (Wickham et al. 2019).

Exudate analysis

To explore the effect of organic and inorganic N sources on LMWOA exudation in **Paper IV**, chemical analysis and characterisation of LMWOAs were performed. Identity of LMWOAs was determined by Liquid Chromatography Tandem Mass Spectrometry (LC-MS-MS).

Network analysis

To relate exudates to DEGs and identify potential correlations in **Paper IV**, co-occurrence network analysis was performed. Data were scaled (exudates multiplied by 100, FPKM values by 1000) and analysed using SCNIC software, which uses compositional data to generate and analyse co-occurrence networks (Shaffer, Thurimella et al., 2023), with Pearson correlations. Pairwise correlations with an R2 value below 0.75 were removed. Network connections were then grouped into modules.

6.4 Results and Discussion

In boreal forests, plants rely on their associated ECM fungi for N, P and base cation acquisition from both organic matter and mineral substrates. To ensure adequate nutrient supply, whilst minimising energy waste through excessive C allocation, the regulation of mineral weathering and N mobilisation must be tightly controlled. Transporter genes of N and base cation uptake are likely to be up- or down-regulated depending on the substrate type and its nutrient composition. Similarly, the production and exudation of LMWOAs by ECM fungi are likely to be adjusted according to the target nutrient source, leading to corresponding changes in the regulation of related genes.

*Strong transcriptional response to treatments in **Paper III** and **Paper IV***

Base cation uptake resulting from mineral weathering has been shown in multiple ECM species (Balogh-Brunstad *et al.*, 2008; Fahad *et al.*, 2016; ***Paper II***), and although mineral weathering is a slow process that takes place over the timescale of millennia (Finlay et al. 2020), ECM fungi must still quickly detect and respond to fluctuations in nutrient availability and efficiently mobilise and take up nutrients. In **Paper III**, we aimed to deepen our understanding of base cation transporter gene regulation during the early stages of mineral weathering. Differences in global gene expression were visualised by PCA of regularised log-transformed raw counts for each sample (Fig. 16a). We found a strong effect of not only mineral treatment on gene expression, but also of *S. bovinus* isolate, timepoint and whether or not *S. bovinus* was growing in symbiosis (MRPP: A = 0.6165, P = 0.001). A total of 1091 genes were differentially expressed across all contrasts (Table 1), and, in general, there was a greater number of up-regulated DEGs. Of the identified DEGs, more genes related to base cation transport were up-regulated than down-regulated, reflecting the effect of mineral treatment and providing evidence for our first hypothesis that base cation transporter genes would be up-regulated in *S. bovinus* grown in the gabbro treatment, compared to *S. bovinus* grown in the limited treatment.

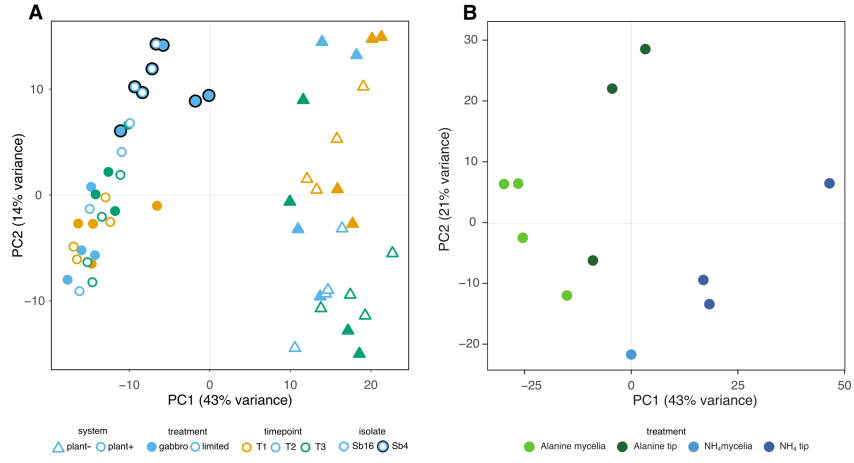

Figure 16. Ordination of samples based on Principal Component Analysis (PCA) of regularised log-transformed raw counts for global gene expression in *S. bovinus* (A), and FPKM values of DEGs in *P. fallax* (B). In (A), shape indicates samples from pure culture systems (plant–, triangle) or symbiotic systems (plant+, circle), gabbro treatment is indicated with a filled shape and limited with a hollow shape, timepoint is indicated by colour, and isolate Sb4 is indicated with a black outer ring around each data point. In (B), tissue type in each treatment is indicated by colour. (*Adapted from* ***Paper III*** *(A) and* ***Paper IV*** *(B)*).

Table 1. Number of DEGs which were up- and down-regulated in the gabbro treatment compared to the limited treatment in each contrast. (*Taken from* ***Paper III***).

Contrast	Up	Down	Total
Sb16 plant+ T1	0	1	1
Sb16 plant+ T2	16	1	17
Sb16 plant+ T3	21	75	96
Sb4 plant+ T2	35	41	76
Sb16 plant– T1	65	35	100
Sb16 plant– T2	422	43	465
Sb16 plant– T3	290	254	544

In contrast to our second hypothesis in **Paper III**, that base cation transporter genes would be more upregulated in symbiotic systems (plant+), we found an overall lower number of DEGs in plant+ systems compared to pure culture systems (plant–) (Table 1), as well as a greater number of up-regulated genes related to base cation transport (Table 3). We speculate that this may have been due to plant influence over expression in *S. bovinus*, resulting in a finetuned and reduced suite of differentially expressed genes (Miyauchi et al. 2020; Martin & van der Heijden 2024). Alternatively, the lower number of DEGs in plant+ systems may be explained by an overall high level of expression of genes related to mineral weathering regardless of treatment. This pattern of constitutively high gene expression was identified in genes related to zinc tolerance in *S. luteus* isolates, and was suggested to be an adaptive strategy to environmental conditions (Smith et al. 2024). In support of our third hypothesis that there would be intraspecific variation in gene expression in response to mineral treatment, we found a large difference in both the number and the identity of DEGs between the Sb16 plant+ and the Sb4 plant+ contrasts at timepoint 2, which aligns with findings of previous studies (Sawyer et al. 2003; Johnson et al. 2012; Plett et al. 2015a, 2021; Stuart et al. 2023).

The regulation of N mobilisation and uptake by ECM fungi at the community, species, and individual level, as well as in relation to N transfer to host plants, has been the focus of several studies (Stuart & Plett 2019; Pérez et al. 2022). Knowledge of the regulation of LMWOA production and exudation in response to differing N sources, however, is lacking. In **Paper IV**, we aimed to deepen our understanding of C release and LMWOA production and exudation in ECM symbiosis in response to organic and inorganic N sources. We found a strong separation of samples by both N treatment and tissue type when visualising FPKM values of DEGs by PCA (Fig. 16b), suggesting an N treatment and a tissue type effect on gene expression in *P. fallax*. In total, 1214 DEGs were identified, with the largest number reported for the N treatment contrast in ECM root tip tissue (Table 2), suggesting not only the importance of the type of N source in ECM fungi, but also that there may be specific genes involved in the transfer and metabolism of either organic or inorganic N that are differentially expressed in the interface between *P. fallax* and *P. sylvestris*. It has been shown that in symbiosis, genes involved in C and N metabolism are up-regulated (Martin et al. 2008; Ceccaroli et al. 2011; Doré et al. 2017), and hundreds of plant host-induced genes have been identified , further supporting the interpretation that the differentially expressed genes in the ECM root tips of different N treatments may be related to N transport and metabolism. The tissue contrast in the alanine treatment also had a large number of up- and down-regulated DEGs, reflecting the substantial difference in function of the two tissue types.

Table 2. Number of DEGs that were up- and down-regulated in tip tissue compared to mycelial tissue in the alanine treatment (first row), in tip tissue compared to mycelial tissue in the ammonium treatment (second row), in alanine treatment compared to ammonium treatment in the tip tissue (third row), and in alanine treatment compared to ammonium treatment in the mycelial tissue (fourth row). (*Taken from* ***Paper IV***).

Contrast	Up	Down	Total
AlaTipAlaMyc	22	214	236
NH4ClTipNH4ClMyc	57	93	150
AlaTipNH4ClTip	176	211	387
AlaMycNH4ClMyc	57	8	65

*Regulation of Ca transport in the mineral treatment in **Paper III***

In **Paper III**, 18 genes related to base cation transport were found to be differentially expressed (Table 3), predominantly in the plant– contrasts. The *S. bovinus* plant– timepoint 2 contrast had the greatest number of base cation related DEGs compared to all other contrasts, and the majority of base cation related genes were up-regulated. Furthermore, in up-regulated genes of the *S. bovinus* 16 plant– timepoint 2 contrast, several cation binding GO terms and a GO term for transport were enriched, reinforcing hypothesis one that base cation transporter genes would be up-regulated in response to mineral treatment, and indicating that mineral weathering was occurring, even within the short timescale of this experiment.

Transcriptional regulation of base cation transporter genes was observed in genes belonging to both base cation transporter gene families that showed significant expansions in **Paper II** and those that did not, supporting hypothesis four. The Ca proton exchanger gene (gene_11569), which was up-regulated in all *S. bovinus* 16 plant– contrasts, is part of the Ca^{2+}:cation (CaCA) transporter gene superfamily (Saier 2006; Martin et al. 2008; Saier et al. 2009, 2014, 2016, 2021; Kohler et al. 2015; Plett et al. 2015b), and plays a key role in Ca homeostasis by facilitating rapid Ca^{2+} transport (Miseta et al. 1999; Waight et al. 2013; Niu et al. 2023). In **Paper II**, the CaCA superfamily did not exhibit significant gene copy number changes; however, the ankyrin gene family, which coordinates the localisation of CaCA transporters (Li et al. 1993; Mohler et al. 2003, 2005), possessed significant expansions and contractions and was positively correlated with Ca uptake by fungi, including *S. bovinus*, grown in the gabbro treatment in **Paper II**. This may indicate that both direct transcriptional regulation of Ca transporter genes and gene copy number of associated genes influence Ca uptake, supporting hypothesis four. Interestingly, a gene encoding a Ca-transporting ATPase (gene_1948) was down-regulated in the *S. bovinus* 16 plant– timepoint 3 contrast, suggesting the utilisation of alternative Ca uptake pathways by *S. bovinus*.

Table 3. Gene ID, logFC, and gene product name of DEGs related to base cation transport found in each contrast. (*Taken from* ***Paper III***).

Gene ID	LogFC	Gene product name	Contrast
gene_11101	4.02	FTH1 iron permease	Sb16 plant– T1
gene_10592	2.65	cytochrome P450	Sb16 plant– T2
gene_11095	2.53	Fet5 multicopper oxidase	Sb16 plant– T2
gene_11101	2.42	FTH1 iron permease	Sb16 plant– T2
gene_11095	2.27	Fet5 multicopper oxidase	Sb16 plant– T1
gene_11564	2.16	hypothetical protein	Sb16 plant– T2
gene_7286	1.98	cytochrome P450	Sb16 plant– T3
gene_11569	1.94	calcium proton exchanger	Sb16 plant– T2
gene_7286	1.84	cytochrome P450	Sb16 plant– T2
gene_11569	1.76	calcium proton exchanger	Sb16 plant– T1
gene_9219	1.74	Mg transporter-E protein	Sb16 plant– T2
gene_8708	1.72	Mg transporter-E protein	Sb16 plant– T2
gene_8714	1.61	Mg transporter-E protein	Sb16 plant– T1
gene_8724	1.50	Mg transporter-E protein	Sb16 plant– T3
gene_10074	1.44	Mg transporter-E protein	Sb16 plant– T2
gene_13201	1.41	heavy metal translocatin	Sb16 plant– T2
gene_11564	1.38	hypothetical protein	Sb16 plant– T1
gene_10592	1.37	cytochrome P450	Sb16 plant– T3
gene_11569	1.35	calcium proton exchanger	Sb16 plant– T3
gene_13393	1.34	cytochrome P450	Sb16 plant– T3
gene_9629	1.27	hypothetical protein	Sb16 plant– T2
gene_13393	1.27	cytochrome P450	Sb16 plant– T2
gene_11564	1.20	hypothetical protein	Sb16 plant– T3
gene_13201	1.18	heavy metal translocatin	Sb16 plant– T3
gene_5094	1.15	Mg transporter-E protein	Sb16 plant– T3
gene_8714	1.07	Mg transporter-E protein	Sb16 plant– T2
gene_9629	-2,71	hypothetical protein	Sb16 plant– T3
gene_9260	-2.09	Fet3 ferroxidase	Sb16 plant– T1
gene_9260	-1.89	Fet3 ferroxidase	Sb16 plant– T2
gene_9260	-1.80	Fet3 ferroxidase	Sb16 plant– T3
gene_9259	-1.65	iron permease FTR1	Sb16 plant– T1
gene_1948	-1,4	Ca-transporting ATPase	Sb16 plant– T3
gene_8714	-1.26	Mg transporter-E protein	Sb4 plant+ T2
gene_9259	-1.21	iron permease FTR1	Sb16 plant– T2
gene_3325	-1.14	Na Bile acid symporter containing protein	Sb4 plant+ T2
gene_9260	-1.05	Fet3 ferroxidase	Sb16 plant+ T3

*Regulation of Mg transport in the mineral treatment in **Paper III***

In **Paper I**, plant and fungal uptake of Mg was demonstrated, with δ^{26}Mg signatures indicating that the mineral B horizon, which was dominated by *S. bovinus*, was the primary source. In **Paper II**, Mg uptake by fungi was also demonstrated, as well as significant evolutionary expansions and contractions of the Mg^{2+} transporter-E (MgtE) gene family (Smith et al. 1995). Copy numbers of this gene family were shown to be significantly positively correlated with Mg uptake by fungi, including *S. bovinus*, in the gabbro, granite and limited treatments. In *S. bovinus*, 57 members of the MgtE gene family are present, and six of those were found to be differentially expressed in response to mineral treatment in **Paper III**. In the *S. bovinus* 16 plant– contrasts, four MgtE genes were up-regulated at timepoint 2, two at timepoint 3, and one at timepoint 1 (Table 3), supporting our first hypothesis that base cation transporter genes would be up-regulated in response to mineral treatment and indicating that Mg uptake is at least partially regulated by transcription. The low number of MgtE genes that were found to be differentially expressed, in comparison to the 57 MgtE genes in the *S. bovinus* genome, may indicate that expansions in this family are related to a diversification in gene function. This may result in the gene products of certain genes being better adapted to specific fungal tissues, life stages, and environmental conditions than others (Copley 2020). One MgtE gene was also down-regulated in the *S. bovinus* 4 plant+ timepoint 2 contrast, which may indicate either a difference in the nutrient requirements of *S. bovinus* when in symbiosis with *P. sylvestris*, or perhaps a delayed response to mineral treatment when *S. bovinus* is in symbiosis, coupled with a difference in the environmental conditions of the two different experimental systems.

*Limiting Fe toxicity in the mineral treatment in **Paper III***

In certain environmental conditions, the concentration of vital nutrients like Fe can become too high and potentially toxic to fungi and plants (Winterbourn 1995). To combat this, plants and fungi can take up excess

nutrients and transport them into their vacuole (Gupta & Outten 2020). In response to mineral treatment, the iron permease FTR1 gene (gene_9259) was down-regulated in the *S. bovinus* 16 plant– timepoint 1 and 2 contrasts, and the Fet3 ferroxidase gene (gene_9260) was down-regulated in all *S. bovinus* 16 plant– contrasts. These genes belong to the iron/lead transporter (ILT) gene family (Debut et al. 2006; Saier 2006; Saier et al. 2009, 2014, 2016, 2021), and together, they constitute the high-affinity reductive Fe uptake system localised to the plasma membrane, where Fe(III) is reduced to Fe(II) by the multicopper oxidase Fet3, and subsequently transported into the cell via the FTR1 iron permease (Askwith et al. 1994; Stearman et al. 1996; Kosman 2010; Tamayo et al. 2025). The down-regulation of these genes, whilst in contradiction to our first hypothesis, indicates that extracellular Fe concentrations in the mineral treatment are greater than in the limited treatment due to mineral weathering, and that *S. bovinus* reduced Fe uptake to avoid toxic intracellular Fe levels.

In the *S. bovinus* 16 plant– contrast, the Fet5 multicopper oxidase gene and the FTH1 iron permease gene were up-regulated at timepoints 1 and 2. These genes are also part of the ILT gene family and, like Fet3 and FTR1, constitute a high-affinity reductive Fe uptake system; however, this system is localised to the vacuole membrane (Spizzo et al. 1997; Urbanowski & Piper 1999; Singh et al. 2006). This system is likely to transport Fe into the cytoplasm and not into the vacuole (Singh et al. 2006). The up-regulation of these genes, in combination with the down-regulation of the Fet3 and FTR1 genes, may point to the intricacies of Fe homeostasis. The ILT gene family had no significant expansions or contractions in **Paper II**, suggesting that gene copy number is not likely to be a contributor to the regulation of these genes' expression, supporting hypothesis four.

*Exudation of LMWOAs is greatly affected by N source in **Paper IV***

In Paper IV, 18 LMWOAs were quantified to assess exudation profiles by *P. fallax* in symbiosis with *P. sylvestris* in response to different N sources. Exudation was higher in the organic N treatment compared to the inorganic N treatment, with a significant effect on N source on 17 out of 18 analysed LMWOAs, and a total LMWOA concentration of 87.4 ± 8.8 µM in the alanine treatment, compared to just 7.2 ± 0.4 µM in the ammonium treatment (*see **Paper IV**, Table 1*), suggesting that the addition of 5 mM NH_4Cl negatively impacted exudation compared to the organic N source. High concentrations of extracellular ammonium, which can result from inorganic N fertilisation and deposition, have been shown to be toxic and negatively impact ECM fungal community composition and function, affecting mycelial production, nutrient uptake and nutrient cycling in forests (Fransson et al. 2000; Lilleskov et al. 2002; Ekblad et al. 2013).

In both N treatments, oxalate was the most abundant LMWOA, contributing 87 % of total LMWOAs in the alanine treatment and 50 % in the ammonium treatment. This is consistent with previous reports of oxalate being the dominant exudate produced by ECM fungi (Plassard & Fransson 2009). Oxalate was followed by pyruvate, which made up 6 % of total LMWOAs in the alanine treatment and 29 % in the ammonium treatment; and malonate, making up 3% in the alanine treatment and 6 % in the ammonium treatment (Fig. 17a). Variation in LMWOA profiles across the samples was visualised by PCA, with the first principal component accounting for 99.8% of the ordination. Despite observed variation in exudate profiles between individual samples within N treatments, all ammonium samples clustered closely together and were distinctly separated from the alanine treatments (Fig. 17b). The impact of N source on LMWOA profiles in **Paper IV** is consistent with previous studies demonstrating that organic N sources significantly influence the exudation of organic acids, monosaccharides, and dissolved organic C (Fransson & Johansson 2010) and the stimulatory and inhibitory effects of nitrate and ammonium, respectively, on oxalic acid production (Plassard & Fransson 2009).

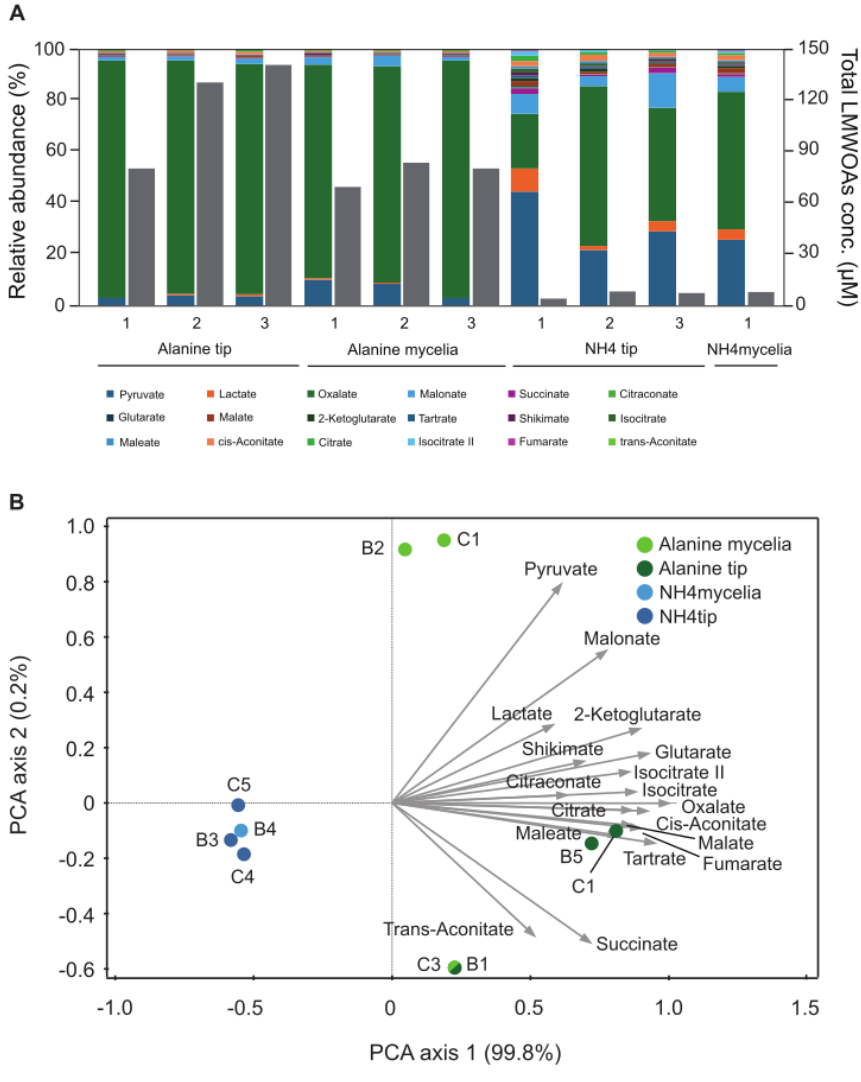

Figure 17. Exudation of LMWOAs by *P. fallax* in symbiosis with *P. sylvestris* in alanine and ammonium treatments. (A) shows the relative abundance and total concentrations of 18 LMWOAs. LMWOA identity is indicated by colour. (B) shows a PCA of LMWOA exudate profiles of samples in alanine or ammonium treatments. Colours indicate the N treatment and tissue type. For the C3 + B1 sample, the tissue types were combined. (*Taken from* ***Paper IV***).

