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RESEARCH ARTICLE

Decomposition of spruce wood and release of volatile organic compounds depend on decay type, fungal interactions and enzyme production patterns

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One sentence summary: Fungal decay type, either brown rot or white rot, determines not only the degree of degradation of wood carbohydrates but also the pattern of VOCs released by fungal decay action.

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ABSTRACT

Effect of three wood-decaying fungi on decomposition of spruce wood was studied in solid-state cultivation conditions for a period of three months. Two white rot species (*Trichaptum abietinum* and *Phlebia radiata*) were challenged by a brown rot species (*Fomitopsis pinicola*) in varying combinations. Wood decomposition patterns as determined by mass loss, carbon to nitrogen ratio, accumulation of dissolved sugars and release of volatile organic compounds (VOCs) were observed to depend on both fungal combinations and growth time. Similar dependence of fungal species combination, either white or brown rot dominated, was observed for secreted enzyme activities on spruce wood. Fenton chemistry suggesting reduction of Fe^{3+} to Fe^{2+} was detected in the presence of *F. pinicola*, even in co-cultures, together with substantial degradation of wood carbohydrates and accumulation of oxalic acid. Significant correlation was perceived with two enzyme activity patterns (oxidoreductases produced by white rot fungi; hydrolytic enzymes produced by the brown rot fungus) and wood degradation efficiency. Moreover, emission of four signature VOCs clearly grouped the fungal combinations. Our results indicate that fungal decay type, either brown or white rot, determines the loss of wood mass and decomposition of polysaccharides as well as the pattern of VOCs released upon fungal growth on spruce wood.

Keywords: wood-decaying fungi; wood biodegradation; white rot; brown rot; VOCs; CAZymes; oxalic acid; iron reduction; *Agaricomycetes*; *Fomitopsis pinicola*; *Phlebia radiata*; *Trichaptum abietinum*

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INTRODUCTION

In the forests, saprotrophic fungi of *Basidiomycota* are largely responsible for carbon cycling and decomposition of wood (Rayner and Boddy 1988; Lundell *et al.* 2014). Dead wood is one of the most demanding microhabitats for living organisms due to its recalcitrant physical and chemical properties (Baldrian 2017). In dead wood and woody debris, the fungal community is under transformation along with advancement of wood decay (Rayner and Boddy 1988). For example, in naturally decomposing spruce wood on a boreal forest site, brown rot fungi dominating in early decay stages were replaced with white rot saprotrophic species upon later stages of decay (Rajala *et al.* 2015). A large study compiling data from fruiting body inventories at forest sites has revealed that some fungal species are more dominant in their occurrence and effect on the wood-colonizing species consortium (Ottosson *et al.* 2014).

Inter- or intra-species hyphal interactions of saprotrophic fungi are dynamic and complex phenomena (Rayner and Boddy 1988; Boddy and Hiscox 2016; Baldrian 2017). Fungal interactions are categorized as mutualistic, neutral or combative in character (Boddy 2000). In fungal communities, interactions are dynamic, and fungal composition apparently affects production of secreted enzymes in combinatory cultures (Hiscox *et al.* 2010; Mali *et al.* 2017). It is assumed that the dynamic mycelial encounters may be advantageous to biodegradation of wood (Mali *et al.* 2017).

In order to access nutritional carbon from dead wood as the substrate for growth, wood-decaying *Basidiomycota* fungi have adopted unique strategies for decomposition of the wood lignocellulose components—cellulose, hemicellulose, pectin and lignin—including an array of diverse enzymes and metabolites (Lundell *et al.* 2014; Rytioja *et al.* 2014b). Secreted enzymes of wood-decaying fungi play an essential role in degradation of wood and lignocelluloses (Eastwood *et al.* 2011; Floudas *et al.* 2012). White rot fungi are able to decompose and modify all wood components by expression of CAZymes (carbohydrate active enzymes) and various oxidoreductases (Lundell, Mäkelä and Hildén 2010; Floudas *et al.* 2012; Yakovlev *et al.* 2013; Riley *et al.* 2014; Rytioja *et al.* 2014b). Recent studies have indicated that the array and activities of enzymes secreted by white rot and brown rot fungi depend on hyphal extension and deviate at different stages of wood decay (Presley and Schilling 2017; Presley *et al.* 2018).

Basidiomycota of the systematic class *Agaricomycetes* comprise wood and litter decomposing white rot species that demonstrate effective secretion of CAZymes and the unique ability to modify wood lignin (Floudas *et al.* 2012; Lundell *et al.* 2014; Riley *et al.* 2014; Kuuskeri *et al.* 2016). *Agaricomycetes* brown rot fungi are as well able to efficiently decompose wood carbohydrates but cause only minor structural changes in lignin units (Baldrian and Valášková 2008; Niemenmaa, Uusi-Rauva and Hatakka 2008; Hatakka and Hammel 2010) evidently due to their lack of the essential class-II peroxidases (Eastwood *et al.* 2011; Floudas *et al.* 2012). It has been concluded that brown rot decay of wood cellulose proceeds at first non-enzymatically by substantial secretion of oxalic acid and generation of extracellular radical oxygen species via Fenton chemistry (Xu and Goodell 2001; Baldrian and Valášková 2008; Eastwood *et al.* 2011; Shah, Mali and Lundell 2018), which is then followed by secretion of hydrolytic enzymes for decomposition of plant cell wall polysaccharides (Zhang *et al.* 2016; Presley and Schilling 2017). In addition, acidic conditions apparently enhance fungal enzyme activities against wood components (Hatakka and Hammel 2010; Lundell *et al.* 2014).

Upon fungal colonization of wood by hyphal extension, decomposition of the compact plant cell wall lignocellulose composite is initiated resulting with breakage of covalent bonds, and release of dissolved sugars and lignin-derived aromatic compounds (Hatakka and Hammel 2010; Lundell *et al.* 2014; Rytioja *et al.* 2014b). Decomposition of the compact woody tissue may result in release of volatile organic compounds (VOCs) from wood or generated as fungal metabolites (Evans *et al.* 2008; Müller *et al.* 2013; Isidorov, Tyszkiewicz and Piroznikow 2016). Encompassing studies on emission and distribution of VOCs in the forest ecosystems have principally focussed on carbon flow and tree physiology, as well as on the impact on lower atmosphere aerosol chemistry and climate change (Paasonen *et al.* 2013).

Warming climate has an impact on forest ecosystems affecting both plants and microorganisms. Forest VOC emissions are estimated to increase, whereas forest soil and its microorganisms may serve as VOC sink (Asensio *et al.* 2007; Yuan *et al.* 2009). Sources of forest VOCs may be abiotic or biotic, such as dead wood and plant biomasses, other organic material on the forest floor, living trees and other plants, as well as plant-associated and soil-inhabiting microbes (Leff and Fierer 2008). Forest VOCs are originating primarily from decomposing organic matter and secondly, a multitude of VOCs are actively emitted by living plants and plant roots (Paavolainen, Kitunen and Smolander 1998; Isidorov and Jdanova 2002; Asensio *et al.* 2007). Previous studies have undoubtedly recognized the importance of forest microbial communities and their activities in VOC emissions (Isidorov and Jdanova 2002; Leff and Fierer 2008). Due to the difficulty to distinguish VOCs produced by fungi from the chemically alike compounds released from woody debris, identification of fungal produced MVOCs (microbial VOCs) (Bäck *et al.* 2010) has mainly succeeded under laboratory conditions on artificial minimal media.

