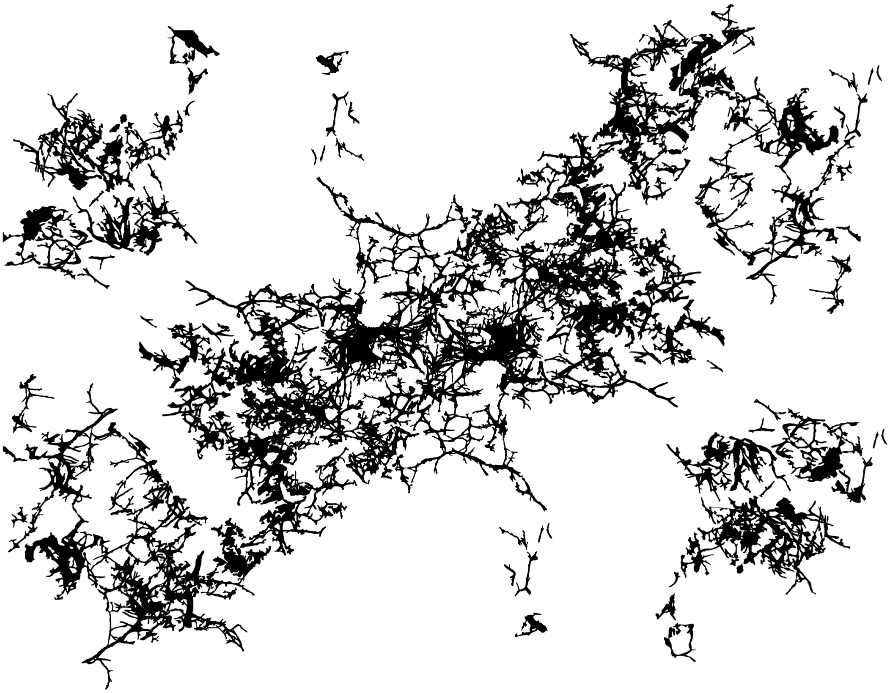
Acta Universitatis Agriculturae Sueciae

Doctoral Thesis No. 2026:29
Faculty of Natural Resources and Agricultural Sciences

Ectomycorrhizal decomposers – enzymes, ecology, and evolution

Erica E. Packard



Ectomycorrhizal decomposers – enzymes, ecology, and evolution

Erica E. Packard
Faculty of Natural Resources and Agricultural Sciences
Department of Soil and Environment
Uppsala

SLU
SWEDISH UNIVERSITY
OF AGRICULTURAL
SCIENCES

DOCTORAL THESIS
Uppsala 2026

Acta Universitatis Agriculturae Sueciae
2026:29

Cover: Mycelium uncovered (Artwork: Erica Packard)

ISSN 1652-6880
ISBN (print version) 978-91-8124-246-1
ISBN (electronic version) 978-91-8124-276-8
https://doi.org/10.54612/a.4fafsjqsnn

Swedish University of Agricultural Sciences, Department of Soil and Environment, Uppsala, Sweden

Print: SLU Grafisk service, Uppsala 2026

Ectomycorrhizal decomposers – enzymes, ecology, and evolution

Abstract

Ectomycorrhizal fungi are central to nutrient cycling and carbon dynamics in forest soils. Some ectomycorrhizal species, here called ectomycorrhizal decomposers, have maintained genes from their saprotrophic ancestors encoding for potent oxidative enzymes known as manganese peroxidases. This thesis aimed to deepen the understanding of ectomycorrhizal decomposers and their role in soil organic matter dynamics, with a focus on *Cortinarius* species. Specifically, I aimed to elucidate the mechanisms and implications of ectomycorrhizal manganese peroxidases using a combination of molecular methods (including ITS metabarcoding, *in silico* genome analysis, and metatranscriptomics), enzyme assays, and heterologous expression of recombinant enzymes.

Recombinant manganese peroxidases from *Cortinarius* were found to oxidise organic compounds. Further, expression of manganese peroxidase genes *in situ* was correlated with expression of genes related to acquisition of organic nitrogen and phosphorus. These results are key steps forward in linking the genomic capacity of ectomycorrhizal decomposers to their suggested role in mining nutrients from recalcitrant soil organic matter. In addition, gene expression data suggested that oxidative decomposition was not associated with a high energy demand, at least relative to other mycelial activities. On the ecosystem scale, it appeared that certain ectomycorrhizal species were particularly important decomposers, with considerable trait variation at the genus-level. The species assigned here exhibited niche differentiation with respect to forest age and soil fertility.

In nitrogen-limited boreal forests, the duality of mycorrhizal ecology and potent decomposer capacity is an especially important trait. As anthropogenic climate change progresses, understanding how ectomycorrhizal decomposers influence soil organic matter in different environments may be fundamental for predicting the capacity for boreal forest soils to sequester carbon and sustain tree productivity.

Keywords: Ectomycorrhizal fungi, manganese peroxidases, soil organic matter, enzymes, metatranscriptomics, *Cortinarius*, decomposition, niches, nitrogen cycling, heterologous expression

Mykorrhizabildande nedbrytare – enzymer, ekologi, och evolution

Sammanfattning

Ektomykorrhizasvampar är centrala för skogsmarkens närings- och kolomsättning. Vissa mykorrhizasvamparter har behållit genetisk kapacitet att tillverka kraftfulla oxidativa enzymer, så kallade manganperoxidaser, från saprotrofa anfäder. Denna avhandling syftar till att fördjupa kunskapen om mykorrhizabildande nedbrytare och deras roll i omsättning av organiskt material, med ett fokus på släktet *Cortinarius*. Jag har studerat hur ektomykorrhizasvampars manganperoxidaser fungerar och påverkar marken genom en kombination av molekylära metoder (ITS sekvensering, genomanalys och metatranskriptomik), enzymanalyser, och tillverkning av enzymer i genmodifierad jäst.

Manganperoxidaser från *Cortinarius* uppvisade kapacitet att oxidera organiska föreningar. Transkription av manganperoxidasgener *in situ* var korrelerad med uttryck av gener involverade i upptag av organiskt kväve och fosfor. Dessa resultat är viktiga steg på vägen för att kunna länka den genetiska kapaciteten hos mykorrhizabildande nedbrytare till deras föreslagna roll i omsättning av svårnedbrytbart organiskt material i marken. Utöver detta visade genuttrycksdata att oxidativ nedbrytning inte var kopplat till hög energikostnad, åtminstone inte i relation till andra processer som i svamparnas mycel. På ekosystemnivå tycktes vissa arter vara särskilt viktiga nedbrytare, med stor variation på släktesnivå. Dessa identifierade arter uppvisade tecken på nischdifferentiering med avseende på skogsålder och markbördighet.

I kvävebegränsade boreala skogar verkar det vara av stor betydelse att kunna kombinera mykorrhizasymbios med förmåga att producera kraftfulla nedbrytande enzymer. Det är viktigt att förstå hur svampar med dessa egenskaper påverkar omsättning av organiskt material för att kunna förutsäga effekter av klimatförändringar på boreala skogars kapacitet att binda in kol och producera trädbiomassa.

nyckelord: Ektomykorrhizasvamp, manganperoxidaser, organiskt material i marken, enzymer, metatranskriptomik, *Cortinarius*, nedbrytning, ekologiska nischer, näringomsättning

Dedication

To Melanie and Dan

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. Packard, E.E., Pérez-Izquierdo, L., Clemmensen, K.E., Dahlberg, A., Spohn, M., Stendahl, J., Lindahl, B.D. (2025). Ectomycorrhizal decomposers and their niche(s) in boreal forests. Functional Ecology, 39 (8), 1998-2014. https://doi.org/10.1111/1365-2435.70085

II. Packard, E.E. & Jörgensen, K. (2025). Targeting Mn-dependent peroxidase activities - considerations and optimizations. Fungal Ecology, 80, 101492. https://doi.org/10.1016/j.funeco.2025.101492

III. Packard, E.E., Berrin, JG., Floudas, D., Haon, M., Martin, F., Lindahl, B.D. Ligninolytic potential of heterologous manganese peroxidase from the ectomycorrhizal fungal species *Cortinarius aurae.* (manuscript)

IV. Packard, E.E., Clemmensen, K.E., Gangiah, T.K., Jörgensen, K., Lindahl, B.D. Using targeted metatranscriptomics to investigate oxidative decomposition mechanisms by *Cortinarius ominosus*. (manuscript)

Paper I is open access under CC BY-NC-ND 4.0 and Paper II is open access under CC BY 4.0.

The contribution of Erica E. Packard to the papers included in this thesis was as follows:

I. Contributed to the planning of analyses with co-authors. Performed data analyses and interpreted the results together with the co-authors. Wrote the manuscript together with co-authors and was responsible for correspondence with journal.

II. Conceptualised and designed the study with co-author. Performed laboratory experiments and data analysis with co-author. Wrote the manuscript together with the co-author.

III. Contributed to the planning of study together with co-authors. Performed the laboratory work and data analysis. Interpreted the results and wrote the manuscript together with co-authors.

IV. Designed and planned the study together with co-authors. Conducted the field and laboratory work. Performed the data analysis with support from co-authors. Interpreted the results and wrote the manuscript together with co-authors.

Abbreviations

(m)RNA	(messenger) Ribonucleic acid
AA*	Auxiliary Activities (family i.e. AA2)
ABTS	2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
C	Carbon
DMAB	3-(dimethylamino)benzoic acid
DNA	Deoxyribonucleic acid
GH	Glycoside hydrolase
H_2O_2	Hydrogen peroxide
ITS	Internal transcribed spacer
MBTH	3-Methyl-2-benzothiazolinone hydrazine hydrochloride monohydrate
MnP	Manganese peroxidase
N	Nitrogen
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
UV	Ultraviolet

AI declaration

Artificial intelligence was actively avoided in all aspects of preparing this thesis. No part of the texts or analyses (here and in the manuscripts) were modified or generated using AI.

1. Soil fungal ecology

Fungi are essential to nutrient cycling and carbon dynamics in soils, and especially in boreal forests, where there is a relatively high fungal to bacterial biomass ratio (Fierer *et al*., 2009; Lindahl & Clemmensen, 2017). The many different roles that soil fungi play in ecosystem processes is influenced by the wide variation in traits across the breadth of fungal diversity. Their diversity, complexity of ecology, and semi-cryptic nature is a challenge to study but also suggests there is so much yet to be uncovered.

How a fungus lives and grows can be difficult to conceptualise in comparison to the perhaps more obvious body structure of plants and animals. Filamentous fungi grow in a 3-dimensional web of hyphae with indeterminate shape, structure, and "body" size (Figure 1; Fricker *et al.* 2017). They can reallocate their cytoplasm within this hyphal network, allowing for continued growth and exploration of patchy resources (Cairney, 1992). While expanding through the soil, they acquire nutrients and energy, interact with the community of bacteria and archaea, and modify the abiotic properties of their environment.

Typically, it is useful to categorise fungi based on their trophic mode: saprotrophic, acquiring energy through decomposition of organic matter; pathotrophic, acquiring energy at the expense of other living organisms; and biotrophic, acquiring energy through symbiosis. This categorisation may be necessary from a practical stance, but it is also challenged by the flexibility of fungi that often cannot be placed neatly into one box. These blurred lines become clear when we investigate fungi that tend to live symbiotically.

Figure 1. The mycelia of a *Cortinarius* species (light yellow) and other unknown fungi (white) in the soil organic layer.

1.1 Ectomycorrhizal fungi

A key component of the boreal forest ecosystem is the symbiosis between ectomycorrhizal fungi and their host trees. Within this symbiosis, the plant host transfers 20-50 % of its photosynthates to the fungus through its roots (Hobbie & Hobbie, 2006). The mycobiont provides its host with increased access to limiting nutrients through extension of its hyphae beyond the root depletion zone and mobilises nutrients that are in forms otherwise not accessible to the plant. Although ectomycorrhizal fungi form symbiosis with only a small fraction of terrestrial plant species, ca. 2-3% (Brundrett & Tedersoo, 2018), they associate with the dominant boreal tree families such as Pinaceae and Betulaceae (as well as some temperate and tropical families), resulting in a distribution that covers most of the northern hemisphere. A tree may host hundreds of different fungal partners simultaneously (Bruns, 1995), and fungal mycelia may colonise several trees with overlapping root systems (Beiler *et al.*, 2010). Ectomycorrhizal fungi are hyper-diverse with over 20,000 species, mostly from the phyla Basidiomycota and Ascomycota (Rinaldi *et al.*, 2008). The earliest fossilised evidence of ectomycorrhizal roots dates to 52 million years ago (Mya; Beimforde *et al.*, 2011); however, it is suggested that the origins of this lifestyle could have evolved at least 200 Mya with the origin of Pinaceae during the Jurassic period. Ectomycorrhizal symbiosis is estimated to have convergently evolved ~ 80 different times from different saprotrophic ancestors, giving rise to ectomycorrhizal fungi dispersed across many different lineages with a diversity of traits (Hibbett *et al.*, 2000; Tedersoo *et al.*, 2010; Strullu-Derrien *et al.*, 2018).

The ectomycorrhizal fungus has several distinct features when forming symbiosis (Figure 2; Smith & Read, 2008). The symbiotic organ – where exchange between the fungus and plant occurs – of ectomycorrhizas is the Hartig net. This is a dense layer of specialised fungal hyphae that grows between the epidermal and cortical cells of fine root tips and creates the high surface area necessary for exchange between partners (Peterson & Massicotte, 2004). Ectomycorrhizal fungi also produce a sheath around the fine root tips, forming what is called the mantle, a structure that can vary in thickness, colour, and texture between species. Further, ectomycorrhizal symbiosis modifies the growth of the short roots, increasing branching and suppressing root hairs. Ectomycorrhizal fungi extend extraradical hyphae beyond the root. In some cases, extraradical mycelia can be extensive, forming dense mycelial mats and/or aggregated cords of hyphae (sometimes called rhizomorphs), growth forms that are often categorised as "medium-distance fringe", "long-distance" and/or "mat" types (Agerer, 2001). These hyphal cords, like those of saprotrophic fungi (Boddy, 1999), are associated with transport and recycling of nutrients (and water) over longer distances (Cairney, 1992). In other cases, only shorter and/or diffuse hyphae extend from the root, often categorised as "contact", "short-distance" and/or "medium-distance smooth" types.

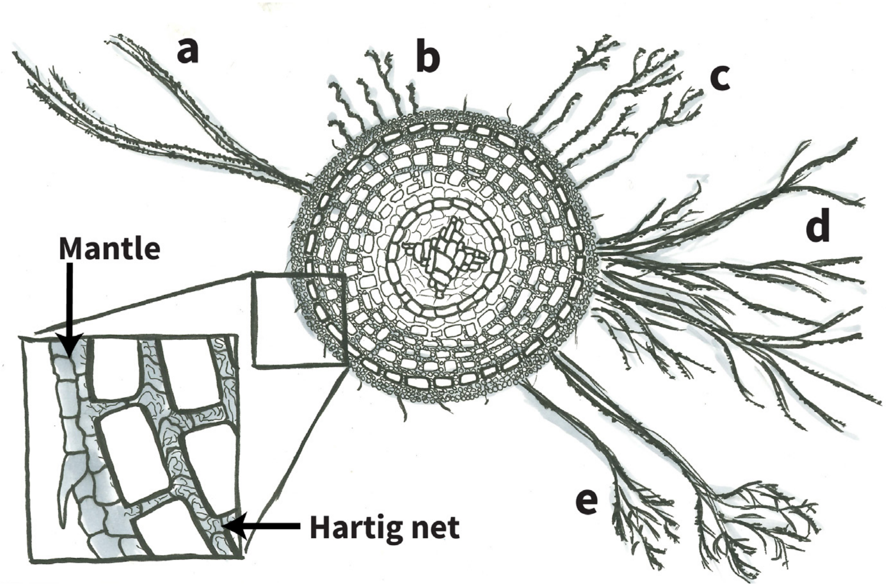


Figure 2. Illustration of ectomycorrhizal root cross-section with different examples of extraradical exploration types *sensu* Agerer (2001). a) long-distance, b) contact, c) short-distance, d) medium-distance fringe, and e) medium-distance smooth. Inset with arrows indicating location of Hartig net between root cortical cells and mantle on surface of root. Illustration by: Wiebke Mareile Heinze

In part due to their diversity, ectomycorrhizal fungi vary in their ability to acquire and take up different sources of nutrients. Nitrogen (N) utilisation from organic sources was first suggested by A.B Frank, the "father" of mycorrhizal research (Frank 1885, as translated by Trappe 2005), but then for the larger part of the 20th century the general opinion and research surrounded the acquisition of only inorganic N sources by ectomycorrhizal fungi (Finlay *et al.*, 1992; Nehls & Plassard, 2018). In the mid-80s, evidence, mostly from petri-dish experiments but also some experiments with inoculated seedlings, was accumulating to support the use of organic N (amino acids, peptides) by at least some ectomycorrhizal fungi (Abuzinadah *et al.*, 1986; Abuzinadah & Read, 1986, 1989; Bending & Read, 1996). While ectomycorrhizal fungi have a preference for ammonium (NH^{4+}), they are also capable of taking up nitrate (NO^{3-}), urea, amino acids, and small polypeptides to varying degrees (Finlay *et al.*, 1992; Chalot & Brun, 1998; Plassard *et al.*, 2000). Boreal forest soils are acidic and generally N-limited, with most N present in organic forms. Therefore, ectomycorrhizal fungi forage for N by releasing extracellular enzymes into the soil. The capacity for ectomycorrhizal fungi to mobilise organic N varies between species, in part due to their evolution from ancestral saprotrophs with different characteristics. Though N is often the focus in boreal forest soils, ectomycorrhizal fungi are also important for the phosphorus (P) acquisition of their plant hosts (Cairney, 2011). Ectomycorrhizal fungi can produce low molecular weight organic acids that contribute to the release of inorganic P absorbed to mineral surfaces. But, as in the case with N, most P is in organic forms such as inositol phosphates (e.g. phytic acid), phosphate esters and diesters that can be hydrolysed by phosphatases (Nehls & Plassard, 2018). The capacity to mobilise P and the contribution to host P acquisition varies between ectomycorrhizal species in a similar way as is for N (Plassard *et al.*, 2011). As a result of their interspecific variability, ectomycorrhizal fungi vary across many different niches according to their sensitivity to changes in the form and availability of nutrients.

While the ectomycorrhizal colonisation on conifer roots is usually thought of as obligate, the exchange between partners is not a simple mutualistic relationship. The contribution of ectomycorrhizal fungi to the nutrition of their hosts is considered essential, but transfer to the plant fluctuates and the metabolic requirements of the fungus need to be met as well (Jones & Smith, 2004). Mycorrhizal fungal traits, such as decomposition capacity, growth rate, and carbon use efficiency, vary broadly across species and subsequently they also diverge in their strength as mutualists (Hoeksema & Kummel, 2003; Jörgensen *et al.*, 2025). In cases of nutrient scarcity, a tree may allocate more carbon (C) belowground, yet this may further aggravate N limitations when mycorrhizal fungi are supplementing their own growth first (Näsholm *et al.*, 2013). Additionally, if ectomycorrhizal fungi were also utilizing some soil organic C as facultative saprotrophs (Courty *et al.*, 2007; Talbot *et al.*, 2008), their strength as a mutualist would decrease; yet there is scarce support to the suggestion that ectomycorrhizal fungi do this in meaningful quantities (Durall *et al.*, 1994; Hobbie *et al.*, 2013). This raises the issue that the mycorrhizal symbiosis must be viewed on a sliding spectrum from parasitism to mutualism (Jones & Smith, 2004) and perhaps also saprotrophism to biotrophism (Koide *et al.*, 2008). It doesn't appear that plants can preferentially allocate C to roots with fungi that supply more N (Mayerhofer *et al.*, 2021), however, whether plants adjust their C allocation to more favourable symbionts is debated (Bunn *et al.*, 2024). The regulation of belowground exchange processes (i.e. between fungal Hartig net and root cells) is largely unknown (as reviewed in Nehls and Plassard, 2018; Smith and Read, 2008; Stuart and Plett, 2020). Further, this is complicated by that it is technically challenging to study whether mycorrhizal fungi with increased foraging capacity are more C demanding for plant hosts, and whether there are trade-offs when N limitation is alleviated. Studies from N fertilisation and deposition experiments consistently support that certain groups, particularly cord-forming mycorrhizal fungi, are sensitive to exogenous N (Lilleskov *et al.*, 2019; Jörgensen *et al.*, 2022). Presumably, when N is more readily available, mycorrhizal fungi that mine for N at a higher C cost become less competitive than mycorrhizal fungi that can absorb inorganic N more readily.

The balance between ectomycorrhizal and saprotrophic fungi influences nutrient cycling. When N is limiting, as saprotrophic fungi decompose litter, C is respired and N is immobilised into their tissues. As a result of saprotrophic decomposition, the C:N ratio declines, over time but also with increasing depth (Clemmensen *et al.*, 2013; Kyaschenko *et al.*, 2019). In the deeper more decomposed organic horizons with lower C:N ratio, where soil organic matter becomes more energy demanding to decompose for saprotrophs, ectomycorrhizal fungi that are supplied C by their host gain a competitive advantage. Because of this competitive disadvantage in deeper soil horizons, saprotrophic fungi tend to be constrained to the fresher litter layers, resulting in a vertical stratification of ectomycorrhizal and saprotrophic fungi (Lindahl *et al.*, 2007; Kyaschenko *et al.*, 2017a). Particularly in the surface litter, mycorrhizal competition with saprotrophic fungi is thought to reduce C turnover, in a process referred to as the ‘Gadgil effect’ (Gadgil & Gadgil, 1975; Averill *et al.*, 2014; Bödeker *et al.*, 2016; Fernandez & Kennedy, 2016; Sterkenburg *et al.*, 2018). In addition, it is suggested that over time as more N is stored in fungal biomass and recalcitrant mycorrhizal residues, forest productivity declines, contributing to ecosystem retrogression (Näsholm *et al.*, 2013; Clemmensen *et al.*, 2013). However, cord-forming saprotrophic fungi, and presumably ectomycorrhizal fungi with similar growth strategies, are efficient at recycling their mycelium (Boddy, 1999), which may minimize the stress of N immobilisation (Clemmensen *et al.*, 2015). Further, it is possible that, if certain ectomycorrhizal fungi have sufficient capacity to mobilise N from recalcitrant organic matter, they may be able to counterbalance the process of ecosystem retrogression (Phillips *et al.*, 2013; Clemmensen *et al.*, 2015). Indeed, modelling by Baskaran *et al.* (2017) suggested that ectomycorrhizal driven degradation of soil organic matter, under low N conditions, may promote tree growth.

1.1.1 The genus *Cortinarius*

The ectomycorrhizal genus *Cortinarius* is one of the largest fungal genera and is the most species rich genus within the order Agaricales. The family Cortinariaceae is within the Agaricoid clade (VI), which includes mostly saprotrophic families such as Agaricaceae, Psathyrellaceae, Inocybaceae, and Strophariaceae *s. str.*, but also a few families containing ectomycorrhizal species, such as Hymenogastraceae and Hydnangiaceae (Matheny *et al.*, 2006). Within Sweden, *Cortinarius* can dominate boreal soils (Sterkenburg *et al.*, 2015) and there are ~ 800 recorded species in Scandinavia (Knudsen & Vesterholt, 2008). While *Cortinarius* was originally one of only two genera in Cortinariaceae (split into 7 subgenera), a recent in-depth phylogenetic study has resolved the genus into ten genera: *Cortinarius*, *Phlegmacium, Thaxterogaster, Calonarius, Aureonarius, Cystinarius, Volvanarius, Hygronarius, Mystinarius,* and *Austrocortinarius* (Liimatainen *et al.*, 2022). Within the new narrower definition of *Cortinarius* there are over 2,000 species, 11 subgenera (*Dermocybe, Illumini, Leprocybe, Iodolentes, Orellani, Telmonia, Infracti, Camphorati, Myxacium, Paramyxacium*, and *Cortinarius*), and *C. iodyes* remains the type species. Some of the most distinct morphological features of the family are sporocarps with a web-like partial veil called a cortina and rust-brown spores (Figure 3). In regard to their mycelium, *Cortinarius* species are cord-forming and can have long lived perennial mycelium, which may spread over several square meters (Dahlberg, 2001). Because the genus is so large and functionally diverse it is difficult to generalize characteristics across the whole genus, however, *Cortinarius* species are often considered to be nitrophobic (Lilleskov *et al.*, 2019) and late-successional (Visser, 1995; Kyaschenko *et al.*, 2017b), supported by strong evidence of their declining abundances following forestry (clear-cutting/fertilizing; Lilleskov *et al.*, 2002; Jones *et al.*, 2012; Hay *et al.*, 2015; Kyaschenko *et al.*, 2017b; Sterkenburg *et al.*, 2019; Jörgensen *et al.*, 2022; Lindahl *et al.*, 2026) and wildfires (LeDuc *et al.*, 2013; Sun *et al.*, 2015; Pérez-Izquierdo *et al.*, 2021).

Figure 3. Sporocarps of different species from the family Cortinariaceae.

2. Carbon and nitrogen dynamics in boreal forest soils

2.1 An overview of the boreal forest

With increasing atmospheric carbon dioxide (CO_2) concentrations resulting from anthropogenic climate change, the importance of understanding C cycling is ever increasing. Boreal forests, which cover approximately 11 % of terrestrial surfaces (Bonan & Shugart, 1989), are estimated to store ~ 272 PgC (Pan *et al.*, 2011). Although the aboveground biomass of trees is a considerable pool of C, it is proportionally small compared to the amount of C stored belowground (~ 60 % in the soil; Pan *et al.*, 2011). The potential of controlling soil C storage as a method for offsetting atmospheric CO_2 in cultivated and uncultivated soils is uncertain (Janzen, 2006). Carbon storage and other characteristics of the boreal forest are driven not only by climatic controls but also belowground influences (Averill *et al.*, 2014; Lindahl & Clemmensen, 2017). Because of N limitation in boreal forest soils, soil C turnover and tree productivity are tightly coupled with internal N cycling by soil organisms (Dynarski & Houlton, 2020). It is suggested that the fungal community plays an equally, if not more, important role in regulating the aboveground plant community assemblage and belowground C sink than climatic controls in the boreal forest (Read & Perez-Moreno, 2003; Lindahl & Clemmensen, 2017).

Neither above- nor belowground pools are stable over long periods of time – especially in the context of climate change. Overall, it is expected that with increased warming under climate change, terrestrial ecosystems will generate positive feedbacks that further increase atmospheric CO_2 and global temperatures (Heimann & Reichstein, 2008; Chen *et al.*, 2020). A metanalysis by Terrer *et al.* (2021) reported that soil organic C stocks are predicted to decline in ectomycorrhizal systems, despite increases in plant biomass, purportedly because of increases in nutrient mining by associated microbiota that accelerate soil organic C losses. This is congruent with the modelled predictions at the ecosystem scale, when the potential for ectomycorrhizal fungi to decay soil organic matter is considered (Baskaran *et al.*, 2017). However, the soil C sink and N cycling are inextricably linked. It is hypothesised that terrestrial ecosystems, including forests, may experience progressive N limitation during elevated atmospheric CO_2 (Luo *et al.*, 2004), despite human inflicted eutrophication of environments on the global scale. Two main, potentially co-occurring, mechanisms have been proposed to explain this potential decline in N availability: 1) increased plant growth that would increase N uptake and reduce soil N availability and 2) increases in the C:N ratio of plant tissues that would exasperate N limitation of soil organisms and promote N immobilisation. In boreal forests, this may be further intensified by increased C investment to mycorrhizal fungi that mobilise soil organic N (Drake *et al.*, 2011; Bassett *et al.*, 2026). Still there is a lot of uncertainty, much of which stems from a deficient understanding of the microbial processes influencing soil C turnover and the interaction with aboveground processes (Heimann & Reichstein, 2008; Schmidt *et al.*, 2011). Moving beyond models that are focussed on plant processes and towards the inclusion of microbial processes can strengthen soil organic C predictions, both specifically in boreal forests and globally (Lindahl *et al.*, 2002; Wieder *et al.*, 2013). Nevertheless, introducing the intricate details of the interactions between mycorrhizal fungi and soil organic C is still mostly limited to models of a smaller scale (Orwin *et al.*, 2011; Baskaran *et al.*, 2017).

Fungal communities are dynamic across large-scale fertility gradients on the global scale. In transition from boreal to temperate forests (or grassland), plant available nutrients increase, and ectomycorrhizal fungi may be disfavoured in exchange for saprotrophic fungi (and arbuscular mycorrhizal fungi; Read, 1991). However, across the boreal forest, increasing soil fertility tends to maintain or increase ectomycorrhizal abundance and decreases in abundance occur primarily following artificial introduction of N (Sterkenburg *et al.*, 2015; Lindahl & Clemmensen, 2017). In boreal forest soils, ectomycorrhizal fungi tend to dominate in abundance over saprotrophic fungi, but once the soil becomes very acidic and extremely N limiting, such as in transition to heathland, stress tolerant ericoid mycorrhizal fungi become favoured (Lindahl & Clemmensen, 2017; Fanin *et al.*, 2022). Yet the niches of ectomycorrhizal species vary across this large-scale ecosystem gradient based on differences in traits across the guild. The ectomycorrhizal fungi that are cord-forming and nitrophobic tend to be more abundant in older and less fertile forests, while ectomycorrhizal fungi with fast growing and diffuse mycelia tend to be more abundant in more nutrient-rich or recently disturbed forests (Lilleskov *et al.*, 2002).

2.2 Decomposition in the organic layer

To understand the cycling of C and N, it is necessary to know the plant and microbial macromolecules that are the dominant part of soil organic matter. Cellulose and lignin are the two most prevalent biopolymers. Cellulose is a polysaccharide, made of long chains of 1,4-β-glycosidic linked glucose that make up the cell walls of plants (Kögel-Knabner, 2002). Cellulose is found in complexes with lignin and hemicelluloses in plant cell walls. Lignin is a structurally complex cross-linked polymer with three basic monomer types (sinapyl alcohol, coniferyl alcohol, and p-coumaryl alcohol), which can be linked in many possible ways (Heitner *et al.*, 2010). To form lignin, these monolignols are oxidatively polymerised by plant peroxidases. The heterogenic structure of lignin provides rigidity to plants and hinders the hydrolysis of linked cellulose and hemicellulose. Tannins are one of the dominant secondary compounds in higher plants. A range of organic N compounds including protein and chitin can form recalcitrant complexes when precipitated by tannins (Adamczyk *et al.*, 2017). Although tannins exhibit high structural diversity, these polyphenols can be separated into two main groups, hydrolysable and non-hydrolysable tannins (Kögel-Knabner, 2002; Adamczyk *et al.*, 2017).

In contrast, microbial tissues have a distinct composition in comparison to plant tissues and generally have a lower C:N ratio. Chitin and 1,3-β-glucans are the dominant polysaccharides of fungal cell walls. Chitin is a polymer formed of 1,4-β-N-acetylglucosamine (Gow *et al.*, 2017) and contains approximately 5.9 - 6.4 % N (Tshinyangu & Hennebert, 1996). Glucans are glycosidic-linked glucose monomers like cellulose, but the type of linkages and the carbon attachments are different than is present in cellulose and the composition of glucans is variable in fungal cell walls (e.g. β-1-3, β-1-6, α-1-3). Melanin is a polymer of a similar nature to lignin, formed in many fungi for protective purposes but also for functions relating to host invasion by pathogenic fungi (Butler & Day, 1998; Eisenman & Casadevall, 2012). In the cell walls of fungi, melanin appears as granular particles, unlike the sheet-like structures of lignin, which has led some to believe that chitin may act as a ‘scaffold’ for melanin (Butler & Day, 1998; Eisenman & Casadevall, 2012).

These compounds are decomposed in different processes. Polysaccharides (both plant and fungal) are degraded in a series of hydrolytic reactions with glycoside hydrolases (GH), which are enzymes specific for breaking the polymers at different sites (i.e. endo – hydrolysing in the middle of a chain or exo – hydrolysing from the terminal end of a chain). On the other hand, the hydrophobic and heterogeneous chemical structures of lignins, melanins, and tannins requires decomposition by oxidative enzymes that can act non-specifically on the diverse chemical bonds of these compounds (Kirk & Farrell, 1987; Heitner *et al.*, 2010). At least in the case of wood-decomposition, these oxidative and hydrolytic stages can occur in succession, both in that the enzyme profile of a single fungal species can shift as decomposition progresses and that different species with complementary (or competitive) strategies can interact (Kuuskeri *et al.*, 2016; Zhang *et al.*, 2016; Mali *et al.*, 2017). The tissues from plants in the boreal forests have higher concentrations of lignin and polyphenols than plants from temperate forests, which results in acidic and hydrophobic litter that can limit decomposition. Further, the composition of fungal tissues varies not only between different species but also depending on environmental conditions, resulting in fungal necromass (i.e. dead fungal biomass) with varying degrees of chemical recalcitrance related to its melanin content and perhaps the composition and linkages of different glucans (Fernandez & Koide, 2012, 2014; Fernandez *et al.*, 2016; Siletti *et al.*, 2017; Fernandez & Kennedy, 2018; Maillard *et al.*, 2026). Tannins can further hinder decomposition of roots and fungal necromass through interactions with proteins and chitin (Adamczyk *et al.*, 2011, 2019).

The influential factors in soil organic matter turnover have been of interest since at least the early 20th century. While microbial influences have been recognised as important since the beginning, the traditional paradigm was that soil organic matter formation is controlled by the introduction of fresh leaf litter and 'humification' – the abiotic *de novo* synthesis of humic substances (Melillo *et al.*, 1982; Cornwell *et al.*, 2008; Schmidt *et al.*, 2011; Blankinship *et al.*, 2018). The chemical composition of a plant substrate, or its 'quality', in combination with climatic conditions were thought to be the main regulators of how readily a compound was stabilised. While litter quality is a factor, the current perspective is that soil organic matter cycling and stabilisation is driven by many simultaneous microbial processes. An emerging framework proposes that, rather than chemical protection promoting C storage, labile C sources that are readily processed by microorganisms promote the formation of long-term C storage in mineral soils (Cotrufo *et al.*, 2013; Liang *et al.*, 2017). Both C inputs from plants that are externally modified by microorganisms (*ex vivo*) and C that is incorporated into microbial biomass (*in vivo*) are subsequently physical protected when forming associations with mineral aggregates (Liang *et al.*, 2017). Mineral associated organic matter (MAOM) is considered a relatively stable pool of C that is important for grasslands and temperate forests (Cotrufo *et al.*, 2019). In contrast, in boreal forests there is a larger proportion of particulate organic matter (POM), as C accumulates in organic layers with little to no minerals. This means that C protection related to inherent chemical structure must still be an important process in boreal forests (Lindahl & Clemmensen, 2017). Therefore, whether these complex chemical structures are actually 'recalcitrant' in the soil depends on the presence of capable fungal species (Lindahl *et al.*, 2021).

3. Evolution and biochemistry of decomposition enzymes

Extracellular oxidative reactions are essential to the decomposition of chemically complex soil organic matter. For example, lignin-cellulose complexes are large and hydrophobic, which does not permit transport to the intracellular environment, and the many diverse bonds of lignin are non-hydrolysable. Fungi that grow on wood are typically categorised as "white-" or "brown-rot" based on their capacity to and the manner in which they degrade wood, and this is connected to their genetic capacity for ligninolytic enzymes (Floudas, 2021). Fungi deemed as brown-rot can non-enzymatically initiate oxidation through hydroxyl radicals in a process referred to as Fenton-chemistry (Arantes & Goodell, 2014). While this brown-rot mechanism can perhaps oxidise lignin, it cannot fully decompose lignin to the same degree that the mechanism of white-rot fungi can. The perhaps most potent form of oxidative reactions, characteristic of white-rot fungi, is generated by class II peroxidases, a class of enzymes with a heme prosthetic group, wherein H_2O_2 is the first oxidant (Kirk & Farrell, 1987; Lundell *et al.*, 2010). In addition, most fungi (and plants and bacteria) can also produce phenol oxidases (i.e. laccases), which are enzymes that oxidize phenolic compounds through the reduction of oxygen (Lundell *et al.*, 2010). However, laccases have a relatively low redox-potential, and therefore, their relative importance for lignin degradation versus their many other cellular functions (e.g. pigmentation and hyphal fusion/division) is debated (Baldrian, 2006).

While lignin modification is not limited to the Agaricomycetes, it is the dominant fungal class that is capable of decomposition with class II peroxidases. The ancestor of Agaricomycetes likely had ligninolytic capacity with multiple class II peroxidase genes and pathways for H_2O_2 production (Floudas *et al.*, 2012). Bayesian phylogenetic analysis estimates that the evolution of this class and the origin of the first ligninolytic enzyme (a manganese peroxidase) occurred ~ 240-295 Mya, near the transition from the Carboniferous to the Permian period (Floudas *et al.*, 2012; Ruiz-Dueñas *et al.*, 2021). This is congruent with the proposal that a lag between the evolution of lignin and the evolution of efficient fungal decomposers lead to the accumulation of organic deposits formed during the late Carboniferous

(Taylor & Osborn, 1996). Multiple gene expansions and contractions of class II peroxidases over time resulted in white- and brown-rot fungi dispersed throughout several different taxonomic orders (Strullu-Derrien *et al.*, 2018). Recently, focus has expanded beyond wood decomposers towards decomposition by saprotrophs of a wider variety of lifestyles (i.e. forest- and grass-litter decomposers) and mycorrhizal fungi (Kohler *et al.*, 2015; Floudas *et al.*, 2020; Ruiz-Dueñas *et al.*, 2021). While the enzymatic machinery of litter decomposers and mycorrhizal fungi partly overlap with wood decomposers, the outcome (i.e. the products produced and their chemical structures) of soil organic matter decomposition may be different (Floudas *et al.*, 2022). It is suggested that although fungi of the same lifestyle may have different phylogenetic origins, they have converged to have similar repertoires of plant cell wall degrading enzymes (Ruiz-Dueñas *et al.*, 2021).

Class II peroxidases are split into several subclasses based on their mechanism and capacity for initiating oxidation: Manganese peroxidases (EC 1.11.1.13) that oxidise organic matter through oxidation of Mn^{2+} to Mn^{3+} (Figure 4), lignin peroxidases (EC 1.11.1.14) that oxidise small phenolics directly with an exposed long-range electron transfer residue, versatile peroxidases (EC 1.11.1.16) that are capable of oxidation through both mechanisms, and general peroxidases that share homology but do not have the known functional residues required for ligninolytic activity. Versatile peroxidases and lignin peroxidases diverged from an ancestral manganese peroxidase (Ruiz-Dueñas *et al.*, 2021). These enzymes all fall within the broader auxiliary activity family two (AA2) class of carbohydrate active enzymes (CAZymes; https://www.cazy.org/; Drula *et al.*, 2022), which also includes intracellular peroxidases (e.g. cytochrome c peroxidases; EC 1.11.1.5) that are produced by many different organisms.

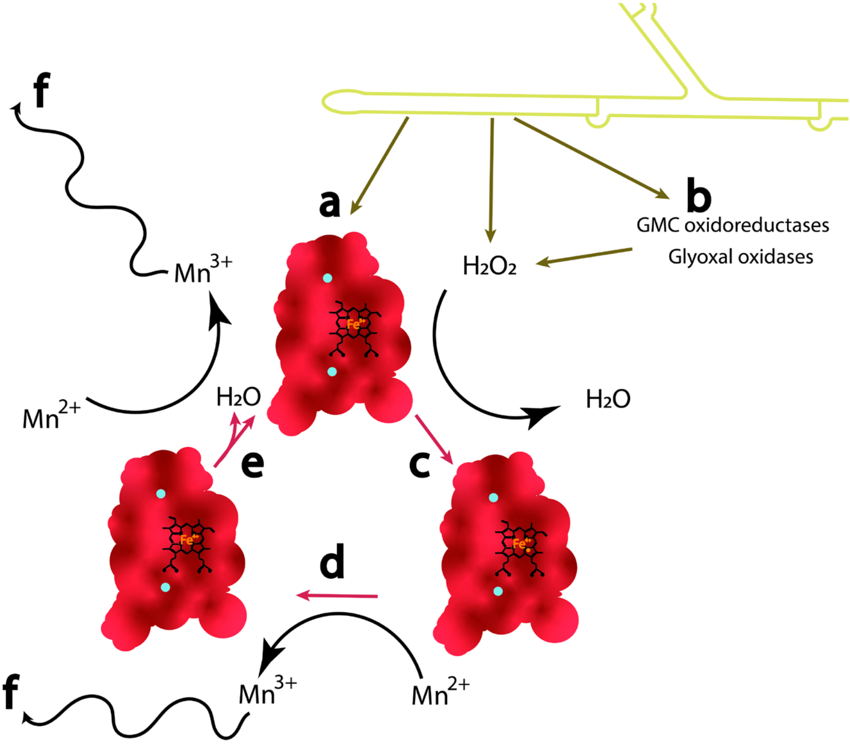


Figure 4. Schematic of the manganese peroxidase catalytic cycle. Manganese peroxidase (in resting state) is secreted by a fungus (a). H_2O_2 is either intracellularly produced and secreted or extracellularly produced by Glucose-methanol-choline (GMC) oxidoreductases and/or glyoxal oxidases (b). The central heme group of the enzyme first bonds with H_2O_2, then as the oxygen-oxygen bond between manganese peroxidase (MnP) and H_2O_2 are broken, two electrons are transferred forming water and a Fe^{4+} oxo-porphyrin-radical complex in MnP-compound I (c; Hofrichter, 2002). In two stages, MnP-Compound I is reduced back to resting state by divalent manganese resulting in a 2:1 stoichiometric production of trivalent manganese (Wariishi *et al.*, 1992; Hofrichter, 2002). MnP-compound II is formed in the first stage when a single electron is gained from Mn^{2+} (d; monochelated), and then in the second stage another electron is gained and a second water molecule is formed (e). The released Mn^{3+} is stabilised by chelation with organic acids, such as malonate, oxalate, tartrate, and lactate (Perez & Jeffries, 1992; Kuan *et al.*, 1993). The chelated Mn^{3+} complex is highly soluble and can diffuse towards organic substrates (f), which can be a range of phenolic and aromatic compounds. To "recharge" the MnP, it must be oxidised again by H_2O_2.

Hydrogen peroxide is required for the oxidative process by all class II peroxidases (as well as for non-enzymatic Fenton-chemistry reactions) and there are several potential pathways for H_2O_2 production (Mattila *et al.*, 2022). Hydrogen peroxide may be produced intracellularly through several metabolic pathways and then exported (potentially by aquaporins), although extracellular transport for this pathway remains unknown (Bienert & Chaumont, 2014). However, it is more likely that H_2O_2 is produced extracellularly. It has been observed that glyoxal oxidase (EC 1.1.3.15; AA5_1), a member of the copper radical oxidase (CRO) class, is important for extracellular H_2O_2 production by the white-rot fungus *P. chrysosporium* (Kersten & Kirk, 1987). This is an aerobic process, as glyoxal oxidases reduce O_2 to H_2O_2 while oxidising simple aldehydes, in which the products (organic acids) may also serve as chelates for manganese ions. Another potential extracellular pathway is the glucose-methanol-choline (GMC; AA3) oxidoreductase family, which also reduces O_2 to H_2O_2 but instead with a broader variety of reductants (e.g. aryl-alcohol, methanol, glucose; Sützl *et al.*, 2018; Mattila *et al.*, 2022). GMC oxidoreductases may be the main pathway for brown-rot fungi, as they lack glyoxal oxidase genes. Further, despite that alcohol (methanol) oxidase (AA3_3) is typically considered intracellular and crucial for the methanotrophic activity of yeasts, it has been suggested that a variant of alcohol oxidase may be a potential important pathway for some brown-rot species (Daniel *et al.*, 2007; Mattila *et al.*, 2022). H_2O_2 can also be formed from coupled reactions of NADPH oxidase and membrane-bound superoxide dismutase, which catalyses the reaction of super oxide to H_2O_2 (Mattila *et al.*, 2022). The production of reactive oxygen species (mostly H_2O_2) is necessary for oxidative decomposition, but it likely needs to be in delicate balance with mitigation of oxidative stress.

3.1 Manganese peroxidases

The first manganese peroxidases (MnPs) isolated in the 1980s, shortly after the first lignin peroxidases, were short and long MnPs from *Phanerochaete chrysosporium* (as reviewed in Kirk and Farrell, 1987). Both long- and short-MnPs have three residues forming the Mn^{2+} oxidation site: two glutamic and one aspartic acid (EED; Ruiz-Dueñas *et al.*, 2013; Lundell *et al.*, 2014). These 'classical' MnPs, originally observed in wood-decomposing Polyporales, have also been described in several families within Agaricales and Russulales. Variation among residues forming the Mn^{2+} oxidation site initially led to characterisation of 'atypical' MnPs (Floudas *et al.*, 2012; Hildén *et al.*, 2014), however, these 'atypical' MnPs are now recognised to belong to one of at least three sub-groups of short-MnPs (Ruiz-Dueñas *et al.*, 2021). In the largest group of MnPs with alternative Mn^{2+} oxidation sites, there are only two coordinating residues, one glutamic and one aspartic acid (ED), with the second glutamic acid replaced typically with serine (Sánchez-Ruiz *et al.*, 2024). The ED group, which diverged from a short-MnP common ancestor ~ 150 Mya, is present in the typical white-rot fungus *Stereum hirsutum* (Russulales) but otherwise mostly in Agaricales. The DED group, only observed in *Peniophora* sp. (Russulales), has an aspartic acid replacement for glutamic acid. The other characterised short-MnPs have a glutamic acid replacement as in the DED group, but the second aspartic acid is also replaced (as in the ED group but typically rather with a glycine residue than with serine) and a glutamine residue near the C-terminal forms the third residue in the Mn^{2+} oxidation site (DDQ). The DDQ group is present in several species across Agaricales, particularly in the family Strophariaceae *s. str.* (predominantly litter decomposers), but not in any typical wood-decomposing saprotrophs.

Manganese peroxidases are predicted to play a substantial role in the regulation of soil organic C stocks. The concentration of available manganese has been correlated with the presence of thin organic layers, less soil organic C, and an increase in litter decomposition rates in both European and North American conifer forests (Berg *et al.*, 2007; Stendahl *et al.*, 2017; Kranabetter *et al.*, 2019). Further, experimental additions of manganese increased decomposition rates and the production of CO_2 (Trum *et al.*, 2011; Sun *et al.*, 2021). These correlations at the community and ecosystem scale are strengthen by observations of fungi recruiting and oxidizing Mn^{2+} at the site of active decomposition on pine needles (Keiluweit *et al.*, 2015). These findings corroborate the idea that soil organic C turnover is strongly regulated by the availability of reduced Mn^{2+} for manganese-dependant oxidation of organic matter, at least in organic soils. Oxidation by Mn^{3+} produced by MnPs can 'enzymatically combust' ^{14}C-labelled lignin completely to CO_2 extracellularly (Kirk & Farrell, 1987; Hofrichter *et al.*, 1999; Hofrichter, 2002; Šnajdr *et al.*, 2010) but fragmentation of soil organic matter and how products from this process are utilised by soil organism *in situ* is not known. Lignin decomposition products (such as phenols) are unlikely to be used as metabolic C, considering that they are not easily assimilated (del Cerro *et al.*, 2021). Manganese peroxidases may increase access to cellulose and hemicellulose that can then be synergistically acted on by other hydrolytic enzymes (produced by the same fungus or other soil organisms; Kuuskeri *et al.*, 2016; Barbi *et al.*, 2020), and/or MnPs may reduce the hydrophobicity of soil organic matter (Hofrichter, 2002).

4. The crux - Ectomycorrhizal decomposers

Ectomycorrhizal fungi intimately connect above- and belowground nutrient cycling and are influential in soil organic matter dynamics (Zak *et al.*, 2019). A large proportion of the C input into forest soils is from belowground contributions mediated by mycorrhizal fungi (Clemmensen *et al.*, 2013). The amount of photosynthates that are transferred to mycorrhizal symbionts is variable but substantial (Högberg *et al.*, 2010; reviewed in Frey, 2019). Root derived C can be built into standing fungal biomass or metabolised into exudates that are released into the soil via the fungal mantle and/or the extraradical hyphae extending beyond the rhizosphere (See *et al.*, 2022). The predominant paradigm until at least the 1990s was that mycorrhizal fungi were solely sinks of C into the soil. Nonetheless, some early evidence pointed to the decomposition capacity of ectomycorrhizal fungi. For example, *Pinus* and *Larix* seedlings grown in mesocosms were "re-greened" when their fungal symbionts proliferated on patches of organic matter (Finlay & Read, 1986a,b) and in the field, Griffiths *et al.* (1990) observed that the formation of *Hysterangium* mycelial mats in the organic layer of Douglas-fir forests coincided with increases in respiration, mineralizable N, and enzyme activity. At the time it was unclear if organic matter was directly acted on by ectomycorrhizal fungi or whether there was facilitation of decomposer fungi. In 1999, when support for 'saprotrophic' capacities in ectomycorrhizal fungi was just starting to surface (Cairney & Burke, 1998), one of the first MnP genes identified from an ectomycorrhizal fungus was amplified from *Tylospora fibrillosa* using PCR fragment analysis (Chambers *et al.*, 1999). Nearly a decade later, field observations of differences in the C:N ratio and ^{15}N natural abundance between layers of spatially separated mycorrhizal and saprotrophic fungi supported the idea of mycorrhizal fungi having a direct role in decomposition (Lindahl *et al.*, 2007). Shortly after, the genome sequencing of *Laccaria bicolor* revealed a drastic reduction in plant cell wall degrading enzymes and no genetic evidence of class II peroxidases, increasing the suspicion that ectomycorrhizal fungi are of little significance for soil organic matter decomposition (Martin *et al.*, 2008). However, more extensive PCR fragment analysis revealed evidence for a spectrum of class II peroxidases in a wide range of ectomycorrhizal fungi and many from the genus *Cortinarius* (Bödeker *et al.*, 2009). While, in general, the evolution of ectomycorrhizal fungal lineages has been linked to a contraction of genes encoding for plant cell wall degrading enzymes concurrent with the expansion of genes regulating symbiosis (Kohler *et al.*, 2015), some lineages have retained MnP genes. Since initiation of the 1000 fungal genomes project, more ectomycorrhizal species with peroxidase genes have been revealed, such as nine MnP gene copies identified in the genome of *Cortinarius* (*Phlegmacium*) *glaucopus*, three in *Hebeloma cylindrosporum*, ~ 1-3 in *Lactarius* and *Russula* spp., 25 in *Gautieria morchelliformis*, and four in *Hysterangium stoloniferum* (Miyauchi *et al.*, 2020). Knowing which ectomycorrhizal species have the potential for decomposition remains challenging, given that *in vitro* experiments are limited to only a few species that do not produce peroxidases (i.e. from the genera *Paxillus*, *Laccaria*, and *Pisolithus*), and that it is not possible to provide genomic information for every species.

Although some ectomycorrhizal fungi may have capacity to decompose soil organic matter ‘like saprotrophs’ (Koide *et al.*, 2008), the effect they have on soil organic matter dynamics is distinct from that of saprotrophs, as they do not need to metabolise the released C (Lindahl & Tunlid, 2015). Rather, it is proposed that ectomycorrhizal decomposers primarily benefit from oxidative decomposition through increased access to nitrogen (Lindahl & Tunlid, 2015; Shah *et al.*, 2016). Thus, the exact fate for organic compounds that are not utilised by ectomycorrhizal fungi remains unknown (Tunlid *et al.*, 2022). However, the degree to which ectomycorrhizal fungi are relevant for soil organic matter decomposition is still debated (Zak *et al.*, 2019). The suspected residues of the Mn^{2+} oxidation site are present in most identified genes, and there is evidence of at least some of these genes being transcribed (Bödeker *et al.*, 2014; Barbi *et al.*, in review); yet, there is no direct evidence that all or some of these genes are capable of breaking down soil organic matter in the same manner as saprotrophic fungi (Pellitier & Zak, 2018). Here and throughout the thesis, the term ‘ectomycorrhizal decomposers’ is used to refer to ectomycorrhizal fungi with the capacity to decay organic matter (i.e. change physical properties, transform into smaller compounds, and/or modify functional groups) using oxidative enzymes.

