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Decomposition of soil organic matter by ectomycorrhizal fungi: Mechanisms and consequences for organic nitrogen uptake and soil carbon stabilization

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A major fraction of nitrogen (N) in boreal forest soils is found in organic forms associated with soil organic matter (SOM) and mineral particles. The capacity of ectomycorrhizal (ECM) fungal symbionts to access this N is debated, considering that these fungi have lost many of the genes for decomposing organic matter that were present in their saprotrophic ancestors. To gain a molecular-level understanding of the N-mining processes in ECM fungi, we developed an experimental approach where the processes of decomposition were studied in parallel with the changes in the structure and properties of the organic matter. We showed that ECM fungi have significant capacities to assimilate organic N associated with SOM and mineral surfaces. The decomposition mechanisms differ between species, reflecting the lignocellulose decomposition mechanisms found in their saprotrophic ancestors. During N-mining, the ECM fungi processed the SOM to a material with increased adsorptive properties to iron oxide mineral particles. Two pathways contributed to these changes: Extracellular modifications of the SOM and secretion of mineral surface reactive metabolites. Some of these metabolites have iron(III)-reducing activities and can participate in extracellular Fenton reactions and redox reactions at iron oxide mineral surfaces. We conclude that the traditional framework for understanding organic N acquisition by ECM fungi from recalcitrant SOM must be extended to a framework that includes how those decomposition activities affect the stabilization and reactivity of mineral-associated SOM. The activity through these complex networks of reactions is decisive for the overall effect of ECM fungal decomposition on nutrients and C-cycling in forest ecosystems.

KEYWORDS

ectomycorrhizal fungi, decomposition, soil C stabilization, iron oxide minerals, nitrogen acquisition, metabolites

Introduction

Primary production in boreal forest ecosystems is commonly limited by nitrogen (N) availability (Högberg et al., 2017). A large fraction of the N in these soils is present in organic forms, primarily as proteinaceous compounds, but also as heterocyclic N molecules (Nannipieri and Paul, 2009; Leinweber et al., 2013), that result from the decomposition of plant, microbe, and animal tissues. This organic N is complexed with other organic molecules present in soil organic matter (SOM), including cellulose, hemicellulose, chitin, lignin, lipids, polyphenols, and other biomolecules (Simpson et al., 2007). The bioavailable fraction of organic N, i.e., compounds that can be taken up by plants and microbes, is considered to consist of water-soluble molecules of relatively small size. This fraction includes amino acids and small peptides, and plants have been shown to directly assimilate amino acids (Näsholm et al., 2009). However, dissolved N compounds account for a small fraction of the organic N pool (Jones et al., 2004). Although there is evidence that plants can increase the available N pool by secretion of proteases and subsequent proteolysis (Godlewski and Adamczyk, 2007; Adamczyk et al., 2008; Paungfoo-Lonhienne et al., 2008), for trees in boreal forests, fungal root symbionts including ectomycorrhizal (ECM) fungi are thought to play a key role in this process (Smith and Read, 2008; Högberg et al., 2017). To access the organic N compounds present in soil, ECM fungi must have the capacity to decompose at least partly the SOM complexes where N is embedded. ECM fungi have evolved several times from saprotrophic ancestors (Kohler et al., 2015; Tedersoo and Smith, 2017). The ancestral decomposition strategies likely resembled the enzymatic system for the decomposition of lignocellulose in white-rot (WR) wood-decaying fungi, the two-step mechanism involving hydroxyl radicals ($\cdot\text{OH}$) generated by Fenton chemistry and hydrolytic enzymes in brown-rot (BR) wood-decaying fungi (Hatakka and Hammel, 2010), or enzymatic decomposition systems in litter decomposers, presumably similar to those of WR fungi (Floudas et al., 2020; Ruiz-Dueñas et al., 2020). The convergent loss of genes encoding plant cell wall-degrading enzymes (PCWDEs) in ECM fungi has been used as an argument against the view that ECM fungi have a significant capacity to decompose SOM (Pellitier and Zak, 2018). However, some genes coding for PCWDEs have been conserved and these display very diverse types and numbers across ECM lineages (Kohler et al., 2015), which suggest that ECM fungi have retained and adapted some features of the decomposition mechanisms found in their saprotrophic ancestors. Moreover, ECM lineages have retained the genes involved in the decomposition of microbial cell walls (Miyachi et al., 2020), which suggests that ECM fungi might selectively target compounds of microbial origin that can be rich in N, such as chitin and proteins.

