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Interactions of Basidiomycota brown rot and white rot fungi: temporal and spatial effects in decay metabolism and wood degradation

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DOCTORAL DISSERTATION

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Cover: Left: Finnish forest, Middle: Tree trunk colonized by *Fomitopsis pinicola* and *Trichaptum abietinum*, Right: Tree trunk colonized by *Phlebia radiata*

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Mattila HK, Kačar D, Mali TLE, Lundell TK, 2018. Lignocellulose bioconversion to ethanol by a fungal single-step consolidated method tested with waste substrates and co-culture experiments. *AIMS Energy* 6(5):866-879. doi:10.3934/energy.2018.5.866

Veloz Villavicencio E, Mali T, Mattila HK, Lundell T, 2020. Enzyme activity profiles produced on wood and straw by four fungi of different decay strategies. *Microorganisms* 8:73. doi:10.3390/microorganisms8010073

Mäki M, Mali T, Hellén H, Heinonsalo J, Lundell T, Bäck J, 2021. Deadwood substrate and species-species interactions determine the release of volatile organic compounds by wood-decaying fungi. *Fungal Ecology* 54:101106. doi:10.1016/j.funeco.2021.101106

ABBREVIATIONS

AA	Auxiliary activities
AAO	Aryl-alcohol oxidase
ABTS	2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
AOX	Alcohol oxidase
CAZy, CAZymes	Carbohydrate-active enzymes
CDH	Cellobiose dehydrogenase
CE	Carbohydrate esterases
CROX	Copper radical oxidases
DNA	Deoxyribonucleic acid
DyP	Dye-decolorizing peroxidases
ECM	Ectomycorrhizal
FAME	Fatty acid methyl ester
FBCC	Fungal Biotechnology Culture Collection
GAOX	Galactose oxidase
GC-MS	Gas chromatography-mass spectrometry
GH	Glycoside hydrolases
GLOX	Glyoxal oxidase
GMC	Glucose-methanol-choline
GOX	Glucose oxidase
GT	Glycosyl transferases
HTP	Heme-thiolate peroxidases
HUBCRI	Helsinki University Biodiversity Collections Research Infrastructure
ITS	Internal transcribed spacer
LiP	Lignin peroxidase
LMW	Low molecular weight
LPMO	Lytic polysaccharide monooxygenases
MCO	Multicopper oxidase
ME	Malt extract
MEA	Malt extract agar
MnP	Manganese peroxidase
mVOC	Microbial volatile organic compoud
PDH	Pyranose dehydrogenase
PL	Polysaccharide lyases
POX	Pyranose oxidase
RNA-Seq	Ribonucleic acid-sequencing
ROS	Radical oxygen species
qRT-PCR	Real-time qRT-PCR – real-time quantitative reverse transcription polymerase chain reaction
SEM	Scanning electron microscopy
TEM	Transmission electron microscopy
UPO	Unspecific peroxidase
VOC	Volatile organic compound
VP	Versatile peroxidase

ABSTRACT

Boreal forest ecosystems expand over continents and comprise almost one third of the Earth's forest carbon stock. Boreal forests include mainly coniferous trees, and Norway spruce (*Picea abies*) is the second most abundant tree species in the forests of Finland. Basidiomycota fungi, especially brown rot fungi, cause degradation in the wood of coniferous trees in the Northern hemisphere. In forest deadwood, microbial species consortium is under continuous transition, which may be seen in changes of the anatomical structure and chemical composition of decaying wood. Therefore, the fungal species assembly in deadwood has an impact on the cycling of carbon and other nutrients in forest ecosystems.

In the research of this doctoral thesis, I first studied the species-species interactions between a common brown rot fungus of our boreal forests, *Fomitopsis pinicola*, and five white rot fungi *Phlebia radiata*, *Junghuhnia luteoalba*, *Trichaptum abietinum*, *Porodaedalea laricis* and *Phellinus ferrugineofuscus*, all belonging to the systematic class Agaricomycetes in the fungal phylum Basidiomycota, and able to grow on Norway spruce deadwood.

In mycelial interactions, the fungal species consortium influenced the production of wood-decaying enzyme activities, including oxidoreductases (MnP and laccase) and hydrolytic enzymes (endoglucanase, xylanase, β-glucosidase) as well as enzymes important for hyphal interactions and re-cycling of cellular components (chitinase and peptidase) (**I**, **II**). The change in spruce wood decay type and efficiency during fungal interactions was also observed with detection of iron reduction capacity, which is indicative for brown rot Fenton chemistry.

In the subsequent studies, a three-species set of the fungi were selected for longer cultivations on spruce wood. The effect of the white rot fungi *P. radiata* and *T. abietinum* on mycelial growth of *F. pinicola* and its brown rot decay mechanism was observed in three months on spruce wood shavings (**II**). *F. pinicola* demonstrated aggressive growth and rapid wood decay, which, however, were subjective to suppression by the presence of white rot fungi, especially *P. radiata*. Together with specific responses on enzyme activities and iron reduction ability, the amounts of dissolved metabolites (oxalic acid, and sugars released from wood) pointed to the switch of brown rot decay towards white rot decay type with respect to fungal growth and interaction time (**II**, **III**).

With observations on fungal responses, their decay type and efficiency in wood decomposition, interaction studies allowed analyses of volatile organic compounds (VOCs) from fungal single-species and combination cultures on spruce wood (**II**). Among the set of identified VOCs, mainly terpenes and terpenoids released from wood by fungal action, a few signature compounds for brown rot decay and white rot decay type could be emphasized.

This tendency of early brown rot – later white rot was further confirmed by the one-year long incubation of the brown rot and white rot fungi together on thin spruce wood veneer slices (**III**). Detailed analysis of the mycelial interaction zones on the spruce veneer slices showed responses at enzyme

production level (e.g., higher manganese peroxidase activity in the interaction zone between *P. radiata* and *F. pinicola*) and at gene expression level analysed by RNA-extraction and real-time qRT-PCR. The brown rot fungus *F. pinicola* had a positive effect on the expression of transcripts of specific oxidoreductase-encoding genes (*mnp2*, *lip2*, *lpmo1*) of *P. radiata.* Similar responses were not observable with the hydrolytic enzyme-encoding genes (*eg1*, *xyn1*, *bgl1*). However, considering brown rot decay, the white rot fungi *P. radiata* and *T. abietinum* both negatively influenced the expression of the ferric reductase encoding gene (*fr1*) of *F. pinicola*. These results highlight that in the late-stage interaction and decay of wood, here after one year of fungal growth, the white rot fungi start to dominate and convert the decay type from brown rot towards white rot in wood.

TIIVISTELMÄ

Pohjoinen havumetsävyöhyke, Taiga, on koko pohjoisen pallonpuoliskon yhtenäinen havumetsäalue, johon on sitoutunut melkein kolmannes maapallon metsien hiilivarannosta. Metsäkuusi (*Picea abies*) on Suomen metsien toiseksi yleisin puulaji. Kantasienet, erityisesti ruskolahottavat lajit, ovat merkittävimpiä havupuiden lahottajia pohjoisella pallonpuoliskolla. Metsien lahopuissa oleva mikrobiyhteisö kehittyy ja muuttuu koko ajan. Tämän lajivaihtelun voi nähdä muutoksina lahoavan puuaineksen rakenteessa ja kemiallisessa koostumuksessa. Lahopuiden mikrobiyhteisöjen rakenteella onkin vaikutusta hiilen ja muiden aineiden kierrolle metsien ekosysteemeissä.

Väitöskirjatutkimuksessani tutkin puuta lahottavia kantasieniin (Basidiomycota) kuuluvia varsinaisia avokantaisia (Agaricomycetes) lajeja, jotka esiintyvät luonnossa metsäkuusella tai kykenevät lahottamaan sitä. Tutkin metsiemme erittäin yleisen ruskolahoa aiheuttavan kantokäävän (*Fomitopsis pinicola*) ja viiden valkolahottajan: rusorypykän (*Phlebia radiata*), kermakarakäävän (*Junghuhnia luteoalba*), kuusenkynsikäävän (*Trichaptum abietinum*), ruostekäävän (*Phellinus ferrugineofuscus*) ja kuusenkäävän (*Porodaedalea laricis*) välistä vuorovaikutusta.

Tutkimuksessani huomasin, että rihmastollisessa yhteiskasvatuksessa sienilajien yhdistelmällä oli vaikutusta puuta lahottavien entsyymien (hapetus-pelkistysentsyymit, hydrolyyttiset entsyymit) sekä vuorovaikutustilanteissa ja omien solujen komponenttien kierrätyksessä käytettävien entsyymien aktiivisuuteen. Puunlahotustavalla ja sienten välisellä vuorovaikutuksella oli merkitystä raudanpelkistyskykyyn, joka viittaa muutoksiin rihmastojen tuottamaan ei-entsymaattiseen, ruskolahottavaan fentoninkemiaan.

Sienten välistä vuorovaikutusta tutkivissa kolme kuukautta kestävissä kasvatuksissa kuusen sahanpurulla keskityttiin kolmeen lajiin: valkolahottaviin rusorypykkään (*P. radiata*), kuusenkynsikääpään (*T. abietinum*) sekä ruskolahottavaan kantokääpään (*F. pinicola*). Kantokääpä yksinään osoittautui erittäin voimakkaaksi rihmastolliseksi kasvajaksi ja kuusipuuaineksen ruskolahottajaksi. Rusorypykällä ja kuusenkynsikäävällä oli hidastavaa vaikutusta kantokäävän puunlahotustehoon ja rihmastolliseen kasvuun. Puunlahotusentsyymien aktiivisuuksien, raudanpelkistyskyvyn, liuenneiden aineenvaihduntatuotteiden ja sienten välistä vuorovaikutusta seurattiin lisäksi vuoden ajan yhteiskasvatuksissa kuusipuuviiluilla. Tänä aikana yhteiskasvatuksien puunlahotustavassa oli huomattavissa muutos ruskolahotuksesta valkolahotukseen.

Edellä mainittujen lahottajasienten aineenvaihdunnan sekä lahotustavan ja -tehon muutoksien lisäksi tutkittiin haihtuvia orgaanisia yhdisteitä sienten yhteiskasvatuksista sekä yhden sienen kasvatuksista kuusen sahanpurulla. Pääasiassa sienten aktiivisen toiminnan seurauksena puuaineksesta vapautui paljon haihtuvia orgaanisia yhdisteitä, etupäässä terpenejä ja terpenoideja, joista muutama tunnistettu yhdiste korreloi puunlahotustavan eli ruskolahotuksen tai valkolahotuksen kanssa.

Tämä lahotustavan muutos ruskolahosta valkolahotukseen kuusipuuaineksen lahoamisen edetessä varmistettiin ohuilla kuusipuuviiluilla vuoden mittaisilla yhden sienilajin sekä kahden tai kolmen sienilajin yhteiskasvatuk-

silla. Sienilajien reaktioita tutkittiin tarkemmin määrittämällä edellä mainittuja entsyymiaktiivisuuksia rihmastollisen vuorovaikutuksen alueelta ja vuorovaikutusalueen molemmilta puolilta erikseen. Reaktioita tarkasteltiin myös erityisten puutalahottavien entsyymien geenien ilmentymisen tasolla RNA-eristyksen ja real-time qRT-PCR-menetelmän avulla. Kantokäävällä oli positiivinen vaikutus rusorypykän hapetus-pelkistysentsyymejä koodaavien geenien ilmentämiseen, kun taas samaa vaikutusta ei huomattu hydrolyyttisten entsyymien ilmentymisessä. Valkolahottavilla rusorypykällä ja kuusenkynsikäävällä sen sijaan oli negatiivinen vaikutus kantokäävän ferrireduktaasientsyymin ilmentämiseen, mikä viittaa muutoksiin kantokäävän raudanpelkistysaktiivisuudessa ja ruskolahotyskyvyssä.

Edellä mainitut tulokset viittaavat siihen, että myöhemmässä, pidemmälle ehtineessä puunlahoamisen vaiheessa valkolahottajat syrjäyttävät ruskolahottajat pääasiallisina lahottajina, jolloin puunlahotusmekanismi myös muuttuu ruskolahotuksesta valkolahotukseen.

1 Introduction

Almost one third of the total carbon stock of the Earth's forests is stored in Northern latitudes, in the Taiga boreal forest ecosystems (Pan et al. 2011). Boreal forests are located between the arctic tundra and temperate forests. Globally, forest trees are carbon sinks due to fixing atmospheric carbon dioxide, leading to mitigation of climate change. It has been estimated that 10-20 % of aboveground biomass in mature forests is stored in deadwood (Brown 2002). This makes deadwood an important carbon sink and part of the carbon cycle. Of the forests in Finland, an areal share of 13 % was preserved with management reported in 2019 (Ruoka- ja luonnonvaratilastojen e-vuosikirja 2020). The vegetation in Boreal forests is dominated by coniferous trees.

In Finland, the conifers Norway spruce (*Picea abies*) and Scots pine (*Pinus sylvestris*) are the two most common tree species in the country's forests (Tomppo et al. 2011). Norway spruce (*Picea abies*) is the second most abundant tree species, making up 30 % of tree coverage (Ruoka- ja luonnonvaratilastojen e-vuosikirja 2020). Norway spruce occurs commonly in Finland except in the most northern part of the country (Fagerstedt et al. 1996) and is widely used as a renewable natural resource and feedstock in the wood processing industry. From the industrial, ecological and forestry points of view, it is therefore important to study fungi able to inhabit, decompose and recycle deadwood of Norway spruce.

Boreal forests create versatile microenvironments. Deadwood is one of the most challenging microhabitats due to its recalcitrant physical and chemical properties (Rayner and Boddy 1988, Baldrian 2017). Saprotrophic Basidiomycota fungi are the organisms mainly responsible for the decomposition of deadwood and carbon cycling thereof (Rayner and Boddy 1988, Lundell et al. 2014). Unique species consortiums in such a habitat are constantly developing and adjusting to the changes in biotic and abiotic factors affecting their living environment (Stokland et al. 2012).

In this doctoral thesis, the species-species interactions of wood decay Basidiomycota fungal species were studied between the brown rot fungus *Fomitopsis pinicola* and five white rot fungi *Phlebia radiata*, *Junghuhnia luteoalba*, *Trichaptum abietinum*, *Porodaedalea laricis* and *Phellinus ferrugineofuscus*. The fungal species are common decayers of spruce deadwood in Finland. Interspecific interactions and wood decay processes were studied with the production of enzyme activities and secreted metabolites, together with measurements of hyphal growth. Additionally, iron reduction capacity, an indication of brown rot Fenton chemistry, was measured. Specific attention was given to the interactions between the species *F. pinicola*, *P. radiata* and *T. abietinum* on spruce deadwood over 3-month-duration and one-year cultivations. In these interactions, volatile organic compounds (VOCs), mainly released from wood by fungal action, and

changes in the structural and chemical properties of wood were detected. To highlight the mycelial interactions in detail, the expression of selected wood decay enzyme encoding genes of *P. radiata* and *F. pinicola* were studied in single-species and combinatory cultivations on spruce wood. The results from this study contribute to a better understanding of fungal interactions and the progress of wood decay by Basidiomycota fungi.

1.1 Spruce deadwood as substrate and habitat

A traditional way of studying the fungal diversity in forest ecosystems uses fruit body inventories (Ovaskainen et al. 2010). However, with molecular biology methods like DNA sequencing, a deeper insight into the taxon-level diversity of fungal hyphae in deadwood, plant litter, soil and for instance in living plants can be obtained. Fruit bodies (basidiocarps) are developed by fungi of phylum Basidiomycota when they need to propagate, spread into new habitat and exchange genetic material in the form of sexual spores. In decomposing deadwood, often no signs of fungal habitation as hyphae are visible on the surface until fruit bodies emerge.

In naturally decaying coniferous deadwood at a Finnish forest site, most of the fungal species identified by DNA sequencing analysis belonged to the phylum Basidiomycota, whereas approximately 40 % of the identified species represented the phylum Ascomycota (Rajala et al. 2010). This study revealed that the most common species of wood decay fungi in Finland's forests are the polyporoid Basidiomycota species *Amylostereum areolatum*, *Antrodia serialis*, *Coniophora puteana*, *Exidia glandulosa*, *Fomitopsis pinicola*, *Phellinus nigrolimitatus*, *Phellinus viticola*, *Postia caesia* and *Veluticeps abietina* (Rajala et al. 2010). The same authors noticed that at earlier stages of wood decay over half (52 %) of the inhabiting fungal species represented Ascomycota and as the decay proceeded, were succeeded by Basidiomycota species (Rajala et al. 2012). In decaying Norway spruce deadwood, fungal diversity increases as decay progresses (Rajala et al. 2011). Fungal species, essential to wood biodegradation, and able to decompose or modify all wood components efficiently, however, belong to Basidiomycota, class Agaricomycetes (Floudas et al. 2012, Lundell et al. 2014, Baldrian 2017). Saprotrophic fungal species are dominant at early stages of wood decay and as decay advances in the spruce deadwood, the abundance of Basidiomycota ectomycorrhizal species increases (Rajala et al. 2012).

In addition, in fresh plant litter deposited on the boreal forest floor, saprotrophic litter-decomposing fungi are the dominant species (Santalahti et al. 2016). As the litter decomposes into humus and humic substances, ectomycorrhizal fungi then start to dominate. In coniferous forest soils the most active and effective decomposers are not necessarily the most abundant species present (Baldrian et al. 2012). Some ectomycorrhizal fungal species

residing in forest soil and tree roots can take nutrients directly from the mycelia of saprotrophic wood decay fungi (Lindahl et al. 1999, Buée et al. 2007).

A diversity of bacteria also colonize deadwood, although their role in wood degradation is marginal in comparison to fungal activities (Baldrian 2017). It has been suggested that through bacterial enzymatic degradation of wood, it is easier for wood decay fungi to colonize deadwood (Clausen 1996). In pioneering studies, however, it was noticed that bacteria were present in deadwood already decayed by fungi, including the brown rot species *Fomitopsis pinicola*, whereas bacteria were absent in the intact xylem of non-decayed wood (Blanchette and Shaw 1978). One explanation is that bacteria may have mutualistic symbiosis with wood decay fungi (Blanchette 1988).

More recent studies of fungal–bacterial interactions in deadwood have shown that some wood decay fungi, such as the white rot species *Phanerochaete chrysosporium*, have an impact on the diversity and number of bacterial taxa living in the same habitat (Hervé et al. 2014). It has been proposed that divergent nitrogen-fixing bacteria associated with wood decay fungi in deadwood could help nitrogen supply for the fungus (Larsen et al. 1978, Hoppe et al. 2014). Even though bacteria may take part in the wood decay process for instance by secreting lignocellulolytic enzymes including oxidoreductases, the principal decomposers of wood are saprotrophic Basidiomycota fungi. Among them the white rot species are able to degrade all wood components including lignin (Hatakka & Hammel 2010, Floudas et al. 2012, Lundel et al. 2014).