Both natural (wildfire, insect outbreaks, windfall, etc.) and anthropogenic disturbances (forestry practices, N deposition, etc.) in forests make rapid alterations to the microbial communities and the turnover of soil organic C. Disturbances such as these account for a substantial proportion of the variability in forest C sequestration (Magnani *et al.*, 2007). Likewise, clear-cutting of forests can have severe impacts on the biodiversity of ectomycorrhizal fungi, particularly of rare species (Varenius *et al.*, 2016; Sterkenburg *et al.*, 2019; Rianhard *et al.*, 2025). Wildfire, clear-cuts, and fertilisation all tend to mobilise N, at least in the intermediate term; therefore, fungal communities dominated by ruderal fungi are favoured following disturbances (Barker *et al.*, 2013; Kohout *et al.*, 2018; Pérez-Izquierdo *et al.*, 2021). On the other hand, ectomycorrhizal fungi with higher C requirements and slower growth are expected to be especially sensitive to reductions in belowground input of symbiotic C resulting from increased N mineralisation (Agerer, 2001; Lilleskov *et al.*, 2011; van der Linde *et al.*, 2018; Wasyliw & Karst, 2020). Given the assumption that H_2O_2 production is energetically demanding (Kirk & Farrell, 1987; Shimizu *et al.*, 2005; Lindahl & Tunlid, 2015), ectomycorrhizal decomposers may be especially C demanding (Defrenne *et al.*, 2019; Argiroff *et al.*, 2022). This is partially supported by evidence of reduced MnP activity when bioavailable N increases (Bödeker *et al.*, 2014; Jörgensen *et al.*, 2024). While disturbance is a natural part of the ecosystem dynamic, human activities and especially intensive clear-cutting and replanting may alter soil biodiversity and C cycling in an irreversible way (Lindahl *et al.*, 2026).

Support for the relevance of *Cortinarius* to decomposition of soil organic matter has come from several lines of evidence. The abundance of *Cortinarius* DNA and transcription of *Cortinarius* MnP genes have been correlated with estimated MnP activity (or MnP genes) in forest soils, suggesting that these enzymes are produced and active *in situ* (Bödeker *et al.*, 2014; Barbi *et al.,* in review; Pellitier & Zak, 2021). Indirectly, the loss of *Cortinarius* species following disturbances (wildfire or removal of roots) has been correlated with a reduction in MnP activity (Kyaschenko *et al.*, 2017b; Sterkenburg *et al.*, 2018; Pérez-Izquierdo *et al.*, 2021). Similarly, a negative correlation has been observed between *Cortinarius* relative abundance and topsoil C stocks across forests treated by thinning and/or fertilisation (Jörgensen *et al.*, 2022). On an even broader scale, across Sweden, the genus was found to be weakly correlated with decreasing soil organic C stocks (Lindahl *et al.*, 2021). The reduction in C stocks was better predicted by the presence of *Cortinarius acutus* sl. than for the genus as a whole, supporting the idea that the capacity for oxidative decomposition may vary throughout the genus and that fungal communities, while diverse, are not entirely functionally redundant (Allison & Martiny, 2008). It is possible that ectomycorrhizal fungi, such as *Cortinarius*, may be able to counterbalance the process of ecosystem retrogression (Clemmensen *et al.* 2015), if the degree of decomposition outrivals that of saprotrophic competitors (Näsholm *et al.*, 2013; Phillips *et al.*, 2013). Understanding which *Cortinarius*, and other ectomycorrhizal, species are relevant for direct soil organic matter decomposition is important, especially considering the potential sensitivity of this group to disturbance.

5. Objectives

The overall objective of this thesis was to deepen the understanding of the mechanisms and implications of oxidative decomposition by ectomycorrhizal decomposers – with a focus on *Cortinarius* species. The thesis can be split into several key questions:

I. Which ectomycorrhizal fungi are key decomposers and is this trait phylogenetically conversed at the genus-level? What is the niche(s) of these ectomycorrhizal decomposers and are they sensitive to forest management? (**Paper I**)

II. Are manganese peroxidases produced by ectomycorrhizal decomposers functionally equivalent to saprotrophic manganese peroxidases? (**Paper III**)

III. Do ectomycorrhizal fungi increase access to N in recalcitrant soil organic matter through expression of manganese peroxidase genes? (**Paper IV**)

IV. Is oxidative decomposition energetically expensive? What are the potential H_2O_2 production pathways for ectomycorrhizal decomposers? (**Paper IV**)

Additionally, the methodology of manganese peroxidase enzyme assays was assessed, as it pertains to all studies included in the thesis (**Paper II**).

6. Methods

This thesis used a broad "toolbox" of methods to assess the enzymes, ecology, and evolution of ectomycorrhizal decomposers (Figure 5). **Paper I** used a combination of molecular metabarcoding data and manganese peroxidase enzyme assays on soil organic layer extracts to assign potentially important ectomycorrhizal decomposers (objective I). Then in the second part of **paper I**, I used metabarcoding data from the Swedish Forest Soil Inventory to assess the niche(s) of these assigned ectomycorrhizal decomposer taxa (objective I). **Paper II** was an assessment of the methodology of manganese peroxidase enzyme assays, including enzymes extracted from a fungus grown in pure culture. In **paper III**, heterologous expression of recombinant enzymes was used to produce and study the ligninolytic capacity of manganese peroxidases from an ectomycorrhizal fungus (objective II). These recombinant manganese peroxidases were purified from the heterologous host, *Pichia pastoris*, and tested for activity using various enzyme assays. To examine gene expression patterns related to oxidative decomposition (objectives III and IV), **paper IV** used genomic data from *Cortinarius* species combined with metatranscriptomic data that was collected from organic soils dominated by *C. ominosus* mycelia.

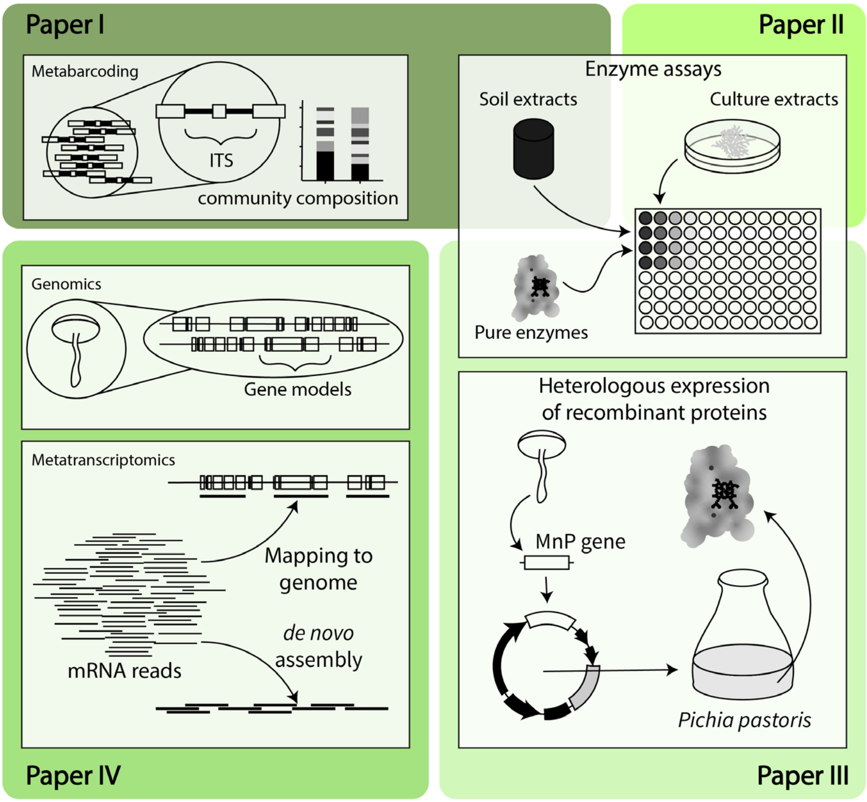


Figure 5. Summarisation of methods used and their distribution amongst papers.

6.1 Enzyme assays

Measuring enzyme activity using colorimetric assays is an affordable and high-throughput method that can help to connect fungal (or bacterial) activities to soil biochemical processes. These enzyme assays measure the change in absorbance of chemical compounds that are proxies for the substrates of the enzymes of interest. Hydrolytic enzyme assays tend to be more straightforward given their relatively high substrate specificity. However, for oxidative enzymes such as peroxidases and phenol oxidases, which are less specific, there exists several challenges (German *et al.*, 2011). An ideal enzyme assay substrate should be stable, soluble, react readily with the enzyme but not with the soil, and be colourless in the absence of the enzyme. Further, once oxidised, it should remain stable and absorb at a wavelength that does not overlap with background absorbance from the soil extract. For assaying potential MnP activity there are several substrate choices, such as 3-(3,4-Dihydroxyphenyl)-L-alanine (L-Dopa; Sinsabaugh *et al.*, 1992), 3-Methyl-2-benzothiazolinone hydrazine hydrochloride monohydrate (MBTH) and 3-(dimethylamino)benzoic acid (DMAB; Ngo & Lenhoff, 1980), 3,30,5,50-tetramethylbenzidine (TMB; Johnsen & Jacobsen, 2008), 2,6-dimethoxyphenol (2,6-DMP), and 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS; Floch *et al.*, 2007). The MBTH/DMAB substrates (used in **papers I, II** and **III**) have a coupled reaction, where MBTH is first oxidised by Mn^{3+} (or the enzyme) then it reacts with DMAB to form an indamine dye. Nevertheless, none of the listed substrates meet the above criteria perfectly (Sinsabaugh, 2010). These substrates do not react specifically with MnPs but rather with all types of peroxidases and phenol oxidases. Therefore, the difference between reactions with and without the presence of H_2O_2 is required to distinguish peroxidase and phenol oxidase activity (Sinsabaugh, 2010) and addition/exclusion of Mn^{2+} is required to distinguish manganese-dependant activity from total peroxidase activity (Glenn & Gold, 1985; Arnstadt *et al.*, 2016).

There remain several other considerations that apply to all enzyme assays. First, given that the assays are generally performed in buffers under non-limiting and optimal conditions of pH and temperature, which usually are not representative of the *in situ* conditions, the measured activity is only an estimate of potential activity (Wallenstein & Weintraub, 2008). Secondly, soils prepared for enzyme assays are often homogenised to some degree. This can release intracellular enzymes that may also react with the substrate, as well as target enzymes that were stabilised in interactions with organic matter and minerals (Nannipieri, 2006). Overall, this tends to lead to an overestimation of enzyme activity.

In addition to the above colorimetric enzyme assays, there are several other assays that can be used to assess the oxidative activity of pure enzymes (as done in **paper III**). The formation of Mn^{3+} by MnPs can be assessed more directly by measuring the absorbance of Mn^{3+}-malonate complexes in the UV-range around 270 nm (Wariishi *et al.*, 1992). For **paper III**, this assay was set up in a 96-well plate in 200 µl reactions with 10 µl of purified enzyme, 50 mM sodium malonate pH 4.5, and 0.2 mM MnSO4 and then initiated with 10 µl of H_2O_2. Then the reaction was measured every 15 s for the duration of the reaction's linear phase, immediately following H_2O_2 addition. Oxidation of the monolignol coniferyl alcohol can also be monitored at UV-ranges, as has been applied in studies assessing the capacity for plant peroxidases to polymerize lignin (Koutaniemi *et al.*, 2005). To assess oxidation of coniferyl alcohol by enzymes produced in **paper III**, assays were arranged in a similar manner (as the Mn^{3+}-malonate assay) but with the addition of 0.5 mM coniferyl alcohol suspended in 5 mM sodium malonate. Changes to the UV-spectrum of coniferyl alcohol (210-340 nm) were followed by repeated measurements over several days.

Absorbance should ideally be converted to a standardised unit for comparison, such as Enzyme units (U) or product concentration (German *et al.*, 2011). This can be done by preparing a standard curve with an enzyme of known enzymatic rate (as done in **paper I**) or by preparing a standard curve of the enzyme product. In **paper II**, we developed another type of standard curve using Mn(III) acetate, essentially a non-enzymatic source of Mn^{3+}. The reaction of Mn(III) acetate with the substrates is spontaneous, and a standard curve can be created for each substrate to convert absorbance at a given wavelength to the corresponding μmol of Mn^{3+} required to produce that absorbance. This standard curve works under the assumption that the substrates are oxidised in a 1:1 ratio with Mn^{3+} and it is more appropriate to consider the measure as Mn^{3+} μmol *equivalents*, given that it can also be used to convert the absorbance in reactions without manganese present.

6.2 "Omics"

Molecular biology and particularly high-throughput sequencing has revolutionised fungal ecology (Peay, 2014). Investigating DNA and RNA allows one to observe the fungal community undisturbed and *in situ*. The following methods all employ similar principles of extracting DNA and/or RNA from environmental samples (soil or sporocarps) followed by sequencing to essentially "read" genetic material that encodes for biological processes within an organism.

6.2.1 Metabarcoding

Next-generation sequencing of a "barcode" gene can be used to evaluate the community of soil organisms present in an environmental sample. This provides a major advantage over traditional methods that relied on culturing and single species Sanger sequencing – both of which can only capture a small portion of the total diversity. The ideal barcode is of consistent length and copy number between species, has a mutation rate that matches the rate of speciation, and is surrounded by conserved regions that general primers can anneal to (Kauserud, 2023). Although these idealised conditions are not met, the best and commonly used barcoding region for fungi is the internal transcribed spacer (ITS) region (Schoch *et al.*, 2012). This region is split into two sections, ITS1 and ITS2, located between the 18S, 5.8S, and 28S ribosomal RNA genes. Primer choice and PCR optimisation play an important role in controlling implicit sequencing bias derived from variations between taxa in their primer annealing specificity and ITS length. In this thesis, the sequencing was done using PacBio technology, which can generate longer reads relative to the earlier Illumina platform and is, in general, less susceptible to biases in the relative community composition (Castaño *et al.*, 2020).

In **paper I**, the goal was to analyse species assigned as ectomycorrhizal decomposers in a regional-scale study for their niche in relation to forest stand age and soil fertility in a national-scale inventory. Therefore, consistent operational taxonomic units (OTUs) that were trackable across these two scales were required. To generate OTUs for analyses performed on the regional- and national-scale, all pools of amplified and sequenced ITS2 markers were analysed together using the bioinformatics pipeline SCATA (scata.mykopat.slu.se; Ihrmark *et al*., 2012). Within the pipeline, sequences were quality controlled, filtered, and then clustered into OTUs using single-linkage clustering. The threshold for clustering was set at 98.5 % sequence similarity required to enter a cluster. The ideal sequencing clustering threshold should group together sequences from the same species; however, given that different lineages have differing rates of mutation, this often results in some species being separated into two or more OTUs and what are likely several different species within the same genus being grouped together as one OTU. This is especially challenging in the case of known species complexes and in this thesis, I sometimes refer to species as “taxon/taxa” to acknowledge that it may be either a species or a group of closely related species that are being referred to. Without being able to determine whether two OTUs are truly different species, the concept of a “species hypothesis” is frequently used in fungal ecology (Abarenkov *et al.*, 2024).

6.2.2 Genomics

The first mycorrhizal genome, *Laccaria bicolor*, was sequenced in 2008 initiated by the Joint Genome Institute (JGI; Martin *et al.*, 2008). Genomics provides *in silico* information on the functional capacity of an organism based on its gene content. While gene presence and counts can be informative for inferring ecological roles and assessing phenotypic traits (Grigoriev *et al.*, 2014), there is still a large gap between potential and actual gene expression, especially for eukaryotic organisms (Barbi *et al.*, 2020). Genomic information can also provide a reference for identifying sequences from environmental samples (as done in **paper IV**).

To have genomic reference material for **paper IV**, seven new genomes of different *Cortinarius* species were generated based on sporocarp material that I collected in the fall of 2023 from forests in the Uppsala region. Large fragment gDNA was extracted, in addition to total RNA, and this material was sequenced, assembled, and annotated by JGI for inclusion in the Mycocosm database (https://mycocosm.jgi.doe.gov/). Prediction and annotation of genes was based on homology of the sequences with other characterised (and putative) genes, alignment with transcriptomic data (RNA), and the presence of specific functional domains. Proteins can be classified based on enzyme commission (EC) number (McDonald & Tipton, 2014) and EuKaryotic Orthologous Groups (KOGs; Koonin *et al.*, 2004). Further, there are databases used for classification of specific types of proteins: the carbohydrate-active enzymes database (CAZy; Drula *et al.*, 2022) database for glycoside hydrolases (GH) and associated auxiliary activity (AA) enzymes, the Transporter Classification Database (TCDB; tcdb.org; Saier *et al.*, 2021) for membrane transport enzymes, and the MEROPS peptidase database for proteolytic enzymes (https://www.ebi.ac.uk/merops/; Rawlings *et al.*, 2018). Therefore, putative function can be assigned, but predicted function and actual activity do not always align. In addition, the predicted gene models annotated in the pipeline can have issues, such as truncation and missed intron/exons. Therefore, in **paper IV**, known genes of interest were further manually curated for accuracy.

Phylogenies of key genes in the decomposition process were made from the newly sequenced *Cortinarius* genomes and other reference sequences from JGI (or other reference databases such as NCBI, ENA or UniProt), by downloading the gene catalogue protein sequences and aligning them using

ClustalW. Sequences were checked for appropriate functional residues and phylogenies were built using maximum likelihood (performed in MEGA v12; Kumar *et al.*, 2024).

6.2.3 Metatranscriptomics

Metatranscriptomics relies on sequencing of the mRNA pool to get a snapshot of what genes are being transcribed and quantify how much they are being transcribed. This approach allows analysis of the entire community of mRNA, without PCR related bias (unlike single-gene analysis). Further, because mRNA is quickly degraded after translation, it can be assumed that mRNA transcripts detected in the sample are representative of current activity (unlike metagenomic approaches, as DNA is more stable in soils). The mRNA sequences can either be mapped to available annotated genomes (as done in **paper IV**) or can be assembled *de novo* to hypothetical gene contigs (Kuske *et al.*, 2015). Still, there are limitations in taxonomic assignments of mRNA genes even to the genus level in environmental samples that contain hundreds of active species (Kuske *et al.*, 2015).

The possibilities of ectomycorrhizal metatranscriptomics have come a long way in a short period of time, from being limited to assessing only the transcription of a few genes from select species (Liao *et al.*, 2014) to much wider taxonomic assignment (Auer *et al.*, 2024). However, some technical challenges still apply specifically to metatranscriptomics at several different methodological stages. The sensitivity of RNA to degradation means that samples must be kept frozen and handled in a time-sensitive manner. In **paper IV**, we decided to flash freeze the soil directly with liquid N before sampling rather than simply placing the samples into dry ice after sampling. Further, mRNA is only a small proportion of the total RNA and therefore mRNA needs to be enriched prior to sequencing. This can be done in two ways: poly(A) tail selection or ribosomal RNA removal. The later approach was taken in **paper IV**, as the environmental RNA may be prone to fragmentation that could lead to a 3’ bias during poly(A) selection (Adiconis *et al.*, 2013).

In **paper IV**, to assess gene expression related to oxidative decomposition, a more targeted metatranscriptomic approach was used. The gene expression of one fungal species *in situ* was mapped back to its genome, as is done in laboratory transcriptomic studies with a single “model” species. Further, I strived to take a more hypothesis driven approach in this study, by first investigating only the transcript abundance of genes for which I had made *a priori* hypotheses, to avoid the pitfall of inductionism that can accompany more holistic metatranscriptomic approaches based on data-driven pattern screening (Prosser, 2020). My approach is also unique in that I did not attempt to connect the patterns in transcript abundance to environmental variables but rather attempted to correspond changes in the expression of specific genes that coincided with the transcription of MnP genes. In this way, I am aiming to interpret not the response of the fungus directly to the environment, but shifts in expression patterns related to the mechanisms of oxidative decomposition.

6.3 Heterologous production of recombinant proteins

Laboratory experiments allow for in-depth investigation into the mechanisms of specific proteins; however, many mycorrhizal fungi are slow growing and difficult to isolate. Much of the research on mycorrhizal fungi is done on a small selection of “model” species that can be more readily cultured such as *Laccaria bicolor*, *Paxillus involutus* and *Suillus* spp.. On the other hand, *Cortinarius* species are rarely used for laboratory experiments. In addition, regardless of selected species, the relevance of laboratory pure-culture studies for actual processes that occur *in situ* is questionable, considering that the behaviour of a mycorrhizal fungus grown with readily supplemented glucose is likely not the same as when provided C in symbiosis. To forgo the study of peroxidases from a mycorrhizal fungus grown in pure culture, heterologous expression of recombinant proteins was used in **paper III**. In this way, the enzyme of interest can be produced and experimented with, independently of growing the fungus of interest (i.e. *Cortinarius*).

Heterologous expression is the insertion of a gene into a host organism to investigate the activity of that specific gene. Often the expression host is bacteria (e.g. *Escherichia coli*) or, in this case, the yeast *Pichia pastoris* (current name *Komagataella phaffii*). Using a eukaryotic host is advantageous for fungal proteins because it has a more similar protein-folding mechanism to the origin fungus than bacteria, it efficiently secretes the recombinant protein, which improves purification, and it grows quickly, generating a high cell density and therefore higher yield of the recombinant protein (Haon *et al.*, 2015). The vector pPICZαA, which contains the alcohol oxidase promotor and a Zeocin resistance marker can be used to create an inducible expression system. In the absence of glucose, *Pichia* metabolises methanol, which requires expression of alcohol oxidase (Cregg *et al.*, 1989) and consequently induces the expression of the recombinant protein. The Zeocin resistance marker allows for successfully transformed *Pichia* cells to be screened.

The protocols described here (and used in **paper III**) are as established by Invitrogen (EasySelect^{TM} Pichia) and modified by Haon *et al.* (2015). Once candidate genes were selected, they were modified before synthesis by codon-optimisation for the expression host, and the signal peptide was replaced with the native *Pichia* secretion signal. Further, a histidine tag was added to the N-terminus of the protein to aid in purification. The synthetic genes were first linearised into the pPICZαA vector at specific restriction sites (Pmel in this case). Then the linearised vectors were transformed into the expression host using electroporation – a method that temporarily reduces the permeability of the cell membrane allowing for the vector to enter. There are two stages for inducible recombinant production with *Pichia*. First, *Pichia* is grown in flasks with a glucose-based liquid medium to achieve a high-cell density, then the medium is replaced with a glucose-free medium, and the feeding is switched to methanol, which induces the transcription of the recombinant protein associated with the alcohol oxidase promoter. After three days of induction, the yeast cells are removed (through centrifugation and size filtration) and the recombinant protein can be purified from the culture supernatant. Purification of histidine tagged proteins was performed using a nickel coated Sepharose column (HisTrap^{TM}, Cytiva), wherein the histidine tag binds to the column during loading and can then be eluted with an imidazole concentration gradient, allowing for separation from endogenous *Pichia* proteins.

Expression of recombinant proteins has the advantage of, 1) enabling study of proteins originating from organisms that are difficult to grow and 2) production of a protein in sufficient quantity and purity to allow for biochemical characterisation. However, by isolating a protein from its original host the potential impacts of localisation and post-translational modification are neglected. For oxidative enzymes with heme co-factors some specific challenges exist. Within a *Pichia* expression system, it is possible to get folded proteins with heme incorporation, unlike *E. coli* expression systems where the unfolded proteins accumulate in insoluble inclusion bodies that must then be *in vitro* folded (Whitwam & Tien, 1996; Gu *et al.*, 2003). However, hemin supplementation during induction is necessary to provide sufficient amounts needed for proper incorporation during folding (Krainer *et al.*, 2015), and this can cause some issues in the downstream analyses.

Several methods were implemented to check the characteristics of the recombinant enzymes. To estimate the size and purity of the isolated enzyme, SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was used. In SDS-PAGE, the denatured proteins migrate through the gel following an applied electric field allowing for separation based on the mass, which can then be compared to a "ladder" of known protein mass. For quantification, absorbance at 280 nm was measured using a spectrophotometer (i.e. Nanodrop) and then, based on the expected mass and molar absorption coefficient of the protein, the absorbance was converted to $\mu g\ \mu l^{-1}$. The Bradford (Coomassie brilliant blue G-250) assay was also used, wherein the protein concentration was determined based on a colorimetric change measured at 595 nm. Likewise, the fluorescence-based Qubit^{TM} Protein Broad Range (Invitrogen) assay was used to measure protein concentration. Further, for heme-containing proteins, the Reinheitzahl value, absorbance at ~ 403 nm (wavelength of Soret peak) divided by absorbance at 280 nm, was determined to estimate the purity. Typically, native peroxidase enzymes exhibit Reinheitzahl values ≥ 3, where higher values indicate better incorporation of the heme group (Lin *et al.*, 2018). In **paper III**, I complemented these above methods with tandem-mass spectrometry (done by SciLifeLab, Stockholm). Tandem-mass spectrometry isolates and identifies proteins by sorting and analysing the ionised proteins based on their mass-to-charge ratio in a two-stage mass spectrometer. This method aided in distinguishing which endogenous *Pichia* proteins remained in my purified enzyme extracts and what proportion of the total protein content was the recombinant protein of interest.

7. Project descriptions

7.1 Paper I

Paper I was a combination of two main studies, one at the regional-scale that aimed to assign ectomycorrhizal decomposer taxa and one at the national-scale that aimed to evaluate the niche(s) of these assigned taxa.

Exploring for key ectomycorrhizal decomposers – regional-scale

Currently most analyses that try to delineate a group of ectomycorrhizal fungi involved in decomposition focus on the genus-level (Argiroff *et al.*, 2022, 2023), however, it seems probable that the capacity and relative strength of ectomycorrhizal fungi as decomposers varies within genera (Bödeker *et al.*, 2014; Lindahl *et al.*, 2021). To fill this gap the main hypothesis addressed was:

- if the fungal community composition is correlated with estimated manganese peroxidase activity on the mycelial scale, then some species of ectomycorrhizal fungi would be positively correlated with community-level peroxidase activity

Estimated manganese peroxidase activity and fungal community composition data were used from a prior study that focussed on spatial patterns and sampling methodology. The study sites were established in 12 forests in the Uppsala region, which included both young and old and pine- and spruce-dominated stands. Thirty-eight samples were collected at each site with a spatially nested hierarchal sample distribution, in which samples were organised in 18 equilateral triangles with increasing side lengths ranging from 0.1 to 10 m.

In brief, MnP activity was estimated using the substrates MBTH/DMAB on freeze-dried soils. The reactions were initiated with the addition of H_2O_2 and measured during the linear phase of the reaction (every 3 minutes for 45 minutes) at 590 nm in a 96-well microplate spectrophotometer. The rate of activity for manganese-dependant and total peroxidase activity was normalised to enzyme units per gram of organic matter. The fungal community composition was determined by Pacbio sequencing of the ITS2 region using the primers gITS7 and a 3:1 mix of ITS4 and ITS4arch with unique 8-base pair sample identification tags. The number of PCR cycles was optimised to reduce length-bias by reducing the number of cycles to as few as possible.

First, spatial autocorrelation of MnP activity was tested in a linear mixed-effect model. The variation within each sampling triangle was tested with triangle size (i.e. triangle side length) as a fixed factor and stand age and dominant tree species as random factors. Because no spatial autocorrelation was observed even with samples only 0.1 m apart, the samples were treated as independent for further analyses.

To assess variation in log-transformed MnP activity explained by the fungal community composition, a canonical correspondence analysis (CCA) was performed. There was a significant relationship between the fungal community composition and estimated manganese (and total) peroxidase activity, therefore, we further investigated which individual taxon within the core Agaricomycetes (214 species within 75 genera) were correlated with hotspots of peroxidase activity. To do this, I calculated the weighted average enzyme activity (WAE) at both the species- and genus-level using eq. 1 (Bödeker *et al*., 2014). The higher the WAE value, the greater the relative abundance of a given taxon in samples with high peroxidase (both manganese and total) activity. WAE for each taxon was tested in a one-sided permutation test against its own simulated random distribution formed through 10,000 re-samplings of the enzyme activity (E_i). Ectomycorrhizal species with a p-value (un-adjusted) below 0.1 (one-sided test), with genomic evidence of peroxidase capacity within the genus, were assigned as potential ectomycorrhizal decomposers. I evaluated whether there was a relationship between the genetic capacity within a genus (i.e. the average number of gene copies of class II peroxidases based on JGI available genomes) and the determined WAE using Kendall's Rank correlation test.

$$WAE = \frac{\sum_{i=1}^{n} E_i P_i}{\sum_{i=1}^{n} P_i} \qquad \text{Eq. 1}$$

(E_i is peroxidase activity in a given sample *i*; P_i is the relative abundance of a genus/species in sample *i*; *n* is the total number of samples)

Niche(s) of ectomycorrhizal decomposers in Sweden – national-scale

If ectomycorrhizal decomposers are C demanding symbionts, then it may be expected that they would be especially sensitive to disturbances that increase available N and therefore shift the competitive outcomes with ectomycorrhizal fungi that acquire inorganic N more readily for a lower C cost (Lilleskov *et al.*, 2011; Hagenbo *et al.*, 2019; Argiroff *et al.*, 2022). The main hypothesis addressed was:

- ectomycorrhizal decomposers, as assigned in the regional-scale analysis, would be positively correlated with forest stand age and negatively with soil fertility

The assigned ectomycorrhizal decomposers from the regional-scale study (described above) were then investigated across a similar climatic range in Sweden as the regional-scale study (temperature sum between 900-1400) to investigate their niche in relation to forest stand age and soil fertility (data from the Swedish Forest and Forest Soil Inventories). A subset of the inventory sites that were productive forests (annual growth > 1 m^3 ha^{-1}) with a mean stand age > 10 years and ≥ 90 % conifer basal area were selected. For soil fertility, an index was created using the first principal component axis of a principal component analysis of soil pH and N/C ratio.

First, a Fisher's exact test was used to test whether there were differences in the frequency of occurrence of the individual ectomycorrhizal decomposer taxon between pine- and spruce-dominated stands. Then the relative abundance (log-transformed) of ectomycorrhizal decomposers as a group, when present, was tested against dominant tree species using a linear model. The change in relative abundance (when present) and frequency of occurrence of the ectomycorrhizal decomposers as a group were tested with linear mixed-effect models and generalised linear mixed-effect models with a binomial linking function, respectively, with stand age and soil fertility as fixed factors and sequencing depth as a random factor. Sequencing depth was included as a random factor because there was a switch from the Pacbio RSII platform to the Sequel 1 platform since the initiation of metabarcoding as a part of the Swedish Forest Soil Inventory. For the regional dataset, sequenced in 2021, the Sequel 1 platform was used. These linear models were done with stand age either as a continuous variable or as a categorical variable, i.e. split into two classes: younger stands with mean stand age < 70 years (likely to have been previously clear-cut) and older stands with mean stand age > 70 (likely to have longer forest continuity). Frequencies of occurrence of individual taxon were tested against soil fertility and stand age using generalised linear models with a binomial linking function.

7.2 Paper II

Paper II aimed to optimise the MnP assay with two main objectives: 1) compare different enzyme assay substrates and their capacity to distinguish manganese-dependant activity and 2) investigate the implications of sample preparation on the release of intracellular peroxidases, specifically pertaining to the degree of mechanical disruption of living tissues. First, the assay H_2O_2 concentration was optimised using commercially available horse radish peroxidase and recombinant MnP from *Phanerochaete chrysosporium*. Then four substrates MBTH/DMAB, 2,6-DMP, L-Dopa, and ABTS, were compared in terms of sensitivity and capacity to distinguish manganese-dependent and -independent activity using these pure enzymes. For the second objective, *Hypholoma fasiculare* was grown axenically on senesced, then sterilised, pine needles with liquid Modified Melin-Norkrans media for 7 weeks. The fungal cultures were subjected to varying degrees of mechanical disruption in extraction buffer: soaking, gentle shaking, and thorough homogenisation with an Ultra-Turrax dispersing mixer. Three technical replicates of each extraction type were assayed with each of the four enzyme assay substrates. The rate of MnP activity and the proportion of manganese-independent activity at each degree of mycelial disruption were tested using one-way ANOVA.

7.3 Paper III

Debate persists on whether MnPs encoded for and expressed by ectomycorrhizal fungi are functionally equivalent to those of saprotrophic fungi (Pellitier & Zak, 2018). This paper aimed to confirm the proposed ligninolytic capacity of a MnP from *Cortinarius* using heterologous expression in *Pichia pastoris*. The main hypothesis addressed was:

- if manganese peroxidases from *Cortinarius aurae* have ligninolytic capacity, then it is expected that they will be able to oxidise Mn^{2+} to Mn^{3+}, oxidise the organic enzyme assay substrate MBTH, and modify conifer monolignol (as observed through changes to its UV-absorbance spectrum)

Three highly transcribed MnP genes from *Cortinarius* were selected as candidates from a metatranscriptomics study performed in a Swedish boreal forest (Barbi *et al.*, in review). Selecting candidate genes from metatranscripts provides two major benefits: 1) given that the gene is expressed it is likely relevant for the fungus and 2) allowed the study of a gene from a fungus that has not been previously genome sequenced. These genes were first amplified from type-specimen sporocarps (Kew Botanical Gardens vouchers K-M:166960 (*Cortinarius aurae*) and K-M:190268 (*Cortinarius ominosus*)) using transcript specific primers and Sanger sequenced to validate gene sequences (Macrogen Europe). Additionally, one gene from the sequenced genome of *Cortinarius* (*Phlegmacium*) *glaucopus*, which was also present in the Barbi *et al.* (in review) metatranscriptomics study, was selected from the JGI Mycocosm database. The presence of characteristic functional residues was checked by aligning the putative-MnPs against characterised MnPs.

One of the four putative-MnP genes was successfully produced in *Pichia*. The production and purification were optimised to increase yield and reduce excess hemin contamination. The purity and quantity of the recombinant enzyme was evaluated in multiple ways, including Bradford protein quantification, Qubit^{TM} fluorescent broad range protein assay, the Reinheitzahl value ($_A403/_A280$), SDS-PAGE, and tandem mass-spectrometry (SciLifeLab, Stockholm, Sweden). To characterise the activity of the recombinant MnP enzyme, assays were performed based on the formation of Mn^{3+}-malonate, the MBTH/DMAB assay, and UV-spectroscopy of coniferyl alcohol. All assays were performed with *Phanerochaete chrysosporium* MnP as a positive control and denatured enzymes (heated to > 90 °C for ~15 mins) as negative controls. The pH optimum of manganese-dependent activity was assayed with MBTH/DMAB in a 50 mM sodium acetate buffer with pH ranging from 3.6 - 6.0.

7.4 Paper IV

Two main assumptions are made about the ecophysiology of ectomycorrhizal decomposers that influence their potential ecosystem role and outcomes for soil organic matter dynamics: 1) that the main benefit of oxidative decomposition for ectomycorrhizal decomposers is increased access to N bound in recalcitrant organic matter (Lindahl & Tunlid, 2015) and 2) that oxidative decomposition is energetically expensive (Shimizu *et al.*, 2005; Chakrawal *et al.*, 2024). To address these assumptions, three main hypotheses were addressed:

- if nutrient mining is facilitated by MnP, then the expression of genes coding for proteolytic and chitinolytic enzymes (and associated transporters) should be positively correlated with MnP gene expression
- if oxidation by MnP is energetically expensive, then the expression ratio of growth marker genes to respiration marker genes (a proxy for carbon-use-efficiency) should be negatively correlated with MnP gene expression
- if H_2O_2 is produced by the glyoxal oxidase and/or GMC oxidoreductase pathways, then the expression of these genes should be positively correlated with MnP gene expression

Paper IV used metatranscriptomics with the aim to assess differences in the expression of genes related to growth, respiration, H_2O_2 production, and N acquisition relative to the expression of MnP genes. In fall of 2024, organic soil was collected from a mixed pine-spruce forest. To target soil dominated by the mycelia of *Cortinarius* subgenus Dermocybe, soil sampling plots were centred over sporocarps tentatively identified as *C. ominosus*. The sampling was done in twenty 15 x 15 cm grids with 1.5 cm diameter cores, each 3 cm apart (Figure 6). First, liquid N was poured directly over the sampling grid to flash freeze the soil. This aimed to inhibit RNA degradation and prevent disturbance of adjacent soil taken in close proximity. The soil samples were kept individually and returned to the lab on dry ice. Before hand-grinding with mortar and pestle, the samples were freeze-dried. RNA was extracted using LiCl precipitation. The RNA was DNAse treated before and after purification to ensure no contamination by gDNA, then an RNA aliquot was cDNA synthesised for sample selection with digital PCR (dPCR).

Figure 6. Site and sampling design of **paper IV**. Panels left to right: selected forest site, sampling plot centred over *Cortinarius* sporocarp, and sampling design for each plot (black points are soil cores).

Primers specific to *Cortinarius* were designed for MnP and 2-oxoglutarate dehydrogenase genes. 2-oxoglutarate dehydrogenase, which is an enzyme in the tricarboxylic acid (TCA) cycle that catalyses α-ketoglutarate to succinyl-CoA, was selected as a marker for respiration based on experiments by Hasby *et al.* (2021). PCR primers were tested using DNA extracted from sporocarps of *C. ominosus* and *C. purpureus*. The soil samples were initially filtered based on successful amplification of one or both gene markers (as analysed by gel electrophoresis), then transcript abundance (copies μl^{-1}) of the two genes was assessed using dPCR. To maximise variation in MnP gene expression, two samples were selected from each of 15 blocks: one with high MnP/KGD expression ratio and one with low MnP/KGD expression ratio. The variation in the ratio was predominantly driven by variation in the expression of MnP genes. The 30 selected samples were subjected to mRNA sequencing following rRNA depletion (SciLifeLab, Uppsala).

After quality control, transcript sequence reads were mapped to the *C. ominosus* genome and quantified using Salmon v1.10 (Patro *et al*., 2017). Transcript abundance was estimated in Salmon using Transcripts per Million (TPM), which accounts for the influence of transcript length on abundance and sequencing depth. The TPM of specific genes related to my *a priori* hypotheses were extracted based on annotation to EC classes, MEROPS families, and TCDB numbers and correlated with the expression of MnP genes (Table 1). The square root transformed TPM of each gene class (all gene copies aggregated) was tested in a linear mixed-effect model with square root transformed MnP as the fixed factor and plot as a random factor.

An exploratory *post hoc* analysis was performed with all EC classes and N transporters (ammonium, nitrate, urea, amino acids, and peptides) using Ranked Pearson's correlation. The top 10 % positive and negative *post hoc* correlations were investigated further.

Table 1. *A priori* genes of interest investigated in **paper IV**

	Enzyme name	Abbrev.	EC, KOG, MEROPS, or TCDB classification (CAZy if applicable)
Peroxidase activity	Manganese peroxidase	MnP	EC 1.11.1.13 (AA2)
Phenol oxidase activity	Laccase		KOG1263 (AA1_1)
Heme synthesis pathway	5-aminolevulinate synthase	ALAS	EC 2.3.1.37
Citric acid cycle	2-oxoglutarate dehydrogenase	KGD	EC 1.2.4.2
Cell wall formation	β-1,3-glucan synthase	GT48	EC 2.4.1.34 (GT48)
H_2O_2 production	Copper radical oxidase / glyoxal oxidase	CRO/ GLOX	EC 1.2.3.15 (AA5_1)
	GMC AA3_3 (alcohol oxidase)	AOx	AA3_3
	GMC AA3_2 (aryl-alcohol oxidase/ pyranose dehydrogenase-like)	AAO/ PDH	AA3_2
	GMC AA3_2 (glucose dehydrogenase-like)	GDH	AA3_2
Proteolytic activity	Aminopeptidases		EC 3.4.11.-
	Aspartic peptidases		A01
Amino acid transport	Amino acid transporter	AAT	2.A.3.1
	Amino acid/auxin porter	AAAP	2.A.18
	Amino acid/choline transporter	ACT	2.A.3.4
	L-type amino acid transporter	LAT	2.A.3.8
	Yeast amino acid transporter	YAT	2.A.3.10
Peptide transport	Oligopeptide transporter family	OPT	2.A.67
	Peptide transporter family	POT	2.A.17
Chitinolytic activity	Chitin deacetylase		EC 3.5.1.41 (CE4)
	Chitinase		EC 3.2.1.14 (GH18)
	β-N-acetylhexosaminidase	NAG	EC 3.2.1.52 (GH20)
Hydrolytic activity	Glucan 1,3- β-glucosidase		EC 3.2.1.58 (GH5_9, GH5_50, GH17)
	β-glucosidase		EC 3.2.1.21 (GH3)

8. Results and discussion

Manganese peroxidase assay optimisation

Manganese peroxidase assays were central to the studies in this thesis. **Paper II** considered the optimisation and limitations of these enzyme assays and how the methodology affects our interpretations of estimated enzyme activity. The optimal H_2O_2 concentration we determined was within the range of frequently reported H_2O_2 concentrations used in other studies, and we observed that at both the highest and lowest concentrations tested the rate (and end point absorbance) was reduced. This stresses the importance of optimising H_2O_2 concentration to suite the samples from each study to enable more accurate estimations of MnP activity. All substrates (L-Dopa, 2,6-DMP, MBTH/DMAB), apart from ABTS, distinguished between manganese-dependant and -independent activity. In the second part of the experiment, regarding sample preparation, we observed that the measured rate of MnP activity increased with increasing physical disruption during extraction (i.e. homogenisation of the mycelium with buffer), but so did the proportion of manganese-independent activity. This indicates that there is a trade-off between extraction efficiency and the release of non-target intracellular peroxidases. ABTS was the most susceptible to oxidation by manganese-independent peroxidases and therefore I suggest that it is best suited as a substrate for phenol oxidase assays rather than MnP assays. Given that this study was performed on a single fungal species grown in pure culture, it is difficult to extrapolate to soil conditions where mycelial densities vary. However, it would be important to consider in future soil enzyme assays the degree of homogenisation required, based on the desired specificity.

In silico analysis of Cortinarius oxidative capacity

For the seven new *Cortinarius* genomes that I collected material for, *in silico* analysis indicated that MnP gene copy number varied from 0 to 12 and most had the characteristic functional residues of DDQ-type short MnPs (Figure 7). *Cortinarius rubellus*, from the subgenus *Orellani*, was the only species that appeared to not encode for MnPs. It is notable that not only were there several gene copies in all but one species but that gene copy number seemed to vary even between quite closely related species (e.g. six in *C. ominosus* and two in *C. aurantiobasis*). Only one gene copy from *C. pilatii* appeared to have closer sequence similarity to the ED-type short MnPs observed in *Phlegmacium glaucopus*. Unlike previously characterised MnPs (Sánchez-Ruiz *et al.*, 2024), all MnP genes from *Cortinarius* lack a disulfide bridge at the N-terminus. This absent disulfide bridge is also a characteristic of some putative-MnPs encoded by saprotrophs, including *Gymnopilus junonius* (i.e. JGI protein ID 1801496; Ruiz-Dueñas *et al.*, 2021) and *Pluteus cervinus* (i.e. JGI protein ID 845893; Varga *et al.*, 2019). This structural difference could result in changes to the enzyme's catalytic efficiency, specificity, and stability, for which **paper III** provided some evidence, but further detailed analyses would be needed.

Paper IV aimed to assess the potential pathways for H_2O_2 production necessary for MnP activity by ectomycorrhizal decomposers. Based on the gene content of the investigated *Cortinarius* species, glucose-methanol-choline (GMC) oxidoreductases may be the main H_2O_2 pathway, as only *C. fulvescens* (and *P. glaucopus)* appear to encode for a putative glyoxal oxidase gene (Figure S1). Four aryl-alcohol oxidase/pyranose dehydrogenase-like (AAO/PDH-like; AA3_2; Figure S2) genes were encoded for by *C. ominosus*, which is consistent with *P. glaucopus,* which has four putative AAO genes annotated (Miyauchi *et al.*, 2020; Mattila *et al.*, 2022). I also noted that the selected gene model for one of the four AAO/PDH-like gene copies (in *C. ominosus*; JGI ProtID 1984530) was much longer than the others, and upon further inspection I determined that the gene model likely encompassed two physically close but separate genes. While these errors in gene model selection are common and can be resolved through manual annotation, this occurrence highlights the benefits of studying a small selection of genes that can feasibly be inspected on a more detailed level. In addition to AAO/PDH-like genes, six putative alcohol oxidase (AOx; AA3_3) and several putative glucose dehydrogenase-like (GDH-like) genes are encoded by *C. ominosus.* This GMC oxidoreductase gene repertoire is generally consistent with the previous observation that, while litter- decomposing Agaricales have had an expansion of GMC oxidoreductases, ectomycorrhizal species tend to have fewer gene copies (Ruiz-Dueñas *et al.*, 2021). The exact physiological role of copper radical oxidases (other than glyoxal oxidases) and whether they also generate H_2O_2 for peroxidase activity remains unclear (Kersten & Cullen, 2014).

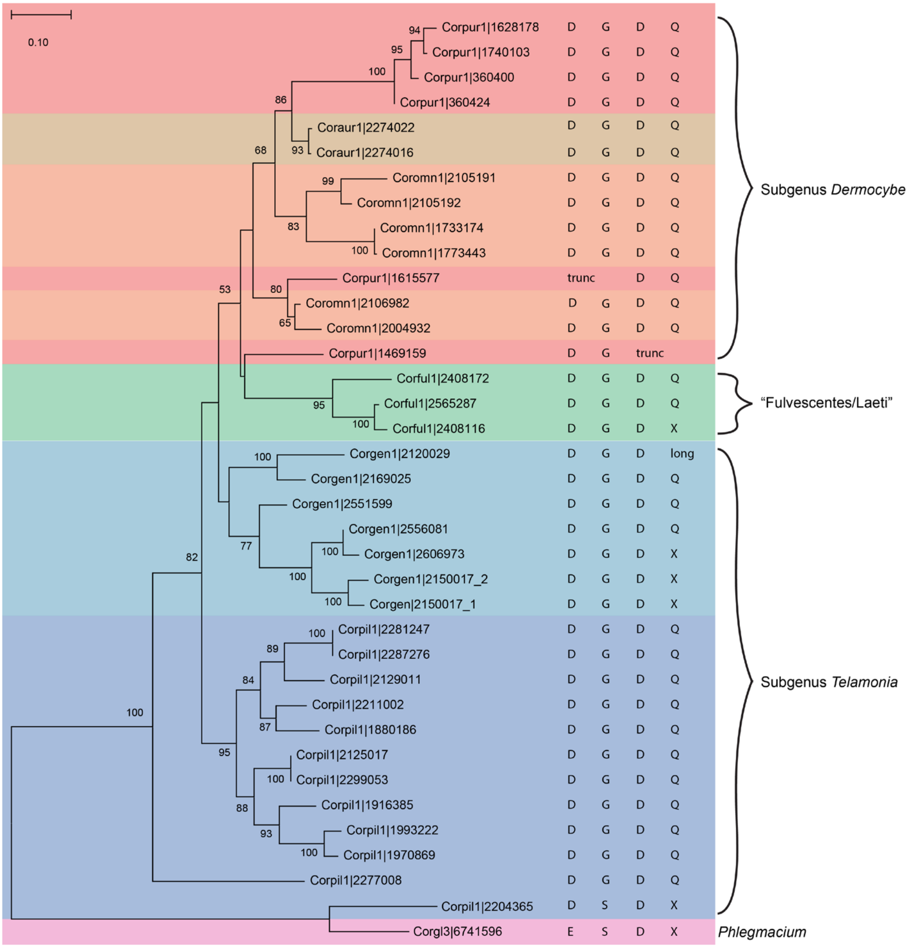


Figure 7. Maximum likelihood tree of manganese peroxidase genes from *Cortinarius* genomes and outgroup from *Phlegmacium glaucopus* (Miyauchi *et al.*, 2020). The names are abbreviated: *Phlegmacium glaucopus*, Corgl; *C. pilatii*, Corpil; *C. gentilis*, Corgen; *C. purpureus*, Corpur; *C. fulvescens*, Corful; *C. ominosus*, Coromn; *C. aurantiobasis*, Coraur. Colour shading indicates the different species included and labelling for the subgroupings within *Cortinarius*. Following the species abbreviation is the JGI protein identification number. The amino acids present in Mn^{2+} oxidation site (with the exception of second residue listed; Sánchez-Ruiz *et al.*, 2024) are denoted to the right of each gene. Amino acids abbreviations: Aspartic, D; Glutamic, G; Glutamine, Q; Serine, S. One gene with longer C-terminus and missing glutamine marked as "long" and otherwise missing glutamine at the C-terminus marked with X. Gene models that are truncated either at beginning or end are marked as "trunc". Bootstrap (adaptive) values are indicated next to nodes.

In **paper I**, we did not find a correlation between the average number of MnP gene copies per genus and the species-specific weighted average enzyme activity (Figure 8). Genomic potential is often used to guide our understanding of the potential for certain species to belong to different functional groups of fungi (Miyauchi *et al.*, 2020; Argiroff *et al.*, 2023), but in this case, I highlight that the number of class II peroxidase genes does not necessarily connect to potential as an ectomycorrhizal decomposer. This was further evident from the expressed transcripts of *C. ominosus* in **paper IV**, where only 2 of the 6 MnP gene copies were highly expressed. There is a potential that these other gene copies are expressed under different conditions than was sampled here, but the possibility that they are mutated in an unknown way and not active (i.e. ghost genes) cannot be excluded.

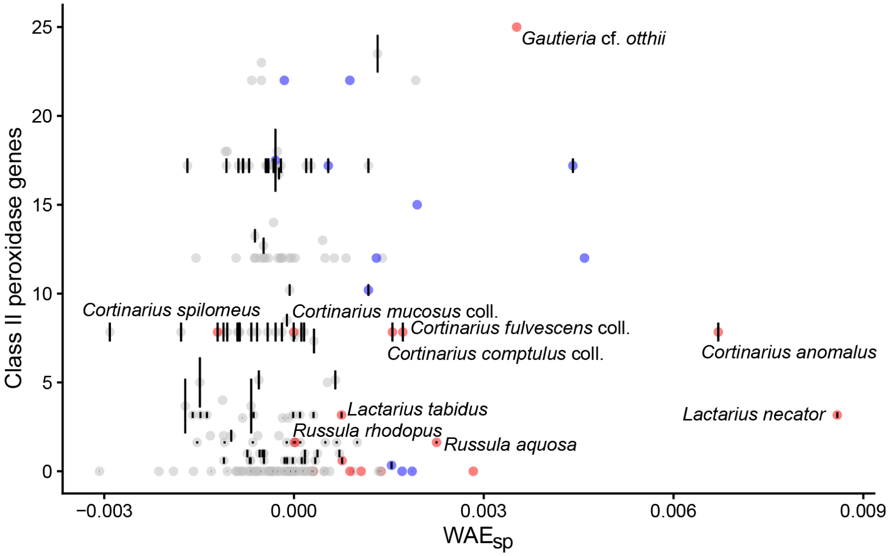


Figure 8. Species-specific weighted average enzyme activity (WAE_{sp}; as determined in **paper I** with the correlative strength between estimated manganese peroxidase activity and the relative abundance of core Agaricomycetes taxa) versus the mean number of class II peroxidases genes per genera. No significant relationship was determined between rank of WAE_{sp} and mean number of class II peroxidase genes per genera as tested using Kendall's Rank test. Coloured points are taxa with WAE_{sp} permutation test P-value < 0.1, where ectomycorrhizal taxa are coloured red and saprotrophic taxa are coloured blue. Ectomycorrhizal taxa assigned as ectomycorrhizal decomposers are labelled by name. Error bars are ± standard error of mean class II peroxidase genes per genus, where no error bars indicate that only one species is represented within the genus.