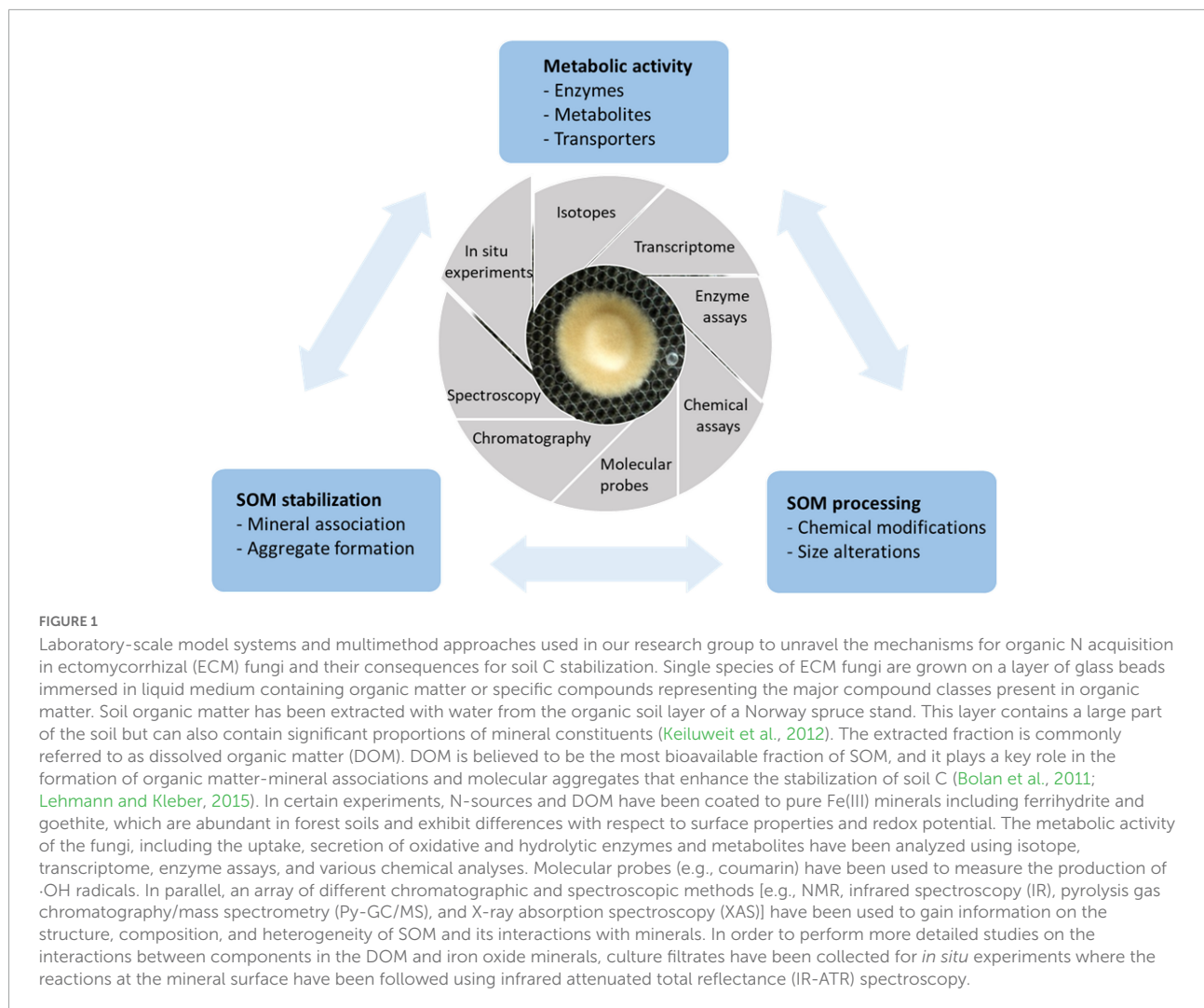
Assuming that the main function of the decomposition activity of ECM fungi is to mobilize nutrients entrapped in