*Few genes relate to LMWOAs in **Paper IV***

Despite the large differences in LMWOA exudation between the alanine and ammonium treatments, only five genes putatively involved in LMWOA production were identified among the DEGs. One gene, annotated to be involved in isocitrate dehydrogenase activity, was up-regulated in ECM root tips in the alanine treatment. Isocitrate dehydrogenase is an enzyme in the tricarboxylic acid (TCA) cycle, a series of biochemical reactions that extract energy from C-rich molecules (Chapman et al. 2020). Given the large disparity in LMWOA production between N treatments, it was expected that there would also be large differences in differential expression of genes related to their production between N treatments; however, this was not the case. One possible explanation for the limited number of identified DEGs is that central pathways like the TCA cycle may be continuously expressed at high levels, reflecting their critical role in C and N metabolism.

Network analyses to determine associations between LMWOAs and genes yielded few patterns of co-occurrence. Out of a total of 602 co-occurrence modules, nine demonstrated relationships between LMWOAs and genes; however, several of these genes were not annotated, and many were not differentially expressed (*see **Paper IV**, Table S1*). Two co-occurrence modules showed co-occurrence of oxalate, 2-ketoglutarate, tartrate, isocitrate and total LMWOAs, which co-occurred with the genes 68967, 811861, and 819119; and glutarate, malate, maleate, cis-aconitate and isocitrate II, which co-occurred with the gene 526370. Neither of these co-occurrence modules corresponds with a specific metabolic pathway. Genes 811861 and 819119 were differentially expressed in the AlaMycNH4ClTip contrast; however, they, along with the other genes in the co-occurrence modules, are not annotated.

6.5 Concluding remarks

Ectomycorrhizal fungi mobilise base cations, N and P from mineral and organic substrates to meet plant nutrient requirements, as well as their own. One mechanism utilised to mobilise nutrients is the production and exudation of LMWOAs. Once mobilised, nutrients must be taken up through specific transporter proteins. These processes must be tightly regulated through several mechanisms, including gene copy number variation and differential gene expression. In **Paper III**, we investigated the transcriptional response of base cation transporter genes in *S. bovinus* in symbiosis and pure culture to mineral treatment. We found several members of the MgtE gene family, which was found to be significantly expanding and significantly positively correlated with Mg uptake by fungal mycelia in **Paper II**, were up-regulated in response to mineral weathering. We also found up-regulation of a Ca transporter gene and up- and down-regulation of several genes involved in Fe transport. Collectively, our findings indicate that base cation transporter genes are differentially expressed during the early stages of mineral weathering, and that their regulation is controlled, at least in part, by both gene copy number and transcriptional mechanisms, which may operate independently or in combination. In **Paper IV**, we explored the transcriptional response of *P. fallax* in symbiosis with *P. sylvestris* to organic and inorganic N sources, as well as their effect on LMWOA production and exudation. A clear effect of N treatment was visible in both the total amount of LMWOAs exuded and the LMWOA profiles. There was also a clear effect of N treatment on gene expression, as well as an effect of tissue type. In particular, a large number of DEGs were found in the N treatment contrast in the ECM root tip tissue, highlighting the importance of N source and a potential difference in expression of genes involved in N transport and metabolism. Unfortunately, the co-occurrence network analysis yielded few results. To make further strides in the study of C release and LMWOA regulation in response to differing N sources, better characterisation of organic acid transporters is required. The findings of **Paper III** and **Paper IV** have implications for the interconnected processes of mineral weathering and N mobilisation, both of which require the regulation of nutrient transporters and the production and exudation of LMWOAs.

7. Conclusions and future perspectives

The fact that ECM fungi play a role in mineral weathering and N mobilisation is clear. They have been shown to produce and exude LMWOAs, protons, free radicals and siderophores (Olsson & Wallander 1998; Wallander & Wickman 1999; Wallander 2000; van Hees et al. 2006; Van Scholl et al. 2006; Fomina et al. 2010; Adeleke et al. 2012; Schmalenberger et al. 2015); to take up base cations (Balogh-Brunstad et al. 2008; Fahad et al. 2016); and to up-regulate K+ transporter genes during mineral weathering (Corratgé et al. 2007; Garcia & Zimmermann 2014; Sun et al. 2019; Pinzari et al. 2022, 2024). The production and exudation of LMWOAs has also been demonstrated during N mobilisation from organic matter (Wang et al. 2017; Tunlid et al. 2022), as well as the production and exudation of extracellular enzymes (Lindahl & Tunlid 2015; Shah et al. 2016), and the employment of Fenton reactions (Nicolás et al. 2019). However, large knowledge gaps remain in how these processes are interconnected, the evolutionary mechanisms driving ECM fungi's mineral weathering capacities, as well as aspects of their regulation.

This thesis aimed to shed light on how mineral weathering, N mobilisation and C allocation influence each other, as well as the role of fungal communities in their integration (**Paper I**). Furthermore, it aimed to elucidate how genes related to base cation uptake, which accompanies mineral weathering, evolved in ECM species with known mineral weathering capabilities (**Paper II**). Moreover, it aimed to identify regulatory mechanisms of base cation transport at the level of gene copy number (**Paper II**) and transcription (**Paper III**). Finally, it aimed to provide insight into the regulation of LMWOA production and exudation and patterns of C release under different N conditions (**Paper IV**).

In **Paper I**, we found that mineral weathering, resulting in the mobilisation of base cations, appeared to be upregulated in response to the greater nutrient demands of the larger plants in the high organic matter availability treatments, compared to the smaller plants of the other treatments. These plants, supplied with larger quantities of N from the larger amount of organic matter available, allocated greater amounts of C to

mycorrhizal fungi in the O and B horizons than smaller plants, further driving N mobilisation and mineral weathering in those respective horizons. The interconnectedness of mineral weathering, N mobilisation, and C allocation calls for more holistic approaches to the future study of these processes. The ECM fungi *Piloderma sphaerosporum* and *Suillus bovinus* were found to dominate the O and B horizons, respectively, implicating them in the mediation of N mobilisation and mineral weathering. The impact of organic matter removal on not only the primary productivity of plants, but also on N mobilisation and mineral weathering, and the subsequent C sequestration associated with these processes, is clear. These results should be incorporated in the development of sustainable forestry practices and climate change mitigation strategies – to maintain plant productivity, nutrient supply and C sequestration and C storage belowground.

In **Paper II**, base cation transporter gene families were found to be rapidly evolving, all of which had expansions and/or contractions in the genus *Suillus*. The expansions and contractions in these transporter gene families potentially relate to the need for base cation uptake, supported by the correlation between mycelial element uptake and gene family copy numbers in the presence of gabbro and granite. The MgtE gene family, in particular, showed significant positive correlations with Mg uptake by fungal mycelia. This gene family was shown to have expanded from several transporter genes of different origins, further indicating rapid evolution. Although each of our hypotheses focused on the *Suillus* genus, significant expansions and base cation uptake were also observed in *Piloderma*. These results provide evidence of the evolutionary mechanisms involved in the proliferation of mineral weathering capabilities in ECM fungi, and also of the role of base cation transporter genes in base cation uptake. Furthermore, they suggest that gene copy number may be one aspect of a more complex system of base cation uptake regulation in ECM fungi.

In **Paper III**, which closely followed on from **Paper II**, several base cation transporter genes were up-regulated in response to mineral treatment in *S. bovinus* grown in pure culture. Six members of the MgtE gene family were found to be differentially expressed, five of which were upregulated under pure culture conditions. A number of other up-regulated genes related to base cation transport also belonged to base cation transporter gene families

found to be significantly expanding in **Paper II**; however, some did not. This suggests that regulation of base cation uptake is complex, with the transport of some base cations appearing to be at least partly regulated at the level of gene copy numbers, some at the level of transcription, and some at both the level of gene copy number and transcription. Contrary to our hypothesis, very few genes related to base cation transport were found to be differentially expressed in *S. bovinus* in symbiosis with *P. sylvestris*, which may be due to a delayed response to mineral treatments compared to *S. bovinus* in pure culture, or constitutively high expression of genes related to mineral weathering and base cation uptake.

In **Paper IV**, LMWOA production and exudation by *P. fallax* in symbiosis with *P. sylvestris* greatly increased in the organic N treatment compared to the inorganic treatment. Gene expression was also influenced by N source, and a large number of genes in the N treatment contrast in ECM root tips were differentially expressed. Unfortunately, a large proportion of DEGs lacked useful annotations, and the co-occurrence network analysis did not generate many useful results, highlighting the need for improved genome annotation and characterisation of genes related to LMWOA production and exudation.

In future, conducting experiments both in the laboratory and field that incorporate analyses of both N mobilisation and mineral weathering would be beneficial. These may include large-scale field experiments comparing these processes and the ECM fungi involved *in situ* in forests where only trunks are harvested and those where whole tree harvesting is practised. In an experiment such as this, not only could analyses of natural abundances of N, C and Mg isotopes be conducted, but also metatranscriptomics. Additionally, pure culture and symbiotic laboratory studies similar to those conducted in **Papers II**, **III**, and **IV**, incorporating a wider range of ECM species would increase our understanding of universal and species-specific ECM traits.

The transcriptional regulation of N mobilisation and transfer to host plants by ECM fungi has been demonstrated (Stuart & Plett 2019; Pérez et al. 2022); however, despite our work here, knowledge of the regulation of LMWOA production and exudation in the context of both N mobilisation

and mineral weathering is still lacking. To aid future studies, efforts should be directed towards improving the annotation of ECM genomes and the characterisation of organic acid transporter proteins in ECM fungi. Further studies into the transcriptional regulation of genes related to mineral weathering, LMWOA production and exudation, and N mobilisation are needed to close the knowledge gap on the regulation of these processes. Of course, gene copy number and transcription are not the only levels of regulation, and as such, future studies should examine other levels of regulation of genes related to both base cation transport and LMWOA production and exudation. Magnesium was a particular focus of this thesis, and further studies into the characterisation of Mg transporter genes and proteins in ECM fungi may prove to be a fruitful place to start.

This thesis has provided useful insight into the integration of mineral weathering and N mobilisation, the evolution of mineral weathering traits in ECM fungi, and the regulation of aspects of mineral weathering and N mobilisation. The effect of organic matter depletion on mineral weathering and N mobilisation, and on the ECM fungi that perform these processes, is stark and particularly relevant in an era of intensifying forestry and climate change. By continuing to expand our understanding of how ECM capabilities evolved to perform these processes, as well as how they are regulated, more information can be gathered to aid sustainable decision-making and direct future research in a meaningful direction with regard to sustainable forestry and climate change mitigation.

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Popular science summary

Ectomycorrhizal (ECM) fungi form mutually beneficial relationships with host plants, by gathering key nutrients, like nitrogen, phosphorus and base cations, and exchanging them for energy-rich molecules like sugars. In Northern boreal forests, ECM fungi play an important role in the biogeochemical processes of mineral weathering and nitrogen mobilisation. Mineral weathering is the breakdown of rock into smaller parts and molecules. These molecules can include phosphorus and base cations, and ECM fungi take them up through specific transporter proteins and transfer them to their host plants. Nitrogen mobilisation involves the breakdown of organic matter into smaller parts and molecules. The main nutrient ECM fungi take up during this process is nitrogen, again through specific transporter proteins, before transferring it to their host plant. These two processes occur in separate parts of the soil and, as such, have frequently been studied separately. In **Paper I** of this thesis, mineral weathering and nitrogen mobilisation are studied together. They were found to have a strong influence on each other, as well as an influence on the amount of energy-rich molecules plants allocated to ECM fungi in different parts of the soil. Plants with access to more nitrogen-rich organic matter were shown to take up more nitrogen and grow bigger, which improved their ability to allocate energy-rich molecules to two parts of the soil where nitrogen mobilisation and mineral weathering happen – the O and B horizons, respectively. Mobilisation of the base cations results from mineral weathering, and the base cation magnesium was shown to be mobilised in the B horizon. Ectomycorrhizal fungi were shown to dominate the soil, and fungal growth in the B horizon significantly increased with plant growth. In the O and B horizons, two ECM species dominated: *Suillus bovinus* and *Piloderma sphaerosporum*. These results show the interconnectedness of mineral weathering, nitrogen mobilisation and allocation of energy-rich molecules by plants.

The evolution of ECM fungi to perform these processes is an important consideration, and whilst the evolution of nitrogen mobilising capabilities has been studied, the evolution of mineral weathering capabilities has not. Nutrients and other molecules must be taken up through specific transporter

proteins, which can be categorised into families based on what molecules they transport and how they transport them. Base cations, for example, are transported by base cation transporter gene families. The number of genes in each of these families for any given species has evolved over millennia, and it affects how a particular species is adapted to its environment. In **Paper II** of this thesis, bioinformatic software was used to study how the number of genes in different transporter gene families in 108 different fungal species has increased or decreased over time. Focus was placed on base cation transporter gene families that transported base cation nutrients, as well as ECM fungi and the ECM genus *Suillus*. Base cation transporter gene families were found to have evolved rapidly, with significant increases and decreases in gene copy numbers, and especially in the genera *Suillus* and *Piloderma*. Alongside the bioinformatic analyses, several fungal species were grown alone in pure culture, with and without minerals, and the concentrations of different base cations they had taken up into their biomass was quantified. Some *Suillus* species were shown to take up base cations from minerals, as were a number of *Piloderma* species. Gene copy numbers of several of the significant base cation transporter families in these fungal species were found to be significantly positively correlated with base cation concentration in fungal biomass. One of these families, the Mg^{2+} transporter-E family, was shown to undergo particularly rapid evolution and was significantly positively correlated with Mg concentrations in fungal biomass. These results suggest that the evolution of mineral weathering capabilities in ECM fungi involved rapid gene copy number changes in base cation transporter gene families.

Mineral weathering and nitrogen mobilisation must be carefully controlled by plants and ECM fungi. Ectomycorrhizal fungi in symbiosis with plants that have large nutrient requirements must be able to increase the amount of nutrients they take up and transfer to their host plant in exchange for energy-rich molecules. Equally, if their associated plants have lesser nutrient requirements, ECM fungi must be able to reduce the amount of nutrients they take up and transfer to their host plants. To do this, components used to break down mineral and organic matter, such as organic acids and enzymes, as well as components used to take up nutrients, such as transporter proteins, must be up-regulated (increase the production) or down-regulated

(decrease the production). In **Paper III** of this thesis, the transcriptional regulation of base cation transporter genes in *Suillus bovinus* in response to growth with minerals was examined. Several genes related to base cation transport were up-regulated in response to growth with minerals. A number of these genes were members of base cation transporter gene families, which were found to be rapidly evolving in **Paper II**. In particular, five members of the Mg^{2+} transporter-E family were upregulated. However, some genes belonged to families that were not rapidly evolving in **Paper II**. These findings suggest that gene copy number and the level of transcription play a role in regulation, sometimes together and sometimes independently of one another. In **Paper IV** of this thesis, the transcriptional regulation of genes in *Piloderma fallax* ECM root tips as well as exploratory mycelia, in symbiosis with Scots pine seedlings in response to organic and inorganic nitrogen sources, was assessed. Genes related to the production and exudation of organic acids, specifically Low Molecular Weight Organic Acids (LMWOAs), as well as patterns of carbon release, were focused on. Many genes were up- and down-regulated in the ECM root tip tissue between the two nitrogen sources. There was also much higher LMWOA production and exudation by *P. fallax* when grown with organic nitrogen, and different LMWOAs were produced between the two nitrogen sources. However, specific transporter genes of organic acids were not found, and a network analysis to assess the relationship between LMWOAs and genes did not find many connections.

The findings of this thesis offer valuable insights into the relationship between mineral weathering and nitrogen mobilisation, the evolution of mineral weathering traits in ECM fungi, and their regulation. The impact of organic matter availability on these processes, and the ECM fungi involved, is significant in the context of intensifying forestry activities and climate change. By furthering our understanding of how ECM fungi evolved to perform these functions and how they are regulated, we can gather more information to support sustainable decision-making and guide future research in sustainable forestry and climate change mitigation.

Populärvetenskaplig sammanfattning

Ektomykorrhizasvampar bildar ett ömsesidigt fördelaktigt samspel med värdväxter, genom vilket de samlar in viktiga näringsämnen som kväve, fosfor och baskatjoner och byter dem mot energirika molekyler som socker. I boreala skogar spelar mykorrhizasvampar en viktig roll i processerna mineralvittring och kvävemobilisering. Mineralvittring är nedbrytningen av berg och mineral i mindre delar och molekyler. Dessa molekyler kan inkludera fosfor och baskatjoner och mykorrhizasvamparna tar upp dem genom specifika transportproteiner och överför dem till sina värdväxter. Kvävemobilisering innebär nedbrytning av organiskt material till mindre delar och molekyler. Det huvudsakliga näringsämnet som mykorrhizasvampar tar upp under denna process är kväve, återigen genom specifika transportörer innan de överförs till sin värdväxt. Dessa två processer sker i separata delar av marken och har ofta studerats separat. I artikel I i avhandlingen studeras mineralvittring och kvävemobilisering tillsammans. Deras inverkan på varandra liksom deras inverkan på mängden energirika molekyler som växterna allokerar till mykorrhizasvampar i olika delar av marken fastställdes. Växter med tillgång till mer kväverikt organiskt material visade sig ta upp mer kväve och växa sig större, vilket förbättrade deras förmåga att allokera energirika molekyler till två delar av marken där kvävemobilisering och mineralvittring sker – det organiska skiktet (O-horisonten) samt anrikningsskiktet (B-horisonten). Ektomykorrhizasvampar dominerade i jordskikten och svamptillväxten i B-horisonten ökade signifikant med växttillväxten. I O- och B-horisonten dominerade två mykorrhizaarter: *Suillus bovinus* och *Piloderma sphaerosporum*. Resultaten visar sammankopplingen mellan mineralvittring, kvävemobilisering och allokering av energirika molekyler av växter.

Utvecklingen av mykorrhizasvampar för att utföra dessa processer är viktig, och även om kvävemobiliseringsförmågan har studerats, har utvecklingen av mineralvittringsförmågan inte gjort det. Näringsämnen och andra molekyler måste tas upp genom specifika transportproteiner som kan kategoriseras i familjer utifrån vilka molekyler de transporterar och hur de transporterar dem. Antalet gener i var och en av dessa familjer för en given art har utvecklats under årtusenden, i samspel med deras miljöanpassningar.

I artikel II i avhandlingen användes bioinformatisk programvara för att studera hur antalet gener i olika transportörgenfamiljer i 108 olika svamparter har blivit större eller mindre under evolutionär tid. Fokus var främst på familjer av baskatjontransportörer, liksom mykorrhizasvampar och mykorrhizasläktet *Suillus*. Baskatjontransportörerna visade sig ha utvecklats snabbt, med betydande ökningar och minskningar av antalet genkopior, speciellt i släktet *Suillus* men även i släktet *Piloderma*. Vid sidan av de bioinformatiska analyserna odlades flera svamparter i renkultur, med och utan mineraler, och koncentrationerna av olika baskatjoner i deras biomassa kvantifierades. Vissa arter av *Suillus* och ett antal *Piloderma* tog upp baskatjoner från mineraler. Antalet genkopior för flera av genfamiljer för baskatjontransportörerna i dessa svamparter visade sig vara signifikant positivt korrelerade med baskatjonkoncentrationen i svampbiomassan. En av dessa familjer, Mg2+ transportör-E-familjen, visade sig genomgå särskilt snabb utveckling och var signifikant positivt korrelerad med Mg-koncentrationer i svampbiomassan. Dessa resultat tyder på att utvecklingen av mineralvittringsförmåga i mykorrhizasvampar involverade förändringar i genkopienummer i genfamiljer för baskatjontransportörerna, och att dessa förändringar har skett snabbt.

Mineralvittring och kvävemobilisering måste kontrolleras noggrant av växter och mykorrhizasvampar. Ektomykorrhizasvampar i symbios med träd som har stort näringsbehov måste kunna öka mängden näringsämnen de tar upp och överför till sin värdväxt i utbyte mot energirika molekyler. På samma sätt om deras associerade växter har mindre näringsbehov måste mykorrhizasvampar kunna minska mängden näringsämnen de tar upp och överför till sina värdväxter. För att göra detta måste verktygen som används för att bryta ner mineral och organiskt material, såsom organiska syror och enzymer, samt upptaget av näringsämnen, såsom transportproteiner, uppregleras (öka produktionen) eller nedregleras (minska produktionen). I artikel III i denna avhandling undersöktes hur regleringen genom transkription av baskatjontransportörer i *Suillus bovinus* svarade på tillväxt med mineraler. Flera gener relaterade till baskatjontransport uppreglerades. Av dessa tillhör ett antal familjer för baskatjontransportörer som visade sig utvecklas evolutionärt snabbt i Paper II. I synnerhet var fem medlemmar av Mg2+ transporter-E-familjen uppreglerade, men vissa gener tillhörde även

familjer som inte utvecklades snabbt i Paper II. Dessa fynd tyder på att antalet genkopior och nivån av transkription spelar en roll i regleringen, ibland tillsammans och ibland oberoende av varandra. I artikel IV i avhandlingen utvärderades den transkriptionella regleringen av gener i mykorrhizarotspetsar och mycel hos *Piloderma fallax* i symbios med tallplantor som svar på organiska och oorganiska kvävekällor. Fokus var på gener relaterade till produktion av organiska syror, särskilt organiska syror med låg molekylärvikt, samt utsöndring. Många gener upp- och nedreglerades i mykorrhizarotspetsarna mellan de två kvävekällorna. Det var också mycket högre produktion och utsöndring av lågmplekylära organiska syror när *P. fallax* odlades med organiskt kväve, och olika organiska syror producerades mellan de två kvävekällorna. Specifika transportörer av organiska syror hittades dock inte, och en nätverksanalys för att bedöma sambandet mellan LMWOAs och gener hittade inte många samband.

Resultaten av avhandlingen ger värdefulla insikter i sambandet mellan mineralvittring och kvävemobilisering, utvecklingen av mineralvittringsegenskaper hos mykorrhizasvampar och deras reglering. Effekten av tillgången på organiskt material på dessa processer och på de involverade mykorhizasvamparna är betydande, särskilt i samband med intensifiering av skogsbruk och klimatförändringar. Genom att öka vår förståelse för hur mykorrhizasvampar utvecklades för att utföra dessa funktioner och hur de regleras, kan vi samla in mer information för att stödja hållbart beslutsfattande och vägleda framtida forskning inom hållbart skogsbruk och klimatanpassningar.