In this study, we challenged the complexity and aimed at opening the role of combinations of wood-decaying white and brown rot fungi in the release and generation of VOCs by adopting spruce wood as growth substrate. Patterns of the secreted enzyme activities were compared to spruce wood decomposition events and emission of the four identified signature VOCs by statistical clustering methods. During three months of cultivation on spruce wood, noticeable changes in fungal enzyme activities and VOC fluxes were observed. Our results point to the significance of fungal growth and interactions for production of enzymes and metabolites that are directing the dynamics and advancement of wood decomposition, and VOC emission profiles.

MATERIALS AND METHODS

Fungal isolates and culture conditions

Isolates of *Basidiomycota* class *Agaricomycetes* order *Polyporales* species *Fomitopsis pinicola* and *Phlebia radiata*, and *Trichaptum abietinum* from the order *Hymenochaetales* were previously confirmed by internal transcribed spacer (ITS)-sequencing (Mali *et al.* 2017) and are deposited at the University of Helsinki HAMBI mBRC FBCC fungal culture sub-collection (https://www.helsinki.fi/en/infrastructures/biodiversity-collections/infrastructures/microbial-domain-biological-resource-centre-hambi)

Table 1. Fungal isolates, and design of the combination cultures on spruce wood.

Species, isolate and identifier		Wood decay type[1]	Species combinations in cultures		
			Single species	Two species	Three species
Fomitopsis pinicola (FBCC1181)	Fp	BR	Fp	FpTa	FpTaPr
Trichaptum abietinum (FBCC0110)	Ta	WR	Ta	FpPr	
Phlebia radiata (FBCC0043)	Pr	WR	Pr	TaPr	

[1]BR = Brown rot, WR = White rot.

(Table 1). The fungal isolates were maintained on malt extract agar (MEA) (2% w V^{-1} malt extract, Biokar Diagnostics, France; 2% w V^{-1} agar, Sigma; pH 5.5) at 25°C, in the dark for one week before initiating the wood cultivations.

Fungal co-cultures and spruce wood cultivations

Fungal isolates were cultivated in single and combinatory solid-state cultures on spruce wood sawdust (Table 1). The Norway spruce (*Picea abies* L. Karst.) wood material originated from a single ca 70 year aged individual tree from Jämijärvi, Finland, felled in May 2016. Oven-dried (60°C, 3 days) spruce wood sawdust was sieved (2 mm mesh size metal sieve) and a 10 g portion was weighted into each 250 mL conical glass flask sealed with a cellulose stopper and metal cap. After autoclaving (121°C, 15 min), the sawdust was moistened with 10 mL of sterile MilliQ-quality water. Moisture content in the wood substrate was maintained during the cultivations by addition of sterile water once a week.

The fungal cultures on spruce wood were initiated by introducing two 5 mm x 5 mm sized mycelial plugs from the pre-cultivated MEA plates into each cultivation flask. The flasks were incubated in the dark at room temperature (20–23°C) for one, two and three months, to follow fungal interactions at the initiation of early stages of wood decay. All cultivations and fungal combinations were performed with four biological replicates (four parallel culture flasks). VOC emissions in the headspace of the culture flasks were measured at the same time points with collecting samples for the enzyme assays. After sample taking, the culture solids (mycelia and spruce sawdust) were stored at −20°C in 50 mL of 25 mM sodium acetate buffer (pH 5.5) for further analyses.

Analyses on released volatile organic compounds (VOCs)

To analyze the VOC production rates of fungal cultures during the decay process, we collected samples from the headspace of the culture flasks (Bäck *et al.* 2010) by analyzing 10 cultures simultaneously for 90 min at room temperature. Flasks were flushed for 20 min with filtered (active carbon trap and MnO_2-coated copper net) ambient air (each flask: 100 mL min^{-1}) in order to remove VOCs and oxidants from the flask headspace before sampling. Samples were collected from the ingoing air and the outflow gas of each culture flask into stainless steel tubes filled with Tenax-TA and Carbopack-B adsorbent (Bäck *et al.* 2010) using a flow rate on 100 mL min^{-1}. The sample tubes were desorbed in the laboratory with a thermodesorption instrument (Perkin-Elmer TurboMatrix 650, Waltham, USA) and analyzed with a gas-chromatograph (Perkin-Elmer Clarus 600, Waltham, USA) connected with a mass selective detector (Perkin-Elmer Clarus 600T, Waltham, USA), using five point calibration with standards in methanol (Aaltonen *et al.* 2011).

Extraction of secreted proteins and oxalic acid

In order to assess fungal activities in the wood sawdust cultures, extracellular proteins were extracted from the solids, stored at −20°C, with 80 mL (in total) of 25 mM sodium acetate buffer, pH 5.5, including 0.2 mM phenylmethane-sulphonyl fluoride (PMSF, Sigma) as protease activity inhibitor, by agitation (200 rpm, 4 h) at 4°C. Amount of extracellular protein was determined by the Bradford assay with bovine serum albumin as reference according to the instructions of the manufacturer (Bio Rad, USA) on 96-well plates recorded with Infinite M200 plate reader (Tecan, Austria). The supernatants were collected by filtering through glass fiber filters (GF-C, Whatman, USA) with vacuum suction and stored at −20°C. The wood culture solids were mixed with 50 mL of 1.5 M HCl and incubated in ultrasound sonication water bath (60°C, 30 min) to release the fungal produced oxalic acid attached to wood solids. After re-filtering through glass fiber filters, the acid extractions were stored at −20°C. The remaining solids were dried overnight at 80°C to estimate the total dry weight of each wood culture (as the sum of the dry mass of wood sawdust and fungal mycelium).

Enzyme activity assays

Activities of the CAZy glycoside hydrolases and auxiliary oxidoreductases were measured from the supernatants of three parallel biological replica flask cultures by using 96-well plastic plates and Infinite M200 spectrophotometer plate reader operated by Magellan software version 7.1 SP1 (Tecan, Austria). Activities of β-glucosidase, endo-β-1,4-glucanase, laccase and manganese peroxidase (MnP) were determined according to the previously adopted assays (Rytioja *et al.* 2014a; Kuuskeri *et al.* 2015). Hydroxyethyl cellulose (Fluka, USA) 1% (w V^{-1}) was used as substrate for endo-β-1,4-glucanase and 1 mM 4-nitrophenyl b-D-glucopyranoside (Merck, Germany) for β-glucosidase activity, respectively. ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), Sigma, USA) was adopted as substrate for laccase activity at pH 4.5, and formation of the radical product was followed at 420 nm, whereas MnP activity at pH 4.5 was determined at 270 nm as oxidation of Mn^{2+} to Mn^{3+} malonate chelates (Hildén *et al.* 2005; Kuuskeri *et al.* 2015). Chitinase and protease activities were determined in the 96-well plate scale by using Victor3 spectrofluorometer (Perkin-Elmer), and MU-conjugated acetylglucosaminide (364 nm excitation, 450 nm emission; Sigma-Aldrich, USA) and albumin-fluorescein iso-thiocyanate conjugate (485 nm excitation, 520 nm emission; Sigma-Aldrich, USA) as substrates, respectively (Shah, Mali and Lundell 2018). For Fe^{3+} reduction capacity assay, 20 mM $FeCl_3$ and 1% (w V^{-1}) ferrozine (3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid monosodium salt hydrate; Sigma-Aldrich, USA) were adopted (Shah, Mali and Lundell 2018). Enzyme activity values were calculated as previously described (Mali *et al.* 2017; Shah, Mali and Lundell 2018) and reported as

(enzyme) activity per g dry weight (of solid culture substrate wood + mycelium) or per μg of ergosterol (produced by hyphae, see below).