organic matter, rather than obtaining metabolic carbon (C) (Lindahl and Tunlid, 2015), an outstanding question then is if these decomposition activities render the remaining organic matter more or less suitable for further decomposition by saprotrophic microbes (Frey, 2019). SOM can be stabilized against microbial decomposition via polymerization reactions that lead to the formation of recalcitrant humic substances (Schnitzer and Monreal, 2011). The humification model for SOM stabilization has been increasingly questioned, and it has been proposed that SOM becomes protected from microbial decomposition by interacting with mineral particles or by being incorporated into molecular aggregates (Lehmann and Kleber, 2015). It follows that the processing of SOM accompanying the N-mining activities in ECM fungi may affect soil C stabilization via several different mechanisms.

A common focus among ecologists examining organic nitrogen acquisition in ECM fungi has been the enzymatic decomposition of SOM including the activity and expression of PCWDEs and proteolytic enzymes. Less attention has been paid to how decomposition by ECM fungi affects the stabilization of soil C. To cover both aspects, we have developed an experimental approach where the decomposition activity and organic N uptake by ECM fungi were examined in parallel with analyzing structural modifications and formation of mineral-stabilized SOM (Figure 1). Coupled with experimental manipulations, space and time-resolved experiments and modeling, our approach has made it possible to make connections between biology and chemistry, and to identify extracellular components and physiological conditions that control organic matter decomposition and N assimilation in ECM fungi. Below, we summarize and discuss some of the key findings from these experiments.

Soil organic matter decomposition—diverse mechanisms but similar function

Spectroscopic analyses together with transcriptome profiling have provided firm experimental evidence that ECM fungi decompose SOM with mechanisms resembling those seen in their saprotrophic ancestors (Rineau et al., 2012; Shah et al., 2016). When five ECM fungi derived from BR, WR, or litter decomposing ancestors were grown on dissolved organic matter (DOM) from the organic soil layer of a Norway spruce stand as the only N source, they all oxidized compounds in the DOM, including polysaccharides and lignin residues (Shah et al., 2016). The observed oxidative modifications of the DOM during decomposition were similar across the examined ECM species, but the extent of the oxidation differed and was related to the growth rate and the rate of N uptake by the ECM fungi. The rapidly growing, long-distance-exploration



types (Agerer, 2001) oxidized DOM and assimilated N to a greater extent than did the slow-growing, short- and medium-distance-exploration types.

Despite the similarity in the chemical transformation of the SOM extract, each species expressed a different set of transcripts encoding enzymes that are typically associated with oxidative degradation of plant cell wall compounds in wood-decaying fungi (Levasseur et al., 2013). The enzymes included: Laccases, for the oxidation of a wide range of organic compounds; glucose-methanol-choline oxidoreductases (GMCOs) and copper-radical oxidases (CROs), for reduction of oxygen to peroxide likely supporting Fenton chemistry and peroxidases (PODs) with extracellular hydrogen peroxide; various PODs and tyrosinases. The fungi also expressed a number of genes encoding extracellular peptidases, presumably involved in the hydrolysis of proteins released from the organic matter. The variation in the expression pattern of such peptidases was smaller than that of oxidoreductases associated with decomposition of lignocellulose (Shah et al., 2016).