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I

New Phytologist

Research

Ectomycorrhizal fungi integrate nitrogen mobilisation and mineral weathering in boreal forest soil

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Summary

- Tree growth in boreal forests is driven by ectomycorrhizal fungal mobilisation of organic nitrogen and mineral nutrients in soils with discrete organic and mineral horizons. However, there are no studies of how ectomycorrhizal mineral weathering and organic nitrogen mobilisation processes are integrated across the soil profile.
- We studied effects of organic matter (OM) availability on ectomycorrhizal functioning by altering the proportions of natural organic and mineral soil in reconstructed podzol profiles containing *Pinus sylvestris* plants, using $^{13}CO_2$ pulse labelling, patterns of naturally occurring stable isotopes (^{26}Mg and ^{15}N) and high-throughput DNA sequencing of fungal amplicons.
- Reduction in OM resulted in nitrogen limitation of plant growth and decreased allocation of photosynthetically derived carbon and mycelial growth in mineral horizons. Fractionation patterns of ^{26}Mg indicated that magnesium mobilisation and uptake occurred primarily in the deeper mineral horizon and was driven by carbon allocation to ectomycorrhizal mycelium. In this horizon, relative abundance of ectomycorrhizal fungi, carbon allocation and base cation mobilisation all increased with increased OM availability.
- Allocation of carbon through ectomycorrhizal fungi integrates organic nitrogen mobilisation and mineral weathering across soil horizons, improving the efficiency of plant nutrient acquisition. Our findings have fundamental implications for sustainable forest management and belowground carbon sequestration.

Introduction

Forests are large and persistent sinks for atmospheric carbon dioxide (CO_2; Pan *et al.*, 2011) and large-scale afforestation and reforestation have been suggested as methods for mitigating climate change through carbon sequestration (Law *et al.*, 2018; Nave *et al.*, 2018; Bastin *et al.*, 2019; Pugh *et al.*, 2019; Domke *et al.*, 2020). However, intensification of forestry (Kastner *et al.*, 2021), including removal of organic residues for bioenergy (Daiogloua *et al.*, 2019), has led to concern about sustainability of base-cation supply (Achat *et al.*, 2018; Akselsson *et al.*, 2019), and better understanding of the ecological and biogeochemical consequences of different management practices is needed (Palmer, 2021).

In forests, symbiotic and decomposer fungi play pivotal roles in sequestration and release of carbon (C; Jones *et al.*, 2009; Clemmensen *et al.*, 2013). The flow of photosynthetically derived C through ectomycorrhizal fungi is important in mobilising nitrogen (N) from organic matter (OM), as well as weathering of mineral substrates (Finlay *et al.*, 2020). There is conflicting evidence concerning whether mobilisation of organic N is linked to net mobilisation or net sequestration of C (Averill *et al.*, 2014; Zak *et al.*, 2019; Clemmensen *et al.*, 2021; Lindahl *et al.*, 2021), but in an evolutionary perspective, there is consensus that ectomycorrhizal symbiosis has intensified mineral weathering and driven drawdown of global CO_2 by enhancing land to ocean calcium (Ca) export and C sequestration into marine carbonates (Quirk *et al.*, 2012, 2014). Enhanced weathering of added silicate rocks has been suggested as a method to mitigate climate change by sequestering C from the atmosphere (Beerling *et al.*, 2020; Kantzas *et al.*, 2022).

In boreal forests, tree growth is driven by mobilisation of (predominantly) organic N and mineral nutrients from vertically stratified soils consisting of an organic (O) horizon overlying an eluvial (E) and a deeper illuvial (B) mineral horizon. Decomposition and weathering in these distinct, but contiguous, soil horizons have been studied, in detail, within essentially separate research traditions by different groups of researchers. This has

hindered mechanistic understanding of how the processes of N mobilisation and biological mineral weathering influence each other, and how these processes are related to patterns of C allocation.

Many studies of ectomycorrhizal fungi have been conducted in the upper, organic horizon, concentrating on fungal mobilisation of nutrients and diversity of fungal communities (Clemmensen *et al.*, 2013, 2015, 2021; Näsholm *et al.*, 2013; Lindahl *et al.*, 2021). Extensive use has been made of stable isotopes of N (Högberg, 1997; Näsholm *et al.*, 2013) to improve understanding of N acquisition and cycling and there is now a broad consensus that, during evolution from saprotrophic ancestors, certain ectomycorrhizal fungi have retained the genetic potential to mobilise N from recalcitrant OM (Lindahl & Tunlid, 2015). Comparatively, few studies have been conducted in the deeper mineral soil horizons, although there are reports that two thirds of mycorrhizal root tips and half of the ectomycorrhizal taxa (Rosling *et al.*, 2003), and half or more of the mycelial biomass (Ekblad *et al.*, 2013) occur in the mineral horizons. Since it was proposed that ectomycorrhizal fungi might weather mineral substrates (Jongmans *et al.*, 1997), there has been increased interest in biological weathering, in particular the role of ectomycorrhizal fungi. The subject has been reviewed from an evolutionary perspective and regarding different spatial scales (Leake & Read, 2017; Finlay *et al.*, 2020). Allocation of C to fungal mycelia results in weathering leading to mobilisation of the base cations and phosphorus (P) essential for plant growth, but existing studies of fungal weathering (see Finlay *et al.*, 2020) are mostly based on individual species inoculated into laboratory microcosms containing artificial substrates. Little mechanistic information is available from natural forest soils, with fungal communities identified *in situ*.

The aim of the present study was to investigate the effects of changes in OM availability in the surface horizon on: (1) plant and fungal mycelial biomass, (2) spatial allocation patterns of plant assimilated C, (3) fungal community composition and the relative abundance and spatial distribution of different functional fungal guilds in soil, and (4) mobilisation and uptake of base cations, P and N. We used microcosms containing different proportions of naturally occurring organic and mineral layers from a boreal forest podzol, creating a gradient of OM availability (Fig. 1a). *Pinus sylvestris* plants were grown in the reconstructed podzol profiles for 14 months. Plants were pulse labelled with $^{13}CO_2$ to study C allocation to different soil horizons and mobilisation of base cations was studied by examining patterns of fractionation of naturally occurring stable isotopes of magnesium (Mg). The composition of fungal communities in different soil horizons was studied using high-throughput DNA sequencing. We hypothesized (1) that the higher availability of OM would increase the amount of N mobilised by ectomycorrhizal fungi and supplied to their plant hosts, and (2) that this would increase plant growth and the supply of photosynthetically derived C allocated to ectomycorrhizal fungi. We further hypothesized (3) that increased allocation of C to ectomycorrhizal fungi would improve their ability to mobilise base cations and P from mineral substrates. Based on evidence that ectomycorrhizal fungi appear to discriminate against uptake of heavy ^{26}Mg (Fahad *et al.*, 2016), we expected (4) that greater uptake of Mg would lead to enrichment of $\delta^{26}Mg$ signatures in soil solution in environments where ectomycorrhizal fungi proliferate, supported by C directly from their host plants.

Materials and Methods

Plant and soil preparation

Pinus sylvestris L. seeds were surface sterilised with 33% hydrogen peroxide for 30 min, rinsed thoroughly with milliQ water, air dried and dispersed into a plant propagator (30 × 20 × 20 cm) filled with 2 l of nonsterile vermiculite. The propagators were incubated in a phytotron 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. The germinating seedlings were incubated under these parameters for *c.* 4 months before use. The seedlings were irrigated weekly with deionised water.

Soil was collected from the organic (O), eluvial (E) and illuvial (B) horizons of a podzol under a well-documented forest at Ivantjärnsheden, Jädraås, central Sweden (60°49′N, 16°30′E, altitude 185 m), located at the border between the Swedish boreal and boreo-nemoral zones (Persson, 1980; Mielke *et al.*, 2022). The forest consists of a homogenous, evenly aged (160 yr) stand of *Pinus sylvestris* L. with a few scattered *Picea abies* (L.) Karst. The understorey consists of the ericaceous dwarf shrubs *Vaccinium vitis-idaea* L. and *Calluna vulgaris* (L.) Hull, with a lower cover of *Empetrum nigrum* L. and *Vaccinium myrtillus* L. and feather moss *Pleurozium schreberi* (Brid.) Mitt. (Bråkenhielm & Persson, 1980). The soil profile consists of a glaciﬂuvial sand podzol, a mor layer (pH 3.0) and a pale eluvial horizon overlying a rust-red illuvial horizon of the mineral soil (pH 4.4–4.8; Bringmark, 1980). The bedrock is composed of granites, sediments and volcanic rocks, which are widespread in Fennoscandia. Organic and mineral horizon soils were homogenised using 5 and 3 mm mesh sieves, respectively. Freshly fallen tree litter, stones, roots and lichens were all removed. Details of the elemental composition of O, E and B horizon soils (Marupakula *et al.*, 2017) are given in Supporting Information Table S1.

Two-compartment mesh bag preparation

To enable harvesting of clean mycelium, free of adhering soil particles (for analysis of elemental and isotopic composition), a two-compartment mesh bag system was developed (see Fig. 1a). The bags consisted of 50 μm nylon mesh with an inner compartment (5 × 4 cm) containing homogenised soil from the substrate the bag was embedded in (6 and 10 g for organic and mineral horizon soils respectively) and surrounded by an outer mesh bag (6 × 5 cm) containing 14 g of 1 mm borosilicate beads (Sigma-Aldrich Co.). These beads were washed with 1 M HCl overnight to eliminate any contamination occurring during manufacturing, rinsed with deionised water until the pH was neutral and dried at 100°C for 48 h. The two mesh bags were

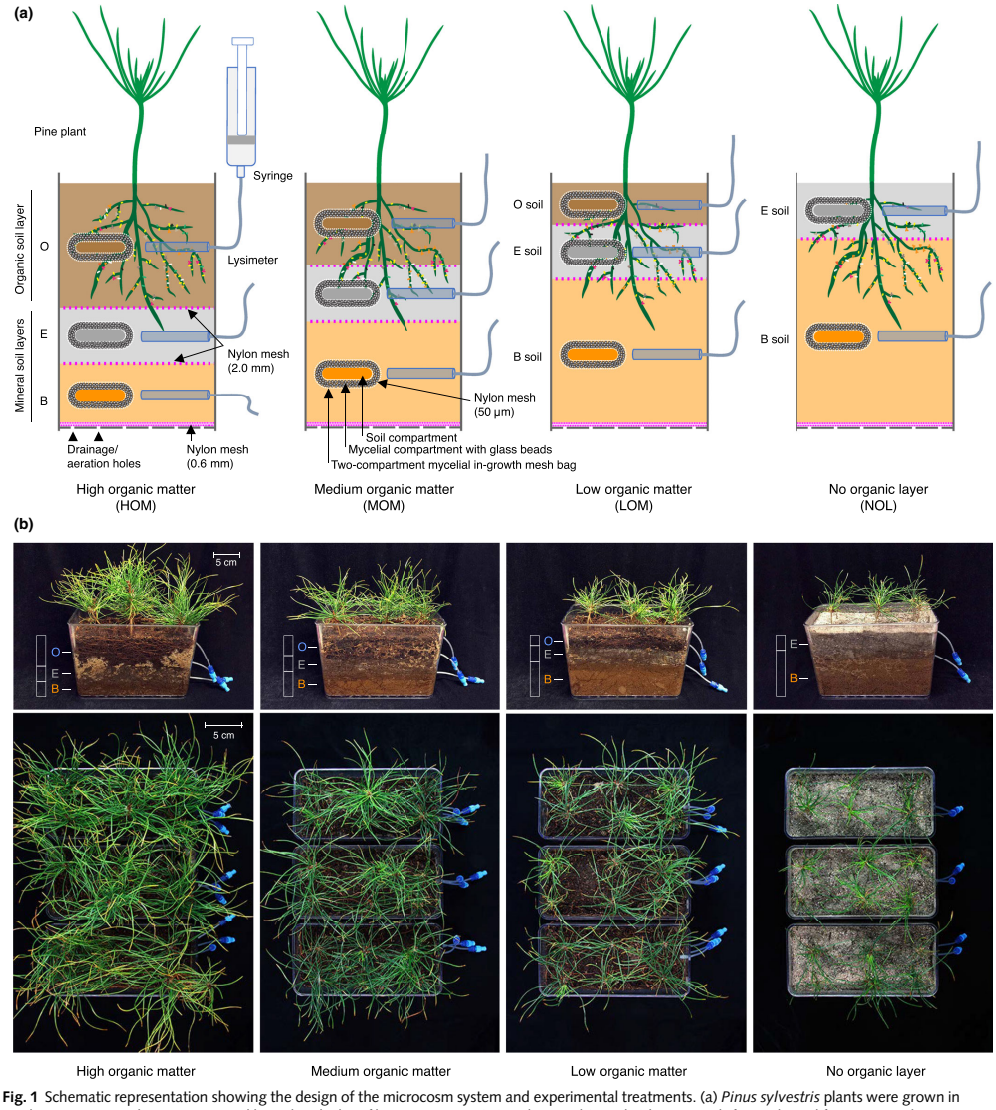

Fig. 1 Schematic representation showing the design of the microcosm system and experimental treatments. (a) *Pinus sylvestris* plants were grown in acrylic containers with a reconstructed boreal podzol profile using organic (O) and mineral (E and B) horizon soils from a boreal forest in Sweden. A gradient of organic matter (OM) availability was created by manipulating the relative amount of O horizon material: HOM, high organic matter; MOM, medium organic matter; LOM, low organic matter; NOL, no organic layer ($n = 3$). Control microcosms without plants were also set up. The O, E and B horizons were separated by 2.0 mm nylon mesh (to facilitate destructive sampling of roots and soils from each horizon at harvest). Lysimeters were inserted in each horizon for soil solution sampling. For harvesting of clean mycelium free of adhering soil particles, two-compartment mycelial in-growth mesh bags were designed – the inner mesh bag contained soil, and the outer 'mycelial compartment' contained glass beads allowing harvesting of clean mycelium. Two mesh bags were embedded in each soil horizon. There were drainage/aeration holes at the base of microcosms and two nylon mesh sheets were placed to prevent loss of soil through the holes. (b) Actual plant growth following 14 months of incubation in phytotron, showing the positive influence of increased amounts of OM.

heat-sealed, and the total weight was recorded. The rationale behind designing the two-compartment mesh bag system was that hyphae/mycelium, after entering the outer mesh bag compartment (containing glass beads), would continue growing towards the central mesh bag (containing soil) to capture nutrients. This approach is expected to result in mycelium with elemental concentrations/composition comparable to mycelium growing in contact with natural soil. Obviously, recovery of clean mycelium from the glass bead-compartment (without contaminating, adhering soil particles) is much easier and harvested mycelium can reliably be used for any downstream chemical/isotopic or molecular analyses.

Microcosms with reconstructed podzol horizons

Freshly sieved soil from the different horizons was transferred into 40 2.1 l (200 × 100 × 142 mm ($L \times W \times H$)) transparent acrylic containers. Two to three 5 mm holes were drilled in the narrow end of each container to allow for soil solution sampling – the number and orientation depending on treatment. Additional holes were drilled in the base for aeration and drainage. Four treatments were designed to create a gradient of OM availability with high, medium and low amounts of OM (HOM, MOM and LOM respectively). No organic layer (NOL) was present in the fourth treatment (see Fig. 1a). This was done by altering the volume of the O horizon. Eight microcosms were constructed per treatment, four containing six *Pinus sylvestris* plants each, and four controls without plants. Before the addition of 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. B horizon soil was then added in variable quantities to keep the overall soil volume constant, 250 g in HOM, 650 g in MOM, 1050 g in LOM and 1450 g in NOL treatments. A nylon mesh sheet (1.0 mm thick, 2.0 mm pore size) was placed on the surface of the B soil maintaining stratification and aiding harvesting (of roots and soil), followed by 800 g E horizon soil regardless of treatment. A second nylon mesh (1.0 mm thick, 2.0 mm pore size) was placed between the E and O horizon soil, which was added in variable quantities depending on treatment (600 g in HOM, 400 g in MOM, 200 g in LOM and 0 g in NOM). The combined total volume of O, E and B horizon soils was the same in every treatment. Two mesh bags and a Rhizon® pore water sampler (Rhizosphere Research Products, the Netherlands) were embedded in each soil layer, facilitating extraction of root/soil-free mycelium and soil solution respectively. Pine seedlings were planted at a standard depth (2–3 cm) either in O horizon (HOM, MOM, LOM treatments) or in E horizon (in the NOL treatment). In Fig. 1(a), the distribution of roots and shoots is schematic, showing that roots were able to grow through the mesh partitions, but does not show differences in growth or distribution related to treatments. Each microcosm was wrapped in aluminium foil and the soil surface was covered with a thick black plastic sheet to prevent exposure of developing mycelia to light and to prevent growth of algae and mosses. The water-holding capacity of each treatment combination of organic and mineral soil was estimated by the construction of four plant-free microcosms before the experiment. Microcosms were incubated in a phytotron for 14 months with a 250 µmol m^{-2} s^{-1} PAR 18 h : 6 h, light : dark cycle and day : night temperature of 18°C : 16°C. Soil moisture was kept constant by gravimetric watering with deionised water. Air moisture was maintained through natural evaporation from randomly placed plastic cups filled with deionised water. The positions of the microcosms were randomised on a weekly basis to reduce the impact of environmental differences within the phytotron.

$^{13}CO_2$ pulse labelling of plants

In the final month of incubation, three replicate microcosms of each treatment were randomly selected for $^{13}CO_2$ pulse labelling, and the remaining microcosms were moved to a separate plant growth room for $\delta^{13}C$ natural abundance measurements. The microcosms were placed in a transparent, airtight acrylic chamber and subjected to $^{13}CO_2$ pulse labelling. The chamber was equipped with fans to promote air circulation, and additional lights to support photosynthesis. Continuous exposure of plants to 99 atom% $^{13}CO_2$ (Cambridge Isotope Laboratories Inc., Andover, MA, USA) occurred in 8 h episodes over the course of 3 d, coinciding with the peak photoperiod. During these 8 h periods, total CO_2 concentration was maintained at an average 480 ppm – measured by an infra-red gas analyser (IRGA; EMG-4; PP Systems, Hitchin, UK). Upon depletion to 300 ppm, additional $^{13}CO_2$ gas was added by manual injection using an airtight syringe. Before the initial pulse of $^{13}CO_2$, plants were allowed to deplete the CO_2 to 200 ppm to promote a higher concentration of $^{13}CO_2$ within the chamber for the duration of the $^{13}CO_2$ exposure. The average rate of $^{13}CO_2$ assimilation by plants was 124 ml h^{-1}, determined by IRGA. Following the 3-d course of $^{13}CO_2$ pulse exposure, microcosms were incubated in a phytotron under normal conditions for a further week – a chase period – allowing assimilated ^{13}C to migrate through the plant to microbial associates in the soil in the form of photoassimilates. After this chase period, microcosms were harvested.

Harvesting of microcosms

Microcosms were harvested destructively. Details on sampling of plant, soil and mycelial compartments, and processing of materials for different analyses are provided in Methods S1. Soil solutions were extracted by centrifugation using modified 60-ml syringe barrels that had been acid washed. Soil samples were centrifuged at 10 000 ***g*** for 1 h at 4°C, and the extracted solutions were filtered with 0.45 µm sterile syringe filters before storing at −20°C. Rhizon® pore water samplers were also used for *in situ* sampling of solutions, but for the final destructive sampling, we used centrifugation. This decision was based on the rationale that ectomycorrhizal hyphae can grow into micropores of mineral particles and microsites of soil aggregates – these sites are generally inaccessible to roots – and are assumed to create hotspots of biogeochemical activity due to allocation of plant host derived C.

Chemical analysis of plants, soils, soil solutions and mycelium

Milled solid samples were digested in concentrated HNO_3, and concentrations of Ca, K, Mg, P, Mn and Sr were determined by inductively coupled plasma mass spectroscopy (ICP-AES). Soil solution pH was also measured.

Stable isotope analysis of ^{13}C, ^{15}N and ^{26}Mg

^{13}C isotope enrichment (or natural abundance), ^{15}N natural abundance, and C and N concentrations were measured in samples of shoots and roots (1.0 and 2.0 mg respectively), organic and mineral soils (1.0 and 6.0 mg respectively) following encapsulation of the materials in tin capsules (Elemental Microanalysis, Devon, UK). Soil solution samples from organic and mineral soils (100 and 500 µl respectively) were dried in tin capsules at 80°C for 2 d. Measurements were performed using an elemental analyzer (EuroEA3024; Eurovector, Milan, Italy) connected to a continuous flow Isoprime isotope ratio mass spectrometer (GV-Instruments, Manchester, UK).

Soil solutions were used for Mg isotope measurements. In brief, aliquots of soil solutions were analysed for pH and major cations (Ca, Mg, K and Na) by ICP-AES, and the remaining solution was evaporated to dryness. Residues were dissolved with 3 ml of concentrated HNO_3 and stored at 4°C until Mg isotope purification. Purified Mg (25–50 µg) samples from soil solution were obtained by ion chromatography using a combination of AGMP1-X8 anion-exchange resin (to eliminate trace elements Fe, Cu, Zn, etc.) with 7 M HCl and AG50W-X12 cation exchange resin (to eliminate major cations) with 1 M HNO_3 (Bolou-Bi *et al.*, 2016). The purified Mg solutions were evaporated to dryness and dried samples were diluted with 0.05 N HNO_3 at 200 ppb before analysis using multicollector-inductively coupled plasma mass spectroscopy (MC-ICP MS). Magnesium isotope ratios were measured using the standard-sample bracketing technique with DSM3 standard solution to correct for the instrument mass bias (Galy *et al.*, 2003).

DNA extraction, PCR amplification and high-throughput sequencing

DNA was co-extracted with RNA from soil samples using RNA PowerSoil Total RNA Isolation Kit and RNA PowerSoil DNA Elution Accessory Kit (MoBio Laboratories, Carlsbad, CA, USA), following the manufacturer's protocol. DNA from mycelium samples was extracted using DNeasy Plant Mini Kit (Qiagen) according to the manufacturer's recommendations. Mycelium was freeze-dried and homogenized by milling in 2 ml tubes, before DNA extraction. DNA concentration was measured by NanoDrop (ND-1000 NanoDrop Technologies, USA) and stored in PCR grade H_2O at −80°C until further processing.