Ergosterol analysis

To estimate fungal growth in the solid wood cultures, ergosterol extraction and quantitation analysis was applied. The wood culture solids as well as uninoculated wood control samples were ground under liquid nitrogen using an A 11 basic Analytical mill (IKA). For ergosterol analysis, 0.5 g of wood powder was extracted in a glass tube using cyclohexane (Niemenmaa, Uusi-Rauva and Hatakka 2006). Dried extracts were dissolved with 250 μL of HPLC grade methanol and filtered through 0.2 μm membrane filters (Chromacol RC, Thermo Scientific, Germany) prior to analysis by HPLC (Agilent 1100, Agilent Technologies) using a Luna C18 column (Phenomenex). Methanol (HPLC grade) was used as eluent at 1 mL min^{-1} and separated ergosterol was detected by following elution chromatogram absorbance at 282 nm (Niemenmaa, Uusi-Rauva and Hatakka 2006). Analytical grade ergosterol (Sigma Aldrich, USA) was dissolved in HPLC grade methanol in a set of μg amounts and run for reference to allow quantitation. For calculations, 0.5 g portion of the milled sample was dried at 80°C for 6 h to estimate the total dry weight of the solids. The ergosterol content was calculated as μg of ergosterol per g dry weight (wood + mycelium).

Oxalic acid and released sugar analyses

The quantities of fungal produced oxalic acid and chelated oxalate anions in the wood cultures were analyzed directly from the liquid-phase samples, and from the acid-extracts by reverse-phase elution using UHPLC equipped with UV-VIS diode-array detector (Mattila, Kuuskeri and Lundell 2017). For concentrations of water-phase dissolved sugars, the quantities of glucose, xylose and cellobiose were analyzed by ion-exchange elution using HPLC equipped with UV-VIS diode-array and refraction index detections (Mattila *et al.* 2018). The samples were thawed and filtered (0.2 μm pore size GHP Acrodisc 13 membrane filters, Gelman) before analysis. Oxalic acid and released sugar concentrations are reported as mmol or μmol of each compound detected per g dry weight of the culture solids (wood + mycelium), or per μg of ergosterol analyzed.

Total carbon and nitrogen measurement

From the milled solids of the wood cultures, a portion was air dried at 60°C for overnight, and the total carbon and nitrogen contents were analyzed by using a Vario Max Elemental analyzer (Elementar Analysensysteme, Germany). From the protein extraction liquid samples, total carbon, total organic carbon and total nitrogen contents were measured with a Total Organic Carbon Analyzer TOC-V CPH (Shimadzu, Japan). Amount of inorganic carbon in the liquid phase was acquired by subtracting the amount of organic carbon from the amount of total carbon in the liquid phase of each culture, acquired with the total organic carbon analyzer.

Statistical analyses

Paired *t*-test was used to determine the significance of changes in enzyme activity values, concentration of secreted oxalic acid, and amount of total carbon and nitrogen adopting the IBM SPSS Statistics 24 software package (IBM Corporation). Boxplot figures were generated adopting SPSS, whereas figure plots of the values of enzyme activities, iron reduction, oxalic acid secretion, wood weight loss and liquid total C concentration were generated with OriginPro 2018 (OriginLab Corporation). For non-metric multidimensional scaling (NMDS) analysis of the data, the ordinations of Bray–Curtis distances were calculated ($n = 100$) with vegan (Oksanen *et al.* 2018) package and visualized by ggplot2 (Wickham 2016) in R software 3.5.1 (R Development Core Team 2016). For all analyses, values of four parallel biological replicate cultures and control samples were adopted.

RESULTS

Oxidoreductase activities

Activities of the lignin-modifying oxidoreductases (manganese peroxidase and laccase) were observed only for single-species cultures of the white rot fungi (Pr and Ta) and in their two-species co-culture (TaPr). Laccase activity was detected in the white rot single cultures at the first time point (after four weeks of cultivation) (Fig. 1A). Lower levels of laccase activity were observed in the white-rot fungal co-culture of PrTa also at later time points. Manganese peroxidase activity was observed on the second and third month of growth on wood in the white-rot cultures, with the highest activity levels measured in the single-species culture of Pr (Fig. 1B). In the two-species culture of TaPr, manganese peroxidase activity was observed already at the first time point, that is after four weeks of growth on spruce wood.

β-Glucosidase and endoglucanase

All fungal co-cultures demonstrated similar pattern of cellulose-decomposition connected β-glucosidase production with an increasing activity pattern during the three-month cultivation time (Fig. 1C). In the single-species culture of Fp or Pr, β-glucosidase activity increased more rapidly than in their co-cultures during the first two months. In contrast to Fp and Pr, the single-culture of Ta, however, showed the lowest activities of β-glucosidase.

The highest β-glucosidase activities were observed after three months of growth on wood by the two-species brown rot—white rot co-cultures including Fp (FpTa and FpPr) (Fig. 1C), indicating strong promotive effect of fungal interactions for β-glucosidase production. When enzyme activity values were normalized against the concentration of ergosterol—reflecting the amount of fungal mycelial biomass generated in the wood cultures—the white rot fungus Pr demonstrated the most efficient production of β-glucosidase (Fig. S3C, Supporting Information). In the co-cultures including the brown rot fungus Fp, the highest level of β-glucosidase activity (nkat (g dw)$^{-1}$) was detected after three months of growth (Fig. 1C), which however, turned to indicate low mycelial efficiency in enzyme production when the activity values were normalized against ergosterol quantities (nkat (μg ergosterol)$^{-1}$) (Fig. S3C, Supporting Information). Regarding the other cellulolytic activity determined—endoglucanase—higher enzyme activity levels were obtained than for β-glucosidase starting from the first sampling time point (Fig. 1D). However, high deviation between the three biological replicates (three parallel culture flasks) does not allow more detailed comparison of the activities generated by the single-species or co-cultures.