Links between organic matter decomposition and the release of organic N, and how these processes are regulated by nutritional cues, have been identified in studies of the ECM fungi *Paxillus involutus* and *Laccaria bicolor*, representing species with BR and litter decomposing ancestors, respectively (Nicolás et al., 2019). In both fungi, assimilation of the N sources followed a temporal pattern. The readily available inorganic N source (i.e., ammonium) was utilized before assimilation of the organic amide-N sources commenced, and these organic N sources were liberated via SOM oxidation. Thus, SOM oxidation was initiated upon depletion of ammonium and ceased when the fungi experienced C limitation. Despite the different evolutionary histories and decomposition mechanisms of the two ECM species, there was functional convergence between them with a common tight link between SOM oxidation and liberation of organic N, and similar nutritional regulation of the processes. This suggests a dual control over the decomposition activities of ECM fungi, i.e., supply of photosynthetic products

from the host plant and the characteristics of the N sources in soil.

Fenton reaction and organic N acquisition in *Paxillus involutus*

SOM decomposition by *P. involutus* is similar to the BR wood-decaying mechanism and involves a two-step process of $\cdot\text{OH}$ oxidation and enzymatic hydrolysis (Nicolás et al., 2019). The $\cdot\text{OH}$ radicals generated by the Fenton reaction also oxidize and thereby facilitate the liberation of N from proteins (Op De Beeck et al., 2018). Moreover, the oxidation of the protein by $\cdot\text{OH}$ radicals is followed by the induction of extracellular proteolytic activity. Notably, oxidized proteins induced a greater extracellular proteolytic activity than did unmodified proteins, suggesting a functional link between oxidation and hydrolysis of protein substrates. The key factors regulating the Fenton reaction during protein acquisition by *P. involutus* were captured in a mathematical model that predicts that the production of $\cdot\text{OH}$ radicals occurs in a burst and that the concentrations of ammonium and Fe(II) are the key factors controlling $\cdot\text{OH}$ production (Op De Beeck et al., 2018). The timing and the onset of the Fenton burst are controlled by a positive feedback mechanism where the fungus senses and responds to degradation products of the oxidized protein substrate.

Transformation of organic matter through anabolic and catabolic processes

During decomposition and assimilation of N, *P. involutus* processes DOM components toward smaller size molecules and molecules with increased polar group content. This increases the propensity of the processed organic matter for adsorption to mineral particles (Wang et al., 2017). These results agree with the conceptual model of SOM formation and stabilization proposed by Lehmann and Kleber (2015). Less expected was the finding that SOM decomposition by *P. involutus* was accompanied by secretion of extensive amounts of low molecular weight compounds (> 10% new biomass C) that also contributed to enhanced mineral retention. NMR analyses of the secreted compounds revealed a range of functional groups, including alkyl, aromatic, phenolic, and carbonyl groups. The secreted compounds were mainly low molecular weight (below 6.5 kDa) metabolites, but also larger size fractions were detected as well as both mineral-surface reactive and non-reactive compounds. Overall, this suggests diverse biological functionalities among the secreted compounds.

Moreover, decomposition of DOM by *P. involutus* generates a modified DOM that displays an increased iron-reducing capacity (Rineau et al., 2012). Recent experiments showed that such processed DOM had a significant capacity to reductively dissolve ferrihydrite and goethite, two common iron-oxide minerals in boreal forest soils (Krumina et al., 2022). Detailed studies of the molecular reactions at the water-iron oxide interfaces using *in situ* IR spectroscopy showed that further oxidation of adsorbed DOM was triggered by adding H_2O_2 , which initiated Fenton reactions both at the iron minerals surface (heterogenous Fenton reactions) and in solution (homogenous Fenton reactions). These processes caused an extensive radical oxidation of the organic matter-iron mineral associations generating a high concentration of surface-associated oxalate. Many soil microorganisms including ECM fungi have the capacity to secrete oxalic acids (Lapeyrie et al., 1987; Jones et al., 2003), and it is thought that oxalate plays a key role in the acquisition of nutrients from mineral particles (Landeweert et al., 2001; Keiluweit et al., 2015). Our results suggest an additional and overlooked extracellular source of oxalic acid in soils that needs to be considered.