The fungal ITS region was PCR amplified using the primers fITS9 (5′-GAACGCAGCRAAIIGYGA-3′; Ihrmark *et al.*, 2012) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′; White *et al.*, 1990). The ITS4 primer contained an 8-base barcode sequence unique for each sample. Details on PCR reagents and amplification conditions are described in Methods S1. The triplicate PCR products from each sample were pooled and cleaned with Agencourt AMPure Kit (Beckman Coulter, Beverly, MA, USA) according to the manufacturer's recommendations. DNA concentration was determined in each sample by Qubit dsDNA HS Assay using a Qubit Fluorometer (Invitrogen). All the PCR products from differently barcoded samples were pooled in equal concentrations and purified using the EZNA Cycle Pure Kit (Omega Bio-Tek, Norcross, GA, USA) and then eluted twice with 50 µl EB. The quality of the resulting pool and fragment size distribution were analysed using 50 Agilent DNA 7500 Kit with the Agilent 2100 Bioanalyzer system (Agilent Technologies, Santa Clara, CA, USA). The pooled sample was sequenced at SciLifeLab, NGI-Uppsala, Sweden, and underwent high throughput sequencing, using 3 SMRT cells of PacBio Sequel System (Pacific Biosciences, San Diego, CA, USA) according to the manufacturer's recommendations. The library preparation and adaptor ligation were also carried out at SciLifeLab, NGI-Uppsala, Sweden.

Bioinformatic and fungal functional guild analyses

In total, 1010 556 reads were obtained from PacBio sequencing of DNA from soil and mycelial samples, and after initial quality filtering and removal of chimaeras 516 998 reads remained. After removal of nonfungal sequences and singletons, 263 435 and 235 355 sequences were obtained from soil and mycelial samples respectively for downstream analysis. The sequencing data were analysed using the QIIME pipeline (Caporaso *et al.*, 2010). In brief, the command 'demultiplex_fasta.py' was used to demultiplex sequences based on barcodes and 'adjust_seq_orientation.py' to reverse complement sequences that remained unassigned. After combining both forward and reverse complemented reads in one file, the command 'split_libraries.py' was used to split libraries according to sample barcodes as listed in the mapping file. Chimeric sequences were identified using VSEARCH (Rognes *et al.*, 2016) in MOTHUR (Schloss *et al.*, 2009) and a UCHIME reference dataset (Nilsson *et al.*, 2015). The sequences were clustered into operational taxonomic units (OTUs) by UCLUST (Edgar, 2010) using the command 'pick_otus.py' with the *denovo* option, as implemented in QIIME, using a 97% sequence similarity criterion. The representative sequences of all OTUs were chosen with command 'pick_rep_sets.py' and an OTU table was constructed with command 'make_otu_table.py'. Individual sequences were classified taxonomically using the 'classify.seqs' command in MOTHUR (Schloss *et al.*, 2009; confidence threshold 80) using the UNITE fungal ITS reference database (Kõljalg *et al.*, 2013). Nonfungal OTUs were removed manually from the OTU table, and the singletons were removed in QIIME using command 'filter_otus_from_otu_table.py'. The filtered OTU table was rarefied using command 'single_rarefaction.py' and from this rarefaction curves were generated using command 'multiple_rarefactions.py'. All samples were rarefied to an equal number of sequences (4493) before further analysis. Taxa summary tables for downstream analyses of sequences were generated using 'summarize_taxa.py', and taxa plots were generated using 'summarize_taxa_through_plots.py'. For

functional guild-based annotation of the OTUs, all OTUs were parsed using FUNGuild tools (Nguyen *et al.*, 2016). The resulting data set contained the following dominant functional guilds: ectomycorrhizal, saprotroph, ericoid mycorrhizal, pathogen and some fungi were assigned to multiple guilds (e.g. ectomycorrhizal-ericoid mycorrhizal, ectomycorrhizal-saprotroph and pathogen-saprotroph). Fungi that could not be classified to any known guild (due to lack of sufficient ecological and/or taxonomic characterisation) were grouped as 'unassigned' and less abundant guilds were grouped as 'others'. All annotations were checked manually against published information on taxonomy, ecology and habitat of different fungal species.

Data analysis and statistics

Effects due to treatments on each parameter were evaluated by analysis of variance (ANOVA), linear regression analysis was used to assess relationship between pairs of selected parameters, and principal component analysis (PCA) was used to assess correlation among several parameters (Jmp Pro 15 statistical package). Using rarefied abundance data, beta diversity patterns were analysed by nonmetric multidimensional scaling (NMDS) ordinations with Bray–Curtis dissimilarity measure. The differences in fungal community composition in O, E and B horizon soils (or mycelial samples from each horizon) within a reconstructed podzol microcosm or in a particular horizon soil across the treatments were estimated using nonparametric analysis of similarity (ANOSIM) and permutational multivariate analysis of variance (PERMANOVA) with 9999 permutations (Past v.4.03 statistical package). The fungal taxa that differed significantly ($P < 0.05$) in relative abundance across the treatments were identified using the command 'group_significance.py' with ANOVA implementation in Qiime (Caporaso *et al.*, 2010).

Results

Increasing availability of OM significantly increased root, shoot and mycelial biomass (Figs 1b, 2a,b), with the largest and statistically strongest effect on mycelial biomass occurring in the mineral B horizon (Fig. 2b,c). There was a 121% increase in mycelial biomass in the treatment with high amounts of OM (HOM) compared with the treatment containing medium amounts of OM (MOM) and 45% of the total mycelial biomass was found in the B horizon. This total increase occurred although the volume of B horizon soil was only 38% of that in the MOM treatment, resulting in a mycelial density in the B horizon ($mg\ cm^{-3}$) that was 3.64 times higher in the HOM treatment than in the MOM treatment (Fig. S1a). Plant N content increased significantly in response to increased amounts of OM (Fig. 2d), suggesting that improved supply of N was driving plant growth. Apart from the fact that the O horizon contained 16 times as much N as the E and B horizons (Table S1), additional support for the idea that the O horizon was the source of the mobilised N is provided by differences in stable isotope signatures. Plants in HOM systems had more depleted $\delta^{15}N$ signatures, which were more like those of soil from the O horizons than those of the mineral horizons (Fig. S1b–d). Interestingly, the plant N use efficiency (shoot biomass production per unit N taken up) was positively related to the mycelial biomass (Fig. 2e), suggesting that plants with access to greater amounts of OM, in addition to more N, also had increased access to other growth-limiting nutrients (essentially base cations and P).

Pulse labelling of ectomycorrhizal *Pinus sylvestris* plants with $^{13}CO_2$ allowed us to monitor the effects of different OM availability on the allocation of recently photosynthetically derived C to plant roots, soil (mycelial biomass) and soil solution (Fig. 3). Increased amounts of ^{13}C were assimilated by the shoots of larger plants (Fig. 3a) and ^{13}C allocation to the soil and soil solution in the B horizon was significantly higher in the HOM systems (Fig. 3b,c). ^{13}C allocation was positively related to the higher mycelial biomass in the B horizon, but not in the O and E horizons (Fig. 3d,e). Moreover, a PCA showed that N use efficiency was strongly correlated with the amount of ^{13}C allocated to O and B horizon soil and soil solutions, with the patterns mainly driven by the HOM systems (Fig. 3f,g).

Correlations between plant base cation content (Fig. 4a) and P content (Fig. 4b) and mycelial biomass were highly significant for the B horizon, significant for the E horizon but not statistically significant for the O horizon. Similar relationships were obtained for the individual base cations Ca, K and Mg (Fig. S2), suggesting that the increased mycelial biomass produced in the mineral horizons, especially the B horizon, played a functional role in mobilising base cations and P that were supplied to the plants. The strong association of plant acquisition of Ca, K, Mg and P with both the mycelial biomass in, and ^{13}C allocation to, the B horizon (as depicted in the PCA; Fig. 4c,d) indicates the importance of C allocation to fungal mycelium for mineral weathering in deeper soil.

Further evidence that mobilisation in the B horizon contributes to improved acquisition of base cations, and Mg in particular, is provided by analyses of $\delta^{26}Mg$ (‰) signatures in the soil solution (Fig. 5). Ectomycorrhizal fungi have been reported to discriminate against heavier isotopes of Mg, such as ^{26}Mg, during Mg^{2+} uptake (Fahad *et al.*, 2016). Accumulation of ^{26}Mg in soil solution (increased $\delta^{26}Mg$ signature) is therefore a sign of active uptake of Mg^{2+}. In the B horizon, we found a significant positive relationship between soil solution $\delta^{26}Mg$ signature and plant/root biomass, plant Mg content and total ^{13}C in soil and soil solution (mycelial biomass was marginally non-significant), but no significant relationships in the O and E horizons (Fig. 5a–e). The higher amounts of ^{13}C and larger amounts of mycelium in the B horizon of HOM systems, coupled with the enrichment of ^{26}Mg in the B horizon soil solution, suggest that plants with access to greater amounts of OM could allocate more C to enhance mobilisation and uptake of Mg in the B horizon. Clear negative relationships between the $\delta^{26}Mg$ signature in soil solution and contents of Ca, Mg, Mn and Sr (Fig. S3a–f) indicated that accumulation of ^{26}Mg in solution was related to higher uptake of not only Mg, but also other elements. In systems without plants (Fig. S3g–l), there were no treatment effects in the B horizon, but some evidence

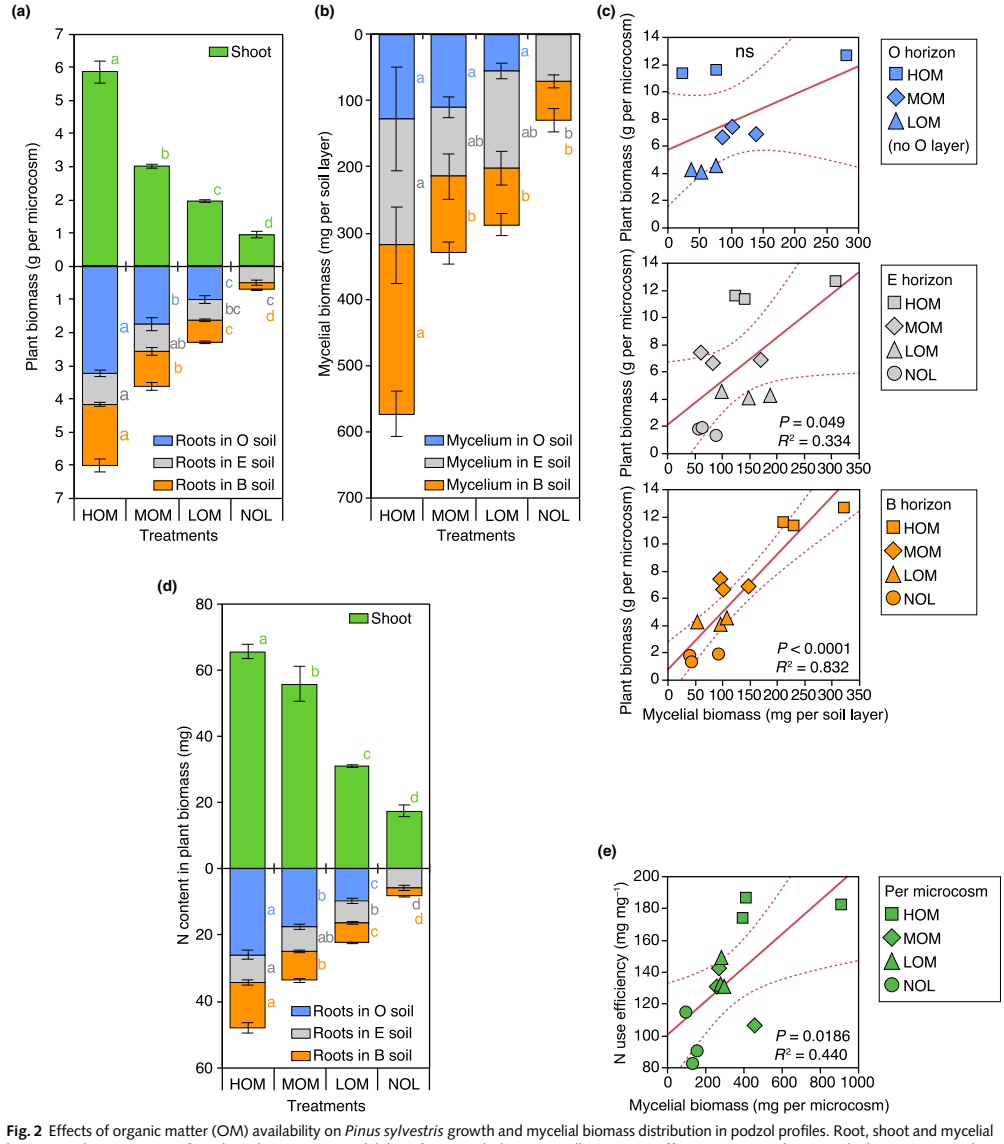

Fig. 2 Effects of organic matter (OM) availability on *Pinus sylvestris* growth and mycelial biomass distribution in podzol profiles. Root, shoot and mycelial biomass (a, b) increase significantly with increasing availability of OM, with the statistically strongest effect occurring in the mineral B horizon (c). Similar significant increases in plant nitrogen (N) content occur in response to increased amounts of OM (d). Nitrogen use efficiency (production of plant biomass per unit N taken up) is improved in plants with adequate access to OM since improved mycelial growth in mineral soil horizons enables access to additional resources (e) in the form of base cations and phosphorus. A gradient of OM availability was created by manipulating the relative amount of O horizon material: HOM, high organic matter; MOM, medium organic matter; LOM, low organic matter; NOL, no organic layer ($n = 3$). Error bars are ±SE and different letters denote differences among means ($P < 0.05$; ANOVA). Dotted lines (c, e) indicate 95% confidence intervals; ns indicates not significant.

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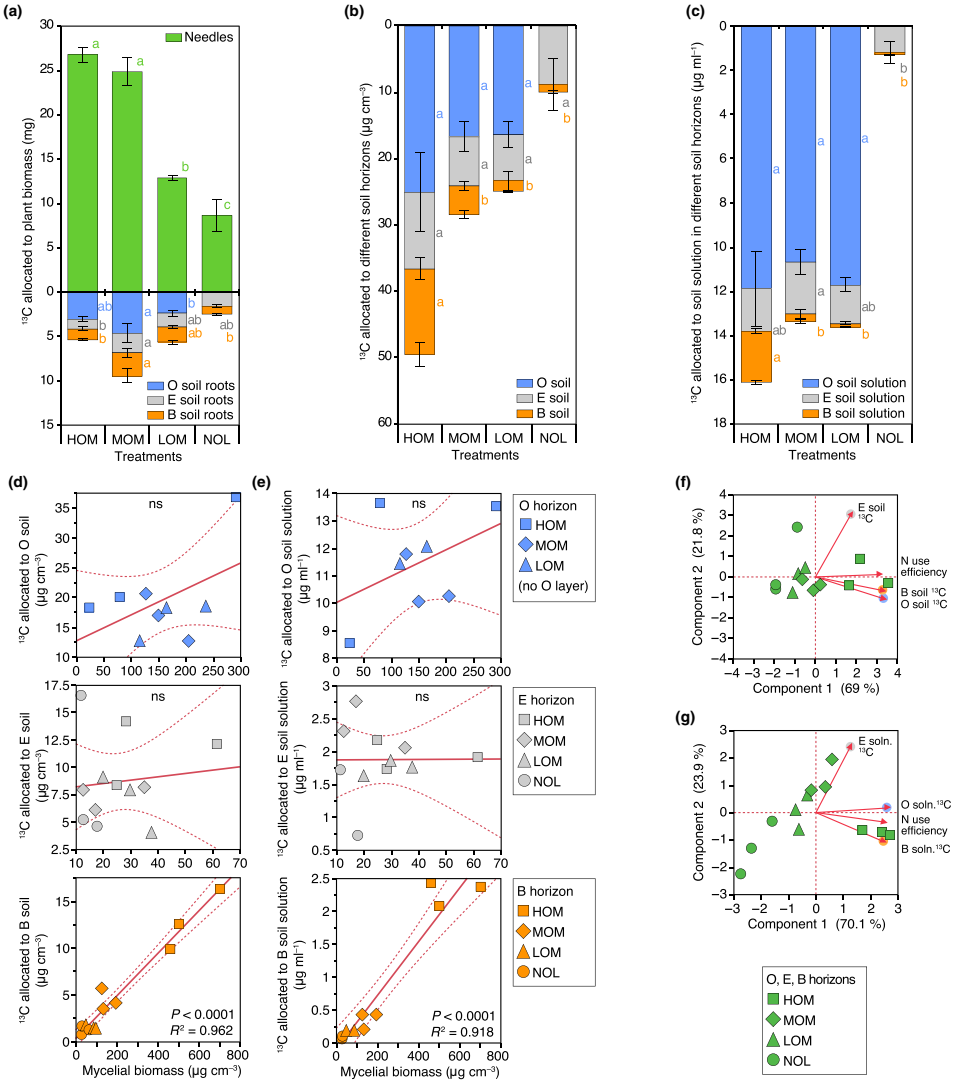

Fig. 3 Allocation of photosynthetically derived carbon to different plant and soil compartments and its relationship with mycelial biomass and nitrogen use efficiency. Ectomycorrhizal *Pinus sylvestris* plants (growing in podzol profiles with varying amounts of O horizon organic matter (OM)) were pulse labelled with $^{13}CO_2$. (a) Allocation of ^{13}C to plant shoots and roots in different soil horizons. (b) Allocation of ^{13}C to organic and mineral soil horizons colonised by mycelium. (c) Allocation of ^{13}C to soil solution in each soil horizon (containing ^{13}C-labelled mycelial and root exudates). (d, e) Linear regression relationships between ^{13}C allocated to soil and soil solution in each soil horizon and mycelial biomass (per cm^3), respectively. (f, g) Principal component analyses illustrate the association of treatment effects with variation in nitrogen use efficiency and ^{13}C content in soil (f) and soil solution (g) in organic and mineral soil horizons. A gradient of OM availability was created by manipulating the relative amount of O horizon material: HOM, high organic matter; MOM, medium organic matter; LOM, low organic matter; NOL, no organic layer ($n = 3$). Histograms (a–c) show mean values (error bars are ±SE), and different letters denote significant treatment differences within compartments ($P < 0.05$; ANOVA). Dotted lines (d, e) indicate 95% confidence intervals; ns indicates not significant.

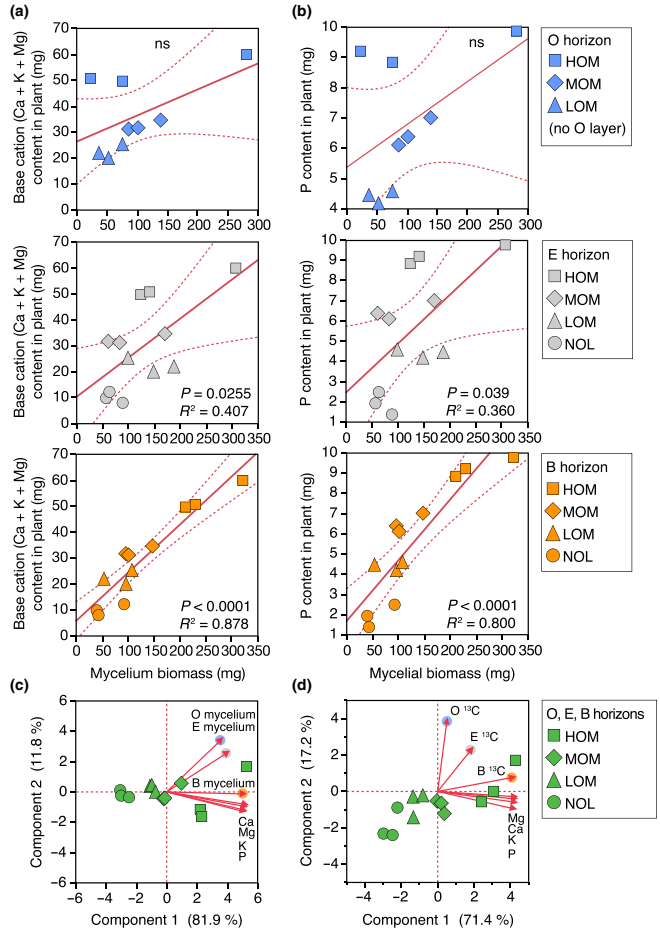

Fig. 4 Positive effects of increasing organic matter (OM) availability on mycelial biomass, carbon allocation and acquisition of base cations and phosphorus by ectomycorrhizal *Pinus sylvestris* plants growing in podzol profiles with varying amounts of O horizon OM. Acquisition of base cations (Ca + K + Mg) (a) and phosphorus (b). Dotted lines (a, b) indicate 95% confidence intervals; ns indicates not significant. Effects are highly significant in the mineral B horizon, less significant in the mineral E horizon but not statistically significant in the O horizon. Principal component analyses show highly significant treatment variation in mycelial biomass (c) and ^{13}C in soil plus soil solution (d) in the B horizon, associated with changes in plant content of Ca, Mg, K and P. A gradient of OM availability was created by manipulating the relative amount of O horizon material: HOM, high organic matter; MOM, medium organic matter; LOM, low organic matter; NOL, no organic layer ($n = 3$).

of nutrient mobilisation by saprotrophic fungi in the O horizon that was proportional to the amount of OM. Elemental analysis of soil samples (Table S1) suggests that significant pools of base cations (in particular Ca) and P exist in the O horizon. However, the $\delta^{26}Mg$ signatures in soil solution (Figs 5, S3a–f) and the proportional distribution of the solubilised elements (Table S2), suggest that it is the elements efficiently removed from the solution in the B horizon, not the O horizon, that accumulated in the plant biomass.