Ligninolytic capacity of a recombinant Cortinarius manganese peroxidase

Previous studies (and **paper IV**) have observed *Cortinarius* MnP transcripts *in situ* (Bödeker *et al.*, 2014; Barbi *et al.*, in review) and have correlated ectomycorrhizal fungal presence to MnP activity (Kyaschenko *et al.*, 2017b; Pérez-Izquierdo *et al.*, 2021; Kranabetter *et al.*, 2025). However, these are indirect forms of evidence that the MnP genes from ectomycorrhizal species encode for enzymes that are active and function as expected. There remains doubt that, even if the genes encoded for by ectomycorrhizal decomposers are actively transcribed, they would have the same ligninolytic capacity as those transcribed by saprotrophs (Pellitier & Zak, 2018). In **paper III**, I provide supporting evidence of oxidative activity by an ectomycorrhizal MnP. The recombinant MnP from *Cortinarius aurae* (hereafter Ca-MnP1) could oxidise Mn^{2+} to Mn^{3+} as well as a common peroxidase enzyme assay substrate (MBTH). Further, changes to the UV-absorbance spectrum of coniferyl alcohol (a constituent of lignin) were observed in reactions with Ca-MnP1. The oxidation of coniferyl alcohol by Ca-MnP1 mirrored that of MnP from *P. chrysosporium*; however, it was not possible to distinguish if the reaction resulted in ring cleavage or polymerisation of the coniferyl alcohol. I had aimed to further investigate the chemical changes to coniferyl alcohol using Fourier-Transformed Infrared Spectroscopy (FT-IR), but the results from my preliminary experiment were unclear. I observed that the coniferyl alcohol reactions performed with Ca-MnP1 when active, denatured, and when active but without available manganese all had similar chemical signatures (as determined by FT-IR) that were unique from the signatures of coniferyl alcohol oxidised by MnP from *P. chrysosporium*. Given the application of relatively new methodology, further follow-up would be needed to assess the validity of this additional experiment.

Nonetheless, the activity of Ca-MnP1 did not fully align as expected with the positive control enzyme from *P. chrysosporium* and it is difficult to disentangle whether these inconsistencies resulted from side-effects of the heterologous expression or functional differences. Ca-MnP1 displayed activity with the MBTH/DMAB assay even in the absence of available manganese. Manganese-independent activity has been observed with other short MnPs of the same functional arrangement (DDQ-type) by Fernández-Fueyo *et al.* (2014) and Sánchez-Ruiz *et al.* (2024), and they suggested that possibly the short C-terminus exposes the reactive site to enable direct oxidation of smaller organic substrates (such as used in my assays). Perhaps with larger and more hydrophobic substrates the manganese-dependant pathway would be more important. However, it is also possible that some non-specific activity is a result of excess hemin bound to the surface of the enzyme. MBTH/DMAB and coniferyl alcohol assays performed with only hemin indicated that hemin could initiate some oxidation even in the absence of the active enzyme. Further, a small proportion of non-biological activity remained even after heat-denaturation. Excess hemin presented issues from early-on in the establishment of our enzyme production and purification methods. Hemin polluted the SDS-page gel and interfered with enzyme quantification using UV absorbance and colorimetric- and florescence-based methods. Attempts were made to reduce excess hemin during induction: decreasing the total amount supplemented, more frequent additions at lower concentrations, and modifying the form of addition from solubilised in NaOH to undissolved hemin. These attempts did not improve the purity and reduced hemin addition led to lower observed enzyme activity. Peroxidases are notoriously challenging to produce recombinantly (Bissaro *et al.*, 2022), and the expression system typically produces much less protein compared to other recombinant CAZymes.

The potential for some manganese-independent activity by *Cortinarius* MnPs influenced the decision in **paper I** to test correlations with both manganese and total peroxidase activity. It was the case that several taxa (with genetic potential for MnPs) had a stronger correlation with total peroxidase activity than with MnP activity, further supporting the idea that not all MnPs are strictly manganese-dependant.

Key ectomycorrhizal decomposers

Over time perspectives have shifted on the capacity of ectomycorrhizal fungi to directly decompose soil organic matter and now there is increasing recognition that at least some ectomycorrhizal fungi may contribute to oxidation of soil organic matter. However, it is often the case that this trait is considered at the genus-level (Pellitier & Zak, 2021; Argiroff *et al.*, 2022), despite known interspecific, and even intraspecific trait variation, among ectomycorrhizal fungi (Bödeker *et al.*, 2014; Dauphin *et al.*, 2025; Lofgren *et al.*, 2025). A lack of functional redundancy in decomposer capacity was highlighted in Lindahl *et al.* (2021), where a group of closely related *Cortinarius* species (*C. acutus* sl.) had a higher correlation with reduced C stocks than the genus as a whole. Along those lines, **paper I** tested whether the trait of (potential) oxidative decomposition was phylogenetically clustered at the genus-level by comparing the correlative strength between MnP activity and relative abundance of a core group of Agaricomycetes taxa at the genus- and species-level. Despite that this approach has many limitations, I observed that, especially when grouping many species from large genera together, the correlative signal between measured enzyme activity and the taxa considered was blurred. Given that *Cortinarius* is a genus with many species of varying characteristics, it should not be surprising that the capacity for oxidative decomposition varies between species (Bödeker *et al*., 2014). However, there are draw-backs to considering the species-level with this correlative approach. For example, the statistical power was limited by the frequency of the taxa considered, wherein if they are too rare there are too few occurrences to correlate with MnP activity and if they are too common, there is noise from occurrences where a fungus is present but not actively producing MnPs. It is likely that there are rarer species that participate in oxidative decomposition but could not be assigned here. Further, the zero-inflated nature of relative abundance data and the large number of species make this type of analysis susceptible to type-I error by multiple testing. Nonetheless, on the bigger scale, it may be sufficient to consider the most frequent taxa, as these are likely to have the strongest impact on soil organic matter dynamics.

In the regional-scale study of **paper I**, ten ectomycorrhizal taxa were assigned as potential key decomposers based on their spatial co-localisation with estimated MnP activity (Figure 8). Half of these taxa were from the genus *Cortinarius*, further highlighting the importance of *Cortinarius* in soil organic matter decomposition. Notably, *Gautieria* cf. *otthii* was also selected, which (along with other fungi with hypogenous sporocarps) has received less attention in previous literature despite its high genetic capacity for MnPs. Interestingly, the analysis also linked some ectomycorrhizal fungi with no known evidence of encoded peroxidase genes to MnP hotspots (e.g. *Thelephora*, *Suillus*). In the case of *Suillus*, a similar correlation was observed in Lindahl *et al.* (2021), where *Suillus variegatus* was correlated with a reduction in C stocks. This opens the question of whether there are interactions at the mycelial scale between MnP producing fungi and other decomposers, and if so, in which way? *Suillus* is likely to have substantial decomposer capacity, but perhaps rather with a Fenton-chemistry reaction (Shah *et al.*, 2016) and could act on the same substrates as *Cortinarius* and other peroxidase producing fungi. This could be a competitive interaction, as oxidation by peroxidases has also been suggested as a mechanism for antagonistic interactions (Score *et al.*, 1997) or potentially even facilitative, if H_2O_2 is being produced and utilised by both fungi.

This approach for assigning important decomposers has several limitations but considering the challenges with other approaches (i.e. strictly genomic- or laboratory-based) it remains one of few options. The co-localisation of fungal relative abundance and MnP activity relies on the assumptions that, 1) there is a relationship between the relative abundance of ITS markers of a species and its enzyme production and 2) that there is spatial-temporal co-localisation of the measured enzyme activity and the fungi present at the time of sampling. Although the first assumption cannot be ignored, it seems probable that fungi producing the key decomposition enzymes would proliferate into these substrates during decomposition and would account for a major part of the community biomass. The second assumption could be violated if there is a temporal disconnect between fungi and the enzymes they produce (i.e. enzymes becoming stabilised in the soil after being released; Nannipieri, 2006; Allison, 2006). In line with this, in **paper II** it was observed that thorough homogenisation (as performed in **paper I**) may distort assessments of extracellular enzyme activity by proportionally increasing non-target intracellular peroxidase activity. However, the observed "patchy" dynamic of MnPs would imply that their activity occurs briefly in "hotspots", and that they are not readily stabilised in the soil. Additionally, a fungus may also be present without producing peroxidases even though it is capable, as was evident in **paper IV**, which adds noise to the correlation between estimated MnP activity and the fungi that produce them.

Niches of ectomycorrhizal decomposers

I did not find support for the hypothesis that ectomycorrhizal decomposers would have a unified niche in older and more nutrient-poor forest stands. Rather, the group that was analysed in the national-scale study was more frequent in "middle-aged" forests (Figure 9a), consistent with the stand age where the maximum ectomycorrhizal species richness is observed across Sweden (Lindahl *et al.* 2026). Also, there was no difference in the frequency of occurrence between forests with a stand age < 70 years with an expected history of clear-cutting and forests with a stand age > 70 years with an expected history of forest continuity. This finding suggests that, as a group, ectomycorrhizal decomposers are not necessarily more sensitive to forestry than other ectomycorrhizal fungi but does not exclude the possibility that rarer species (not analysed here) are sensitive to clear-cutting, as has been observed in other studies (Varenius *et al.*, 2016; Lindahl *et al.*, 2026). It seems likely that there is sufficient niche differentiation in regards to forest stand age and soil fertility, wherein different ectomycorrhizal decomposers can establish and be active in stands of varying age and forest management, at least within the region investigated here.

The assigned group of ectomycorrhizal decomposers was, in general, most frequent in nutrient-poor spruce-dominated stands and nutrient-rich pine-dominated stands (Figure 9b). In the range of forests considered here, tree growth should still be limited by N even in the most fertile soils, and the investigated region is not exposed to high levels of N deposition that might disfavour ectomycorrhizal N mining. Further, it seems likely that in the most nutrient-poor and acidic soils, ectomycorrhizal fungi are replaced by more stress-tolerant fungi, including ericoid mycorrhizal fungi (Clemmensen *et al.*, 2015; Sterkenburg *et al.*, 2015; Fanin *et al.*, 2022). Oxidative decomposition may also be limited in very acidic soils, as the soil pH is far below the optima for MnPs, and in one case MnP activity has been observed to increase with soil fertility in boreal forests (Kyaschenko *et al.*, 2017b). In **paper III**, I observed that the pH optimum of manganese-dependant activity of recombinant Ca-MnP1 was above pH 5. While I expected that Ca-MnP1 would have a lower pH optimum than MnPs produced by the saprotrophic wood-decomposer *P. chrysosporium*, due to adaptation to the low pH soils that *C. aurae* typically grows in (Puissant *et al.*, 2019), this did not appear to be the case. The pH of 98 % of the forest soils included in **paper I** are below the estimated pH optima of Ca-MnP1. It may be that MnPs are only functional outside their optimum to a certain degree and below a certain pH threshold the return on investment declines – as is consistent with my observation that there was almost no activity of Ca-MnP1 at pH 3.6.

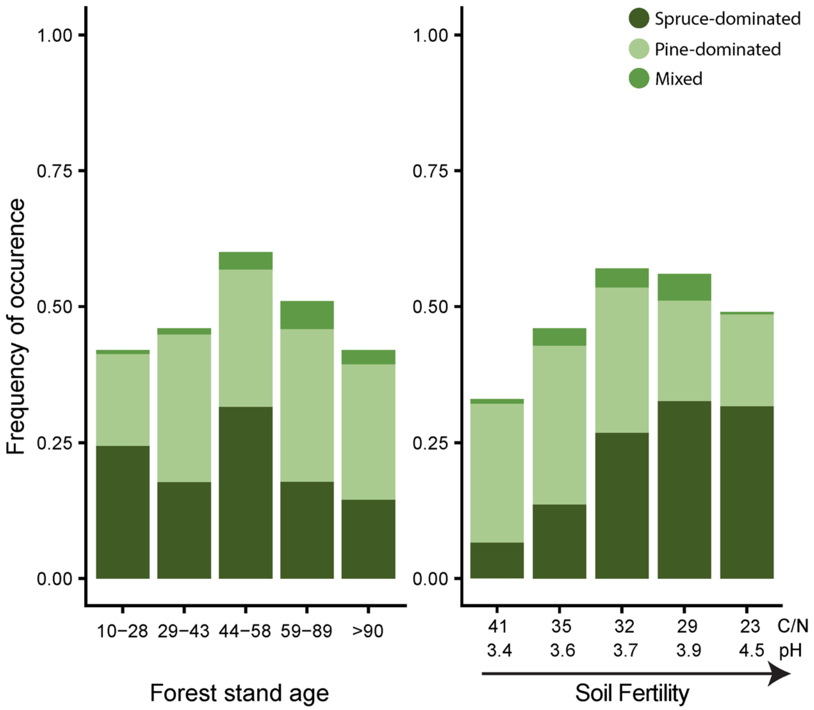


Figure 9. Frequency of occurrence of taxa assigned as ectomycorrhizal decomposers across forest stand age (a) and soil fertility (b). Forests are sectioned into five evenly distributed bins with inclusive age ranges (in panel a) and their mean soil C/N and pH (in panel b) on the x-axes. Bars are coloured by proportion of each forest type within each bin.

Nutrient mining by ectomycorrhizal decomposers

In **paper IV**, I aimed to test the correlative patterns in gene expression by *Cortinarius ominosus* to indicate whether oxidative decomposition is linked to mining for nitrogenous compounds bound in recalcitrant organic matter. The transcript abundance of several *a priori* selected genes related to N acquisition were correlated with the transcript abundance of MnPs on a mycelial scale, supporting my first hypothesis that MnPs facilitate N mining (Table 2). Gene expression of aspartic proteases was observed to be strongly correlated with the expression of MnP genes (Figure 10a; Table 2) and this was driven largely by one predicted extracellular endopeptidase – cathepsin E. Aminopeptidases were also correlated with MnP gene expression but this correlation was only evident during the *post hoc* analysis that investigated individual EC classes of peptidases (amino- and carboxypeptidases) rather than the group as a whole (all EC 3.4.11.-), as was done in the *a priori* specified hypotheses tests. The correlated peptidases included gene copies that both did, and did not, have predicted signal peptides or were potentially membrane bound. Among the most strongly correlated peptidases were glutamate carboxypeptidases I and II (MEROPs family M20A and M28B), which did not appear to have signal peptides (Figure 10b; Table 2), and tripeptidyl aminopeptidases (MEROPs family S53), which included at least one gene variant that was potentially secreted. Overall, the positively correlated genes I observed were congruent with the gene expression patterns of *Paxillus involutus* during initiation of oxidative decomposition (although with Fenton-chemistry, rather than by peroxidases in that case; Rineau *et al.*, 2012) in response to N starvation (Nicolás *et al.*, 2019). This proposed N mining is further strengthened by a strong correlation between the transcript abundance of MnPs and oligopeptide transporters (Figure 10c; Table 2), which are proposed to enable import of larger peptides (3-8 amino acids in length; Lucic *et al.*, 2008). Likewise, the expression of amino acid/auxin porter (AAAP) genes was also positively correlated (Table 2). While many proteins from this class of transporters are vacuolar transporters (Lucic *et al.*, 2008), alignment of the two most highly expressed AAAP genes by *C. ominosus* indicated homology with the AAAP subgroup of Fungal oligopeptide transporters (FOT), which are proposed membrane dipeptide transporters (Figure 10d; Damon *et al.*, 2011). Considered all together, the expression pattern of extracellular aspartic proteases (endopeptidases), intracellular peptidases, and polypeptide transporters would suggest that proteins are degraded to smaller polypeptides extracellularly, imported into the cell, then further depolymerized to amino acids. Taking up larger peptides intact rather than depolymerizing them extracellularly could possibly be a mechanism to reduce competition with bacteria and other fungi, which may otherwise exploit recently released nutrients.

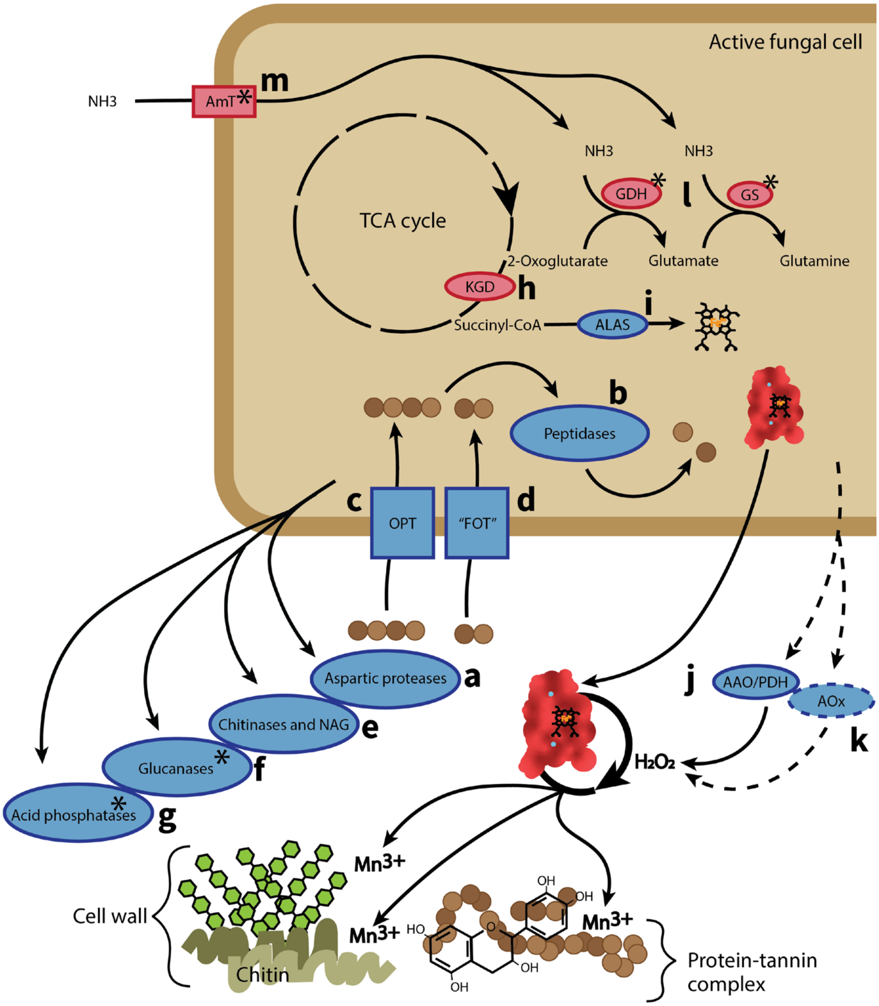


Figure 10. Conceptualisation of the results from **paper IV**. Expression of genes correlated with the expression of manganese peroxidase genes. Negatively correlated gene products are in red circles and positively correlated gene products are in blue circles: (a) aspartic proteases; (b) peptidases; (c) oligopeptide transporter, OPT; (d) fungal oligopeptide transporter, FOT (classified as amino acid/auxin transporter; AAAP); (e) chitinases and β-N-acetylhexosaminidase, NAG; (f) acid phosphatases; (g) glucanases; (h) 2-oxoglutarate dehydrogenase, KGD; (i) 5-aminolevulinate synthase, ALAS; (j) aryl-alcohol oxidases / pyranose dehydrogenases-like, AAO/PDH; (k) alcohol oxidases, AOx; (l) glutamate dehydrogenase, GSH and glutamine synthase, GS; (m) ammonium transporter, AmT. Dashed lines indicate uncertainty in the secretion of alcohol oxidases (AOx) and its role in H_2O_2 production. Asterisks indicate relationships determined in *post hoc* analyses. Statistics of *a priori* selected genes are presented in Table 2.

Gene expression of chitinases and NAGs were positively correlated with MnP gene expression (Figure 10e; Table 2), while chitin deacetylase gene expression was not (Table 2). Given that previous studies have observed a positive relationship between chitinase gene expression and the mobilisation of nitrogen from ^{15}N-labelled chitin (Maillard *et al.*, 2023), my results could support that manganese peroxidases facilitate the access to fungal cell wall chitin and therefore an additional source of organic nitrogen. In addition, several different glucanases were also positively correlated with MnP in the *post hoc* analysis, including α-glucosidase and glucan 1,3-α-glucosidase from GH31, and glucan endo-1,3-α-glucosidase from GH71 (Figure 10f). Microbial cell wall degrading enzymes, including glucanases, which are widely encoded for by ectomycorrhizal fungi (Miyauchi *et al.*, 2020), could be important for increasing access to chitin that is linked with glucans. Further, in a study by Maillard *et al.* (2026), fungal necromass derived from older and more N starved mycelia had increasing 1,3-β-glucan branching and a higher proportion of 1,3-α-glucans. They found that "older" necromass decayed substantially slower, which in their experiment was not related to melanin content but rather other biochemical traits. If decomposition of fungal necromass is sometimes limited by cell wall components other than melanin, the positive correlation we observed may support that MnPs aid in "opening-up" fungal necromass for further decomposition by hydrolases. There was no correlation between β-glucosidase (GH3; Table 2), which releases sugars from cellulose and is essential for saprotrophism, and MnP gene expressions. Therefore, it is likely that ectomycorrhizal fungi do not act as facultative saprotrophs, as has been proposed previously (Talbot *et al.*, 2008). Both the hypothesis tests and the explorative analyses support the first hypothesis of **paper IV**, that oxidative decomposition by *Cortinarius* facilitates nitrogen mining (Lindahl & Tunlid, 2015).

Table 2. Linear regression of transcript abundance (square root transformed TPM) of *a priori* selected genes versus manganese peroxidase gene expression with plot as a random factor, sorted by correlation estimate. P-values were corrected for multiple testing with False Discovery Rate (FDR). P-values < 0.05 are in bold and < 0.1 are in italics. Abbreviations in Table 1.

gene	Estimate	Std.error	t-value	p-value
OPT (2.A.67) *	0.664	0.064	10.459	**<0.001**
Aspartic proteases *	0.410	0.05	8.124	**<0.001**
AAAP (2.A.18) *	0.220	0.046	4.765	**<0.001**
Chitinase*	0.061	0.014	4.511	**<0.001**
ACT	0.056	0.039	1.456	0.246
GMC AA3_3 – AOx*	0.050	0.024	2.092	*0.085*
ALAS *	0.032	0.009	3.735	**0.001**
GMC AA3_2 – AAO/PDH-like*	0.026	0.006	4.201	**<0.001**
Glucan 1,3-β-glucosidase *	0.019	0.014	1.403	0.253
Aminopeptidases *	0.011	0.014	0.796	0.562
CUE (i.e. GT48/KGD)	0.009	0.003	2.517	**0.037**
NAG *	0.009	0.004	2.037	*0.092*
GT48	0.001	0.011	0.125	0.943
AAT	0.001	0.009	0.154	0.943
YAT	0.001	0.053	0.016	0.987
CRO	-0.006	0.016	-0.405	0.794
β-glucosidase	-0.012	0.008	-1.486	0.246
Chitin deacetylase	-0.013	0.017	-0.74	0.562
KGD ǂ	-0.015	0.006	-2.368	**0.049**
LAT	-0.016	0.008	-2.175	*0.072*
GMC AA3_2 – GDH-like	-0.020	0.016	-1.257	0.287
Laccases	-0.022	0.012	-1.848	0.129

*within top 10% of rank Pearson's correlation – positive

ǂwithin top 10% of rank Pearson's correlation – negative

Ectomycorrhizal fungi are known to be able to mobilise both organic and inorganic P, yet most focus is centred around N acquisition. In the *post hoc* analysis, acid phosphatase and phosphodiesterase gene expressions were positively correlated with MnP gene expression, indicating that P, not only N, is mined from organic matter (Figure 10g). This would be consistent with the observations of Griffiths *et al.* (1990), where soils colonized by ectomycorrhizal mats of *Hysterangium*, a genus that contains species encoding for manganese peroxidases (Miyauchi *et al.*, 2020), had higher activity of acid phosphatases in comparison to adjacent uncolonized soils. In comparison to forests exposed to N fertilisation alone, Jörgensen *et al.* (2021) observed that combined N and P fertilisation resulted in a further increase in soil C stocks and a reduction in the activity of acid phosphatases (Jörgensen *et al.*, 2022). Although Jörgensen *et al.* (2022) did not observe any significant reduction in manganese peroxidase activity, these evidences could still partially support that mobilisation of P contributes to a reduction in C stocks under P limited conditions. While P is not considered as a limiting nutrient in most boreal forests, ectomycorrhizal decomposers may also play an important role in more nutrient-rich forests where P is potentially limiting (Mayer *et al.*, 2023).

One challenge is determining the appropriate (but also logistically feasible) scale to study the questions of interest. In the case of **paper IV**, the interest was variation in gene expression within a mycelium, i.e. the centimetre scale variation. Yet, even within the small dimensions of my individually sampled soil cores (1.5 cm diameter) there is almost certainly a mixture of *C. ominosus* hyphae with different activities. The most obvious being that there is a mixture of hyphae exchanging nutrients in the Hartig net and extraradical hyphae interacting with the soil. In laboratory conditions, the transcriptomic profile of hyphae changes during the formation of symbiosis, including the expression of CAZymes potentially associated with the modifications to plant cell walls (Veneault-Fourrey *et al.*, 2014). For example, the upregulation of oligopeptide transporters by *Pisolithus microcarpus* (Dauphin *et al.*, 2025) and *Lactarius deliciosus* (Tang *et al.*, 2021) in symbiotic tissue perhaps suggests that these transporters could also be involved in nutrient exchange with roots. Therefore, while it seems likely that the overwhelming proportion of transcripts originate from hyphae exploring the soil, observed patterns in expression may be confounded by gene expression related to other processes.

Together, the relationships of MnP transcript abundance with that of chitinases, proteases, peptidases, phosphatases, and glucanases signify that oxidative decomposition is a generally “explosive” process that liberates many different compounds for further degradation and assimilation (Figure 10). Likely, this release of nutrients also contributes to priming of other bacteria and fungi in the mycorrhizosphere. As proposed originally by Kirk and Farrel (1987), oxidative decomposition may directly lead to the extracellular "combustion" of some organic compounds all the way to CO_2, but some liberated smaller organic molecules are probably also metabolised by the associated microbiota. The question still remains open on the exact fate of released organic compounds that are not utilised by the ectomycorrhizal fungus.

Carbon-use-efficiency and H_2O_2 production

My second hypothesis in **paper IV** was that if oxidative decomposition is energetically demanding there would be a negative relationship between MnP gene expression and a gene-marker proxy for carbon-use-efficiency (i.e. the expression ratio of growth (GT48) to respiration (KGD) marker genes; Hasby *et al.*, 2021). However, the opposite was observed, and the positive relationship was driven by a negative correlation between the expression of MnP and KGD genes (Table 2; Figure 10h), and no shift in the expression of GT48 genes. KGD is one of the enzymes in the TCA cycle, which is the metabolic cycle that generates energy through respiration and the production of NADH. Therefore, by proxy, respiration was likely to be lower when MnP gene expression was high. My exploratory *post hoc* investigation revealed that expressions of all other TCA cycle genes were either not correlated or, likewise, negatively correlated (aconitase; EC 4.2.1.3) with MnP transcript abundance. In line with this, an *in vitro* litter decomposition study by Barbi *et al.* (2020) observed that *Gymnopus androsaceus* did not have a lower carbon-use-efficiency (estimated in a similar manner as in **paper IV**) when expression of peroxidase genes was higher. This partially supports my observation that oxidative decomposition is not especially energy demanding relative to other processes. However, these results conflict with a previous *in vitro* study on *P. chrysosporium* where the authors observed an up-regulation of KGD and the preceding TCA cycle genes upon exposure to vanillin (a degradation product of lignin; Shimizu *et al.*, 2005). They proposed that ligninolytic activity induces a metabolic shift from the glyoxylate pathway to the TCA cycle to increase production of NAD(P)H and replenish succinyl-CoA, which is needed as a precursor for the heme synthesis pathway. I observed that ALAS (an enzyme in the heme synthesis pathway) was positively correlated with MnP transcript abundance (Table 2; Figure 10i), indicating at least consistency in the importance of up-regulation of heme synthesis for peroxidase hemecofactors; however, no direct coupling with increased gene expression in the TCA cycle was observed.

The assumption that H_2O_2 production is the main contributor to the high energy cost of ectomycorrhizal decomposers, is based on that H_2O_2 must be constantly replenished and that there must be a sufficient supply of electron-donor substrates for H_2O_2 generating enzymes (Chakrawal *et al.*, 2024). While there is indirect evidence to support that ectomycorrhizal decomposers are particularly energy demanding, this is mostly because they are generally more sensitive to reductions in C allocation from their hosts compared to non-decomposers (van der Linde *et al.*, 2018; Wasyliw & Karst, 2020). Meanwhile, there is not substantial physiological evidence to support this proposed energy cost. Relatively little experimental work has been done surrounding H_2O_2 production, and many knowledge gaps exist, not only for ectomycorrhizal decomposers but for saprotrophic fungi as well (Janusz *et al.*, 2017). The sources of substrates for glyoxal oxidase (e.g. glycolaldehyde, glyoxal, methylglyoxal) and GMC oxidoreductases (e.g. aryl-alcohol, glucose, cellobiose, methanol) have been proposed to be products of ligninolytic activity (e.g. methanol derived from demethylation of lignin) that are cycled to perpetuate the action of peroxidases (Kersten & Kirk, 1987; Daniel *et al.*, 2007). Therefore, it may be incorrect to assume that, if either of these classes of enzymes are used for H_2O_2 production, the process would be energy demanding after it has been initiated. Further, this raises the question that if substrates for glyoxal oxidase and GMC oxidoreductases are derived from ligninolysis, then from where do the substrates originate if an ectomycorrhizal fungus is mining nutrients from organic compounds other than lignin (e.g. tannins and/or melanins)?

As proposed in my third hypothesis in **paper IV,** the expression of AAO/PDH-like (AA3_2) genes was positively correlated with MnP gene expression (Figure 10j), suggesting that the GMC oxidoreductase pathway could be a potential H_2O_2 production pathway for *C. ominosus* (in line with that glyoxal oxidases are absent in the *C. ominosus* genome; Figure S1). Glyoxal oxidase was suggested to be a H_2O_2 production pathway for *Paxillus involutus,* a Fenton-chemistry decomposer, (Rineau *et al.*, 2013), but it seems likely that ectomycorrhizal decomposers from different lineages (i.e. Agaricales versus Boletales) have different mechanisms. In addition to AAO/PDH-like genes, the gene expression of alcohol oxidases (AOx), which are also from the GMC oxidoreductase family, tended to be positively correlated with MnP gene expression (Figure 10k). However, AOx, unlike many other GMC oxidoreductases, is generally not excreted (Mattila *et al.*, 2022) and for the most abundant AOx transcript in **paper IV** there was only weak *in silico* evidence that it may have extracellular activity. Gene characterisation and immunofluorescence staining of *Gloeophyllum trabeum* AOx by Daniel *et al.* (2007) indicated that AOx was excreted despite lacking a N-terminus signal peptide, rather they implied that the unique C-terminus may be related to its localisation. The transcribed AOx genes from *C. ominosus* do not appear to share homology with the characterised *G. trabeum* AOx at the C-terminus, however *C. ominosus* AOx also lacks a peroxisome targeting signal, which could indicate that it is localised elsewhere (e.g. excreted). Therefore, AOx may be a manner for H_2O_2 production for ectomycorrhizal decomposers, but AAO/PDH-like genes are more probable candidates. Nonetheless, further laboratory experiments, including proteomics, would be required to support these results.

Within the “absorber to miner” trait spectrum proposed by Jörgensen *et al.* (2025), low *apparent* carbon-use-efficiency is considered a trait of fungi towards the “miner” side of the spectrum. *Apparent* carbon-use-efficiency in this sense represent the accumulation of hyphal biomass per acquired C and is an integration of growth and hyphal turnover/mortality over time, amongst different species (Manzoni *et al.*, 2018). My results would suggest that N mobilisation from recalcitrant organic matter using MnPs, does not strongly trade-off with C use efficiency. *Cortinarius* and other ectomycorrhizal decomposers, may still have low *apparent* carbon-use-efficiency but it may be a result of other components of their life strategy, e.g. high mortality, rather than actual metabolic carbon-use-efficiency in the stricter sense.

A post hoc speculation

I observed that MnP gene expression did not seem to be energetically expensive, rather the transcript abundance of two genes coding for enzymes in the TCA cycle were negatively correlated with MnP transcript abundance. This raises the question of what processes were energy/carbon demanding in the MnP "coldspots"? In the *post hoc* analysis of **paper IV**, glutamate dehydrogenase (EC 14.1.4) and glutamine synthase (EC 6.3.1.2) gene expression was negatively correlated with MnP gene expression (Figure 10l). These enzymes are associated with the assimilation of ammonia into glutamate and glutamine – the primary sink for N in fungal tissues (Chalot & Brun, 1998) – as in the below reactions:

$$\alpha\text{-ketoglutarate} + NH3 \leftrightarrow \text{glutamate}$$
$$\text{glutamate} + NH3 \rightarrow \text{glutamine}$$

This could suggest that *C. ominosus* assimilates inorganic N when MnP gene expression is low, which is reinforced by a negative correlation with ammonium transporter genes (Figure 10m), of which the most highly expressed has sequence similarity with a high-affinity ammonium transporter characterised in *Hebeloma cylindrosporum* (Javelle *et al.*, 2003). This does not imply that ammonium is abundant, as ammonium transporters are thought to be up-regulated during low ammonium availability (Javelle *et al.*, 2003), and ammonium is usually very scarce in boreal forest soils. I speculate, in line with the proposed hypothesis of Wallander (1995), that inorganic N assimilation is linked to a high C demand, as the fungus must allocate more C skeletons for amino acid synthesis (Bidartondo *et al.*, 2001). In contrast, during decomposition by MnPs, amino acids derived from organic N sources can be more readily recycled and assimilated without a direct C cost. Glutamine--fructose-6-phosphate transaminase (EC 2.6.1.16), a rate limiting enzyme in the *de novo* synthesis of N-acetylglucosamine (a precursor of chitin in the fungal cell wall), was also negatively correlated with MnP transcript abundance, further connecting the proposed assimilation of N to the metabolism of aminosugars. The expression patterns of glutamine--fructose-6-phosphate transaminase, glutamate dehydrogenase, and glutamine synthase are consistent with those of *P. involutus* grown on organic matter extracts with readily available C (Rineau *et al.*, 2013), indicating some similarity in regulation of N assimilation pathways.

While this idea of increased C demand for inorganic N assimilation is appealing, it is not possible to infer the actual outcomes of these enzyme activities. For example, glutamate dehydrogenase is a bidirectional enzyme and could be either producing or consuming α-ketoglutarate. Further, respiration (or just KGD expression) only provides one of the two parts of C use considered in C efficiency: respiration and growth. There is no way to evaluate if C demand actually fluctuates within a mycelium. It could be that C demand is evenly distributed across the mycelium while C use is different, i.e. in MnP "hotspots" the C is allocated to production of enzymes and in "coldspots" it is allocated to assimilation of inorganic N. These hypotheses are based on loosely threaded pieces of evidence, and I have no (or little) information about the environmental conditions, the realised function of these expressed transcripts, or post-translational control. However, the aim of the exploratory analysis was to develop new testable hypotheses for future experiments, and I see that this may be an interesting path forward.

I propose that within the "absorber" to "miner" spectrum of species traits, as suggested by Jörgensen *et al.* (2025), we should also consider spatial trait variation within individual mycelia. I visualize that within a single mycelium there are patches of organic nutrient mining coinciding with high MnP gene expression and in other patches there is high inorganic N absorption (Figure 11). The results from **paper I**, regarding the lack of spatial autocorrelation on the 0.1 m scale, and the dPCR data on MnP gene expression across all samples from **paper IV**, provide additional support that "hotspots" of MnP activity occur on a small-scale (centimetre) within a single mycelium. Potentially, in these "hotspots", organic N is primarily taken up as peptide and chitin oligomers. In this phase, the main C demand may be the production of enzymes and exudates required for oxidation of recalcitrant organic matter and subsequent depolymerisation of organic macromolecules. Other soil organisms may be primed by this mobilisation and likely continue to depolymerise and mineralise nutrients. Successively, as the mycelium expands further, this patch may become more inorganic nutrient containing, and then eventually the fungal hyphae senesce and N is recycled.

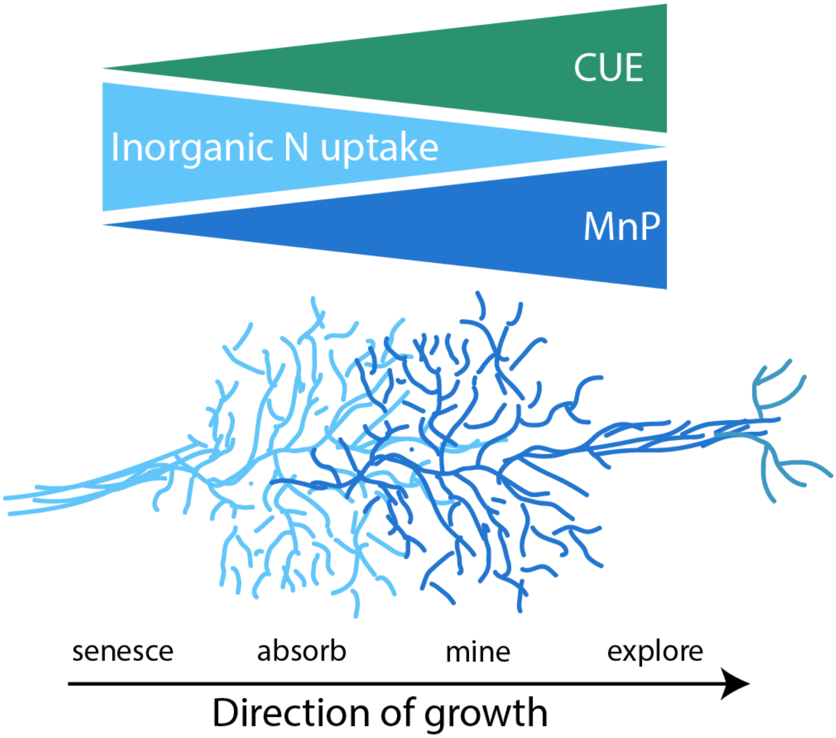


Figure 11. Postulated dynamic of mycelium scale carbon-use-efficiency in relation to manganese peroxidase (MnP) gene expression and inorganic nitrogen (N) uptake. Within a single mycelium, as growth progresses, there is a positive relationship between MnP transcript abundance and carbon-use-efficiency (CUE) and a negative relationship between inorganic N assimilation and CUE.

9. Conclusions and moving forward

This thesis provided several results that were, sometimes, contrasting to my previous expectations:

- I hypothesised that ectomycorrhizal decomposers would have their niche in older and more nutrient-poor forest soils and be more sensitive to history of clear-cutting than other ectomycorrhizal fungi. However, the niches of ectomycorrhizal decomposers were broader than expected, and there may be sufficient niche diversification to promote the perpetuation of N cycling across forest of varying management. This result implies that forestry may not be as much of threat for ectomycorrhizal decomposers, as previously proposed (e.g. in Kyaschenko *et al.* 2017b), especially in the case of more frequent species (perhaps generalists).
- The MnP gene encoded for by *Cortinarius aurae* is capable of Mn^{2+} oxidation and modification of a lignin constituent, but the activity was not strictly manganese-dependant. Available manganese seems to be important for the regulation of C stocks (Stendahl *et al.*, 2017), suggesting that manganese-dependant activity is likely still important for soil organic matter turnover. However, the potential for manganese-independent activity may be advantageous for soil decomposers if manganese availability fluctuates.
- The expression of proteolytic and chitinolytic genes was tightly linked to the expression of MnP genes by *Cortinarius omniosus*, suggesting that oxidative decomposition may be important in mining for N from recalcitrant soil organic matter (Lindahl & Tunlid, 2015). I also observed correlations with acid phosphatases and glucanases, suggesting that MnPs may also increase access to chitin in fungal necromass and organic P.
- Oxidative decomposition by MnPs may not be as energy demanding as previously proposed (Chakrawal *et al.*, 2024), at least not relative to other mycelial activities. This must be reconciled with the observations of declining MnP activity with increasing exogenous N.

Given the difficulties in culturing and experimentally manipulating most ectomycorrhizal fungi, I used many different approaches to investigate the ecophysiology of ectomycorrhizal decomposers. I combined studies on the ecosystem and mycelial scales using a broad “toolbox” of methods. This approach can hopefully aid in expanding typical “model” species from species grown *in vitro* to ecologically relevant species *in situ*. With this in mind, laboratory experiments that evaluate relationships between genomic capacity, gene expression and regulation, enzyme activity and biochemical transformation are very important, as they provide supporting background information required for the interpretation of ecological data. I felt that some of the largest uncertainties in this thesis (but mostly in **paper IV**) were related to the potential disconnect between predicted gene functions and realised enzyme activity. **Paper II** provided an example of trying to narrow this gap for at least one gene, and here I succeeded in confirming the proposed oxidative activity of a recombinant MnP from an ectomycorrhizal fungus. This methodology is promising but requires further development, including testing different MnP genes and other expression systems. With a higher performing expression system, it may be possible to investigate the structural changes to organic matter exposed to these recombinant enzymes and/or assess whether they can liberate N from tannin-protein complexes.

The data gathered in this thesis opens the possibility for many other analyses. The new genomes are still largely un-investigated, and I think it will be fruitful to continue exploring the mechanism of oxidative decomposition by *Cortinarius* species. Further, expanding on phylogenomics and relationships within the genus in terms of genetic capacity and ecological niches could be particularly interesting. Likewise, in **paper IV**, the transcripts of only one species were analysed, but with total mRNA sequencing there is the opportunity to investigate other organisms (other fungi, bacteria, or tree roots) and their interaction with *Cortinarius* mycelia. For example, whether there is priming of bacteria and other fungi in MnP “hotspots” could be addressed or whether there is variation in the expression of plant invertase genes, potentially indicating fluctuations in sugar transport to ectomycorrhizal fungi.

References

Abarenkov K, Nilsson RH, Larsson K-H, Taylor AFS, May TW, Frøslev TG, Pawlowska J, Lindahl B, Põldmaa K, Truong C, *et al.* 2024. The UNITE database for molecular identification and taxonomic communication of fungi and other eukaryotes: sequences, taxa and classifications reconsidered. *Nucleic Acids Research* **52**: D791–D797.

Abuzinadah RA, Finlay RD, Read DJ. **1986**. The role of proteins in the nitrogen nutrition of ectomycorrhizal plants: II. Utilization of protein by mycorrhizal plants of *Pinus contorta*. *New Phytologist* **103**: 495–506.

Abuzinadah RA, Read DJ. **1986**. The role of proteins in the nitrogen nutrition of ectomycorrhizal plants. I. Utilization of peptides and proteins by ectomycorrhizal fungi. *New Phytologist* **103**: 481–493.

Abuzinadah RA, Read DJ. **1989**. The role of proteins in the nitrogen nutrition of ectomycorrhizal plants. IV. The utilization of peptides by birch (*Betula pendula* l.) infected with different mycorrhizal fungi. *The New Phytologist* **112**: 55–60.

Adamczyk B, Adamczyk S, Smolander A, Kitunen V. **2011**. Tannic acid and Norway spruce condensed tannins can precipitate various organic nitrogen compounds. *Soil Biology and Biochemistry* **43**: 628–637.

Adamczyk B, Karonen M, Adamczyk S, Engström MT, Laakso T, Saranpää P, Kitunen V, Smolander A, Simon J. **2017**. Tannins can slow-down but also speed-up soil enzymatic activity in boreal forest. *Soil Biology and Biochemistry* **107**: 60–67.

Adamczyk B, Sietiö OM, Biasi C, Heinonsalo J. **2019**. Interaction between tannins and fungal necromass stabilizes fungal residues in boreal forest soils. *New Phytologist* **223**: 16–21.

Adiconis X, Borges-Rivera D, Satija R, DeLuca DS, Busby MA, Berlin AM, Sivachenko A, Thompson DA, Wysoker A, Fennell T, *et al.* 2013. Comparative analysis of RNA sequencing methods for degraded or low-input samples. *Nature Methods* **10**: 623–629.

Agerer R. **2001**. Exploration types of ectomycorrhizae: A proposal to classify ectomycorrhizal mycelial systems according to their patterns of differentiation and putative ecological importance. *Mycorrhiza*: 107–114.

Allison SD. **2006**. Soil minerals and humic acids alter enzyme stability: implications for ecosystem processes. *Biogeochemistry* **81**: 361–373.

Allison SD, Martiny JBH. **2008**. Resistance, resilience, and redundancy in microbial communities. *Proceedings of the National Academy of Sciences of the United States of America* **105**: 11512–11519.

Arantes V, Goodell B. **2014**. Current understanding of brown-rot fungal biodegradation mechanisms: A review. In: ACS Symposium Series.

Deterioration and Protection of Sustainable Biomaterials. American Chemical Society, 3–21.

Argiroff WA, Zak DR, Pellitier PT, Upchurch RA, Belke JP. **2022**. Decay by ectomycorrhizal fungi couples soil organic matter to nitrogen availability. *Ecology Letters* **25**: 391–404.

Argiroff WA, Zak DR, Upchurch RA, Pellitier PT, Belke JP. **2023**. Fungal community composition and genetic potential regulate fine root decay in northern temperate forests. *Molecular Ecology* **32**: 2005-2021.

Arnstadt T, Hoppe B, Kahl T, Kellner H, Krüger D, Bässler C, Bauhus J, Hofrichter M. **2016**. Patterns of laccase and peroxidases in coarse woody debris of *Fagus sylvatica*, *Picea abies* and *Pinus sylvestris* and their relation to different wood parameters. *European Journal of Forest Research* **135**: 109–124.

Auer L, Buée M, Fauchery L, Lombard V, Barry KW, Clum A, Copeland A, Daum C, Foster B, LaButti K, *et al.* 2024. Metatranscriptomics sheds light on the links between the functional traits of fungal guilds and ecological processes in forest soil ecosystems. *New Phytologist* **242**: 1676–1690.

Averill C, Turner BL, Finzi AC. **2014**. Mycorrhiza-mediated competition between plants and decomposers drives soil carbon storage. *Nature* **505**: 543–545.

Baldrian P. 2006. Fungal laccases – occurrence and properties. *FEMS Microbiology Reviews* **30**: 215–242.

Barker JS, Simard SW, Durall DM, Jones MD. 2013. Ectomycorrhizal fungal community assembly on regenerating Douglas-fir after wildfire and clearcut harvesting. *Oecologia* **172**: 1179–1189.

Barbi F, Menzel U, Simone D, Niskanen T, Lindahl BD. In review. Ectomycorrhizal Cortinarius species dominate fungal Mn-peroxidase gene expression in the soil of a boreal forest. *New phytologist.*

Barbi F, Kohler A, Barry K, Baskaran P, Daum C, Fauchery L, Ihrmark K, Kuo A, LaButti K, Lipzen A, *et al.* 2020. Fungal ecological strategies reflected in gene transcription - a case study of two litter decomposers. *Environmental Microbiology* **22**: 1089–1103.

Baskaran P, Hyvönen R, Berglund SL, Clemmensen KE, Ågren GI, Lindahl BD, Manzoni S. **2017**. Modelling the influence of ectomycorrhizal decomposition on plant nutrition and soil carbon sequestration in boreal forest ecosystems. *New Phytologist* **213**: 1452–1465.

Bassett KR, Hupperts SF, Jämtgård S, Östlund L, Fridman J, Perakis SS, Gundale MJ. **2026**. Rising atmospheric CO_2 reduces nitrogen availability in boreal forests. *Nature* **650**: 629–635.

Beiler KJ, Durall DM, Simard SW, Maxwell SA, Kretzer AM. **2010**. Architecture of the wood-wide web: *Rhizopogon* spp. genets link multiple Douglas-fir cohorts. *New Phytologist* **185**: 543–553.

Beimforde C, Schäfer N, Dörfelt H, Nascimbene PC, Singh H, Heinrichs J, Reitner J, Rana RS, Schmidt AR. **2011**. Ectomycorrhizas from a Lower Eocene angiosperm forest. *New Phytologist* **192**: 988–996.

Bending GD, Read DJ. **1996**. Nitrogen mobilization from protein-polyphenol complex by ericoid and ectomycorrhizal fungi. *Soil Biology and Biochemistry* **28**: 1603–1612.

Berg B, Steffen KT, McClaugherty C. **2007**. Litter decomposition rate is dependent on litter Mn concentrations. *Biogeochemistry* **82**: 29–39.

Bidartondo MI, Ek H, Wallander H, Söderström B. **2001**. Do nutrient additions alter carbon sink strength of ectomycorrhizal fungi? *New Phytologist* **151**: 543–550.

Bienert GP, Chaumont F. **2014**. Aquaporin-facilitated transmembrane diffusion of hydrogen peroxide. *Biochimica et Biophysica Acta (BBA) - General Subjects* **1840**: 1596–1604.

Bissaro B, Kodama S, Nishiuchi T, Díaz-Rovira AM, Hage H, Ribeaucourt D, Haon M, Grisel S, Simaan AJ, Beisson F, *et al.* **2022**. Tandem metalloenzymes gate plant cell entry by pathogenic fungi. *Science Advances* **8**: eade9982.

Blankinship JC, Berhe AA, Crow SE, Druhan JL, Heckman KA, Keiluweit M, Lawrence CR, Marín-Spiotta E, Plante AF, Rasmussen C, *et al.* **2018**. Improving understanding of soil organic matter dynamics by triangulating theories, measurements, and models. *Biogeochemistry* **140**: 1–13.

Boddy L. **1999**. Saprotrophic cord-forming fungi: meeting the challenge of heterogeneous environments. *Mycologia* **91**: 13–32.

Bödeker ITM, Clemmensen KE, de Boer W, Martin F, Olson Å, Lindahl BD. **2014**. Ectomycorrhizal Cortinarius species participate in enzymatic oxidation of humus in northern forest ecosystems. *New Phytologist* **203**: 245–256.

Bödeker ITM, Lindahl BD, Olson Å, Clemmensen KE. **2016**. Mycorrhizal and saprotrophic fungal guilds compete for the same organic substrates but affect decomposition differently. *Functional Ecology* **30**: 1967–1978.

Bödeker ITM, Nygren CMR, Taylor AFS, Olson Å, Lindahl BD. **2009**. ClassII peroxidase-encoding genes are present in a phylogenetically wide range of ectomycorrhizal fungi. *ISME Journal* **3**: 1387–1395.

Bonan GB, Shugart HH. 1989. Environmental factors and ecological processes in boreal forests. *Ann. Rev. Ecol. Syst.* **20**: 1–28.

Brundrett MC, Tedersoo L. **2018**. Evolutionary history of mycorrhizal symbioses and global host plant diversity. *New Phytologist* **220**: 1108–1115.

Bruns TD. **1995**. Thoughts on the processes that maintain local species diversity of ectomycorrhizal fungi. *Plant and Soil* **170**: 63–73.

Bunn RA, Corrêa A, Joshi J, Kaiser C, Lekberg Y, Prescott CE, Sala A, Karst J. **2024**. What determines transfer of carbon from plants to mycorrhizal fungi? *New Phytologist* **244**: 1199–1215.

Butler MJ, Day AW. **1998**. Fungal melanins: a review. *Canadian Journal of Microbiology* **44**: 1115–1136.

Cairney JWG. **1992**. Translocation of solutes in ectomycorrhizal and saprotrophic rhizomorphs. *Mycological Research* **96**: 135–141.

Cairney JWG. **2011**. Ectomycorrhizal fungi: the symbiotic route to the root for phosphorus in forest soils. *Plant and Soil* **344**: 51–71.

Cairney JWG, Burke RM. **1998**. Do ecto- and ericoid mycorrhizal fungi produce peroxidase activity? *Mycorrhiza* **8**: 61–65.

Castaño C, Berlin A, Brandström Durling M, Ihrmark K, Lindahl BD, Stenlid J, Clemmensen KE, Olson Å. **2020**. Optimized metabarcoding with Pacific biosciences enables semi-quantitative analysis of fungal communities. *New Phytologist* **228**: 1149–1158.

del Cerro C, Erickson E, Dong T, Wong AR, Eder EK, Purvine SO, Mitchell HD, Weitz KK, Markillie LM, Burnet MC, *et al.* **2021**. Intracellular pathways for lignin catabolism in white-rot fungi. *Proceedings of the National Academy of Sciences* **118**: e2017381118.

Chakrawal A, Lindahl BD, Manzoni S. **2024**. Modelling optimal ligninolytic activity during plant litter decomposition. *New Phytologist* **243**: 866–880.

Chalot M, Brun A. **1998**. Physiology of organic nitrogen acquisition by ectomycorrhizal fungi and ectomycorrhizas. *FEMS Microbiology Reviews* **22**: 21–44.

Chambers SM, Burke RM, Brooks PR, Cairney JWG. **1999**. Molecular and biochemical evidence for manganese-dependent peroxidase activity in *Tylospora fibrillosa*. *Mycological Research* **103**: 1098–1102.

Chen J, Elsgaard L, Groenigen KJ, Olesen JE, Liang Z, Jiang Y, Lærke PE, Zhang Y, Luo Y, Hungate BA, *et al.* **2020**. Soil carbon loss with warming: New evidence from carbon-degrading enzymes. *Global Change Biology* **26**: 1944–1952.