Nitrogen acquisition from mineral-associated proteins

Approximately 20–50% of the SOM in boreal forest soils is associated with reactive minerals (Kramer and Chadwick, 2018). In particular, the protein fraction is known to bind strongly to such minerals (Kleber et al., 2007), and a common idea is that protein desorption must precede enzymatic degradation (Mikutta et al., 2007; Jilling et al., 2018). Accordingly, mineral-associated proteins are rarely considered as bioavailable N for soil microorganisms (Jilling et al., 2018). This assumption is challenged by recent results showing that the ECM fungi *P. involutus*, *Hebeloma cylindrosporium*, and *Piloderma olivaceum* assimilate N from iron mineral-associated proteins by hydrolyzing the protein directly at the mineral surface without initial desorption (Wang et al., 2020, 2021). This proteolytic surface activity was moderated by the secretion of low-molecular-weight metabolites that conditioned the mineral surface and thereby facilitated proteolysis. The chemical structures of these metabolites have not been characterized in detail, but IR spectroscopy indicated a chemical diversity that differed between ECM fungal species (Wang et al., 2020, 2021).

Discussion

The recent research summarized above emphasizes the need to develop a framework for organic N acquisition in ECM fungi that includes the effect of SOM decomposition activities on the stabilization of organic matter in addition to the