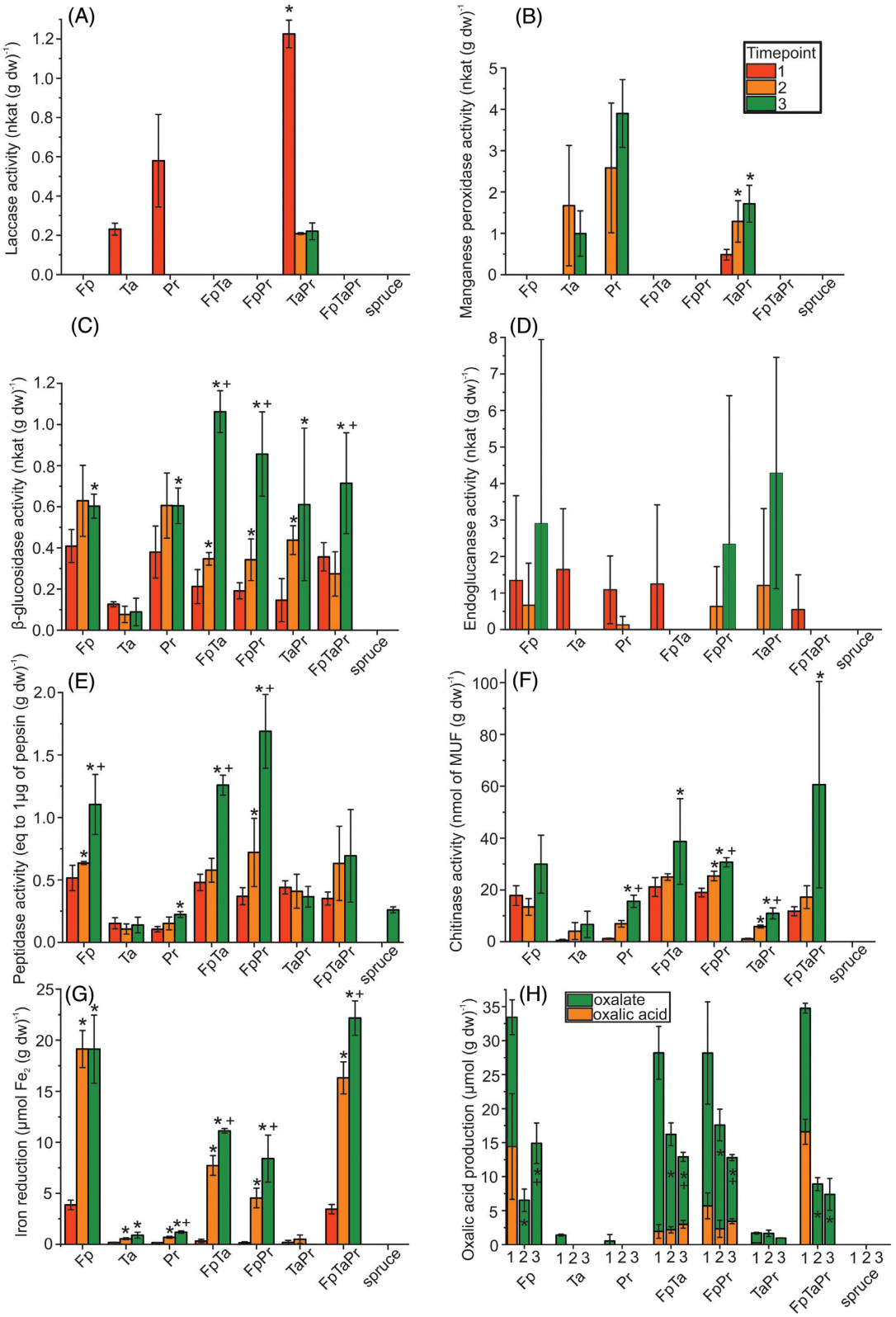

Figure 1. (A) Laccase, (B) manganese peroxidase, (C) β-glucosidase, (D) endoglucanase, (E) peptidase, (F) chitinase activities, (G) iron reduction and (H) concentration of accumulated oxalic acid in the fungal cultures on spruce wood calculated in relation to total dry weight of the culture solids at each time point (cultivation months 1, 2, 3). Mean average values ($n = 3$, three parallel cultures) with standard deviation are presented at each time point. $*$ = time point 2 or 3 differs significantly ($P \leq 0.01$) from time point 1, $+$ = time point 3 differs significantly ($P \leq 0.01$) from time point 2. Fungal species abbreviations, see Table 1.

Peptidase and chitinase activities produced on wood

The highest values of extracellular acidic protein-degrading (peptidase) activity produced on spruce wood substrate was observed in the single-species cultures, and in the two-species co-cultures including the brown rot fungus Fp (combinations FpTa and FpPr), especially in the end of cultivation after three months (Fig. 1E). When the results were normalized against ergosterol quantities, the white rot two-species combination TaPr showed the highest efficiency in the production of acidic protease activity (Fig. S3E, Supporting Information).

Similar pattern was observed with fungal chitinase activities (Fig. 1F), and the highest values were detected in the presence of the brown rot fungus Fp (cultures of Fp, FpPr, FpTa, FpTaPr). However, after normalizing the activity values against ergosterol content in the cultures, the white rot fungus Pr was the most efficient in secretion of chitinase in comparison to the production of fungal biomass.

Iron reduction and production of oxalic acid

Highest values of iron reduction were observed in the Fp brown rot single-species culture as well as in the three-species co-culture of FpTaPr (Fig. 1G). In contrast, the white rot fungal co-culture TaPr as well as the single-species cultures Ta and Pr demonstrated the lowest activities in reduction of Fe^{3+} ions. After normalizing the values with ergosterol content in the cultures, the same trend was obvious: the highest efficiency in iron reduction was attained by the brown rot fungus Fp (Fig. 3G, Supporting Information).

The most prominent production of extracellular oxalic acid was also detected in the single-species brown rot culture Fp (up to 35 μmol (g dw)$^{-1}$), whereas the two white rot species Pr and Ta demonstrated scarcely detectable concentrations of extracellular oxalic acid (Fig. 1H). The highest concentration of oxalic acid/oxalate was measured in the three-species co-culture of FpTaPr (up to 35 μg g^{-1} dry weight of wood), followed by the two-species co-cultures of FpTa and FpPr.

Notable is that the highest concentrations of free oxalic acid produced by the brown rot fungus Fp were detected after the first four weeks of cultivation (first month of growth), with values then declining until the end of the cultivation (Fig. 1H). This is partially explained by the findings obtained after extracting the wood culture solids with strong mineral acid (HCl); the acid extraction released additional high concentrations of oxalic acid, which were apparently bound to wood lignocellulloses as dissociated oxalate anions (Fig. 1H). However, after summing the mass of the Fp secreted free oxalic acid and bound oxalate, it may be concluded that a substantial portion (5/6) of oxalic acid accumulating during the first month of fungal growth has disappeared both from the liquid phase and the solid wood substrate. The disappearance may be explained by extensive Fenton reactions consuming oxalate (see Discussion).