Clemmensen KE, Bahr A, Ovaskainen O, Dahlberg A, Ekblad A, Wallander H, Stenlid J, Finlay RD, Wardle DA, Lindahl BD. **2013**. roots and associated fungi drive long-term carbon sequestration in boreal forest. *Science* **339**: 1615–1618.

Clemmensen KE, Finlay RD, Dahlberg A, Stenlid J, Wardle DA, Lindahl BD. **2015**. Carbon sequestration is related to mycorrhizal fungal community shifts during long-term succession in boreal forests. *New Phytologist* **205**: 1525–1536.

Cornwell WK, Cornelissen JHC, Amatangelo K, Dorrepaal E, Eviner VT, Godoy O, Hobbie SE, Hoorens B, Kurokawa H, Pérez-Harguindeguy N, *et al.* **2008**. Plant species traits are the predominant control on litter decomposition rates within biomes worldwide. *Ecology Letters* **11**: 1065–1071.

Cotrufo MF, Ranalli MG, Haddix ML, Six J, Lugato E. **2019**. Soil carbon storage informed by particulate and mineral-associated organic matter. *Nature Geoscience* **12**: 989–994.

Cotrufo MF, Wallenstein MD, Boot CM, Denef K, Paul E. **2013**. The Microbial Efficiency-Matrix Stabilization (MEMS) framework integrates plant litter decomposition with soil organic matter stabilization: do labile plant inputs form stable soil organic matter? *Global Change Biology* **19**: 988–995.

Courty P-E, Bréda N, Garbaye J. **2007**. Relation between oak tree phenology and the secretion of organic matter degrading enzymes by *Lactarius quietus* ectomycorrhizas before and during bud break. *Soil Biology and Biochemistry* **39**: 1655–1663.

Cregg JM, Madden KR, Barringer KJ, Thill GP, Stillman CA. **1989**. Functional characterization of the two alcohol oxidase genes from the yeast *Pichia pastoris. Molecular and Cellular Biology* **9**: 1316–1323.

Dahlberg A. **2001**. Community ecology of ectomycorrhizal fungi: an advancing interdisciplinary field. *New Phytologist* **150**: 555–562.

Damon C, Vallon L, Zimmermann S, Haider MZ, Galeote V, Dequin S, Luis P, Fraissinet-Tachet L, Marmeisse R. **2011**. A novel fungal family of oligopeptide transporters identified by functional metatranscriptomics of soil eukaryotes. *The ISME Journal* **5**: 1871–1880.

Daniel G, Volc J, Filonova L, Plíhal O, Kubátová E, Halada P. **2007**. Characteristics of *Gloeophyllum trabeum* Alcohol Oxidase, an extracellular source of H_2O_2 in brown rot decay of wood. *Applied and Environmental Microbiology* **73**: 6241–6253.

Dauphin B, de Freitas Pereira M, Croll D, Cardoso Anastácio T, Fauchery L, Guinet F, Dutra Costa M, Martin F, Peter M, Kohler A. **2025.** Genetic variation among progeny shapes symbiosis in a basidiomycete with poplar. *New Phytologist* **258**: 157-177.

Defrenne CE, Philpott TJ, Guichon SHA, Roach WJ, Pickles BJ, Simard SW. **2019**. Shifts in ectomycorrhizal fungal communities and exploration types relate to the environment and fine-root traits across interior douglas-fir forests of western Canada. *Frontiers in Plant Science* **10**: 643.

Deluca TH, Boisvenue C. **2012**. Boreal forest soil carbon: distribution, function and modelling. *Forestry: An International Journal of Forest Research* **85**: 161–184.

Drake JE, Gallet-Budynek A, Hofmockel KS, Bernhardt ES, Billings SA, Jackson RB, Johnsen KS, Lichter J, McCarthy HR, McCormack ML, *et al.* **2011**. Increases in the flux of carbon belowground stimulate nitrogen uptake and sustain the long-term enhancement of forest productivity under elevated CO2. *Ecology Letters* **14**: 349–357.

Drula E, Garron M-L, Dogan S, Lombard V, Henrissat B, Terrapon N. **2022**. The carbohydrate-active enzyme database: functions and literature. *Nucleic Acids Research* **50**: D571–D577.

Durall DM, Todd AW, Trappe JM. **1994**. Decomposition of ^{14}C-labelled substrates by ectomycorrhizal fungi in association with Douglas fir. *New Phytologist* **127**: 725–729.

Dynarski KA, Houlton BZ. **2020**. Isotopic constraints on plant nitrogen acquisition strategies during ecosystem retrogression. *Oecologia* **192**: 603–614.

Eisenman HC, Casadevall A. **2012**. Synthesis and assembly of fungal melanin. *Applied Microbiology and Biotechnology* **93**: 931–940.

Fanin N, Clemmensen KE, Lindahl BD, Farrell M, Nilsson M-C, Gundale MJ, Kardol P, Wardle DA. **2022**. Ericoid shrubs shape fungal communities and suppress organic matter decomposition in boreal forests. *New Phytologist* **236**: 684–697.

Fernandez CW, Kennedy PG. **2016**. Revisiting the 'Gadgil effect': do interguild fungal interactions control carbon cycling in forest soils? *Source: The New Phytologist* **209**: 1382–1394.

Fernandez CW, Kennedy PG. **2018**. Melanization of mycorrhizal fungal necromass structures microbial decomposer communities. *Journal of Ecology* **106**: 468–479.

Fernandez CW, Koide RT. **2012**. The role of chitin in the decomposition of ectomycorrhizal fungal litter. *Ecology* **93**: 24–28.

Fernandez CW, Koide RT. **2014**. Initial melanin and nitrogen concentrations control the decomposition of ectomycorrhizal fungal litter. *Soil Biology and Biochemistry* **77**: 150–157.

Fernandez CW, Langley JA, Chapman S, McCormack ML, Koide RT. **2016**. The decomposition of ectomycorrhizal fungal necromass. *Soil Biology and Biochemistry* **93**: 38–49.

Fernández-Fueyo E, Ruiz-Dueñas FJ, Martínez MJ, Romero A, Hammel KE, Medrano FJ, Martínez AT. **2014**. Ligninolytic peroxidase genes in the oyster mushroom genome: heterologous expression, molecular structure, catalytic and stability properties, and lignin-degrading ability. *Biotechnology for Biofuels* **7**: 2.

Fierer N, Strickland MS, Liptzin D, Bradford MA, Cleveland CC. 2009. Global patterns in belowground communities. *Ecology Letters* **12**: 1238–1249.

Finlay RD, Frostegard A, Sonnerfeldt A-M. **1992**. Utilization of organic and inorganic nitrogen sources by ectomycorrhizal fungi in pure culture and in symbiosis with *Pinus contorta* Dougl. ex Loud. *New Phytologist* **120**: 105–115.

Finlay RD, Read DJ. **1986a**. The structure and function of the vegetative mycelium of ectomycorrhizal plants. I. Translocation of ^{14}C-labelled carbon between plants interconnected by a common mycelium. *New Phytologist* **103**: 143–156.

Finlay RD, Read DJ. **1986b**. The structure and function of the vegetative mycelium of ectomycorrhizal plants. II. The uptake and distribution of phosphorus by mycelial strands interconnecting host plants. *New Phytologist* **103**: 157–165.

Floch C, Alarcon-Gutiérrez E, Criquet S. **2007**. ABTS assay of phenol oxidase activity in soil. *Journal of Microbiological Methods* **71**: 319–324.

Floudas D. **2021**. Chapter Two - Evolution of lignin decomposition systems in fungi. In: Morel-Rouhier M, Sormani R, eds. Wood degradation and ligninolytic fungi. Advances in Botanical Research. Academic Press, 37–76.

Floudas D, Bentzer J, Ahrén D, Johansson T, Persson P, Tunlid A. **2020**. Uncovering the hidden diversity of litter-decomposition mechanisms in mushroom-forming fungi. *The ISME Journal* **14**: 2046–2059.

Floudas D, Binder M, Riley R, Barry K, Blanchette RA, Henrissat B, Martínez AT, Otillar R, Spatafora JW, Yadav JS, *et al.* **2012**. The paleozoic origin of enzymatic lignin decomposition reconstructed from 31 fungal genomes. *Science* **336**: 1715–1719.

Floudas D, Gentile L, Andersson E, Kanellopoulos SG, Tunlid A, Persson P, Olsson U. **2022**. X-ray scattering reveals two mechanisms of cellulose microfibril degradation by filamentous fungi. *Environmental Microbiology* **88**: e00995-22.

Frank B. **2005**. On the nutritional dependence of certain trees on root symbiosis with belowground fungi (an English translation of A.B. Frank's classic paper of 1885). *Mycorrhiza* **15**: 267–275.

Frey SD. **2019**. Mycorrhizal fungi as mediators of soil organic matter dynamics. *Annual Review of Ecology, Evolution, and Systematics* **50**: 237–259.

Fricker MD, Heaton LLM, Jones NS, Boddy L. **2017**. The mycelium as a network. *Microbiology Spectrum* **5**.

Gadgil RL, Gadgil PD. **1975**. Suppression of litter decomposition by mycorrhizal roots of *Pinus radiata*. *New Zealand Journal of Forestry Science* **5**.

German DP, Weintraub MN, Grandy AS, Lauber CL, Rinkes ZL, Allison SD. **2011**. Optimization of hydrolytic and oxidative enzyme methods for ecosystem studies. *Soil Biology and Biochemistry* **43**: 1387–1397.

Glenn JK, Gold MH. **1985**. Purification and characterization of an extracellular mn(ll)-dependent peroxidase from the lignin-degrading basidiomycete, *Phanerochaete chrysosporium*. *Archives of Biochemistry and Biophysics* **242**: 329–341.

Gow NAR, Latge J-P, Munro CA. **2017**. The fungal cell wall: structure, biosynthesis, and function. *Microbiology Spectrum* **5**: 5.3.01.

Griffiths RP, Caldwell BA, Cromack Jr. K, Morita RY. **1990**. Douglas-fir forest soils colonized by ectomycorrhizal mats. I. Seasonal variation in nitrogen chemistry and nitrogen cycle transformation rates. *Canadian Journal of Forest Research* **20**: 211–218.

Grigoriev IV, Nikitin R, Haridas S, Kuo A, Ohm R, Otillar R, Riley R, Salamov A, Zhao X, Korzeniewski F, *et al.* **2014**. MycoCosm portal: gearing up for 1000 fungal genomes. *Nucleic Acids Research* **42**: D699–D704.

Gu L, Lajoie C, Kelly C. **2003**. Expression of a *Phanerochaete chrysosporium* manganese peroxidase gene in the yeast *Pichia pastoris*. *Biotechnology Progress* **19**: 1403–1409.

Hagenbo A, Hadden D, Clemmensen KE, Grelle A, Manzoni S, Mölder M, Ekblad A, Fransson P. **2019**. Carbon use efficiency of mycorrhizal fungal mycelium increases during the growing season but decreases with forest age across a *Pinus sylvestris* chronosequence. *Journal of Ecology* **107**: 2808–2822.

Haon M, Grisel S, Navarro D, Gruet A, Berrin J-G, Bignon C. **2015**. Recombinant protein production facility for fungal biomass-degrading enzymes using the yeast *Pichia pastoris*. *Frontiers in Microbiology* **6**: 1002.

Hasby FA, Barbi F, Manzoni S, Lindahl BD. **2021**. Transcriptomic markers of fungal growth, respiration and carbon-use efficiency. *FEMS Microbiology Letters* **368**: fnab100.

Hay TN, Phillips LA, Nicholson BA, Jones MD. **2015**. Ectomycorrhizal community structure and function in interior spruce forests of British Columbia under long term fertilization. *Forest Ecology and Management* **350**: 87–95.

Heimann M, Reichstein M. **2008**. Terrestrial ecosystem carbon dynamics and climate feedbacks. *Nature* **451**: 289–292.

Heitner C, Dimmel D, Schmidt J. **2010**. *Lignin and Lignans: Advances in Chemistry*. Baton Rouge, USA: Taylor & Francis Group.

Hibbett DS, Gilbert L-B, Donoghue MJ. **2000**. Evolutionary instability of ectomycorrhizal symbioses in basidiomycetes. *Nature* **407**: 506–508.

Hildén K, Mäkelä MR, Steffen KT, Hofrichter M, Hatakka A, Archer DB, Lundell TK. **2014**. Biochemical and molecular characterization of an atypical manganese peroxidase of the litter-decomposing fungus *Agrocybe praecox*. *Fungal Genetics and Biology* **72**: 131–136.

Hobbie JE, Hobbie EA. **2006**. N15 in symbiotic fungi and plants estimates nitrogen and carbon flux rates in Arctic Tundra. *Ecology* **87**: 816–822.

Hobbie EA, Ouimette AP, Schuur EAG, Kierstead D, Trappe JM, Bendiksen K, Ohenoja E. **2013**. Radiocarbon evidence for the mining of organic nitrogen from soil by mycorrhizal fungi. *Biogeochemistry* **114**: 381–389.

Hoeksema JD, Kummel M. 2003. Ecological persistence of the plant-mycorrhizal mutualism: a hypothesis from species coexistence theory. *The American Naturalist* **162**: S40–S50.

Hofrichter M. 2002. Review: lignin conversion by manganese peroxidase (MnP). *Enzyme and Microbial Technology* **30**: 454–466.

Hofrichter M, Vares K, Scheibner K, Galkin S, Sipilä J, Hatakka A. 1999. Mineralization and solubilization of synthetic lignin by manganese peroxidases from *Nematoloma frowardii* and *Phlebia radiata*. *Journal of Biotechnology* **67**: 217–228.

Högberg MN, Briones MJI, Keel SG, Metcalfe DB, Campbell C, Midwood AJ, Thornton B, Hurry V, Linder S, Näsholm T, et al. 2010. Quantification of effects of season and nitrogen supply on tree below-ground carbon transfer to ectomycorrhizal fungi and other soil organisms in a boreal pine forest. *New Phytologist* **187**: 485–493.

Ihrmark K, Bödeker ITM, Cruz-Martinez K, Friberg H, Kubartova A, Schenck J, Strid Y, Stenlid J, Brandström-Durling M, Clemmensen KE, et al. 2012. New primers to amplify the fungal ITS2 region - evaluation by 454-sequencing of artificial and natural communities. **FEMS** *Microbiology Ecology* **82**: 666–677.

Janusz G, Pawlik A, Sulej J, Świderska-Burek U, Jarosz-Wilkołazka A, Paszczyński A. 2017. Lignin degradation: microorganisms, enzymes involved, genomes analysis and evolution. *FEMS Microbiology Reviews* **41**: 941–962.

Janzen HH. 2006. The soil carbon dilemma: Shall we hoard it or use it? *Soil Biology and Biochemistry* **38**: 419–424.

Javelle A, Morel M, Rodríguez-Pastrana B-R, Botton B, André B, Marini A-M, Brun A, Chalot M. 2003. Molecular characterization, function and regulation of ammonium transporters (Amt) and ammonium-metabolizing enzymes (GS, NADP-GDH) in the ectomycorrhizal fungus *Hebeloma cylindrosporum*. *Molecular Microbiology* **47**: 411–430.

Johnsen AR, Jacobsen OS. 2008. A quick and sensitive method for the quantification of peroxidase activity of organic surface soil from forests. *Soil Biology and Biochemistry* **40**: 814–821.

Jones MD, Phillips LA, Treu R, Ward V, Berch SM. 2012. Functional responses of ectomycorrhizal fungal communities to long-term fertilization of lodgepole pine (*Pinus contorta* Dougl. ex Loud. var. latifolia Engelm.) stands in central British Columbia. *Applied Soil Ecology* **60**: 29–40.

Jones MD, Smith SE. 2004. Exploring functional definitions of mycorrhizas: Are mycorrhizas always mutualisms? **1109**: 1089–1109.

Jörgensen K, Clemmensen KE, Fransson P, Manzoni S, Wallander H, Lindahl BD. 2025. A trait spectrum linking nitrogen acquisition and carbon use of ectomycorrhizal fungi. *New Phytologist* **246**: 2425-2434

Jörgensen K, Clemmensen KE, Wallander H, Lindahl BD. **2024**. Ectomycorrhizal fungi are more sensitive to high soil nitrogen levels in forests exposed to nitrogen deposition. *New Phytologist* **242**: 1725–1738.

Jörgensen K, Granath G, Lindahl BD, Strengbom J. **2021**. Forest management to increase carbon sequestration in boreal Pinus sylvestris forests. *Plant and Soil* **466**: 165–178.

Jörgensen K, Granath G, Strengbom J, Lindahl BD. **2022**. Links between boreal forest management, soil fungal communities and below-ground carbon sequestration. *Functional Ecology* **36**: 392–405.

Kauserud H. **2023**. ITS alchemy: On the use of ITS as a DNA marker in fungal ecology. *Fungal Ecology* **65**: 101274.

Keiluweit M, Nico P, Harmon ME, Mao J, Pett-Ridge J, Kleber M. **2015**. Long-term litter decomposition controlled by manganese redox cycling. *Proceedings of the National Academy of Sciences of the United States of America* **112**: E5253–E5260.

Kersten P, Cullen D. 2014. Copper radical oxidases and related extracellular oxidoreductases of wood-decay Agaricomycetes. *Fungal Genetics and Biology* **72**: 124–130.

Kersten PJ, Kirk TK. **1987**. Involvement of a new enzyme, glyoxal oxidase, in extracellular H_2O_2 production by *Phanerochaete chrysosporium*. *American Society of Microbiology* **169**: 2195–2201.

Kirk TK, Farrell RL. **1987**. Enzymatic ‘combustion’: the microbial degradation of lignin. *Annual Review of Microbiology* **41**: 465–505.

Knudsen H, Vesterholt J. **2008**. *Funga Nordica: Agaricoid, Boletoid and Cyphelloid Genera*. Copenhagen: Nordsvamp.

Kögel-Knabner I. **2002**. The macromolecular organic composition of plant and microbial residues as inputs to soil organic matter. *Soil Biology and Biochemistry* **34**: 139–162.

Kohler A, Kuo A, Nagy LG, Morin E, Barry KW, Buscot F, Canbäck B, Choi C, Cichocki N, Clum A, *et al.* 2015. Convergent losses of decay mechanisms and rapid turnover of symbiosis genes in mycorrhizal mutualists. *Nature Genetics* **47**: 410–415.

Kohout P, Charvátová M, Štursová M, Mašínová T, Tomšovský M, Baldrian P. **2018**. Clearcutting alters decomposition processes and initiates complex restructuring of fungal communities in soil and tree roots. *The ISME Journal* **12**: 692–703.

Koide RT, Sharda JN, Herr JR, Malcolm GM. **2008**. Ectomycorrhizal fungi and the biotrophy–saprotrophy continuum. *New Phytologist* **178**: 230–233.

Koonin EV, Fedorova ND, Jackson JD, Jacobs AR, Krylov DM, Makarova KS, Mazumder R, Mekhedov SL, Nikolskaya AN, Rao BS, *et al.* 2004. A comprehensive evolutionary classification of proteins encoded in complete eukaryotic genomes. *Genome Biology* **5**: R7.

Koutaniemi S, Toikka MM, Kärkönen A, Mustonen M, Lundell T, Simola LK, Kilpeläinen IA, Teeri TH. 2005. Characterization of basic p-coumaryl and coniferyl alcohol oxidizing peroxidases from a lignin-forming *Picea abies* suspension culture. *Plant Molecular Biology* **58**: 141–157.

Krainer FW, Capone S, Jäger M, Vogl T, Gerstmann M, Glieder A, Herwig C, Spadiut O. **2015**. Optimizing cofactor availability for the production of recombinant heme peroxidase in *Pichia pastoris*. *Microbial Cell Factories* **14**: 4.

Kranabetter JM. **2019**. Increasing soil carbon content with declining soil manganese in temperate rainforests: is there a link to fungal Mn? *Soil Biology and Biochemistry* **128**: 179–181.

Kranabetter JM, Innes F, Norris CE, Philpott TJ, Lacourse T, Hawkins BJ. **2025**. Diverging soil peroxidase activity under ectomycorrhizal versus arbuscular mycorrhizal conifers with increasing C:N and exchangeable manganese. *Soil Biology and Biochemistry* **209**: 109913.

Kuan IC, Johnson KA, Tien M. **1993**. Kinetic analysis of manganese peroxidase. The reaction with manganese complexes. *Journal of Biological Chemistry* **268**: 20064–20070.

Kumar S, Stecher G, Suleski M, Sanderford M, Sharma S, Tamura K. 2024. MEGA12: Molecular Evolutionary Genetic Analysis Version 12 for adaptive and green computing. *Molecular Biology and Evolution* **41**: msae263.

Kuske CR, Hesse CN, Challacombe JF, Cullen D, Herr JR, Mueller RC, Tsang A, Vilgalys R. **2015**. Prospects and challenges for fungal metatranscriptomics of complex communities. *Fungal Ecology* **14**: 133–137.

Kuuskeri J, Häkkinen M, Laine P, Smolander O-P, Tamene F, Miettinen S, Nousiainen P, Kemell M, Auvinen P, Lundell T. **2016**. Time-scale dynamics of proteome and transcriptome of the white-rot fungus *Phlebia radiata*: growth on spruce wood and decay effect on lignocellulose. *Biotechnology for Biofuels* **9**: 192.

Kyaschenko J, Clemmensen KE, Karltun E, Lindahl BD. **2017a**. Below-ground organic matter accumulation along a boreal forest fertility gradient relates to guild interaction within fungal communities. *Ecology Letters* **20**: 1546–1555.

Kyaschenko J, Clemmensen KE, Hagenbo A, Karltun E, Lindahl BD. **2017b**. Shift in fungal communities and associated enzyme activities along an age gradient of managed *Pinus sylvestris* stands. *ISME Journal* **11**: 863–874.

Kyaschenko J, Ovaskainen O, Ekblad A, Hagenbo A, Karltun E, Clemmensen KE, Lindahl BD. **2019**. Soil fertility in boreal forest relates to root-driven nitrogen retention and carbon sequestration in the mor layer. *New Phytologist* **221**: 1492–1502.

LeDuc SD, Lilleskov EA, Horton TR, Rothstein DE. **2013**. Ectomycorrhizal fungal succession coincides with shifts in organic nitrogen availability and canopy closure in post-wildfire jack pine forests. *Oecologia* **172**: 257–269.

Liang C, Schimel JP, Jastrow JD. 2017. The importance of anabolism in microbial control over soil carbon storage. *Nature Microbiology* **2**: 17105.

Liao H-L, Chen Y, Bruns TD, Peay KG, Taylor JW, Branco S, Talbot JM, Vilgalys R. **2014**. Metatranscriptomic analysis of ectomycorrhizal roots reveals genes associated with *Piloderma–Pinus* symbiosis: improved methodologies for assessing gene expression in situ. *Environmental Microbiology* **16**: 3730–3742.

Liimatainen K, Kim JT, Pokorny L, Kirk PM, Dentinger B, Niskanen T. **2022**. Taming the beast: a revised classification of Cortinariaceae based on genomic data. *Fungal Diversity* **112**: 89–170.

Lilleskov EA, Fahey TJ, Horton TR, Lovett GM. **2002**. Belowground ectomycorrhizal fungal community change over a nitrogen deposition gradient in Alaska. *Ecology* **83**: 104–115.

Lilleskov EA, Hobbie EA, Horton TR. **2011**. Conservation of ectomycorrhizal fungi: exploring the linkages between functional and taxonomic responses to anthropogenic N deposition. *Fungal Ecology* **4**: 174–183.

Lilleskov EA, Kuyper TW, Bidartondo MI, Hobbie EA. **2019**. Atmospheric nitrogen deposition impacts on the structure and function of forest mycorrhizal communities: A review. *Environmental Pollution* **246**: 148–162.

Lin MI, Nagata T, Katahira M. **2018**. High yield production of fungal manganese peroxidases by E. coli through soluble expression, and examination of the activities. *Protein Expression and Purification* **145**: 45–52.

Lindahl BD, Clemmensen KE. **2017**. Fungal ecology in boreal forest ecosystems. In: Martin F, ed. Molecular Mycorrhizal Symbiosis. John Wiley & Sons, Inc, 387–404.

Lindahl BD, Clemmensen KE, Stendahl J, Dahlberg A. **2026.** Long-term effects of clear-cutting forestry on ectomycorrhizal fungi in boreal forest. *New Phytologist* **n/a**.

Lindahl BD, Ihrmark K, Boberg J, Trumbore SE, Högberg P, Stenlid J, Finlay RD. **2007**. Spatial separation of litter decomposition and mycorrhizal nitrogen uptake in a boreal forest. *New Phytologist* **173**: 611–620.

Lindahl BD, Kyaschenko J, Varenius K, Clemmensen KE, Dahlberg A, Karltun E, Stendahl J. **2021**. A group of ectomycorrhizal fungi restricts organic matter accumulation in boreal forest. *Ecology Letters* **24**: 1341–1351.

Lindahl BO, Taylor AFS, Finlay RD. **2002**. Defining nutritional constraints on carbon cycling in boreal forests – towards a less `phytocentric' perspective. *Plant and Soil* **242**: 123–135.

Lindahl BD, Tunlid A. 2015. Ectomycorrhizal fungi - potential organic matter decomposers, yet not saprotrophs. *New Phytologist* **205**: 1443–1447.

van der Linde S, Suz LM, Orme CDL, Cox F, Andreae H, Asi E, Atkinson B, Benham S, Carroll C, Cools N, *et al.* 2018. Environment and host as large-scale controls of ectomycorrhizal fungi. *Nature* **558**: 243–248.

Lofgren L, Maillard F, Michaud T, Gredeby A, Tunlid A, Kennedy PG. 2025. Diverse nitrogen acquisition strategies of conifer-associated ectomycorrhizal fungi shape unique responses to changing nitrogen regimes. *Frontiers in Plant Science* **16**: 1666003**.**

Lucic E, Fourrey C, Kohler A, Martin F, Chalot M, Brun-Jacob A. **2008**. A gene repertoire for nitrogen transporters in *Laccaria bicolor*. *New Phytologist* **180**: 343–364.

Lundell TK, Mäkelä MR, Hildén K. **2010**. Lignin-modifying enzymes in filamentous basidiomycetes – ecological, functional and phylogenetic review. *Journal of Basic Microbiology* **50**: 5–20.

Lundell TK, Mäkelä MR, de Vries RP, Hildén KS. **2014**. Chapter Eleven - genomics, lifestyles and future prospects of wood-decay and litter-decomposing Basidiomycota. In: Martin FM, ed. Fungi. Advances in Botanical Research. Academic Press, 329–370.

Luo Y, Su B, Currie WS, Dukes JS, Finzi A, Hartwig U, Hungate B, McMurtrie RE, Oren R, Parton WJ, *et al.* 2004. Progressive nitrogen limitation of ecosystem responses to rising atmospheric carbon dioxide. *BioScience* **54**: 731–739.

Magnani F, Mencuccini M, Borghetti M, Berbigier P, Berninger F, Delzon S, Grelle A, Hari P, Jarvis PG, Kolari P, *et al.* 2007. The human footprint in the carbon cycle of temperate and boreal forests. *Nature* **447**: 848–850.

Maillard F, Kohler A, Morin E, Hossann C, Miyauchi S, Ziegler-Devin I, Gérant D, Angeli N, Lipzen A, Keymanesh K, *et al.* 2023. Functional genomics gives new insights into the ectomycorrhizal degradation of chitin. *New Phytologist* **238**: 845–858.

Maillard F, Lopes Ramos D, Zhang B, Ahlawat A, Gill AL, Troein C, Hedenström M, Sparrman T, Persson P, Tunlid A. **2026**. Mycelial biomass growth stage at death determines fungal necromass decay dynamics. *Soil Biology and Biochemistry* **214**: 110079.

Mali T, Kuuskeri J, Shah F, Lundell TK. **2017**. Interactions affect hyphal growth and enzyme profiles in combinations of coniferous wood-decaying fungi of Agaricomycetes. *PLOS ONE* **12**: e0185171.

Manzoni S, Čapek P, Porada P, Thurner M, Winterdahl M, Beer C, Brüchert V, Frouz J, Herrmann AM, Lyon SW, *et al.* 2018. Reviews and syntheses: Carbon use efficiency from organisms to ecosystems - Definitions, theories, and empirical evidence. *Biogeosciences* **15**: 5929–5949.

Martin F, Aerts A, Ahrén D, Brun A, Danchin EGJ, Duchaussoy F, Gibon J, Kohler A, Lindquist E, Pereda V, *et al.* 2008. The genome of *Laccaria bicolor* provides insights into mycorrhizal symbiosis. *Nature* **452**: 88–92.

Matheny PB, Curtis JM, Hofstetter V, Aime MC, Moncalvo J-M, Ge Z-W, Yang Z-L, Slot JC, Ammirati JF, Baroni TJ, *et al.* 2006. Major clades of agaricales: A multilocus phylogenetic overview. *Mycologia* **98**: 982–995.

Mattila H, Österman-Udd J, Mali T, Lundell T. **2022**. Basidiomycota fungi and ros: genomic perspective on key enzymes involved in generation and mitigation of reactive oxygen species. *Frontiers in Fungal Biology* **3**: 837605.

Mayer M, Matthews B, Sandén H, Katzensteiner K, Hagedorn F, Gorfer M, Berger H, Berger TW, Godbold DL, Rewald B. 2023. Soil fertility determines whether ectomycorrhizal fungi accelerate or decelerate decomposition in a temperate forest. *New Phytologist* **239**: 325-339.

Mayerhofer W, Schintlmeister A, Dietrich M, Gorka S, Wiesenbauer J, Martin V, Gabriel R, Reipert S, Weidinger M, Clode P, *et al.* 2021. Recently photoassimilated carbon and fungus-delivered nitrogen are spatially correlated in the ectomycorrhizal tissue of *Fagus sylvatica*. *New Phytologist* **232**: 2457–2474.

McDonald AG, Tipton KF. **2014**. Fifty-five years of enzyme classification: advances and difficulties. *The FEBS Journal* **281**: 583–592.

Melillo JM, Aber JD, Muratore JF. 1982. Nitrogen and Lignin Control of Hardwood Leaf Litter Decomposition Dynamics. *Ecology* **63**: 621–626.

Miyauchi S, Kiss E, Kuo A, Drula E, Kohler A, Sánchez-García M, Morin E, Andreopoulos B, Barry KW, Bonito G, *et al.* 2020. Large-scale genome sequencing of mycorrhizal fungi provides insights into the early evolution of symbiotic traits. *Nature Communications* **11**: 5125.

Nannipieri P. **2006**. Role of stabilised enzymes in microbial ecology and enzyme extraction from soil with potential applications in soil proteomics. In: Nannipieri P, Smalla K, eds. Nucleic Acids and Proteins in Soil. Berlin, Heidelberg: Springer, 75–94.

Näsholm T, Högberg P, Franklin O, Metcalfe D, Keel SG, Campbell C, Hurry V, Linder S, Högberg MN. **2013**. Are ectomycorrhizal fungi alleviating or aggravating nitrogen limitation of tree growth in boreal forests? *New Phytologist* **198**: 214–221.

Nehls U, Plassard C. **2018**. Nitrogen and phosphate metabolism in ectomycorrhizas. *New Phytologist* **220**: 1047–1058.

Ngo TT, Lenhoff HM. **1980**. A sensitive and versatile chromogenic assay for peroxidase and peroxidase-coupled reactions. *Analytical Biochemistry* **105**: 389–397.

Nicolás C, Martin-Bertelsen T, Floudas D, Bentzer J, Smits M, Johansson T, Troein C, Persson P, Tunlid A. **2019**. The soil organic matter decomposition mechanisms in ectomycorrhizal fungi are tuned for liberating soil organic nitrogen. *ISME Journal* **13**: 977–988.

Op De Beeck M, Troein C, Peterson C, Persson P, Tunlid A. **2018**. Fenton reaction facilitates organic nitrogen acquisition by an ectomycorrhizal fungus. *New Phytologist* **218**: 335–343.

Orwin KH, Kirschbaum MUF, St John MG, Dickie IA. **2011**. Organic nutrient uptake by mycorrhizal fungi enhances ecosystem carbon storage: a model-based assessment. *Ecology Letters* **14**: 493–502.

Pan Y, Birdsey RA, Fang J, Houghton R, Kauppi PE, Kurz WA, Phillips OL, Shvidenko A, Lewis SL, Canadell JG, et al. 2011. A large and persistent carbon sink in the world's forests. *Science* **333**: 988–993.

Patro R, Duggal G, Love MI, Irizarry RA, Kingsford C. **2017**. Salmon provides fast and bias-aware quantification of transcript expression. *Nature Methods* **14**: 417–419.

Peay KG. **2014**. Back to the future: natural history and the way forward in modern fungal ecology. *Fungal Ecology* **12**: 4–9.

Pellitier PT, Zak DR. **2018**. Ectomycorrhizal fungi and the enzymatic liberation of nitrogen from soil organic matter. *New Phytologist* **217**: 68–73.

Pellitier PT, Zak DR. 2021. Ectomycorrhizal fungal decay traits along a soil nitrogen gradient. *New Phytologist* **232**: 2152–2164.

Perez J, Jeffries TW. **1992**. Roles of manganese and organic acid chelators in regulating lignin degradation and biosynthesis of peroxidases by Phanerochaete chrysosporium. *Applied and Environmental Microbiology* **58**: 2402–2409.

Pérez-Izquierdo L, Clemmensen KE, Strengbom J, Granath G, Wardle DA, Nilsson MC, Lindahl BD. **2021**. Crown-fire severity is more important than ground-fire severity in determining soil fungal community development in the boreal forest. *Journal of Ecology* **109**: 504–518.

Peterson RL, Massicotte HB. **2004**. Exploring structural definitions of mycorrhizas, with emphasis on nutrient-exchange interfaces. *Canadian Journal of Botany* **82**: 1074–1088.

Phillips RP, Brzostek E, Midgley MG. **2013**. The mycorrhizal-associated nutrient economy: A new framework for predicting carbon-nutrient couplings in temperate forests. *New Phytologist* **199**: 41–51.

Plassard C, Bonafos B, Touraine B. **2000**. Differential effects of mineral and organic N sources, and of ectomycorrhizal infection by *Hebeloma cylindrosporum*, on growth and N utilization in *Pinus pinaster*. *Plant, Cell and Environment* **23**: 1195–1205.

Plassard C, Louche J, Ali MA, Duchemin M, Legname E, Cloutier-Hurteau B. **2011**. Diversity in phosphorus mobilisation and uptake in ectomycorrhizal fungi. *Annals of Forest Science* **68**: 33–43.

Prosser JI. **2020**. Putting science back into microbial ecology: A question of approach. *Philosophical Transactions of the Royal Society B: Biological Sciences* **375**: 20190240.

Puissant J, Jones B, Goodall T, Mang D, Blaud A, Gweon HS, Malik A, Jones DL, Clark IM, Hirsch PR, *et al.* 2019. The pH optimum of soil exoenzymes adapt to long term changes in soil pH. *Soil Biology and Biochemistry* **138**: 107601.

Rawlings ND, Barrett AJ, Thomas PD, Huang X, Bateman A, Finn RD. **2018**. The MEROPS database of proteolytic enzymes, their substrates and inhibitors in 2017 and a comparison with peptidases in the PANTHER database. *Nucleic Acids Research* **46**: D624–D632.

Read DJ. **1991**. Mycorrhizas in ecosystems. *Experientia* **47**: 376–391.

Read DJ, Perez-Moreno J. **2003**. Mycorrhizas and nutrient cycling in ecosystems – a journey towards relevance? *New Phytologist* **157**: 475–492.

Rianhard OM, Kranabetter JM, Penkova LV, Durall DM, Jones MD. 2025. Ectomycorrhizal fungal community succession and fragmentation across forest edges nearly three decades postharvest. *Plants, People, Planet* **7:** 1510–1523

Rinaldi AC, Comandini O, Kuyer TW. **2008**. Ectomycorrhizal fungal diversity: separating the wheat from the chaff. *Fungal Diversity* **33**: 1–45.

Rineau F, Shah F, Smits MM, Persson P, Johansson T, Carleer R, Troein C, Tunlid A. **2013**. Carbon availability triggers the decomposition of plant litter and assimilation of nitrogen by an ectomycorrhizal fungus. *ISME Journal* **7**: 2010–2022.

Rineau F, Roth D, Shah F, Smits M, Johansson T, Canbäck B, Olsen PB, Persson P, Grell MN, Lindquist E, et al. 2012. The ectomycorrhizal fungus *Paxillus involutus* converts organic matter in plant litter using a trimmed brown-rot mechanism involving Fenton chemistry. Environmental Microbiology **14**: 1477–1487.

Ruiz-Dueñas FJ, Barrasa JM, Sánchez-García M, Camarero S, Miyauchi S, Serrano A, Linde D, Babiker R, Drula E, Ayuso-Fernández I, *et al.* 2021. Genomic analysis enlightens Agaricales lifestyle evolution and increasing peroxidase diversity. *Molecular biology and evolution* **38**: 1428–1446.

Ruiz-Dueñas FJ, Lundell T, Floudas D, Nagy LG, Barrasa JM, Hibbett DS, Martínez AT. **2013**. Lignin-degrading peroxidases in Polyporales: an evolutionary survey based on 10 sequenced genomes. *Mycologia* **105**: 1428–1444.

Saier MH, Reddy VS, Moreno-Hagelsieb G, Hendargo KJ, Zhang Y, Iddamsetty V, Lam KJK, Tian N, Russum S, Wang J, *et al.* 2021. The Transporter Classification Database (TCDB): 2021 update. *Nucleic Acids Research* **49**: D461–D467.

Sánchez-Ruiz MI, Santillana E, Linde D, Romero A, Martínez AT, Ruiz-Dueñas FJ. **2024**. Structure–function characterization of two enzymes from novel subfamilies of manganese peroxidases secreted by the lignocellulose-degrading Agaricales fungi *Agrocybe pediades* and *Cyathus striatus*. *Biotechnology for Biofuels and Bioproducts* **17**.

Schimel J. **1995**. ecosystem consequences of microbial diversity and community structure. In: Chapin FS, Körner C, eds. Ecological Studies. Arctic and alpine biodiversity: patterns, causes and ecosystem consequences. Berlin, Heidelberg: Springer, 239–254.

Schmidt MWI, Torn MS, Abiven S, Dittmar T, Guggenberger G, Janssens IA, Kleber M, Kögel-Knabner I, Lehmann J, Manning DAC, *et al.* 2011. Persistence of soil organic matter as an ecosystem property. *Nature* **478**: 49–56.

Schoch CL, Seifert KA, Huhndorf S, Robert V, Spouge JL, Levesque CA, Chen W, Bolchacova E, Voigt K, Crous PW, *et al.* 2012. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. *Proceedings of the National Academy of Sciences of the United States of America* **109**: 6241–6246.

Score AJ, Palfreyman JW, White NA. **1997**. Extracellular phenoloxidase and peroxidase enzyme production during interspecific fungal interactions. *International Biodeterioration & Biodegradation* **39**: 225–233.

See CR, Keller AB, Hobbie SE, Kennedy PG, Weber PK, Pett-Ridge J. **2022**. Hyphae move matter and microbes to mineral microsites: Integrating the hyphosphere into conceptual models of soil organic matter stabilization. *Global Change Biology* **28**: 2527-2540.

Shah F, Nicolás C, Bentzer J, Ellström M, Smits M, Rineau F, Canbäck B, Floudas D, Carleer R, Lackner G, *et al.* 2016. Ectomycorrhizal fungi decompose soil organic matter using oxidative mechanisms adapted from saprotrophic ancestors. *New Phytologist* **209**: 1705–1719.

Shah F, Rineau F, Canbäck B, Johansson T, Tunlid A. **2013**. The molecular components of the extracellular protein-degradation pathways of the ectomycorrhizal fungus *Paxillus involutus*. *New Phytologist* **200**: 875–887.

Shimizu M, Yuda N, Nakamura T, Tanaka H, Wariishi H. **2005**. Metabolic regulation at the tricarboxylic acid and glyoxylate cycles of the lignin-degrading basidiomycete *Phanerochaete chrysosporium* against exogenous addition of vanillin. *PROTEOMICS* **5**: 3919–3931.

Siletti CE, Zeiner CA, Bhatnagar JM. **2017**. Distributions of fungal melanin across species and soils. *Soil Biology and Biochemistry* **113**: 285–293.

Sinsabaugh RL. **2010**. Phenol oxidase, peroxidase and organic matter dynamics of soil. *Soil Biology and Biochemistry* **42**: 391–404.

Sinsabaugh RL, Antibus RK, Linkins AE, McClaugherty CA, Rayburn L, Repert D, Weiland T. **1992**. Wood decomposition over a first-order watershed: Mass loss as a function of lignocellulase activity. *Soil Biology and Biochemistry* **24**: 743–749.

Smith SE, Read DJ. **2008**. *Mycorrrhizal symbiosis*. San Diego: Elsevier Science & Technology.

Šnajdr J, Steffen KT, Hofrichter M, Baldrian P. **2010**. Transformation of ^{14}C-labelled lignin and humic substances in forest soil by the saprobic basidiomycetes *Gymnopus erythropus* and *Hypholoma fasciculare*. *Soil Biology and Biochemistry* **42**: 1541–1548.

Stendahl J, Berg B, Lindahl BD. **2017**. Manganese availability is negatively associated with carbon storage in northern coniferous forest humus layers. *Scientific Reports* **7**: 1–6.

Sterkenburg E, Bahr A, Durling MB, Clemmensen KE, Lindahl BD. **2015**. Changes in fungal communities along a boreal forest soil fertility gradient. *The New Phytologist* **207**: 1145–1158.

Sterkenburg E, Clemmensen KE, Ekblad A, Finlay RD, Lindahl BD. **2018**. Contrasting effects of ectomycorrhizal fungi on early and late stage decomposition in a boreal forest. *ISME Journal* **12**: 2187–2197.

Sterkenburg E, Clemmensen KE, Lindahl BD, Dahlberg A. **2019**. The significance of retention trees for survival of ectomycorrhizal fungi in clear-cut Scots pine forests. *Journal of Applied Ecology* **56**: 1367–1378.

Strullu-Derrien C, Selosse M-A, Kenrick P, Martin FM. **2018**. The origin and evolution of mycorrhizal symbioses: from palaeomycology to phylogenomics. *New Phytologist* **220**: 1012–1030.

Stuart EK, Plett KL. **2020**. Digging Deeper: In search of the mechanisms of carbon and nitrogen exchange in ectomycorrhizal symbioses. *Frontiers in Plant Science* **10**: 1–11.

Sun H, Santalahti M, Pumpanen J, Köster K, Berninger F, Raffaello T, Jumpponen A, Asiegbu FO, Heinonsalo J. **2015**. Fungal community shifts in structure and function across a boreal forest fire chronosequence. *Applied and Environmental Microbiology* **81**: 7869–7880.

Sun T, Yu C, Berg B, Wei Z, Wang L, Liu X, Feng C, Wu Z, Bai W, Zhang L. **2021**. Empirical evidence that manganese enrichment accelerates decomposition. *Applied Soil Ecology* **168**: 104148.

Sützl L, Laurent CVFP, Abrera AT, Schütz G, Ludwig R, Haltrich D. **2018**. Multiplicity of enzymatic functions in the CAZy AA3 family. *Applied Microbiology and Biotechnology* **102**: 2477–2492.

Talbot JM, Allison SD, Treseder KK. **2008**. Decomposers in disguise: Mycorrhizal fungi as regulators of soil C dynamics in ecosystems under global change. *Functional Ecology* **22**: 955–963.

Tang N, Lebreton A, Xu W, Dai Y, Yu F, Martin FM. **2021**. Transcriptome Profiling Reveals Differential Gene Expression of Secreted Proteases and

Highly Specific Gene Repertoires Involved in Lactarius–Pinus Symbioses. *Frontiers in Plant Science* **12**: 714393.

Taylor TN, Osborn JM. **1996**. The importance of fungi in shaping the paleoecosystem. *Review of Palaeobotany and Palynology* **90**: 249–262.

Tedersoo L, May TW, Smith ME. **2010**. Ectomycorrhizal lifestyle in fungi: Global diversity, distribution, and evolution of phylogenetic lineages. *Mycorrhiza* **20**: 217–263.

Terrer C, Phillips RP, Hungate BA, Rosende J, Pett-Ridge J, Craig ME, van Groenigen KJ, Keenan TF, Sulman BN, Stocker BD, *et al.* 2021. A trade-off between plant and soil carbon storage under elevated CO_2. *Nature* **591**: 599–603.

Trum F, Titeux H, Cornelis JT, Delvaux B. **2011**. Effects of manganese addition on carbon release from forest floor horizons. *Canadian Journal of Forest Research* **41**: 643–648.

Tshinyangu KK, Hennebert GL. **1996**. Protein and chitin nitrogen contents and protein content in *Pleurotus ostreatus* var. columbinus. *Food Chemistry* **57**: 223–227.

Tunlid A, Floudas D, Op De Beeck M, Wang T, Persson P. 2022. Decomposition of soil organic matter by ectomycorrhizal fungi: Mechanisms and consequences for organic nitrogen uptake and soil carbon stabilization. *Frontiers in Forests and Global Change* **5**: 934409**.**

Vanden Wymelenberg A, Sabat G, Mozuch M, Kersten PJ, Cullen D, Blanchette RA. **2006**. structure, organization, and transcriptional regulation of a family of copper radical oxidase genes in the lignin-degrading Basidiomycete *Phanerochaete chrysosporium*. *Applied and Environmental Microbiology* **72**: 4871–4877.

Varenius K, Kårén O, Lindahl B, Dahlberg A. **2016**. Long-term effects of tree harvesting on ectomycorrhizal fungal communities in boreal Scots pine forests. *Forest Ecology and Management* **380**: 41–49.

Varga T, Krizsán K, Földi C, Dima B, Sánchez-García M, Sánchez-Ramírez S, Szöllősi GJ, Szarkándi JG, Papp V, Albert L, *et al.* 2019. Megaphylogeny resolves global patterns of mushroom evolution. *Nature Ecology & Evolution* **3**: 668–678.

Veneault-Fourrey C, Commun C, Kohler A, Morin E, Balestrini R, Plett J, Danchin E, Coutinho P, Wiebenga A, de Vries RP, *et al.* 2014. Genomic and transcriptomic analysis of *Laccaria bicolor* CAZome reveals insights into polysaccharides remodelling during symbiosis establishment. *Fungal Genetics and Biology* **72**: 168–181.

Visser S. **1995**. Ectomycorrhizal fungal succession in jack pine stands following wildfire. *New Phytologist* **129**: 389–401.

Wallander H. **1995**. A new hypothesis to explain allocation of dry matter between mycorrhizal fungi and pine seedlings in relation to nutrient supply. In:

Nilsson LO, Hüttl RF, Johansson UT, eds. Nutrient Uptake and Cycling in Forest Ecosystems: Proceedings of the CEC/IUFRO Symposium Nutrient Uptake and Cycling in Forest Ecosystems Halmstad, Sweden, June, 7–10, 1993. Dordrecht: Springer Netherlands, 243–248.

Wallenstein MD, Weintraub MN. **2008**. Emerging tools for measuring and modeling the *in situ* activity of soil extracellular enzymes. *Soil Biology and Biochemistry* **40**: 2098–2106.

Wariishi H, Valli K, Gold MH. **1992**. Manganese(II) oxidation by manganese peroxidase from the basidiomycete Phanerochaete chrysosporium. Kinetic mechanism and role of chelators. *Journal of Biological Chemistry* **267**: 23688–23695.

Wasyliw J, Karst J. **2020**. Shifts in ectomycorrhizal exploration types parallel leaf and fine root area with forest age. *Journal of Ecology* **108**: 2270–2282.

Whitwam R, Tien M. **1996**. Heterologous expression and reconstitution of fungal Mn peroxidase. *Archives of Biochemistry and Biophysics* **333**: 439–446.

Wieder WR, Bonan GB, Allison SD. **2013**. Global soil carbon projections are improved by modelling microbial processes. *Nature Climate Change* **3**: 909–912.

Zak DR, Pellitier PT, Argiroff WA, Castillo B, James TY, Nave LE, Averill C, Beidler KV, Bhatnagar J, Blesh J, *et al.* 2019. Exploring the role of ectomycorrhizal fungi in soil carbon dynamics. *New Phytologist* **223**: 33–39.

Zhang Y, Hobbie SE, Schlesinger WH, Berg B, Sun T, Zhu J. **2024**. Exchangeable manganese regulates carbon storage in the humus layer of the boreal forest. *Proceedings of the National Academy of Sciences* **121**: e2318382121.

Zhang J, Presley GN, Hammel KE, Ryu J-S, Menke JR, Figueroa M, Hu D, Orr G, Schilling JS. **2016**. Localizing gene regulation reveals a staggered wood decay mechanism for the brown rot fungus *Postia placenta*. *Proceedings of the National Academy of Sciences of the United States of America* **113**: 10968–10973.

Popular science summary

A group of fungi living in forest soils form symbiosis with the roots of trees. These fungi – known as ectomycorrhizal fungi – aid in increasing access to pools of nutrients that would otherwise be unavailable to the trees. Sugars fixed in photosynthesis are transferred from the trees to these fungal symbionts growing on the fine root tips, providing them with energy. The symbiotic transfer of carbon to mycorrhizal fungi contributes a large proportion of the carbon that enters the soil, but how much becomes stored depends on the balance between long-term stabilisation and decomposition by soil organisms (leading to release of CO_2 to the atmosphere). Ectomycorrhizal fungi can acquire inorganic nitrogen, which is scarce in boreal forest soils, but notably also organic nitrogen, which accumulates in the topsoil of boreal forests. However, this organic nitrogen is often bound in soil organic matter that is difficult to degrade and requires potent enzymes to be released. Some ectomycorrhizal fungi – here called ectomycorrhizal decomposers – have the capacity to produce these necessary enzymes, called manganese peroxidases. Ectomycorrhizal decomposers are thought to play an important role in regulating the cycling of nutrients and carbon in forest soils. In this thesis, I aimed to deepen our understanding of ectomycorrhizal fungi regarding their capacity to decompose soil organic matter and the role they play in forest soils.

To assign potentially important ectomycorrhizal decomposers, I performed an analysis combining DNA-based identification of species and measurements of enzyme activity. We suggest that not all species within a genus should be considered to have the same potential as decomposers, which in terms of protecting species that are important for the soil ecosystem means we need to better understand traits at a species-level. Further, using the Swedish Forest Soil Inventory, we found that these species have their niches in a variety of forests, not only in older forests with low forestry impact. While it was expected that ectomycorrhizal decomposers would be more sensitive to disturbances in comparison to other ectomycorrhizal fungi, this was not the case for all species, suggesting that forestry may not pose as much of a threat to this ecosystem process. However, we only assessed the niche of a small group of relatively common fungi, and many species are negatively impacted by intense forest management.

There is often a gap between what a fungus is predicted to be able to do (based on its genes) and what it actually does. In this case, ectomycorrhizal decomposers are predicted to be able to decompose soil organic matter, but testing whether this is true remains challenging. To confirm the predicted activity of manganese peroxidases produced by ectomycorrhizal decomposers, I produced these enzymes using a method where the manganese peroxidase gene from an ectomycorrhizal fungus was inserted into an easy-to-grow yeast. This yeast was then used to produce the enzyme in the laboratory. The manganese peroxidase produced in the yeast was functional, but the enzyme activity seemed to be different than that of typical manganese peroxidases, in that the enzyme did not necessarily require manganese to function. This is one step towards confirming that ectomycorrhizal fungi can decompose soil organic matter, but more investigation is needed.

I used RNA-sequencing, which provides a snapshot of which enzymes (and other proteins) a fungus is producing, to test some key assumptions about ectomycorrhizal decomposers. First, I aimed to test whether ectomycorrhizal decomposers use manganese peroxidases to increase access to nitrogen bound in organic matter; a necessary step to maintain nutrient cycling between the soil and trees. I found that there was a clear positive relationship between the use of manganese peroxidase genes and genes for enzymes that break down organic sources of nitrogen (and phosphorus).This suggests that ectomycorrhizal decomposers may be able to maintain nutrient cycling and tree productivity in nitrogen-limited soils, but this mining for nutrients may also restrict the potential for soil carbon storage. Second, I aimed to test whether there is a high energy cost for decomposition with manganese peroxidases. It may be that ectomycorrhizal decomposers are more costly symbiotic partners that are not supported by the tree when nitrogen is more readily available. However, I did not find support for this hypothesis and rather observed that decomposition of hard-to-break-down organic matter does not appear as energetically costly as other processes occurring in the mycelium.