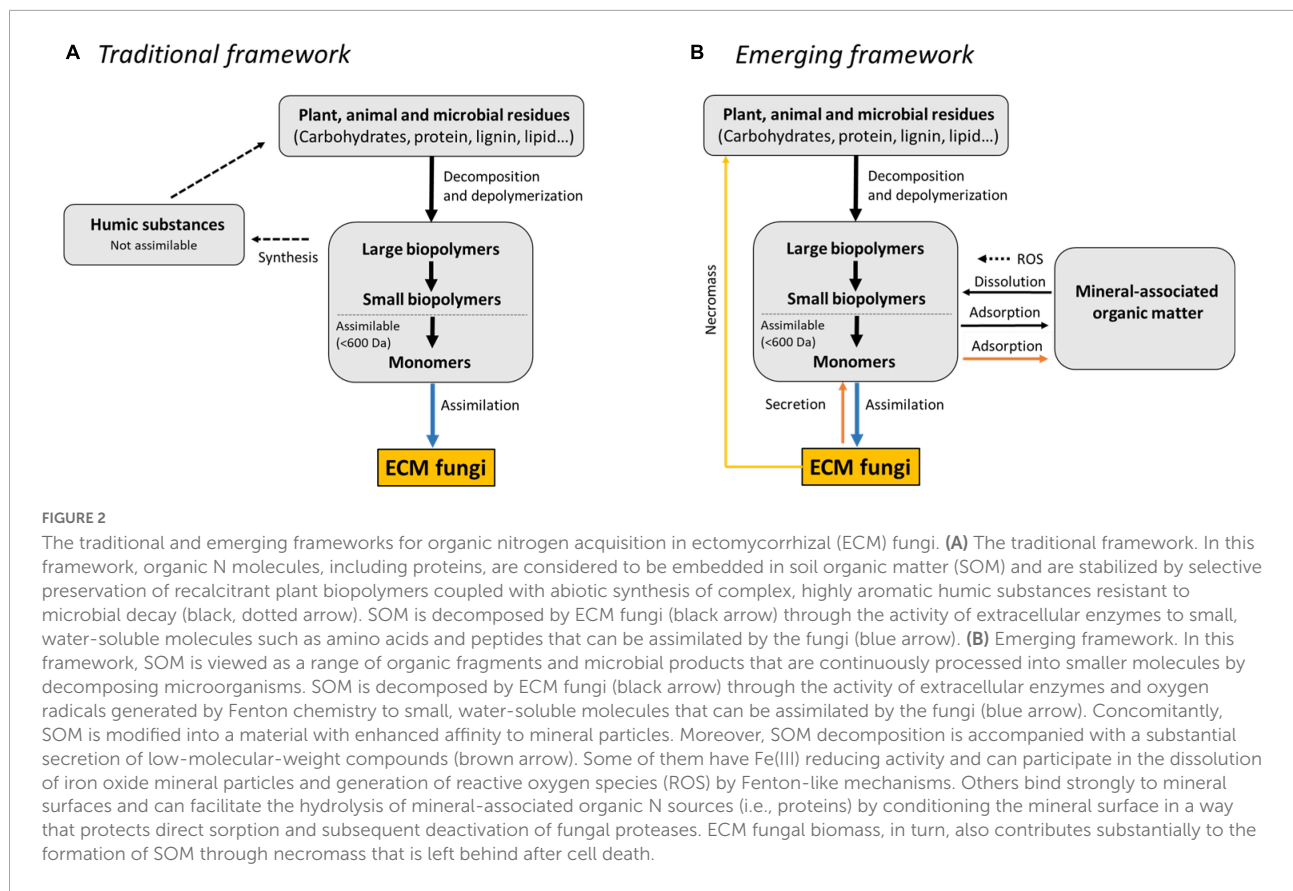


FIGURE 2

The traditional and emerging frameworks for organic nitrogen acquisition in ectomycorrhizal (ECM) fungi. **(A)** The traditional framework. In this framework, organic N molecules, including proteins, are considered to be embedded in soil organic matter (SOM) and are stabilized by selective preservation of recalcitrant plant biopolymers coupled with abiotic synthesis of complex, highly aromatic humic substances resistant to microbial decay (black, dotted arrow). SOM is decomposed by ECM fungi (black arrow) through the activity of extracellular enzymes to small, water-soluble molecules such as amino acids and peptides that can be assimilated by the fungi (blue arrow). **(B)** Emerging framework. In this framework, SOM is viewed as a range of organic fragments and microbial products that are continuously processed into smaller molecules by decomposing microorganisms. SOM is decomposed by ECM fungi (black arrow) through the activity of extracellular enzymes and oxygen radicals generated by Fenton chemistry to small, water-soluble molecules that can be assimilated by the fungi (blue arrow). Concomitantly, SOM is modified into a material with enhanced affinity to mineral particles. Moreover, SOM decomposition is accompanied with a substantial secretion of low-molecular-weight compounds (brown arrow). Some of them have Fe(III) reducing activity and can participate in the dissolution of iron oxide mineral particles and generation of reactive oxygen species (ROS) by Fenton-like mechanisms. Others bind strongly to mineral surfaces and can facilitate the hydrolysis of mineral-associated organic N sources (i.e., proteins) by conditioning the mineral surface in a way that protects direct sorption and subsequent deactivation of fungal proteases. ECM fungal biomass, in turn, also contributes substantially to the formation of SOM through necromass that is left behind after cell death.

commonly considered enzymatic mechanisms and mobilization of N from recalcitrant SOM (Figure 2). We have identified two processes in ECM fungi that affect the adsorptive properties of SOM: *Ex vivo* modifications where extracellular enzymes and oxygen radicals attack and transform DOM into a material with enhanced propensity to associate with mineral particles; *in vivo* turnover pathway where the fungi synthesize and secrete mineral-surface reactive metabolites (Liang et al., 2017). Apart from metabolites, dead ECM hyphae (i.e., necromass) may significantly contribute to the formation of stable organic matter in forest soils (Godbold et al., 2006; Clemmensen et al., 2013). Necromass is formed through the *in vivo* turnover pathway when mycelial growth is arrested due to stress conditions in the environment, typically C-limitation (Ekblad et al., 2013). Experiments suggest that the decomposing activities of ECM fungi can show diverse responses to prolonged C limitation (Nicolás et al., 2019), which emphasize that further studies are needed to establish links between microbial growth and SOM turnover, and how these processes are controlled by nutrient- and C conditions. It is also clear that different ECM fungi differ in the way they decompose SOM. Hence, the molecular mechanisms involved in SOM decomposition need to be characterized in more ECM fungi to get a better overview of the functional diversity of decomposition systems employed by ECM fungi across a wide phylogenetic range.