VOC profiles

Of the array of released VOCs, four identified compounds (6-methyl-5-heptene-2-one, methyl 3-furoate, terpinolene, α-humulene) were apparently emitted in the cultures in relation to the fungal decay type (either brown rot or white rot) and according to the duration of cultivation. Altogether, over 50 emitted VOCs were identifiable in the fungal cultures on spruce wood (Table S1, Supporting Information). Three of the four identified signature VOCs showed positive correlation (R^2 values between 0.66 and 0.76) apparent with distinct fungal activities (Fig. 2). Increasing concentrations of released dissolved glucose correlated with accumulation of the sesquiterpene compound α-humulene (Fig. 2A), whereas the brown rot characteristic ability for iron reduction demonstrated strong positive correlation with accumulation of the monoterpene compound terpinolene (δ-terpinene) (Fig. 2B). In turn, total amount of fungal secreted proteins detected in the cultures after two and three months of cultivation correlated significantly with accumulation of the volatile compound methyl 3-furoate (methyl furan-3-carboxylate) (Fig. 2C).

The OVOC (oxidized VOC) 6-methyl-5-heptene-2-one was recognized primarily after one month of fungal growth, at the first time point, with the most intense release in the brown rot Fp single-species and co-culture of FpTa (Table S1, Supporting Information). Time-dependent release of another OVOC, one of the signature compounds—methyl 3-furoate—was emitted primarily during the later growth and decay phases (after months 2 and 3), and in significant amounts in the white rot fungal cultures (Ta, Pr, TaPr).

In the white rot cultures, more secreted proteins were produced, which is also seen in the positive correlation between the OVOC methyl 3-furoate (Fig. 2C). Release of the two OVOC compounds demonstrate clear specificity of compound release according to the fungal decay type and growth in wood (decomposition trait affects to specificity of VOC release), which is also subjected to the advancement of wood decomposition. The monoterpene terpinolene and sesquiterpene α-humulene were emitted in large quantities only in the cultures including the brown rot fungus Fp, with proportional increase in quantity over time among total emission of monoterpenes and sesquiterpenes, respectively (Table S1, Supporting Information).

Secreted proteins, ergosterol and nitrogen analyses in the cultures

The amount of ergosterol increased significantly in all fungal cultures during the cultivation period of three months, except in the single-species culture of the white rot fungus Pr (Fig. 3A), thus signifying smaller amount of hyphae generated in spruce wood by this fungus. According to ergosterol content, the brown rot fungus Fp was the most prominent in hyphal growth and biomass production on spruce wood, also in the co-cultures. Ergosterol contents in the cultures correlated positively with the total dry weight loss of the wood culture solids (Pearson coefficient = 0.83).

On the contrary to fungal biomass production estimated by ergosterol quantities, amounts of extracellular proteins produced in the cultures were the highest in the white rot fungal cultures, both in single-species and co-cultures of Pr and Ta (Fig. 3B). Noticeable is that in the presence of the brown rot species Fp, remarkably lower amounts of extracellular proteins were detected. After the first month of growth, the protein levels stayed even under the detection limit in the cultures of Fp and FpTaPr.

Concentrations of extractable and water dissolved total nitrogen were the highest in the brown rot fungus Fp including cultures (Fig. 3C), thus following similar time-evolving pattern as was observed with ergosterol quantities (Fig. 3A). In the white rot fungal cultures, half of the levels of total dissolved nitrogen were reached as compared to the brown rot fungus Fp including cultures (Fig. 3C). Water-dissolved total nitrogen concentrations demonstrated positive correlation (Pearson

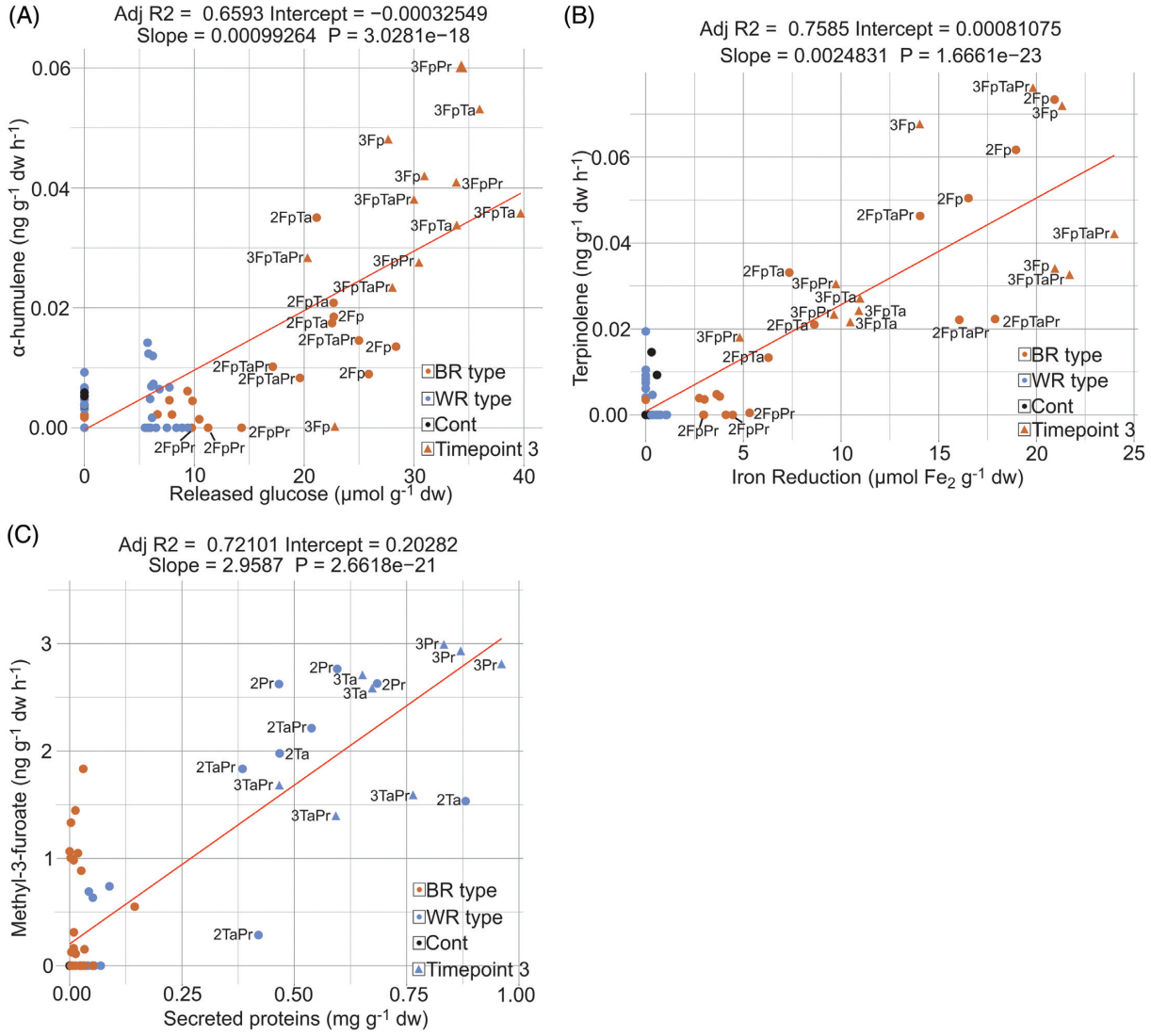

Figure 2. Correlation between fungal activities and three signature VOCs detected in the headspace of the spruce wood—fungal culture flasks: (A) α-humulene and released dissolved glucose, (B) terpinolene and iron reduction capacity and (C) methyl 3-furoate and total amount of secreted proteins. In the panels, the cultivation time points at months 2 (spheres) and 3 (triangles) are depicted. Fungal species abbreviations, see Table 1.

correlation coefficient $r = 0.9$) with total dry mass loss of the wood cultures, thereby indicating the influence of fungal activities and decomposition of the solid substrate (spruce wood sawdust).