Deadwood, apart from being a habitat for fungi and prokaryotes, is a habitat for invertebrates (Stokland et al. 2012), which can disperse fungal spores and hyphal fragments either incidentally or by symbiosis (Boddy and Jones 2008). They consume decaying woody material, fruit bodies, spores or mycelia of wood decay fungi, and bacteria (Stokland et al. 2012). Termites are known to cultivate fungi inside deadwood, and Ascomycota species *Xylaria nigripes* is known to be associated with termite nests (Rogers 1979). In the same way, a few fungal saprotrophic species living in soil, plant litter and deadwood can utilize invertebrates. For instance, the Basidiomycota white rot species *Pleurotus ostreatus* has been shown to kill and consume nematodes (Thorn and Barron 1984).

Table 1. Examples of wood decay and other saprobic forest fungi of Basidiomycota and Ascomycota with different lifestyles.

Lifestyle	Fungal species	Finnish name/ English name	Taxonomy	Habitat/ substrate	Additional information	Reference
WR	*Phlebia radiata*	Rusorypykkä/ Wrinkled crust	Basidiomycota, Agaricomycetes, Polyporales, Meruliaceae	Deciduous and coniferous deadwood	Promising in conversion of plant biomass waste and lignocelluloses. Degrades lignin. Produces ethanol.	Lundell 1993, Kuuskeri et al. 2016, Mäkinen et al. 2019, Mattila et al. 2020
WR	*Phlebiopsis gigantea*	Harmaa-orvakka	Basidiomycota, Agaricomycetes, Polyporales, Phanerochaetaceae	Coniferous wood of *Picea abies* and *Pinus sylvestris*	Used for biocontrol against *H. annosum* and *H. parviporum*	Kotiranta et al. 2009, Terhonen et al. 2013, Hori et al. 2014
WR	*Obba rivulosa (Physisporinus rivulosa)*	Talikääpä	Basidiomycota, Agaricomycetes, Polyporales, Gelatoporiaceae	Coniferous deadwood	Fruiting bodies are found on charred wood after forest fires. Biopulping by selective delignification.	Hakala et al. 2004, Miettinen et al. 2016
WR, P	*Heterobasidion parviporum*	Kuusenjuurikääpä	Basidiomycota, Agaricomycetes, Russulales, Bondarzewiaceae	*P. abies*	Severe tree pathogen, causes large economic losses	Niemelä 2005, Olson et al. 2012, Terhonen 2015
	Heterobasidion annosum	Männynjuurikääpä Root rot		*P. sylvestris*		
SRT	*Schizophyllum commune*	Halkihelttä/ Split gill	Basidiomycota, Agaricomycetes, Agaricales, Schizophyllaceae	Deciduous and coniferous deadwood	Habitats also woody foundations and construction waste.	Kotiranta et al. 2009, Ohm et al. 2010
BR, P	*Fomitopsis pinicola*	Kantokääpä/ Red-belted conk	Basidiomycota, Agaricomycetes, Polyporales, Fomitopsidaceae	*Picea abies*, other conifers, deciduous wood	Pathogenic against wood seedlings	Ortiz-Santana et al. 2013, Floudas et al. 2012

BR	*Serpula lacrymans*	Lattiasieni	Basidiomycota, Agaricomycetes, Boletales, Serpulaceae	Wooden houses, construction wood	In imperfectly ventilated foundations of wooden houses	Eastwood et al. 2011, Kauserud et al. 2012
LDF	*Agaricus bisporus*	Herkkusieni/ Button mushroom	Basidiomycota, Agaricomycetes, Agaricales, Agaricaceae	Forest and garden floor soil organic matter, litter	Commercially cultivated for consumption	Morin et al. 2012, Royse 2014
ECM	*Paxillus involutus*	Pulkkosieni/ Brown roll-rim	Basidiomycota, Agaricomycetes, Boletales, Paxillaceae	Symbiont of a broad range of different trees	Very poisonous. Saprobic brown rot type activity.	Rineau et al. 2012
SR	*Trichoderma reesei*		Ascomycota, Sordariomycetes, Hypocreales, Hypocreaceae	Cellulose containing materials	In commercial use for production of cellulolytic and hemicellulolytic enzymes	Martinez et al. 2008, Druzhinina et al. 2010
SR, P	*Xylaria spp*		Ascomycota, Sordariomycetes, Xylariales, Xylariaceae	Living trees or deadwood	Saprotrophs and parasites, can cause root rot, associated with fungus cultivating termites	Rogers 1979
S, P	*Ophiostoma spp*		Ascomycota, Sordariomycetes, Ophiostomatales, Ophiostomataceae	Elm trees	Causes Dutch elm disease. Resin lipid degrading. Dark melanin pigmented hyphae.	Forgetta et al. 2013, Comeau et al. 2014

WR = white rot of wood, SRT = soft rot type of wood decay, BR = brown rot of wood, LDF = litter-decomposing (fungus), ECM = ectomycorrhizal symbiont, SR = soft rot decay of woody debris, S = staining of wood, P = plant pathogen

1.2 Deadwood colonization by fungi

Decaying forest deadwood and woody debris are colonized by diverse fungal species with different lifestyles (Table 1). Deadwood-inhabiting fungi may be wood decayers, litter decomposers, ectomycorrhizal fungi, plant pathogens, mycoparasites, or endophytes. The latter may exist as either filamentous or yeast-like forms in the woody material, and e.g., in decaying tree roots. In this chapter, the focus is on the Basidiomycota saprotrophic wood decay fungi, which extend their hyphae inside wood through the empty lumen space of the long wood tracheids (Eriksson et al. 1990).

Wood decay fungal species can colonize living wood or deadwood by the dispersal of asexual or sexual spores, or sclerotia, or directly by hyphal extension and penetration into the xylem (Rayner and Boddy 1988, Boddy and Hiscox 2016). Fungal competition over environmental resources e.g., nutrition or territory depends on whether the territory is occupied (primary resource capture) or not (secondary resource capture) (Cooke and Rayner 1984). Wood decay fungi have many strategies to survive in environments they inhabit. These strategies can be categorized according to three different factors: stress, disturbance, and competitors, as well as their combinations (Boddy and Hiscox 2016). Wood decay fungi can be categorized roughly into early, secondary, and late-secondary colonizers (Boddy and Hiscox 2016). Early colonizers arrive and colonize deadwood before the arrival of other fungal species. Secondary and late-secondary colonizers arrive after the deadwood habitat has been occupied by early colonizers.

When filamentous fungi have colonized a territory, for instance deadwood or soil, their hyphae will remain in the habitat for as long as the specific location supplies nutrition. Fungi will find new territories either by mycelial extension into a new area or by forming fruit bodies for spore generation and dispersal. These might also happen for other reasons, such as unsuitable changes of abiotic factors or other competing species occupying the same territory (Boddy and Hiscox 2016). Changes in abiotic factors or other species arriving to the same territory may also eliminate the first arriving fungal species.

Properties of the woody material (e.g., moisture, density, carbon to nitrogen ratio, nitrogen availability, lignin properties, and decay stage) have an effect on the developing fungal community in decaying wood (Rajala et al. 2011). Moreover, these properties alter as wood biodegradation and fungal colonization advances. Additionally, fungal properties, such as spore germination and mycelial extension rate, and capability of using available organic compounds have an impact on the forming fungal community and its transitions (Cooke and Rayner 1984).

1.3 Fungal interactions

In deadwood and woody debris, the fungal community undergoes transformation along with the advancement of wood decay (Rayner and Boddy 1988). A study concentrating on Boreal forest sites observed that brown rot fungi dominate in the early decay stages of decomposing Norway spruce wood (Rajala et al. 2015). In later stages of decay however, brown rot fungi were replaced with white rot saprotrophic species and the species richness increased at the same time. A large study with data from several fruit body inventories of Nordic boreal forest sites revealed that some fungal species dominate more than others in the succession of the species consortium in deadwood (Ottosson et al. 2014). It has been shown that the history of fungal community assembly affects the appearance of successive fungal species and therefore, composition of the future community. Furthermore, transitions in the fungal community influence both the substrate and abiotic factors, hence affecting function of the ecosystem (Heilmann-Clausen and Boddy 2005, Fukami et al. 2010).

Competition over environmental resources can be divided into mutualistic, neutral or combative hyphal interactions (Rayner and Boddy 1988). During interspecific combative interactions, hyphae of the interacting species can replace or exceed the first species. When either one of the interacting fungal species cannot replace each other, a mycelial “deadlock” occurs (Hiscox et al. 2018). Species of wood decay fungi have different strategies to react in combat. The upregulation of genes involved in the biosynthesis of secondary metabolites in the brown rot fungus *Gloeophyllum trabeum* in response to a competitor was observed in a recent study (Presley et al. 2020). In the same study, a broad selection of oxidoreductase encoding genes was upregulated in the brown rot fungus *Rhodonia placenta* as a response. In another, ecological study of fungi on decaying tree stumps, it has been suggested that in later stages of wood decay, fungal interactions are not that combative in nature, contrary to early wood decay stage interactions (Van der Wal et al. 2015).

1.3.1 Enzymes and metabolites in interactions

Combative hyphal interactions can proceed either at a distance, through close hyphal interference or through mycelial contact (Boddy 2000). Interaction at a distance may involve water diffusible metabolites and volatile organic compounds, whereas in close hyphal interference, the diffusible organic compounds are most likely the ones involved (Woodward and Boddy 2008). Basidiomycota fungi are known to produce secondary metabolites with antifungal substances (Florianowicz 2000). Fungal interactions and the fungal community structure influence the production of enzyme activities (Baldrian 2004, Hiscox et al. 2010, Mali et al. 2017). During gross mycelial contact, the produced enzymes, such as laccase, have an apparent role in the interaction

(Boddy 2000, Baldrian 2004). Some wood decay fungi have also been observed to generate thick mycelial blocks during hyphal interaction (Boddy 2000, Mali et al. 2017).

In a study of chitinase activities in wood decay fungi, the authors noticed that chitinase activity produced by *Hypholoma capnoides* increased during its secondary colonization of wood, indicating degradation of the hyphal cell wall of the previous colonizer, *F. pinicola* (Lindahl and Finlay 2005). In mycoparasitism, fungal hyphae of one species penetrate the mycelium of the second species to obtain nutrients either biotrophically or necrotrophically. Mycoparasitism can be the sole method of nutrient intake or a temporary strategy for the fungus giving an advantage in occupying the territory (Boddy 2000).

1.3.2 Volatile organic compounds produced by fungi

The distribution of emitted VOCs in the forests originating from abiotic sources, from woody material or from plants and microbes, is still largely unknown (Leff and Fierer 2008). Field studies have shown that VOCs originate mainly from decomposing organic material or from living plants and plant roots (Paavolainen et al. 1998, Isidorov and Jdanova 2002, Asensio et al. 2007). The importance of microbial communities and their activity in VOC emissions has also been recognized (Isidorov and Jdanova 2002, Leff and Fierer 2008). When saprotrophic fungi colonize deadwood by hyphal extension, VOCs are produced and released from the degrading wood or as a consequence of fungal metabolism (Evans et al. 2008, Müller et al. 2013, Hung et al. 2015, El Ariebi et al. 2016, Isidorov et al. 2016, Mäki et al. 2021).

A wide array of fungal mVOCs (microbial Volatile Organic Compounds) are produced through primary and secondary metabolism (Korpi et al. 2009). Many factors such as fungal species, growth substrate, nutrients, temperature, moisture, and VOCs in the air or in the growth substrate influence fungal growth and therefore, also influence mVOC production by fungi (Korpi et al. 2009). As mentioned previously, fungal-produced mVOCs have an impact on fungal species-species interactions (Hynes et al. 2007, Woodward and Boddy 2008). Hyphal interaction between the Basidiomycota Agaricomycetes species *Resinicium bicolor* and *Hypholoma fasciculare* had an impact on the produced sesquiterpenes (Hynes et al 2007). Fungal mVOCs of soil-borne fungi may stimulate a positive or negative response in plant growth (Morath et al. 2012, Ditengou et al. 2015). Fungi produce mVOCs to also attract invertebrates. For instance, the wood decay white rot fungus *Trametes versicolor* produces mVOCs to attract beetles that feed on fruit bodies of the fungi (Drilling and Dettner 2009).

Distinguishing fungal produced mVOCs from the VOCs released from woody debris is extremely challenging and, therefore fungal mVOC identification has been mainly studied under laboratory conditions using well-known minimal media as growth substrates (Evans et al. 2008, Korpi et al.

2009, Bäck et al. 2010). Despite the difficulties, signature mVOCs and mVOC profiles from fungal species of the phyla Basidiomycota and Ascomycota have been identified (Lemfack et al. 2017). Some signature mVOCs identified from the brown rot fungus *F. pinicola* have not been detected in other fungal species or in bacteria, suggesting that these compounds are species-specific (Table 2) (Rösecke et al. 2000, Lemfack et al. 2017). As the amount of mVOCs from different fungal and bacterial species is expanding in line with the number of studied taxa, the species-specific mVOCs might become rare. For the identification of microbial species, particularly for bacteria, fatty acid methyl ester (FAME) profiling is a standardized method which is applied for specific phenotype fingerprinting to confirm species level identity (Willers et al. 2015). Identification of fungi on a species level has been attempted by analysing mycelial produced mVOCs (Rösecke et al. 2000, Evans et al. 2008, Korpi et al. 2009 Bäck et al. 2010, Willers et al. 2015). However, a lack of standardization of growth media and collection of VOC samples complicate comparison of the results and the creation of a reliable library of fungal mVOC profiles.

Table 2. Signature VOCs extracted from fruit bodies of the brown rot fungus *Fomitopsis pinicola*. For comparison, detection of the compounds from wood decay, ectomycorrhizal and Ascomycota species is included. Data retrieved from the mVOC database*.

	Signature VOCs of *Fomitopsis pinicola**	Pub Chem ID	BR				WR				ECM			A
			Fb	Go	Ac	Am	Pc	Pe	Gl	Ts	Pi	Lb	Ao	Tr
Aliphatic alcohols, ketones, aldehydes	(E,E)-2,4-decadienal	5283349		x				x	x	x				
	1-octanol	957	x											
	(Z)-2-octen-1-ol	5364959	x											
	3-octanol	11527	x											
	1-octen-3-ol	18827	x	x		x		x		x	x	x		x
	n-heptanal	8130							x	x				
	3-dodecanone	15229												
	3-undecanone	75189												
Terpenes	(Z)-Biformene	56927938												
	1-epi-cubenol	12046149	x				x	x						
	(E)-β-farnesene	5281517				x			x		x	x		x
	6-protoilludene	15939655												
	7,13-abietadiene	6432211												
	α-barbatene	14037349												
	α-cadinol	10398656					x	x						
	α-copaene	70678558												
	α-pinene	6654	x						x					
	α-ylangene	20055075							x			x		
	abietatriene	443470												
	β-copaene	57339298												
	Camphene	6616												
	Camphor	2537			x				x					
	Cembrene a	5281384												
	γ-cadinene	92313			x							x		
	Limonene	22311						x					x	x
	Longifolene	289151												
	sativene	11830550												
	Trans-calamenene	6429022												
	Trans nerolidol	5284507		x			x	x	x	x				
	α-cubebene	86609												
	α-longipinene	520957												
	α-muurolene	12306047										x		
	(-)-β-barbatene	14109421								x				
	β-calacorene	529621												
	Trans/β-caryophyllene	5281515									x	x		
	γ-amorphene	12313019												
	δ-cadinene	441005	x		x	x			x		x	x		
	γ-muurolene	12313020			x	x								
Aromatic compounds	2-pentylfurane	19602				x	x	x	x	x	x	x		
	Furfural	7362												
	Benzaldehyde	240	x							x				
	Phenyl acetaldehyde	998							x	x				
	Methyl 4-methoxyphenyl acetate	90266		x										

*Rösecke et al. 2000, Lemfack et al. 2017
Gray= found only in *Fomitopsis pinicola*, not yet found in other microorganisms in the mVOC database
BR = brown rot species, WR = white rot species, ECM = ectomycorrhizal species, A = Ascomycota species, Pi = *Paxillus involutus*, Tr = *Trichoderma reesei*, Lb = *Laccaria bicolor*, Ac = *Antrodia cinnamomea*, Pc = *Pleurotus cystidiosus*, Pe = *Pleurotus eryngii var. tuoliensis*, Am = *Armillaria mellea*, Gl = *Ganoderma lucidum*, Fb = *Fomitopsis betulina*, Ts = *Trametes suaveolens*, Go = *Gleophyllum odoratum*, Ao = *Amanita ovoide*

1.4 Chemical and structural composition of spruce wood

The coniferous softwood tracheid cell wall is composed of primary and secondary (S1, S2 and S3) cell wall layers (Eriksson et al. 1990). Between adjacent cells is a pectin rich middle lamella. The primary cell wall contains cellulose, hemicellulose, pectin, proteins, and water. The secondary cell wall layers in coniferous softwood contain cellulose (41 %), hemicelluse (27 %), lignin (30 %) and small amounts of pectin (2 %) (Eriksson et al. 1990, Albersheim 2011). Wood sugar residues of the polysaccharides in the sapwood, outermost part of softwood xylem, are glucose (41.6 %), xylose (5.2 %) and mannose (13.6 %) (Eriksson et al. 1990).

Cellulose is a long unbranched polysaccharide chain of β-1,4-linked glucose molecules (Sjöström 1993). The long cellulose chains are tightly packed by hydrogen bonding into approximately 40 chain containing units called microfibrils. Microfibrils are oriented differentially in the secondary cell wall layers S1, S2 and S3. In a fully matured xylem, most of the cellulose chains are crystalline, making a tough, ordered microfibril (lamellae) structure of the wood cell walls.

Hemicellulose is a branched polysaccharide heteropolymer with a backbone of either β-1,4-linked glucose, mannose or xylose (Albersheim 2011). Short side branches containing sugar, sugar acid, sugar acid ester or acetylated sugar units are attached to the hemicellulose backbone (Albersheim 2011). The main hemicellulose of coniferous softwood like spruce wood is galactoglucomannan (Eriksson et al. 1990, Ebringerová et al. 2005). Hemicellulose units are connected to cellulose with hydrogen bonds (Albersheim 2011).

Lignin is a complex, aromatic, high-molecular weight polymeric compound. Lignin has three types of aromatic subunits: *p*-hydroxyphenyl, guaiacyl and syringyl rings, with variable linkages, such as ether and carbon-carbon bonds (Eriksson et al. 1990, Boerjan 2003, Albersheim 2011). The aromatic subunits are formed from phenylpropanoid monolignol precursors: *p*-coumaryl, coniferyl and sinapyl alcohols (Boerjan et al. 2003).

Cellulose, hemicellulose, and lignin generate a compact lignocellulose complex in the wood cell walls. Lignin is connected to the carboxyl groups of hemicellulose by ether and benzyl ester linkages (Albersheim 2011). Lignin has a role in increasing the toughness of woody cell walls and repelling water from the lignifying cell walls. Most of the lignin is located inside the secondary cell wall, especially in the S2 layer, but the middle lamellae are also highly lignified (Eriksson et al. 1990). The spruce wood cell wall contains approximately 27.5% lignin mainly composed of guaiacyl units, with the *p*-hydroxyphenyl/guaiacyl ratio being > 0.07 (Albersheim 2011).