Microbial processing of organic matter may also contribute to the formation of stabilized SOM by producing compounds with an increased propensity to become incorporated into molecular aggregates (Lehmann and Kleber, 2015). However, little is known about the molecular aggregates present in the organic matter extracted from boreal forest soils. A recent study from our group shows that ca. 40–70% of the total organic C in DOM extracted from boreal forest is present in a colloidal size fraction (Meklesh et al., 2022). Important challenges for future research are to examine whether nutrients present in colloidal fractions are accessible to ECM fungi and whether the decomposing activity of ECM fungi will alter the colloidal fractions of organic matter.

The significance of metabolites in decomposition processes has been questioned by soil scientists considering that soil microorganisms do not have the energy and nutrient resources needed for synthesizing metabolites in quantities required for their actions (Lehmann and Kleber, 2015). This may be true for saprotrophic fungi that encounter metabolic C limitations. However, the situation is different for ECM fungi, which can receive an extensive amount of energy from the host plants (Högberg and Högberg, 2002). The importance of metabolites has been highlighted in the so-called unbutton model where low molecular weight organic acids, such as citric and oxalic acids produced by ECM fungi, are suggested to play an important role

in releasing nutrients from SOM aggregates. The acids disrupt the aggregates by forming complexes with network-forming metal ions (Clarholm et al., 2015). Our research suggests that ECM fungi secrete metabolites that can facilitate nutrient acquisition with other mechanisms involving Fenton chemistry and redox reactions at mineral surfaces. These metabolites work in concert with extracellular enzymes, and most likely ECM fungi have evolved mechanisms to keep the reactants and their products close to the hyphae. Extracellular polymeric substances (EPS) may be important functional components that assure that extracellular decomposition reactions occur at the right time and in the right place (Op De Beeck et al., 2020, 2021).

A recent discovery is the large capacity of ECM fungi with diverse evolutionary origins to reductively dissolve iron minerals (Wang et al., 2020, 2021). The mechanisms of these electron-transfer reactions are not known, but in agreement with mechanisms identified in iron-reducing bacteria (Glasser et al., 2017), we hypothesize that electrons are transferred from the cell to the mineral surface by small, secreted molecules, i.e., extracellular electron shuttles (EESs). This is supported by experiments showing that reductive dissolution of solid iron(III) complexes by *P. involutus* is accompanied with secretion of iron(III) reducing metabolites (Shah et al., 2021). The reductive dissolution of iron(III) minerals may, in the presence of O₂ and/or H₂O₂, trigger the production of highly reactive oxygen species via Fenton-like reactions (Garrido-Ramirez et al., 2010; Krumina et al., 2022). Thus, the capacity to oxidize SOM by ·OH may be much more widespread among ECM fungi than has so far been anticipated. A recent study based on the analyses of reactive Mn(III) and Fe(II/III) species and oxidative activities suggests that the prevalence of Mn- vs. Fe-mediated oxidative mechanisms in forest soils varies with soil depth; Mn-mediated oxidation predominates in surface soil layers and Fe-mediated oxidation in deeper, mineral-containing soil layers (Jones et al., 2020). This differentiation correlates with the vertical distribution of fungal communities where saprotrophic litter decomposing fungi inhabit surface soil layers while ECM fungi predominate in deeper layers, following the colonization depth of the roots they grow on (Lindahl et al., 2007). We hypothesize that the success of ECM fungi in colonizing deeper soil layers is partly due to their ability to control iron redox chemistry, and exploiting this for oxidative decomposition and nutrient acquisition. This hypothesis is also supported by observations made on the landscape level, as Mn availability and the production of Mn-peroxidases are negatively correlated to carbon storage across different northern and temperate forests (Stendahl et al., 2017; Kranabetter et al., 2021). In temperate forests, the switch from Mn-peroxidases to Fenton-mediated decomposition with increased soil weathering resulted not only in deeper organic horizons but also a twofold increase in mineral soil C content (Kranabetter et al., 2021).