Wood decomposition and carbon distribution

Total dry weight of the solid spruce wood substrate apparently decreased during the three-month period of cultivation most prominently in the brown rot fungus Fp dominated single and co-cultures (Fig. 4A) signifying the rapid and efficient destruction of wood carbohydrates by the brown rot decay mechanism. Most rapid mass loss occurred during the second month of Fp brown rot fungal growth (time point 2). In the brown rot–white rot co-cultures (FpTa and FpPr), the rapid decline of wood dry weight occurred later, during the third cultivation month, indicating of fungal hyphal interactions causing some delay in the growth and physiological activities of Fp.

In intact spruce wood, the total carbon (total C) content was determined as approximately 50% (Fig. 4B). Percentual relative amount of total carbon (as the sum of carbon in the solid particles and water-dissolved C in the buffer extracts versus wood dry weight) in the cultures seemed to increase within three months of fungal growth (Fig. 4B). The brown rot–white rot co-culture of FpPr showed the highest relative carbon content (51.6%) at the end of the cultivation.

After the first month of fungal growth, dissolved carbon compounds accumulated in all fungal cultures (Fig. 4C). Noticeable is the fast rate of decomposition of wood to soluble C compounds in the single-species cultures of the brown rot fungus Fp during the first two months of cultivation. In the co-culture of FpTa, the most rapid release of dissolved C compounds occurred during the second month of growth but later in the co-culture of FpPr. With summing the solid and water-dissolved C (Fig. 4C), it is estimated that even up to 20% of the initial solid organic C of spruce wood was converted to gaseous C compounds, including inorganic CO_2 generated by the fungi upon growth on wood, and as wood-released VOCs. The highest proportions of generated volatile C were generated in the brown rot Fp fungal cultures.

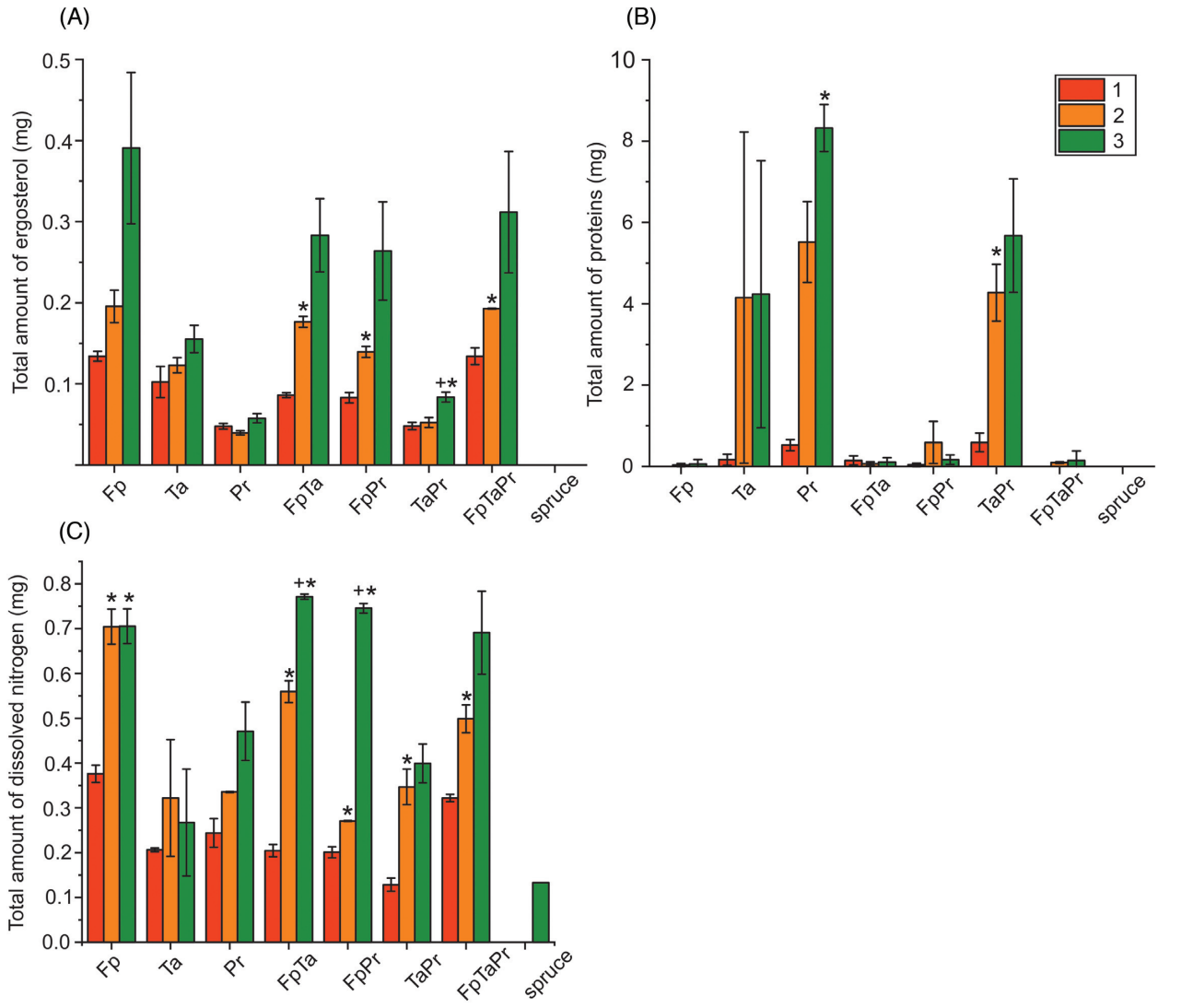

Figure 3. Total amount of (A) ergosterol, (B) secreted proteins and (C) dissolved nitrogen measured in the culture extracts during three months, at each time point 1, 2 and 3 months of fungal growth on spruce wood. Mean average values ($n = 3$, three parallel cultures) with standard deviation are presented at each time point. $*$ = time point 2 or 3 differs significantly ($P \leq 0.01$) from time point 1, $+$ = time point 3 differs significantly ($P \leq 0.01$) from time point 2. Fungal species abbreviations, see Table 1.