Overall, 704 fungal taxa were distinguished. Nonmetric multidimensional scaling ordinations of fungal community composition in the O, E and B horizons (Figs 6, S4a) illustrate strongly significant statistical differences between soil horizons, as well as significant treatment effects in both the O and B horizons. The changed community composition in the B horizon was particularly strongly associated with variation in plant contents of Ca, Mg, K, N and P, as indicated by the lengths of the vectors (Fig. 6a). Linear regression analyses showed a significant positive relation between total plant biomass and the relative abundance of the ectomycorrhizal guild (Fig. 6b) in the B horizon, while the saprotrophic guild in the same horizon (Fig. 6c) showed a negative relation with plant biomass. The occurrence of fungal guilds in other horizons showed no relation to plant biomass. The contrasting relationships of plant biomass with the relative abundance of ectomycorrhizal and saprotrophic fungi in the B horizon are most likely explained by competition between the

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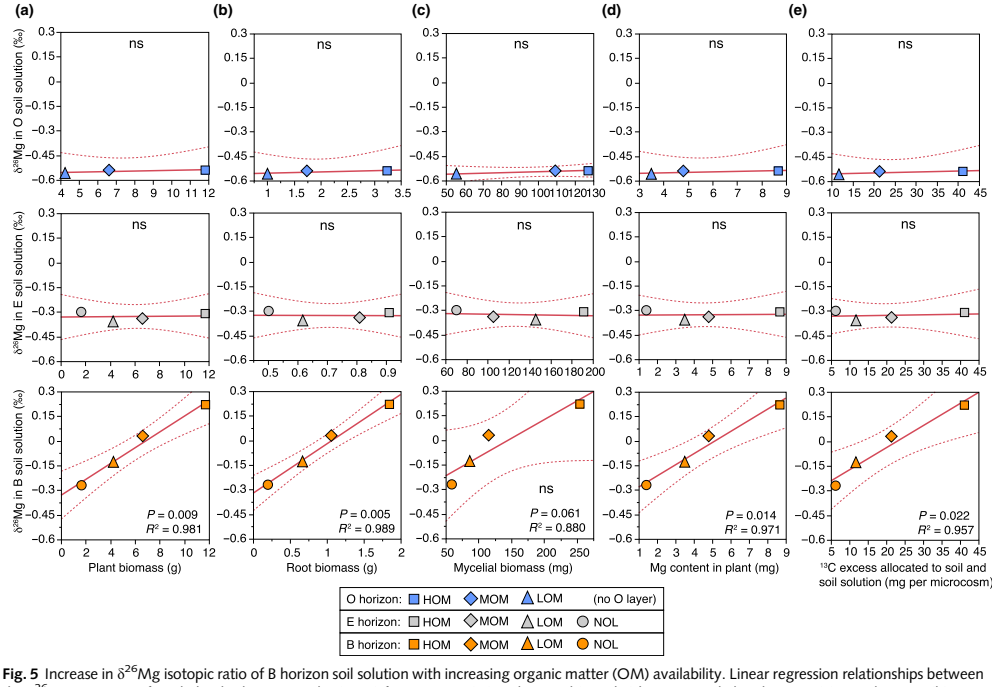

Fig. 5 Increase in $\delta^{26}Mg$ isotopic ratio of B horizon soil solution with increasing organic matter (OM) availability. Linear regression relationships between the $\delta^{26}Mg$ signature of pooled soil solution samples ($n = 3$) from organic (O) and mineral (E and B) horizons and plant biomass (a), root biomass (b), mycelial biomass (c), Mg content in plant biomass (d) and ^{13}C content in soil and soil solution (e) demonstrate statistically significant positive relationships for all parameters in the B horizon, except mycelial biomass, which was marginally nonsignificant ($P < 0.061$). The data suggest that the positive effects of increasing OM availability on the parameters caused increased plant growth, carbon allocation and mineral weathering, leading to increased Mg uptake. The relationships are not significant in the O and E horizons. Ectomycorrhizal *Pinus sylvestris* plants were grown in podzol profiles with a gradient of OM availability that was created by manipulating the relative amount of O horizon material: HOM, high organic matter; MOM, medium organic matter; LOM, low organic matter; NOL, no organic layer. Dotted lines indicate 95% confidence intervals; ns indicates not significant.

two guilds, since available C in OM is low in the B horizon, but ectomycorrhizal fungi can receive C allocated from their plant hosts. The high relative abundance of ectomycorrhizal fungi in the B horizon, particularly in the HOM treatment where C allocation is increased, suggests that the base cation and P mobilisation is driven primarily by ectomycorrhizal fungi. The 25 most abundant taxa in soil accounted for *c.* 90% of all sequences, and the impact of OM reduction in the O horizon was strongest on relative abundance of these taxa in the mineral soil, in which 14 and 7 taxa were significantly impacted in the B and E horizons respectively, and only three taxa were significantly influenced in the O horizon (Fig. S5). This suggests that fungi in deeper mineral soil are more sensitive to changes in OM than taxa in the top organic horizon. The ectomycorrhizal species *Piloderma sphaerosporum* was the most abundant species in the O horizon, while *Suillus bovinus* was the most abundant in the E and B horizons (Fig. S5). Two-compartment mesh in-growth bags were inserted into each soil horizon for mycelial biomass determination. These were intensively colonised by 441 fungal taxa in total, dominated by *P. sphaerosporum* and *S. bovinus*, with the ectomycorrhizal fungal guild accounting for over 80% of the analysed amplicons (Fig. S6). Total plant contents of Ca, K, Mg and P were positively correlated with the proportional abundance of ectomycorrhizal amplicons in the B horizon (Fig. S7). The proportional abundance of ectomycorrhizal amplicons in the B horizon was also negatively correlated with the soil solution pH (Fig. S8).

Discussion

Human appropriation of net primary production is increasingly being driven by changes in land use intensity rather than changes in land use area (Kastner *et al.*, 2021) and intensification of forestry is no exception to this trend. Soil OM is generally assumed to be important to forest productivity, but its direct influence has been difficult to demonstrate because it is both a cause and effect

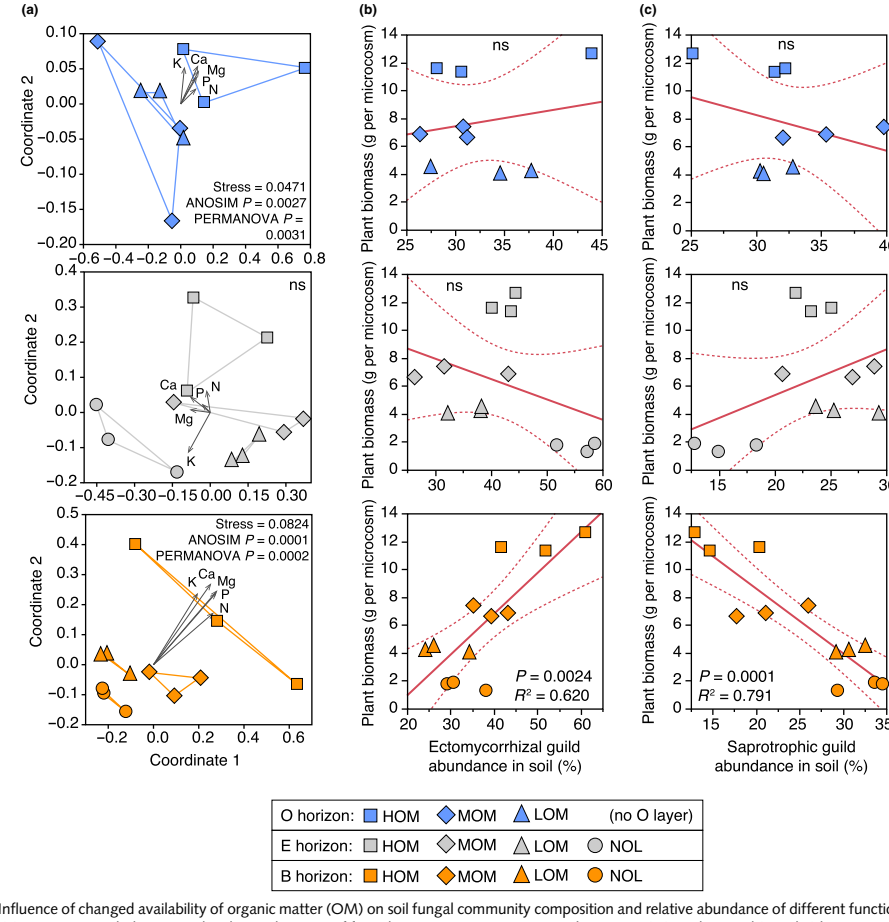

Fig. 6 Influence of changed availability of organic matter (OM) on soil fungal community composition and relative abundance of different functional guilds. (a) Nonmetric multidimensional scaling ordinations of fungal community composition in the organic (O), and mineral (E and B) horizons of podzol profiles in relation to changes in OM availability from high organic matter (HOM) to medium organic matter (MOM) to low organic matter (LOM) to no organic layer (NOL) ($n = 3$). Significant treatment differences ($P < 0.05$) occur in the O and B horizons, and the arrow lengths indicate the strength of the contribution of different elements to the overall treatment differences (strongest in the B horizon). (b, c) Linear regression relationships between total plant biomass and the relative abundance of the ectomycorrhizal and saprotrophic fungal guilds, respectively. There is a highly significant positive relationship for the ectomycorrhizal guild and a highly significant negative relationship for saprotrophs in the B horizon, but no significant relationships in other horizons. Dotted lines indicate 95% confidence intervals; ns indicates not significant.

of productivity and the relationship is complex and site-specific (Grigal & Vance, 2000). The present study provides novel evidence, based on fractionation of stable isotopes, of ectomycorrhizal weathering and uptake of naturally occurring base cations from mineral forest soil by indigenous fungal communities. This weathering is driven by sinks generated by ectomycorrhizal mobilisation of N from the organic horizon, and our experiments show clearly how changes in OM availability in the O horizon influence fungal C allocation to deeper mineral horizons, as well as the abundance of different functional fungal guilds. Ectomycorrhizal fungi play an important role in mobilising N from organic substrates and our results support the well-established idea that photosynthetically derived C transferred to the ectomycorrhizal fungal symbionts drives this process (Bending & Read, 1995; Rineau *et al.*, 2013; Shah *et al.*, 2016; Nicolás *et al.*, 2018).

Selective allocation of photosynthetically derived C through ectomycorrhizal fungal networks to patches of added minerals has been shown in experimental microcosms inoculated with single fungal species (Rosling *et al.*, 2004; Smits *et al.*, 2012) and shown to increase the weathering of apatite and subsequent uptake of P by plants (Smits *et al.*, 2012). However, there are few mechanistic studies of mycorrhizal weathering in natural soils. Uptake and transport of Mg through fungal hyphae to *Picea abies* seedlings inoculated with the mycorrhizal fungus *Paxillus involutus* have been shown in sand-filled microcosms supplied with ^{25}Mg-labelled nutrient solution (Jentschke *et al.*, 2000), but the present experiment demonstrates the capacity of indigenous fungal communities to mobilise naturally occurring Mg from mineral soil and transport it to tree seedlings. The higher relative proportion of base cations and P remaining in the O horizon solution (Table S2) are consistent with previous studies in which accumulation of organic P has been reported in O horizon/humus layer of boreal forests (Giesler *et al.*, 2002, 2004; Vincent *et al.*, 2022), and different geochemical explanations have been proposed. Both Al and Fe appear to have a stabilising effect that protects organic P from microbial degradation (Vincent *et al.*, 2012), and this could be one effect slowing mycelial uptake from this horizon. We speculate that even though the base cations and P are in a dissolved state in the O horizon solution, they may be bound in relatively recalcitrant organic complexes requiring more intensive enzymatic degradation of dissolved OM. High competition with saprotrophs in the O horizon may also restrict successful competition for, and removal of P from soil solution by ectomycorrhizal fungi. In the present study, the soil solution pH was negatively correlated with the proportional abundance of ectomycorrhizal amplicons in the B horizon to the (Fig. S8) suggesting that the mobilisation and uptake of base cations and P may have been associated with acidification due to secretion of organic acids by the predominantly ectomycorrhizal fungi colonising that horizon (Van Hees *et al.*, 2005). This result is also consistent with pure culture studies demonstrating the superior ability of ectomycorrhizal fungi, compared with saprotrophic species, to mobilise Mg from granite particles (Fahad *et al.*, 2016). The improved access to base cations and P, enabled by the increased mycelial colonisation of the B horizon soil may explain the improved N use efficiency of plants in treatments with greater OM availability.

The way in which C allocation through ectomycorrhizal mycelial networks integrates N mobilisation in the uppermost O horizon and mineral weathering in the deeper B horizon is summarised in Fig. 7. Our results demonstrate that weathering of base cations in the B horizon can be upregulated in response to demand induced by improved N supply from the O horizon. In both processes, C allocation from the host plant to mycorrhizal fungi plays an essential role. Conversely, it is apparent that, where

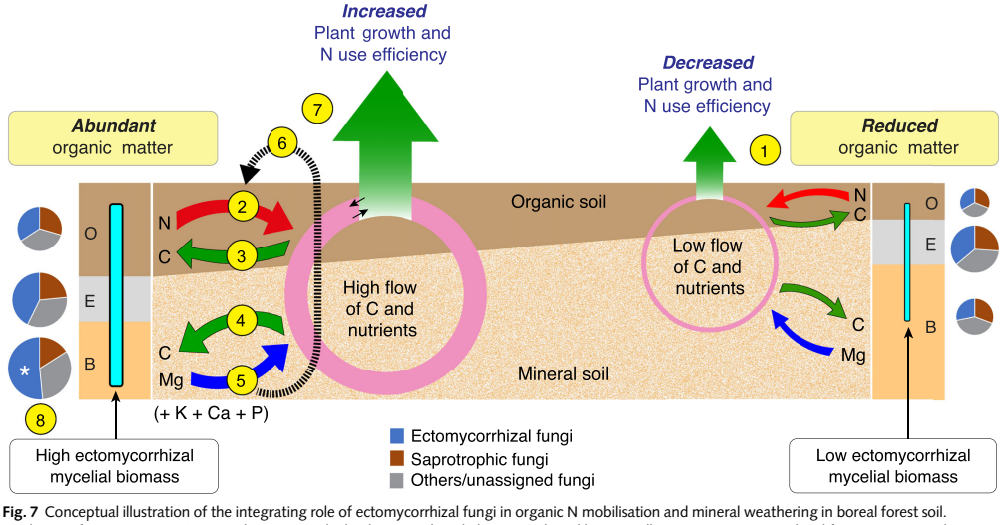

Fig. 7 Conceptual illustration of the integrating role of ectomycorrhizal fungi in organic N mobilisation and mineral weathering in boreal forest soil. Depletion of organic matter (OM) reduces N supply, leading to reduced plant growth and lower C allocation to ectomycorrhizal fungi, restricting their ability to mobilise base cations from the mineral soil (1). Increased availability of OM improves N supply (2), increasing plant growth and C allocation to ectomycorrhizal fungi (3, 4), enabling them to mobilise increased amounts of Mg from the B horizon soil (5). The increased supply of Mg (and other base cations and P) improves the N use efficiency of the plants (6) – demonstrating how ectomycorrhizal fungi integrate N mobilisation and weathering across soil horizons to improve plant growth (7). The pie charts (8) indicate the proportional abundance of fungal amplicons in different categories and their diameter is proportional to total fungal biomass, demonstrating that, when there is abundant OM, the increased mycelial biomass in the B horizon is primarily due to the ectomycorrhizal guild (indicated by the asterisk).

N supply is limited by low availability of OM, supply of base cations may be more limited by reduced weathering of minerals from deeper horizons rather than by the reduced stocks of base cation containing organic residues *per se*. Measured amounts of Mg in litterfall are small in relation to tree uptake (Helmisaari, 1995) and suggest that the mineral soil weathering potential demonstrated in the present study is crucial for Mg availability. This is underlined by the rapid uptake of Mg from soil solution in the B horizon (Table S2).

Terrestrial weathering of silicates, leading to production of marine carbonates, is thought to have driven long-term sequestration of C, leading to drawdown of global CO_2 levels (Berner, 1997) and enhanced rock weathering (ERW) has been suggested as a possible method to increase sequestration of atmospheric C as part of an integrated strategy to mitigate climate change through CO_2 reduction (Taylor *et al.*, 2016; Beerling *et al.*, 2018, 2020). Our results suggest that enhanced weathering will require adequate N supply from organic substrates, or some other source that does not reduce belowground C allocation, since N fertilization with inorganic fertilisers can reduce the belowground flux of C to soil biota, including ectomycorrhizal fungi (Högberg *et al.*, 2010). Repeated harvesting of organic residues from forests as biofuel may reduce the density of mycorrhizal roots (Mahmood *et al.*, 1999) and the results of the present study suggest that colonisation of deeper mineral soils may also be disrupted, with concomitant effects on base cation supply. Such negative effects may be counteracted by site preparation methods that increase the amount of OM around the roots and have been shown to have long-term (30 yr) positive effects on plant survival and tree production (Hjelm *et al.*, 2019).

Ectomycorrhizal fungi have been postulated to play an important part in weathering, and even in pedogenesis (Leake & Read, 2017), but there are few detailed studies of mycorrhizal weathering using natural soils. Microcosm studies with added minerals and microscopic investigation of mineral surfaces suggest that ectomycorrhizal fungi can modify mineral surfaces (Quirk *et al.*, 2012) and that allocation of C to the fungal mycelium colonising in-growth cores filled with basalt is related to the rate of calcium silicate dissolution from the basalt (Quirk *et al.*, 2014). The fungi in the latter experiment were not identified but enhanced weathering of added apatite patches and uptake of mobilised P have been shown in microcosms using *P. sylvestris* seedlings inoculated with the ectomycorrhizal fungus *Paxillus involutus* and growing under axenic conditions (Smits *et al.*, 2012). In our study, we found that high availability of N from OM increased plant C allocation to mycorrhizal mycelial biomass in deeper mineral horizons, resulting in increased mobilisation of Mg, Ca, K and P, and we identified the fungal communities involved, but we did not quantify long-term C sequestration. The ectomycorrhizal taxa *P. sphaerosporum and S. bovinus* that were dominant in organic and mineral horizons respectively, are common in boreal forest soils (Marupakula *et al.*, 2017, 2021). Some *Suillus* spp. have been found to play a role in weathering of apatite, biotite or granite (Wallander *et al.*, 2003; Balogh-Brunstad *et al.*, 2008; Fahad *et al.*, 2016) while *Piloderma* spp. have been reported to produce extracellular proteases and can improve the ability of *Pinus sylvestris* to utilise N from organic sources such as proteins (Heinonsalo *et al.*, 2015) and can also mobilise base cations and P from granite (Fahad *et al.*, 2016). However, many of these studies are based on pure culture systems, and generalisations about the functional role of individual species should be made with caution, since a high degree of phenotypic plasticity can be exhibited under different environmental conditions.

The climate mitigation effects of enhanced weathering of silicates are likely to be influenced by different biological processes (Vicca *et al.*, 2022). Symbiotic N fixation has been demonstrated to drive mobilisation of rock-derived nutrients (Perakis & Pett-Ridge, 2019), and it is likely that the effect we demonstrate here involving N mobilisation by ectomycorrhizal fungi will be even bigger since the mobilisation of N from organic substrates by ectomycorrhizal fungi is a fundamental and well-documented process driving tree growth (Lindahl & Tunlid, 2015). Interestingly, the short-term changes in availability of OM in our study appear to have the same effect as the long-term changes, including the evolution of larger, better developed mycelial systems and build-up of OM, that have been postulated to drive the development of biological weathering over evolutionary time (Leake & Read, 2017; Finlay *et al.*, 2020). Belowground C allocation to the rhizosphere leads to formation of stable soil C more efficiently than aboveground inputs of C (Sokol & Bradford, 2019), and associations between microbially derived OM and minerals form a large pool of slowly cycling C (See *et al.*, 2022). See *et al.* (2022) argue that there is a need for more spatially explicit information on environmental drivers of fungal mycelial exploration since fungi have a strong influence on the amounts and type of C deposited on minerals. The present study demonstrates increased mycelial C allocation to deeper mineral soil, and interactions between plant-derived C compounds and mineral soil leading to mobilisation of base cations, but further studies are required to examine the precise mechanisms involved, and the long-term stability of C ultimately associated with the minerals. Although weathering of minerals, not stabilisation of OM, was the primary focus of the present study, our results do provide new, spatially explicit information on the distribution, species composition and patterns of C distribution in ectomycorrhizal fungal mycelia colonising boreal forest soils, as well as new information on factors that drive C allocation, mycelial growth, fungal mobilisation- and plant uptake of mineral nutrients.

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to the life, work and memory of Prof. Torgny Unestam (1931–2023), friend, mentor and colleague at SLU.

Competing interests

None declared.

Author contributions

SM and RDF conceived the original idea. SM designed and constructed the microcosms. ZF and SM performed the experiment. ZF processed samples for chemical/isotopic analyses and collected the data. SM and ZF performed the statistical analyses with input from RDF. SM performed the molecular and bioinformatic analyses, with input from KK. EBB-B performed the ^{26}Mg analyses, with input from SJK and KB. AE performed the ^{13}C and ^{15}N analyses. SM and RDF supervised ZF and KK. SM, AE and RDF drafted the manuscript with input from all other authors.

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Data availability

The PacBio sequencing data files have been submitted to the European Nucleotide Archive (ENA) at EMBL-EBI (www.ebi.ac.uk) and are available under the study accession no. PRJEB59040. Experimental data of this study are available via figshare (doi: 10.6084/m9.figshare.22012829).

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Effects of organic matter availability on mycelial biomass and $\delta^{15}N$ signatures of plants and soils.

Fig. S2 Positive effects of increasing organic matter availability on mycelial biomass and the subsequent positive effect on acquisition of Ca, K and Mg.

Fig. S3 Variation in the $\delta^{26}Mg$ isotopic ratio of soil solution in organic and mineral soil horizons in relation to soil solution content of different elements.

Fig. S4 Fungal community composition of organic and mineral horizon soils and mycelial in-growth mesh bags.

Fig. S5 Influence of different availability of organic matter on fungal taxa, saprotrophic and ectomycorrhizal guild interactions, and the role of ectomycorrhizal fungi in base cation and P mobilisation in mineral B horizon soil.

Fig. S6 Effects of changing soil organic matter availability on relative abundance of fungal taxa and functional guilds in in-growth mesh bags.

Fig. S7 Relationships between elemental contents of plants and the abundance of ectomycorrhizal and saprotrophic guilds in soil.