Pectin is composed of the heteropolysaccharides homogalacturonan, xylogalacturonan, rhamnogalacturonan I and rhamnogalacturonan II, depending on the plant species and tissues (Albersheim 2011). The pectic

heteropolysaccharides in Norway spruce wood are homogalacturonan and rhamnogalaturonan I (Kim and Daniel 2017). Homogalacturonan is a linear polymer compound composed of 1,4-linked galacturonic acid. In xylogalacturonan, xylose is β-(1,3)-linked to the homogalacturonan backbone. The rhamnogalacturonan I backbone is composed of α-(1,2)-linked rhamnosyl and α-(1,4)-linked galactosyluronic acid, with sugars attached to the rhamnosyl residues. In rhamnogalacturonan II polymers, rare sugars are attached to the α-(1,4)-linked galactosyluronic acid backbone (Vincken et al. 2003).

1.5 Fungal mechanisms of wood decay

Saprotrophic fungi of the taxonomic class Agaricomycetes (phylum Basidiomycota) can be divided into two groups according to their lifestyles: wood decay or litter-decomposing fungi. In addition to the saprotrophic species, the class Agaricomycetes includes ectomycorrhizal, soil inhabiting fungi, and a few plant pathogenic species (Hibbett et al. 2007, Floudas et al. 2012, Nagy et al. 2016). Wood decay and wood-inhabiting fungi are generally divided into Basidiomycota white rot and brown rot fungi, and Ascomycota soft rot and blue stain fungi (Table 1) (Eriksson et al. 1990, Stokland et al. 2012, Lundell et al. 2014). Over 2000 different (phylum Ascomycota and phylum Basidimycota) fungal species associated with deadwood have been identified in Nordic countries (Stokland et al. 2012). White rot and brown rot fungal species have their own unique ways of degrading or modifying wood components (cellulose, hemicellulose, lignin and pectin) (Lundell et al. 2014). It has been suggested that hemicellulose is degraded early on during brown rot decay of wood, whereas in white rot decay of wood, lignin is degraded first before the decomposition of cellulose (Hastrup et al. 2012b).

In addition, expression of wood decay enzyme encoding genes in some Basidiomycota fungal species has been observed to be substrate-specific and influenced by the wood properties (Wu et al. 2019). Traditionally, the wood decay fungi of Agaricomycetes have been classified as white rot or brown rot species according to their different repertoires of secreted, plant biomass degrading enzymes, and visual observation of the degraded wood (Floudas et al. 2012, Lundell et al. 2014). However, whole-genome sequencing of wood decay fungi havs revealed that differentiation between these two wood decay types is not so obvious for all taxa, and there exists, in fact, a continuum of wood decomposition mechanisms in fungi (Riley et al. 2014). Some fungi show more intermediate wood decay characteristics due to several individual evolutionary events from the white rot to brown rot lifestyle, when e.g., class-II peroxidase encoding genes have been lost among Agaricomycotina, resulting in the reduced ability of brown rot fungi to modify lignin (Riley et al. 2014, Floudas et al. 2015, Nagy et al. 2016). Lignin loss relative to the density loss of decaying wood is promising method to show and distinguish the

diversity of rot types, falling between white rot and brown rot, from the actual decaying wood (Schilling et al. 2020).

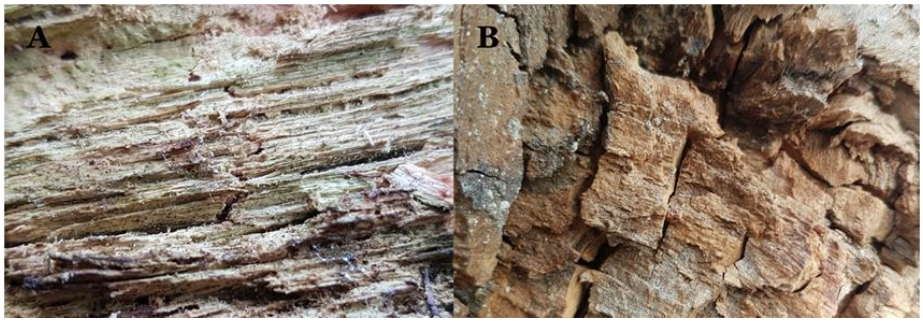

Figure 1. A) white rot decay of wood (Betula sp.) B) brown rot of birch wood (Mali T, 2021)

1.5.1 White rot

White rotted wood is pale in color, soft and fibrous (Figure 1A). According to comparative genomics on species of Agaricomycetes fungi, white rot type of wood decay was most likely an ancestral mechanism of wood decomposition by saprotrophic Basidiomycota fungi (Floudas et al. 2012, Ruiz-Dueñas et al. 2013, Riley et al. 2014). Lignin degradation has been estimated to occur 295 (195-399) million years ago around the end of the Permian and Carboniferous periods (Floudas et al. 2012). Secreted enzymes are essential in wood degradation by white rot fungi (Table 3). White rot fungi decompose and modify all wood components with an array of secreted carbohydrate active enzymes (CAZymes) and oxidoreductases (Lundell et al. 2010, Floudas et al. 2012, Yakovlev et al. 2013, Kuuskeri et al. 2016). From saprotrophic organisms, only white rot and litter-decomposing fungi are able to degrade or modify lignin efficiently with their ability to produce high-redox class-II oxidoreductases such as lignin peroxidase, manganese peroxidase and versatile peroxidase (Hofrichter et al. 2010, Lundell et al. 2014). White rot fungal species decompose or degrade lignin to access cellulose and hemicellulose for energy, since they cannot utilize lignin as a carbon source (Eriksson et al. 1990, Lundell et al. 2014).

Some white rot fungi degrade cellulose, lignin and hemicellulose simultaneously, whereas some species are called “selective lignin degraders” since they apparently first attack lignin and hemicellulose before they start to degrade cellulose (Hatakka 2001, Baldrian 2008). Lignin degradation can occur either only in the middle lamellae or in all wood cell wall layers, depending on the degradation strategy of the white rot fungal species (Eriksson et al. 1990, Riley et al. 2014, Nagy et al. 2016). Previously, it was considered that the most efficient degraders of lignin are white rot fungal species which, in nature, occur in deciduous deadwood (Baldrian 2008).

However, there are wood decay polyporoid white rot species preferentially and rapidly occupying coniferous wood, such as the severe tree pathogen *Heterobasidion* spp. inhabiting roots and xylem in living Scots pine and Norway spruce trees (Olson et al. 2012). *Phlebiopsis gigantea* is a common white rot fungus of Agaricomycetes and an exceptional biocontrol against *Heterobasidion* spp. root-rot due to its ability to occupy the cut wood surfaces, thus preventing invasion by the pathogenic fungus (Terhonen 2015).

1.5.2 Brown rot

Brown rotted wood is dark and brown in color, and cracks in cube-like manner on lengthways to the wood fibers (Figure 1B). The brown color is mainly a result of modified lignin and the accumulation of iron in the decomposed wood (Jellison et al. 1997). Brown rot fungi have genetically lost a few CAZy wood decay enzyme families during evolution within several orders in the subphylum Agaricomycotina, including the order Polyporales, making brown rot fungi a very diverse group of species (Eastwood et al. 2011, Floudas et al. 2012, Ruiz-Dueñas et al. 2013, Riley et al. 2014).

Instead, brown rot fungi have non-enzymatic approaches to decomposing wood lignocellulose components (Arantes and Goodell 2014). Brown rot fungi are able to modify lignin to some extent, for instance by demethylation (Niemenmaa et al. 2008a, Lundell et al. 2010, Eastwood et al. 2011, Arantes et al. 2012), but lack the lignin-targeting high-redox oxidoreductases, the class-II peroxidases of CAZy AA2 class (Eastwood et al. 2011, Floudas et al. 2012, Ruiz-Dueñas et al. 2013). However, laccase or laccase like multicopper oxidase encoding genes are found in their genomes (Riley et al. 2014).

It is generally agreed upon that brown rot fungi decompose cellulose and hemicellulose primarily non-enzymatically via Fenton chemistry, by the secretion of oxalic acid and generating radical oxygen species (ROS) (Xu and Goodell 2001, Eastwood et al. 2011, Arantes et al 2012). Brown rot fungi may also alter the crystallinity of wood cell wall cellulose chains, since the total amount of crystalline cellulose changes temporally during brown rot decay of wood (Howell et al. 2009). Regarding the generation of extracellular H_2O_2 for radical forming Fenton chemistry, several pathways have been previously suggested for the extracellular reduction of O_2 to H_2O_2 (Hyde and Wood 1997, Kerem et al. 1999, Daniel et al. 2007). In brown rot fungal degradation of wood, methanol is produced by demethylation of the lignin substructures (Niemenmaa et al. 2008a). The fungi are believed to rely on e.g., low molecular weight mediator (LMW) compounds and oxidants to loosen the wood cell wall matrix for easier infiltration of hydrolytic enzymes (Suzuki et al. 2006, Goodell et al. 2017).

Brown rot fungal species are found to occupy both coniferous and deciduous wood, but they are more common in dead coniferous softwood in the northern hemisphere (Goodell et al. 2003, Baldrian 2008, Binder et al. 2013, Riley et al. 2014). The most common species of brown rot fungi, in the

Northern and Eastern areas of Finland has been the order Polyporales species *Fomitopsis pinicola* according to fruit body inventories during years 1998-2004 (Niemelä 2005).

1.5.3 Other Basidiomycota rot types

Apart from traditionally categorized white rot and brown rot fungi, wood-inhabiting species falling in between these two categories are also described in the Basidiomycota class Agaricomycetes (Riley et al. 2014). *Schizophyllum commune* has been traditionally categorized as a white rot fungus but it lacks high-redox class II peroxidase encoding genes (Ohm et al. 2010). However, the fungus has a cellulose dehydrogenase encoding gene and an extremely wide array of cellulase, hemicellulase and pectinase encoding genes, including lytic polysaccharide monooxygenases. *S. commune* secretes an extraordinary variety of different hemicellulases and pectinases compared to other wood decay fungi, making it a very capable wood decayer, even though it is weak at degrading lignin (Zhu et al. 2016). Despite the versatile and expanded CAZymes arsenal, *S. commune* possesses a brown rot type ability for Fenton chemistry (Zhu et al. 2016).

Litter decomposing fungi are efficient degraders of soil organic matter, which is concentrated with humic substances, and is comprised mostly from above- and below ground plant matter (Björklund and Mello 2012). In a Scots pine boreal forest site, the soil organic matter has been observed to contain up to 10 times more nitrogen than the tree biomass (Korhonen et al. 2013). Litter decomposing fungi are able to grow in the presence of humic substances, unlike most wood decay fungi (Morin et al. 2012). Litter decomposing fungi can also degrade all wood cell wall components, similarly to white rot fungi, and they usually have more genes encoding pectin modifying enzymes compared to wood decay fungi (Tanesaka et al. 1993, Morin et al. 2012, Rytioja et al. 2014).

Ectomycorrhizal fungi, apart from being plant-symbionts, have the ability to marginally decompose woody material (Tanesaka et al. 1993, Rineau et al. 2012, Martin et al. 2016, Shah et al. 2016). Ectomycorrhizal fungi have diverged from saprotrophic fungi several times during their evolution (Kohler et al. 2015, Martin et al 2016, Martino et al. 2018). Even though many ectomycorrhizal fungi lack high-redox class-II peroxidases, some species can produce and secrete other oxidative enzymes, apparently to modify lignin to some extent (Shah et al. 2016). Similarly, some ectomycorrhizal fungi can decompose soil organic matter non-enzymatically via Fenton chemistry (Rineau et al. 2012). Normally, ectomycorrhizal fungi receive carbon from their host plant but it has been suggested that if they do not receive enough, they could use their saprotrophic abilities to aid in retrieving carbon from soil organic matter (Talbot et al. 2008).

1.5.4 Ascomycota rot types

Fungi in the phylum Ascomycota may cause soft rot on woody materials and deadwood surfaces, which is visually observed as gray, soft and spongy. Soft rot fungi are able to decompose cellulose and hemicellulose enzymatically under moist conditions (Dix and Webster 1995). Soft rot fungi have shown to have more pectinolytic enzyme encoding genes than Basidiomycota white rot or brown rot fungi (Kameshwar and Qin 2018). Fungi from the Ascomycota genus *Xylaria* are widely spread in living trees and in deadwood (Table 1) and are also known to cause root rot in apple trees (Rogers 1979).

Some species in the phylum Ascomycota are causing staining on wounded or recently dead wood (Stokland et al. 2012). These staining fungi utilize soluble sugars, starches, lipids and proteins since they cannot decompose complex lignocellulose components (Stokland et al. 2012). Staining fungi are known to discolor timber wood, and a few are known to cause severe tree diseases. Dutch elm disease is caused by staining fungi *Ophiostoma* spp., as are so called blue-stain and canker stain diseases caused by staining fungi (Butin et al. 1995). The relationships between deadwood and deadwood associated Ascomycota fungi have not yet been studied in as much depth as, for example, Basidiomycota fungi.

1.6 Fungal enzymes important for life in deadwood

CAZymes are produced by fungi in the enzymatic conversion of lignocellulose (Lombard et al. 2014, Lundell et al. 2014, Rytioja et al. 2014). These CAZymes are responsible for the breakdown of glycoconjugates, and oligo- and polysaccharides. Information on these enzymes is collected into the Carbohydrate Active EnZYmes database CAZy (www.cazy.org) (Lombard et al. 2014). The enzymes are categorized into enzyme classes according to their structure and molecular mechanism. The CAZymes are divided into glycoside hydrolases (GH), glycosyl transferases (GT), polysaccharide lyases (PL), carbohydrate esterases (CE) and a family of auxiliary activities (AA), including oxidoreductases, which are directly or indirectly active on carbohydrates and therefore important for the conversion of lignocellulose (Table 3).

Table 3. Extracellular enzymes involved in wood decomposition and produced by wood decay fungi. Enzymatic activities, their target lignocellulose components and principal mechanism in wood decay are mentioned.

Enzyme name	EC number	CAZy-class	Target	Enzyme function
Cellulase (Endoglucanase)	3.2.1.4	GH5, 6, 7, 9, 12, 44, 45	C	Cleaves glycosyl bonds of cellulose chains in amorphic regions
Cellobiohydrolase	3.2.1.91	GH6	C	Cuts non-reducing ends of cellulose (produces cellobiose)
Cellobiohydrolase	3.2.1.176	GH7	C	Cuts reducing ends of cellulose (produces cellobiose)
β-glucosidase	3.2.1.21	GH1, 3, 5	C	Hydrolysis of terminal, non-reducing β-D-glucosyl residues with release of β-D-glucose
Endo-1,4-β-xylanase	3.2.1.8	GH5, 10, 11	H	Cleaves (Endohydrolysis of) xylan backbone
Xylan 1,4-β-xylosidase	3.2.1.37	GH1, 3, 43	H	Hydrolysis of xylobiose and non-reducing terminus of xylooligosaccharides
α-1,4-Galactosidase	3.2.1.22	GH27, 36	H	Hydrolysis of terminal, non-reducing α-D-galactose residues
β-galactosidase	3.2.1.23	GH1, 2	H	Hydrolysis of terminus, non-reducing β-D-galactose residues in hemicellulose branches
β-mannosidase	3.2.1.25	GH1, 2, 5	H	Hydrolysis of non-reducing terminus β-D-mannose residues in mannans
β -glucuronidase	3.2.1.31	GH1, 2	H	Hydrolysis of the ends of hemicellulose branches
α-glucuronidase	3.2.1.139	GH67,115	H	Hydrolysis of the ends of hemicellulose branches
α -fucosidase	3.2.1.51	GH29, 95	H	Hydrolysis of the ends of hemicellulose branches
α -Arabinofuranosidase	3.2.1.55	GH51, 54	H	Hydrolysis of terminal non-reducing α-L-arabinofuranoside in hemicellulose branches
Mannan endo-1,4-β-mannosidase	3.2.1.78	GH5	H	Endohydrolysis of mannose in mannans, galactomannans and glucomannans
Xyloglucan-specific endo-β-1,4-glucanase	3.2.1.151	GH5, 12, 74	H	Endohydrolysis of xyloglucan, resulting xyloglucan oligosaccharides
Endopolygalacturonases	3.2.1.15	GH28	P	Endohydrolysis of homogalacturonan backbone
Exopolygalacturonases	3.2.1.67	GH28	P	Exohydrolysis from the non-reducing end of homogalacturonan backbone
Xylogalacturonan hydrolase	3.2.1.-		P	Cleaves xylogalacturonan
Endorhamnogalacturonase	3.2.1.171	GH28	P	Endohydrolysis from the reducing end of rhamnogalacturonan backbone
Exorhamnogalacturonase	3.2.1.-	GH28	P	Exohydrolysis of rhamnogalacturonan backbone

Enzyme name	EC number	CAZy-class	Target	Enzyme function
Unsaturated glucuronyl hydrolase	3.2.1.-	GH88	P	Hydrolysis of glucuronyl
Unsaturated rhamnogalacturonan hydrolase	3.2.1.172	GH105	P	Hydrolysis of rhamnogalacturonan I
Chitinase	3.2.1.14	GH18, 19, 23, 48	Ch	Random endohydrolysis of glycosidic linkages in chitin
Feruloyl esterase	3.1.1.73	CE1	H, P, L	Cleaves ferulic acid from the end of hemicellulose branches
Acetyl xylan esterase	3.1.1.72	CE1, 5, 6, 7	H	Cleaves acetyls
Pectin methyl esterase	3.1.1.11	CE8	P	Cleaves methyl group from galacturonic acid
Rhamnogalacturonan acetyl esterase	3.1.1.-	CE12	P	Cleaves galacturonic ends
Pectin acetyl esterase	3.1.1.-	CE12	P	Cleaves acetyl groups from galacturonic acid
Rhamnogalacturonan lyase	4.2.2.23	PL4, 11	P	Cleaves rhamno-galacturonan I domains in ramified hairy regions of pectin
Laccase	1.10.3.2	AA1_1	L	Oxidation of phenols
Multicopper oxidase		AA1	L	Oxidation of phenols and other related substances
Manganese peroxidase	1.11.1.13	AA2	L	Oxidation of Mn^{2+} to Mn^{3+}
Versatile peroxidase	1.11.1.16	AA2	L	Oxidation of phenols and Mn^{2+} to Mn^{3+}
Lignin peroxidase	1.11.1.14	AA2	L, VA	Oxidation of aromatic rings
Glucose methanol choline family Aryl alcohol oxidase Alcohol oxidase Pyranose oxidase Glucose oxidase Cellobiose dehydrogenase	 1.1.3.7 1.1.3.13 1.1.3.10 1.1.3.4 1.1.99.18	AA3	L, C	Production of H_2O_2 through oxidation of: aryl alcohol methanol glucose glucose cellobiose, semiquinones, Fe^{3+}
Copper radical oxidase family Glyoxal oxidase Galactose oxidase	 1.2.3.15 1.1.3.9	AA5	L	Production of H_2O_2 through oxidation of glyoxal, glycerol galactose
Lytic polysaccharide monooxygenases	1.14.99.54 1.14.99.56	AA9 (GH61) AA15	C, H, P, Ch	Oxidation of crystalline cellulose microfibril regions at reducing or non-reducing ends
Dye decolorizing peroxidase	1.11.1.19	(AA2)	L	Non-phenolic lignin model dimers
p-benzoquinone reductase (quinone reductase)	1.6.5.6	AA6	I	Reduction of quinones and aromatic compounds
Hemethiolate peroxidase	1.11.1.-	(AA2)	L	Oxidation of veratryl alcohol and aromatic compounds

gray = activities measured in this thesis study
Lombard et al. 2014, Lundell et al. 2014, Rytioja 2014, Henrissat 1991, Henrissat and Bairoch 1993, Davies and Henrissat 1995, Henrissat and Bairoch 1996, Henrissat and Davies 1997, Lombard et al. 2010, Levasseur et al. 2013
C = Cellulose, H = Hemicellulose, P = Pectin, L = Lignin, VA = Veratryl alcohol, I = Intracellular, Ch = Chitin

1.6.1 Carbohydrate active hydrolytic enzymes

Glycoside hydrolases (GH) are enzymes which hydrolyse glycosidic bonds between a carbohydrate and either another carbohydrate or a non-carbohydrate molecule (Henrissat 1991, Henrissat and Bairoch 1993, Davies and Henrissat 1995, Henrissat and Bairoch 1996, Henrissat and Davies 1997). Carbohydrate esterases cleave ester linkages, releasing the attached acyl or alkyl groups (Lombard et al. 2010), whereas Polysaccharide lyases cleaves polysaccharide polymers of uronic acid.