Together the studies in this thesis suggest that ectomycorrhizal decomposers are influential in nutrient cycling and organic matter decomposition, but that more work is necessary to fully understand the implications for large-scale process, such as tree productivity and carbon storage.

Populärvetenskaplig sammanfattning

Vissa svampar som lever i skogsmark bildar symbios med trädens rötter. Dessa svampar, så kallade ektomykorrhizasvampar, ökar trädens tillgång till svåråtkomliga näringsämnen. Mykorrhizasvamparnas mobilisering av näringsämnen möjliggörs av det kol (energi) de får av träden i form av socker. Transporten av kol till svamparna i symbiosen tillför kol till marken, men hur mycket kol som slutligen lagras in i marken avgörs av balansen mellan andelen som stabiliseras långsiktigt och hur mycket kol som avgår som koldioxid när markorganismer bryter ned organiskt material. Ektomykorrhizasvampar kan ta upp oorganiska former av kväve, vilka förekommer i låga koncentrationer i boreal skogsmark, men också organiskt kväve som ackumuleras i stora mängder i skogarnas humuslager. Det organiska kvävet är ofta bundet i svårnedbrytbart organiskt material, och kraftfulla enzymer krävs för att kvävet ska bli tillgängligt. Vissa mykorrhizasvampar har kapacitet att tillverka sådana enzymer; manganperoxidaser, och tros spela en viktig roll i omsättning av näringsämnen och kol i skogsmark. I denna avhandling har jag studerat mykorrhizabildande svampars kapacitet för nedbrytning och därigenom öka förståelsen för deras roll i skogsmark.

Jag kombinerade DNA-baserad identifiering av svamparter med mätningar av enzymaktiviteter för att identifiera potentiella mykorrhizabildande arter med nedbrytarkapacitet. Studiens slutsats var att arter inom ett släkte verkar ha olika kapacitet att använda manganperoxidaser. Detta tyder på att fördjupad kunskap om enskilda arters egenskaper behövs för att kunna bevara de som är viktiga för skogsekosystemens funktion. Vidare verkade mykorrhizabildande nedbrytare ha en relativt bred ekologisk nisch; de förekom inte bara i gamla skogar utan även i brukade bestånd. Trots att vi förväntade oss att nedbrytar-ektomykorrhiza skulle vara känsliga för intensivt skogsbruk hittade vi inget stöd för det antagandet, vilket indikerar att kalavverkning potentiellt inte utgör något tydligt hot mot denna ekosystemprocess. Vi undersökte dock bara en liten andel en artrik grupp svampar, och många arter påverkas negativt av intensivt skogsbruk.

Ofta finns en diskrepans mellan de funktioner svampar antas kunna utföra (baserat på deras genetiska potential) och vilka funktioner de faktiskt utför. Mykorrhizabildande nedbrytare förväntas kunna bryta ned organiskt material men det är svårt att undersöka om så verkligen är fallet. För att testa om deras manganperoxidaser faktiskt kan bryta ned organiskt material tillverkade vi dessa enzymer genom att genetiskt transformera en jästsvamp (genom att överföra mykorrhizasvampens gener). Det tillverkade manganperoxidaset var funktionellt, men dess funktion verkade skilja sig från den hos typiska manganperoxidaser genom att det fungerade även utan tillgång till mangan. Detta styrker antagandet om att vissa mykorrhizasvampar kan bryta ned organiskt material, men mer forskning behövs.

För att testa antaganden om mykorrhizabildande nedbrytares ekologi och funktion använde vi oss av RNA-sekvensering, vilket ger en ögonblicksbild av vilka enzymer (och andra proteiner) en svamp producerar. Först testade vi om svamparna använder manganperoxidaser för att öka tillgängligheten av kväve som är bundet i organiskt material, vilket utgör ett nödvändigt steg för att upprätthålla markens näringsomsättning och transporten av kväve till träden. Vi observerade ett tydligt positivt samband mellan uttryck av gener som kodar för manganperoxidaser och gener som kodar för nedbrytning av organiska molekyler med högt innehåll av kväve eller fosfor. Detta tyder på att mykorrhizabildande nedbrytare bidrar till att driva näringsomsättning och produktionen av trädbiomassa i näringsfattiga skogar, men att omsättningen sker på bekostnad av markens kolförråd. Vidare undersökte vi om nedbrytning med hjälp av manganperoxidaser är en energikrävande process. Mykorrhizasvampar med nedbrytarförmåga kan alltför dyra (sockerkrävande) symbiospartners för träden när kvävetillgängligheten är god. Vi fann dock inget stöd för denna hypotes; nedbrytningen av svårnedbrytbart organiskt material verkade vara lika energikrävande som andra processer som pågick i svampens mycel.

Sammantaget styrker dessa studier bilden av mykorrhizabildande nedbrytare som viktiga för skogens näringsomsättning och nedbrytning, men det finns mycket mer att lära om deras påverkan på storskaliga processer, såsom trädproduktivitet och markens kolinlagring.

Acknowledgements

I am grateful every day for the network of people who have surrounded me with positive energy, kindness, and inspiration. Life and especially a PhD is easier when you have the support of others.

I want to thank you, *Björn*, for your endless encouragement and understanding. I appreciate that you always had time to work through the "upward-spiral" with me. Your philosophy of science has really inspired me.

I had an excellent co-supervisory team: *Karina*, *Dimitri*, and *Marie*. Thank you for each contributing your unique skills and ideas. Thank you for being available to bounce ideas and troubleshoot problems. The mentorship I received from you was an important part of my journey. While not a supervisor *per se*, *Karolina*, you were such an important guide and friend for me throughout my PhD. So glad I can count on you to "nerd-out" and to vent with when needed.

How fortunate I am to have been a part of such an amazing soil biology group: *Tamlyn*, *Péter*, *Karolina*, *Samia*, *Eva Krab*, *Franzi*, *Margaux, Tobias* and the whole soil ecology journal club crew. Fieldwork, conferences, lab struggles, the ups and downs of writing, courses, and everything else in between was better because I felt a part of a team. The soil ecology journal club was my favourite place to be. I always felt it was a safe space to ask the "stupid" question and even sometimes to speak before I fully formulated my thoughts. I learned the most being a part of the discussions we had and I am grateful I had this space to grow. Thank you to everyone in the Mykopat lab, a place that felt so welcoming, and especially to *Katta*, without you I would still be hesitating to start my RNA extractions. There are so may brilliant and kind scientists that I got the chance to interact with at SLU and abroad. I am thankful for everyone who has helped me with the big and small problems.

There have been so many friends and colleagues at SLU who have made my experience enjoyable both at work and outside. Thank you to all my fellow PhD students and early career researchers, past and present. I never wanted to work from home because I got so much energy from interacting with everyone daily at fika, lunch, or simply passing in the hallways. I was especially fortunate to find friends as lovely and supporting as *Elsa*, *Eva*, *Mareile*, *Péter*, *Tamlyn*, *Viki*, *Yaana*, and *Ylva*. From heart-to-heart chats, weekend trips, to just general shenanigans, you have all made my time as a PhD student fun. I want to thank the newly founded running "SLUgs" and the founding members, *Tom*, *Gustavo*, and *Celina*. And to you, *Viki*, a special thanks for being my partner in “type-2 fun”. I am not sure what I would do without you.

Melanie and *Dan*, thank you for helping me start off on the right foot and following along my PhD journey. Your enthusiasm and joy are what sparked my love for the research that is now a part of who I am, and I am so grateful.

Last but not least, I want to thank my family, who maybe still isn’t sure exactly what I am doing but listens anyways. Everyone has always supported my dreams even when they took me far away. I am grateful (at least most of the time) that the apple didn’t fall far from the tree. Thank you, *Maa* and *Dad*, for being steady and supportive. Thank you, *Craig*, for the thoughtful and (surprisingly) wise advice only an older brother can provide. To *Brooke* and *Kelly*, not family by blood but by choice, thank you for being there for me through thick and thin.

Appendix

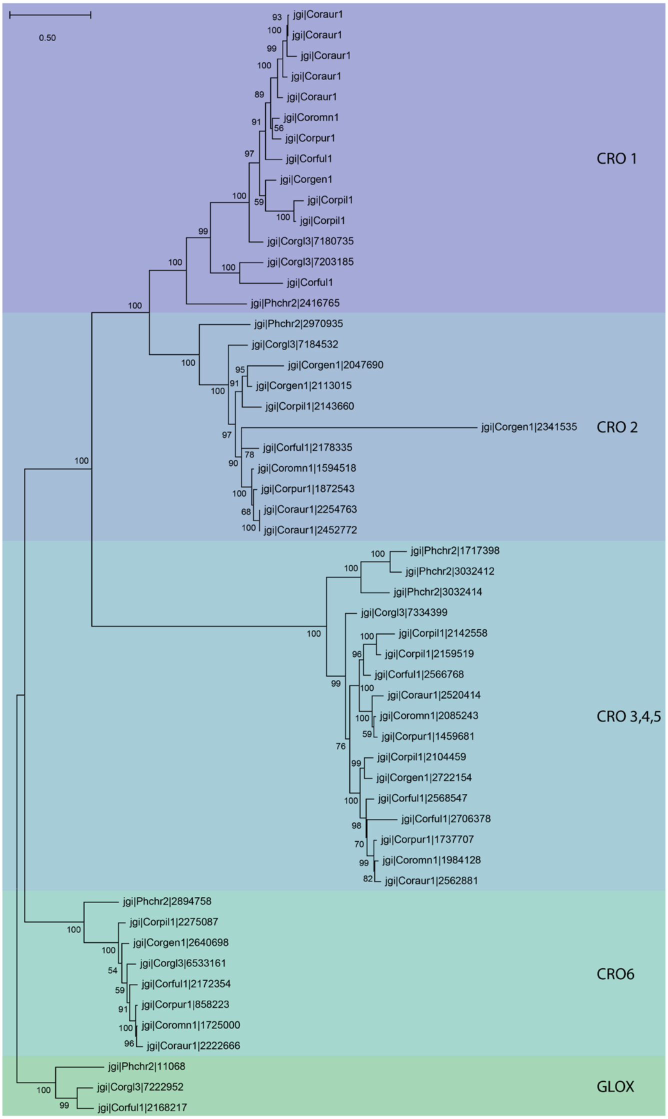


Figure S1. Phylogenetic assignment of copper radical oxidases (CRO; GLOX = glyoxal oxidase) in new *Cortinarius* genomes using maximum-likelihood tree with reference sequences from *Phlegmacium glaucopus* (Corgl; Miyauchi *et al*., 2020) as classified by Mattila *et al*. (2022) and *Phanerochaete chrysosporium* (Phchr; Vanden Wymelenberg *et al*., 2006). Bootstrap values are indicated next to nodes (500 replicates).

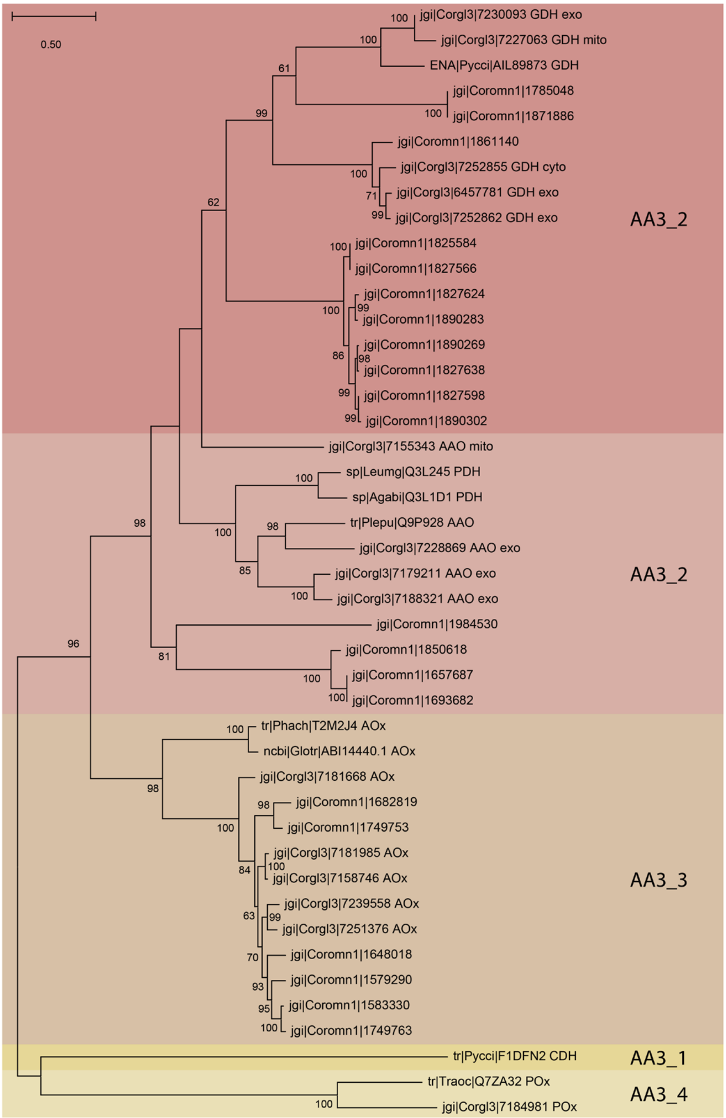


Figure S2. Phylogenetic assignment of Glucose-methanol-choline (GMC) oxidoreductases in *Cortinarius ominosus* using maximum-likelihood tree with reference sequences from *Phlegmacium glaucopus* (Corgl; Miyauchi *et al*., 2020) as classified by Mattila *et al*. (2022), *Phanerochaete chrysosporium* (Phach), *Pleurotus pulmonarius* (Plepu), *Pycnoporus cinnabarinus* (Pycci), *Leucoagaricus meleagris* (Leumg), *Agaricus bisporus* (Agabi), and *Gloeophyllum trabeum* (Glotr; Daniel *et al*., 2007). Following the species name abbreviation is the JGI, NCBI, ENA or UniProt protein identification number. Bootstrap values are indicated next to nodes (500 replicates).

Received: 20 December 2024 | Accepted: 22 May 2025
DOI: 10.1111/1365-2435.70085

BRITISH ECOLOGICAL SOCIETY Functional Ecology

RESEARCH ARTICLE

Ectomycorrhizal decomposers and their niche(s) in boreal forests

Erica E. Packard[1] | **Leticia Pérez-Izquierdo**[2] | **Karina E. Clemmensen**[3] | **Anders Dahlberg**[3] | **Marie Spohn**[1] | **Johan Stendahl**[1] | **Björn D. Lindahl**[1]

[1]Department of Soil and Environment, Swedish University of Agricultural Sciences, Uppsala, Sweden
[2]Basque Centre for Climate Change (BC3), Scientific Campus of the University of the Basque Country, Leioa, Spain
[3]Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences, Uppsala, Sweden

Correspondence
Erica E. Packard
Email: erica.packard@slu.se

Funding information
Naturvårdsverket, Grant/Award Number: 802-0148-18; Svenska Forskningsrådet Formas, Grant/Award Number: 2020-01105

Handling Editor: Adam Frew

Abstract

1. Ectomycorrhizal fungi that produce oxidative enzymes—ectomycorrhizal decomposers—may limit soil carbon stocks while maintaining forest productivity in nutrient-poor forest soils by mobilising nitrogen from organic matter. Yet, these fungi are difficult to study in laboratory experiments. Here, we used a correlation-based analysis of field-measured properties to study traits of these unculturable fungi.
2. Two datasets were used to test hypotheses on the effect and response traits of ectomycorrhizal decomposers. Based on samples at the centimetre scale, correlations between fungal abundances and manganese peroxidase activity were tested, enabling assignment of potential important taxa. In a national scale inventory, the niche(s) of the assigned ectomycorrhizal decomposers, concerning mean stand age and soil fertility, were investigated.
3. We found 10 ectomycorrhizal taxa that were significantly co-localised with manganese peroxidase hotspots. Collectively, in pine-dominated forests these taxa were most frequent in relatively young stands with more fertile soils, whereas in spruce-dominated forests, they were most frequent in stands with more nutrient-poor soils. However, individual taxa varied in their responses.
4. There was evidence for niche variation related to stand age and soil fertility among the 10 investigated taxa, suggesting that they do not share one common niche and that ectomycorrhizal decomposers may contribute to oxidative decomposition across a variety of forests with different management histories.

KEYWORDS
ectomycorrhizal fungi, effect traits, forest management, fungal communities, manganese peroxidases, response traits, soil inventory, soil nutrients

[Correction added on 17 June 2025, after first online publication: Section headings are renumbered.]

1 | INTRODUCTION

Decomposition of organic matter by oxidative enzymes, such as manganese peroxidases (Mn-peroxidases), is a crucial step in carbon and nutrient cycling in organic forest soils (Jones et al., 2020; Kellner et al., 2014; Kranabetter, 2019; Stendahl et al., 2017; Talbot et al., 2013). In the presence of Mn^{2+} and H_2O_2, Mn-peroxidases oxidise organic compounds that are resistant to hydrolysis, such as lignins, melanins and tannin complexes (Kirk & Farrell, 1987). Previous observations of negative correlations between available manganese and carbon stocks have been attributed to oxidation by Mn-peroxidases (Kranabetter, 2019; Stendahl et al., 2017; Zhang et al., 2024). Therefore, oxidative decomposition is of interest when modelling ecosystem and global carbon (C) cycling (Baskaran et al., 2017; Chen et al., 2018; McGuire & Treseder, 2010; Moorhead & Sinsabaugh, 2006). The capacity to produce Mn-peroxidases is phylogenetically dispersed across the fungal class Agaricomycetes, including lineages that evolved ectomycorrhizal symbiosis (Floudas et al., 2012; Ruiz-Dueñas et al., 2021). Current research on soil C stabilisation focuses on interactions of microbially processed C with minerals and physical protection of C in aggregates (Lehmann & Kleber, 2015; Schmidt et al., 2011). However, the uppermost soil horizon in boreal forests consists of organic matter with insignificant mineral content (Lindahl et al., 2007). Thus, intrinsic biochemical properties of the litter, chemical stabilisation and the occurrence of fungi with enzymes capable of decomposing persistent organic matter are important factors in regulating C and nutrient dynamics in the organic layer. However, the key fungal species that are relevant for oxidative decomposition and regulation of these enzymes remain largely unknown.

Evidence of oxidative decomposition in the presence of ectomycorrhizal *Hydnellum* (Hintikka & Näykki, 1967) and *Hysterangium* (Cromack et al., 1988; Entry et al., 1991) mats was observed as early as the late 1960s, but recent research on ectomycorrhizal fungi with oxidative capacity (i.e. ectomycorrhizal decomposers) has focused on the genus *Cortinarius* (Bödeker et al., 2014; Defrenne et al., 2023; Lindahl et al., 2021; Pellitier & Zak, 2021). Peroxidase encoding genes have also been detected in some species from the ectomycorrhizal genera *Russula* and *Lactarius* (Russulales), *Tylospora* and *Piloderma* (Atheliales), *Hebeloma* and *Hygrophorus* (Agaricales), and *Gomphus* and *Gautieria* (Gomphales), but are apparently absent in species from the orders Boletales, Cantharellales, Sebacinales and Thelephorales (Bödeker et al., 2009, 2014; Chambers et al., 1999; Miyauchi et al., 2020). However, it cannot be inferred that peroxidases produced by ectomycorrhizal fungi are relevant for decomposition based on gene presence alone (Barbi et al., 2020). Yet, *Cortinarius* Mn-peroxidase genes were found to be actively transcribed in forest soils (Bödeker et al., 2014; Hasby, 2022), and large reductions (over 80% decrease) in Mn-peroxidase activity were observed following the removal of ectomycorrhizal fungi by root trenching (Sterkenburg et al., 2018) or fire-induced tree mortality (Pérez-Izquierdo et al., 2021), suggesting that active enzymes are produced and quantitatively relevant by at least some species within the genus. Both Lindahl et al. (2021) and Bödeker et al. (2014) observed that there may not be strong phylogenetic conservation of Mn-peroxidase production within genera and that taxonomic affiliation alone is also not sufficient to infer relevance of ectomycorrhizal species for decomposition. Thus, the key species of 'ectomycorrhizal decomposers' still remain to be pinpointed. Ectomycorrhizal fungi display a high degree of patchiness at the meter scale (Pickles et al., 2010), but expression of Mn-peroxidases is not likely to be evenly distributed throughout mycelia. Hotspots of Mn-peroxidase activity occur not only at the micrometre scale between fungal hyphae and their substrate (Keiluweit et al., 2015) but also at the centimetre-to-metre scale within single fungal mycelia (Šnajdr et al., 2008). The patchiness of both ectomycorrhizal communities and enzyme activities opens up possibilities to assign likely decomposers through correlative inference. Such correlative studies linking enzyme activity with the presence of specific fungi on the spatial scale of individual mycelia are one of the few possible approaches to assign ectomycorrhizal decomposers (Bödeker et al., 2014), since most ectomycorrhizal fungi cannot be cultivated.

The mobilisation of persistent organic nitrogen by ectomycorrhizal fungi through oxidative enzyme activity has a major influence on ecosystems (an effect trait) but also simultaneously modulates the ectomycorrhizal fungal niche (a response trait; Koide et al., 2014). Response traits refer to the properties of organisms that define how they respond to their environment, i.e. their ecological niches, while effect traits refer to traits that influence abiotic conditions and other species in their surrounding environment (Lavorel & Garnier, 2002). Effect traits that have major relevance for ecosystem processes and a low degree of redundancy, i.e. are linked/synonymous with a narrow set of response traits in certain species, are particularly important to understand fungal influences on ecosystem processes, and how these will be altered by environmental change (Allison & Martiny, 2008; Crowther et al., 2014; Koide et al., 2014). In Sweden, most forests are intensely managed through clear-cutting and replanting resulting in even-age stands. Mean stand age has a marked effect on the soil nutrient status and fungal communities (Kyaschenko et al., 2017; Twieg et al., 2007; Wallander et al., 2010). Ectomycorrhizal fungal community composition may shift in response to changes in the below-ground input of symbiotic C, and there is evidence of variation among ectomycorrhizal fungi in their C requirements and growth rate (Jörgensen et al., 2023; Saikkonen et al., 1999). Presumably, decomposition by Mn-peroxidases has a high energy requirement due to concurrent production of H_2O_2 (Hagenbo et al., 2019; Kirk & Farrell, 1987; Lindahl & Tunlid, 2015; Shimizu et al., 2005). In contrast, strong constraints on nutrient availability in older forest stands may increase the competitive advantage that ectomycorrhizal decomposers have compared with ectomycorrhizal fungi that lack this capability, in spite of larger C costs (Hagenbo et al., 2018; Kyaschenko et al., 2017). During periods of elevated mineralisation (in younger stands following tree harvest or wildfires) and N deposition (fertilisation and atmospheric N deposition), plant below-ground C allocation decreases (Högberg et al., 2003), which as proposed by van der Linde et al. (2018), may hamper ectomycorrhizal decomposers that grow slowly and have high C requirements (Agerer, 2001; Jörgensen et al., 2024; Wasyliw & Karst, 2020). Furthermore, variation in inherent soil fertility affects peroxidase activity (Jörgensen et al., 2024). Therefore, in comparison with other ectomycorrhizal fungi, ectomycorrhizal decomposers should be particularly sensitive to declines in below-ground C supply and physical disturbances to the soil (Argiroff et al., 2022; Defrenne et al., 2019; Lilleskov et al., 2002; Lindahl et al., 2021).

In line with the reasoning above, Lilleskov et al. (2011) proposed that ectomycorrhizal fungi that tend to form hydrophobic mycelial cords (e.g. *Cortinarius* species) or dense mycelial mats (e.g. many members of the family Bankeraceae and subclass Phallomycetidae) are associated with enhanced organic matter decomposition and are preferably found in nutrient-poor soils. This framework has been commonly applied to suggest that ectomycorrhizal decomposers may have their main niche in older, nutrient-poor forests, leading to the perpetuation of organic matter turnover, even as N-limitation intensifies during ecosystem retrogression (Clemmensen et al., 2015; Dynarski & Houlton, 2020). Thus, there is a risk that stand-replacing forestry, through host-tree removal and increased soil nutrient availability, may interfere with the functionality of this group of fungi (Kyaschenko et al., 2017; Lindahl et al., 2021).

The objective of this study was to pinpoint the most important ectomycorrhizal taxa related to local Mn-peroxidase activity (as an effect trait) and to identify the niche of these taxa (their response traits) with respect to forest stand age and soil fertility. Our *a priori* hypotheses were tested in two connected steps (Figure 1). A regional dataset with fine-scaled sampling was used to study the Mn-peroxidase activity as an effect trait of ectomycorrhizal decomposers, based on our first hypothesis:

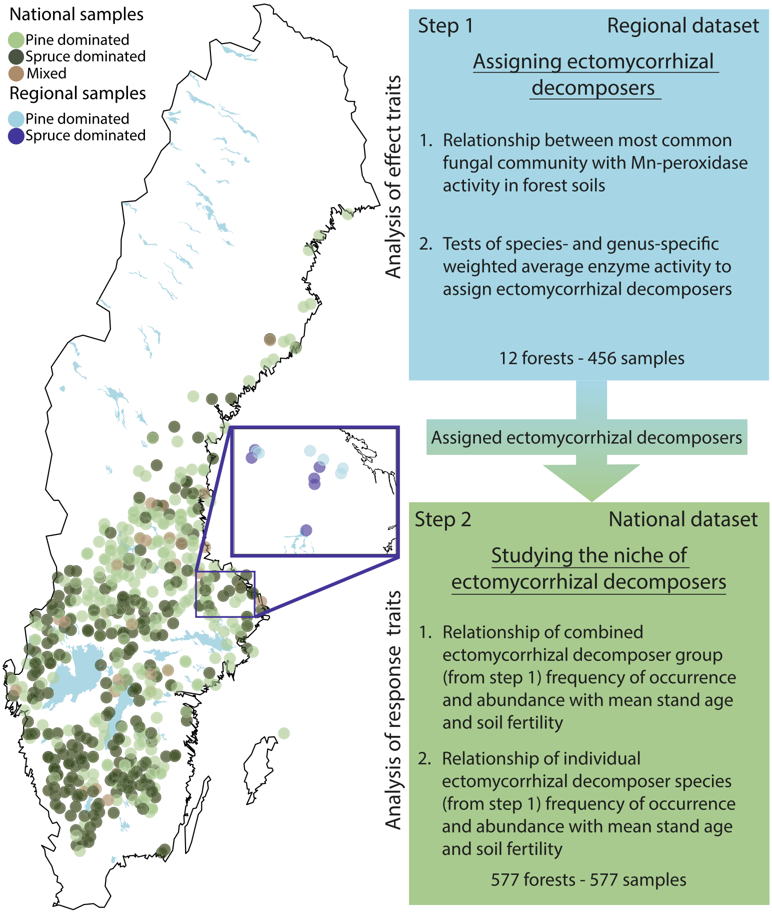


FIGURE 1 Distribution of 12 regional and 577 national sampling sites within Sweden and structure of data analysis. The regional samples were collected for a study focussed on local spatial heterogeneity and are used in Step 1 (Figure S1). The national samples were collected as a part of the Swedish Forest Soil Inventory and are used in Step 2. The national sites have >90% basal area of pine and spruce, and were categorised as pine-dominated or spruce-dominated (>60% basal area either pine or spruce). Stand age ranged from 15 to >80 years for the regional sites and from 10 to 215 years for the national sites (Figure S2).

1. That fungal community composition would be predictive of Mn-peroxidase activity on the mycelial scale (cm to m), and that some ectomycorrhizal species from genera with documented Mn-peroxidase genes would correlate positively with community-level Mn-peroxidase activity.

Subsequently, a national dataset was used to study the realised niche of these fungi, based on our second hypothesis:

2. That ectomycorrhizal Mn-peroxidase-producing fungi, as defined in our first analysis, would correlate positively with forest stand age and negatively with soil fertility.

2 | MATERIALS AND METHODS

2.1 | Soil sampling and analyses

2.1.1 | Regional dataset

Soil samples were collected from 12 boreal forests differing in dominant tree species and stand age (Figure 1; Table S1). No permits were required for fieldwork. In early October 2019, we collected 38 soil cores (2.5 cm in diameter) at different spatial distances from each other (0.1–10 m) following a hierarchical nested sampling design implemented in each of the 12 forests (456 samples total; Figure S1). This design was intended for a separate study focused on assessing spatial heterogeneity of various soil parameters on the meter scale (Lindahl et al., 2023), but here we focused on links between Mn-peroxidase activity and fungal communities. Upon collection, all green plant parts and any mineral soil were removed, retaining only the organic mor layer (the O-horizon, including litter). The soils were stored at −20°C from the time of sampling till further analyses. Samples were freeze-dried, weighed and milled. Total C and N were determined with an Isotope ratio mass spectrometer (DeltaV; Thermo Fisher Scientific, Bremen, Germany) coupled to an elemental analyser (Flash EA 2000; Thermo Fisher Scientific). Soil pH was measured in a 1:5 (v:v) ratio of dry soil to deionised water slurry with a 744 pH meter (Metrohm, Herisau, Switzerland).

Potential enzyme activity of Mn-peroxidase was measured in freeze-dried soils (Valášková & Baldrian, 2006) from the regional dataset using 3-Methyl-2-benzothiazolinone hydrazine hydrochloride monohydrate (MBTH) and 3-(dimethylamino)benzoic acid (DMAB) in a coupled colorimetric assay (Supporting Information). In brief, activity was measured in 200 μL reactions containing 50 μL of soil extracted enzymes in 50 mM sodium acetate (pH 5) and 150 μL of reaction solution containing 0.05 mM MBTH, 2.5 mM DMAB, 25 mM sodium lactate (pH 4.5), 25 mM sodium succinate (pH 4.5), 0.25 mM H_2O_2 and either 0.1 mM $MnSO_4$ or 0.2 mM EDTA (i.e. no reactive Mn present). The changes in absorbance were measured at 590 nm in a SpectraMax Plus 384 Microplate Reader (Molecular Devices, Sunnyvale, USA) over 45 min to determine the rate of activity. To determine Mn-dependent activity, reactions with EDTA were subtracted from reactions with $MnSO_4$ (Baldrian et al., 2000; Ngo & Lenhoff, 1980). Total peroxidase activity was determined as the difference in the rate of activity between reactions with and without H_2O_2.

2.1.2 | National dataset

As a part of the Swedish Forest Soil Inventory, soil samples across all of Sweden were collected from sites chosen based on a grid design (Fridman et al., 2014). We used a subset of 577 sites in similar climatic conditions to the regional sites that were sampled between 2014 and 2021 (Temperature sum between 900 and 1400; Odin et al., 1983; Figure 1). We further restricted to only productive forests (annual growth >1 $m^3 ha^{-1}$) with a mean stand age greater than 10 years (a different subset of the samples was previously used in Lindahl et al., 2021). This age threshold was made to avoid confounding effects of recent clear-cutting forestry on the ectomycorrhizal community (Wallander et al., 2010). Stand age was assessed as the average tree age weighted by their basal area (Nilsson et al., 2013). Furthermore, we selected only coniferous sites (90% of the basal area as *Pinus sylvestris* L. and/or *Picea abies* L.) with either pine or spruce dominance. No permits were required for the use of data from these field sites.

Soil was collected separately for analyses of soil parameters and fungal communities as described in Lindahl et al. (2021). In brief, tree parameters were measured within a 10 m radius circle, and within that, two concentric circles were sampled for fungal communities (1 m radius) and soil properties (0.6 m radius). For the fungal community, samples from the uppermost 10 cm of the soil were pooled from five points, and for the soil properties, a 10 cm diameter corer was used to sample the organic layer until at least 1.5 L was collected (1–9 cores). The soil was frozen at −20°C within 6 days of sampling (on average) and then freeze-dried and finely ground with a ball mill (Bertin Technologies; Montigny-le-Bretonneux; France). C and N concentrations were measured from dry sieved soil using an elemental analyser (TruMac CN; LECO, St. Joseph, USA), and soil pH was determined with an Aquatrode Plus Pt1000 pH meter (Metrohm) in a 1:2.5 (dry weight: volume) ratio with deionised water.

2.2 | Fungal community analysis

DNA was extracted from 100 mg of homogenised soil from both sample sets using the NucleoSpin® Soil kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. ITS2 markers were PCR amplified using fungal specific primers gITS7 (Ihrmark et al., 2012) and a 3:1 mix of the two reverse primers ITS4 (White et al., 1990) and ITS4arch (Kyaschenko et al., 2017), fitted with unique 8-bp sample identification tags (Clemmensen et al., 2023). PCR reactions (50 μL volume) were run: 5 min at 94 (regional samples) or 95°C (national samples) for denaturation, 20–35 cycles of 30 s at

94 or 95°C, 30 s at 56°C and 30 s at 72°C, and a final elongation period at 72°C for 7 min. To reduce fragment length bias, the number of PCR cycles was minimised to obtain 'weak but visible' bands on electrophoresis gels (Castaño et al., 2020). For samples from the national dataset, all PCR products were run in duplicates that were pooled before cleaning with AMPure (Beckman Coulter, Indianapolis, USA). PCR products from each sample of the regional dataset were purified with Sera-Mag magnetic carboxylate modified particles (Hydrophobic; GE Healthcare, Chicago, USA), measured with Qubit fluorometer (Thermo Fisher Scientific) and mixed in equal concentrations into six composite pools before purifying pools with E.Z.N.A cycle pure kit (Omega bio-tek; Nocross, USA). BioAnalyzer was used to check the amplicon size distribution (Agilent Technologies, Santa Clara, USA). Adaptor ligation and PacBio (Pacific Biosciences, Menlo Park, USA) sequencing were performed by SciLifeLab NGI (Uppsala, Sweden) using one SMRT cell per pool. National samples collected between 2014 and 2016 were sequenced on the RSII platform and samples collected between 2017 and 2021, as well as all regional samples, were sequenced with Sequel 1 (both PacBio technologies).

Sequence quality control and clustering into species hypothesis (hereafter species) (Kõljalg et al., 2013) was performed in the SCATA pipeline (Ihrmark et al., 2012). Only sequences with tag matching at both ends, and >90% match with both primers were accepted. After removal of primer sequences and global singletons, pairwise comparisons were made with USEARCH (Edgar, 2010) followed by single-linkage clustering, with 98.5% similarity required for sequences to enter clusters. An internal annotated reference database was included during clustering to assign sequences to species, with additional manual identification performed with UNITE (Abarenkov et al., 2024).

2.3 | Statistics

Data analysis was performed in R v4.4.1 (R Core Team, 2025). The significance level used was $\alpha = 0.05$ for two-sided tests and $\alpha = 0.1$ for one-sided tests. We used dplyr (Wickham et al., 2023), ggplot2 (Wickham, 2016), factoextra (Kassambara & Mundt, 2020), patchwork (Pedersen, 2022), tmap (Tennekes, 2018) and vegan (Oksanen et al., 2022) for data processing and visualisations. Scripts for analysis are available on Zenodo.

2.3.1 | Regional dataset: Relationships between Mn-peroxidase activity and fungal community composition

To test for spatial autocorrelation in Mn-peroxidase activity between cores, the standard deviation of the three points in each triangle (Figure S1) was calculated. We used a linear model to test the relationship between variation in Mn-peroxidase activity (scaled and centred by site) within triangles and the size of triangles (0.1, 0.3, 1.0, 3.0 and 10.0 m), dominant host-tree species (spruce or pine) and stand age (young or old) as categorical explanatory factors, including the interactions between triangle size and tree species or stand age. Variation in Mn-peroxidase activity within triangles was not significantly related to triangle size, that is the distance between cores (ANOVA; $df = 4$; $F = 0.91$; $p = 0.460$). Spatial autocorrelation was not observed at any scale, so patchiness of enzyme activity should occur at scales <0.1 m. Therefore, the distance between cores was not considered, and each core was treated as an independent sample with respect to Mn-peroxidase activity in subsequent analyses.

We aimed to test whether the most frequent species in the regional samples (1212 species from the total fungal community that were present in ≥10 of the 456 samples and ≥3 of the 12 sites) explained variation in Mn-peroxidase activity (Figure S3). The square root-transformed relative abundances (Hellinger-transformation) of the fungal communities were analysed in a canonical correspondence analysis (CCA) with log-transformed Mn-peroxidase activity as a predictor, which was evaluated with PERMANOVA with 1000 permutations constrained to within sites to focus on within-site variation.

2.3.2 | Regional dataset: Exploring for ectomycorrhizal decomposers

To assign species as potential ectomycorrhizal decomposers, we determined the species-specific weighted average enzyme activity (WAE_{sp}; Equation 1; Bödeker et al., 2014) of the most frequent Agaricomycetes species (214 species of any ecology, i.e. ectomycorrhizal and saprotrophic). A higher WAE_{sp} indicates species with higher relative abundance in samples with high peroxidase activity. For this, Mn-peroxidase and total peroxidase activity (untransformed) were first centred by site (value minus the arithmetic mean) to reduce inter-site variation but maintain intra-site variation. To assess whether the trait was phylogenetically clustered at the genus level, we taxonomically aggregated species by genera and repeated the assessments of weighted average enzyme activity (WAE_{gen}; Equation 2).

$$\text{WAE}_{\text{sp}} = \frac{\sum_{i=1}^{n} E_i P_{si}}{\sum_{i=1}^{n} P_{si}}. \tag{1}$$

$$\text{WAE}_{\text{gen}} = \frac{\sum_{i=1}^{n} E_i P_{gi}}{\sum_{i=1}^{n} P_{gi}}. \tag{2}$$

(E_i is peroxidase activity in sample i; P_{si} is the relative abundance of the species in sample i; P_{gi} is the relative abundance of the genus in the sample; n is the total number of samples (456)).

Each species and/or genus was evaluated with a one-sided permutation test for their relationship with both Mn-peroxidase and total peroxidase activity. A simulated random distribution of each *WAE* was formed by 10,000 re-samplings of the enzyme activity (E_i) within Equation (1) or (2), and each observed WAE was evaluated against its simulated distribution. *p*-values were determined based on the sum of observations in the simulated distributions that were ≥ the test statistic (WAE_{sp} or WAE_{gen}) divided by the total number of permutations. WAE_{sp} and WAE_{gen} were also tested to see which taxa had a negative relationship with peroxidase hotspots (i.e. sum of observations in the simulated distributions that were ≤ the test statistic).

Ectomycorrhizal species with an uncorrected *p*-value <0.1, for either Mn-peroxidase and/or total peroxidase activity, were assigned as ectomycorrhizal decomposers for subsequent analysis of their niche at the national scale. In addition, we chose a more exclusive approach for assigning ectomycorrhizal decomposers that also excluded ectomycorrhizal species lacking Class-II peroxidase genes (at the genus level) based on currently available genomic data and fungi of unknown or saprotrophic ecologies.

For all sequenced species within the Agaricomycetes genera observed in this study, the number of genes coding for Class-II peroxidases was extracted from Miyauchi et al. (2020) and the Joint Genome Institute (mycocosm.jgi.doe.gov; Balasundaram et al., 2018; Branco et al., 2015; Eastwood et al., 2011; Gaskell et al., 2017; Grigoriev et al., 2014; Harder et al., 2024; Kohler et al., 2015; Lebreton et al., 2022; Lofgren et al., 2021; Looney et al., 2022; Martinez et al., 2009; Miyauchi et al., 2020; Nagy et al., 2016). We determined the mean number of Class-II peroxidase genes per genus. In cases where no genomes were sequenced within a genus, data from species within the same family were used. Kendall's Rank correlation test was used to test whether WAE_{sp} correlated with the mean number of Class-II peroxidase genes across species within the genera.

2.3.3 | National dataset: Niche(s) of ectomycorrhizal decomposers

Soil pH and N/C were strongly co-linear, so these variables were incorporated into a principal component analysis (after centring and scaling), where the first PC axis was interpreted as a soil fertility index (Figure S4). Fisher's exact test was used to test whether the ectomycorrhizal decomposer group and the individual assigned species occurred more frequently in pine- or spruce-dominated forests. A linear model with (log-transformed) relative abundance of the ectomycorrhizal decomposer group, when present, and dominant tree species as a fixed factor was used to test whether their abundance differed between forest cover types. For subsequent analyses, the data were analysed both across all forest cover types (577 sites) and split into spruce-dominated (253 sites) and pine-dominated (295 sites) datasets (sites with 50/50 host species dominance were omitted).

Mean stand age and the soil fertility index were used as independent predictor variables to model the niche of the combined ectomycorrhizal decomposer group assigned in the regional dataset. Separate models were made with stand age either as a continuous log-transformed variable or a categorical variable (young [<70 years] or old [>70 years]). Forests with a stand age less than 70 years have likely been clear-cut, while forests with a stand age more than 70 years likely have a longer forest continuity (Antonson & Jansson, 2011; Lundmark et al., 2013). By testing stand age in two ways, we aimed to assess whether the history of clear-cutting or mean stand age was more relevant to the niche of ectomycorrhizal decomposers. In both presence/absence (frequency of occurrence) and relative abundance models, the sequencing platform was included as a random factor. Frequency of occurrence of the combined assigned ectomycorrhizal decomposer group was tested with generalised linear mixed models (*glmer* from lme4; Bates et al., 2015) with binomial linking functions, and relative abundance (log-transformed) was tested with linear mixed models (*lmer* from lme4). The relative abundance of the ectomycorrhizal decomposer group, when present, was tested either as a proportion of the total fungal community or of the ectomycorrhizal community. In cases where data appeared unimodal, we tested polynomial terms for both stand age and the soil fertility index. Evaluation of multicollinearity between predictor variables (soil fertility and stand age) was evaluated (*vif* function from car; Fox & Weisberg, 2019) and was unproblematic, as variance inflation factors were less than three in all models tested. Adjusted R^2 were determined with the r.*squaredGLMM* function from the MuMIn package (Bartoń, 2023).

Correlations between stand age or soil fertility with the presence/absence of individuals of ectomycorrhizal decomposer species were evaluated with generalised linear models with a binomial linking function. *p*-values were adjusted for multiple testing using the false discovery rate method (Benjamini & Hochberg, 1995).

3 | RESULTS

3.1 | Regional dataset: Mn-peroxidase activity as a trait of ectomycorrhizal decomposers

Fungal community composition was correlated with Mn-peroxidase (PERMANOVA; df = 1; proportion explained of CCA = 0.003; $F = 1.28$; $p \leq 0.001$) and total peroxidase activity (PERMANOVA; df = 1; proportion explained of CCA = 0.003; $F = 1.27$; $p = 0.004$) at the scale of individual soil cores. Twenty-nine fungal taxa had significantly higher WAE_{sp} than expected by random variation, of which 16 were ectomycorrhizal (Table 1). Ten of the 29 taxa lacked Class-II peroxidase genes: *Coniophora puteana*, *Postia ptychogaster*, *Serpula himantioides*, *Sistotrema* sp., *Suillus luteus*, *Tomentellopsis submollis*, *Thelephora terrestris*, *Thelephora longisterigmata*, *Thelephora eucoerulea* and *Tylospora asterophora* (Shah et al., 2016). Nine of the 29 taxa were saprotrophic species of known ligninolytic capacity: two *Trechispora* taxa (Trechisporales); *Galerina* sp., *Galerina calyptrata*, *Gymnopilus penetrans*, *Mycena rubromarginata* and *Mycena sanguinolenta* (Agaricales); *Ganoderma lucidum* (Polyporales); and *Rescinicium bicolor* (Hymenochaetales) (Floudas, 2021). *Coniophora puteana*, *Postia ptychogaster* and *Serpula himantioides* are considered 'brown-rot' saprotrophs, while *Sistotrema* sp. has uncertain ecology. The assigned ectomycorrhizal decomposers consisted of 10 taxa:

TABLE 1 Fungal taxa with significantly higher estimated specific Mn-peroxidase and/or total peroxidase activity than expected by random variation tested each with one-sided permutation tests.

			MnP			Total peroxidase		
Species	**UNITE species hypothesis**	**Mean Class-II peroxidase genes ± SE (number of species in genus with published genomes)**	***p*-value**	**WAEsp rank order**	**WAEgen rank order**	***p*-value**	**WAEsp rank order**	**WAEgen rank order**
Lactarius necator	**SH0961100.10FU**	**2.8 ± 0.2 (10)**	**<0.001**	**1**	**15**[a]	**0.029**	**4**	**25**
Cortinarius anomalus	**SH1017270.10FU**	**9 (1)**	**0.022**	**2**	**58**		**16**	**56**
Trechispora sp.2	SH0841098.10FU	12 (1)[b]	0.023	3	21	0.057	6	38
Mycena rubromarginata	SH0748954.10FU	17.2 ± 0.4 (25)	0.03	4	36	0.099	17	36
Gautieria* cf. *otthii	**SH0932000.10FU**	**25 (1)**	**0.029**	**5**	**1**[a]	**0.067**	**11**	**3**[a]
Suillus luteus	**SH0946483.10FU**	**0 (22)**	**0.019**	**6**	**20**	**0.026**	**7**	**15**
Russula aquosa	**SH0944250.10FU**	**1.63 ± 0.2 (8)**	**0.026**	**7**	**23**		**27**	**28**
Rescinicium bicolor	SH0860289.10FU	15 (0)	0.064	8	2[a]		24	6
Coniophora puteana	SH0883820.10FU	0 (2)	0.069	10	6[a]	0.068	15	4[a]
***Cortinarius fulvescens* coll.**	**SH1017507.10FU**	**9 (1)**	**0.080**	**11**	**58**	**0.072**	**14**	**56**
Serpula himantioides	SH0992263.10FU	0 (3)	0.092	12	4[a]		28	7
***Cortinarius comptulus* coll.**	**SH0986335.10FU**	**9 (1)**	**0.059**	**13**	**58**	**0.007**	**2**	**56**
Postia ptychogaster	SH0755398.10FU	0 (2)	0.094	14	7		35	12
Trechispora sp.1	SH0888744.10FU	12 (1)[b]	0.076	15	21		32	38
Tomentellopsis submollis	**SH0936881.10FU**	**0 (2)**[b]	**0.099**	**16**	**33**		**44**	**46**
Ganoderma lucidum	SH0762718.10FU	10.2 ± 0.3 (5)	0.062	21	26		42	16
Thelephora terrestris	**SH0919166.10FU**	**0 (2)**	**0.045**	**23**	**30**		**43**	**23**
Thelephora eucoerulea	**SH0920796.10FU**	**0 (2)**		**26**	**30**	**0.060**	**12**	**23**
Galerina calyptrata	SH0994936.10FU	22 (1)		27	12	0.028	8	8
Tylospora asterophora	**SH0826300.10FU**	**0 (5)**[b]		**29**	**16**	**0.084**	**26**	**13**
Lactarius tabidus	**SH0961123.10FU**	**2.8 ± 0.2 (10)**	**0.084**	**30**	**15**[a]		**55**	**25**
Mycena sanguinolenta	SH0748984.10FU	17.2 ± 0.4 (25)		37	36	0.021	18	36
Sistotrema sp.	SH1030361.10FU	0 (1)		49	74	0.055	13	57
Russula rhodopus	**SH0956387.10FU**	**1.63 ± 0.2 (8)**		**73**	**23**	**0.003**	**1**	**28**
***Cortinarius mucosus* coll.**	**SH1017686.10FU**	**9 (1)**		**78**	**58**	**0.031**	**10**	**56**
Galerina sp.	SH0886132.10FU	22 (1)		99	12	0.026	9	8
Gymnopilus penetrans	SH0886174.10FU	15 (1)		117	46	0.010	5	2[a]
Thelephora longisterigmata	**SH0918425.10FU**	**0 (2)**		**125**	**51**	**0.007**	**3**	**26**
Cortinarius spilomeus	**SH1017671.10FU**	**9 (1)**		**196**	**58**	**0.093**	**20**	**56**

Note: All species hypotheses with an unadjusted p-value <0.1 are shown. Ectomycorrhizal taxa are in bold (guild assignment in accordance with FungalTraits database; Põlme et al., 2020). Taxa considered as potential ectomycorrhizal decomposers are highlighted in grey. $\mathrm{WAE}_{\mathrm{sp}}$ and $\mathrm{WAE}_{\mathrm{gen}}$ are ranked from low (stronger correlation) to high (weaker correlation). Mean number of Class-II peroxidase genes per species within genera is extracted from Miyauchi et al. (2020) and the Joint Genome Institute Mycocosm portal.

[a] Significant at the genus level.

[b] Gene counts from within family.

Cortinarius anomalus, *C. comptulus* coll., *C. fulvescens* coll., *C. mucosus* coll., *C. spilomeus*, *Gautieria* cf. *otthii*, *Lactarius necator*, *L. tabidus*, *Russula aquosa* and *R. rhodopus*.

Mean Class-II peroxidase gene counts per genus (or per family in certain cases) and WAE_{sp} did not correlate across the 214 studied Agaricomycete species (Kendall's rank; $z=-0.155$; $p=0.877$) or 76 genera (Kendall's rank; $z=-0.153$; $p=0.878$). The share of genera that had a significant relationship with estimated Mn-peroxidase activity (WAE_{gen}) was smaller (9 of 75 genera) than that of species (WAE_{sp}; 29 of 214 species). The nine genera significantly related to Mn-peroxidase and/or total peroxidase activity (WAE_{gen} with unadjusted *p*-values <0.1) were *Gautieria*, *Coniophora*, *Lactarius*, *Postia*, *Naucoria*, *Rescinicium*, *Gymnopilus*, *Clavulina* and *Serpula* (Table S2). *Gautieria*, *Rescinicium* and *Serpula* were represented by only one species each (thus WAE_{gen} and WAE_{sp} did not differ). Other genera containing taxa with significant WAE_{sp}, *Cortinarius*, *Galerina*, *Ganoderma*, *Mycena*, *Russula*, *Sistotrema*, *Suillus*, *Thelephora*, *Tomentellopsis*, *Trechispora* and *Tylospora*, were not significant at the genus level (WAE_{gen}).

There were 44 taxa that were negatively co-localised with Mn-peroxidase activity hotspots (Table S3). Genera that contained both positively and negatively correlated species included *Cortinarius*, *Lactarius*, *Mycena*, *Russula*, *Sistotrema*, *Thelephora* and *Trechispora*.

3.2 | National dataset: Niche(s) of ectomycorrhizal decomposers

As a group, the assigned ectomycorrhizal decomposers were present in 47.6% of the samples of the national inventory (individual taxa average 7.3 ± 4.6%; range between 1% and 15%), and when present, they accounted for on average 1.2% of the total fungal sequences and 4.5% of the ectomycorrhizal sequences. There were 473 ectomycorrhizal species hypotheses identified in the national inventory.

The assigned ectomycorrhizal decomposer group was collectively more frequent (Fisher's exact; Figure S6a; odds ratio = 2.90; 95% CI = 2.02–4.18; $p \leq 0.001$) and had a marginally higher relative abundance (ANOVA; Figure S6b; df = 1; $F=3.48$; $p=0.063$) in spruce-dominated forests. However, *C. mucosus* coll. was 2.2 times more frequent in pine-dominated forests (Figure S7; Table S4).