Fig. S8 Relationships between pH of soil solution and the relative abundance of ectomycorrhizal and saprotrophic guilds in soil.

Methods S1 Harvesting of microcosms; PCR amplification conditions.

Table S1 Elemental composition of soil samples from each of the organic (O), eluvial (E) and illuvial (B) horizons in a boreal forest podzol profile.

Table S2 Total and proportional (%) distribution of solubilised elements in plant biomass, mycelial biomass and soil solution in microcosms containing *Pinus sylvestris* plants growing in a reconstructed boreal forest podzol.

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Transporter gene family evolution in ectomycorrhizal fungi in relation to mineral weathering capabilities

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Abstract. The role of ectomycorrhizal (ECM) fungi in biological mineral weathering is increasingly recognised, although the quantitative significance of microbially mediated mineral dissolution for plant growth is debated. Species within the ECM genus *Suillus* are found to preferentially inhabit mineral soils and are frequently reported to possess mineral weathering capabilities. Though studies growing ECM fungi with minerals have shown heightened mycelial nutrient content compared to growth without minerals, the mechanistic understanding of nutrient mobilisation and uptake associated with weathering remains largely unknown. We examined copy numbers of 173 transporter gene families present in 108 Agaricomycetes species, and analysed evolutionary expansions and contractions of base cation transporter gene families across the phylogeny. We also quantified mycelial base cation uptake by ECM species in the genera *Suillus* and *Piloderma*, and two saprotrophic fungi, when grown in pure culture with and without minerals. We hypothesised that 1) greater base cation uptake is dependent on evolutionary expansions in copy-numbers of base cation transporter genes, 2) mineral weathering results in base cation uptake by *Suillus* growing in pure culture with mineral additions, 3) base cation uptake by *Suillus* growing in pure culture with mineral additions will be greater compared to other fungal species, and 4) base cation uptake will correlate with base cation transporter gene family copy numbers. We showed that 25 transporter gene families are significantly expanding and contracting in the genus *Suillus*, 10 of which correspond to base cation transporter families and two of which are accessories to base cation transport, highlighting the importance of base cation uptake and transport in the life strategy of *Suillus* species. Additionally, there are significant expansions in the Fungal Mating-type Pheromone Receptor (MAT-PR) Family in *Suillus* species, suggesting that these species are adapting to their environment. For all elements there were examples of higher mycelial concentrations after growth in pure culture with mineral additions as compared to the nutrient limited treatment for *Suillus* species but also for some *Piloderma* species, confirming that mineral weathering resulted in base cation uptake in pure culture. For 40% of the significantly expanding and/or contracting base cation gene transporters families, copy numbers were significantly correlated with uptake of mineral elements, and most of

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the significant correlations were positive. This suggested that members of the genus *Suillus* are rapidly evolving, and that the expansions and/or contractions in these transporter families are likely to be related to the requirement of base cation uptake, underpinned by the finding that mycelial uptake of elements in the presence of minerals often increased with base cation transporter family copy numbers. Further work into other levels of regulation, e.g. through transcriptional regulation of transporter proteins, would be useful to gain a deeper understanding of base cation uptake regulation and its role in mineral weathering by *Suillus* species, and ECM fungi in general.

1 Introduction

In boreal forests biological mineral weathering is driven by plant-associated ectomycorrhizal (ECM) fungi, which form the dominant type of symbiosis, primarily with trees of the family Pinaceae (Smith and Read, 2008). The ECM symbiosis plays key roles in plant nutrient acquisition, carbon (C) partitioning and biogeochemical cycles by exchanging nutrients mobilised by ECM fungi from the soil with photosynthetically derived C from the host plant (Clemmensen et al., 2013; Genre et al., 2020; Van Der Heijden et al., 2015). Essential mineral nutrients, or base cations, and phosphorus (P) and nitrogen (N) are primarily mobilised by mineral weathering and decomposition of organic matter, respectively (Finlay et al., 2020; Hoffland et al., 2004; Tunlid et al., 2022). Boreal forest soils are stratified podzols, with an overlying organic (O), a middle mineral eluvial (E), and a typically thicker underlying mineral illuvial (B) soil horizon, which form due to low temperatures and pH, recalcitrant plant litter, slow decomposition rates, and the absence of earthworms resulting in a lack of mixing (van Breemen et al., 2000; Lundström et al., 2000). The O horizon is the main source of N (Hobbie and Horton, 2007) and holds the greatest biological activity, with the highest density of short roots, and ECM mycelial biomass in similar quantities to that of fine roots (Wallander et al., 2001). ECM fungi dominate the soil fungal communities both in the fermentation and humus layers of the O horizon, and in the E and B horizons (Bödeker et al., 2016; Lindahl et al., 2007; Marupakula et al., 2021). Mobilisation of base cations via mineral weathering by ECM fungi occurs mainly in the E and B horizons (van Breemen et al., 2000; Jongmans et al., 1997; Mahmood et al., 2024) and even though fine root density is lower compared to the O horizon, due to the large volume the mineral soil contains up to two thirds of ECM root tips, up to half of all ECM taxa across a boreal forest profile, and many ECM taxa which are not found elsewhere (Rosling et al., 2003). Although decomposition of organic matter to acquire N and mineral weathering to acquire base cations predominantly occur separately in discrete soil horizons, these processes are integrated. Mahmood et al., (2024) demonstrated that weathering in the mineral soil was driven by sinks generated by ECM mobilisation of N from the O horizon, and that changes in organic matter availability in the O horizon influenced fungal C allocation to deeper mineral horizons, as well as the abundance of different functional fungal guilds. Weathering of base cations in the B horizon can be upregulated in response to demand induced by improved N supply from the O horizon to both the plant and the ECM fungus, but when organic matter is depleted, both N acquisition and mineral weathering rates are reduced. Mahmood *et al.*, (2024) also showed that mobilisation and uptake of

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magnesium (Mg), which acts as an activator for many enzymes and plays a key role in photosynthesis (Black et al., 2007; Bose et al., 2011), occurred primarily in the deeper mineral horizon and was driven by carbon allocation to ECM mycelium. Intense forestry practices and the increasing demand for bioenergy result in a reduction of organic matter supplied to the O horizon and the return of nutrients, like N, P and base cations, to the soil (Akselsson et al., 2007, 2019; Klaminder et al., 2011; Moldan et al., 2017). These practices are suggested to be unsustainable for long-term plant nutrition, to alter fungal communities and to negatively impact mineral weathering rates and base cation supply (Finlay et al., 2020; Joki-Heiskala et al., 2003; Rähn et al., 2023). Therefore, a better understanding of mineral weathering, base cation uptake and the key role ECM fungi play in these processes is essential in the development of more sustainable forestry practices.

Fungi can mobilise base cations from mineral soils by physical force and extrusion of low molecular weight organic acids (LMWOAs), free radicals, protons and siderophores, which consort together to weather minerals (Finlay et al., 2020; Fomina et al., 2010; Schmalenberger et al., 2015). In particular, LMWOAs are known to play a significant role and mineral weathering by ECM fungi has been demonstrated in a number of studies (Calvaruso et al., 2013; Drever and Stillings, 1997; Paris et al., 1996; Schmalenberger et al., 2015; van Scholl et al., 2006; Wallander, 2000). Members of the genus *Suillus*, many of which grow well in culture and are commonly used in experiments, have been frequently reported to perform mineral weathering and are found primarily in the B horizon in boreal forests (Lofgren et al., 2024; Mahmood et al., 2024; Marupakula et al., 2021). In the presence of minerals, several *Suillus* species have been observed to increase production and exudation of LMWOAs, a pattern not seen in the absence of minerals (Adeleke et al., 2012; Olsson and Wallander, 1998; Wallander and Wickman, 1999). *Suillus* species have also been shown to take up base cations, for example potassium (K^+), magnesium (Mg^{2+}) and iron (Fe^{2+}) was taken up by *S. tomentosus* growing in liquid culture with the mineral biotite (Balogh-Brunstad et al., 2008a), and *S. variegatus* was shown to take up Mg^{2+} in pure culture with granite rock (Fahad et al., 2016). Mahmood *et al.*, (2024) found that Mg^{2+} was taken up from B horizon soil solution in reconstructed podzol microcosms containing *Pinus sylvestris* seedlings growing in natural forest soils in which *S. bovinus* was the most abundant taxon. Other mineral weathering ECM species include *Paxillus involutus*, which when grown in pure culture has been shown to weather biotite (Bonneville et al., 2009), chlorite (Gazze et al., 2012), muscovite (Van Scholl et al., 2006), and phlogopite (Paris et al., 1995, 1996), and *Pisolithus tinctorius* which has been shown to increase mineral weathering rates when in symbiosis with *Pinus resinosa* seedlings (Balogh-Brunstad et al., 2008b). These previous studies however, relied on abundance data and chemical analyses of ECM fungal biomass, plant biomass, or growth medium of experimental systems, and did not focus on the mechanism or regulation of base cation uptake.

Regulation of base cation uptake through transporter proteins can occur at the genomic, transcriptional, post-transcriptional, translational and post-translational level (Mukhopadhyay et al., 2024). One mechanism of regulating transporter protein

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expression is copy number variation of a protein encoding gene. This has been shown to drive genome evolution and environmental adaptation (Steenwyk and Rokas, 2017; Tralamazza et al., 2024). Wapinski *et al.* (2007) found that genes related to growth and maintenance show little duplication or loss in copy number, but genes related to stress and environment interactions show much greater fluctuations in copy number. Genes encoding transporter proteins belong to the latter group, thus, it is reasonable to expect lifestyle and niche dependent fluctuations in copy number as a form of adaptation and regulation of transport. Fungal comparative genomic studies have highlighted the importance of gene family copy number in the emergence of the ECM lifestyle from saprotrophic ancestors (Kohler et al., 2015; Miyauchi et al., 2020). Gene family copy numbers of plant cell wall degrading enzymes, important in fungal-mediated decomposition of plant material, have been observed to be markedly reduced in ECM fungi compared to their saprotrophic ancestors, and gene copy numbers of symbiosis-induced secreted proteins have been shown to expand in ECM fungi (Miyauchi et al., 2020). Studies focusing on regulation of base cation transporter genes are scarce, however, upregulation of K^+ transporter genes during mineral weathering has been identified in *Hebeloma cylindrosporum* (Corratgé et al., 2007; Garcia et al., 2014), *Amanita pantherina* (Sun et al., 2019) and *Paxillus involutus* (Pinzari et al., 2022). Whilst there are some studies into the regulation of base cation transporter genes, there are currently no studies investigating the evolution of base cation transporter genes involved in ECM-mediated mineral weathering.

Here, we investigated the evolution of base cation transporter gene families in the Agaricomycotina, with a particular focus on the genus *Suillus*. We hypothesised that 1) greater base cation uptake is dependent on evolutionary expansions in copy-numbers of base cation transporter genes, 2) mineral weathering results in base cation uptake by *Suillus* growing in pure culture with mineral additions, 3) base cation uptake by *Suillus* growing in pure culture with mineral additions will be greater compared to other fungal species, and 4) base cation uptake will correlate with base cation transporter gene family copy numbers. Using bioinformatics we explored evolutionary expansions and contractions in base cation transporter gene families, and using experimental approaches we determined base cation uptake by five species of *Suillus* (*S. bovinus*, *S. granulatus*, *S. grevillei*, *S. luteus* and *S. variegatus*), and a selection of other ECM and saprotrophic fungi that were grown in pure culture with and without mineral additions. This study aims to shed light on the evolution of base cation transporter genes and the importance of their copy numbers in relation to mineral weathering and base cation uptake by ECM fungi and in particular, the genus *Suillus*.

2 Materials and methods

2.1 Bioinformatics

2.1.1 Agaricomycotina phylogeny

A phylogeny of 108 published Agaricomycotina species was inferred using genome data downloaded from JGI MycoCosm (https://mycocosm.jgi.doe.gov/mycocosm/species-tree/tree;aY7X8o?organism=agaricomycotina) (Sup. Tab. 1). *Tremella*

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mesenterica (Tremellomycetes) and *Dacryopinax primogenitus* (Dacrymycetes) were used as outgroups. Orthogroups were identified with OrthoMCL v2.0.9 (Chen et al., 2006) with the settings percentMatchCutoff=50 and evalueExponentCutoff=-5. Single copy orthogroups that were present in at least 50% of the taxa were chosen for the subsequent analyses. Multiple sequence alignment was done with MAFFT v7.407 (Katoh et al., 2005) using the auto settings after which poorly aligned regions were removed with TrimAL v1.4.1 (Capella-Gutiérrez et al., 2009) with a gap threshold setting of -gt 0.1. Individual gene alignments were concatenated with genestitcher v3 (https://github.com/ballesterus/Utensils). Finally, a maximum likelihood phylogeny was reconstructed with IQ-TREE v2. (Nguyen et al., 2015) using the MFP+MERGE arguments to select the best-fit substitution model using ModelFinder (Kalyaanamoorthy et al., 2017) and with 1000 ultrafast bootstraps (Hoang et al., 2018).

2.1.2 Molecular dating

The resulting phylogenetic tree was time calibrated with r8s v1.81 (Sanderson, 2003) using the penalised likelihood method, POWELL optimisation and fossil constrained cross validation. Multiple smoothing parameters were tested before selecting a value of 0.01 which gave the best cross validation score. The root of Agaricomycotina was fixed to 436 million years ago (MYA). A suilloid ectomycorrhizal fungi fossil was used to calibrate the Suillaceae node (~50-100 MYA) (LePage et al., 1997) and the fossil *Palaeoagaricites antiquus* was used to calibrate the Agaricales node (~105-210 MYA) (Poinar and Buckley, 2007). The resulting ultrametric tree was then visualised and edited with inkscape (https://inkscape.org).

2.1.3 Analysis of transporter gene family evolution

Transporter gene family annotations were downloaded from JGI MycoCosm (https://mycocosm.jgi.doe.gov/mycocosm/annotations/browser/tcdb/summary;FdfSB_?p=agaricomycotina) (Tab. S2), which are categorised to three levels of criteria, following the Transporter Classification Database (TCDB) system (Saier, 2006; Saier et al., 2009, 2014, 2016, 2021). 173 transporter gene families which were present in the analysed taxa were categorised as either being involved in base cation transport or not. Both categories were included in the analysis to facilitate a comparative evaluation of base cation transporter gene evolution. In addition, transporter families which may be rapidly evolving were carefully evaluated, as this together with the presence of a high number of copies of base cation transporter genes, may indicate an increase in mineral weathering capabilities. This data was analysed by principal component analysis (PCA) using CANOCO 5.10 (Microcomputer Power, Ithaca, NY, USA; Braak and Smilauer, 2012) for visualization of relationships between transporter gene family copy numbers and fungal taxa. Additionally, to detect significant differences in copy number among transporter gene families, the influence of taxa and transporter gene family on copy number was tested using a two-way analysis of variance (ANOVA), and copy numbers for each of the transporter gene families were compared using Tukey's HSD in R (packages: AICcmodavg (Mazerolle, 2023), emmeans (Lenth, 2024), multcomp, multcompView (Hothorn et al., 2008)). The same data was used to estimate gene family evolution of transporters across the

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phylogeny using CAFE5 v5.1 (Mendes et al., 2020). Due to the birth-death model employed by CAFE5 transporter gene families with zero gene copies at the root were excluded, reducing the analysed transporter gene families from 173 to 114. CAFE5 was unable to run when within-family size variation exceeded 80 copy numbers. Due to this limitation, we ran CAFE5 with a scaled version of the copy number data. Raw gene copy numbers of transporter gene families were scaled to values ranging between 0 and 80 and then rounded up to the nearest whole number in R (packages: tidyverse (Wickham et al., 2019), scales (Wickham et al., 2024)). This dataset is referred to as the "full" dataset. CAFE5 was also run on a smaller dataset with non-scaled copy numbers to account for any error introduced by the scaling, which included 28, taxa selected as they or their close relatives were present in the pure culture experiment. These taxa were extracted from the original Agaricomycotina tree using R (packages: Geiger (Harmon et al., 2008), ape v5.8 (Paradis and Schliep, 2019)) and will hereby be referred to as the "partial family" dataset. For this dataset, the number of transporter gene families included in CAFE5 analyses was 126.

Superfamilies of transporter genes which were significantly expanding or contracting in the partial family dataset were further separated into transporter families with four levels of criteria, as these gene superfamilies had high copy numbers and incorporated a broad range of functions making it difficult to determine their precise role. This dataset is hereby referred to as the "partial subfamily" dataset, with 51 transporter gene families included in CAFE5 analyses (Tab. S3). The partial family and partial subfamily datasets were run in CAFE5 again with the 28 taxa tree. The resulting data was then filtered in two ways to either include only significant expansions and contractions of all transporter gene families, or to include only significant expansions and contractions of base cation transporter gene families and accessories to base cation transporter gene families, using the python script CafeMiner (https://github.com/EdoardoPiombo/CafeMiner/tree/main). Trees were visualised using CafePlotter (https://github.com/moshi4/CafePlotter).

2.1.4 The Mg^{2+} transporter-E (MgtE) family phylogeny

Magnesium is an important base cation which is essential to photosynthesis (Black et al., 2007) and an activator in many enzymes (Bose et al., 2011), and has been previously found to be mobilised and taken up by ECM fungi from mineral soils (Fahad et al., 2016; Mahmood et al., 2024). As such, we inferred the evolutionary relationships of proteins in the Mg^{2+} transporter-E (MgtE) gene family (TCDB ID 1.A.26). A phylogenetic tree of all MgtE family protein sequences present in the 28 taxa of the partial family dataset was constructed. Protein sequences were extracted from the corresponding taxa proteomes using SAMTOOLS (Danecek et al., 2021) the tree was then reconstructed using the same methods as in section 2.1.1 and visualised in R using the ggtree package (Wang et al., 2020; Yu, 2020; Yu et al., 2017).

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2.2 Base cation uptake in pure culture

As members of the ECM genus *Suillus* have been reported to possess mineral weathering capabilities the genus was studied in greater depth. Isolates of *Suillus* species, along with the ECM genus *Piloderma* and two saprotrophic fungal species for comparison, were grown in pure culture in axenic systems with and without mineral additions to determine base cation uptake during mineral weathering.

2.2.1 Fungal isolates

A total of 23 fungal isolates, covering 11 species and two isolates identified to genus level, were used in the study (Tab. S4). Members of the ECM genus *Suillus* were isolated in summer and autumn of 2022 from morphologically identified fruitbodies collected in Central and Southern Sweden. From these a subset of four *S. bovinus*, three *S. granulatus*, one *S. grevillei*, three *S. luteus* and three *S. variegatus* isolates were selected based on geographical spread and growth performance. The genus *Piloderma* was selected as an ECM comparison to *Suillus*, as members of this group are also common in boreal forests and found both in the organic and mineral horizons (Mahmood et al., 2024; Marupakula et al., 2021). Six *Piloderma* isolates, including *P. byssinum*, *P.* aff. *fallax*, *P. olivaceum*, *P. sphaerosporum*, *Piloderma* sp. 1 and *Piloderma* sp. 2, were obtained from the culture collection maintained at the Department of Forest Mycology and Plant Pathology, SLU. In addition, two saprotrophic brown-rot wood decaying species, *Coniophora puteana* and *Serpula lacrymans* (obtained from Geoffry Daniel at the Department of Forest Biomaterials and Technology, Wood Science, SLU), were included as comparison to the ECM lifestyle. Prior to the experiment, all isolates were grown on Modified Melin-Norkrans (MMN) media (Marx, 1969) for 12 and 5 weeks for ECM and saprotrophic fungi, respectively.

2.2.2 Experimental conditions

To measure base cation uptake during mineral weathering, fungal isolates were grown in 9 cm Petri dish closed axenic systems with and without mineral additions, as previously described by (Fahad et al., 2016). Granite and gabbro rock material was selected as mineral sources as they commonly occur in bedrock in Sweden (Holtstam et al., 2004), and are proposed to have differing weathering rates, with gabbro weathering faster than granite (Bazilevskaya et al., 2013; Fritz, 1988). Granite is a complex of K feldspar, muscovite mica and quartz and gabbro is a complex of sodium (Na)/calcium (Ca) feldspar, amphibole, pyroxene and olivine (Goldich, 1938). These minerals contain essential base cations, such as Ca, iron (Fe), K, Mg and Na, which are required for growth. Granite and gabbro material was provided by Swerock AB, Ängelholm, Sweden, and originated from Stora Almby and Harbo, respectively. Pulverised rock material was sieved under flowing deionised water to retain a size fraction of 63-250 mm to maximise surface area. No smaller fraction was included to ensure the material remained in its mineral form and that fungi must weather the sand to access the nutrients. Sand was sonicated

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for 10 minutes and sieved again to dislodge and remove any debris adhering to the grains. The sand was then placed in a 1 L cylinder with small holes at the base and placed under deionised flowing water for 3 days to remove any loosely bound ions. Two treatments with granite and gabbro, with 30 replicates per isolate, were established. A primary mineral free layer of 20 mL agar media containing $(NH4)_2HPO_4$ 0.22 g/L, thiamine-HCl 100 mg/L, glucose 5 g/L, malt extract 5 g/L and agar 12 g/L served as a C and N source. A secondary 5 mL layer of the same media with the addition of 20 g/L granite or gabbro, respectively, was added after the first layer had set. In both cases the pH was adjusted to 5.5 (Fahad et al., 2016).

Two controls with 30 replicates per isolate were also established. These were termed "limited", which provided a minimal mineral nutrient source, and "rich", which provided a more abundant mineral nutrient source. The limited control had the same contents as the primary layer described above and was used as a base level comparison against the mineral treatments. The rich control contained $CaCl_2.2H_2O$ 0.05 g/L, NaCl 0.025 g/L, $MgSO_4.7H_2O$ 0.15 g/L, $(NH4)_2HPO_4$ 0.22 g/L, KH_2PO_4 0.5 g/L, $FeCl_3.6H_2O$ 0.012 g/L, thiamine-HCl 100 mg/L, glucose 5 g/L, malt extract 5 g/L and agar 12 g/L, thus providing all essential nutrients necessary for growth. It provided a comparison of base cation uptake between conditions where weathering is required to access base cations and where base cations are readily available. Both were set to pH 5.5. Table 1 shows the concentration of Ca, Fe, K, Mg, Na and P present in each of the treatments relative to the limited treatment. Cellophane was thoroughly rinsed in deionised water and autoclaved, after which it was laid on each plate to prevent mycelial growth into the agar to aid harvesting, but allow transfer of soluble compounds.