To decompose cellulose effectively, various endoglucanase cellulases, cellobiohydrolases and β-glucosidases are required (Table 3). Cellulases are needed to hydrolyze β-1,4-links in amorphic cellulose chain regions at random positions. Cellobiohydrolases cut either from the non-reducing ends (GH6) or reducing ends (GH7) of cellulose chains, producing cellobiose. β-glucosidase is needed to hydrolyze cellobiose into β-D-glucose.

To cut the hemicellulose xylan backbone endo-1,4-β-xylanases and xylan 1,4-β-xylosidases are needed. Many enzymes of CAZy GH and CE classes take part in breaking linkages of hemicellulose branches (Table 3). Pectin degradation by wood decay Basidiomycota fungi occurs mainly via hydrolytic enzymes belonging to GH28. Pectin active enzyme encoding genes of Basidiomycota have also been found to represent CAZy family PL4.

1.6.2 Carbohydrate active oxidoreductases

CAZy class AA (auxiliary activities) contains several different protein families of oxidoreductases, including lignin-modifying enzymes as well as cellulose and other polysaccharides attacking lytic polysaccharide monooxygenases (LPMOs) (Table 3) (Levasseur et al. 2013). Not all these enzymes act on carbohydrates, but since lignin is strongly associated with plant carbohydrates, AA class enzymes are part of CAZymes (Levasseur et al. 2013). In addition, the AA class includes a few protein families of different oxidases, which are enzymes usually producing hydrogen peroxide by reducing of molecular oxygen.

Laccase and other multicopper oxidases (MCOs) belong to CAZy family AA1 (Table 3). These enzymes use diphenols and other substances, such as diamines, phenolic oligomers, and toxic compounds, as electron donors and oxygen as an acceptor (Thurston 1994, Baldrian 2006, Hildén et al. 2009). MCOs cannot directly oxidize or attack lignin but, with the help of mediator compounds, laccases can affect the phenolic and non-phenolic structures (Hatakka and Hammel 2010). Apart from lignin depolymerization, laccases are important for fungi in hyphal fusions, spore and basidiocarp formation, as well as in pigment synthesis (Thurston 1994, Baldrian 2006). Laccase has a role in species-species interactions. Laccase activity has been observed to increase during interactive situations between wood decay fungi (Baldrian 2004). Besides laccases, wood decay fungi secrete several different oxidases to produce H_2O_2, most likely for the peroxidases to function (Mäkelä 2009).

Heme-containing class II peroxidases (Table 3), such as manganese peroxidases (MnP), versatile peroxidases (VP) and lignin peroxidases (LiP), belong to CAZy family AA2. These high-redox class II peroxidases and their respective genes are found in white rot and litter-decomposing fungi and a few ECM fungi (Floudas et al. 2012, Lundell et al. 2014, Riley et al. 2014, Kohler et al. 2015). MnP enzymes predicted in the genomes of species of Agaricomycetes vary substantially and can be short, long, extra-long or atypical (Ruiz-Dueñas et al. 2013). Class II peroxidases are glycosylated, globular proteins with a heme cofactor as the electron transfer center (Hofrichter et al. 2010). MnP and VP enzymes contain an Mn binding site, whereas LiP and VP have a specific tryptophan radical center (Ruiz-Dueñas et al. 2009, Hofrichter at al. 2010, Lundell et al. 2010). Hydrogen peroxide is used as an electron acceptor, with a release of two water molecules and two-electron oxidation of the second, reducing substrate (Hofrichter et al. 2010). Class-II peroxidases require acidic conditions to function (Levasseur et al. 2013, Lundell et al. 2014).

In addition to class II peroxidases, dye-decolorizing peroxidases (DyPs) and hemethiolate peroxidases (HTPs) are found in white rot and litter-decomposing fungi (Table 3) (Hofrichter et al. 2010). DyPs have low substrate specificity and can oxidize non-phenolic lignin model dimers and veratryl alcohol (Liers at al. 2013, Salvachúa et al. 2013). HTPs, which include unspecific peroxygenases (UPOs) and chloroperoxidase-like enzymes ,can oxidize veratryl alcohol and aromatic compounds which can originate from lignin or other plant extractives (Ullrich et al. 2004, Kinne et al. 2010).

Lytic polysaccharide monooxygenases (LPMOs) of CAZy AA9 and other families oxidize cleave glucosidic bonds (Quinlan et al. 2011). LPMO enzymes are found in fungi, bacteria, and viruses (Johansen 2016). and may be inactivated by either O_2 or H_2O_2 (Bissaro et al. 2017). During LPMO binding to the surface of crystalline cellulose, non-bound LPMOs can produce H_2O_2, thus resulting in self-inactivation (Bissaro et al. 2017, Li et al. 2019).

A recent study showed that LPMO enzymes may promote AA2 class-II peroxidases to lignin model compound degradation (Li et al. 2019). In the study, is suggested that non-bound LPMO produces H_2O_2 for class II peroxidases to degrade lignin units, making it easier for LPMO enzymes to bind to crystalline cellulose (Li et al. 2019). Other studies have shown that LPMOs can use lignin derivatives as a reductant in decomposition reactions of cellulose (Kracher et al. 2016, Brenelli et al. 2018).

1.6.3 Assisting enzymes producing H_2O_2

Fungal generation of H_2O_2 for wood decay reactions proceeds via secreted enzymes or other extracellular substances (Ferreira et al. 2015). Hydrogen peroxide may be produced enzymatically by fungi. Regarding wood decay, hydrogen peroxide is required in oxidative lignin degradation in white rot and in non-enzymatic conversion of lignocellulose in brown rot (Table 3). As an

example, white rot fungi in the genus *Pycnoporus* secrete hydrogen peroxide-producing enzymes and class-II peroxidases together with AA9 LPMOs concurrently (Miyauchi et al. 2020). In this system, *Pycnoporus* spp. can utilize produced H_2O_2 immediately, protecting its hyphae from the oxidative damage caused by hydrogen peroxide.

Fungal enzymes of the CAZy family AA3 aryl-alcohol oxidase (AAO), glucose oxidase (GOX), pyranose oxidase (POX), alcohol oxidase (AOX), pyranose dehydrogenase (PDH) and cellobiose dehydrogenase (CDH) belong to the glucose-methanol-choline (GMC) oxidoreductase superfamily and generate H_2O_2 (Table 3) (Levasseur et al. 2013). Enzymes in the GMC superfamily have a role in lignocellulose modification. GMC enzymes use flavin-adenine dinucleotide as a cofactor and possess the corresponding binding domain (Henriksson et al. 2000, Ludwig et al. 2010, Levasseur et al. 2013). Genes encoding enzymes in the GMC superfamily can generally be found in fungi and the genomes of Agaricomycetes, including white rot and brown rot fungi (Ferreira et al. 2015).

Within the GMC superfamily enzymes, signal peptides indicating protein secretion are found in the predicted AAO, a few GOX, PDH and CDH enzymes but not from AOX or POX (Ferreira et al. 2015). Extracellular activity of AOX has been detected, and the enzyme seemingly oxidizes methanol to produce H_2O_2 (Daniel et al. 2007). AOX may be important for brown rot Fenton chemistry, which is why an alternative way of secreting AOX has been proposed (Daniel et al. 2007). In the enzyme pyranose oxidase POX, signal peptide in the translated protein model has not been discovered, but an alternative way of protein secretion has been proposed as well (Ferreira et al. 2015). Among the various AA3 enzymes, CDH is an extracellular enzyme and can for instance act as an electron donor for LPMOs and generate H_2O_2 (Tan et al. 2015, Johansen 2016).

Glyoxal oxidase (GLOX) and galactose oxidase (GAOX) belong to CAZy AA5 and another protein family of copper radical oxidases (CROX), also producing H_2O_2 (Table 3) (Levasseur et al. 2013). Glyoxal, which is one of the substrates for GLOX, has been proposed to be produced in fungal decay of lignocellulose by Fenton chemistry or by peroxidation of linoleic acid (Watanabe et al. 2001, Manini et al. 2006, Kersten and Cullen 2014).

1.6.4 Fungal chitin and protein degrading activities

Chitinase enzyme encoding genes and chitinase activity are important for filamentous fungi since chitin is a fundamental biopolymer in the fungal cell walls as well as in the cuticle of many insects and crustaceous animals (Phillips 2017). Chitinases, in general, are secreted enzymes included in the CAZy classification (Table 3). However, not all chitinases contain a signal peptide, and the secretion pathway for some chitinases is still unknown (Langner and Göhre 2016). Chitinase activity may be targeted at the fungal cell wall for recycling hyphal building blocks and nutrients under non-optimal

growth conditions, during interactions, or in mycoparasitism against another fungus' mycelium (Seidl 2008, Langner and Göhre 2016).

Fungi secrete hydrolytic enzymes, such as proteases, into their environment to cut proteins into peptides and amino acids as a source of nitrogen, carbon and sulphur (Snyman et al. 2019). Fungi regulate production and secretion of proteases as a response to environmental factors such as nitrogen, carbon, and sulphur availability, pH, and temperature. Protease activity has an important role in the virulence of some pathogenic fungi such as the human pathogen *Candida albicans* and many plant-pathogenic species (Hörger and van der Hoorn 2013, Snyman et al. 2019). Proteases are needed for sporulation, conidial discharge, hyphal fusion, enzyme modification, regulation of gene expression and to obtain nutrition (Rao et al. 1998). In previous studies, it has been suggested that proteases are used to recycle the fungus' own secreted enzymes for nitrogen supply, especially in a low-nitrogen environment such as wood (Abuzinadah and Read 1986, Alfaro et al. 2014, Kuuskeri et al. 2016).

1.7 Non-enzymatic conversion of lignocellulose

1.7.1 Fenton chemistry

The wood cell wall is a well-buffered acidic environment, having an approximate pH value of 5.5. It is generally considered that for efficient degradation of wood, non-enzymatic Fenton chemistry is required since enzymes secreted by fungi are too large to penetrate intact wood cell walls (Arantes et al. 2009). In Fenton chemistry, soluble Fe^{2+} ions and H_2O_2 are needed for the generation of ROS, in this case, the free hydroxyl radicals ($^{\cdot}OH$). It is implicated that hydroxyl radicals are the major oxidants contributing to depolymerization of wood polysaccharides (Arantes et al. 2011).

For functional Fenton chemistry reactions ($H_2O_2 + Fe^{2+} + H^+ \rightarrow H_2O + Fe^{3+} + {}^{\cdot}OH$), acidic conditions are a prerequisite. Furthermore, the generated Fe^{3+} ions must be reduced back to Fe^{2+} together with a constant supply of H_2O_2. For effective brown rot decay of wood via Fenton chemistry, several mechanisms have been proposed (Arantes and Goodell 2014). Laboratory experiments have suggested that fungal-created Fenton chemistry could operate with the assistance of low molecular weight organic compounds, especially phenols and quinones, which may be produced by the fungi or generated from decomposing lignin and other aromatic units in wood (Goodell et al. 1997, Kerem et al. 1999, Arantes and Goodell 2014). In these phenol-quinone recycling reactions, laccase enzymes have been proposed to be involved (Hirano et al. 1997, Jensen et al. 2001, Suzuki et al. 2006, Baldrian and Valaskova 2008, Wei et al. 2010).

1.7.2 Fungal staggered mechanism of wood decay

Brown rot fungi degrade wood non-enzymatically through oxidative Fenton chemistry, but they also secrete a broad array of polysaccharide-degrading CAZy hydrolytic enzymes that are sensitive to oxidation by the radicals and ROS created in the reactions (Kerem et al. 1999, Martinez et al. 2009, Ryu et al. 2011). Fenton reactions may also harm the growing fungal hyphae. Hence, Fenton chemistry and enzymatic hydrolysis of polysaccharides are processes that cannot function in close proximity during wood decomposition and therefore, either spatial or temporal separation of the reactions and events is needed.

Temporal (time-based) separation of the two mechanisms (radical-driven oxidation and enzymatic degradation) has been demonstrated for the brown rot fungi *Postia placenta* and *Gloeophyllum trabeum* (Zhang et al. 2016, Presley et al. 2018). When occupying the wood space, mycelial growth of the brown rot fungus creates a two-step oxidative-hydrolytic mechanism to decay wood. The upregulation of oxidoreductase enzyme-activity encoding genes has been observed early in brown rot decay, followed later by upregulation of hydrolytic enzyme-activity encoding genes (Zhang et al. 2016). In this so called “staggered mechanism” of brown rot, at first the intact wood cell walls experience an oxidative attack, leading to the wood cell wall structure loosening. This loosening effect thereby promotes infiltration by the hydrolytic enzymes in order to accelerate degradation of polymeric cellulose and hemicelluloses (Zhang et al. 2016).

1.7.3 Role of laccase and low molecular weight compounds

Low molecular weight (LMW) fungal produced compounds are proposed to not only function as charge-transfer mediators in degradation of lignin by laccases (see chapter 1.6.2) but also to promote Fenton chemistry by reducing Fe^{3+} ions to Fe^{2+} (Goodell et al. 1997, Kerem et al. 1999). These LMW compounds could penetrate through the wood cell wall lignocellulose matrix, unlike enzymes (Goodell et al. 1997, Hirano et al. 2000). It has been demonstrated that wood cell walls degraded by brown rot fungi have these molecules in the S2 layer (Jellison et al. 1997). Fungal-produced LMW compounds have been proposed to reduce Fe^{3+} ions to Fe^{2+}, chelate iron ions and transfer the complexes into wood cell walls for Fenton chemistry to act (Hirano et al. 2000). For example, one potential LMW compound, 2,5-dimethoxyhydroquinone, has been discovered to be produced by several brown rot fungal species (Newcombe et al. 2002, Wei et al. 2010, Karripally et al. 2013).

Laccase enzymes, in turn, have been proposed to oxidize the phenolic LMW hydroquinones to semiquinones, which may act as reductants for the promotion of Fenton chemistry (Wei et al. 2010). Fungal-produced LMW compounds may also increase production of hydroxyl radicals in the wood cell walls (Hirano et al. 2000). The LMW molecules diffuse through the innermost

S3 layer of the secondary wood cell wall into the S2 and S1 layers, and middle lamellae where extracellular production of hydroxyl radicals takes place (Hirano et al. 2000). The involvement of secreted LMW compounds to produce H_2O_2 non-enzymatically for Fenton chemistry has also been indicated in previous studies (Enoki et al. 1997). Lignin monomers (coniferyl and sinapyl alcohol) and related compounds (2,3-dihydroxybenzoic acid, 2-methoxy-4-propylphenol, eugenol, trans-ferulic acid, isoeugenol, guaiacol) have the ability to reduce Fe^{3+} to Fe^{2+}, while this capability was not observed in wood polysaccharides (e.g., xylan, mannan, Avicel crystalline granulated cellulose) (Tamaru et al. 2019).

The same study indicated that intact wood lignocellulose surfaces have the capacity to reduce iron and may generate ROS (Tamaru et al. 2019). Close to the hyphae, in very low pH conditions, fungal-produced phenolate and hydroquinone LMW compounds are weaker chelators for iron ions than oxalate (see the next chapter). This prevents Fe^{3+} reduction from occuring at low pH and protects the hyphae from oxidative damage by Fenton reactions (Goodell et al. 1997, Arantes et al. 2009).

1.7.4 Role of oxalic acid and pH gradient

Oxalic acid is produced by microbes, fungi, and plants, and it occurs extensively in nature, but mainly in its oxalate anion form chelating cations and forming potassium, sodium, and calcium oxalate deposits (Traquiar 1987, Dutton and Evans 1996). Oxalic acid is synthesized by fungi through the tricarboxylic acid cycle, or by glyoxylate cycle in the glyoxysomes, and secreted as a metabolic waste which is not utilized for energy (Espejo and Agosin 1991, Dutton and Evans 1996, Mäkelä et al. 2010). Carbon and nitrogen sources, carbon to nitrogen ratio, presence of carbonate ions and pH of the surrounding environment all affect the production of oxalic acid by fungi (Dutton and Evans 1996).

In studies with brown rot species *P. placenta* and *G. trabeum*, rapid production of oxalic acid was observed first, after which oxalate concentration decreased to a steady level, indicating that production of oxalic acid and its decomposition are differentially regulated during wood decay (Zhang et al. 2016, Presley et al. 2018, Zhang et al. 2019,). Regulation of extracellular oxalate concentration by fungi is proposed to occur through actions of the enzyme oxalate decarboxylase (Micales 1997, Mäkelä et al. 2010). The detection of formic acid in decayed wood supports the notion that oxalate decarboxylase activity controls oxalate concentration (Hastrup et al. 2012a, Presley et al. 2018).

Production of oxalate by brown rot fungi peaks during wood decay. However, the severe decrease in pH suggests an alternative acidification method, potentially after decarboxylation of oxalate to carboxylate anion (Presley et al. 2018). Brown rot fungi have specific means to solubilize insoluble Fe^{3+} oxide-hydroxide from plant tissues and to reduce to Fe^{2+}

especially for Fenton reactions. The Fe-O bond in the hydroxides is weakened by protons, indicating that pH value has an impact on the dissolution of iron oxides (Lee et al. 2006).