Across all the included forests, the group of assigned ectomycorrhizal decomposers tended to have a peak in frequency of occurrence in mature secondary forests (Figure 2a). The decline in the oldest forest stands was supported by a negative and significant polynomial term. However, the increase at early succession (~10–45 years; linear term) had weak statistical support (Table 2). This was also the case in pine-dominated stands but not in spruce-dominated stands (Figure 2b,c). There were no significant differences in the frequency

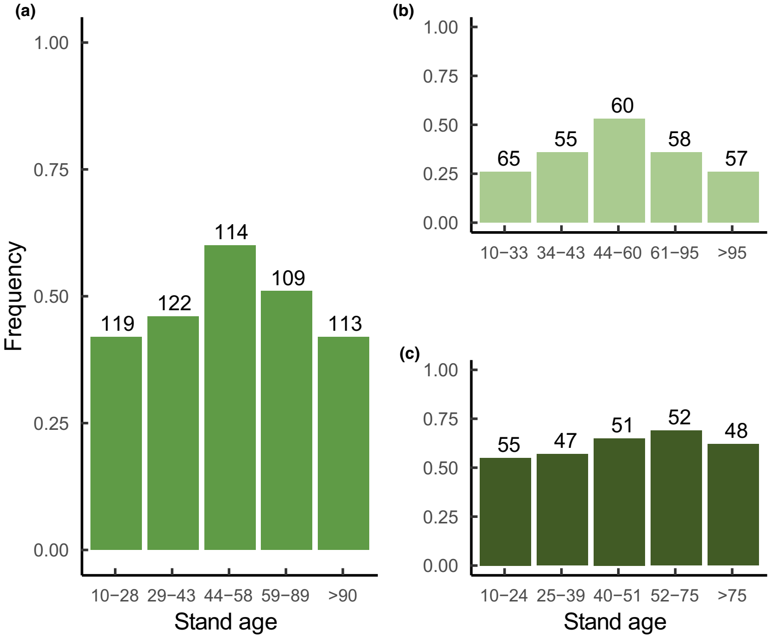


FIGURE 2 Frequency of occurrence of species assigned as ectomycorrhizal decomposers across forest stand ages in (a) all forests, (b) pine-dominated and (c) spruce-dominated forests. Stand age is sectioned into five evenly distributed bins (for graphical representation) with their inclusive age ranges shown on the *x*-axis. Numbers on top of bars indicate the total number of forests in each age range. Statistics are presented in Table 2.

TABLE 2 Correlations between combined frequency of occurrence of species assigned as ectomycorrhizal decomposers and stand age and soil fertility with sequencing platform as a random effect analysed by generalised linear mixed models.

		Stand age (log-transformed years)			Stand age (old vs. young)		
		Estimate (SE)	*p*-value	Model Pseudo delta R^2	Estimate (SE)	*p*-value	Model Pseudo delta R^2
All forests (577 sites)	Intercept	(+) 0.341 (0.28)		0.10	(−) 0.150 (0.24)		0.03
	Age	(+) 0.110 (0.09)	0.240		(−) 0.067 (0.19)	0.728	
	Age^2	**(−) 0.279 (0.08)**	**<0.001**		n.a		
	Soil fertility index	**(+) 0.329 (0.09)**	**0.001**		(+) 0.070 (0.7)	0.298	
	Soil fertility index^2	**(−) 0.157 (0.04)**	**<0.001**		n.a		
Pine-dominated (295 sites)	Intercept	(−) 0.257 (0.26)		0.14	(−) 0.642 (0.24)		0.05
	Age	(+) 0.199 (0.16)	0.210		(−) 0.132 (0.28)	0.633	
	Age^2	**(−) 0.531 (0.14)**	**<0.001**		n.a		
	Soil fertility index	**(+) 0.308 (0.12)**	**0.007**		**(+) 0.345 (0.11)**	**0.002**	
Spruce-dominated (253 sites)	Intercept	(+) 0.424 (0.30)		0.09	(+) 0.401 (0.31)		0.08
	Age	(+) 0.150 (0.14)	0.284		(−) 0.032 (0.33)	0.924	
	Soil fertility index	**(−) 0.337 (0.10)**	**<0.001**		**(−) 0.356 (0.10)**	**<0.001**	

Note: Polynomial terms of stand age (age^2) and/or soil fertility (soil fertility index^2) were tested when data appeared unimodal. Soil fertility index represents the first axis of a principal component analysis (PCA) with soil pH and N/C from all sites. *p*-values <0.05 are in bold.

of occurrence of the group between young or old forest stands (Table 2). While no individual species had a significant response to stand age in pine-dominated forests, in spruce-dominated forests some species responded, but in contrasting directions. Frequency of occurrence of *Cortinarius fulvescens* coll. was higher in older stands and *C. spilomeus* was lower (Figure S5; Table S5). *Cortinarius comptulus* coll., *Lactarius necator* and *Russula aquosa* were also marginally more frequent in older forests, yet *Russula rhodopus* tended to be less frequent ($p < 0.1$; Table S5).

The frequency of occurrence of the group had a unimodal response to soil fertility (Figure 3a). In spruce-dominated forests, they collectively declined with increasing soil fertility; yet, they had the opposite relationship to soil fertility in pine-dominated forests (Figure 3b,c; Table 2). As in the response to stand age, individual species also responded in varying directions to soil fertility. The individual taxa most strongly driving the overall pattern were *C. comptulus* and *C. fulvescens* in spruce-dominated forests (Figure 4b,d; Table S5) and *G.* cf. *otthii* and *R. aquosa* in pine-dominated forests (Figure 4a,c; Table S5).

There was no relationship between the relative abundance of the group of ectomycorrhizal decomposers, when present, when assessed either as a proportion of the whole fungal community or relative to other ectomycorrhizal fungi (Tables S6 and S7).

4 | DISCUSSION

4.1 | Mn-peroxidase activity as a trait of ectomycorrhizal decomposers

Manganese peroxidase activity was highly variable (Figure S3) but did not display spatial autocorrelation at scales >10 cm across the 12 forests studied here. Nevertheless, enzyme activities correlated with fungal community composition, and we explore this covariation to assign potential key ectomycorrhizal decomposers. The 10 taxa highlighted here should be relevant for oxidative decomposition of organic matter in Swedish forest soils. We note that our assignment of taxa is limited by the local context of the regional study; thus, we cannot extrapolate with certainty across all boreal forests. In line with previous observations (Bödeker et al., 2014; Lindahl et al., 2021; Pellitier & Zak, 2021), species from the genus *Cortinarius* made up half of the highlighted ectomycorrhizal decomposers in this study. Furthermore, *Gautieria* stands out as having a particularly high number of Class-II peroxidase genes (Miyauchi et al., 2020) and can be grown in pure culture with insoluble protein–tannin complexes as the sole nitrogen source, suggesting a capacity to mobilise recalcitrant nitrogen (Griffiths & Caldwell, 1992), which is consistent with a high WAE_{sp}. The ecology of the truffle-like fungus *Gautieria* is understudied, but production of peroxidases in combination with a tendency to produce dense mycelial mats would suggest that they could have a strong localised influence on the decomposition of soil organic matter. *Russula* and *Lactarius* species tend to have a relatively modest number of Class-II peroxidase genes (Table 1). Their mycorrhizal structures are morphologically classified as contact exploration types (Agerer, 2001), yet they tend to proliferate in organic ingrowth bags (Jörgensen et al., 2023), which together with a high WAE_{sp} of some members of these genera goes against the idea that organic nutrient mobilisation is primarily a trait of cord and/or mat-forming fungi. Among Agaricomycetes (ectomycorrhizal and saprotrophic), the trait of Mn-dependant oxidation did not appear to be phylogenetically clustered at the genus level: aggregation of species within genera weakened statistical patterns, particularly for more species-rich genera. Furthermore, several genera contain species with both high and low WAE_{sp} (e.g. *Cortinarius* and *Mycena*). It seems possible that not all species within a genus have active

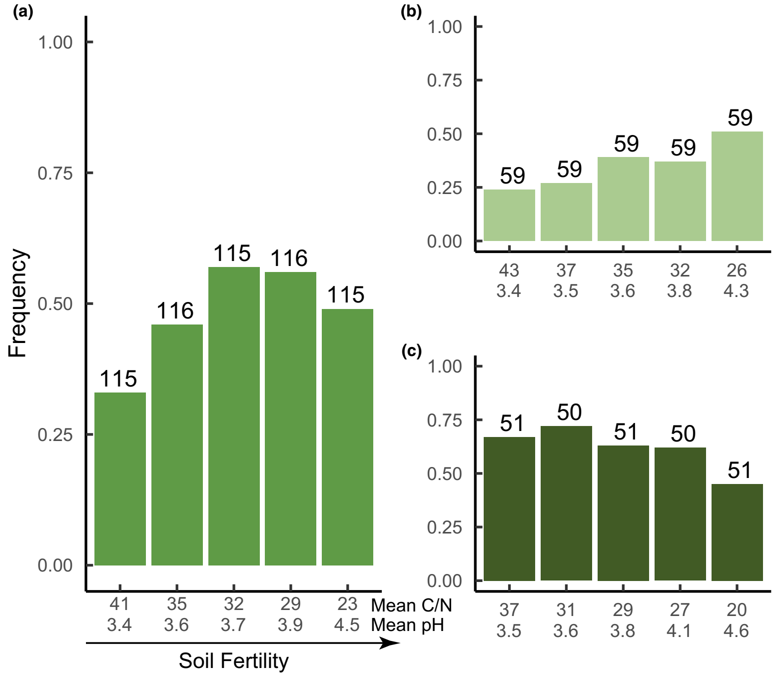


FIGURE 3 Frequency of occurrence of species assigned as ectomycorrhizal decomposers across soil fertility in (a) all forests, (b) pine-dominated and (c) spruce-dominated forests. Soil fertility is sectioned into five evenly distributed bins (for graphical representation) with their mean C/N and pH shown on the *x*-axis. Numbers on top of bars indicate the total number of forests in each soil fertility bin. Note that the range of mean C/N and pH is different between pine-dominated and spruce-dominated forests. Statistics are presented in Table 2.

Mn-peroxidase genes or expressed them under the observed conditions. This is not unexpected given that gene loss and duplication occurs at a slower pace than trait differentiation (Hess et al., 2021; Kohler et al., 2015). In particular, substantial production of Mn-peroxidases is not likely to be a general effect trait for all *Cortinarius* species, but has to be assessed at the species level, in concordance with Lindahl et al. (2021).

Fungal traits may be studied more directly and experimentally in pure culture (albeit under unrealistic conditions), but for fungi that are not amendable to isolation, correlations with field-measured properties, as used here, remain one of the few options. Although the fungal community composition explained a minor proportion of the variation in Mn-peroxidase activity, there was a link to the local composition of fungal species, as posed by our first hypothesis. The longevity of peroxidases in soil is largely unknown (Allison, 2006; Sinsabaugh, 2010), and the precision of our method depends on temporal and spatial synchronisation between Mn-peroxidase activity and the fungi that produce them. Localisation of extracellular enzymes to hotspots rather than an even distribution throughout mycelia may also contribute unexplained variation (Lindahl & Finlay, 2006), as supported by the observed lack of Mn-peroxidase spatial autocorrelation across the different spatial scales. Further, most species are infrequent, leading to low predictability. Presence of ITS sequences, especially at low relative abundances, could also be attributed to inactive fungal propagules. With this in mind, we selected a more lenient α value for our one-sided permutation tests, aiming to strike a balance between type-I and type-II error. Species from genera with a generally high number of Class-II peroxidase genes in genome-sequenced representative species did not necessarily have a high WAE_{sp} (except *Gautieria* cf. *otthii*). Thus, assigning decomposition traits to unculturable fungi is possible via statistical inference, but there are certainly many less frequent ectomycorrhizal decomposers that were not detected, even with this large sampling effort. Despite challenges in assigning traits based on community-level enzyme activity and ITS relative abundances, we found some signal, especially for the more frequent taxa, which may anyway be the most important for understanding ecosystem processes.

The assignment of traits based on correlative data can be susceptible to false positives. However, the probability of correctly assigning a trait seems reasonably high, given that 10 out of 16 ectomycorrhizal species assigned in our permutation-based analysis of WAE_{sp} also have evidence of genetic capacity within the genus, despite that most ectomycorrhizal fungi do not (Miyauchi et al., 2020).

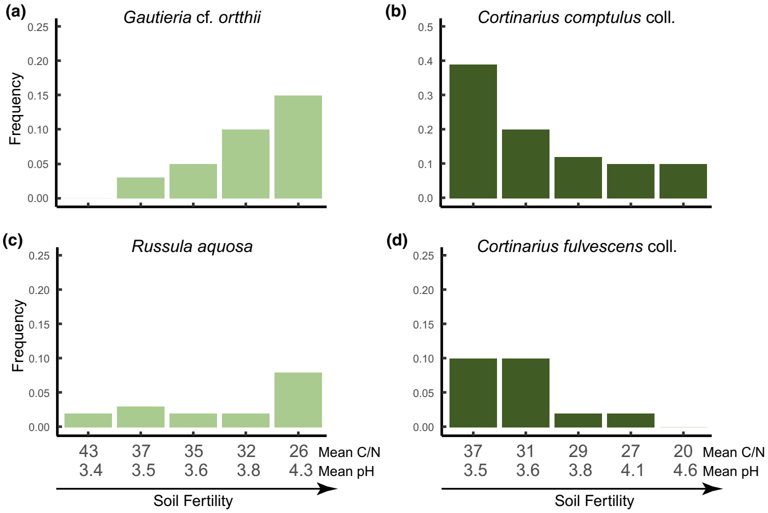


FIGURE 4 Frequency of occurrence (a) *Gautieria* cf. *otthii*, (b) *C. comptulus* coll., (c) *Russula aquosa* and (d) *C. fulvescens* coll. in pine-dominated (a, c; light-green) and spruce-dominated (b, d; dark-green) forests with varying soil fertility. Soil fertility is sectioned into five evenly distributed (59 pine-dominated forests or 50/51 spruce-dominated forests each) bins with the mean C/N and soil pH shown on the *x*-axis. Note that range of C/N and soil pH is different between pine-dominated and spruce-dominated forests. All show a significant relationship between frequency and soil fertility (*p*-value <0.05). Statistics are presented in Table S5.

Notably, *Suillus luteus* and several *Thelephorales* spp. were co-localised with Mn-peroxidase hotspots, yet do not have evidence of Class-II peroxidase genes. They may co-occur with peroxidase-producing fungi through several means of facilitation. Presumably, niche modification occurs during soil organic matter decomposition, as it does during wood decomposition (Hiscox et al., 2015). Furthermore, fungi lacking Mn-peroxidases may scavenge for degradation products of oxidative decomposition that they otherwise would not be able to access. In addition, *Suillus luteus*, which probably has decomposition mechanisms similar to Fenton reactions of 'brown-rot' fungi (Shah et al., 2016), could take advantage of H_2O_2 produced by Mn-peroxidase producers for their own non-enzymatic decay mechanisms (or vice versa, i.e. provide H_2O_2 that is hijacked by ectomycorrhizal decomposers).

4.2 | Niche(s) of ectomycorrhizal decomposers

We found no support for our hypothesis that previous clear-cutting would have long-lasting negative impacts on the presence of ectomycorrhizal decomposers. Actually, they occurred more frequently in mature secondary forests established after clear-cutting than in forests with mostly no history of clear-cutting. Despite that no consistent response to stand age was observed for all of the ectomycorrhizal decomposers in spruce-dominated forests, it was apparent that individual species tended to have their niches in either older or younger forests. Further, this varied between species in the same genus, highlighting that there is not only wide variation in response traits within genera but also that fungi with predicted similar effect traits (i.e. oxidative capacity) vary widely in their response to their environment (Koide et al., 2014). While some ectomycorrhizal decomposers, such as *Cortinarius acutus* s.l., are found increasingly present in older nutrient-poor forest stands (Lindahl et al., 2021), it is evident that this is not the rule for all. Splitting of stand age into categories of forests older or younger than 70 years explained less variation in the frequency of ectomycorrhizal decomposers than stand age as a continuous variable. Although both models had low explanatory power, this may be an important distinction in terms of forest management, as this suggests that, at least for the fungi investigated, whether a forest has been clear-cut or not may not be more important for fungal assemblages than the stand age per se.

While we expected, based on the prevalent view (Argiroff et al., 2022; Lilleskov et al., 2019; Lindahl et al., 2021), that ectomycorrhizal decomposers would be restricted to the least fertile soils, this was not the case. There was a unimodal response to soil fertility across all forests included in our study with a maximum at intermediate fertility. The pattern across all forests reflects that pine-dominated forest soils tend to be less fertile than spruce-dominated forest soils (pine mean C:N ratio = 34 ± 8, $n = 295$; spruce mean C:N ratio = 29 ± 6, $n = 253$), such that the upward slope in Figure 3a corresponds to increasing frequency in more fertile pine-dominated forest soils (Figure 3b) and the downward slope corresponds to decreasing frequency in more fertile spruce-dominated forest soils (Figure 3c). The decline of ectomycorrhizal decomposers in fertile spruce forest soils is in line with the idea that they are sensitive to higher available nitrogen, as in temperate forests (Argiroff et al., 2022). Counter to our expectations, all individual ectomycorrhizal decomposer taxa, except *Cortinarius mucosus* coll., were more common in spruce-dominated forests, and many taxa tended to be more frequent in fertile soils, some significantly so. In the most acidic and least fertile soils, ectomycorrhizal decomposers may be replaced by ericoid mycorrhizal fungi or other more stress-tolerant fungi (Fanin et al., 2022; Sterkenburg et al., 2015). Pine forests also tend to have coarser soil textures, lending to drier and drought-prone soils, and ectomycorrhizal communities are influenced by these microclimatic conditions (Castaño et al., 2018). Yet, all these forests are within the organic nutrient cycling state (*sensu* Phillips et al., 2013), and it is possible that ectomycorrhizal decomposers, even in more nutrient-rich boreal forests, play an important role in maintaining soil fertility (Clemmensen et al., 2015; Jörgensen et al., 2024).

When present, the abundance of the ectomycorrhizal decomposers relative to the total fungal or ectomycorrhizal community did not significantly relate to stand age or forest soil fertility. This also points to limitations in modelling relative abundances of a small group of individual species. While relative abundance data may give an indication of whether these ectomycorrhizal fungi are present as just a few spores or as dense mycelium, our objective to assess the niche(s) of these fungi is similarly achieved through a frequency-based analysis, which indicates whether or not they are present in a given environment. Although some species may respond negatively to tree removal and high nutrient availability (as we hypothesised), there may be broad enough niche variation among ectomycorrhizal decomposers to sustain nutrient and carbon dynamics, even in previously clear-cut and/or nutrient-rich forests, as is also found to be the case with ectomycorrhizal fungi producing hydrolytic enzymes (Jones et al., 2010; Walker et al., 2016).

In conclusion, correlation-based analyses of community-level peroxidase activity and fungal community composition may expand our knowledge of key decomposer taxa and their niches, but several challenges remain, some of which may be addressed by metatranscriptomics (Auer et al., 2024). The effect trait of oxidative decomposition with Mn-peroxidases is likely not conserved within genera, which makes trait assignment through extrapolation from closely related species problematic, and a way forward may be to focus on the most frequent species. The group of 10 ectomycorrhizal decomposers assigned here may have the potential to influence C stocks and N cycling in boreal forests, as they were represented in nearly half of the samples. We found little support for our hypothesis that ectomycorrhizal decomposers, as a group, would have their niche in older forest stands with less fertile soils (at least not in the context of this study). Rather, our findings suggest that there is large niche variation among ectomycorrhizal fungi with oxidative decomposer capacity in boreal forests. Ectomycorrhizal decomposers are likely also constrained by the most acidic and nutrient-poor forest soils. Additionally, certain ectomycorrhizal decomposers (e.g. *Gautieria* species) may not only be adapted to higher fertility conditions but also maintain ecosystem fertility by contributing to the decomposition of persistent soil organic matter (Jörgensen et al., 2024). Furthermore, the trait of ectomycorrhizal oxidative decomposition has a degree of redundancy in relation to stand age and soil fertility, with different species maintaining this function in different forest types. Thus, stand-replacing forestry may not pose as much of a threat for ectomycorrhizal decomposition over the long term, as previously proposed (Kyaschenko et al., 2017; Lindahl et al., 2021).

AUTHOR CONTRIBUTIONS

Björn D. Lindahl, Anders Dahlberg, Johan Stendahl and Erica E. Packard conceptualised the study. Leticia Pérez-Izquierdo performed field sampling and laboratory analyses of the regional dataset. Erica E. Packard compiled the data, performed all statistical analyses and wrote the first draft of the manuscript. Karina E. Clemmensen, Anders Dahlberg, Björn D. Lindahl, Erica E. Packard, Leticia Pérez-Izquierdo, Marie Spohn and Johan Stendahl contributed to the interpretation of results and revisions of the manuscript.

ACKNOWLEDGEMENTS

This work was supported by the Swedish Research Council FORMAS (grant no.: 2020-01105) and the Swedish Environmental Protection Agency (grant no.: 802-0148-18), both awarded to Björn Lindahl. The Swedish Forest Soil Inventory is a part of the national environmental monitoring commissioned by the Swedish Environmental Protection Agency. We graciously thank Karolina Jörgensen for feedback on the manuscript, Jeremie Orliac for laboratory work on enzyme assays, Mikael Jeppson and Tuula Niskanen for help to clarify the taxonomic status of *Gautieria* and *Cortinarius*, and anonymous reviewers for their comments.

CONFLICT OF INTEREST STATEMENT

The authors declare no competing financial interests.

DATA AVAILABILITY STATEMENT

The raw sequence data analysed during this study are available on NCBI SRA under the accessions numbers: PRJNA693127 and PRJNA1088460, for national and regional datasets, respectively. The R code for this analysis is available at Zenodo: https://doi.org/10.5281/zenodo.15343882 (Packard, 2025).

ORCID

Erica E. Packard https://orcid.org/0000-0002-3175-090X
Leticia Pérez-Izquierdo https://orcid.org/0000-0002-5200-8157
Karina E. Clemmensen https://orcid.org/0000-0002-9627-6428
Anders Dahlberg https://orcid.org/0000-0002-3669-6797
Marie Spohn https://orcid.org/0000-0002-1010-7317
Johan Stendahl https://orcid.org/0000-0002-9944-0297
Björn D. Lindahl https://orcid.org/0000-0002-3384-4547

REFERENCES

Abarenkov, K., Nilsson, R. H., Larsson, K.-H., Taylor, A. F. S., May, T. W., Frøslev, T. G., Pawlowska, J., Lindahl, B., Põldmaa, K., Truong, C., Vu, D., Hosoya, T., Niskanen, T., Piirmann, T., Ivanov, F., Zirk, A., Peterson, M., Cheeke, T. E., Ishigami, Y., … Kõljalg, U. (2024).

The UNITE database for molecular identification and taxonomic communication of fungi and other eukaryotes: Sequences, taxa and classifications reconsidered. *Nucleic Acids Research*, *52*(D1), D791–D797. https://doi.org/10.1093/nar/gkad1039

Agerer, R. (2001). Exploration types of ectomycorrhizae: A proposal to classify ectomycorrhizal mycelial systems according to their patterns of differentiation and putative ecological importance. *Mycorrhiza*, *11*, 107–114. https://doi.org/10.1007/s005720100108 2001

Allison, S. D. (2006). Soil minerals and humic acids alter enzyme stability: Implications for ecosystem processes. *Biogeochemistry*, *81*(3), 361–373. https://doi.org/10.1007/s10533-006-9046-2

Allison, S. D., & Martiny, J. B. H. (2008). Resistance, resilience, and redundancy in microbial communities. *Proceedings of the National Academy of Sciences of the United States of America*, *105*, 11512–11519. https://doi.org/10.1073/pnas.0801925105

Antonson, H., & Jansson, U. (Eds.). (2011). *Agriculture and forestry in Sweden since 1900: Geographical and historical studies*. The Royal Swedish Academy of Agriculture and Forestry.

Argiroff, W. A., Zak, D. R., Pellitier, P. T., Upchurch, R. A., & Belke, J. P. (2022). Decay by ectomycorrhizal fungi couples soil organic matter to nitrogen availability. *Ecology Letters*, *25*(2), 391–404. https://doi.org/10.1111/ele.13923

Auer, L., Buée, M., Fauchery, L., Lombard, V., Barry, K. W., Clum, A., Copeland, A., Daum, C., Foster, B., LaButti, K., Singan, V., Yoshinaga, Y., Martineau, C., Alfaro, M., Castillo, F. J., Imbert, J. B., Ramírez, L., Castanera, R., Pisabarro, A. G., … Martin, F. M. (2024). Metatranscriptomics sheds light on the links between the functional traits of fungal guilds and ecological processes in forest soil ecosystems. *New Phytologist*, *242*(4), 1676–1690. https://doi.org/10.1111/nph.19471

Balasundaram, S. V., Hess, J., Durling, M. B., Moody, S. C., Thorbek, L., Progida, C., LaButti, K., Aerts, A., Barry, K., Grigoriev, I. V., Boddy, L., Högberg, N., Kauserud, H., Eastwood, D. C., & Skrede, I. (2018). The fungus that came in from the cold: Dry rot's pre-adapted ability to invade buildings. *The ISME Journal*, *12*(3), 791–801. https://doi.org/10.1038/s41396-017-0006-8

Baldrian, P., Der Wiesche, C., Gabriel, J., Nerud, F., & Zadražil, F. (2000). Influence of cadmium and mercury on activities of ligninolytic enzymes and degradation of polycyclic aromatic hydrocarbons by *Pleurotus ostreatus* in soil. *Applied and Environmental Microbiology*, *66*(6), 2471–2478. https://doi.org/10.1128/AEM.66.6.2471-2478.2000

Barbi, F., Kohler, A., Barry, K., Baskaran, P., Daum, C., Fauchery, L., Ihrmark, K., Kuo, A., LaButti, K., Lipzen, A., Morin, E., Grigoriev, I. V., Henrissat, B., Lindahl, B. D., & Martin, F. (2020). Fungal ecological strategies reflected in gene transcription—A case study of two litter decomposers. *Environmental Microbiology*, *22*(3), 1089–1103. https://doi.org/10.1111/1462-2920.14873

Bartoń, K. (2023). *MuMIn: Multi-model inference. R package version 1.47.5* [computer software]. https://CRAN.R-project.org/package=MuMIn

Baskaran, P., Hyvönen, R., Berglund, S. L., Clemmensen, K. E., Ågren, G. I., Lindahl, B. D., & Manzoni, S. (2017). Modelling the influence of ectomycorrhizal decomposition on plant nutrition and soil carbon sequestration in boreal forest ecosystems. *New Phytologist*, *213*(3), 1452–1465. https://doi.org/10.1111/nph.14213

Bates, D., Mächler, M., Bolker, B., & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, *67*(1), 1–48. https://doi.org/10.18637/jss.v067.i01

Benjamini, Y., & Hochberg, Y. (1995). Controlling the false discovery rate: A practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society*, *57*(1), 289–300.

Bödeker, I. T. M., Clemmensen, K. E., de Boer, W., Martin, F., Olson, Å., & Lindahl, B. D. (2014). Ectomycorrhizal Cortinarius species participate in enzymatic oxidation of humus in northern forest ecosystems. *New Phytologist*, *203*(1), 245–256. https://doi.org/10.1111/nph.12791

Bödeker, I. T. M., Nygren, C. M. R., Taylor, A. F. S., Olson, Å., & Lindahl, B. D. (2009). ClassII peroxidase-encoding genes are present in a phylogenetically wide range of ectomycorrhizal fungi. *ISME Journal*, *3*(12), 1387–1395. https://doi.org/10.1038/ismej.2009.77

Branco, S., Gladieux, P., Ellison, C. E., Kuo, A., LaButti, K., Lipzen, A., Grigoriev, I. V., Liao, H.-L., Vilgalys, R., Peay, K. G., Taylor, J. W., & Bruns, T. D. (2015). Genetic isolation between two recently diverged populations of a symbiotic fungus. *Molecular Ecology*, *24*(11), 2747–2758. https://doi.org/10.1111/mec.13132

Castaño, C., Berlin, A., Brandström Durling, M., Ihrmark, K., Lindahl, B. D., Stenlid, J., Clemmensen, K. E., & Olson, Å. (2020). Optimized metabarcoding with Pacific biosciences enables semi-quantitative analysis of fungal communities. *New Phytologist*, *228*(3), 1149–1158. https://doi.org/10.1111/nph.16731

Castaño, C., Lindahl, B. D., Alday, J. G., Hagenbo, A., Martínez de Aragón, J., Parladé, J., Pera, J., & Bonet, J. A. (2018). Soil microclimate changes affect soil fungal communities in a Mediterranean pine forest. *New Phytologist*, *220*(4), 1211–1221. https://doi.org/10.1111/nph.15205

Chambers, S. M., Burke, R. M., Brooks, P. R., & Cairney, J. W. G. (1999). Molecular and biochemical evidence for manganese-dependent peroxidase activity in *Tylospora fibrillosa*. *Mycological Research*, *103*(9), 1098–1102. https://doi.org/10.1017/S095375629900831X

Chen, J., Luo, Y., van Groenigen, K. J., Hungate, B. A., Cao, J., Zhou, X., & Wang, R. (2018). A keystone microbial enzyme for nitrogen control of soil carbon storage. *Science Advances*, *4*(8), eaaq1689. https://doi.org/10.1126/sciadv.aaq1689

Clemmensen, K. E., Finlay, R. D., Dahlberg, A., Stenlid, J., Wardle, D. A., & Lindahl, B. D. (2015). Carbon sequestration is related to mycorrhizal fungal community shifts during long-term succession in boreal forests. *New Phytologist*, *205*(4), 1525–1536. https://doi.org/10.1111/nph.13208

Clemmensen, K. E., Ihrmark, K., Durling, M. B., & Lindahl, B. D. (2023). Sample preparation for fungal community analysis by high-throughput sequencing of barcode amplicons. In F. Martin & S. Uroz (Eds.), *Microbial environmental genomics (MEG)* (2nd ed., pp. 37–64). Springer US. https://doi.org/10.1007/978-1-0716-2871-3_3

Cromack, K., Fichter, B. L., Moldenke, A. M., Entry, J. A., & Ingham, E. R. (1988). Interactions between soil animals and ectomycorrhizal fungal mats. *Agriculture, Ecosystems & Environment*, *24*(1), 161–168. https://doi.org/10.1016/0167-8809(88)90063-1

Crowther, T. W., Maynard, D. S., Crowther, T. R., Peccia, J., Smith, J. R., & Bradford, M. A. (2014). Untangling the fungal niche: The trait-based approach. *Frontiers in Microbiology*, *5*, 579. https://doi.org/10.3389/fmicb.2014.00579

Defrenne, C. E., Moore, J. A. M., Tucker, C. L., Lamit, L. J., Kane, E. S., Kolka, R. K., Chimner, R. A., Keller, J. K., & Lilleskov, E. A. (2023). Peat loss collocates with a threshold in plant–mycorrhizal associations in drained peatlands encroached by trees. *New Phytologist*, *240*(1), 412–425. https://doi.org/10.1111/nph.18954

Defrenne, C. E., Philpott, T. J., Guichon, S. H. A., Roach, W. J., Pickles, B. J., & Simard, S. W. (2019). Shifts in ectomycorrhizal fungal communities and exploration types relate to the environment and fine-root traits across interior douglas-fir forests of western Canada. *Frontiers in Plant Science*, *10*, 643. https://doi.org/10.3389/fpls.2019.00643

Dynarski, K. A., & Houlton, B. Z. (2020). Isotopic constraints on plant nitrogen acquisition strategies during ecosystem retrogression. *Oecologia*, *192*(3), 603–614. https://doi.org/10.1007/s00442-020-04606-y

Eastwood, D. C., Floudas, D., Binder, M., Majcherczyk, A., Schneider, P., Aerts, A., Asiegbu, F. O., Baker, S. E., Barry, K., Bendiksby, M., Blumentritt, M., Coutinho, P. M., Cullen, D., de Vries, R. P., Gathman, A., Goodell, B., Henrissat, B., Ihrmark, K., Kauserud, H., …

Watkinson, S. C. (2011). The plant cell wall-decomposing machinery underlies the functional diversity of forest fungi. *Science (New York, N.Y.)*, *333*(6043), 762–765. https://doi.org/10.1126/science.1205411

Edgar, R. C. (2010). Search and clustering orders of magnitude faster than BLAST. *Bioinformatics*, *26*(19), 2460–2461. https://doi.org/10.1093/bioinformatics/btq461

Entry, J. A., Donnelly, P. K., & Cromack, K. (1991). Influence of ectomycorrhizal mat soils on lignin and cellulose degradation. *Biology and Fertility of Soils*, *11*(1), 75–78. https://doi.org/10.1007/BF00335839

Fanin, N., Clemmensen, K. E., Lindahl, B. D., Farrell, M., Nilsson, M.-C., Gundale, M. J., Kardol, P., & Wardle, D. A. (2022). Ericoid shrubs shape fungal communities and suppress organic matter decomposition in boreal forests. *New Phytologist*, *236*(2), 684–697. https://doi.org/10.1111/nph.18353

Floudas, D. (2021). Chapter two—Evolution of lignin decomposition systems in fungi. In M. Morel-Rouhier & R. Sormani (Eds.), *Advances in botanical research* (Vol. *99*, pp. 37–76). Academic Press. https://doi.org/10.1016/bs.abr.2021.05.003

Floudas, D., Binder, M., Riley, R., Barry, K., Blanchette, R. A., Henrissat, B., Martínez, A. T., Otillar, R., Spatafora, J. W., Yadav, J. S., Aerts, A., Benoit, I., Boyd, A., Carlson, A., Copeland, A., Coutinho, P. M., De Vries, R. P., Ferreira, P., Findley, K., … Hibbett, D. S. (2012). The paleozoic origin of enzymatic lignin decomposition reconstructed from 31 fungal genomes. *Science*, *336*(6089), 1715–1719. https://doi.org/10.1126/science.1221748

Fox, J., & Weisberg, S. (2019). *An R companion to applied regression* (3rd ed.). Sage Publications.

Fridman, J., Holm, S., Nilsson, M., Nilsson, P., Ringvall, A. H., & Ståhl, G. (2014). Adapting National Forest Inventories to changing requirements—The case of the Swedish National Forest Inventory at the turn of the 20th century. *Silva Fennica*, *48*(3), 1095. https://doi.org/10.14214/sf.1095

Gaskell, J., Kersten, P., Larrondo, L. F., Canessa, P., Martinez, D., Hibbett, D., Schmoll, M., Kubicek, C. P., Martinez, A. T., Yadav, J., Master, E., Magnuson, J. K., Yaver, D., Berka, R., Lail, K., Chen, C., LaButti, K., Nolan, M., Lipzen, A., … Cullen, D. (2017). Draft genome sequence of a monokaryotic model brown-rot fungus Postia (Rhodonia) placenta SB12. *Genomics Data*, *14*, 21–23. https://doi.org/10.1016/j.gdata.2017.08.003

Griffiths, R. P., & Caldwell, B. A. (1992). Mycorrhizal mat communities in forest soils. In D. J. Read, D. H. Lewis, A. H. Fitter, & I. J. Alexander (Eds.), *Mycorrhizas in ecosystems* (pp. 98–105). C.A.B. International.

Grigoriev, I. V., Nikitin, R., Haridas, S., Kuo, A., Ohm, R., Otillar, R., Riley, R., Salamov, A., Zhao, X., Korzeniewski, F., Smirnova, T., Nordberg, H., Dubchak, I., & Shabalov, I. (2014). MycoCosm portal: Gearing up for 1000 fungal genomes. *Nucleic Acids Research*, *42*(D1), D699–D704. https://doi.org/10.1093/nar/gkt1183

Hagenbo, A., Hadden, D., Clemmensen, K. E., Grelle, A., Manzoni, S., Mölder, M., Ekblad, A., & Fransson, P. (2019). Carbon use efficiency of mycorrhizal fungal mycelium increases during the growing season but decreases with forest age across a *Pinus sylvestris* chronosequence. *Journal of Ecology*, *107*(6), 2808–2822. https://doi.org/10.1111/1365-2745.13209

Hagenbo, A., Kyaschenko, J., Clemmensen, K. E., Lindahl, B. D., & Fransson, P. (2018). Fungal community shifts underpin declining mycelial production and turnover across a *Pinus sylvestris* chronosequence. *Journal of Ecology*, *106*(2), 490–501. https://doi.org/10.1111/1365-2745.12917

Harder, C. B., Miyauchi, S., Virágh, M., Kuo, A., Thoen, E., Andreopoulos, B., Lu, D., Skrede, I., Drula, E., Henrissat, B., Morin, E., Kohler, A., Barry, K., LaButti, K., Salamov, A., Lipzen, A., Merényi, Z., Hegedüs, B., Baldrian, P., … Kauserud, H. (2024). Extreme overall mushroom genome expansion in *Mycena* s.s. irrespective of plant hosts or substrate specializations. *Cell Genomics*, *4*(7), 100586. https://doi.org/10.1016/j.xgen.2024.100586

Hasby, F. (2022). *Impacts of clear-cutting on soil fungal communities and their activities in boreal forests—A metatranscriptomic approach* [Doctoral thesis]. Swedish University of Agricultural Sciences.

Hess, J., Balasundaram, S. V., Bakkemo, R. I., Drula, E., Henrissat, B., Högberg, N., Eastwood, D., & Skrede, I. (2021). Niche differentiation and evolution of the wood decay machinery in the invasive fungus *Serpula lacrymans*. *The ISME Journal*, *15*(2), 592. https://doi.org/10.1038/s41396-020-00799-5

Hintikka, V., & Näykki, O. (1967). *Notes on the effects of the fungus Hydnellum ferrugineum (FR.) Karst. on forest soil and vegetation* (Vol. *62*). Metsäntutkimuslaitos.

Hiscox, J., Savoury, M., Müller, C. T., Lindahl, B. D., Rogers, H. J., & Boddy, L. (2015). Priority effects during fungal community establishment in beech wood. *The ISME Journal*, *9*(10), 2246–2260. https://doi.org/10.1038/ismej.2015.38

Högberg, M. N., Bååth, E., Nordgren, A., Arnebrant, K., & Högberg, P. (2003). Contrasting effects of nitrogen availability on plant carbon supply to mycorrhizal fungi and saprotrophs—A hypothesis based on field observations in boreal forest. *The New Phytologist*, *160*(1), 225–238. https://doi.org/10.1046/j.1469-8137.2003.00867.x

Ihrmark, K., Bödeker, I. T. M., Cruz-Martinez, K., Friberg, H., Kubartova, A., Schenck, J., Strid, Y., Stenlid, J., Brandström-Durling, M., Clemmensen, K. E., & Lindahl, B. D. (2012). New primers to amplify the fungal ITS2 region—Evaluation by 454-sequencing of artificial and natural communities. *FEMS Microbiology Ecology*, *82*(3), 666–677. https://doi.org/10.1111/j.1574-6941.2012.01437.x

Jones, M. D., Twieg, B. D., Ward, V., Barker, J., Durall, D. M., & Simard, S. W. (2010). Functional complementarity of Douglas-fir ectomycorrhizas for extracellular enzyme activity after wildfire or clearcut logging. *Functional Ecology*, *24*(5), 1139–1151. https://doi.org/10.1111/j.1365-2435.2010.01699.x

Jones, M. E., Lacroix, R. E., Zeigler, J., Ying, S. C., Nico, P. S., & Keiluweit, M. (2020). Enzymes, manganese, or iron? Drivers of oxidative organic matter decomposition in soils. *Environmental Science and Technology*, *54*(21), 14114–14123. https://doi.org/10.1021/acs.est.0c04212

Jörgensen, K., Clemmensen, K. E., Wallander, H., & Lindahl, B. D. (2023). Do ectomycorrhizal exploration types reflect mycelial foraging strategies? *New Phytologist*, *237*(2), 576–584. https://doi.org/10.1111/nph.18566

Jörgensen, K., Clemmensen, K. E., Wallander, H., & Lindahl, B. D. (2024). Ectomycorrhizal fungi are more sensitive to high soil nitrogen levels in forests exposed to nitrogen deposition. *New Phytologist*, *242*(4), 1725–1738. https://doi.org/10.1111/nph.19509

Kassambara, A., & Mundt, F. (2020). *factoextra: Extract and visualize the results of multivariate data analyses*. [computer software]. https://CRAN.R-project.org/package=factoextra

Keiluweit, M., Nico, P., Harmon, M. E., Mao, J., Pett-Ridge, J., & Kleber, M. (2015). Long-term litter decomposition controlled by manganese redox cycling. *Proceedings of the National Academy of Sciences of the United States of America*, *112*(38), E5253–E5260. https://doi.org/10.1073/pnas.1508945112

Kellner, H., Luis, P., Pecyna, M. J., Barbi, F., Kapturska, D., Krüger, D., Zak, D. R., Marmeisse, R., Vandenbol, M., & Hofrichter, M. (2014). Widespread occurrence of expressed fungal secretory peroxidases in forest soils. *PLoS One*, *9*(4), e95557. https://doi.org/10.1371/journal.pone.0095557

Kirk, T. K., & Farrell, R. L. (1987). Enzymatic “combustion”: The microbial degradation of lignin. *Annual Review of Microbiology*, *41*, 465–505.

Kohler, A., Kuo, A., Nagy, L. G., Morin, E., Barry, K. W., Buscot, F., Canbäck, B., Choi, C., Cichocki, N., Clum, A., Colpaert, J., Copeland, A., Costa, M. D., Doré, J., Floudas, D., Gay, G., Girlanda, M., Henrissat, B., Herrmann, S., … Martin, F. (2015). Convergent losses of decay mechanisms and rapid turnover of symbiosis genes in mycorrhizal mutualists. *Nature Genetics*, *47*(4), 410–415. https://doi.org/10.1038/ng.3223

Koide, R. T., Fernandez, C., & Malcolm, G. (2014). Determining place and process: Functional traits of ectomycorrhizal fungi that affect both community structure and ecosystem function. *New Phytologist*, *201*(2), 433–439. https://doi.org/10.1111/nph.12538

Kõljalg, U., Nilsson, R. H., Abarenkov, K., Tedersoo, L., Taylor, A. F. S., Bahram, M., Bates, S. T., Bruns, T. D., Bengtsson-Palme, J., Callaghan, T. M., Douglas, B., Drenkhan, T., Eberhardt, U., Dueñas, M., Grebenc, T., Griffith, G. W., Hartmann, M., Kirk, P. M., Kohout, P., … Larsson, K. H. (2013). Towards a unified paradigm for sequence-based identification of fungi. *Molecular Ecology*, *22*(21), 5271–5277. https://doi.org/10.1111/mec.12481

Kranabetter, J. M. (2019). Increasing soil carbon content with declining soil manganese in temperate rainforests: Is there a link to fungal Mn? *Soil Biology and Biochemistry*, *128*, 179–181. https://doi.org/10.1016/j.soilbio.2018.11.001

Kyaschenko, J., Clemmensen, K. E., Hagenbo, A., Karltun, E., & Lindahl, B. D. (2017). Shift in fungal communities and associated enzyme activities along an age gradient of managed *Pinus sylvestris* stands. *ISME Journal*, *11*(4), 863–874. https://doi.org/10.1038/ismej.2016.184

Lavorel, S., & Garnier, E. (2002). Predicting changes in community composition and ecosystem functioning from plant traits: Revisiting the Holy Grail. *Functional Ecology*, *16*(5), 545–556. https://doi.org/10.1046/j.1365-2435.2002.00664.x

Lebreton, A., Tang, N., Kuo, A., LaButti, K., Andreopoulos, W., Drula, E., Miyauchi, S., Barry, K., Clum, A., Lipzen, A., Mousain, D., Ng, V., Wang, R., Dai, Y., Henrissat, B., Grigoriev, I. V., Guerin-Laguette, A., Yu, F., & Martin, F. M. (2022). Comparative genomics reveals a dynamic genome evolution in the ectomycorrhizal milk-cap (*Lactarius*) mushrooms. *The New Phytologist*, *235*(1), 306–319. https://doi.org/10.1111/nph.18143

Lehmann, J., & Kleber, M. (2015). The contentious nature of soil organic matter. *Nature*, *528*(7580), 60–68. https://doi.org/10.1038/nature16069

Lilleskov, E. A., Fahey, T. J., Horton, T. R., & Lovett, G. M. (2002). Belowground ectomycorrhizal fungal community change over a nitrogen deposition gradient in Alaska. *Ecology*, *83*(1), 104–115. https://doi.org/10.1890/0012-9658(2002)083[0104:BEFCCO]2.0.CO;2

Lilleskov, E. A., Hobbie, E. A., & Horton, T. R. (2011). Conservation of ectomycorrhizal fungi: Exploring the linkages between functional and taxonomic responses to anthropogenic N deposition. *Fungal Ecology*, *4*(2), 174–183. https://doi.org/10.1016/j.funeco.2010.09.008

Lilleskov, E. A., Kuyper, T. W., Bidartondo, M. I., & Hobbie, E. A. (2019). Atmospheric nitrogen deposition impacts on the structure and function of forest mycorrhizal communities: A review. *Environmental Pollution*, *246*, 148–162. https://doi.org/10.1016/j.envpol.2018.11.074

Lindahl, B. D., & Finlay, R. D. (2006). Activities of chitinolytic enzymes during primary and secondary colonization of wood by basidiomycetous fungi. *New Phytologist*, *169*(2), 389–397. https://doi.org/10.1111/j.1469-8137.2005.01581.x

Lindahl, B. D., Ihrmark, K., Boberg, J., Trumbore, S. E., Högberg, P., Stenlid, J., & Finlay, R. D. (2007). Spatial separation of litter decomposition and mycorrhizal nitrogen uptake in a boreal forest. *New Phytologist*, *173*(3), 611–620. https://doi.org/10.1111/j.1469-8137.2006.01936.x

Lindahl, B. D., Kyaschenko, J., Varenius, K., Clemmensen, K. E., Dahlberg, A., Karltun, E., & Stendahl, J. (2021). A group of ectomycorrhizal fungi restricts organic matter accumulation in boreal forest. *Ecology Letters*, *24*(7), 1341–1351. https://doi.org/10.1111/ele.13746

Lindahl, B. D., Pérez-Izquierdo, L., Dahlberg, A., Hallin, S., Krab, E. J., & Stendahl, J. (2023). *DNA-baserad övervakning av biodiversitet i svensk skogsmark—Provtagning, provhantering och analysmetoder*. Naturvårdsverket.

Lindahl, B. D., & Tunlid, A. (2015). Ectomycorrhizal fungi—Potential organic matter decomposers, yet not saprotrophs. *New Phytologist*, *205*(4), 1443–1447. https://doi.org/10.1111/nph.13201

Lofgren, L. A., Nguyen, N. H., Vilgalys, R., Ruytinx, J., Liao, H.-L., Branco, S., Kuo, A., LaButti, K., Lipzen, A., Andreopoulos, W., Pangilinan, J., Riley, R., Hundley, H., Na, H., Barry, K., Grigoriev, I. V., Stajich, J. E., & Kennedy, P. G. (2021). Comparative genomics reveals dynamic genome evolution in host specialist ectomycorrhizal fungi. *The New Phytologist*, *230*(2), 774–792. https://doi.org/10.1111/nph.17160

Looney, B., Miyauchi, S., Morin, E., Drula, E., Courty, P. E., Kohler, A., Kuo, A., LaButti, K., Pangilinan, J., Lipzen, A., Riley, R., Andreopoulos, W., He, G., Johnson, J., Nolan, M., Tritt, A., Barry, K. W., Grigoriev, I. V., Nagy, L. G., … Martin, F. M. (2022). Evolutionary transition to the ectomycorrhizal habit in the genomes of a hyperdiverse lineage of mushroom-forming fungi. *New Phytologist*, *233*(5), 2294–2309. https://doi.org/10.1111/nph.17892

Lundmark, H., Josefsson, T., & Östlund, L. (2013). The history of clear-cutting in northern Sweden—Driving forces and myths in boreal silviculture. *Forest Ecology and Management*, *307*, 112–122. https://doi.org/10.1016/j.foreco.2013.07.003

Martinez, D., Challacombe, J., Morgenstern, I., Hibbett, D., Schmoll, M., Kubicek, C. P., Ferreira, P., Ruiz-Duenas, F. J., Martinez, A. T., Kersten, P., Hammel, K. E., Vanden Wymelenberg, A., Gaskell, J., Lindquist, E., Sabat, G., Bondurant, S. S., Larrondo, L. F., Canessa, P., Vicuna, R., … Cullen, D. (2009). Genome, transcriptome, and secretome analysis of wood decay fungus *Postia placenta* supports unique mechanisms of lignocellulose conversion. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(6), 1954–1959. https://doi.org/10.1073/pnas.0809575106

McGuire, K. L., & Treseder, K. K. (2010). Microbial communities and their relevance for ecosystem models: Decomposition as a case study. *Soil Biology and Biochemistry*, *42*(4), 529–535. https://doi.org/10.1016/j.soilbio.2009.11.016

Miyauchi, S., Kiss, E., Kuo, A., Drula, E., Kohler, A., Sánchez-García, M., Morin, E., Andreopoulos, B., Barry, K. W., Bonito, G., Buée, M., Carver, A., Chen, C., Cichocki, N., Clum, A., Culley, D., Crous, P. W., Fauchery, L., Girlanda, M., … Martin, F. M. (2020). Large-scale genome sequencing of mycorrhizal fungi provides insights into the early evolution of symbiotic traits. *Nature Communications*, *11*(1), 5125. https://doi.org/10.1038/s41467-020-18795-w

Moorhead, D. L., & Sinsabaugh, R. L. (2006). A theoretical model of litter decay and microbial interaction. *Ecological Monographs*, *76*(2), 151–174. https://doi.org/10.1890/0012-9615(2006)076[0151:ATMOLD]2.0.CO;2

Nagy, L. G., Riley, R., Tritt, A., Adam, C., Daum, C., Floudas, D., Sun, H., Yadav, J. S., Pangilinan, J., Larsson, K.-H., Matsuura, K., Barry, K., Labutti, K., Kuo, R., Ohm, R. A., Bhattacharya, S. S., Shirouzu, T., Yoshinaga, Y., Martin, F. M., … Hibbett, D. S. (2016). Comparative genomics of early-diverging mushroom-forming fungi provides insights into the origins of lignocellulose decay capabilities. *Molecular Biology and Evolution*, *33*(4), 959–970. https://doi.org/10.1093/molbev/msv337

Ngo, T. T., & Lenhoff, H. M. (1980). A sensitive and versatile chromogenic assay for peroxidase and peroxidase-coupled reactions. *Analytical Biochemistry*, *105*(1), 389–397. https://doi.org/10.1016/0003-2697(80)90475-3

Nilsson, P., Cory, N., Fridman, J., & Kempe, G. (2013). *Skogsdata 2013: Aktuella uppgifter om de svenska skogarna från Riksskogstaxeringen. Tema: Olika mått på skogens ålder och trädslagssammansättning (Skogsdata)*. Institutionen för skoglig resurshushållning, Sveriges lantbruksuniversitet.

Odin, H., Eriksson, B., & Pertuu, K. (1983). *Temperature climate maps for Swedish forestry* (Vol. 57). Swedish University of Agricultural Sciences.