	Ca (g/L)	Fe (g/L)	K (g/L)	Mg (g/L)	Na (g/L)	P (g/L)
gabbro	0,1995	0,2770	0,0418	0,1113	0,0141	0,0085
granite	0,0238	0,1396	0,0115	0,0169	0,0041	0,0049
rich	0,0136	0,0248	0,1437	0,0244	0,0000	0,1138

Table 1 - Amount of additional base cation and phosphorus (P) nutrients in the gabbro, granite and rich medias compared to the limited media. Base cations include calcium (Ca), iron (Fe), potassium (K), magnesium (Mg) and sodium (Na). Concentrations given in g/L.

All isolates were grown in 24 h darkness at 25° C apart from *S. lacrymans* which was grown at 20° C. Due to differing growth rates, ECM isolates were grown for nine weeks, *C. puteana* for five weeks and *S. lacrymans* for three weeks. Biomass was harvested and washed twice in sterile double distilled water (ddH_2O) by adding 5 mL ddH_2O, briefly shaking and centrifuging for 10 minutes at 1500 rpm (Beckman GS-6, California, USA). Following this, biomass of individual replicates was freeze-dried, weighed and milled. To obtain enough biomass for elemental analysis replicates were then pooled into 50 mL falcon tubes to give three samples per isolate per treatment. Ten 5 mm glass beads were added to each tube, and they were loaded into a 50 mL tube adapter (Retsch MM 400, Haan, Germany) and milled for 15-30 minutes at 30 Hz until a fine powder was obtained.

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2.2.3 Elemental analyses and statistics

Biomass samples were sent to the James Hutton Institute, Aberdeen, UK for elemental analyses. C and N analysis (Mettler MT5 Microbalance, Mettler-Toledo GmbH, Laboratory & Weighing Technologies, Greifensee, Switzerland and Thermo Finnigan Elemental Analyser FlashEA 1112 Series, Thermo Fisher Scientific, Massachusetts, USA), nitric acid microwave digestion (PerkinElmer Titan MPS Microwave Sample Preparation System, PerkinElmer, Massachusetts, USA) and ICP-EOS analysis (AVIO 500 ICP-OES, PerkinElmer, Massachusetts, USA) of Ca, Fe, K, Mg, Na and P was conducted on fungal biomass, cellophane, gabbro and granite.

Individual biomass per plate and elemental concentration in biomass pools from each isolate and treatment were used for statistical analysis, which was performed using the statistical software R v4.3.2 (R Core Team, 2023). The 'Tidyverse' suite v2.0.0 was used for most data processing and visualisation (Wickham et al., 2019). Carbon and N measurements were used to calculate C:N ratios. Linear models were fitted for each element to estimate the effects of isolate, treatment, and their interactive effect on the individual response variables This was repeated for biomass and C:N ratio measurements, with species in place of isolate. These effects were tested with the 'emmeans' v1.10.4 package (Lenth, 2024) and compact letter displays were determined with the 'multcomp' v1.4-26 packages (Hothorn et al., 2008). This was followed by pairwise comparisons of elemental concentration between treatments within each isolate, and biomass and C:N ratio between treatments within each species and between species within each treatment, using Tukey's HSD. Weathering was defined as a significantly higher element concentration after growth with gabbro and/or granite as compared to the limited treatment, based on the linear models' output.

Serpula lacrymans and *C. puteana* were excluded from biomass analyses based on individual plates due to difficulties harvesting complete biomass. The following isolates and treatments included fewer than three replicates for elemental analyses due to a lack of biomass; *P. byssinum* and *S. lacrymans* all treatments (1 replicate), isolates Sl2 and Sl3 limited treatment (1 replicate), and Sl3 gabbro treatment (2 replicates).

To evaluate whether base cation uptake by *Suillus* growing with mineral additions was greater compared to other fungal isolates linear models were fitted to estimate the differences in the ratio of individual element concentration in mycelia growing in gabbro and granite treatments compared to the limited treatment, with isolate and treatment as explanatory factors. This was tested with the 'emmeans' v1.10.4 package (Lenth, 2024) and results are reported as means with confidence intervals.

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Correlations between base cation transporter gene family copy numbers and means of mycelial elemental concentration for all isolates in each mineral treatment and the control treatments were tested using Spearman's correlation. Base cation transporter gene families were restricted to those which were reported as significantly expanding or contracting in the CAFE5 analyses. The TCDB substrate search tool (https://tcdb.org/progs/?tool=substrate#/) was used to determine if any base cation transporter gene families were specifically involved in the transport of Ca, Fe, K, Mg, Na and P. Isolates without transporter family copy number data were excluded (*S. granulatus*, *S. grevillei*, *P.* aff. *fallax*, *Piloderma* sp. 1 and *Piloderma* sp. 2).

3 Results

3.1 Phylogenetic Analyses

3.1.1 Agaricomycotina phylogeny

The phylogenetic inference of 108 Agaricomycotina genomes was performed using 152 single copy orthologs that were present in at least 50% of the taxa, which after concatenation resulted in a supermatrix with 54,895 amino acids. Most nodes recovered a bootstrap value of 100 (Fig. S1).

3.1.2 Transporter gene family copy numbers of 108 Agaricomycotina taxa

There are significantly ($p < 0.05$) more gene copy numbers of the major facilitator (MFS) gene superfamily (TCDB ID 2.A.1), the Mg^{2+} transporter-E (MgtE) gene family (TCDB ID 1.A.26), the ATP-binding cassette (ABC) gene superfamily (TCDB ID 3.A.1), the peroxisomal protein importer (PPI) gene family (TCDB ID 3.A.20) and the mitochondrial carrier (MC) gene family (TCDB ID 2.A.29) compared to other transporter gene families (Fig. S2). Of these, the MSF gene superfamily, the MgtE gene family, the ABC gene superfamily and the MC gene family are involved in base cation transport. The MSF gene superfamily has notably high copy numbers in all 108 taxa, ranging from 61 to 294. The highest copy number for a single species is in the MgtE gene family with 563 copies in *Tulasnella calospora*. Visualisation of transporter gene family copy numbers by PCA showed a clear clustering of the families Suillaceae and Russulaceae which are distinct from each other and all other taxa (Fig. 1a). Among the 30 most contributing transporter gene families, 18 are base cation transporter gene families (Fig 1b).

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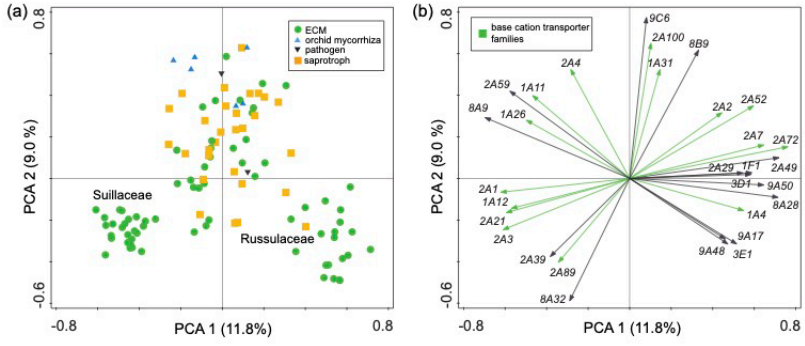

Figure 1 - Variation in transporter gene family composition in 108 Agaricomycotina taxa, based on Principle Component Analysis of copy numbers of 173 transporter gene families and visualized by (a) sample and (b) species plots showing the 30 transporter gene families with the greatest contribution to the ordination of taxa. Lifestyle (a) is coded by shape and colour of sample points, and in (b) base cation transporter families are indicated in green. The first two axes together explained 20.8% of a total variation of 9586.4.

3.1.3 Transporter gene family evolution

Thirteen out of 114 analysed transporter gene families in the full dataset were found to have significant expansions and contractions (Fig. S3-15), six of which are base cation transporter gene families and one of which is a base cation transport accessory family (Tab. S2). In the partial family dataset, 22 of the 126 analysed transporter gene families had significant expansions and contractions (Fig. S18-39), with nine being base cation transporter gene families and 2 being base cation transport accessory families (Tab. S2). All significantly expanding or contracting transporter gene families in the full dataset are in the genus *Suillus* (Fig. S3-15). Other clades with higher density of expansions and contractions are the families Atheliaceae and Russulaceae (Fig. S16). In the partial family dataset, where 23 of the 28 taxa are *Suillus* species, there is a loss of three and an addition of 12 transporter gene families which are expanding and/or contracting significantly in the genus *Suillus*, when compared to the full dataset (Tab. S2). In the partial subfamily dataset, 15 of 51 analysed transporter gene families were shown to be significantly expanding and contracting (Tab. S3). Of these, ten are involved in base cation transport (Fig. S41-56, Tab. S3).

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3.1.3.1 Base cation transporter gene family evolution

Within the genus *Suillus*, several branches show significant expansions and contractions in base cation transporter gene families in the full dataset (Fig S17), with the most frequent transporter gene families being the MgtE gene family (Fig. S3) and the MSF gene superfamily (Fig. S5). This pattern is reflected in the partial family dataset with 14 branches showing significant expansions and contractions in base cation transporter gene families (Fig. 2). The MgtE gene family (Fig. S19) is again one of the most frequent gene families, along with the β-amyloid cleaving enzyme 1gene family (TCDB ID 1.A.33) (Fig. S20). In the partial subfamily dataset, which included subdivisions of transporter gene superfamilies which were significantly expanding or contracting in the partial family dataset, there are significant expansions and contractions in branches leading to 14 internal nodes (Fig. S56), with the drug:H^+ antiporter-1 gene family (TCDB ID 2.A.1.2) (Fig. S42) and the pleiotropic drug resistance gene family (TCDB ID 3.A.1.205) (Fig. S50) as the most frequent gene families. There are significant expansions and contractions within the clade containing Suillus species in the full dataset (Fig S17), with the MSF gene superfamily (Fig. S5) and the ABC gene superfamily (TCDB ID 3.A.1) (Fig. S7) being the most frequent ones. In the partial family dataset, there are also many significant expansions and contractions in the clade containing *Suillus* species (Fig. 2). The most frequent gene families are again the MSF gene superfamily (Fig. S21) and the ABC gene superfamily (Fig. S26), with the addition of the MgtE gene family (Fig. S19) and the β-amyloid cleaving enzyme 1 gene family (Fig. S34). Within the genus the highest number of expansions and contractions in base cation transporter gene families in the full dataset occur in the branches leading to the ancestral node of *S. variegatus*, *S. tomentosus*, *S. hirtellus* and *S. fuscotomentosus*; and *S. cothurnatus*, *S. lakei*, and *S.* cf *subluteus* (Fig. S17). In the partial family dataset, *S. cothurnatus*, *S. lakei*, and *S.* cf *subluteus* again are inferred to have high numbers of expansions and contractions, as well as branches leading to the ancestral node of *S. placidus* and *S. weaverae/grevillei*; the ancestral node of *S. occidentalis, S. luteus* and *S. brevipes*; the ancestral node of *S.* cf *subluteus* and *S. subalutaceus*; and *S. decipiens*, *S. pictus/spraguei*, *S. plorans*, *S. subalutaceus*, *S. subaureus* and *S. variegatus* (Fig. 2).

Branches within the *Piloderma* clade also exhibit a number of expansions and contractions in both the full and the partial family datasets (Fig. S17 and Fig. 2, respectively) and, similar to *Suillus*, the most frequent base cation transporter gene family is the MgtE gene family (Fig. S3 and S19, respectively). *Piloderma byssinum* in particular has similarly high numbers of expansions in base cation transporters in both datasets (Fig. S17 and Fig. 2), and *P. olivaceum* shows high numbers of contractions in the partial family dataset (Fig. 2). Branches leading to the saprotrophic fungi *C. puteana* and *S. lacrymans* show low numbers of significant expansions and contractions in base cation transporter gene families in general, with *C. puteana* displaying one expansion in the partial family dataset (Fig. 2) and *S. lacrymans* displaying one contraction in the full dataset (Fig. S17) and two contractions and one expansion in the partial family dataset.

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Other branches leading to nodes with high numbers of significant expansions and contractions in base cation transporter gene families from the full dataset include those leading to the ancestral node of the genus *Lactarius*; the ancestral node for *Russula vinacea*, *R. ochroleuca*, *R. rugulosa* and *R. emetica*; the ancestral node for *Paxillus involutus* and *P. ammoniavirescens*; the ancestral node for *Fibulorhizoctonia psychrophila*, *Piloderma olivaceum*, *P. byssinum* and *P. sphaerosporum*; and *Amanita rubescens*, *Gymnopus androsaceus, Fibulorhizoctonia psychrophila*, *Rhizopogon vesiculosus*, *Pisolithus microcarpus*, *Melanogaster broomeianus*, *Paxillus involutus*, *Lactarius indigo*, *L. sanguifluus*, *L. deliciosus*, *L. hengduanensis*, *L. pseudohatsudake*, *L. akahatsu* and *Ceratobasidium* sp. *anastomosis* (Fig. S17).

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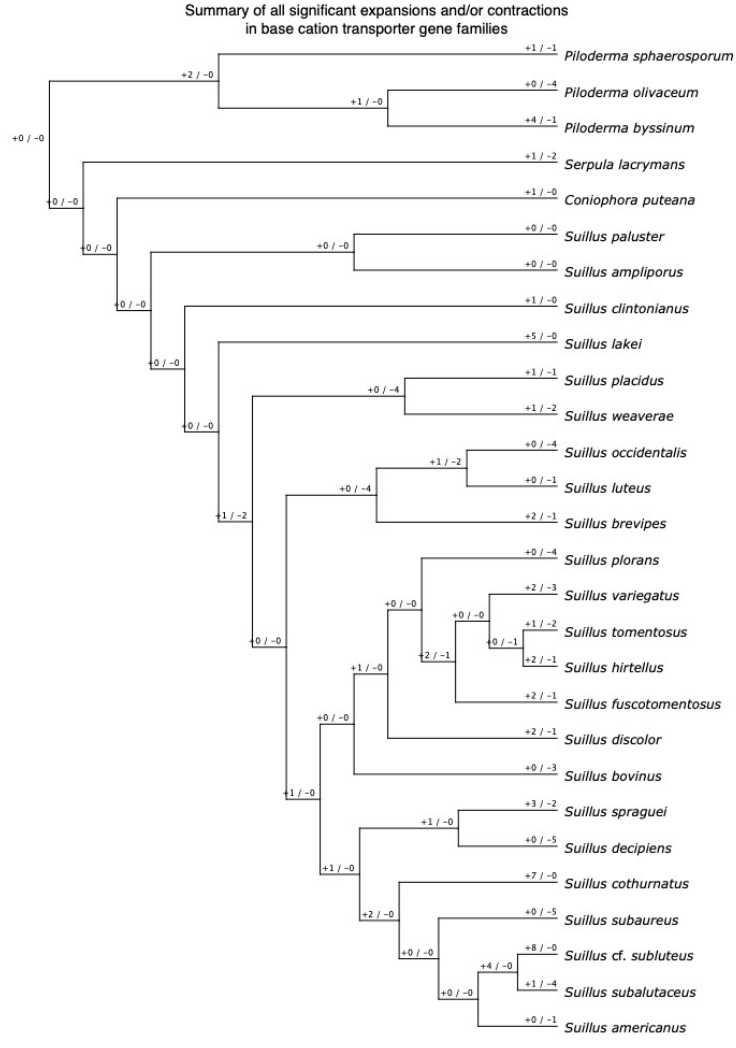

370 Figure 2 - Phylogenomic tree showing total of significant expansions and contractions of all base cation transporter gene families in the partial family dataset (28 taxa selected as they or their close relatives were present in the pure culture experiment). Branch-length not to scale.

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3.1.3.2 Fungal mating-type pheromone receptor (MAT-PR) transporter gene family evolution

Expansions and contractions in transporter family genes related to sexual reproduction can have implications in the rate of evolution and adaptation, which may lead to changes in mineral weathering capabilities of taxa. The fungal mating-type pheromone receptor (MAT-PR) gene family (TCDB ID 9.B.45) was inferred to be significantly expanding in the branch leading to the ancestral node of *S. occidentalis, S. luteus* and *S. brevipes*; and in *S. clintonianus*, *S. variegatus*, *S. tomentosus* and *S.* cf. *subluteus* in the full dataset (Fig. S15). In the partial family dataset, significant expansions were found in the branch leading to the ancestral node of *S. occidentalis, S. luteus* and *S. brevipes*; the branch leading to the ancestral node of *S. plorans*, *S. variegatus*, *S. tomentosus*, *S. hirtellus* and *S. fuscotomentosus* and *S. discolor*; and the branch leading to the ancestral node of *S. variegatus*, *S. tomentosus*, *S. hirtellus* and *S. fuscotomentosus*; in addition to *S. clintonianus*, *S. variegatus*, *S. tomentosus*, *S. cothurnatus*, *S. pictus/spraguei*, *S. brevipes*, *S. occidentalis*, *S. placidus*, *S. ampliporus* and *S.* cf. *subluteus* (Fig. S39). Significant contractions were inferred in the branch leading to *S. luteus* in the full dataset and in those leading to *S. lakei*, *S. weaverae/grevillei*, *S. luteus*, *S. hirtellus*, *S. bovinus*, *S. decipiens* and *S. subaureus* in the partial family dataset. Additionally, in the full dataset there is a large significant expansion in *R. Emetica*.

3.1.4 Mg^{2+} transporter-E (MgtE) gene family evolution

A phylogenetic tree of protein sequences belonging to the MgtE gene family and present in the partial family dataset was reconstructed in order to understand whether there has been an expansion of one homologous transporter gene in the genus *Suillus*, or if there are several transporter genes of different origin. The phylogeny shows several clades of MgtE genes present in each of the taxa (Fig. S57).

3.2 Base cation uptake by mycelia grown in pure culture

3.2.1 Correlation between base cation transporter gene family copy number and mycelial base cation concentration

Of the 25 base cation transporter gene families found to be significantly expanding and/or contracting, a total of six base cation transporter gene families that are involved specifically in the transport of Ca, Fe, K, Mg, Na and P, and five other base cation transporter gene families which are more generally reported as base cation transporting without being specifically linked to transport of a particular base cation, showed significant correlations between gene family copy number and base cation uptake. There was a total of 28 significant correlations between mycelial concentration of base cations and copy numbers of base cation transporter gene families across species within the different treatments. Most of the significant correlations were positive with increasing copy numbers resulting in larger base cation uptake, but some were also negative (Fig. 3 and S58-68).

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Mycelial Mg concentration was significantly positively correlated with genome copy numbers of the MgtE gene family in the gabbro, granite and limited treatments, and there was a positive correlation in the rich treatment (Fig. 3a-d, respectively). There was a significant positive correlation in the limited treatment for Ca concentration (Fig. 3g), no correlations in the gabbro and the granite treatments and a negative correlation in the rich treatment (Fig. 3h). There were also negative
410 correlations in all treatments for Fe concentration (Fig. 3i-l), though not significant.

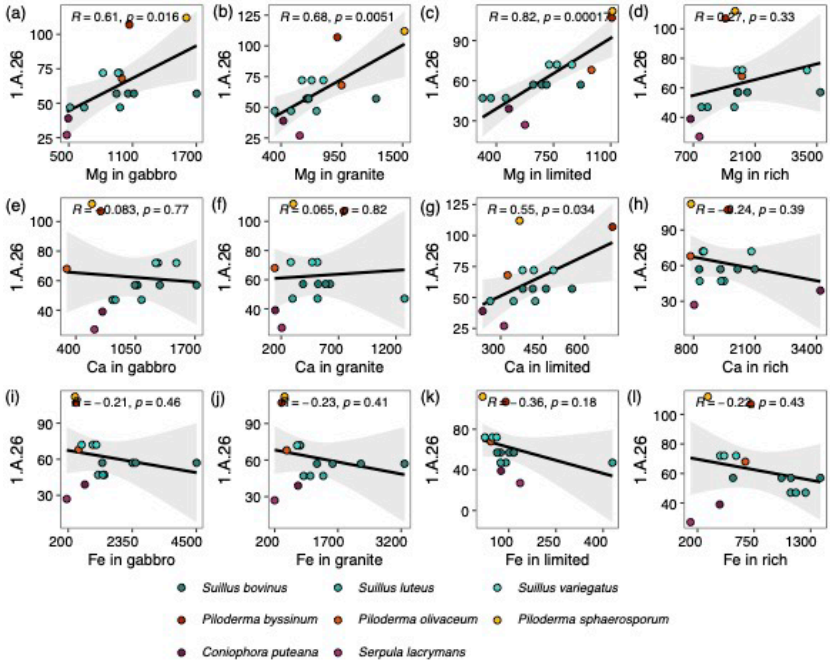

Figure 3 - Correlations between mycelial magnesium (Mg), calcium (Ca) and iron (Fe) concentrations (mg/kg) and transporter gene copy numbers. Element concentration is given as the estimated mean values in eight different fungal species grown in pure culture in gabbro, granite, limited and rich treatments, and the gene copy numbers of the Mg2+ transporter-E
415 family (TCDB ID: 1.A.26) in the corresponding fungal genomes.