Oxalic acid is capable of binding and solubilizing Fe^{3+} and Fe^{2+} ions ($Fe(C_2O_4)_3^{3-}$ or $Fe(C_2O_4)_2^-$) (Arantes et al. 2009). Iron is chelated at low pH levels (pH 2) in high oxalic acid concentrations near the brown rot fungal hyphae, which extend in the wood cell lumen area (Goodell et al. 1997). Iron chelation suppresses the Fenton reaction, thus protecting the fungal hyphae from oxidative damage (Shimada et al. 1994, Shimada et al. 1997). Fe-oxalate complexes are proposed to diffuse through the pH gradient towards the less acidic, higher pH zones in the wood (Hyde and Wood 1995, Goodell 2003). A higher pH value and lower oxalate to Fe molar ratio, further away from the hyphae, enable temporary transfer of Fe^{3+} ions closer to cellulose at the wood cell wall (Arantes et al. 2009). Controlled concentrations of oxalate apparently maximize Fenton reactions and minimize oxidative damage to the secreted enzymes and fungal hyphae (Varela and Tien 2003, Presley et al. 2018).

The Fe^{3+}-oxalate complexes are quite stable in darkness, and direct reduction of Fe^{3+} to Fe^{2+} by oxalate anions requires light (Hyde and Wood 1995, Goodell et al. 1997). Light cannot penetrate solid wood and therefore, oxalate cannot act as an iron-reducing direct catalyst for Fenton chemistry inside decaying wood. Although oxalic acid is not directly involved in the redox reactions in wood biodegradation, it has an assisting role in production of ROS hydroxyl radicals (Kaneko et al. 2005).

Oxalic acid has other important qualities in fungal decay of wood, aside from being a metabolic waste and assisting in Fenton chemistry. Oxalic acid is believed to be one of the main factors in copper tolerance for some species of fungi (Sazanova et al. 2015, Hietala et al. 2016). Copper compounds are normally very effective against fungal hyphae in their habitat. Oxalate anions can chelate copper atoms, forming copper oxalates and deposits, thereby immobilizing copper and decreasing the pH value of the surroundings, thus protecting fungal hyphae (Sutter et al. 1983, Tang et al. 2013).

2 Aims and objectives of the study

The purpose of this doctoral study was to explore and illustrate the interactions of wood decay Basidiomycota from different perspectives. In publications I-II and in manuscript III, a set of wood decay fungal species isolates were studied under experimental conditions and on woody substrates, primarily on Norway spruce wood. The aim was to observe the events and effects of fungal mycelial interactions on wood degradation. Fungal metabolic activities like production of enzyme activities and oxalic acid and emission of VOCs were measured in cultivations on wood for several weeks and up to one year of incubation. The research focus was on fungal interactions, and how these interactions influence the advancement of wood decay processes.

The research hypotheses were:
1. Species-species interacting fungi produce higher activities of wood-decay enzymes and are therefore more rapid in decomposing Norway spruce wood than individual, non-interacting fungal species.
2. White rot and brown rot fungi demonstrate specific strategies of wood decomposition which is seen in altering profiles of enzymes, metabolites, and VOCs.
3. The specific strategy of wood decomposition is in transition directed by a succession of dominating fungal species as wood decays.

The specific objectives of the publications (**I-II**) and manuscript (**III**) were:
- To study how different species of white rot fungi affect hyphal growth rate, wood degradation enzyme activities, and the production of oxalic acid in the brown rot species *Fomitopsis pinicola* (**I**)
- To observe how the brown rot fungus *F. pinicola* affects hyphal growth rate, wood degradation enzyme activities and culture acidity of five different white rot fungal species (**I**)
- To explore the decomposition of Norway spruce wood and fungal enzyme activities in single-species and combination cultures of *F. pinicola*, and two white rot species *Phlebia radiata* and *Trichaptum abietinum* (**II**).
- To discover volatile organic compounds (VOCs) that could be descriptive of white rot or brown rot decomposition of wood (**II**).
- To observe the metabolic activities of fungi produced in the interaction zones during late-stage decomposition of Norway spruce wood (**III**).
- To study the gene expression of specific enzymes important to wood decay by white rot and brown rot fungi in the interaction zones on spruce wood (**III**).
- To explore chemical and anatomical differences in spruce xylem after one year of inhabitation and decomposition by the combination of *F. pinicola* and the white rot fungi *P. radiata* and *T. abietinum* (**II** and **III**).

3 Summary of Materials and Methods

The experimental setup and description of the analytical methods used in this doctoral study are explained in detail in publications **I** and **II**, and manuscript **III,** and are shown in Table 4. Fungal species and isolates used in the study are presented in Table 5.

Table 4. Methods used in this doctoral study.

Method	Description	Article
Fungal cultivations	On agar media for growth rate determination of single-species and co-cultures	I
	Liquid medium for single- and co-cultures	I
	Semi-solid wood medium for single- and co-cultures	I
	Solid state spruce wood single- and co-cultures	II, III
Enzyme activity measurements	Endoglucanase	I, II
	β-glucosidase	I, II
	Xylanase	I
	Laccase	I, II, III
	Manganese peroxidase	I, II, III
	Chitinase	II
	Peptidase	II
Iron reduction capacity		II, III
DNA extraction		I
PCR amplification	ITS1 and ITS2	I
Phylogenetic sequence analysis		I
UHPLC analysis of fungal metabolites	Oxalic acid concentrations	I, II, III
Extracellular protein extraction and concentration		II, III
Trapping of VOCs		II
GC-MS	VOCs	II
Ergosterol extraction and HPLC analysis		II
Elementar analysis	Total nitrogen from solid wood and liquid phase samples	II
	Total carbon from solid wood and liquid phase samples	II
RNA extraction and purification		III
qRT-PCR	Species specific amplicons of selected genes	III
Klason and acid soluble lignin		III
Microscopy of wood	TEM, SEM	III
Phylogenetic analysis		I
Statistical analyses	SPSS (t-test, ANOVA)	I, II
	R (PCA, NMDS, t-test, linear regression, ANOVA)	I, II, III

Table 5. Fungal species and isolates studied in the experiments.

Species identity, abbreviation, and taxonomic order	HAMBI-FBCC identifier of the isolate	Natural substrate deadwood in the forest	Wood decay type	Wood decay stage for colonization	Publication or manuscript	Species and wood decay stage reference
Fomitopsis pinicola Fp (Polyporales)	FBCC1181	C, D	BR	Early	I, II, III	Holmer et al. 1997, Ortiz-Santana et al. 2013
Phlebia radiata Pr (Polyporales)	FBCC0043	D	WR	Early, secondary	I, II, III	Rayner et Boddy 1988, Rajala et al. 2010
Junghuhnia luteoalba Jl (Polyporales)	FBCC1472	C	WR	Middle	I	Sippola and Renvall 1999
Trichaptum abietinum Ta (Hymenochaetales)	FBCC0110	C, D	WR	Early	I, II, III	Ovaskai-nen et al. 2010
Porodaedalea laricis Pl (Hymenochaetales)	FBCC0768	C	WR	Early	I	Jönsson et al. 2008
Phellinus ferrugineofuscus Pf (Hymenochaetales)	FBCC0945	C	WR	Middle	I	Holmer et al. 1997

C = Coniferous wood, D = Deciduous wood, BR = Brown rot fungus, WR = White rot fungus

4 Results and Discussion

4.1 Fungal identification by ITS phylogeny (I)

The six species of Basidiomycota fungi of class Agaricomycetes studied were originally isolated from fruit bodies found on decaying deadwood at boreal forest sites in Finland (Table 5). The fungal isolates originate from decaying Norway spruce (*Picea abies*) deadwood, except *Phlebia radiata* FBCC0043, which was isolated from decaying Grey alder (*Alnus incana*) wood **(I**, Kuuskeri et al. 2016, Shah et al. 2018). The isolates are kept as hyphal cultures in the FBCC subcollection of the Microbial Domain Biological Resource Centre HAMBI (HAMBI 2020), which is part of the Biodiversity Collections Research Infrastructure (HUBCRI) in the Helsinki Institute of Life Science, University of Helsinki.

The fungal isolates were identified at the species level by phylogenetic analysis of their ribosomal DNA ITS region (ITS1+ITS2) (**I**). Species identity of the class Agaricomycetes order Polyporales isolates *Fomitopsis pinicola* FBCC1181 (Fp), *Phlebia radiata* FBCC0043 (Pr) and *Junghuhnia luteoalba* FBCC1472 (Jl), and the order Hymenochaetales isolate *Trichaptum abietinum* FBCC0110 (Ta) remained as previously described. Isolates FBCC0768 and FBCC0945 were taxonomically revised into the species *Porodaedalea laricis* (Pl) and *Phellinus ferrugineofuscus* (Pf) respectively, in the order Hymenochaetales (**I**).

4.2 Fungal growth in single-species and combination cultures (I, II)

Hyphal growth rates of five white rot fungal species and the brown rot species *F. pinicola* were measured in addition to how the white rot fungi affected the hyphal growth rate of *F. pinicola* and *vice versa*. On malt extract agar plate cultivations of the six selected fungi, *P. radiata* was the most prominent, based on hyphal extension rate (Table 6) (**I**). On the ABTS agar plate, *F. pinicola* and *P. radiata* were the fastest, exhibiting mycelial growth over twice as fast as *T. abietinum*. However, on malt extract liquid medium within two months of cultivation, *F. pinicola* produced the highest amount of mycelial biomass (in dry weight), followed by *P. radiata* and *T. abietinum* (**I**). This pinpoints the effect of cultivation conditions on mycelial growth in filamentous fungi.

When cultivated on solid Norway spruce wood shavings, the brown rot fungus *F. pinicola* was the most prominent in mycelial growth, followed by *T. abietinum*. *P. radiata* produced substantially less biomass according to ergosterol content in the solid-state cultures (Table 6) (**II**). However, these results may be considered only suggestive since ergosterol content in respect to total hyphal biomass is not constant between different species of fungi (Gessner and Chauvet 1993, Niemenmaa et al. 2008b). The accumulation of ergosterol is influenced, for instance, by growth substrate, culture atmospheric

conditions, and temperature. To overcome these distorting factors in the analysis, a conversion factor is needed for each species and isolate to obtain an estimate of fungal biomass (Gessner and Chauvet 1993). Moreover, measurement of fungal hyphae as a biomass growing in solid wood to obtain the conversion value is impossible in practice. Additionally, different fungal growth stages cause mycelial ergosterol content to vary (Gao et al 1993).

Table 6. Hyphal growth on malt-extract and ABTS agar media and mycelial growth on Norway spruce wood shavings according to ergosterol content

Fungus	MEA plate (mm/day) (publ. I)	ABTS agar plate (mm/day) (publ. I)	ME liquid medium (mg of mycelial dry weight in two months*) (publ. I)	Spruce wood shavings (total amount of ergosterol in mg*) (publ. II)		
				1 month	2 months	3 months
Fp	5.5	5.1	112.6 ± 0.013	134.1 ± 5.0	195.6 ± 16.3	390.7 ± 76.1
Ta	5.8	2.1	39.4 ± 0.004	102.5 ± 15.7	123.1 ± 7.5	155.4 ± 13.8
Pr	8.8	4.8	60.4 ± 0.01	47.9 ± 2.8	39.7 ± 2.2	57.6 ± 4.6
Jl	4.5	1.7	41.2 ± 0.011	NA	NA	NA
Pl	1.7	1.0	51.2 ± 0.003	NA	NA	NA
Pf	3.0	1.5	42 ± 0.004	NA	NA	NA

*Mean value of three biological replicate cultures with standard deviation
MEA = malt-extract agar, ME = malt extract; NA = Not Assessed

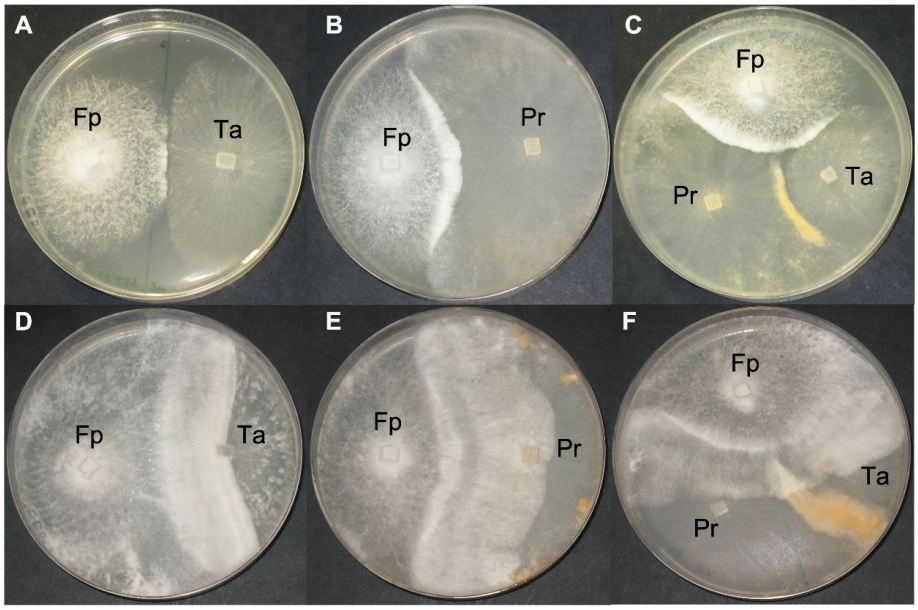

Figure 2. Combination cultures of *F. pinicola* (Fp) and *T. abietinum* (Ta; A and D), *F pinicola* and *P. radiata* (Pr; B and E) and *F. pinicola*, *T. abietinum* and *P. radiata* (C and F) on cultivation days 7 (A-C) and 17 (D-F) on malt extract agar medium incubated in darkness at 25 °C.

In the fungal combination cultures on ME or ABTS agar plates (**I**), *F. pinicola* was the most prominent in hyphal extension. Its mycelium exceeded the white rot fungal mycelia, forming more dense white extension fronts of hyphae within one week of growth (Figure 2). The outcome of the interaction cannot be stated, however, since it was impossible to check whether the mycelia under the mycelium of *F. pinicola* was still viable on the agar plates. For instance, hyphae of *J. luteoalba* invaded the agar medium, so this fungus may have survived. In boreal forests, fruit bodies of *T. abietinum* are often found on the same decaying deadwood spruce tree trunks as *F. pinicola* (Ovaskainen et al. 2010), indicating a possible mutualistic lifestyle for these two species. Thus, a similar mutualistic survival strategy may have occurred with Ta against Fp, or in the other species of spruce inhabiting white rot fungi tested (Table 6).

P. radiata formed dense mycelial pale yellow-orange pigmented blocks against some of the other white rot fungi, but not against the brown rot fungus *F. pinicola* (Figure 2). Similar species-dependent mycelial block formation and pigmentation in fungal mycelium has been observed before (Chi et al. 2007). Pigmentation of the mycelium could be due to biosynthesis of melanin and enhanced phenol oxidase activity (Henson et al. 1999, Boddy 2000). Melanins are dark green to black-coloured biopolymers on fungal hyphae, spores and macrostructures like fruit bodies, and can be synthesized by laccases and tyrosinases (Baldrian 2006, Cordero and Casadevall 2017). Melanins apparently function as protective barriers against other fungal species during interaction (Baldrian 2006, Zaidi et al. 2014, Cordero and Casadevall 2017). However, in the case of *P. radiata*, the pale yellow orange colour indicates formation of other molecules rather than melanins at the mycelial front.

4.3 Enzyme activities (I, II, III)

The effect of different white rot fungal species on the wood-degradation related enzyme activities of *F. pinicola*, and *vice versa*, were studied on solid and liquid growth substrates and media. Another purpose was to study the fungal produced enzyme activities in the species-species mycelial interaction zones on Norway spruce wood veneer slices. As the activities of selected extracellular enzymes were measured, clear differences between the fungal species on the growth substrates and media were observed, either on single species or combinatory cultures (**I, II and III**).

The total amount of secreted proteins was higher in cultivations that included the white rot fungal species *T. abietinum* Ta and/or *P. radiata* Pr without the presence of the brown rot species *F. pinicola* Fp, conducted on spruce wood shavings for three months (**II**). The amount of secreted proteins was low in the single-species culture of *F. pinicola*, with a minor increase over the period of three months (**II**).

Wood decay by white rot fungi is an enzymatic process, whereas brown rot fungi principally use non-enzymatic decomposition mechanisms (Xu and Goodell 2001, Eastwood et al. 2011, Arantes et al. 2012, Floudas et al. 2012, Lundell et al. 2014). Therefore, it was expected that Fp secretes substantially less extracellular proteins into wood in comparison to the white rot fungi. In

our previous study on *F. pinicola* isolates cultivated on liquid medium, this feature of low protein secretion capacity was observed (Shah et al. 2018).

After one year of cultivation on spruce wood veneer slices, however, the amount of secreted proteins was low in the white rot fungal cultures (Pr or Ta) or in their co-cultures with the brown rot fungus Fp (**III**). The presence of brown rot fungus seemed to increase the total amount of secreted proteins in the co-cultures. This may indicate a transition of the wood decay type towards enzymatic degradation in the combination cultures and on-going, or even enhanced, production of the wood-attacking enzymes by the interacting mycelia. In the combination cultures (FpPr, FpTa, FpPrTa) on spruce wood veneer slices, the late-stage decay was seen to predominantly turn into white rot (Figure 3).

In naturally decaying Norway spruce wood logs, the number of brown rot fungal species was highest during the early stages of wood decay, while white rot fungal species were detected more steadily throughout the decay gradient, peaking later than the brown rot fungal species (Rajala et al. 2015). In the same study, the authors found that *F. pinicola* was mainly present in the earlier and middle stages of wood decay. Fungal activity results obtained in this study, and the change in dominating wood decay rot type from brown rot to white rot as wood decomposition advances are supported by the findings of previous ecological findings.