Reductive dissolution of mineral particles also facilitates the mobilization of other nutrient sources than N; in particular

phosphorus (P). In soils, a major fraction of P is associated with Fe(III) minerals (Arai and Sparks, 2007). Several studies have shown that ECM fungi can access mineral-bound P (Wallander et al., 1997; Rosling, 2009), but the precise mechanism(s) are still being debated. A recent study has demonstrated that mineral-associated P can be released via reductive dissolution promoted by Fe(III) reducing bacterial metabolites (McRose and Newman, 2021). We propose that a similar mechanism may contribute to the mobilization of P by ECM fungi.

It should be noted that organic N mining and stabilization of soil C by ECM fungi in boreal forest soils include several processes and mechanisms that have not been examined in our experiments. For example, the production of fungal necromass might have a profound effect on soil C stabilization (Fernandez et al., 2016), and the release of low-molecular-weight compounds by mycorrhizal fungal exudation may stimulate the decomposition activities of saprotrophic microorganisms (Phillips et al., 2012). Recent research has shown that soil C sources might be stabilized not only by association with mineral particles but also by interactions with root-derived tannins (Adamczyk, 2021). Moreover, competition with free-living saprotrophic fungi may potentially suppress SOM decomposition (the so-called Gadgil effect) (Fernandez and Kennedy, 2016).

To conclude, laboratory-scale experiments with single ECM fungal species and multimethod analytical approaches have increased our current understanding of the complex mechanisms underlying soil organic N acquisition in ECM fungi, including their impacts on SOM stabilization. The activity of these pathways is likely to be strongly dependent on the soil geochemistry, the presence of other microorganisms, and the allocation of host photosynthate to the ECM mutualists (Frey, 2019; Zak et al., 2019). High priority must be given to identifying molecular markers that can be used for probing the activity in the decomposing pathways of ECM fungi in more complex laboratory systems and in the field. We believe that experiments in standardized laboratory systems coupled with transcriptomics and advanced spectroscopic methods will accelerate the discovery of molecular markers that can provide functional links between ECM fungal activities, SOM dynamics, and organic N uptake. Such markers will significantly increase our knowledge on the extent to which ECM fungi get access organic N embedded in SOM and adsorbed on mineral particles *in situ*. Combined with measurements of the fungal transfer of this N to the plant host, the research has important implications for understanding the role of ECM fungi to plant growth, and the cycling of C and N in boreal forest ecosystems. Key research questions to be addressed by future research include:

- Is SOM decomposition by ECM fungi the result of partial retention of ancestral mechanisms or development of novel mechanisms?

- How is SOM decomposition by ECM fungi regulated by the host C supply and the nutrient conditions in the soil environment?
- How do ECM physiology and growth strategy affect SOM decomposition and N transfer to the host plant?
- Are specific SOM molecules targeted by the decomposition activities of ECM fungi, and what are the chemical characteristics of ECM-decomposed SOM?
- Are nutrients present in the colloidal fraction of DOM available to ECM fungi and does DOM decomposition change the colloidal properties of DOM?
- How prevalent is the ECM capacity to reductively dissolve iron-oxide minerals and what are the consequences for Fenton-based oxidation of organic matter in boreal forest soils? (SOM chemistry).
- How can mechanisms and reaction pathways from laboratory-scale experiments accurately describing SOM decomposition by ECM fungi be incorporated into models that predict soil carbon budgets?

Author contributions

AT wrote the first draft of the manuscript. DF, MO, TW, and PP wrote sections of the manuscript. All authors

contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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