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., Minchin, P. R., O'Hara, R. B., Simpson, G. L., Solymos, P., Stevens,

M. H. H., Szoecs, E., & Wagner, H. (2022). *vegan: Community ecology package* [computer software]. https://cran.r-project.org/package=vegan

Packard, E. E. (2025). eepackard/Ecto_decomposers: v1.0. *Zenodo*, https://doi.org/10.5281/zenodo.15343882

Pedersen, T. L. (2022). *patchwork: The composer of plots* [computer software]. https://CRAN.R-project.org/package=patchwork

Pellitier, P. T., & Zak, D. R. (2021). Ectomycorrhizal fungal decay traits along a soil nitrogen gradient. *New Phytologist*, *232*(5), 2152–2164. https://doi.org/10.1111/nph.17734

Pérez-Izquierdo, L., Clemmensen, K. E., Strengbom, J., Granath, G., Wardle, D. A., Nilsson, M. C., & Lindahl, B. D. (2021). Crown-fire severity is more important than ground-fire severity in determining soil fungal community development in the boreal forest. *Journal of Ecology*, *109*(1), 518. https://doi.org/10.1111/1365-2745.13529

Phillips, R. P., Brzostek, E., & Midgley, M. G. (2013). The mycorrhizal-associated nutrient economy: A new framework for predicting carbon-nutrient couplings in temperate forests. *New Phytologist*, *199*(1), 41–51. https://doi.org/10.1111/nph.12221

Pickles, B. J., Genney, D. R., Potts, J. M., Lennon, J. J., Anderson, I. C., & Alexander, I. J. (2010). Spatial and temporal ecology of scots pine ectomycorrhizas. *New Phytologist*, *186*(3), 755–768. https://doi.org/10.1111/j.1469-8137.2010.03204.x

Põlme, S., Abarenkov, K., Henrik Nilsson, R., Lindahl, B. D., Clemmensen, K. E., Kauserud, H., Nguyen, N., Kjøller, R., Bates, S. T., Baldrian, P., Frøslev, T. G., Adojaan, K., Vizzini, A., Suija, A., Pfister, D., Baral, H.-O., Järv, H., Madrid, H., Nordén, J., … Tedersoo, L. (2020). FungalTraits: A user-friendly traits database of fungi and fungus-like stramenopiles. *Fungal Diversity*, *105*(1), 1–16. https://doi.org/10.1007/s13225-020-00466-2

R Core Team. (2025). *R: A language and environment for statistical computing* [computer software]. R Foundation for Statistical Computing. https://www.R-project.org/

Ruiz-Dueñas, F. J., Barrasa, J. M., Sánchez-García, M., Camarero, S., Miyauchi, S., Serrano, A., Linde, D., Babiker, R., Drula, E., Ayuso-Fernández, I., Pacheco, R., Padilla, G., Ferreira, P., Barriuso, J., Kellner, H., Castanera, R., Alfaro, M., Ramírez, L., Pisabarro, A. G., … Martínez, A. T. (2021). Genomic analysis enlightens Agaricales lifestyle evolution and increasing peroxidase diversity. *Molecular Biology and Evolution*, *38*(4), 1428–1446. https://doi.org/10.1093/molbev/msaa301

Saikkonen, K., Ahonen-Jonnarth, U., Markkola, A. m., Helander, M., Tuomi, J., Roitto, M., & Ranta, H. (1999). Defoliation and mycorrhizal symbiosis: A functional balance between carbon sources and below-ground sinks. *Ecology Letters*, *2*(1), 19–26. https://doi.org/10.1046/j.1461-0248.1999.21042.x

Schmidt, M. W. I., Torn, M. S., Abiven, S., Dittmar, T., Guggenberger, G., Janssens, I. A., Kleber, M., Kögel-Knabner, I., Lehmann, J., Manning, D. A. C., Nannipieri, P., Rasse, D. P., Weiner, S., & Trumbore, S. E. (2011). Persistence of soil organic matter as an ecosystem property. *Nature*, *478*(7367), 49–56. https://doi.org/10.1038/nature10386

Shah, F., Nicolás, C., Bentzer, J., Ellström, M., Smits, M., Rineau, F., Canbäck, B., Floudas, D., Carleer, R., Lackner, G., Braesel, J., Hoffmeister, D., Henrissat, B., Ahrén, D., Johansson, T., Hibbett, D. S., Martin, F., Persson, P., & Tunlid, A. (2016). Ectomycorrhizal fungi decompose soil organic matter using oxidative mechanisms adapted from saprotrophic ancestors. *New Phytologist*, *209*(4), 1705–1719. https://doi.org/10.1111/nph.13722

Shimizu, M., Yuda, N., Nakamura, T., Tanaka, H., & Wariishi, H. (2005). Metabolic regulation at the tricarboxylic acid and glyoxylate cycles of the lignin-degrading basidiomycete *Phanerochaete chrysosporium* against exogenous addition of vanillin. *Proteomics*, *5*(15), 3919–3931. https://doi.org/10.1002/pmic.200401251

Sinsabaugh, R. L. (2010). Phenol oxidase, peroxidase and organic matter dynamics of soil. *Soil Biology and Biochemistry*, *42*(3), 391–404. https://doi.org/10.1016/j.soilbio.2009.10.014

Šnajdr, J., Valášková, V., Merhautová, V., Herinková, J., Cajthaml, T., & Baldrian, P. (2008). Spatial variability of enzyme activities and microbial biomass in the upper layers of *Quercus petraea* forest soil. *Soil Biology and Biochemistry*, *40*(9), 2068–2075. https://doi.org/10.1016/j.soilbio.2008.01.015

Stendahl, J., Berg, B., & Lindahl, B. D. (2017). Manganese availability is negatively associated with carbon storage in northern coniferous forest humus layers. *Scientific Reports*, *7*(1), 1–6. https://doi.org/10.1038/s41598-017-15801-y

Sterkenburg, E., Bahr, A., Durling, M. B., Clemmensen, K. E., & Lindahl, B. D. (2015). Changes in fungal communities along a boreal forest soil fertility gradient. *The New Phytologist*, *207*(4), 1145–1158. https://doi.org/10.1111/nph.13426

Sterkenburg, E., Clemmensen, K. E., Ekblad, A., Finlay, R. D., & Lindahl, B. D. (2018). Contrasting effects of ectomycorrhizal fungi on early and late stage decomposition in a boreal forest. *ISME Journal*, *12*(9), 2187–2197. https://doi.org/10.1038/s41396-018-0181-2

Talbot, J. M., Bruns, T. D., Smith, D. P., Branco, S., Glassman, S. I., Erlandson, S., Vilgalys, R., & Peay, K. G. (2013). Independent roles of ectomycorrhizal and saprotrophic communities in soil organic matter decomposition. *Soil Biology and Biochemistry*, *57*, 282–291. https://doi.org/10.1016/j.soilbio.2012.10.004

Tennekes, M. (2018). tmap: Thematic maps in R. *Journal of Statistical Software*, *84*(6), 1–39. https://doi.org/10.18637/jss.v084.i06

Twieg, B. D., Durall, D. M., & Simard, S. W. (2007). Ectomycorrhizal fungal succession in mixed temperate forests. *New Phytologist*, *176*(2), 437–447. https://doi.org/10.1111/j.1469-8137.2007.02173.x

Valášková, V., & Baldrian, P. (2006). Estimation of bound and free fractions of lignocellulose-degrading enzymes of wood-rotting fungi *Pleurotus ostreatus*, *Trametes versicolor* and *Piptoporus betulinus*. *Research in Microbiology*, *157*(2), 119–124. https://doi.org/10.1016/j.resmic.2005.06.004

van der Linde, S., Suz, L. M., Orme, C. D. L., Cox, F., Andreae, H., Asi, E., Atkinson, B., Benham, S., Carroll, C., Cools, N., De Vos, B., Dietrich, H.-P., Eichhorn, J., Gehrmann, J., Grebenc, T., Gweon, H. S., Hansen, K., Jacob, F., Kristöfel, F., … Bidartondo, M. I. (2018). Environment and host as large-scale controls of ectomycorrhizal fungi. *Nature*, *558*(7709), 243–248. https://doi.org/10.1038/s41586-018-0189-9

Walker, J. K. M., Ward, V., & Jones, M. D. (2016). Ectomycorrhizal fungal exoenzyme activity differs on spruce seedlings planted in forests versus clearcuts. *Trees*, *30*(2), 497–508. https://doi.org/10.1007/s00468-015-1239-7

Wallander, H., Johansson, U., Sterkenburg, E., Brandström Durling, M., & Lindahl, B. D. (2010). Production of ectomycorrhizal mycelium peaks during canopy closure in Norway spruce forests. *New Phytologist*, *187*(4), 1124–1134. https://doi.org/10.1111/j.1469-8137.2010.03324.x

Wasyliw, J., & Karst, J. (2020). Shifts in ectomycorrhizal exploration types parallel leaf and fine root area with forest age. *Journal of Ecology*, *108*(6), 2270–2282. https://doi.org/10.1111/1365-2745.13484

White, T. J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In T. J. White, T. Bruns, S. Lee, & J. Taylor (Eds.), *PCR protocols: A guide to methods and applications* (pp. 315–322). Academic Press, Inc. https://doi.org/10.1016/B978-0-12-372180-8.50042-1

Wickham, H. (2016). *ggplot2: Elegant graphics for data analysis*. Springer-Verlag. https://ggplot2.tidyverse.org

Wickham, H., François, R., Henry, L., Müller, K., & Vaughan, D. (2023). *dplyr: A grammar of data manipulation* [computer software]. https://CRAN.R-project.org/package=dplyr

Zhang, Y., Hobbie, S. E., Schlesinger, W. H., Berg, B., Sun, T., & Zhu, J. (2024). Exchangeable manganese regulates carbon storage in the humus layer of the boreal forest. *Proceedings of the National Academy of Sciences*, *121*(13), e2318382121. https://doi.org/10.1073/pnas.2318382121

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Data S1: Manganese peroxidase (MnP) and total peroxidase (Perox) activity per gram organic matter in soil samples collected for regional study.

Table S1: Descriptions of the 12 regional sites located in South-Central Sweden.

Table S2: List of genera with significantly higher estimated specific Mn-peroxidase and/or total peroxidase activity than expected by random variation tested each with a one-sided permutation test.

Table S3: List of taxa with significantly lower estimated specific Mn-peroxidase and/or total peroxidase activity than expected by random variation tested each with a one-sided permutation test.

Table S4: Results of Fisher's exact test of differences in frequency of individual ectomycorrhizal decomposers in pine- or spruce-dominated forest.

Table S5: Correlations between frequency of occurrence of species assigned as ectomycorrhizal decomposers and stand age and soil fertility analysed by generalized linear models.

Table S6: Correlations between combined relative abundance of the assigned ectomycorrhizal decomposers, when present, and stand age and soil fertility with sequencing platform as a random effect analysed by linear mixed model.

Table S7: Correlations between combined relative abundance of the assigned ectomycorrhizal decomposers among ectomycorrhizal fungi, when present, and stand age and soil fertility with sequencing platform as a random effect analysed by linear mixed model.

Figure S1: Sampling design at each of the twelve sites from the small-scale dataset.

Figure S2: Distribution of 12 small-scale (inset) and 577 national sampling sites within Sweden.

Figure S3: Manganese peroxidase activity (change in absorbance per minute per gram of organic matter across the 12 regional plots).

Figure S4: Principal Components Analysis of N/C ratio, soil pH, spruce percent basal area, and stand age of 548 large-scale samples.

Figure S5: Frequency of occurrence of select individual ectomycorrhizal decomposers across forest stand age in spruce-dominated forests.

Figure S6: Differences in (a) frequency of occurrence of the assigned ectomycorrhizal decomposer species group and (b) relative abundance of the assigned ectomycorrhizal decomposers as a proportion of the total fungal community, when present between dominant tree species.

Figure S7: Frequency of occurrence of individual assigned ectomycorrhizal decomposer species in pine- versus spruce-dominated forests.

How to cite this article: Packard, E. E., Pérez-Izquierdo, L., Clemmensen, K. E., Dahlberg, A., Spohn, M., Stendahl, J., & Lindahl, B. D. (2025). Ectomycorrhizal decomposers and their niche(s) in boreal forests. *Functional Ecology*, *39*, 1998–2014. https://doi.org/10.1111/1365-2435.70085

Ectomycorrhizal decomposers and their niche(s) in boreal forests

Erica E. Packard[1], Leticia Pérez-Izquierdo[2], Karina E. Clemmensen[3], Anders Dahlberg[3], Marie Spohn[1], Johan Stendahl[1], Björn D. Lindahl[1]

[1]Swedish University of Agricultural Sciences, Department of Soil and Environment, 75007 Uppsala, Sweden
[2]Basque Centre for Climate Change (BC3), Scientific Campus of the University of the Basque Country, 48940 Leioa, Spain
[3]Swedish University of Agricultural Sciences, Department of Forest Mycology and Plant Pathology, 75007 Uppsala, Sweden

Correspondence: erica.packard@slu.se

Supplemental tables

Table S1 Descriptions of the 12 regional sites located in South-Central Sweden. Thirty-eight samples were collected at each site following the sampling design shown in Figure S1. The younger forests (15-20 years) were established by planting after clear-cutting, whereas the older forests contained trees that were > 80 years old, indicating a history of continuous tree cover.

Site Type	Dominant tree species	Understory species	Vegetation types	Forest age (years)
Young Pine (3 sites)	*Pinus sylvestris L.*	*Vaccinium vitis-idaea L.* *Pleurozium schreberi (B.) Mitt.* *Deschampsia flexuosa L.* *Festuca ovina L.*	Shrub Moss Grass Grass	15-20
Old Pine (3 sites)	*Pinus sylvestris L.*	*Vaccinium vitis-idaea L.* *Pleurozium schreberi (B.) Mitt.*	Shrub Moss	> 80
Young Spruce (3 sites)	*Picea abies* L.	*Vaccinium myrtillus* L. *Hylocomium splendens* (Hedw.) Schimp. *Deschampsia flexuosa L.* *Festuca ovina L.*	Shrub Moss Grass Grass	15-20
Old Spruce (3 sites)	*Picea abies* L.	*Vaccinium myrtillus* L. *Hylocomium splendens* (Hedw.) Schimp.	Shrub Moss	> 80

Table S2 List of genera with significantly higher estimated specific Mn-peroxidase and/or total peroxidase activity than expected by random variation tested each with a one-sided permutation test. All genera with an unadjusted p-value < 0.1 are shown. Ectomycorrhizal genera are in bold. Genera containing species considered as potential ectomycorrhizal decomposers are highlighted in grey. WAE_{gen} is ranked from low (stronger correlation) to high (weaker correlation). Mean number of Class-II peroxidase genes per species within genera are extracted from Miyauchi *et al*. (2020) and the Joint Genome Institute Mycocosm portal.

Genus	Number of species clusters within the genus	Mean Class-II peroxidase genes ± SE (number of species in genus with published genomes)	Mn-peroxidase		Total peroxidase	
			p-value	WAE_{gen} rank order	p-value	WAE_{gen} rank order
Gautieria	**1**	**25 (1)**	**0.0286**	**1**	**0.0673**	**3**
Lactarius	**25**	**2.8 ± 0.2 (10)**	**0.0504**	**15**		**25**
Coniophora	4	0 (2)	0.0768	6	0.0706	4
Gymnopilus	2	15 (1)		46	0.0102	2
Postia	3	0 (2)	0.0984	7		12
Rescinicium	1	15 (1)	0.064	2		6
Serpula	1	0 (3)	0.0915	4		7
Clavulina*	**5**	**0 (0)**		**5**	**0.0233**	**1**
Naucoria*	**2**	**13.3 ± 9.6 (3)†**	**0.0559**	**3**		**10**

† Gene counts from within family

* No species from within the genus significant

Table S3 List of taxa with significantly higher estimated specific Mn-peroxidase and/or total peroxidase activity than expected by random variation tested each with a one-sided permutation test. All taxa with an unadjusted p-value < 0.1 are shown. Ectomycorrhizal taxa are in bold. WAE_{sp} is ranked from high (stronger correlation) to low (weaker correlation). Mean number of Class-II peroxidase genes per species within genera are extracted from Miyauchi *et al*. (2020) and the Joint Genome Institute Mycocosm portal.

Species	UNITE species hypothesis	Mn-peroxidase		Total peroxidase	
		p-value	*WAEsp* rank order	p-value	*WAEsp* rank order
Amanita porphyria	**SH0996683.10FU**	**0.0098**	**206**	**0.0121**	**205**
***Amphinema* sp.**	**SH0943289.10FU**	**0.0983**	**191**	**0.0923**	**186**
Ampulloclitocybe clavipes	SH0760706.10FU	0.0437	155	0.0511	147
Ceraceomyces eludens	SH1006121.10FU		145	0.064	197
Ramariopsis sp.	SH0955512.10FU	0.0833	194		167
Coprinellus micaceus	SH1003896.10FU	0.0234	184	0.004	195
Cortinarius* aff. *obtusus	**SH1017247.10FU**	**0.0699**	**210**	**0.0346**	**210**
Cortinarius armeniacus	**SH0986739.10FU**		**149**	**0.0764**	**192**
Cortinarius casimiri	**SH0986663.10FU**	**0.0127**	**213**	**0.0118**	**214**
Cortinarius spilomeus	**SH1017671.10FU**	**0.0792**	**196**		**20**
Hebeloma laterinum	**SH0995162.10FU**		**90**	**0.0546**	**198**
Inocybe rufoalba	**SH0865010.10FU**	**0.0211**	**212**	**0.0045**	**213**
***Laccaria laccata* coll.1**	**SH0976157.10FU**	**0.0286**	**209**	**0.0269**	**207**
***Laccaria laccata* coll.2**	**SH0976157.10FU**		**160**	**0.0039**	**200**
Lactarius camphoratus	**SH0960918.10FU**	**0.0272**	**200**	**0.055**	**188**
Lactarius fennoscandicus	**SH0960988.10FU**	**0.0581**	**201**		**177**
***Lactarius glyciosmus* coll.**	**SH0961100.10FU**		**80**	**0.0704**	**180**
Lactarius rufus	**SH0961123.10FU**	**0.0884**	**156**		**109**
Lactarius torminosus	**SH0960964.10FU**	**0.0181**	**207**	**0.0072**	**209**
***Leccinum* sp.**	**SH0918039.10FU**	**0.0934**	**181**		**159**
Lycoperdon pyriforme	SH1012772.10FU	0.0013	199	0.0065	189
Lycoperdon subumbrinum coll.	SH1012760.10FU		109	0.0302	181
Mycena sp.	SH0948001.10FU	0.0983	188	0.0606	190
Mycena amicta	SH0760377.10FU		178	0.0275	199
Mycena aurantiomarginata	SH0748985.10FU	0.0475	208		110
Mycena cinerella	SH0948046.10FU	0.0743	131		111
***Piloderma* sp.**	**SH1024083.10FU**		**139**	**0.0798**	**175**
Piloderma olivaceum	**SH1024182.10FU**	**0.0789**	**144**	**0.0738**	**139**
Piloderma sphaerosporum	**SH1024220.10FU**	**0.0414**	**135**		**104**
Polyozellus mucidulus	**SH0991672.10FU**		**123**	**0.0453**	**201**
Pseudotomentella mucidula	**SH0991678.10FU**		**64**	**0.0684**	**196**
Rhodocollybia butyracea f. *asema*	SH0830119.10FU	0.0621	192		132
***Russula emetica* coll.**	**SH0956252.10FU**	**0.0126**	**204**	**0.0082**	**206**
Russula favrei	**SH0957080.10FU**		**74**	**0.0522**	**194**
Serendipita sp.1	SH0796279.10FU	0.0713	151	0.0285	162
Serendipita sp.2	SH0734306.10FU	0.0833	177		151
Sistotrema sp.	SH0766568.10FU	0.0259	214	0.0699	211
Sistotrema sp.	SH0837147.10FU	0.037	211	0.0159	212

Thelephora alnii	**SH0919137.10FU**	**0.0788**	**161**		**115**
Thelephora eucoerulea	**SH0920796.10FU**		**167**	**0.0767**	**170**
***Tomentella (Thelephora) bryophila* coll.**	**SH0921838.10FU**	**0.0906**	**197**	**0.0489**	**208**
Tomentella (Thelephora) ellisii	**SH1017569.10FU**	**0.0469**	**203**	**0.0453**	**203**
Trechispora cohaerens	SH0942492.10FU	0.0682	205	0.0935	202
Xylodon asperus	SH1003274.10FU		189	0.0518	204

Table S4 Results of Fisher's exact test of differences in frequency of individual ectomycorrhizal decomposers in pine- or spruce-dominated forest. Odd's ratio greater than one indicate higher frequency of occurrence in spruce-dominated forests and less than one indicate higher frequency of occurrence in pine-dominated forests. P-values adjusted for multiple tests with False Discovery Rate method. P-values < 0.05 are in bold.

	n	Odd's ratio (CI)	p-value	p-value adjusted
Cortinarius comptulus coll.	77	0.99 (0.56-1.79)	1	1
Gautieria cf. *otthii*	81	2.72 (1.48-5.17)	**<0.001**	**0.006**
Lactarius necator	49	1.33 (0.67-2.73)	0.422	0.602
Cortinarius fulvescens coll.	20	1.01 (0.36-2.96)	1	1
Lactarius tabidus	27	0.82 (0.34-2.02)	0.681	0.852
Russula aquosa	32	1.56 (0.67-3.86)	0.337	0.562
Cortinarius anomalus	58	1.84 (0.95-3.70)	**0.068**	0.183
Cortinarius mucosus coll.	29	0.26 (0.10-0.63)	**0.001**	**0.006**
*Russula rhodopus**	6	Na	Na	Na
Cortinarius spilomeus	19	2.68 (0.82-11.41)	0.091	0.183

*only present in spruce-dominated forests

Table S5 Correlations between frequency of occurrence of species assigned as ectomycorrhizal decomposers and stand age and soil fertility analysed by Generalized Linear Models. P-values adjusted for multiple tests with False Discovery Rate method. P-values < 0.05 are in bold and < 0.1 are in italic.

			Stand age (log-transformed years)			Soil fertility index		
		n	Estimate (SE)	p-value	p-value adjusted	Estimate (SE)	p-value	p-value adjusted
Pine dominated (295)	*Cortinarius comptulus* coll.	31	-0.189 (0.19)	0.320	0.913	0.060 (0.16)	0.716	0.719
	Gautieria cf. *otthii*	20	-0.025 (0.23)	0.913	0.913	0.509 (0.17)	**0.002**	**0.032**
	Lactarius necator	17	0.157 (0.25)	0.538	0.913	0.417 (0.18)	**0.021**	*0.087*
	Cortinarius fulvescens coll.	8	0.249 (0.37)	0.501	0.913	-0.494 (0.44)	0.256	0.390
	Lactarius tabidus	12	-0.200 (0.29)	0.495	0.913	0.229 (0.23)	0.315	0.504
	Russula aquosa	10	0.127 (0.33)	0.698	0.913	0.616 (0.22)	**0.004**	**0.032**
	Cortinarius anomalus	17	0.240 (0.26)	0.352	0.913	0.249 (0.19)	0.197	0.390
	Cortinarius mucosus coll.	20	-0.045 (0.23)	0.846	0.913	-0.125 (0.22)	0.577	0.638
	Cortinarius spilomeus	4	0.122 (0.51)	0.812	0.913	0.330 (0.36)	0.360	0.504
Spruce dominated (253)	*Cortinarius comptulus* coll.	46	0.315 (0.17)	*0.062*	*0.097*	-0.751 (0.19)	**<0.001**	**<0.001**
	Gautieria cf. *otthii*	61	-0.119 (0.15)	0.419	0.465	-0.002 (0.11)	0.984	0.991
	Lactarius necator	32	0.368 (0.20)	*0.062*	*0.097*	0.046 (0.13)	0.727	0.991
	Cortinarius fulvescens coll.	12	0.885 (0.35)	**0.010**	**0.030**	-1.137 (0.42)	**0.006**	**0.004**
	Lactarius tabidus	15	0.389 (0.28)	0.161	0.220	-0.174 (0.22)	0.429	0.758
	Russula aquosa	22	0.539 (0.24)	**0.025**	*0.068*	-0.128 (0.18)	0.470	0.758
	Cortinarius anomalus	41	-0.146 (0.17)	0.396	0.465	-0.135 (0.13)	0.315	0.758
	Cortinarius mucosus coll.	9	-0.180 (0.34)	0.597	0.596	-0.230 (0.29)	0.433	0.758
	Russula rhodopus	6	-0.841 (0.46)	*0.065*	*0.097*	0.070 (0.28)	0.804	0.991
	Cortinarius spilomeus	15	-0.820 (0.29)	**0.005**	**0.030**	0.001 (0.19)	0.992	0.991

Table S6 Correlations between combined relative abundance of the assigned ectomycorrhizal decomposers, when present, and stand age and soil fertility with sequencing platform as a random effect analysed by Linear Mixed Model. Soil fertility index represents the first axis of a PCA with soil pH and N/C.

		Stand age (log-transformed years)			Stand age (old vs young)		
		Estimate (SE)	p-value	Model Pseudo delta R_2	Estimate (SE)	p-value	Model Pseudo delta R_2
Pine dominated (295 sites)	intercept	(-) 2.421 (0.11)			(-) 2.390 (0.11)		
	Age	(-) 0.093 (0.08)	0.242	0.05	(-) 0.161 (0.13)	0.233	0.05
	Soil fertility index	(-) 0.009 (0.05)	0.864		(-) 0.013 (0.05)	0.788	
Spruce dominated (253 sites)	intercept	(-) 2.330 (0.05)			(-) 2.315 (0.06)		
	Age	(+) 0.021 (0.06)	0.698	0.01	(-) 0.085 (0.12)	0.485	0.01
	Soil fertility index	(-) 0.043 (0.05)	0.357		(-) 0.054 (0.05)	0.241	

Table S7 Correlations between combined relative abundance of the assigned ectomycorrhizal decomposers among ectomycorrhizal fungi, when present, and stand age and soil fertility with sequencing platform as a random effect analysed by Linear Mixed Model. Soil fertility index represents the first PC axis of a PCA with soil pH and N/C ratio.

		Stand age (log-transformed years)			Stand age (old vs young)		
		Estimate (SE)	p-value	Model Pseudo delta R_2	Estimate (SE)	p-value	Model Pseudo delta R_2
Pine dominated (295 sites)	intercept	(-) 1.765 (0.06)			(-) 1.731 (0.07)		
	Age	(-) 0.092 (0.08)	0.246	0.02	(-) 0.168 (0.13)	0.210	0.02
	Soil fertility index	(+) 0.026 (0.05)	0.601		(+) 0.021 (0.05)	0.678	
Spruce dominated (253 sites)	intercept	(-) 1.723 (0.06)			(-) 1.738 (0.07)		
	Age	(+) 0.066 (0.05)	0.223	0.02	(+) 0.021 (0.12)	0.858	0.01
	Soil fertility index	(+) 0.012 (0.05)	0.778		(-) 0.0004 (0.05)	0.993	

Supplemental Figures

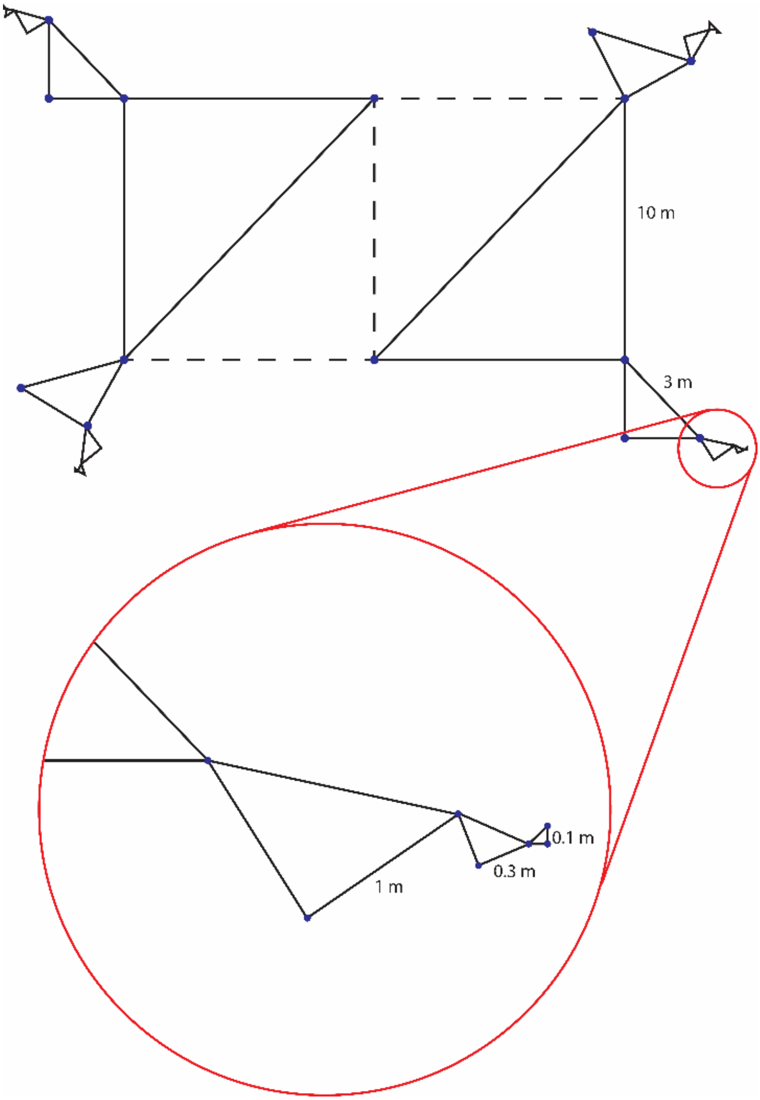


Fig. S1 Sampling design at each of the twelve sites from the small-scale dataset. The design consists of two side-by-side 10 m triangles with smaller triangles extending from each “outside” corner. The triangles decrease in size from 10 m to 0.1 m. From each corner a 2.5 cm diameter core was sampled (shown as blue dots), resulting in 38 cores per site.

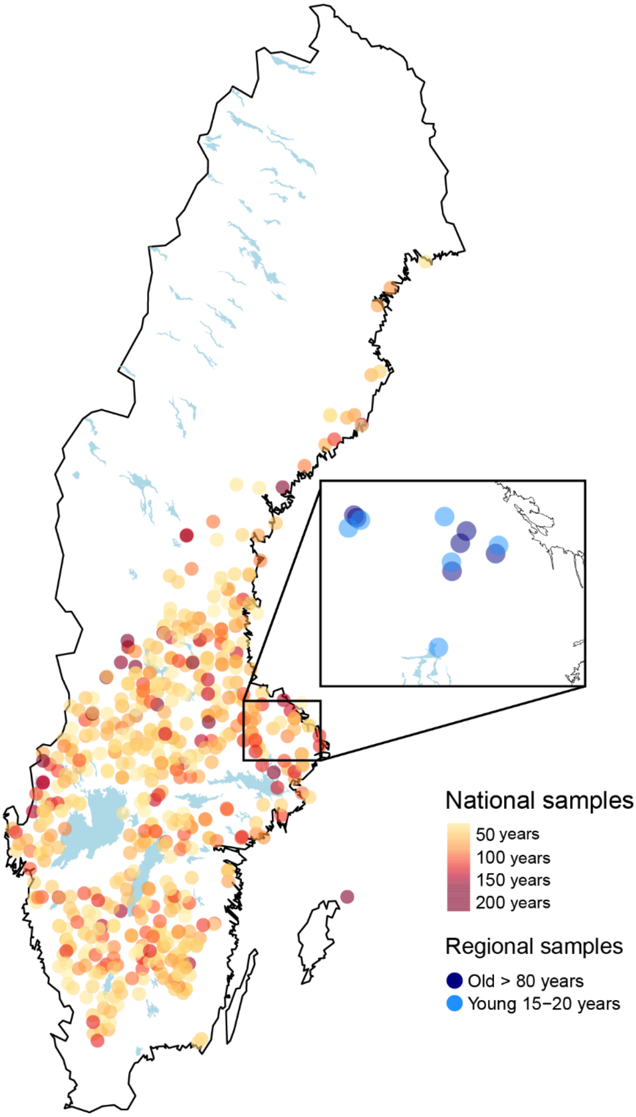


Fig. S2 Distribution of 12 small-scale (inset) and 577 national sampling sites within Sweden. The large-scale dataset was collected as a part of the Swedish National Forest Inventory and the regional dataset was collected for a study focussed on spatial heterogeneity of fungal communities and Mn-peroxidase activity. The minimum stand age for national samples is 10 years and the maximum age is 215 years. In the large-scale dataset, forests age under 70 years have likely regenerated following clear-cut logging whereas forests over 70 years have a history of longer forest continuity.

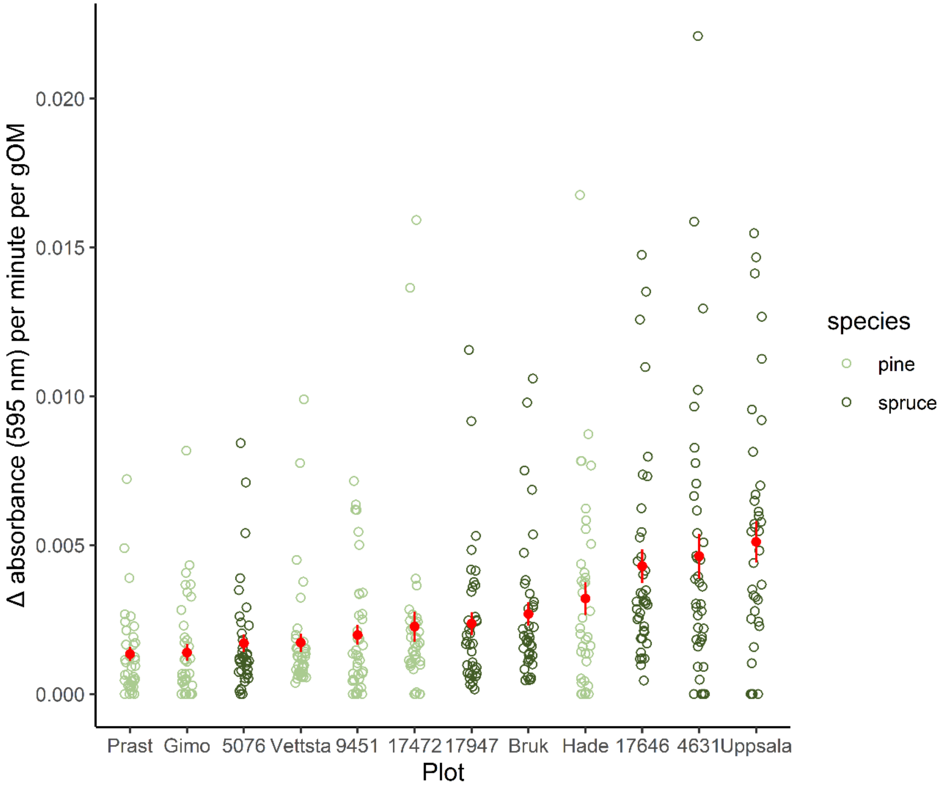


Fig. S3 Manganese peroxidase activity (change in absorbance per minute per gram of organic matter across the 12 regional plots. Enzyme activity is measured using the coupled colorimetric reaction of DMAB and MBTH. Each point is estimated enzyme activity from an individual soil core. Red points represent mean ± SE. n = 38

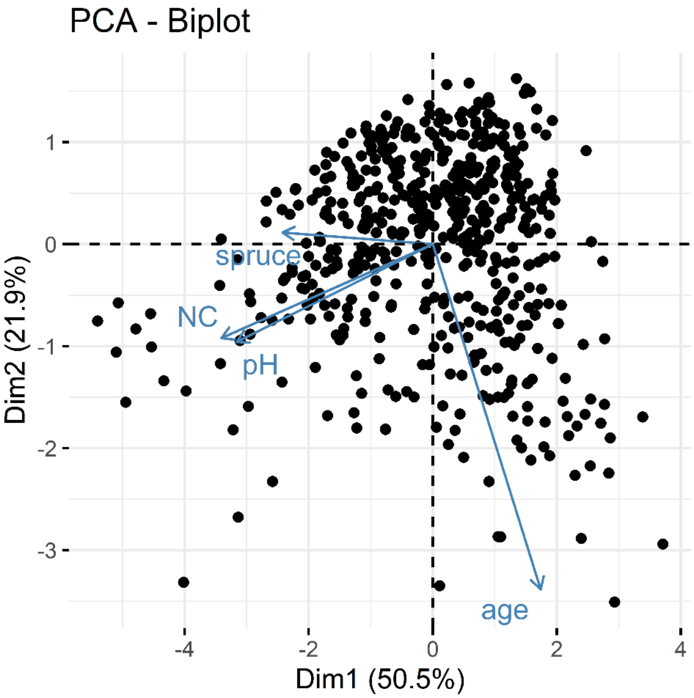


Fig. S4 Principal Components Analysis of N/C ratio, soil pH, spruce percent basal area, and stand age of 548 large-scale samples. Soil pH and NC ratio are highly collinear.

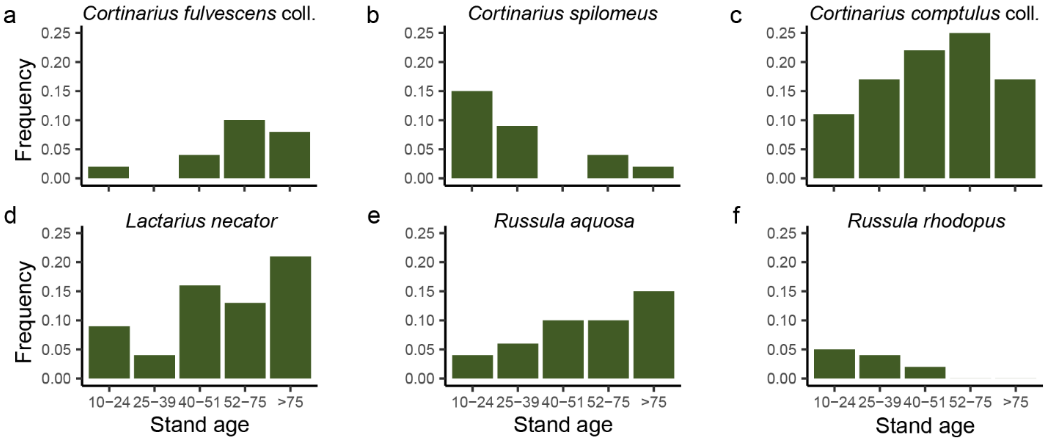


Fig. S5 Frequency of occurrence of select individual ectomycorrhizal decomposers across forest stand age in spruce-dominated forests. Stand age is sectioned into five evenly distributed bins (for graphical representation; 47-55 forests each). Statistics are presented in Table S5.

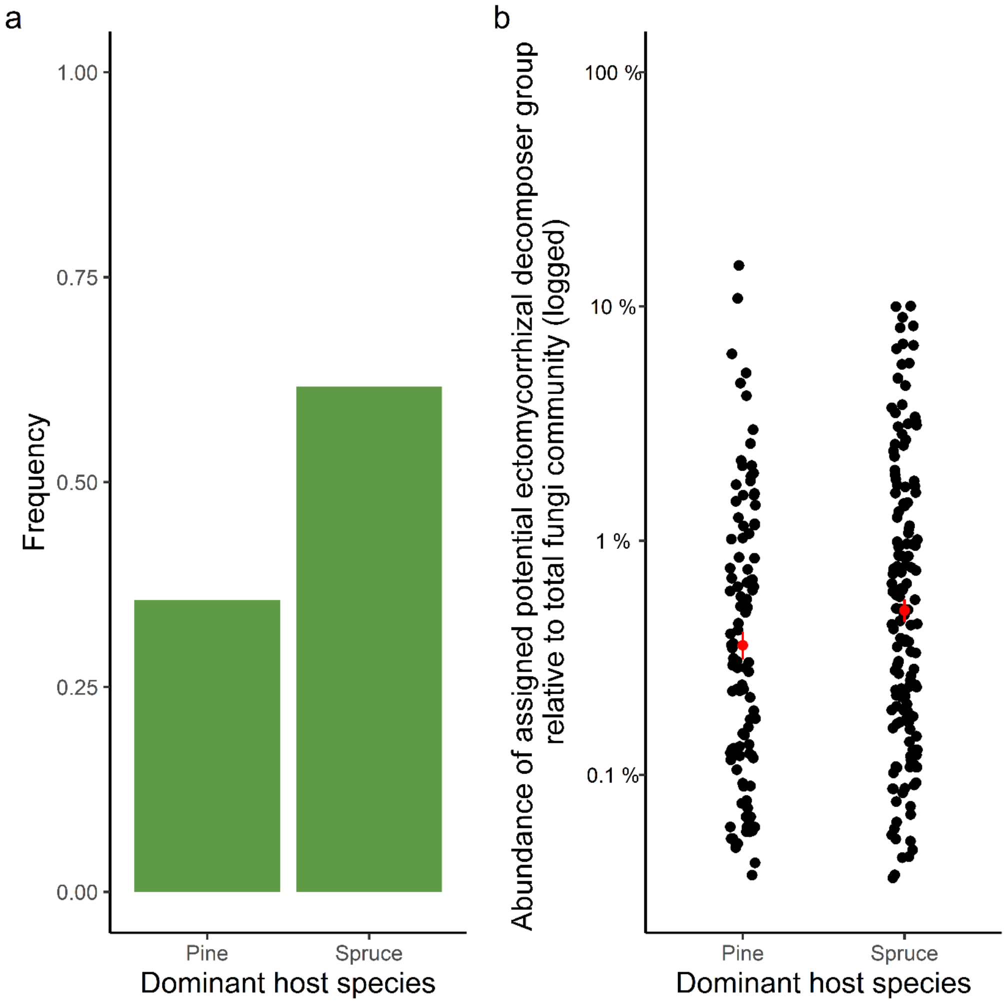


Fig. S6 Differences in (a) frequency of occurrence of the assigned ectomycorrhizal decomposer species group and (b) relative abundance of the assigned ectomycorrhizal decomposers as a proportion of the total fungal community, when present between dominant tree species. In (b), red symbols represent mean ± SE. n = 295 for pine-dominated and n = 253 for spruce-dominated.

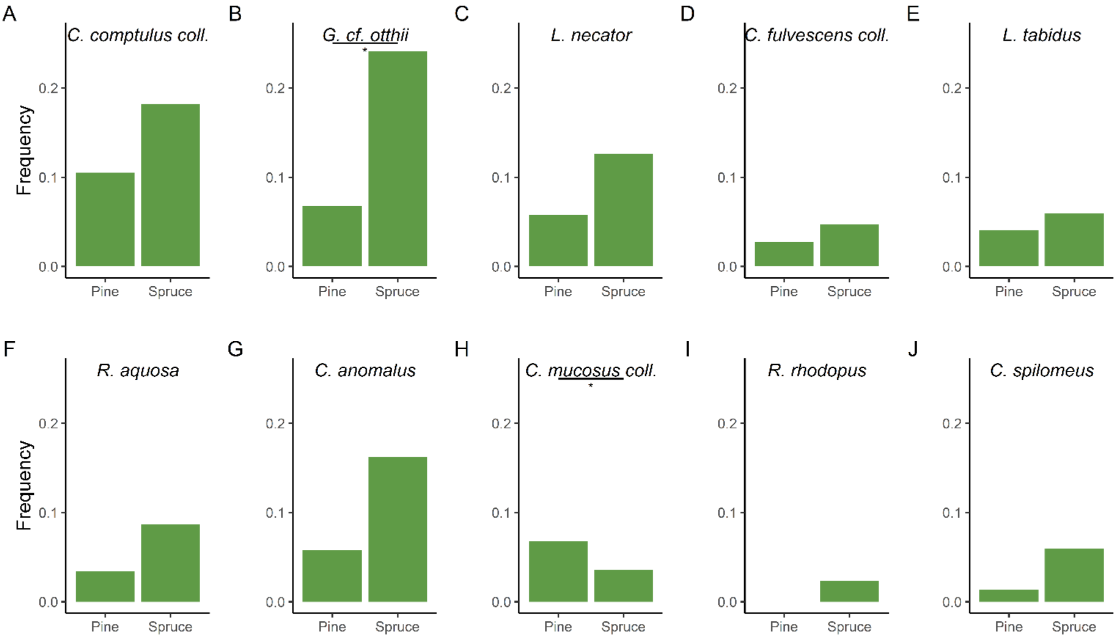


Fig. S7 Frequency of occurrence of individual assigned ectomycorrhizal decomposer species in pine- versus spruce-dominated forests. The asterisks indicate significant (p-value < 0.05) differences between pine-dominated and spruce-dominated forests. Statistics are present in Table S4.

Fungal Ecology 80 (2026) 101492

Contents lists available at ScienceDirect

Fungal Ecology

journal homepage: www.elsevier.com/locate/funeco

Targeting Mn-dependent peroxidase activities - considerations and optimizations

E.E. Packard, K. Jörgensen *

Department of Soil and Environment, Swedish University of Agricultural Sciences, Box 7014, SE-750 07, Uppsala, Sweden

A R T I C L E I N F O

Handling Editor: Prof. Linda van Diepen

Keywords:
2,6-DMP
ABTS
Decomposition
DMAB
Enzyme assay
Extracellular enzymes
L-DOPA
MBTH

A B S T R A C T

Estimating enzyme activity is a widely applied method for understanding the activity of organisms and their impact on biogeochemical cycles. In forest soils, fungal oxidative enzymes, such as manganese peroxidases, are key regulators of carbon stocks. Here we investigate whether MBTH/DMAB assays, targeting manganese peroxidase activities, are impacted by the degree of fungal cell disruption during extraction from pure culture. Further, we assess whether substrates 2,6-DMP, ABTS, and L-DOPA can distinguish manganese-dependent peroxidase activities under conditions optimized for MBTH/DMAB. Increased mycelial disruption during enzyme extraction increased estimated manganese peroxidase activity, but also the proportion of activity presumed to be from intracellular manganese-independent peroxidases. All substrates could detect peroxidase activities, but their specificity towards manganese peroxidases varied. In particular, ABTS was more readily oxidized by manganese-independent peroxidases. We recommend that extraction methods from soil be adapted to avoid excessive release of internal peroxidases, due to the trade-off between extraction efficiency and assay specificity.

1. Introduction

Soils store a large part of the global carbon stock (Pan et al., 2011), and insight into the processes and mechanisms that regulate turnover of organic matter in the soil is vital for better understanding of biogeochemical cycles. Soil fungi and bacteria secrete extracellular enzymes that hydrolyze or oxidize organic compounds in the soil, thus contributing to regulation of carbon stocks. Generally, hydrolytic enzymes are highly specific towards target compounds of high chemical quality (*i.e.* regular repeating polymers; Bosatta and Ågren, 1991), while oxidative enzymes are less specific and can target more complex molecules with heterogeneous structures, such as lignins (Baldrian, 2008). Due to the ubiquity of extracellular enzymes in decomposition processes, high-throughput and comparably cheap enzyme assays have been developed to estimate enzyme activities. Enzyme assays are used to describe activities of microbial communities and explore links to carbon and nutrient dynamics (Sinsabaugh et al., 2002) and in particular several studies have found links between fungal communities and oxidative enzyme activity (Šnajdr et al., 2008; Bödeker et al., 2014; Entwistle et al. 2017, 2018; Kyaschenko et al., 2017a; Sterkenburg et al., 2018; Kranabetter et al., 2021; Pérez-Izquierdo et al., 2021; Jörgensen et al., 2022) with implications on the size and turnover of the soil carbon stock (Kyaschenko et al., 2017b). Because of the high diversity of enzymes, both intracellular and extracellular, it is important that assays can reliably target specific enzymes of significance in biogeochemical transformations (Nannipieri et al., 2018).

There are multiple classes of oxidative enzymes, including peroxidases (EC 1.11.1). These can be produced both intra- and extracellularly by a diverse array of soil organisms and utilize peroxides as electron acceptors. Yet, only a subset of fungi, within the class Agaricomycetes, secrete a range of ligninolytic class-II peroxidases (Floudas et al., 2012), such as manganese peroxidases (EC 1.11.1.13), lignin peroxidases (EC 1.11.1.14), and versatile peroxidases (EC 1.11.1.16). Manganese peroxidases are exclusively extracellular and oxidize Mn^{2+} to Mn^{3+}, with hydrogen peroxide (H_2O_2) as an electron acceptor. Mn^{3+}, in turn, can oxidize a wide variety of substrates including phenolic and other aromatic compounds, non-specifically (Kirk and Farrell, 1987). Exchangeable manganese has been implicated as one of the most important regulators of carbon stocks in boreal and temperate forest soils (Stendahl et al., 2017; Kranabetter, 2019; Zhang et al., 2024), suggesting that manganese-dependant oxidation of organic compounds and the fungi that produce them are fundamental to our understanding of soil carbon

---

* Corresponding author.
E-mail address: karolina.jorgensen@slu.se (K. Jörgensen).

https://doi.org/10.1016/j.funeco.2025.101492
Received 10 January 2025; Received in revised form 5 December 2025; Accepted 12 December 2025

storage.

Most assays of oxidative enzymes are colorimetric and estimate potential enzyme activity by utilizing substrates that produce dyes when they are oxidized by an enzyme. By measuring the difference in dye formation (absorbance at a substrate specific wavelength) between samples incubated with or without H_2O_2 in the reaction, peroxidase activity is distinguished from that of phenol oxidases (such as laccases EC 1.10.3.2). To further distinguish manganese-dependent peroxidase activity, assays can be adapted to exploit that manganese peroxidases rely solely on oxidation mediated through Mn^{3+} (Glenn and Gold, 1985; Baldrian et al., 2000; Arnstadt et al., 2016a). Manganese-dependent peroxidases can thus be targeted by comparing rates of product formation between reactions where a manganese source is present, or where free manganese ions are inhibited through the addition of a chelator. This approach, with inclusion of $MnSO_4$ or EDTA, has previously been used for methods based on the coupled oxidation of 3-methyl-2-benzothiazolinone hydrazone hydrochloride monohydrate (MBTH) and 3-(dimethylamino)benzoic acid (DMAB; Ngo and Lenhoff, 1980; Daniel et al., 1994), and for 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS; Arnstadt et al., 2016a). Similar adaptations for 3-(3,4-Dihydroxyphenyl)-L-alanine (L-DOPA) and 2,6-dimethoxyphenol (2,6-DMP) have not been developed. Comparisons of enzyme assay substrates has largely been focused on ABTS and L-DOPA (Sinsabaugh, 2010; Bach et al., 2013), with very few studies including MBTH/DMAB or 2–6,DMP (Castillo et al., 1994; García-Esquivel et al., 2021).

Typically, enzyme assays are done in buffer solutions under optimal pH and temperature conditions, with non-limiting concentrations of reagents to maximize the assay efficiency. Since H_2O_2 is crucial for the functioning of peroxidases, we test whether the concentration of H_2O_2 in the reaction affects reaction rates. Moreover, samples are often finely homogenized when doing assays, which may release “stabilized” enzymes that were chemically or spatially immobilized (Wallenstein and Weintraub, 2008; Buckley et al., 2019). Thus, one important caveat is that these assays measure “potential activities” of enzymes and are likely to overestimate the actual enzyme activities (Wallenstein and Weintraub, 2008; Nannipieri et al., 2012). In addition to the release of stabilized extracellular enzymes, the buffer choice and homogenization process could also release intracellular enzymes (Nannipieri, 2006). Despite this issue often being mentioned in papers that review enzyme assay protocols (Nannipieri et al., 1996; Nannipieri, 2006; Fornasier et al., 2011; German et al., 2011), there has been little investigation into the extent that sample homogenization method influences enzyme activity. Therefore, we assess the potential interference by the presence of intracellular peroxidases released during cell lysis by measuring enzyme activity from pure culture of a manganese peroxidase-producing saprotrophic fungus at different levels of mechanical intensity during extraction. Finally, we evaluate whether the inclusion of $MnSO_4$ or EDTA to specify manganese-dependent peroxidases could also be used under the conditions optimized for MBTH/DMAB with the substrates 2,6-DMP, ABTS, and L-DOPA by measuring activities of horseradish peroxidase and a commercial manganese peroxidase, and from pure culture of a saprotrophic fungus.

We expected that (i) higher extraction intensity would increase the estimated enzyme activities in extracts from a cultured saprotrophic fungus. Further, we expected that (ii) fungal cell lysis due to mechanical disruption of living mycelium would release internal peroxidases, thus increasing the proportional contribution of manganese-independent peroxidases to total peroxidase activity. We also expected that (iii) all substrates could be used to distinguish between manganese-dependent and -independent peroxidase activity. Specifically, we expected that the manganese peroxidase would oxidize the substrates *only* when manganese was present, while manganese-independent peroxidases would oxidize the substrates regardless of manganese presence.

2. Materials and methods

2.1. Enzyme solutions

Recombinant manganese peroxidase from *Phanerochaete chrysosporium* (CAS: 114995-15-2, Sigma-Aldrich, Burlington, MA, USA) was dissolved in ultrapure water and diluted to a stock concentration of 1 U ml^{-1}. Horseradish peroxidase (CAS: 9003-99-0, Sigma-Aldrich, Burlington, MA, USA) was dissolved and diluted in ultrapure water to a stock concentration of 1000 U ml^{-1}. Both stock solutions were further diluted to be used in the assays (two concentrations): manganese peroxidase diluted to 0.008 and 0.005 U ml^{-1}; and horseradish peroxidase diluted to 0.4 and 0.1 U ml^{-1}.

Peroxidases were extracted from pure cultures of *Hypholoma fasciculare* (isolate: MUCL 047611), which was selected as a representative of a filamentous manganese peroxidase-producing decomposer that does not secrete manganese-independent peroxidases (Ruiz-Dueñas et al., 2021). The cultures were grown in 9.6 cm petri dishes with *ca.* 2 g of dried and autoclaved *Pinus sylvestris* L. needles (that were collected when senesced but still attached to branches) and 25 ml of liquid Modified Melin-Norkrans medium (Marx, 1969) for 7 weeks in darkness at 20 °C. We aimed to create a gradient in extraction intensity by adding *ca.* 30 ml of 50 mM sodium acetate buffer (pH 5) to the petri dishes and agitating the culture to different degrees using three different methods. The first level was “soaking” by adding extraction buffer and leaving the culture undisturbed for *ca.* 1 min. The second level was “shaking” where extraction buffer was added and the Petri dish was gently shaken on a vortex at setting 7 (Scientific Industries Vortex-Genie2, Fisher Scientific, Hampton, NH, USA) for *ca.* 1 min without creating visible disruptions to the mycelium. The rationale for this treatment was that it would increase the contact between extraction buffer, mycelium and needles without damaging the mycelium. The final level was “fine homogenization” where the culture was thoroughly homogenized with extraction buffer using a dispersing mixer (T 25 digital ULTRA-TURRAX, IKA-Werke GmbH & Co. KG, Staufen, Germany) for *ca.* 1 min. There were three biological replicates per treatment (soaking, shaking, and homogenization). All enzyme extracts were diluted to a final volume of 50 ml with sodium acetate buffer. A schematic of the experimental set up is shown in Fig. S1.