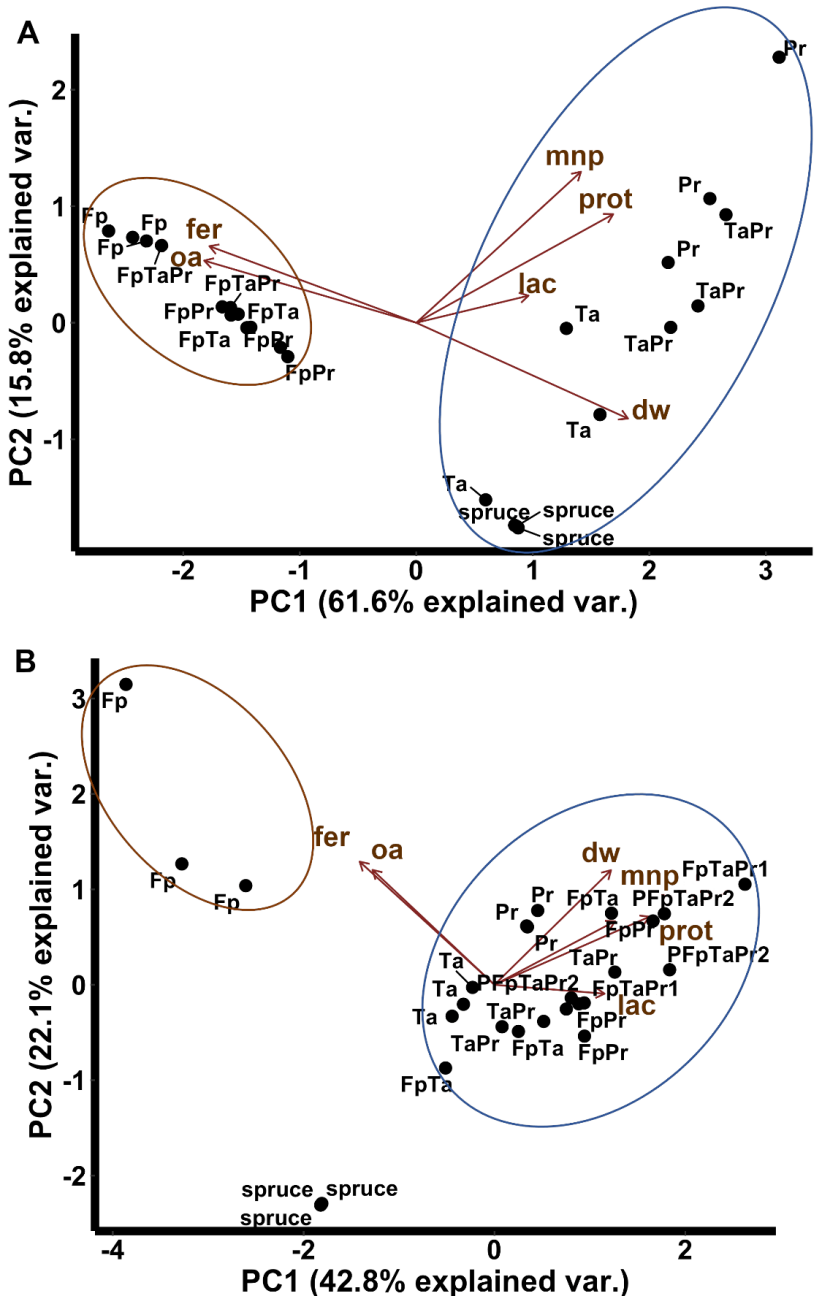

Figure 3. Principal component analysis including clustering of laccase (lac), manganese peroxidase (mnp), iron reduction activity (fer), total oxalic acid and oxalate concentration (oa), amount of proteins (prot) and dry weight compared to non-decayed wood (time point 0; dw). Single-species and combination cultures on A) spruce shavings after three months, and B) spruce wood veneer slices after one year of fungal growth and wood decay. Brown circle demonstrates brown rot type fungal decay of wood, and blue circle demonstrates white rot type. Fp = *F. pinicola*, Ta = *T. abietinum*, Pr = *P. radiata*.

4.3.1 Cellulose and hemicellulose targeting enzymes

Activities of the cellulose and hemicellulose targeting enzymes, β-glucosidase and xylanase were measured weekly from the wood supplemented semi-solid low-nitrogen cultivations (Figure 4 in **I**). The highest activities were detected for the brown rot fungus *F. pinicola*. In the combination cultures with different species of white rot fungi, the trend seemed to be that the presence of the white rot species only diminutively influenced the observed enzyme activity levels.

On the contrary, in the two-month incubation on spruce wood shavings, β-glucosidase activity was observed in the white rot fungal single-species cultures of Ta and Pr (Figure 1 in **II**). In the Ta and Pr single-species cultures, β-glucosidase activity increased during the first two months. However, in the two- and three-species co-cultures, β-glucosidase activity increased for three months. Thus, it may be concluded that on wood substrate, fungal species-species interactions enhance the production of cellulolytic and oligosaccharide attacking CAZymes, even during moderate-stage of decay.

In a recent study, interspecies interactions between the white rot fungus *Bjerkandera adusta* and either of the brown rot fungi *Antrodia sinuosa* or *Gloeophyllum sepiarium* in liquid cultivations, supplemented with coniferous or deciduous wood, had a positive impact on the enzymatic degradation of wood cellulose and hemicellulose components (Sugano et al. 2021). As a conclusion, the production of wood decay enzymes is dependent on the fungal community composition, and white rot – brown rot combinatory cultures may accelerate the decomposition of wood.

4.3.2 Oxidoreductases

Laccase. On wood-supplemented liquid cultivations, the highest laccase activities were observed for the white rot fungi Ta and Pr during cultivation weeks 2-3, after which the activity decreased significantly (Figure 2 in **I**). On spruce wood shavings, laccase activity was observed again for Ta and Pr after four weeks of growth, but not later (**II**, Figure 4A). After one year of cultivation on the spruce wood veneer slices, low laccase activity was observed for both white rot fungi in single-species and interacting co-cultures (**III**, Figure 4B). This supports laccase being generally expressed and secreted by white rot fungi as a response to environmental conditions such as mycelial confrontation with another fungus. However, the presence of the brown rot fungus Fp caused no increase in laccase activity produced by the white rot fungi during the early stages of wood decay (weeks 1-8, **I, II**).

After one year of growth on spruce wood veneer slices, laccase activity increased in the co-cultures (FpPr, PrTa, TaFpPr) compared to the white rot Ta or Pr single-species cultures (**III**). In the brown rot – white rot combination culture FpPr, laccase activity was measurable directly in the interaction zone, with decreasing activities on both sides of the zone (Figure 5). This was not observed in the FpTa co-culture, whereas with the three-species (FpPrTa) combination, laccase activities in both interaction zones (Ta-Fp and Fp-Pr zones) were higher than those detected in Ta or Pr single-cultures. Likewise observed in the previous cultivations (**I, II**), after one year on spruce veneer

slices, co-culture of the two white rot fungi (Pr and Ta) showed increased laccase activity in the interaction zone (**III**). Considering that laccase activity was not observed at any point in the single-species cultures of the brown rot fungus Fp on any media or spruce wood (**I, II**, **III**), it may be assumed that the detected laccase activity in combination cultures was due to active production of the enzyme by the white rot fungi Ta and Pr.

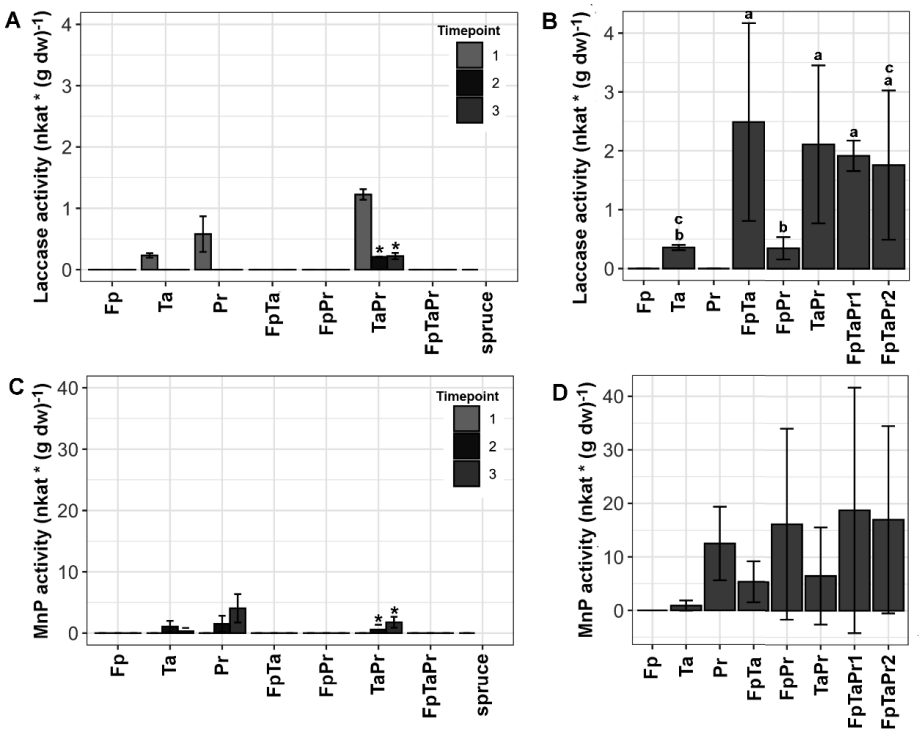

Figure 4. Enzyme activities in the fungal single-species and combination cultures. A) Laccase activity on spruce wood shavings after 1, 2 and 3 months of growth, B) laccase activity on spruce wood veneers after 12 months of cultivation, C) manganese peroxidase (MnP) activity on spruce wood shavings after 1, 2 and 3 months of growth, and D) manganese peroxidase (MnP) activity on spruce wood veneers after 12 months of cultivation. Mean average values (n = 3 parallel cultures) with standard deviation are presented. In A and C) ∗= time point 2 or 3 differs significantly ($P \leq 0.01$) from time point 1 (paired t-test). In B) the small letters indicate statistically significant differences in the zones between the single-species and combination cultures (ANOVA, Tukey test $p < 0.05$). Fp = *F. pinicola*, Ta = *T. abietinum*, Pr = *P. radiata*.

Laccase activity has been shown to increase during fungal interaction in the mycelial interaction zones, proposed to function against VOCs and diffusible organic compounds (Iakovlev and Stenlid 2000, Baldrian 2004, Hiscox et al. 2010). This indicates the need for laccase at different stages of fungal growth and during interactions. Laccases can eliminate toxic compounds produced by competing fungal species during interaction (Baldrian 2006, Boddy and Hiscox 2017). Even though no visible mycelial barriers were observed in the

brown rot – white rot combinations of *F. pinicola* and *P. radiata* on agar media (**I**), increased laccase activity, apparently produced by Pr (**I, II, III**), suggests a competitive interaction situation between these two fungi when growing on spruce wood.

Interestingly, no extracellular laccase activity was detected in the single-species cultures of the brown rot fungus Fp in any cultivation conditions in this study (**I**, **II**, **III**). This was unexpected since several laccase-MCO enzyme encoding genes have been annotated in the genome of *F. pinicola* (Floudas et al. 2012). In another species of brown rot fungi of Agaricomycetes, *Gloeophyllum trabeum*, a predictably non-secreted, most likely cell-wall bound specific laccase enzyme has been annotated (Presley et al. 2020). The respective laccase encoding gene was upregulated in *G. trabeum* in combination with another brown rot fungus *Rhodonia* (*Postia*) *placenta,* but no secreted laccase enzyme or extracellular laccase activity was observed in the study.

Non-secreted, fungal cell wall-bound laccases may have a function in melanin pigmentation of the hyphae (Baldrian 2006, Upadhyay et al. 2016). Melanized cells are more protected against oxidative stress and against competing fungal species during interspecific interactions (Boddy 2000, Cordero and Casadevall 2017). Regarding this, the genome-predicted laccase-MCO enzymes in *F. pinicola* (Riley et al. 2014) may have similar functions, rather than an involvement in mycelial combat and interaction reactions.

Manganese peroxidase. In the liquid medium supplemented with coniferous wood, manganese peroxidase (MnP) activity was observed for the white rot fungal single-species (Ta, Pr, Pl, Jl, Pf) cultures (**I**). As expected, no MnP activity was detected in Fp brown rot single-species cultures on the various media and cultivation conditions applied (**I, II, III**).

In the white rot (Pr and Ta) single-species cultures on spruce wood shavings, MnP activity was observed during the second and third months of growth (weeks 8 and 12), but not earlier (**II**, Figure 4C). Cyclic production of MnP activity was observed in the liquid cultivations of Pr and Ta (Figure 3 in **I**). Therefore, it is possible that on solid spruce wood shavings (**II**), the white rot fungi produced the first MnP activity peak before or after the sampling time on week 4. In the two-species co-culture of Ta and Pr, MnP activity was lower than with Pa alone growing on spruce wood shavings or veneer slices for one year (**II, III**). This indicates a suppression of Pr MnP expression by Ta mycelium.

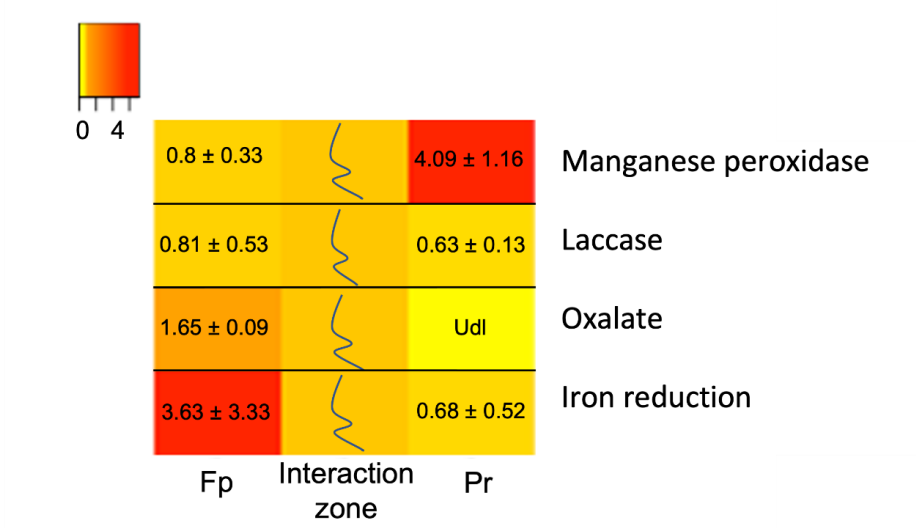

Figure 5. Illustration of relative oxidoreductase activities, concentration of oxalate, and iron reduction activity in different zones of the spruce wood veneer slices inoculated with the brown rot fungus *F. pinicola* (Fp) and the white rot fungus *P. radiata* (Pr). Mean values of three biological replicates with standard deviation are shown. Udl = under detection limit. Adopted from Fig. 5 in **III**.

In the brown rot – white rot co-culture of Fp and Pr growing together on spruce wood shavings for three months, no MnP activity was observed (**II**). On the contrary, after one year of fungal growth on the spruce veneer slices, extracted MnP activity was observed in the mycelial interaction zone, even though the activity was lower than observed for Pr alone (**III**, Figure 4D). MnP activity increased next to the interaction zone (0.5-1.0 cm distance) on the Pr mycelial side (Figure 5). Other studies have shown an increase or decrease in MnP activity in the mycelial interaction zone in the case of two interactive species of wood decay Basidiomycota (Baldrian 2004, Chi et al. 2007, Hiscox et al. 2010).

It is possible that MnP production during species-species interactions may be enhanced to promote the decomposition of wood by fungi rather than having a direct effect on the interaction reactions. In Pr, several MnP enzymes are expressed on spruce wood together with a few LiP enzymes (Kuuskeri et al. 2016), and the MnPs and LiPs of Pr are efficient enzymes for degradation and modification of wood-lignin like compounds (Lundell et al. 1993, Lundell et al. 2017). With other white rot fungi, the expression of MnP activity apparently caused no effect on the outcome of hyphal interaction (Hiscox et al. 2010), which supports the above statement.

4.3.3 Chitinase and peptidase (II)

Chitinase activity was observed to increase over time in all the single-species or combination cultures with *F. pinicola*, *T. abietinum* and *P. radiata* on spruce

wood shavings as growth substrate (Figure 6A). Chitinases have been proposed to be active in the degradation of the fungus' own old mycelium to be utilized later and recycled (Lindahl and Finlay 2005). Chitinase activity has been observed to be higher in the older mycelium than in the elongating hyphal front in the white rot fungus *Phanerochaete chrysosporium* (Karlsson et al. 2016). Similarly, high chitinase activity together with the low amount of mycelial ergosterol noticed previously (Niemenmaa et al. 2008b), and in this thesis study on spruce wood, could indicate efficient cycling of cellular components in the hyphae of *P. radiata.* Chitinase activity increased at the mycelial tip during fungal interspecific interactions, indicating that it has some involvement in degradation of the fungal cell wall of the competing species (Lindahl and Finlay 2005). After three months of hyphal growth, chitinase activity seemed to be slightly higher in the interacting three-species (FpPrTa) co-cultures than in the single-species or two-species cultures. In line with these observations, it is hypothesized that fungal chitinases are used during combative interaction against competing species (Seidl 2008).

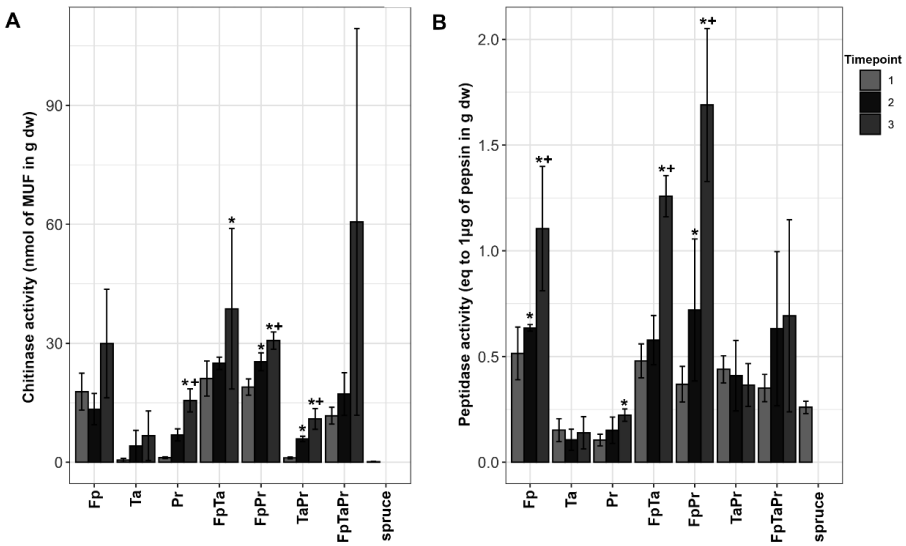

Figure 6. A) Chitinase activities and B) peptidase activities in the fungal single-species and combination cultures on spruce wood shavings after 1, 2 and 3 months of growth. Mean average values (n = 3 parallel cultures) with standard deviation are presented. ∗= time point 2 or 3 differs significantly (P ≤ 0.01) from time point 1, += time point 3 differs significantly (P ≤ 0.01) from time point 2 (paired t-test). Fp = *F. pinicola*, Ta = *T. abietinum*, Pr = *P. radiata*.

In contrast to the observed chitinase activities, the highest activities of protein-degrading acidic peptidase were observed on spruce wood shavings in co-cultures including the brown rot fungus Fp (Figure 6B). Low acidic-peptidase activity was observed with Pr alone, but this increased during the three-month cultivation period. Peptidases are hypothesized to be produced to degrade and re-cycle the fungus' own secreted enzyme proteins, for instance in low-

nitrogen, high-carbon characterized woody environments (Abuzinadah and Read 1986, Alfaro et al. 2014).

Proteases are also secreted for nutrional supply by the degradation of proteins in the environment as well as to regulate of the activity of wood decay enzymes (Kudryavtseva et al. 2008). The production of acidic and other peptidases has been demonstrated in the white rot fungus *P. radiata* during six weeks of growth on spruce wood, with alternating abundances (Kuuskeri et al. 2016). The acidic peptidase activity in the brown rot – white rot two-species (FpPr, FpTa) co-cultures was slightly higher than in the single-species culture of *F. pinicola* (**II**). In three-species (FpPrTa) combinations, peptidase activity, on the contrary, was lower after three months of growth. These results indicate the need for acidic peptidases to act during interspecies interactions, depending on the fungal consortium.

Wood decay fungi have various strategies for recycling their own cellular building elements to support hyphal extension and enzyme production (Rao et al. 1998, Alfaro et al. 2014, Langner and Göhre 2016, Kuuskeri et al. 2016). Deadwood has limited in nitrogen as nutrition for microbes (Baldrian 2017). Degradation of fungal cell wall chitin and extracellular proteins may supply nitrogen from old hyphae and therefore, allow further growth and extension of new hyphae together with production of extracellular enzymes and metabolites required for the decay of wood and plant litter.