Of the dissolved C compounds, concentration of glucose increased in the fungal cultures throughout the cultivation period, with the highest level of accumulation of glucose observed in the single-species and co-cultures with the brown rot fungus Fp (Fig. S2A, Supporting Information). Concentration of dissolved xylose, however, showed a different pattern, and in the white rot fungal cultures of Ta and Pr, concentrations of dissolved xylose decreased signifying active uptake of the pentose sugars by fungal cells. Concentrations of water-dissolved inorganic carbon demonstrated significant increase in all fungal co-cultures as well as in the single-species culture of Fp (Fig. S2B, Supporting Information). The inorganic dissolved carbon concentrations in the fungal cultures demonstrated positive correlation with Fe^{3+} reduction capacity (Pearson coefficient = 0.8).

Fungal activities, released signature VOCs and wood decay

NMDS analysis of the combination of all data from the three cultivation time points on spruce wood reveals assembling of the fungal single-species and co-cultures according to their wood decay types, either brown rot dominated (Fp present) or white rot (Pr, Ta single-species and co-cultures) (Fig. 5). Cultivation time affected the grouping with the tendency of (Fig. S1, Supporting Information), and in relation to fungal growth time, i.e. duration of the cultivation (Fig. 5). Interestingly, the two-species brown rot–white rot fungal co-cultures of FpTa and FpPr located closer to the white rot type characteristics than to the three-species co-culture of FpTaPr at the first time point, after one month of growth on spruce wood.

Most of the ordinates of the later time points (cultivation months 2 and 3) assembled the fungal co-cultures together with the brown rot Fp features. This indicates the high dominance of Fp in spruce wood decomposition, and its brown rot type of decay mechanism and biochemical events in the brown rot–white rot fungal co-cultures under these experimental conditions. However, three of the measured variables (manganese peroxidase enzyme activity, production of secreted proteins, accumulation of the OVOC methyl 3-furoate) noticeably separated the white rot fungal cultures (Ta, Pr, TaPr) from the

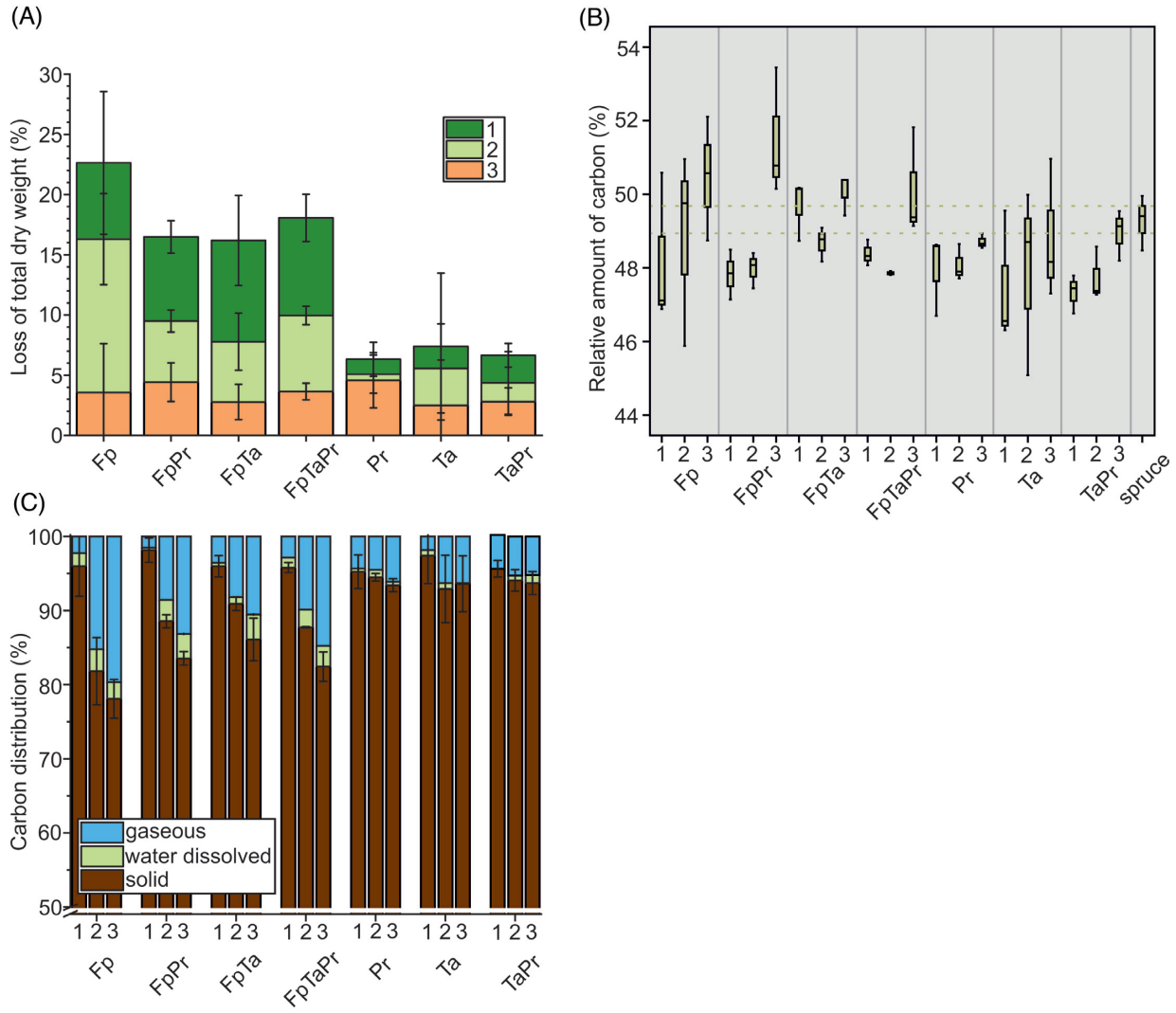

Figure 4. (A) Percent mass loss from three different time points of the spruce wood–fungal cultures, (B) relative content of total carbon (sum of C in the solid particles and water-dissolved C in the buffer extracts) and (C) carbon distribution (at time points 1, 2 and 3 months). Mean average values ($n = 3$, three parallel cultures) with standard deviation are presented at each time point. Fungal species abbreviations, see Table 1.

Fp dominated co-cultures. The NMDS analysis undoubtedly visualizes the clear distinction of fungal physiological decay type and mechanism, either brown rot or white rot, as the directing force for wood decomposition and subsequent release of signature VOCs. Fungal growth and species-association affecting changes were distinguishable at each cultivation time point (NMDS ordination of the variables individually for 1, 2 and 3 months of fungal growth on spruce wood sawdust; Fig. S1, Supporting Information).

DISCUSSION

In this study, interactions of three wood-decaying fungi, one brown rot species (*F. pinicola*) and two white rot species (*P. radiata* and *T. abietinum*) were studied, in two and three-species combinations on Norway spruce sawdust as the solid wood substrate for a cultivation period of three months.

Based on wood mass loss and accumulation of released water-dissolved glucose upon fungal growth, the brown rot fungus *F. pinicola* showed the greatest impact on spruce wood decomposition and degradation efficiency both in single-species and species combination cultures. Our findings point to the rapid growth in wood and primarily non-enzymatic oxidative, Fenton chemistry involving action of *F. pinicola* (Shah, Mali and Lundell 2018) in efficient decomposition of the wood lignocellulose carbohydrates.