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Magnesium mycelial concentration is also significantly positively correlated with copy numbers of the transient receptor potential Ca^{2+}/cation channel (TRP-CC) gene family (TCDB ID 1.A.4) (Fig. S58k), the ABC gene superfamily (Fig. S59g) and the ankyrin gene family (TCDB ID 8.A.28) (Fig. S60o) in the limited treatment, and of the P-type ATPase (P-ATPase) gene superfamily (TCBD ID 3.A.3) in the granite treatment (Fig. S61n); however there is a significant negative correlation with copy numbers of the Ca^{2+}, Mn^{2+}- ATPase gene family (TCDB ID 3.A.3.2) in the limited treatment (Fig. S62k). In the rich treatment, mycelial Na concentration is significantly positively correlated with copy numbers of the anion:cation symporter (ACS) gene family (TCDB ID 2.A.1.14) (Fig. S63h), the H^+- or Na^+-translocating F-type, V-type and A-type ATPase (F-ATPase) gene superfamily (TCDB ID 3.A.2) (Fig. S64d), the Ca^{2+}, Mn^{2+}- ATPase gene family (Fig. S62p), the nucleobase:cation symporter-1 gene family (TCDB ID 2.A.39) (Fig. S65h), and the G-protein-coupled receptor (GPCR) gene family (TCDB ID 9.A.14) (Fig. S66d); and is significantly negatively correlated with copy numbers of the TRP-CC gene family (Fig. S58p). In the gabbro treatment, mycelial Na concentration is also significantly positively correlated with copy numbers of the GPCR gene family (Fig. S66c), and in the gabbro and the limited treatments with the β-amyloid cleaving enzyme 1 (BACE1) gene family (TCDB ID 8.A.32) (Fig. S67q and S67t, respectively). Potassium mycelial concentration is significantly positively correlated with copy numbers of TRP-CC gene family (Fig. S58g) and the P-ATPase gene superfamily (Fig. S61k), and negatively correlated with copy numbers of the ACS gene family (Fig. S63c) in the limited treatment. Calcium mycelial concentration is significantly positively correlated with copy numbers of the amino acid-polyamine-organocation (APC) gene superfamily (TCDB ID 2.A.3) in the gabbro (Fig. S68a) and the limited (Fig. S68c) treatment, the nucleobase:cation symporter-1 gene family in the gabbro treatment (Fig. S65a), the ankyrin gene family in the limited treatment (Fig. S60c), and the BACE1 gene family in the gabbro treatment (Fig. S67a). Mycelial Ca concentration is significantly negatively correlated with copy numbers of the TRP-CC gene family (Fig. S58a) and the ankyrin gene family (Fig. S60a) in the gabbro treatment. There are significant positive correlations between Fe mycelial concentration and copy numbers of the BACE1 gene family in the gabbro (Fig. S67e) and granite (Fig. S67f) treatments. There are no significant correlations between P concentration and copy numbers of P transporting transporter gene families, however, correlations are generally positive regardless of treatment (Fig. S59 and S63).

3.2.2 Mycelial base cation concentration

For mycelial concentration of each base cation measured there were significant ($p < 0.001$) overall effects of isolate, treatment (gabbro, granite, limited or rich) and the interaction between isolate and treatment. For all elements there were examples of higher element concentrations after growth with gabbro and/or granite as compared to the limited treatment.

For example, all *S. bovinus* isolates showed significantly greater Mg mycelial concentration in the gabbro treatment compared to the granite and limited treatment (Fig. S69d). Additionally, one *S.* luteus and one *S. variegatus* isolate, and the

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Piloderma isolates Pf and Ps showed significantly greater Mg mycelial concentrations in the gabbro treatment compared to the limited (Fig. S69d). For Fe, all isolates excluding *P. byssinum* and *S. lacrymans* showed significantly greater Fe mycelial concentrations in the gabbro and the granite treatment compared to the limited (Fig. S69b). For Ca, all *S. bovinus*, *S. variegatus*, two *S. luteus*, one *S. granulatus* and the saprotroph *C. puteana* showed significantly greater mycelial concentrations in the gabbro treatment compared to the limited (Fig. S69a). Two *S. bovinus* isolates and two *Piloderma* isolates showed significantly greater K mycelial concentrations in the gabbro treatment compared to the limited (Fig. S69c), and one *S. bovinus* and one *S. luteus* isolate showed significantly greater P mycelial concentrations in the gabbro compared to the limited treatment (Fig. S69f). All *Suillus bovinus* isolates had significantly greater Na mycelial concentrations in the gabbro and limited treatments compared to the rich treatment, and one *Piloderma* isolate showed a significantly greater Na mycelial concentration in the gabbro treatment compared to the limited treatment (Fig. S69e).

There were also examples of higher element concentration in the rich treatment as compared to the mineral treatments. Nearly all isolates showed significantly greater Ca, Fe and Mg mycelial concentrations in the rich treatment compared to the limited (Fig. S69d). Six *Suillus* isolates and four *Piloderma* isolates showed significantly greater K mycelial concentrations in the rich treatment compared to the limited (Fig. S69c). For Na, four *Suillus* isolates showed significantly lower concentrations and one *S. luteus* isolate showed a significantly higher concentration in the rich compared to the limited (Fig. S69e) For P concentrations, eight *Suillus* isolates and four *Piloderma* isolates showed significantly higher concentrations in the rich compared to the limited treatment (Fig. S69f).

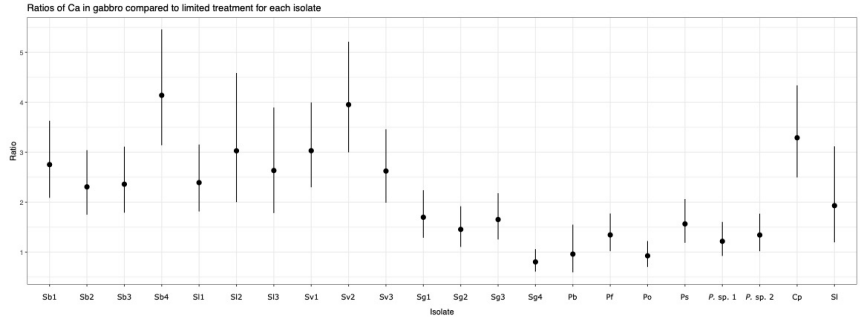

Figure 4 - Ratios of calcium (Ca) concentration (mg/kg) between gabbro and limited treatments for each fungal isolate. Isolates include *Suillus bovinus* (Sb1-4), *S. luteus* (Sl1-3), *S. variegatus* (Sv1-3), *S. granulatus* (Sg1-3), *S. grevillei* (Sg4), *Piloderma byssinum* (Pb), *P.* aff. *fallax* (Pf), *P. olivaceum* (Po), *P. sphaerosporum* (Ps), *Piloderma* sp. 1, *Piloderma* sp. 2,

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Coniophora puteana (Cp), and *Serpula lacrymans* (Sl). Data points represent the mean ratio between Ca concentration in gabbro and in limited for each isolate, with error bars showing the confidence interval.

Comparisons between *Suillus, Piloderma*, and saprotroph isolates of the ratio of individual element concentrations in mycelia grown in mineral treatments compared to the limited treatment showed different patterns (Fig S70, S71). The linear models showed many instances of a significant overall effect ($p < 0.05$) of isolate and treatment on the ratio between concentrations of each element in the mineral and limited treatments. The strongest difference between groups was seen for Ca in the gabbro treatment where linear models showed the ratio between gabbro and limited were significant for all isolates except for one *Suillus* and four *Piloderma* isolates; and the ratios for *Suillus* isolates in general were higher compared to *Piloderma* isolates, and in many cases significantly higher (Fig 4). In the granite treatment the pattern for Ca ratios was similar but weaker, where ratios for only four *Suillus* isolates and one *Piloderma* isolate were significant from the linear models (Fig. S71a). For Fe the isolates showed a similar pattern in ratios in both gabbro and granite, including higher ratios for all *S. bovinus* isolates, two *S. variagatus* isolates, and *Piloderma sphaerosporum* (Fig. S70a and S71b, respectively). This was reflected in the linear models, where only the ratio of the saprotroph *S. lacrymans* was not significant in either the gabbro or the granite treatments. For Mg in the gabbro treatment some *Suillus* isolates had significantly higher ratios compared to some of the *Piloderma* isolates, but *Piloderma sphaerosporum* also had a high ratio (Fig. S70c). In granite the Mg ratios showed less pronounced differences between isolates (Fig. S71d). In the gabbro, the linear models showed that Mg ratios in 11 *Suillus* and two *Piloderma* isolates were significant, and in the granite Mg ratios of only four *Suillus* isolates and one *Piloderma* were significant. The ratios for K, Na and P mostly did not differ between isolates (Fig. S70b and S71c, S70d and S71e, and S70e and S71f, respectively) and linear models showed very few instances of significant ratios in the gabbro and the granite treatments.

3.2.3 Mycelial biomass and C:N ratio

There was a significant overall effect ($p < 0.001$) of species, treatment (gabbro, granite, limited or rich), and their interaction for fungal biomass and C:N ratio. Overall, *Suillus* and *Piloderma* species produced similar amounts of biomass across all treatments (Fig. S72). *Piloderma olivaceum* had significantly higher biomass than all other species in the gabbro treatment, followed by *S. variegatus* which had significantly higher biomass compared to the remaining species. In the granite and rich treatments *P. olivaceum* and *S. variegatus* both had significantly greater biomass than other species, whilst *S. variegatus*

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alone had significantly greater biomass compared to other species in the limited treatment. *Piloderma byssinum* produced significantly less biomass than all other species in all treatments.

For comparisons between treatments within each species *Suillus bovinus* showed a significant increase in biomass in the rich treatment compared to all other treatments (Fig. S73). *Suillus luteus*, *P. olivaceum* and isolates *Piloderma* sp. 1 and sp. 2 produced significantly more biomass in the gabbro, granite and rich treatments compared to the limited treatment, with *Piloderma* sp. 2 producing significantly more biomass in the rich treatment compared to the granite treatment. *Suillus grevillei* produced significantly greater biomass in the granite treatment compared to the limited and *P. byssinum* had significantly higher biomass in the rich treatment compared to the limited.

When comparing C:N ratios between treatments within species *S. luteus* is the only species which displayed a significant difference between treatments, with higher C:N ratio in the rich treatment compared to the limited (Tab. S5a), while all other species were similar. When comparing between species within treatments, *Suillus* species generally had a higher C:N ratio than *Piloderma* species, and saprotrophic species generally had low C:N ratios (Tab. S5b). In particular, *S. variegatus* had a significantly higher C:N ratio than all other species excluding *S. luteus* and *S. granulatus* in the gabbro treatment. This pattern was repeated in the other treatments, excluding *S. granulatus* in the granite and limited treatments, and including *P. olivaceum* in the rich treatment.

4 Discussion

Rapid base cation transporter gene family evolution in *Suillus* species

The requirement for base cations by plants and fungi is a key driver of biological mineral weathering and a number of ECM fungal species have been shown to take part in this process by colonising mineral surfaces, producing LMWOAs and altering soil chemistry (Calvaruso et al., 2013; Gazze et al., 2012; Griffiths et al., 1994; Schmalenberger et al., 2015; van Scholl et al., 2006). The genus *Suillus* is a group of ECM fungi with high host specificity (Lofgren et al., 2021) and the capability to take up base cations from mineral sources (Balogh-Brunstad et al., 2008a; Wallander, 2000). In the ordination based on copy numbers of all transporter gene families, members of the families Suillaceae and Russulaceae clustered separately from each other and other Agaricomycotina species (Fig. 1), and among the most contributing transporter gene families, base cation transporter gene families were highly abundant indicating that this group of transporters may be important in the lifestyle and environmental adaptations of these two groups.

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As a general rule, copy numbers of genes related to cell and organism maintenance and growth remain relatively constant as mutations in these genes would likely reduce fitness, however, copy numbers of genes related to ecological adaptations, including stress, have greater copy number variation as changes in these genes are less likely to reduce fitness and may give rise to advantageous phenotypes (Wapinski et al., 2007). It is therefore expected that transporter genes, which frequently have roles in abiotic and biotic environmental interactions, will display greater variation in copy numbers over time, as a result of expansions and contractions, and that they have important roles in the rapid adaptation to environmental changes and stress. Our analyses of 108 Agaricomycotina genomes confirm significant expansions and contractions in base cation transporter gene families and display a rapid evolution of these transporter gene families in the genus *Suillus*, thereby supporting our first hypothesis that greater base cation uptake is dependent on evolutionary expansions in copy numbers of base cation transporter gene families.

Thirteen transporter gene families in the full dataset, 22 in the partial family dataset and 15 in the partial subfamily dataset were found to have significant expansion and contractions, all of which have significant expansions and contractions in the *Suillus* genus. Differences in the number of transporter families with significant expansions and contractions between datasets are likely due to the scaling of the full dataset and the difference in the number of taxa included. These significant expansions and contractions, suggest that these gene families have rapidly evolved in the genus *Suillus* enabling species within this group to adapt to their environment.

There are also significant expansions in the fungal mating-type pheromone receptor (MAT-PR) family in a number of *Suillus* species, in both the full and partial family datasets, and a large expansion in *Russula emetica* in the full dataset. This transporter gene family is reported to play a role in chemotrophic sensing of host plants (Turrà et al., 2015), and in female sexual development in *Neurospora crassa* (Krystofova and Borkovich, 2006). Sexual reproduction is often favoured in unstable environments or in situations where sources of nutrients may be inconsistent (Nieuwenhuis and James, 2016), and adaptations in sexual populations can occur faster than in asexual populations (Becks and Agrawal, 2012; McDonald et al., 2016; Nieuwenhuis and James, 2016) Therefore, expansions in this transporter family may indicate an increase in the proportion of sexual reproduction performed relative to asexual reproduction, which likely results in populations more rapidly adapting to environmental changes.

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Significantly evolving base cation transporters and their functions

Approximately half of the significantly expanding and contracting transporter gene families found in each dataset are involved in base cation transport, indicating that transport of base cations may be important in adjusting to environmental fluctuations and stress, as genes which vary in copy number are more likely to have roles in adaptation (Wapinski et al., 2007). In addition, expansions and contractions in these transporter families are likely to be related to the requirement of base cation uptake, and their occurrence within taxa, such as the genus *Suillus*, may be an indication of mineral weathering capabilities. Branches leading to *S. lakei*, *S. variegatus, S. cothurnatus* and *S.* cf *subluteus,* as well as two internal branches within the genus *Suillus* possess significant expansions in the MgtE gene family (Fig. S19). Though primarily studied in bacteria and mammals, members of the MgtE gene family are identified as facilitators of Mg^{2+}, Zn^{2+}, Co^{2+}, Ni^{2+}, Fe and Cu influx (Goytain and Quamme, 2005; Smith et al., 1995), and play roles in Mg^{2+} homeostasis (Conn et al., 2011; Franken et al., 2022; Hattori et al., 2009; Hermans et al., 2013). Additionally, it has been found that many organisms have multiple copies of one type of Mg^{2+} transporter gene family, which may explain the relatively low abundance of other Mg^{2+} transporter gene families in our datasets (Franken et al., 2022). In plants, it was reported that there are many gene copies of a Mg^{2+} transporter gene family, the CorA/Mrs2 metal ion transporter (MIT) gene family, and that they are not functionally redundant (Gebert et al., 2009; Schmitz et al., 2013). Our analysis suggests multiple different homologous MgtE genes have undergone expansions, as observed in the phylogenetic gene tree of MgtE gene family members present in the partial dataset (Fig. S57), so rather than having a not functionally redundant transporter, there might be multiple homologues performing the same function or each with with a different function. In the genus *Suillus*, the major facilitator gene superfamily has six significant expansions in both the full and the partial family datasets, three and nine significant contractions in the full and partial family datasets, respectively, and one significant contraction in *P. sphaerosporum* in both datasets (Fig. S5, Fig. S21). This gene superfamily is large and diverse and reported to transport solutes such as sugars and cations (Law et al., 2008).

Although *S. bovinus* possesses two significant contractions in the full dataset, three in the partial family dataset and no expansions in base cation transporter families, it had significantly higher mycelial concentrations of Mg, Ca and Fe in the gabbro treatment compared to the limited treatment. One isolate in particular (Sb1) had significantly higher mycelial concentration of all base cations in the gabbro treatment compared to the limited. This corroborates previous findings, of Mg^{2+} uptake from B horizon soil solution, where *S. bovinus* was the most abundant species (Mahmood *et al.*, 2024). Additionally, *S. bovinus* showed a clear increase in abundance from the O to E to B horizon, a pattern that was repeated in the in-growth mesh bags which were placed in the horizons, indicating the preference of *S. bovinus* toward mineral soils (Mahmood et al., 2024).

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Suillus luteus has no significant expansions or contractions in the full dataset and one contraction in the full and partial family datasets, but had significantly higher mycelial concentrations of Ca and Fe in the gabbro treatment compared to the limited treatment. These findings confirm our hypothesis about mineral weathering resulting in base cation uptake when *S. bovinus* and *S. luteus* grow with gabbro additions. Notably, isolate Sl2 had significantly higher mycelial concentrations of Mg, Ca, Fe and P in the gabbro treatment compared to the limited treatment. *Suillus luteus* has previously been reported to be tolerant to heavy metal exposure, specifically zinc and cadmium which are both cations (Bazzicalupo et al., 2020; Colpaert et al., 2011; Ruytinx et al., 2011, 2017, 2019). These adaptations were concluded to be due to single nucleotide polymorphisms and gene copy number variation in genes involved in heavy metal homeostasis, which includes metal ion transporter genes, in tolerant populations (Bazzicalupo et al., 2020). These findings align with our first hypothesis that adaptation to the environment, such as greater uptake of base cations, can occur through expansions in gene family copy number (Bazzicalupo *et al.*, 2020).

In addition to significant expansions in the MgtE gene family, *S. variegatus* also possesses significant expansions in the nucleobase:cation symporter-1 gene family (TCDB ID 2.A.39) which is associated with Na uptake. In pure culture, *S. variegatus* had significantly higher mycelial Ca and Fe concentrations in the gabbro compared to the limited treatment, further confirming the hypothesis about mineral weathering resulting in base cation uptake when fungi are growing with mineral additions. Previously, *S. variegatus* has been shown to take up K^+ from granite particles (Fahad et al., 2016), to produce high levels of organic acids in the presence of minerals (Olsson and Wallander, 1998; Wallander and Wickman, 1999) and to demonstrate raised levels of weathering when in symbiosis with *Pinus sylvestris* compared to non-mycorrhizal plants (Wallander and Wickman, 1999), ratifying our second hypothesis. In addition to significant expansions in the MgtE gene family, *S.lakei*, *S. cothurnatus* and *S.* cf *subluteus* have particularly high numbers of other base cation transporter gene families with significant expansions in the full and the partial family datasets, which may also indicate a heightened ability to take up base cations and potentially weather minerals, however this needs experimental confirmation.

Proportionally higher base cation uptake of Ca in *Suillus* compared to *Piloderma* indicating greater weathering capabilities

Whether base cation uptake by *Suillus* species growing with mineral additions was greater compared to *Piloderma* species was partly confirmed by comparing the differences in the ratio of element concentrations in the mineral treatments compared

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to the nutrient limited media. Only Ca showed a clear pattern for *Suillus* as a group, but other base cations either showed no pattern or mixed patterns. Taken together this may indicate a greater weathering capability for *Suillus*, especially in the gabbro, which has a higher proportion of weatherable minerals compared to the granite. Though it was expected that *Suillus* isolates would take up more base cations in pure culture than all other isolates, a number of *Piloderma* isolates performed equally well (Fig. S69). Similarly to *Suillus* species, *P. byssinum* and *P. sphaerosporum* had significant expansions in the MgtE gene family (Fig. S3 and S19), indicating that these isolates also are capable of not only taking up base cations, but potentially also of weathering minerals.

Mycelial base cation uptake correlates with base cation transporter gene family copy numbers

We found 28 significant correlations between different elements and copy numbers of base cation transporter gene families (which were significantly expanding or contracting) associated with their uptake, confirming the hypothesis that base cation uptake correlate with base cation transporter gene family copy numbers. For example, copy numbers of the MgtE gene family were shown to have significant positive correlations with Mg uptake in the gabbro, granite and limited treatments and a weak positive correlation in the rich treatment, which may indicate that these genes are only transcribed in conditions where nutrients are limited and mineral weathering is required to access nutrients. The major facilitator gene superfamily showed no significant correlation between gene copy number and base cation uptake, however when further separated into transporter gene families, copy numbers of the anion:cation symporter (ACS) gene family, associated with K, Na and P transport (Farsi et al., 2016; Pavón et al., 2008; Prestin et al., 2014), had a significant positive correlation with Na uptake in the rich treatment. Not all transporter gene families associated with transport of a particular base cation showed a significant positive correlation between gene copy number and uptake of that base cation, for example the TRP-CC gene family and the ACS gene family showed significant negative correlations with Ca uptake in the gabbro treatment and t K uptake in the limited treatment, respectively, which may indicate that another mechanism of regulation, such as increasing rates of transcription of a smaller number of genes, may be favoured. Additionally, there were significant correlations between uptake of specific base cations and gene copy numbers of transporter families which are not associated with the transport of those base cations. For example, the oligopeptide transporter (OPT) family, which is associated with Fe transport (Kurt, 2021; Mousavi et al., 2020; Wintz et al., 2003), was significantly negatively correlated with Mg uptake in the gabbro, granite and limited treatments and may indicate an inhibitory effect of proteins of this family on Mg uptake (data not shown).

Conclusions

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Suillus species which often grow in the B horizon in boreal forests have the capability to take up base cations from mineral sources, and here we tested whether greater base cation uptake is dependent on evolutionary expansions in copy-numbers of base cation transporter gene families. Base cation transporter gene families were among the most abundant type of transporter gene families, with a rapid evolution in the genus *Suillus* in particular. This suggests that the expansions and/or contractions in these transporter gene families are likely related to the requirement of base cation uptake, underpinned by the finding that mycelial uptake of elements in the presence of gabbro and granite, two commonly occurring mineral-rich rock types, often increased with base cation transporter gene family copy numbers. Since the result was not confined to the genus *Suillus* alone, but also detected for the genus *Piloderma*, some species of which are also found growing in mineral soil layers, this type of study using controlled experiments with ECM fungi growing in pure culture, or in symbiosis with a seedling, to confirm the weathering process should be expanded to include more species. Further work into other levels of regulation, e.g. through transcriptional regulation of transporter proteins, would also be useful to gain a deeper understanding of base cation uptake regulation and its role in mineral weathering by *Suillus* species, and ECM fungi in general.

Supplementary Information

Supplementary information is available on FigShare doi: 10.6084/m9.figshare.28016633.

Author Contribution

All authors contributed to conceptualisation of the project. KK and MSG performed bioinformatic analyses. KK and PF carried out experimental work and elemental analyses. KK wrote the manuscript with contribution from all co-authors.

Competing interests

The authors declare that they have no conflicts of interest

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Mineral weathering and nitrogen mobilisation are two key biogeochemical processes in boreal forests. The contribution of ectomycorrhizal fungi in mediating these processes is clear, however key knowledge gaps remain. This thesis explores the interconnectedness of mineral weathering and nitrogen mobilisation, and the role of fungal communities in their mediation; the evolution of mineral weathering traits in ectomycorrhizal fungi; and the regulation of base cation uptake during mineral weathering and the production and exudation of organic acids.

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