4.4 Non-enzymatic activities (I, I, III)

4.4.1 Iron reduction (II, III)

It is recognized that extracellular iron reduction capacity (from Fe^{3+} to Fe^{2+}) is an indication of ongoing Fenton chemistry (Blanchette 1988, Eriksson et al. 1990, Arantes et al. 2009, Shah et al. 2013). In this study, the highest iron reduction capacities were observed in the single-species and co-cultures including the brown rot fungus *F. pinicola* in solid spruce wood shaving cultivations after 2 and 3 months or growth (**II**, Figure 7A). However, after one year of incubation on the spruce wood veneer slices, iron reduction capacity was almost non-detectable in the three-species (TaFpPr) combination culture, and even the single-species veneer culture of Fp demonstrated lower relative activity in iron reduction (**III**, Figure 7B). A similar trend was observed with the brown rot - white rot two-species combinations of Fp with either Ta or Pr: higher iron reduction on spruce wood shavings after 2 and 3 months of growth, but hardly any activity detectable after one year of growth on the veneer slices (**II, III**). In the brown rot – white rot combination culture FpPr, the iron reduction activity was highest on the Fp side of the interaction zone while decreasing towards the Pr mycelium side (Figure 5).

These results may indicate that, on the contrary to the first three months of wood decay, brown rot Fenton chemistry becomes less prevalent in the later stages of decay (after 12 months in the thin spruce veneer slices), while the white rot fungi and decay type become dominant in the interactive combinatory cultures. This transformation from brown rot to white rot decay mechanism is

also supported by the oxidoreductase activities detected in the same spruce veneer slices (**III**). An interesting detail is that in the case of the white rot fungi *T. abietinum* and *P. radiata*, low iron reduction capacity was measured from their single-species cultures on spruce wood both after 2 and 3 months of growth (**II**), as well as after one year of incubation on the veneer slices (**III**). This may result from the production of white rot oxidoreductases or metabolites potentially involved in Fe^{3+} reduction but this is beyond the scope of these experiments and study.

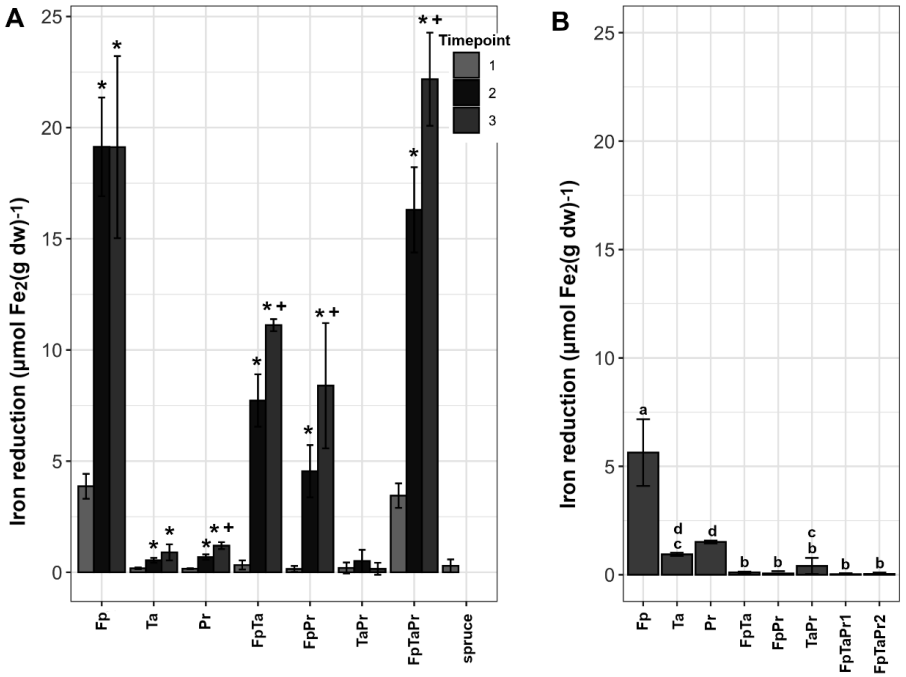

Figure 7. Iron reduction activities in the fungal single-species and combination cultures on A) spruce wood shavings after 1, 2 and 3 months of growth, and B) spruce wood veneers after 12 months of cultivation. Mean average values (n = 3 parallel cultures) with standard deviation are presented. In A) ∗= time point 2 or 3 differs significantly (P ≤ 0.01) from time point 1, += time point 3 differs significantly (P ≤ 0.01) from time point 2 (paired t-test). In B) the small letters indicate statistically significant differences in the zones between the single-species and combination cultures (ANOVA, Tukey test p < 0.05). Fp = *F. pinicola*, Ta = *T. abietinum*, Pr = *P. radiata*.

Recently, an oxidative-enzymatic two step decay mechanism for brown rot decomposition of wood has been suggested (Zhang et al. 2016, Presley et al. 2018). In this "staggered mechanism" (see also Chapter 1.7.2), the brown rot fungus first causes a non-enzymatic pre-treatment of wood with oxidative action, most probably through Fenton chemistry, followed by enzymatic action and hydrolysis of cellulose and hemicellulose polymers into sugars. The findings of this study are in line with the "staggered mechanism" theory. The high values for iron reduction capacity detected on spruce wood at the early stages of *F. pinicola* brown rot decay (months 2 and 3 of fungal growth, **II**) and

the very low values measured at later stages of growth and spruce wood decay (after 1 year, **III**), support the explanation of temporal and spatial separation of oxidative and hydrolytic attacks in the brown rot decay mechanism (Zhang et al. 2016, Presley et al. 2018).

4.4.2 Oxalic acid and pH (I, II, III)

Similar to other brown rot fungi representing various orders in the systematic class Agaricomycetes, the species *F. pinicola*, of the order Polyporales, is a strong producer of oxalic acid (Espejo and Agosin 1991, Mäkelä et al. 2002, Hastrup et al. 2012b, Shah et al. 2018). After 8 weeks of growth in the ME liquid medium, concentrations of 58 mM and 61 mM of oxalic acid accumulated in the *F. pinicola* single-species culture and the brown rot – white rot two-species cultures with *T. abietinum*, respectively (Figure 8) (Table A in Supplementary File 2 in **I**). Extremely high concentrations of produced oxalic acid and tolerance of acidic conditions by *F. pinicola* are most likely connected to its competition method during interspecific interactions (Gramss 2020).

In the two-species and three-species co-cultures including *F. pinicola*, however, production of oxalic acid was higher or lower depending on the white rot species present (Table A in Supplementary File 2 in **I**). *P. radiata* was the only white rot species that was observed to produce some oxalic acid. It is notable that the combination cultures of Fp, including Pr, demonstrated diminutive to zero concentrations of oxalic acid. This may indicate that the oxalate-degrading enzyme oxalate decarboxylase was actively expressed in *P. radiata*, as has been shown in the white rot Polyporales fungus *Dichomitus squalens* (Mäkelä et al. 2009, Mäkelä et al. 2010). These results indicate the capability of *P. radiata* to tolerate aggressive oxalic acid production and consequent acidification of the growth environment by *F. pinicola*.

Acidification of the ME liquid medium followed the same trend with the accumulation of oxalic acid, with the lowest pH value (1.53) measured from the combination of FpTa (**I**). In the spruce wood supplemented cultivations, however, the pH of the culture fluid increased in the white rot fungal cultures, even up to pH 6.2 (in the case of *Phellinus ferrugineofuscus*). In these cultivations, the initial pH value of 4.5 was buffered by sodium succinate. An exception to this was the combination of Fp and *P. laricis*, in which the culture fluid was acidified to pH 3.2 (**I**).

An increase in the culture fluid pH indicates conversion of the produced acids, either chemically or through fungal metabolic activity (Mäkelä et al. 2002). Previously, increases in the culture fluid pH and decreases in the concentration of acids has been observed with fungi able to metabolize low-molecular weight organic acids like succinate, malonate or acetate (Moilanen et al. 1996, Galkin et al. 1998, Hofrichter et al. 1999). Additionally, an increase of pH value may be due to dying mycelium in longer duration cultivations in a liquid medium (Borkovich and Ebbole 2010).

On spruce wood shavings, *F. pinicola* produced the highest amounts of oxalic acid, with concentrations reaching up to 35 μmol * g^{-1} total dry weight, peaking in cultivation week 4 (**II**, Figure 8A). On this substrate, some production of oxalic acid was observed with both white rot fungi Ta and Pa

alone. Change of the growth substrate from the rich ME liquid medium to a more natural substrate (spruce wood) seemed to also induce some oxalic acid production in *T. abietinum* (Figure 8A-C).

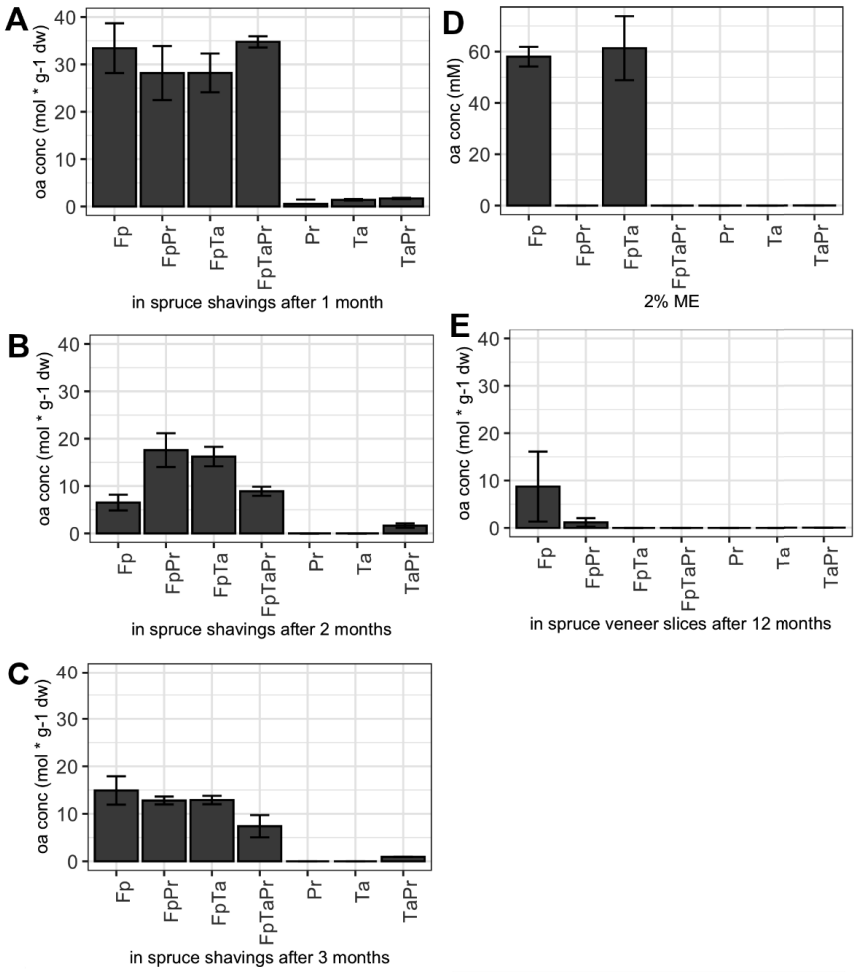

Figure 8. Oxalic acid (oxalic acid and oxalate combined) in mM concentrations detected A) on malt extract liquid medium after 2 months of fungal growth; and on spruce wood shavings after B) 1 month, C) 2 months, D) 3 months of growth, and on spruce wood veneer slices E) after 12 months of incubation. Mean average values (n = 3 parallel cultures) with standard deviation are presented. Fp = *Fomitopsis pinicola*, Ta = *Trichaptum abietinum*, Pr = *Phlebia radiata*.

Most oxalic acid in the spruce wood shaving cultivations (at all time points) was in the form of chelated, wood bound oxalate, whereas dissolved oxalic acid was observed in week 4 in the cultures including *F. pinicola*. The total concentration of oxalate plus oxalic acid was observed to decrease over time. On the spruce wood veneer slices after one year of culturing, wood bound oxalate was only discovered with *F. pinicola* (**III**). Low concentrations were detected from the brown rot – white rot combination of FpPr on the Fp side,

and in the interaction zone (Figure 8E). These results further support the oxidative-enzymatic staggered wood decay mechanism proposed for brown rot type of wood decay (Zhang et al 2016, Presley et al 2018). This is also an indication of the shift in the wood rot type changing from brown rot to white rot during later stage decomposition.

4.4.3 Emission of VOCs on spruce deadwood (II)

Released volatile organic compounds (VOCs) in the headspace of the fungal cultivations on spruce wood shavings were collected after 1, 2 and 3 months of growth and analyzed by GC-MS to see whether descriptive VOCs for white rot or brown rot decomposition could be detected (**II**). Over 50 VOCs were identified from spruce wood cultures (Supplementary Information in **II**, Mäki et al. 2021). Of the set of compounds, four (6-methyl-5-heptene-2-one, methyl 3-furoate, terpinolene, α-humulene) appeared to be emitted in relation to the wood decay type (either brown rot or white rot dominated) and accumulated during cultivation (Figure 9).

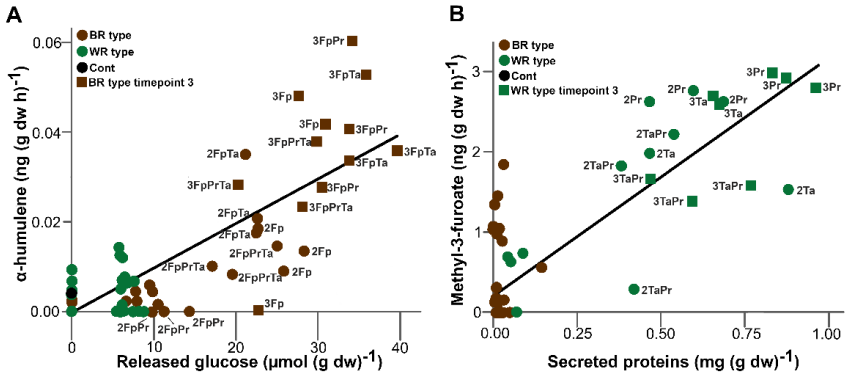

Figure 9. Correlation between two signature VOCs and explanatory fungal activities detected in the fungal cultures grown on spruce wood shavings for three months: (A) α-humulene and released dissolved glucose, and (B) methyl 3-furoate and total amount of secreted proteins. The cultivation time points in months 2 (circles) and 3 (squares) are depicted. Fp = *F. pinicola*, Ta = *T. abietinum*, Pr = *P. radiata*. Adapted from Fig. 2 in **II**.

The proportion of α-humulene among emitted sesquiterpenes increased over time in the single-species and combination cultures including *F. pinicola* (Table 1 in Supplementary Information in **II**). In addition, the total amount of α-humulene increased during the cultivation period. A similar trend was noticed with terpinolene, and its proportional amount among the set of monoterpenes emitted increased in the single-species and combination cultures including the brown rot fungus *F. pinicola*.

Terpinolene is not documented as a signature mVOC of *F. pinicola* (Rösecke et al. 2000, Lemfack et al. 2017) (Table 2). This indicates that the detected terpinolene was more likely released from the decaying spruce wood as a result of brown rot instead of being a new mVOC of *F. pinicola*.

Terpinolenes are common monoterpenes biosynthesized in spruce tree species and constitute a major part of the terpenoid resin VOCs in coniferous wood (Martin et al. 2002).

In previous studies, it has been noticed that interspecific interactions can affect the production of fungal mVOCs, especially sesquiterpenes (Hynes et al 2007). Additionally, the growth substrate influences the emission of VOCs either as fungal metabolites or released from the substrate due to substrate decomposition (Korpi et al. 1998, Hynes et al. 2007, El Ariebi et al. 2016). Enzymatic attacks on competing fungal species may even increase the rate of release of sesquiterpene compounds (Mäki et al. 2021). In another recent study, *F. pinicola* was shown to be able to control the quantities of released monoterpenes, oxygenated compounds and sesquiterpenes from the wood substrate during growth (Gramss 2020).

4.5 Decay-specific gene-expression in late-stage of wood decay (III)

To see if mycelial interaction between the brown rot fungus *F. pinicola* and the white rot fungus *P. radiata* could influence the expression of fungal genes, real-time qRT-PCR analysis was conducted with selected genes encoding enzymes involved in the degradation of wood lignocelluloses (**III**). These enzyme-encoding genes were upregulated according to previous RNA-Seq transcriptomics obtained for both fungi on spruce wood as the growth substrate (Kuuskeri et al. 2016, Mattila et al. 2020, Mäkinen, Shah, Lundell, unpublished data).

The brown rot fungus *F. pinicola* had a positive effect on expression of the oxidoreductase encoding genes (fold change >10 for *mnp2*, *lip2* and *lpmo1* relative to non-interaction situations) in *P. radiata* (Figure 2 in **III**). With *mnp2*, the effect was weaker than the other two oxidoreductase enzyme-encoding genes. For the cellulose and hemicellulose targeted enzyme-encoding genes (*eg1*, *xyn1* and *bgl1*) of *P. radiata*, expression levels either decreased or no change was observed in the interaction zone.

Contrary to the positive effect of the brown rot fungus on white rot fungal expression of the LiP, MnP and LPMO oxidoreductase encoding genes, the presence of the white rot fungi *P. radiata* and *T. abietinum* caused a decrease in expression of the ferric reductase encoding gene *fr1* in *F. pinicola* (Figure 3 in **III**). Expression of the laccase multicopper oxidase (*mco1*) encoding gene was low in the single-species culture of *F. pinicola* and under the detection limit (amplification started after >36 cycles) in the brown rot – white rot combination of *F. pinicola* and *P. radiata*. On the other hand, the presence of the third fungus, the white rot species *T. abietinum* in the three-species combination, seemed to enhance expression of the *F. pinicola mco1* gene.

Cultivation mode, growth media, and wood substrate species have all been shown to influence the overall expression of genes in divergent brown rot fungi, including *F. pinicola* (Wu et al. 2018, Wu et al. 2019). Expression of genes encoding for plant polysaccharide degrading enzymes has been demonstrated to stay more or less the same in the interaction zone in a

combination culture of the two brown rot fungi *R. placenta* and *G. trabeum,* similar to their single-species cultures (Presley et al. 2020). Compared to the results of this study, in which the fungal interaction zones were investigated after one year of cultivation (**III**), the authors studied the brown rot – brown rot fungal interaction zone in an earlier phase of wood colonization and decay (Presley et al. 2020), which may be the reason for not yet noticing any induction of fungal enzymes involved in wood decay.

A mycoparasitic Ascomycota fungal species *Clonostachys rosea* has been observed to have different and species-specific gene expression reponses for some of its genes during interspecific interactions against the other Ascomycota species *Botrytis cinerea* or *Fusarium graminearum* (Nygren et al. 2018). The responses in relative fold changes of some of the selected wood lignocellulose targeted genes (*mnp2* and *lpmo1*), in the white rot fungus *P. radiata* against the brown rot fungus *F. pinicola* and white rot fungus *T. abietinum*, support this finding. Changes in gene expression when a third species is introduced could be due to stress with more species present in the interaction, or a species-specific response against another fungal species.