2.2. Assay procedure

Substrate solutions were prepared in a 5:5:2:2 volumetric ratio with 100 mM sodium lactate buffer (pH = 4.5), 100 mM sodium succinate buffer (pH = 4.5), 1 mM $MnSO_4$ *or* 2 mM EDTA and a colorimetric substrate: 20 mM ABTS, 25 mM L-DOPA, 5 mM 2,6-DMP, *or* 1 mM MBTH/50 mM DMAB (1:1 volumetric ratio). All chemicals were purchased from Sigma-Aldrich (Burlington, MA, USA).

Assays were run with four technical replicates with 50 μl of enzyme solution made from either 10 μl of the diluted commercial enzymes and 40 μl of 50 mM sodium acetate buffer (pH = 5), or 50 μl of the *H. fasiculare* enzyme extract with 140 μl of substrate solution (with or without $MnSO_4$). Extracts from *H. fasiculare* were centrifuged at 2500 rpm for 5 min, and only the supernatant used in assays to avoid interference by mycelia. Either 10 μl of 2.5 mM H_2O_2 (0.125 mM in final reaction) or an additional 10 μl of sodium acetate buffer (to control for non-peroxidase activity) were added to the reaction (Table S1). The H_2O_2 concentration was optimized based on the rate of coupled MBTH/DMAB formation with two concentrations of manganese peroxidase and a range of H_2O_2 concentrations (2.5 mM; 0.5 mM; 0.25 mM; 0.125 mM; 0.063 mM; 0.038 mM, 0.019 mM, 0 mM). Samples were incubated at room temperature in transparent, flat bottomed 96 well plates (Immunoplate, unbinding, SPL Life Sciences, Seoul, Korea) for 30 min in an fluorescence/absorbance spectrophotometer (Tecan Spark, Tecan Group Ltd. Männedorf, Switzerland) with absorbance measured every 30 s at 420 nm (ABTS), 450 nm (L-DOPA), 468 nm (2,6-DMP) or 590 nm

(MBTH/DMAB).

To enable comparison between substrates, and enzymes, standard curves were prepared using Mn(III) acetate to convert absorbance at given wavelengths to Mn^{3+} equivalents in μmol amounts. Separate standard curves were prepared for each enzyme assay substrate. Mn(III) acetate was prepared in 50 mM sodium malonate (pH 4.5) immediately before assay. The standard curves were established with the same reagents and reaction conditions as all other enzyme assays. Dye formation was immediate and substrate-specific absorbance for each concentration of the Mn(III) acetate gradient was measured at a single time-point.

2.3. Data handling

Absorbance at each time point of the 30 min incubation was calculated as the average absorbance of the four technical replicates. To test the effect of extraction intensity on the rate of manganese-independent peroxidase activity, the proportion of activity in reactions without $MnSO_4$ out of the total peroxidase activity (with H_2O_2 present) was calculated for all extraction methods and substrates. Reaction rates were calculated based on the initial linear phase of the incubation, between 0 and 10 min. The data were checked for inconsistency in the linear reaction and measurements that were >1.5 standard deviations from the median of technical replicates were removed. One-way ANOVAs were used to assess whether there were differences between the different extraction methodologies in 1) the proportion of manganese-independent enzyme activity and 2) the reaction rate of manganese-dependant enzyme activity. Tests were done for each substrate separately. To test the sensitivity of the substrates, one-way ANOVAs were used to evaluate whether the reaction rate of manganese-dependent enzyme activity varied between the different substrates, for each extraction method.

Data handling was done using the Tidyverse package (Wickham et al., 2019) and figures were drawn using ggplot2 (Wickham et al., 2020) and patchwork (Pedersen, 2022).

3. Results

Addition of different concentrations of H_2O_2 affected the rate of product formation when using the MBTH/DMAB assay with pure manganese peroxidase from *P. chrysosporium* (Fig. 1). We observed inhibited product formation when H_2O_2 concentration exceeded 0.125 mM in the reaction. The same optimal H_2O_2 concentration was observed for both concentrations of manganese peroxidase.

The rate of manganese-peroxidase activity, *i.e.* the extraction efficiency, increased with more intense extraction of *H. fasiculare* grown on pine needles (ANOVA; F-value = 23.12; df = 2; p = 0.002; Fig. 2A–C), The average proportion of manganese-independent enzyme activity was over 15-fold higher in the thoroughly homogenized extract (0.27 ± 0.03) in comparison to the soaking treatment (0.017 ± 0.01) and 2-fold higher than in comparison to the shaking treatment (0.09 ± 0.03; ANOVA; F value = 64.52; df = 2; p = <0.001; Fig. 2D). Similar indications of release of intracellular peroxidases with fine homogenization were also observed for 2,6-DMP and L-DOPA, but ABTS had a high proportion of manganese-independent enzyme activity regardless of extraction intensity (Fig. S2 and S3A; Table S2). Further, the rate of manganese-dependant enzyme activity varied between substrates, with estimated activity being higher for MBTH/DMAB than the other substrates (Fig. S3B, Table S3).

Manganese peroxidase from *P. chrysosporium* oxidized MBTH/DMAB, L-DOPA, and 2,6-DMP at a rate at least two orders of magnitude higher in the presence of manganese than with EDTA (Table 1, Fig. S4). ABTS was oxidized both in the presence and absence of manganese, but more so with manganese. Horseradish peroxidase oxidized all substrates regardless of presence of manganese.

4. Discussion

When performing the assays to measure potential enzyme activities, concentrations of reagents in the assay are optimized to not be limiting, *i.e.* the rate limiting step should be the enzyme concentration in the extract. We observed that the highest H_2O_2 concentrations, and even a H_2O_2 concentration just 2x the optimal condition, reduced the initial rate of activity and the end-point absorbance (Fig. 1). Presumably, too high a concentration of H_2O_2 may damage the enzyme or reduce Mn^{3+} back to Mn^{2+} before it oxidizes the substrate (Wariishi et al., 1992). At the same time, concentrations below the optimum may have caused depletion of the H_2O_2 and thus reduced the end-point absorbance of the assay. Although this test was done in a pure enzyme solution, the optimal concentration of 0.125 mM falls within the range mentioned in other studies that assay peroxidases in pure culture and environmental samples (0.010–0.250 mM; Table S4). Still, it is advisable to optimize concentrations when assaying potential peroxidase activities.

Increased mechanical disruption of *Hypholoma fasiculare* mycelia grown on pine needles increased the rate of manganese peroxidase activity, suggesting that insufficient extraction can result in underestimation of potential manganese peroxidase activity. It is likely that the hydrophobic mycelium made the efficiency of the soaking treatment low

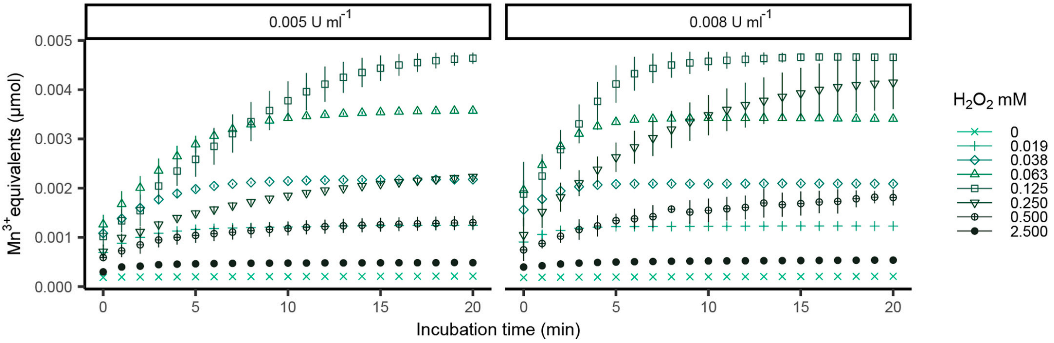


Fig. 1. Mn^{3+} equivalents (μmol) formed over time in reactions with manganese peroxidase from *Phanerochaete chrysosporium* at two concentrations (0.005 U ml^{-1} and 0.008 U ml^{-1}) with varying H_2O_2 concentrations added to initiate the reaction. Points represent average absorbance of four technical replicates, with lines indicating standard deviation of the mean, colors/shapes correspond to different concentrations of H_2O_2. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

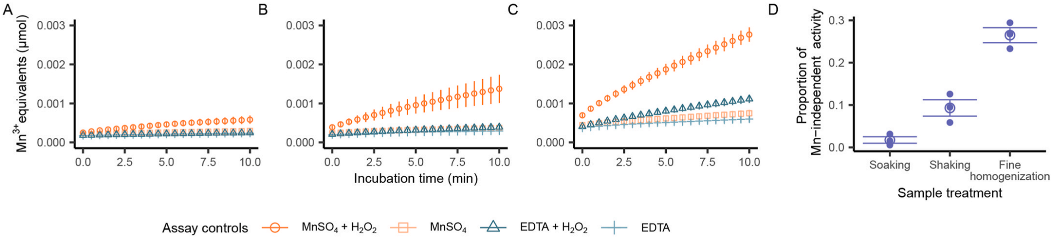


Fig. 2. Peroxidase activities (Mn^{3+} equivalents (μmol) formed per min) in cultures of *Hypholoma fasiculare* grown on sterilized senesced pine needles, assayed with MBTH/DMAB. Enzymes were extracted in 50 mM sodium acetate buffer at different extraction methodologies; (A) soaking the mycelium, (B) gently shaking the mycelium on a vortex (no visible tears in the mycelium) or (C) fine homogenization of mycelium and needles with a dispersing mixer. Points represent average absorbance of three biological replicates with four technical replicates with lines indicating standard deviation of the mean. (D) The proportion of Mn-independent activity relative to total peroxidase activity at the varying levels of extraction intensity of *Hypholoma fasiculare* grown on pine needles in pure-culture. Hollow coloured points represent means of replicates with standard error bars (n = 3).

Table 1

Reaction rates (Mn^{3+} equivalents formed (μmol) per min) of commercial manganese peroxidase (MnP) and horseradish peroxidase (HRP) with four different substrates in the presence of either $MnSO_4$ or EDTA. Reaction rates are calculated as the average slope of four technical replicates within the first 5 min of the reaction being initiated by addition of 0.125 mM H_2O_2.

		Reaction rate (μmol/min)	
		$MnSO_4$	EDTA
MBTH/DMAB	MnP	2.32 x 10^{-04}	5.61 x 10^{-06}
	HRP	1.67 x 10^{-04}	1.71 x 10^{-04}
ABTS	MnP	2.03 x 10^{-05}	1.10 x 10^{-05}
	HRP	7.24 x 10^{-04}	8.05 x 10^{-04}
L-DOPA	MnP	3.43 x 10^{-04}	2.00 x 10^{-06}
	HRP	9.16 x 10^{-06}	3.87 x 10^{-06}
2,6-DMP	MnP	1.14 x 10^{-04}	8.20 x 10^{-08}
	HRP	3.19 x 10^{-06}	2.95 x 10^{-06}

since the extraction buffer barely came in contact with the needles, where one would expect the highest peroxidase activity (Keiluweit et al., 2015). The shaking treatment allowed for more contact between needles and extraction buffer, while fine homogenization allowed for full contact between needles and extraction buffer. While enzyme activities measured from soaked and shaken samples were low, they only captured manganese-dependent oxidation of the substrate, as indicated by a positive reaction rate only when $MnSO_4$ was present (Fig. 2A–B).

However, in the more thoroughly homogenized extract, product formation also occurred in the absence of $MnSO_4$, suggesting that disruption of the mycelium released intracellular manganese-independent peroxidases into the extract (Fig. 2C). The potential release of intracellular peroxidases during sample preparation could contribute to the total measured peroxidase activity and be misinterpreted as extracellular peroxidase activity. Hence, there seems to be a trade-off between extraction efficiency and release of internal peroxidases. However, the degree to which this trade-off is important in more complex samples (e.g. soil samples) is still uncertain, since mycelial densities may vary widely on small spatial scales. Release of intracellular peroxidases could potentially be more of a concern when biomass of soil organisms is high as it tends to be in organic horizons or in soils with high organic matter content (Bastida et al., 2021). Currently, the standard methods for measuring manganese peroxidase activities from soil are based on intensively homogenized “soil slurries” (Daniel et al., 1994; Saiya-Cork et al., 2002) and it is likely that intracellular peroxidases from a range of soil organisms are released during sample preparation (Nannipieri, 2006). Therefore, it would be advisable to interpret total peroxidase activities carefully, since some of the measured enzymes may not originate from the soil matrix, and this may lead to overestimation of the potential for oxidative decomposition in soil samples. Manganese peroxidases are, however, *only* extracellular and the manganese-dependent activity could, therefore, be assumed to be less affected by soil homogenization. Given its sensitivity to the lowest enzyme concentration presented here (the soaking treatment), the MBTH/DMAB assay could likely be performed without fine homogenization of samples to minimize noise from internal peroxidases. Yet, too little homogenization could result in an underestimation of potential enzyme activity, as enzymes may be occluded in soil aggregates. Regardless of substrate choice, extraction methods should be selected based on what is of main interest in a given study and then standardized across samples to avoid variation due to different extraction intensities.

When using the same assay conditions as optimized for MBTH/DMAB, both 2,6-DMP and L-DOPA were oxidized by manganese peroxidases from *P. chrysosporium,* only in the presence of $MnSO_4$, but neither were readily oxidized by horseradish peroxidase (Fig. S4). In contrast, ABTS was readily oxidized by horseradish peroxidase but seemed comparably stable against oxidation by manganese peroxidase (Fig. S4). ABTS has a high redox potential relative to the other substrates (Bach et al., 2013), suggesting that although it can be oxidized by both enzymes, the ABTS radical is susceptible to being reduced back to its colourless state. ABTS assays have been adapted to target manganese peroxidases in coarse woody-debris samples (Arnstadt et al. 2016a, 2016b), but this did not work with our assay conditions. However, other studies have also reported limitations of ABTS as a substrate, including interference with tannic acid (Terrón et al., 2004) and unsuitability for alkaline soils (Bach et al., 2013). Further, Glenn and Gold (1985) noted that ABTS was more readily oxidized by horseradish peroxidase than by manganese peroxidases from *P. chrysosporium*. We observed a high proportion of manganese-independent enzyme activity from extracts of needle cultures, suggesting ABTS could be oxidized directly by manganese peroxidase or by redox mediators other than manganese. This issue is likely more of a concern in soil samples which may be more chemically complex than the fungal-needle cultures used in this experiment. Given these observations, it seems reasonable to conclude that ABTS is not an ideal substrate for measuring manganese peroxidases in soil, but may still be suitable for estimating phenol-oxidase activity (Floch et al., 2007).

While there are apparent limitations of enzyme assays (more thoroughly addressed in German et al., 2011; Nannipieri et al., 2018 and references therein) they are still informative and widely used, since alternative methods, such as microdialysis or other means of sampling soil solution in pore spaces *in situ* are less developed and mainly focus on small spatial scales (Buckley et al., 2019; Levakov et al., 2021). We, therefore, urge awareness of the effects of sample preparation, substrate choice, and H_2O_2 concentration when conducting substrate-based enzyme assays and for thorough reporting of modifications made to assay protocols during optimization. This would help improve our interpretations of the relationship between potential enzyme activities and biogeochemical processes, and lead to better estimations of the contribution of fungal peroxidases to carbon cycling.

CRediT authorship contribution statement

E.E. Packard: Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **K. Jörgensen:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Funding

This work was supported by the Swedish Research Council FORMAS [2020-01105], awarded to Björn D. Lindahl.

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Acknowledgements

We thank Björn D. Lindahl for insightful discussions throughout the planning and writing of this work.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funeco.2025.101492.

Data availability

Data and code to reproduce the results of this study will be available on Dryad and Zenodo upon acceptance. Until then, it is available for review on GitHub (https://github.com/KMJorgensen/PeroxidaseAssayTest).

References

Arnstadt, T., Hoppe, B., Kahl, T., et al., 2016a. Patterns of laccase and peroxidases in coarse woody debris of *Fagus sylvatica*, *Picea abies* and *Pinus sylvestris* and their relation to different wood parameters. Eur. J. For. Res. 135, 109–124. https://doi.org/10.1007/s10342-015-0920-0.

Arnstadt, T., Hoppe, B., Kahl, T., et al., 2016b. Dynamics of fungal community composition, decomposition and resulting deadwood properties in logs of *Fagus sylvatica, Picea abies* and *Pinus sylvestris*. For. Ecol. Manag. 382, 129–142. https://doi.org/10.1016/j.foreco.2016.10.004.

Bach, C.E., Warnock, D.D., Van Horn, D.J., et al., 2013. Measuring phenol oxidase and peroxidase activities with pyrogallol, l-DOPA, and ABTS: effect of assay conditions and soil type. Soil Biol. Biochem. 67, 183–191. https://doi.org/10.1016/j.soilbio.2013.08.022.

Baldrian, P., 2008. Chapter 2 Enzymes of saprotrophic basidiomycetes. In: Boddy, L., Frankland, J.C., van West, P. (Eds.), British Mycological Society Symposia Series. Academic Press, pp. 19–41.

Baldrian, P., In Der Wiesche, C., Gabriel, J., et al., 2000. Influence of cadmium and mercury on activities of ligninolytic enzymes and degradation of polycyclic aromatic hydrocarbons by *Pleurotus ostreatus* in soil. Appl. Environ. Microbiol. 66, 2471–2478. https://doi.org/10.1128/AEM.66.6.2471-2478.2000.

Bastida, F., Eldridge, D.J., García, C., et al., 2021. Soil microbial diversity–biomass relationships are driven by soil carbon content across global biomes. ISME J. 15, 2081–2091. https://doi.org/10.1038/s41396-021-00906-0.

Bödeker, I.T.M., Clemmensen, K.E., de Boer, W., et al., 2014. Ectomycorrhizal *Cortinarius* species participate in enzymatic oxidation of humus in northern forest ecosystems. New Phytol. 203, 245–256. https://doi.org/10.1111/nph.12791.

Bosatta, E., Ågren, G.I., 1991. Dynamics of carbon and nitrogen in the organic matter of the soil: a generic theory. Am. Nat. 138, 227–245.

Buckley, S., Allen, D., Brackin, R., et al., 2019. Microdialysis as an in situ technique for sampling soil enzymes. Soil Biol. Biochem. 135, 20–27. https://doi.org/10.1016/j.soilbio.2019.04.007.

Castillo, M.D., Stenstrom, J., Ander, P., 1994. Determination of manganese peroxidase activity with 3-Methyl-2-benzothiazolinone hydrazone and 3-(Dimethylamino) benzoic acid. Anal. Biochem. 218, 399–404. https://doi.org/10.1006/abio.1994.1198.

Daniel, G., Volc, E., Kubatova, 1994. Pyranose oxidase, a major source of H2O2 during wood degradation by *Phanerochaete chrysosporium*, *Trametes versicolor*, and *Oudemansiella mucida*. Appl. Environ. Microbiol. 60, 2524–2532. https://doi.org/10.1128/aem.60.7.2524-2532.1994.

Entwistle, E.M., Romanowicz, K.J., Argiroff, W.A., et al., 2018. Anthropogenic N deposition alters the composition of expressed class II fungal peroxidases. Appl. Environ. Microbiol. 84, e02816. https://doi.org/10.1128/AEM.02816-17, 17.

Entwistle, E.M., Zak, D.R., Argiroff, W.A., 2017. Anthropogenic N deposition increases soil C storage by reducing the relative abundance of lignolytic fungi. Ecol. Monogr. 88, 225–244. https://doi.org/10.1002/ecm.1288.

Floch, C., Alarcon-Gutiérrez, E., Criquet, S., 2007. ABTS assay of phenol oxidase activity in soil. J. Microbiol. Methods 71, 319–324. https://doi.org/10.1016/j.mimet.2007.09.020.

Floudas, D., Binder, M., Riley, R., et al., 2012. The paleozoic origin of enzymatic lignin decomposition reconstructed from 31 fungal genomes. Science 336, 1715–1719. https://doi.org/10.1126/science.1221748.

Fornasier, F., Dudal, Y., Quiquampoix, H., 2011. Enzyme extraction from soil. In: Methods of Soil Enzymology. John Wiley & Sons, Ltd, pp. 371–383.

García-Esquivel, Y., Mercado-Flores, Y., Anducho-Reyes, M.A., et al., 2021. 3-Methyl-2-benzothiazolinone hydrazone and 3-dimethylamino benzoic acid as substrates for the development of polyphenoloxidase and phenoloxidase activity by zymograms. 3 Biotech 11, 39. https://doi.org/10.1007/s13205-020-02622-6.

German, D.P., Weintraub, M.N., Grandy, A.S., et al., 2011. Optimization of hydrolytic and oxidative enzyme methods for ecosystem studies. Soil Biol. Biochem. 43, 1387–1397. https://doi.org/10.1016/j.soilbio.2011.03.017.

Glenn, J.K., Gold, M.H., 1985. Purification and characterization of an extracellular Mn (ll)-Dependent peroxidase from the lignin-degrading basidiomycete, *Phanerochaete chrysosporium*. Arch. Biochem. Biophys. 242, 329–341. https://doi.org/10.1016/0003-9861(85)90217-6.

Jörgensen, K., Granath, G., Strengbom, J., Lindahl, B.D., 2022. Links between boreal forest management, soil fungal communities and below-ground carbon sequestration. Funct. Ecol. 36, 392–405. https://doi.org/10.1111/1365-2435.13985.

Keiluweit, M., Nico, P., Harmon, M.E., et al., 2015. Long-term litter decomposition controlled by manganese redox cycling. Proc. Natl. Acad. Sci. U. S. A 112, E5253–E5260. https://doi.org/10.1073/pnas.1508945112.

Kirk, T.K., Farrell, R.L., 1987. Enzymatic " Combustion ": the microbial degradation of lignin. Annu. Rev. Microbiol. 41, 465–505.

Kranabetter, J.M., 2019. Increasing soil carbon content with declining soil manganese in temperate rainforests: is there a link to fungal Mn? Soil Biol. Biochem. 128, 179–181. https://doi.org/10.1016/j.soilbio.2018.11.001.

Kranabetter, J.M., Philpott, T.J., Dunn, D.E., 2021. Manganese limitations and the enhanced soil carbon sequestration of temperate rainforests. Biogeochemistry 156, 195–209. https://doi.org/10.1007/s10533-021-00840-5.

Kyaschenko, J., Clemmensen, K.E., Hagenbo, A., et al., 2017a. Shift in fungal communities and associated enzyme activities along an age gradient of managed *Pinus sylvestris* stands. ISME J. 11, 863–874. https://doi.org/10.1038/ismej.2016.184.

Kyaschenko, J., Clemmensen, K.E., Karltun, E., Lindahl, B.D., 2017b. Below-ground organic matter accumulation along a boreal forest fertility gradient relates to guild interaction within fungal communities. Ecol. Lett. 20, 1546–1555. https://doi.org/10.1111/ele.12862.

Levakov, I., Ronen, Z., Siebner, H., Dahan, O., 2021. Continuous in-situ measurement of free extracellular enzyme activity as direct indicator for soil biological activity. Soil Biol. Biochem. 163, 108448. https://doi.org/10.1016/j.soilbio.2021.108448.

Marx, D.H., 1969. The influence of ectotrophic mycorrhizal fungi on the resistance of pine roots to pathogenic infections. II. Production, identification, and biological activity of antibiotics produced by *Leucopaxillus cerealis* var. *piceina*. Phytopathology 59, 411–417.

Nannipieri, P., 2006. Role of stabilised enzymes in microbial ecology and enzyme extraction from soil with potential applications in soil proteomics. In: Nannipieri, P., Smalla, K. (Eds.), Nucleic Acids and Proteins in Soil. Springer, Berlin, Heidelberg, pp. 75–94.

Nannipieri, P., Giagnoni, L., Renella, G., et al., 2012. Soil enzymology: classical and molecular approaches. Biol. Fertil. Soils 48, 743–762. https://doi.org/10.1007/s00374-012-0723-0.

Nannipieri, P., Sequi, P., Fusi, P., 1996. Humus and enzyme activity. In: Humic Substances in Terrestrial Ecosystems. Elsevier, pp. 293–328.

Nannipieri, P., Trasar-Cepeda, C., Dick, R.P., 2018. Soil enzyme activity: a brief history and biochemistry as a basis for appropriate interpretations and meta-analysis. Biol. Fertil. Soils 54, 11–19. https://doi.org/10.1007/s00374-017-1245-6.

Ngo, T.T., Lenhoff, H.M., 1980. A sensitive and versatile chromogenic assay for peroxidase and peroxidase-coupled reactions. Anal. Biochem. 105, 389–397. https://doi.org/10.1016/0003-2697(80)90475-3.

Pan, Y., Birdsey, R.A., Fang, J., et al., 2011. A large and persistent carbon sink in the world's forests. Science 333, 988–993. https://doi.org/10.1126/science.1201609.

Pedersen, T.L., 2022. patchwork: The Composer of Plots. R package version 1.3.2.9000. https://patchwork.data-imaginist.com.

Pérez-Izquierdo, L., Clemmensen, K.E., Strengbom, J., et al., 2021. Crown-fire severity is more important than ground-fire severity in determining soil fungal community development in the boreal forest. J. Ecol. 109, 504–518. https://doi.org/10.1111/1365-2745.13529.

Ruiz-Dueñas, F.J., Barrasa, J.M., Sánchez-García, M., et al., 2021. Genomic analysis enlightens Agaricales lifestyle evolution and increasing peroxidase diversity. Mol. Biol. Evol. 38, 1428–1446. https://doi.org/10.1093/molbev/msaa301.

Saiya-Cork, K.R., Sinsabaugh, R.L., Zak, D.R., 2002. The effects of long term nitrogen deposition on extracellular enzyme activity in an *Acer saccharum* forest soil. Soil Biol. Biochem. 34, 1309–1315. https://doi.org/10.1016/S0038-0717(02)00074-3.

Sinsabaugh, R.L., 2010. Phenol oxidase, peroxidase and organic matter dynamics of soil. Soil Biol. Biochem. 42, 391–404. https://doi.org/10.1016/j.soilbio.2009.10.014.

Sinsabaugh, R.L., Carreiro, M.M., Repert, D.A., 2002. Allocation of extracellular enzymatic activity in relation to litter composition, N deposition, and mass loss. Biogeochemistry 60, 1–24. https://doi.org/10.1023/A:1016541114786.

Šnajdr, J., Valášková, V., Merhautová, V., et al., 2008. Spatial variability of enzyme activities and microbial biomass in the upper layers of *Quercus petraea* forest soil. Soil Biol. Biochem. 40, 2068–2075. https://doi.org/10.1016/j.soilbio.2008.01.015.

Stendahl, J., Berg, B., Lindahl, B.D., 2017. Manganese availability is negatively associated with carbon storage in northern coniferous forest humus layers. Sci. Rep. 7, 1–6. https://doi.org/10.1038/s41598-017-15801-y.

Sterkenburg, E., Clemmensen, K.E., Ekblad, A., et al., 2018. Contrasting effects of ectomycorrhizal fungi on early and late stage decomposition in a boreal forest. ISME J. 12, 2187–2197. https://doi.org/10.1038/s41396-018-0181-2.

Terrón, M.C., López-Fernández, M., Carbajo, J.M., et al., 2004. Tannic acid interferes with the commonly used laccase-detection assay based on ABTS as the substrate. Biochimie 86, 519–522. https://doi.org/10.1016/j.biochi.2004.07.013.

Wallenstein, M.D., Weintraub, M.N., 2008. Emerging tools for measuring and modeling the in situ activity of soil extracellular enzymes. Soil Biol. Biochem. 40, 2098–2106. https://doi.org/10.1016/j.soilbio.2008.01.024.

Wariishi, H., Valli, K., Gold, M.H., 1992. Manganese(II) oxidation by manganese peroxidase from the basidiomycete *Phanerochaete chrysosporium*. Kinetic mechanism and role of chelators. J. Biol. Chem. 267, 23688–23695. https://doi.org/10.1016/s0021-9258(18)35893-9.

Wickham, H., Averick, M., Bryan, J., et al., 2019. Welcome to the tidyverse. J. Open Source Softw. 4, 1686. https://doi.org/10.21105/joss.01686.

Wickham, H., Chang, W., Henry, L., et al., 2020. ggplot2: Create Elegant Data Visualisations Using the Grammar of Graphics.

Zhang, Y., Hobbie, S.E., Schlesinger, W.H., et al., 2024. Exchangeable manganese regulates carbon storage in the humus layer of the boreal forest. Proc. Natl. Acad. Sci. USA 121, e2318382121. https://doi.org/10.1073/pnas.2318382121.

Targeting Mn-dependent peroxidase activities – considerations and optimizations

Erica E. Packard[1] & Karolina Jörgensen[1]

[1]*Swedish University of Agricultural Sciences, Department of Soil and Environment, 75007 Uppsala, Sweden*

Correspondence: karolina.jorgensen@slu.se

Supplemental Figures and Tables

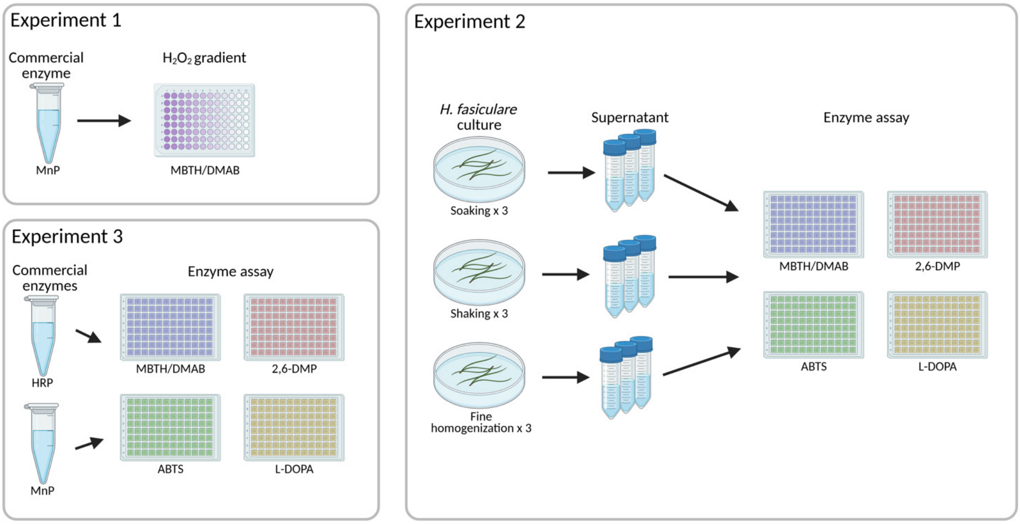


Fig. S1 Experimental design. Experiments one and three used commercial enzymes with the focus on optimizing H_2O_2 concentrations and testing for ability to distinguish manganese-dependant and manganese-independent peroxidase activity using EDTA as a chelator of manganese, respectively. Experiment two used enzymes extracted from *Hypholoma fasiculare* growing on senesced pine needles with a focus on extraction intensity and its impact on interference from intracellular peroxidases.

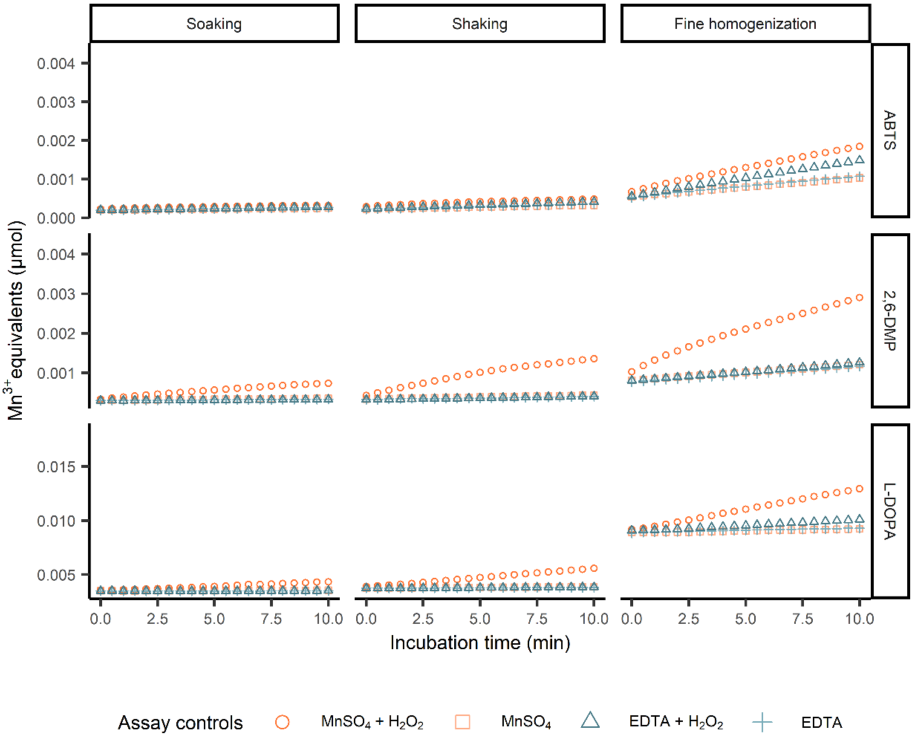


Fig. S2 Peroxidase activities (approximated by Mn^{3+} equivalents formed in µmol) in cultures of *Hypholoma fasiculare* grown on senesced pine needles, assayed with three substrates. Enzymes were extracted in 50 mM sodium acetate buffer at different extraction intensities; soaking the mycelium, gently shaking the mycelium on a vortex (no visible tears in the mycelium) or fine homogenization of mycelium and needles with a dispersing mixer. Points represent average absorbance of three biological replicates with four technical replicates. The reaction rate of Mn-dependant enzyme activity was increased with increasing extraction intensity for all three substrates (Table S2).

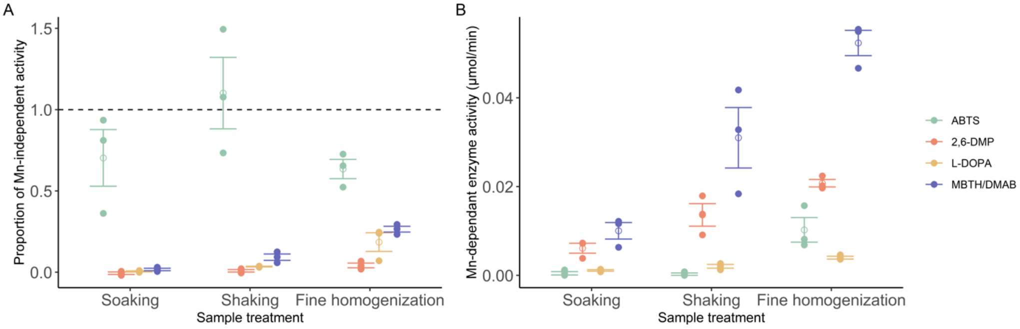


Fig. S3 (A) Proportion of Mn-independent activity relative to total peroxidase activity and (B) rate of Mn-dependant enzyme activity (MnP; µmol min^{-1}) at varying levels of extraction intensity of *Hypholoma fasiculare* grown on senesced pine needles, assayed with four substrates. Enzymes were extracted in 50 mM sodium acetate buffer at different extraction intensities; soaking the mycelium, gently shaking the mycelium on a vortex (no visible tears in the mycelium) or fine homogenization of mycelium and needles with a dispersing mixer. Hollow coloured points represent mean of replicates with standard error bars (n = 3). Values over horizontal dashed line at 1.0, in panel A, represent assays where the rate of activity was higher when manganese was absent than when present. The reaction rate of Mn-dependant enzyme activity differed among substrates and was highest in MBTH/DMAB assays (Table S3).

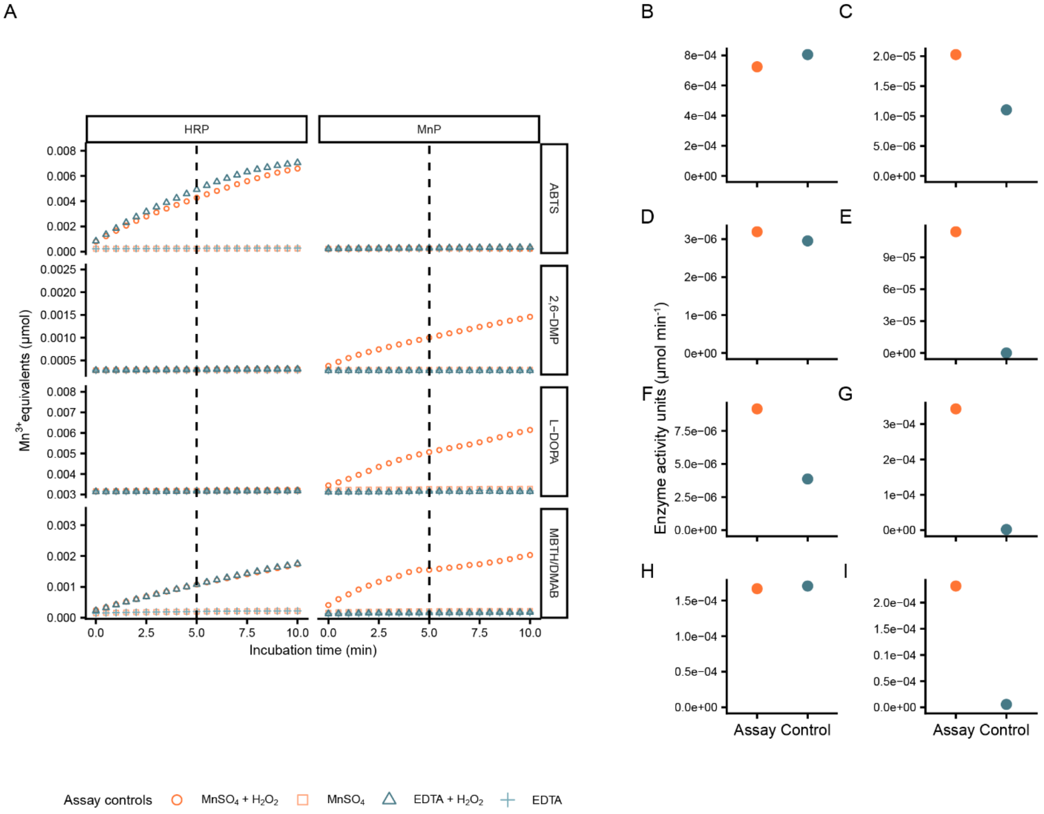


Fig. S4 Comparisons between reactions with either commercial manganese peroxidase (MnP) from *Phanerochaete chrysosporium* or horseradish peroxidase (HRP) assayed with four substrates for estimating potential peroxidase activity. In (A) points represent average absorbance from four technical replicates and vertical dashed line represents the time point at which reaction rates are determined up to. In (B-I) the reaction rates of the four different substrates in the presence of either $MnSO_4$ or EDTA are shown. Reaction rates are calculated as the average slope of four technical replicates within the first five minutes of the reaction being initiated by 0.125 mM H_2O_2. Panels B-I correspond to position of plots in panel A (*i.e.* panel B corresponds to HRP assayed with ABTS).

Table S1. Combinations of reagents used in enzyme assays of commercial enzyme (experiment 1 and 3) and *Hypholoma fasiculare* enzyme extracts (experiment 2)

Activity determined	Reagents		Enzyme	
			Experiment 1 and 3	Experiment 2
Mn-dependent peroxidase activity	140 µl Substrate solution with $MnSO_4$	10 µl H_2O_2	10 µl Commercial enzyme + 40 µl Sodium acetate	50 µl *Hypholoma fasiculare* enzyme extracts
Non-peroxidase dependent oxidation	140 µl Substrate solution with $MnSO_4$	10 µl Sodium acetate	10 µl Commercial enzyme + 40 µl Sodium acetate	50 µl *Hypholoma fasiculare* enzyme extracts
Mn-independent peroxidase activity	140 µl Substrate solution with EDTA	10 µl H_2O_2	10 µl Commercial enzyme + 40 µl Sodium acetate	50 µl *Hypholoma fasiculare* enzyme extracts
Non-peroxidase dependent oxidation	140 µl Substrate solution with EDTA	10 µl Sodium acetate	10 µl Commercial enzyme + 40 µl Sodium acetate	50 µl *Hypholoma fasiculare* enzyme extracts

Table S2. One-way ANOVA results comparing differences in Mn-dependent enzyme activity (Mn^{3+} equivalents (µmol) per min) and the proportion of Mn-independent enzyme activity between different levels of extraction intensity (soaking, shaking and fine homogenization). Results for MBTH/DMAB are presented in main text.

Substrate		df	F-value	p-value
ABTS	Mn-dependant enzyme activity	2	12.40	0.007
	Proportion of Mn-independent enzyme activity	2	2.32	0.179
2,6-DMP	Mn-dependent enzyme activity	2	19.10	0.003
	Proportion of Mn-independent enzyme activity	2	5.59	0.043
L-DOPA	Mn-dependant enzyme activity	2	21.20	0.002
	Proportion of Mn-independent enzyme activity	2	8.43	0.018

Table S3. One-way ANOVA results comparing differences in Mn-dependent enzyme activity (Mn^{3+} equivalents (µmol) per min) between different enzyme assay substrates (ABTS, 2,6-DMP, L-DOPA and MBTH/DMAB).

	df	F-value	p-value
Soaking	3	16.51	<0.001
Shaking	3	15.05	0.001
Fine homogenization	3	111.57	<0.01

Table S4. Summary of select studies that measure peroxidase activity and the concentrations of H_2O_2 applied in the enzyme assays. The mean concentration across studies is 0.114 mM.

Reference	Colorimetric substrate	enzyme soil/location	H_2O_2 addition to reaction	Final H_2O_2 concentration (mM)
Hildén et al., 2013	2,6-DMP	pure cultures	Based on Wariishi et al., 1992	0.1
Liers et al., 2006	2,6-DMP	pure cultures	Based on Bollag et al., 1979	0.1
Heinonsalo et al., 2012	ABTS	pure cultures	10 µl in a 1 ml reaction	0.1
Presley et al., 2018	ABTS	pure cultures	1 mM	1
Mester and Field 1998	ABTS, 2,6-DMP	pure cultures	0.1 mM	0.1
Arnstadt et al., 2016	ABTS	wood	0.1 mM	0.1
Johnsen and Jacobsen 2008	L-DOPA	forest soil	100 µl of 35% H2O2 in 20.1 ml reaction	0.017
Jones et al., 2020	L-DOPA	forest soil	Based on Bach et al., 2013	0.012
Phillips et al., 2014	L-DOPA	forest soil	Based on Saiya-Cork et al., 2002	0.012
Saiya-Cork et al., 2002	L-DOPA	forest soil	10µl of 0.3% in 260 µl reaction	0.012
Bach et al., 2013	L-DOPA, ABTS	broad range of soils	Based on Floch et al., 2007 and Saiya and Cork, 2002	0.012
Algora Gallardo et al., 2021	MBTH/DMAB	forest litter	based on Baldrian et al., 2010	0.01
Bödeker et al., 2014	MBTH/DMAB	forest soil	10 µl of 1mM in 200µl reaction	0.05
Jörgensen et al., 2024	MBTH/DMAB	forest soil	Based on Kyaschenko et al., 2017	0.25
Kranabetter et al., 2021	MBTH/DMAB	forest soil	10 µl of 5 mM in 200µl reaction	0.25
Kyaschenko et al., 2017	MBTH/DMAB	forest soil	0.25 mM	0.25
Šnajdr et al., 2008	MBTH/DMAB	forest soil	based on Ngo and Lenhoff, 1979	0.01
Baldrian et al., 2010	MBTH/DMAB	forest soil	based on Ngo and Lenhoff, 1980	0.01
Sterkenburg et al., 2018	MBTH/DMAB	forest soil	150 µl of 67 µM in 200 µl reaction	0.0525
Šnajdr et al., 2010	MBTH/DMAB	pure cultures	based on Ngo and Lenhoff, 1980	0.01
Baldrian et al., 2000	MBTH/DMAB	pure cultures	based on Ngo and Lenhoff, 1980	0.01
Daniel et al., 1994	MBTH/DMAB	pure cultures	10 µl of 10 mM in 2 ml reaction	0.05

Supplemental References

Algora Gallardo C, Baldrian P, López-Mondéjar R (2021) Litter-inhabiting fungi show high level of specialization towards biopolymers composing plant and fungal biomass. Biol Fertil Soils 57:77–88. https://doi.org/10.1007/s00374-020-01507-3

Arnstadt, T., Hoppe, B., Kahl, T., Kellner, H., Krüger, D., Bässler, C., Bauhus, J., Hofrichter, M., 2016. Patterns of laccase and peroxidases in coarse woody debris of Fagus sylvatica, Picea abies and Pinus sylvestris and their relation to different wood parameters. Eur J Forest Res 135, 109–124. https://doi.org/10.1007/s10342-015-0920-0

Bach CE, Warnock DD, Van Horn DJ, et al (2013) Measuring phenol oxidase and peroxidase activities with pyrogallol, l-DOPA, and ABTS: Effect of assay conditions and soil type. Soil Biology and Biochemistry 67:183–191. https://doi.org/10.1016/j.soilbio.2013.08.022

Baldrian P, In Der Wiesche C, Gabriel J, et al (2000) Influence of Cadmium and Mercury on Activities of Ligninolytic Enzymes and Degradation of Polycyclic Aromatic Hydrocarbons by *Pleurotus ostreatus* in Soil. Appl Environ Microbiol 66:2471–2478. https://doi.org/10.1128/AEM.66.6.2471-2478.2000

Baldrian P, Merhautová V, Petránková M, et al (2010) Distribution of microbial biomass and activity of extracellular enzymes in a hardwood forest soil reflect soil moisture content. Applied Soil Ecology 46:177–182. https://doi.org/10.1016/j.apsoil.2010.08.013

Daniel G, Volc E, Kubatova (1994) Pyranose Oxidase, a Major Source of H2O2 during Wood Degradation by Phanerochaete chrysosporium, Trametes versicolor, and Oudemansiella mucida. Applied and Environmental Microbiology 60:2524–2532. https://doi.org/10.1128/aem.60.7.2524-2532.1994

Floch C, Alarcon-Gutiérrez E, Criquet S (2007) ABTS assay of phenol oxidase activity in soil. Journal of Microbiological Methods 71:319–324. https://doi.org/10.1016/j.mimet.2007.09.020

Heinonsalo J, Kabiersch G, Niemi RM, et al (2012) Filter centrifugation as a sampling method for miniaturization of extracellular fungal enzyme activity measurements in solid media. Fungal Ecology 5:261–269. https://doi.org/10.1016/j.funeco.2011.07.008

Hildén K, Mäkelä MR, Lankinen P, Lundell T (2013) Agaricus bisporus and related Agaricus species on lignocellulose: Production of manganese peroxidase and multicopper oxidases. Fungal Genetics and Biology 55:32–41. https://doi.org/10.1016/j.fgb.2013.02.002

Johnsen AR, Jacobsen OS (2008) A quick and sensitive method for the quantification of peroxidase activity of organic surface soil from forests. Soil Biology and Biochemistry 40:814–821. https://doi.org/10.1016/j.soilbio.2007.10.017

Jones ME, Lacroix RE, Zeigler J, et al (2020) Enzymes, Manganese, or Iron? Drivers of Oxidative Organic Matter Decomposition in Soils. Environmental Science and Technology 54:14114–14123. https://doi.org/10.1021/acs.est.0c04212

Jörgensen K, Clemmensen KE, Wallander H, Lindahl BD (2024) Ectomycorrhizal fungi are more sensitive to high soil nitrogen levels in forests exposed to nitrogen deposition. New Phytologist. https://doi.org/10.1111/nph.19509

Kranabetter JM, Philpott TJ, Dunn DE (2021) Manganese limitations and the enhanced soil carbon sequestration of temperate rainforests. Biogeochemistry 156:195–209. https://doi.org/10.1007/s10533-021-00840-5

Kyaschenko J, Clemmensen KE, Hagenbo A, et al (2017) Shift in fungal communities and associated enzyme activities along an age gradient of managed Pinus sylvestris stands. ISME J 11:863–874. https://doi.org/10.1038/ismej.2016.184

Liers C, Ullrich R, Steffen KT, et al (2006) Mineralization of 14C-labelled synthetic lignin and extracellular enzyme activities of the wood-colonizing ascomycetes Xylaria hypoxylon and Xylaria polymorpha. Appl Microbiol Biotechnol 69:573–579. https://doi.org/10.1007/s00253-005-0010-1

Mester T, Field JA (1998) Characterization of a novel manganese peroxidase-lignin peroxidase hybrid isozyme produced by Bjerkandera species strain BOS55 in the absence of manganese. Journal of Biological Chemistry 273:15412–15417. https://doi.org/10.1074/jbc.273.25.15412

Ngo TT, Lenhoff HM (1980) A sensitive and versatile chromogenic assay for peroxidase and peroxidase-coupled reactions. Analytical Biochemistry 105:389–397. https://doi.org/10.1016/0003-2697(80)90475-3

Phillips LA, Ward V, Jones MD (2014) Ectomycorrhizal fungi contribute to soil organic matter cycling in sub-boreal forests. ISME Journal 8:699–713. https://doi.org/10.1038/ismej.2013.195

Saiya-Cork KR, Sinsabaugh RL, Zak DR (2002) The effects of long term nitrogen deposition on extracellular enzyme activity in an Acer saccharum forest soil. Soil Biology and Biochemistry 34:1309–1315. https://doi.org/10.1016/S0038-0717(02)00074-3

Šnajdr J, Cajthaml T, Valášková V, et al (2011) Transformation of Quercus petraea litter: Successive changes in litter chemistry are reflected in differential enzyme activity and changes in the microbial community composition. FEMS Microbiology Ecology 75:291–303. https://doi.org/10.1111/j.1574-6941.2010.00999.x

Šnajdr J, Steffen KT, Hofrichter M, Baldrian P (2010) Transformation of 14C-labelled lignin and humic substances in forest soil by the saprobic basidiomycetes Gymnopus erythropus and Hypholoma fasciculare. Soil Biology and Biochemistry 42:1541–1548. https://doi.org/10.1016/j.soilbio.2010.05.023

Šnajdr J, Valášková V, Merhautová V, et al (2008) Spatial variability of enzyme activities and microbial biomass in the upper layers of Quercus petraea forest soil. Soil Biology and Biochemistry 40:2068–2075. https://doi.org/10.1016/j.soilbio.2008.01.015

Sterkenburg E, Clemmensen KE, Ekblad A, et al (2018) Contrasting effects of ectomycorrhizal fungi on early and late stage decomposition in a boreal forest. ISME Journal 12:2187–2197. https://doi.org/10.1038/s41396-018-0181-2

Wariishi H, Valli K, Gold MH (1992) Manganese(II) oxidation by manganese peroxidase from the basidiomycete Phanerochaete chrysosporium. Kinetic mechanism and role of chelators. Journal of Biological Chemistry 267:23688–23695. https://doi.org/10.1016/s0021-9258(18)35893-9

ACTA UNIVERSITATIS AGRICULTURAE SUECIAE

DOCTORAL THESIS NO. 2026:29

Ectomycorrhizal fungi are known for cycling nutrients above- and below-ground but less is known about the capacity of some species to decompose recalcitrant soil organic matter. The enzymes involved in oxidative decomposition and the ecology of the ectomycorrhizal fungi that produce them were investigated. The results suggested that ectomycorrhizal decomposers have the potential to oxidise soil organic matter and improve access to nutrients, therefore highlighting the importance of their role in soil carbon and nitrogen dynamics.

Erica E. Packard received her graduate education at the Department of Soil and Environment at the Swedish University of Agricultural Sciences, Uppsala, Sweden. She holds a BSc degree in Ecology and Evolutionary Biology from the University of British Columbia Okanagan, Kelowna, Canada.

ISSN 1652-6880
ISBN (print version) 978-91-8124-246-1
ISBN (electronic version) 978-91-8124-276-8