4.6 Wood properties after fungal decay (II, III)

The effect of fungal interactions on decomposition and on the chemical and anatomical differences of xylem structure on decaying Norway spruce wood were studied by mass loss, dissolved carbon compounds and lignin composition, as well as visually by electron microscopy methods. In fungal cultivations on the spruce wood shavings, aggressive wood decay with rapid mass loss was observed in the single-species and combinatory cultures including the brown rot fungus *F. pinicola* (**II**). Up to a 25 % decrease of the total dry weight was observed in the single-species culture of *F. pinicola*, whereas in the white rot fungal cultures (Pr, Ta, TaPr), decreases of less than 10 % of the total dry weight were observed within three months.

F. pinicola is a primary colonizer of deadwood and living trees, whereas *P. radiata* is an early secondary colonizer of deadwood, and *T. abietinum* often colonizes the same deadwood tree trunks as *F. pinicola* (Rayner and Boddy 1988, Holmer et al. 1997, Ovaskainen et al. 2010, Rajala et al. 2010). Rapid decomposition of spruce wood shavings by *F. pinicola* could be due to its brown rot decay mechanism being more suitable for intact wood than is the case with the secondary colonizer white rot fungal species *P. radiata* and *T. abietinum*.

After 12 months of growth on spruce wood veneer slices, the total dry weight decreased 37 % in the single-species culture of *F. pinicola*, whereas in the white rot (*P. radiata* or *T. abietinum*) single-species cultures, the loss was about half of this value (maximum of 20 %) (Table 7) (**III**). The rate of spruce wood mass loss decreased after three months with *F. pinicola* (**II**), indicating that the easily degradable components of wood had been utilized. Since the spruce wood veneer slice as a growth substrate in the one-year long cultivation was relatively large compared to the spruce wood shavings during three-month cultivation, direct comparison is not possible. When grown on spruce wood veneer slices, it is noticeable that the slices inhabited by the brown rot

fungus *F. pinicola* are drastically more composed after one year compared to after three months (Figure 10; Fp, FpPr, FpTa).

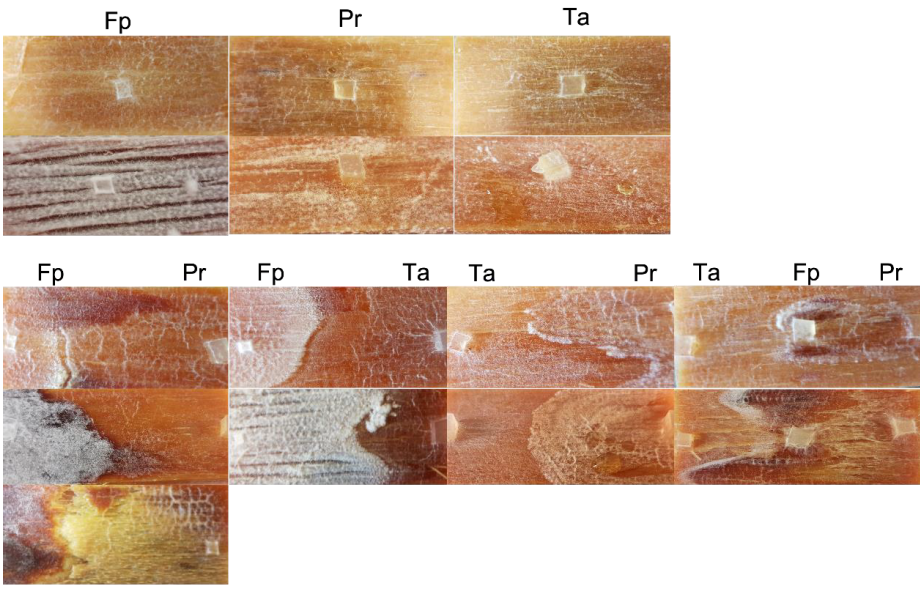

Figure 10. Growth on spruce wood veneer slices in single- and combination cultures of two or three species after 3 months (upper) and 12 months (lower). Fp = *Fomitopsis pinicola*, Ta = *Trichaptum abietinum*, Pr = *Phlebia radiata*

Also notable in the three-month long cultivations on spruce wood shavings including the brown rot fungus *F. pinicola* was that dissolved carbon compounds were released from the wood as degradation products, and were not completely utilized by the fungi, since the accumulation of sugars (carbohydrates) was detected (**II**). Glucose accumulated in the liquid phase of the cultivations, except in the white rot combination culture PrTa, where glucose was observed after two months of growth but not after three months. This indicates an uptake of the released sugars by the white rot fungi.

Xylose dissolved from the intact spruce wood and accumulated in the fungal cultivations (**II**). Signs of xylose utilization by white rot fungi were found, and *P. radiata* is known to utilize pentose sugars such as xylose (Mattila et al. 2020). For instance, in the white rot combination culture PrTa, xylose concentration was observed to decrease and disappear during three months of cultivation (**II**). In addition, the amount of inorganic carbon (respiration products: CO_2, HCO_3^-, CO_3^{2-}) in the water phase accumulated temporally in the single-species and combination cultures including *F. pinicola* (**II**). The accumulation of inorganic carbon in the liquid phase could be due to the aggressive brown rot mechanism of wood decay and rapid fungal growth with intensive respiration.

After one year of decay, the relative amount of Klason lignin in the spruce wood veneer slices increased from about 20 % to over 30 % with the brown rot fungus *F. pinicola* alone, and to over 20 % with the white rot fungus

P. radiata alone (Table 7) (**III**). In a previous study, the relative amount of Klason lignin in spruce wood increased during six weeks of growth and decay by *P. radiata* (Kuuskeri et al. 2016). Enrichment of lignin content is not unusual even with lignin-degrading white rot fungi, since total mass (as dry weight) of the substrate wood decreases as wood decomposes and decay advances.

Even though the relative amount of lignin increased with *P. radiata* alone, calculated total lignin amount decreased approximately 15 % after one year of growth and decay on spruce wood veneer slices. This is in line with previous studies on the ability of *P. radiata* to convert and degrade wood lignin, as well as synthetic and isolated lignins (Moilanen et al. 1996, Hatakka et al. 2002, Niemenmaa et al. 2006, Kuuskeri et al. 2016).

With the brown rot fungus *F. pinicola*, the however, relative amount of Klason lignin remained almost the same in the spruce wood veneer slices within one year of growth and decay, which is indicative of only a limited ability to degrade wood lignin. Interestingly, relative amount of Klason lignin in the brown rot – white rot (Fp – Pr) interaction zone was even higher than with *F. pinicola* alone (Table 7) (**III**). This is an indication of very pronounced utilization of celluloses and hemicelluloses in that area. The recently proposed lignin loss relative to density loss of decaying wood as a method to distinquish rot types in decaying wood (Schilling et al. 2020) is supported by the findings of this study. The proposed relation might better pinpoint the development of dominant rot types in mycelial interaction situations and in naturally decaying deadwood at forest sites.

Table 7. Mass loss, relative amount of Klason lignin and acid soluble lignin in the single-species and interactive combinations of the brown rot fungus *F. pinicola* and the white rot fungus *P. radiata* after one year on spruce wood veneer slices.

Species combination	Mass loss (% of the veneer slice)	Klason lignin (%)	Acid soluble lignin (%)
F. pinicola	37.4 ± 4.7 *	32.2 ± 2.7 *	0.89 ± 0.1 *
P. radiata	20.0 ± 2.9 *	22.7 ± 4.1	0.82 ± 0.05 *
F. pinicola + *P. radiata*	31.7 ± 9.0 *	46.9 ± 3.7 *	1.11 ± 0.09 *
Spruce wood	0.37 ± 0.12	21.3 ± 0.6	0.53 ± 0.001

* Statistically significant difference between the fungal species present compared to intact spruce wood without fungi. Statistical difference as Benjamini-Hochberg adjusted *p*-values (*p*-value < 0.05, linear models).

The high relative lignin content in the brown rot – white rot (Fp – Pr) interaction zone indicates that the presence of *P. radiata* has supported the brown rot of wood by *F. pinicola.* After one year of collaborative existence and interaction in the same wood slice, the decay mechanism was changing from brown rot to white rot. The observed MnP activity, and the positive effect of *F. pinicola* on expression of oxidoreductase enzyme-encoding genes in *P. radiata*, together with decrease in oxalate plus oxalic acid concentration, as well as lower iron reduction capacity, all support this theory.

After one year, the white rot and brown rot fungal strategies of spruce wood decay were observed by using transmission electron microscope (TEM) and scanning electron microscope (SEM) imaging of the wood ultrastructure (**III**, Figure 11). In the resulting images, *F. pinicola* hyphae seemed to penetrate through the wood cell wall from the empty lumen side, and the brown rot fungal attack resulted in noticeable porousity of the wood cell walls compared to ultrastructure of the intact spruce wood (Figure 11A-C and F-G).

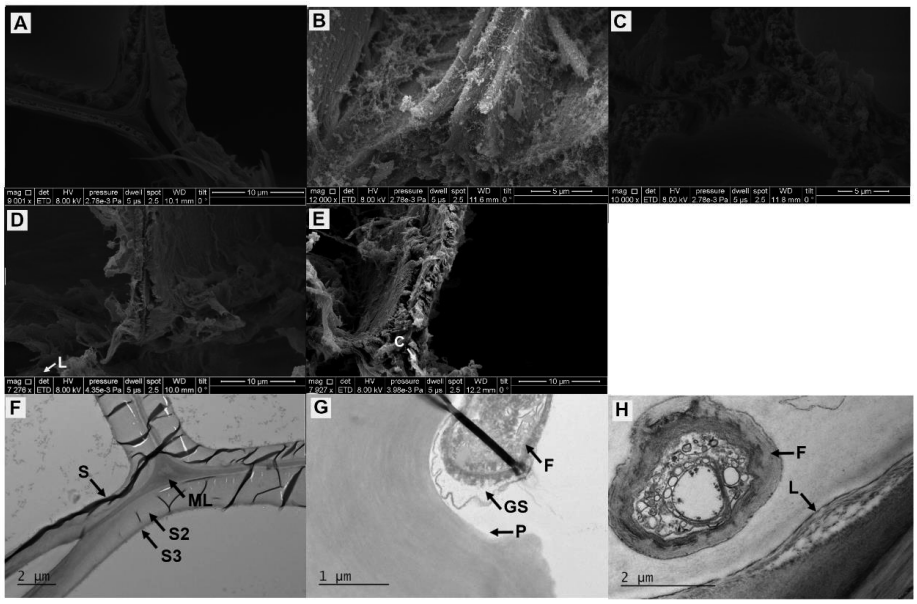

Figure 11. SEM (A-E) and TEM (F-G) images of cross-sections of the spruce veneer slices after 12 months of incubation. A) Non-inoculated wood, B-C) *F. pinicola* colonized, brown rot decayed wood, D) *T. abietinum* colonized, white rot decayed wood, and E) *P. radiata* colonized, white rot decayed wood, F) Non-inoculated wood, G) *F. pinicola* colonized, brown rot decayed wood, H) *P. radiata* colonized, white rot decayed wood. S = dark stretch of polymeric embedding material, ML = middle lamellae, S2 = secondary cell wall layer 2, S3 = secondary cell wall layer 3, F = cross section of fungal hypha, GS = glucan sheath at the hyphal tip, P = signs of degradation of the wood cell wall near the hyphal tip, L = detaching wood cell wall layers). Scale bars are 10 µm (A, D, E), 5 µm (B, C), 2 µm (F, H), 1 µm (G).

In the white rot decay of *P. radiata* and *T. abietinum*, a different pattern is visible. After one year of white rot attack, the hemicellulose and lignin created "glueing matrix" between the cellulose microfibrils is disappearing. As a result, fibrillous cell walls of the tracheids seem loosened, making the wood cell walls able to be peeled off layer by layer. Thinning of the wood cell wall as a result of white rot decay of spruce wood by *P. radiata* has been observed before (Kuuskeri et al. 2016). This specific white rot thinning of the wood cell walls is initiated from the lumen side where the fungal hyphae are extending (Figure 11 D-E and H).

In the wood cell lumen space, fungal hyphae secrete wood-decay and other enzymes together with metabolites as a response to the hyphal brown

rot – white rot fungal interaction (Figure 12). These interactions can involve species-specific or general responses (Nygren et al. 2018). Wood degradation by fungal hyphae from the lumen side of wood cell walls is initiated at the innermost secondary S3 layer. Responses in gene expression, secretion of enzymes and metabolites and other fungal activities in the interaction zones have an effect on wood decay and dominating wood decay type.

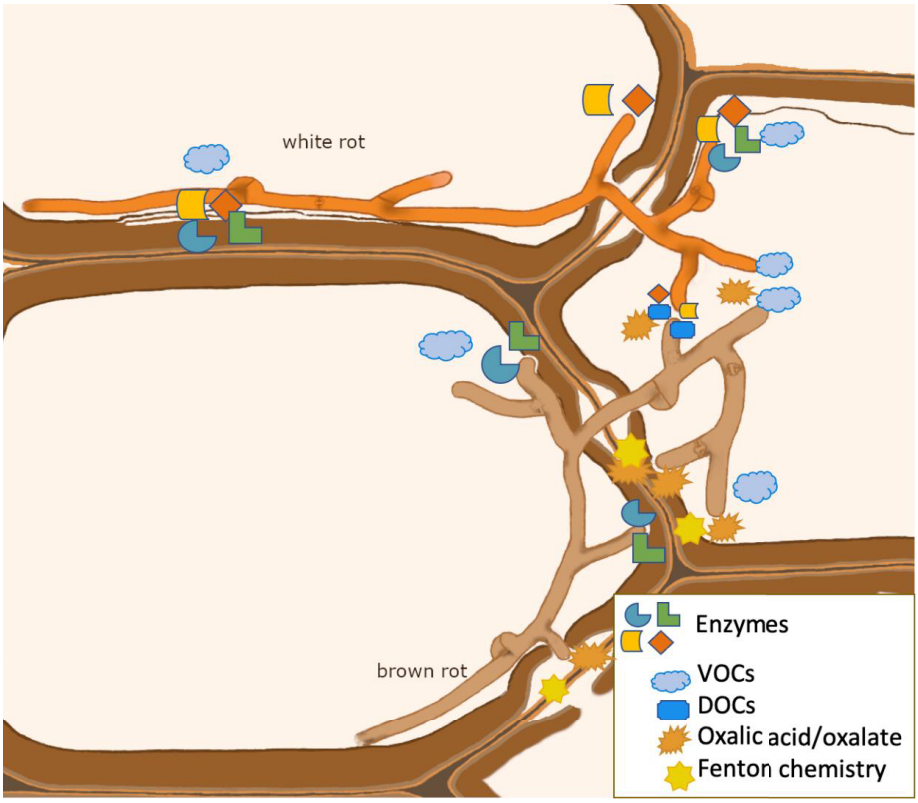

Figure 12. Illustration of wood decay metabolism by white rot and brown rot fungi and factors involved in their interspecific hyphal interactions Dark brown coloured: wood cell wall; light brown coloured: brown rot fungal hyphae; orange coloured: white rot fungal hyphae. Symbols refer to metabolites and biochemical processes explained in detail in thesis Chapters 1 and 4. (Mali T, 2021).

5 Conclusions

This thesis demonstrates that brown rot and white rot wood decay fungi of the class Agaricomycetes, phylum Basidiomycota, tolerate each other in various culture media and cultivation conditions (on wood, in liquid medium, on agar media). Natural preferences of the fungal species for their substrate deadwood type is seemingly not a primary factor influencing their interaction responses. Interspecific interactions between the mycelia were observed not only visually but in secreted enzyme activities and gene expression, production of oxalic acid and iron reduction capacity, emission of volatile organic compounds, and advancement and change of decay type on spruce wood.

Interacting wood decay fungi produced higher activities of wood-decaying enzymes, but not in all combinations or at all time points measured. Contrary to the first hypothesis, wood decay rate was not enhanced by fungal interaction. However, the wood decay pattern was affected and was under transition during the interactive cultivations. This thesis research demonstrates that wood decay fungal interactions are complex processes. The composition of fungal combinations in interactive situations impacts the production of wood decay enzyme activities.

Late-phase decay and interaction events (after 12 months) are partially comparable to the earlier phases of decay (after 1, 2, 3 months) on spruce wood, but not for all measurements. Fungal interactions on deadwood may impact wood decay and order and efficiency in decomposition of the lignocellulose biopolymers (cellulose, hemicellulose, lignin). White rot and brown rot fungal species in this study demonstrated specific wood decomposition strategies, which was observable in both early- and late-phase decay in all combinations and in single-species cultures of the fungi.

Spruce wood is not the preferred deadwood substrate for the white rot fungus *P. radiata* in its natural environments, however, after one year on spruce wood veneer slices, *P. radiata* and the brown rot fungus *F. pinicola* had an impact on each other. As a result, the wood decay type shifted from brown rot to white rot. During early stages of wood decay in the combination culture of *F. pinicola* and *P. radiata* (months 1, 2, 3 on spruce wood shavings), the wood decay mechanism was brown rot type. After one year, a change from a brown rot to white rot decomposition mechanism was detected. The findings of this study further support the recently presented theory of “staggered brown rot mechanism”, with temporal and spatial separation of the oxidative attack followed by production of the hydrolytic enzyme activities for decomposition of wood polysaccharides.

The main finding of this thesis study is that interspecific interactions of wood decay fungi are on-going and changing processes during mycelial growth and conctacts during the advancement of wood decay. Brown rot – white rot fungal interactions determine not only the initial, but also the late-

stage, decay strategy and the outcome of wood decomposition. In the future, transcriptome responses on fungal gene expression should be given more emphasis, not only against competing mycelia but also towards abiotic factors affecting fungal growth and decomposition metabolism. A deeper understanding of especially three-species combinations and fungal species responses to interaction requires more research. The specific signals for gene expression from the interacting species that trigger a species-specific response or a response against the number of species present in the interaction is to be studied.

The findings of this thesis study suggest that the brown rot – white rot fungal interactions could be developed into applications of biological pre-treatment of wood and wood-based wastes. This would allow more sustainable, low-technology carbon-cycling solutions to replace e.g., fossil fuels in the making of biofuels and biopolymers, thereby decreasing environmental threats such as chemical load from the process industry subjected to nature.

This study clearly highlights and gives new information on the dynamic properties of fungal interactions, and the temporal and spatial changes occurring in wood decomposition in combinations of brown rot – white rot fungi. Long-term cultivations and interaction studies are required to investigate how fungal interactions transform and wood decay proceeds. Laboratory scale studies, like those performed in this study, are not directly transferable to natural ecosystems and processes in decaying deadwood. However, they demonstrate the complexity of interspecific fungal interactions under continuous biological and biochemical transitions in decaying wood. Therefore, in the best cases, these types of simulation experiments may gradually enlighten complex ecological phenomena.

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