In addition, the white rot type of wood decay observed in cultures of *P. radiata* and *T. abietinum* as well as in their combinatory culture was distinguishable from the *F. pinicola* brown rot decay especially in the patterns of released VOCs, and production of extracellular proteins and enzyme activities on spruce wood. As previously noticed, the lignin-attacking oxidoreductase (manganese peroxidase and laccase) activities were only produced by the white rot species (Mali *et al.* 2017). In our current study, positive correlation of the amount of secreted proteins including lignin-attacking enzyme activities, and release of the volatile compound methyl-3-furoate (see below) points to a specific white rot mechanism for decomposition of coniferous wood, here Norway spruce. Especially *P. radiata* is an efficient colonizer of dead wood, including spruce, causing an oxidative enzyme burst in the early colonization and decay stage followed by secretion of a large array of CAZymes and visual thinning

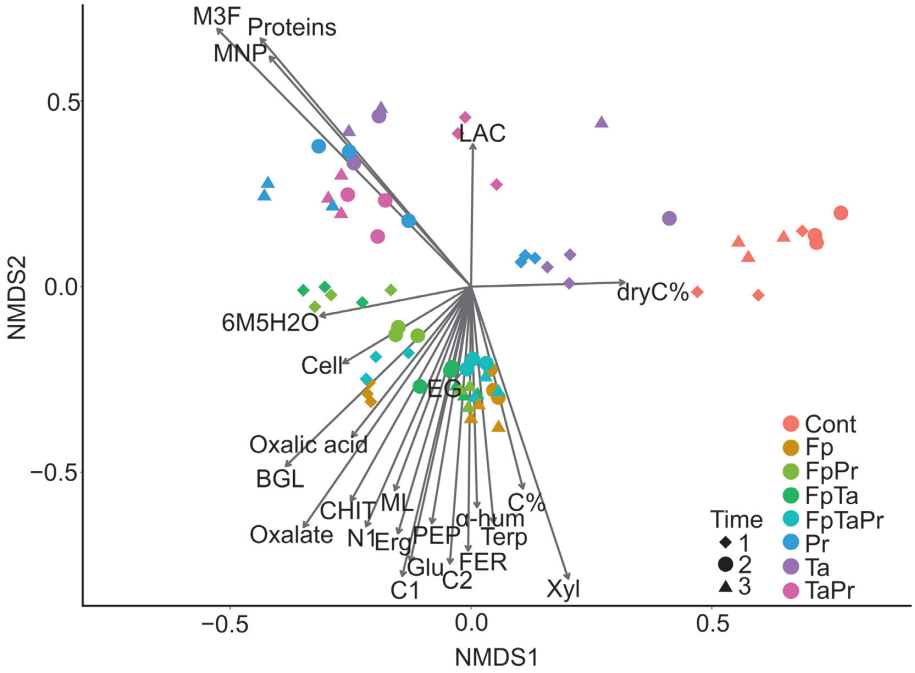

Figure 5. NMDS ordination analysis of all measured variables per total dry weight of the solids of the fungal cultures from the three sample-taking time points (cultivation time months 1, 2 and 3). The variables are as follows: (M3F = methyl 3-furoate, MNP = manganese peroxidase, LAC = laccase, Terp = terpenolene, α-hum = α-humulene, EG = endo-β-1,4-glucanase, FER = iron reduction, C1 = dissolved carbon, C2 = inorganic dissolved carbon, Glu = glucose, PEP = peptidase, Erg = ergosterol, Xyl = xylose, N1 = dissolved nitrogen, CHIT = chitinase, ML = mass loss, BGL = β-glucosidase, Cell = cellobiose, 6M5H2O = 6-methyl-5-heptene-2-one). Cont, intact spruce wood sawdust. The stress of the ordination was 0.1463745. Fungal species abbreviations, see Table 1.

of the wood cell walls (Kuuskeri *et al.* 2016). During six weeks, spruce wood lignin units were already modified by *P. radiata* (Kuuskeri *et al.* 2016). Thus, it is suggested that emergence of the specific VOC methyl-3-furoate is a consequence of wood lignin modification activity.

Considering the significant role of wood-decaying fungi in decomposition of the recalcitrant organic carbon biopolymers in forest ecosystems (Eastwood *et al.* 2011; Floudas *et al.* 2012; Lundell *et al.* 2014; Baldrian 2017), only a few studies have so far been performed with focus on the effect of development of fungal bioconversion and wood decay processes on the diversity and changes in the array of VOCs released from dead wood as substrate (Korpi, Pasanen and Viitanen 1998; Ewen *et al.* 2004; Konuma *et al.* 2015; El Ariebi *et al.* 2016). This is partially due to the difficulties in deviating fungal-biodegradation generated VOCs from endogenously produced fungal MVOCs (microbial VOCs), which range from short-chained alcohols and ketones to terpenes and aromatic secondary metabolic compounds (Gross *et al.* 1989; Ewen *et al.* 2004; Korpi, Järnberg and Pasanen 2009; Hung, Lee and Bennett 2015; Lemfack *et al.* 2018). In fact, a wide array of MVOCs are generated by filamentous fungi as common metabolites and side-products through their biosynthetic pathways (Korpi, Järnberg and Pasanen 2009).

Keeping this in mind, we tried to address the role of wood-decaying brown rot and white rot fungi as VOC producers, both releasing VOCs from their woody substrate as well as synthesizing endogenous MVOCs. Fungal produced VOCs are apparently important signalling molecules upon hyphal species-species (interspecies) interactions (Hynes *et al.* 2007; Ditengou *et al.* 2015), and fungal volatiles may as well affect physiology of other microorganisms and plants in various ecosystems (Hung, Lee and Bennett 2015). Of the four signature VOCs (monoterpene terpinolene, sesquiterpene α-humulene, furan OVOC methyl 3-furoate and a sesquiterpene derived OVOC compound 6-methyl-5-hepetenone), α-humulene was previously identified as the most abundant sesquiterpene emitted by Scots pine root associated *Ascomycota* (Bäck *et al.* 2010). Together with an array of terpenoids, methyl furoates (predicted as linoleic acid oxidation derived products) were identified in another study as MVOC products in beech-wood single-species cultures of a brown rot fungus (*Fomitopsis palustris*) and a white rot fungus (*Trametes versicolor*) (Konuma *et al.* 2015).

In agreement with our findings, majority of the produced volatiles of two wood-inhabiting *Basidiomycota Agaricomycetes* species (*Resinicium bicolor* and *Hypholoma fasciculare*) were recognized as sesquiterpenes upon hyphal interactions (Hynes *et al.* 2007), also when the fungi were cultivated on beech wood (El Ariebi *et al.* 2016). In the latter study, and in analogy with our results, both fungal hyphal combinations (interactions) and growth time affected VOC emission profiling. However, fungal interaction signature compounds were not identified. Despite the technical difficulties in distinguishing emitted fungal VOCs from the VOC flux from decaying wood addressed in previous studies, in this study we were able to identify four volatiles signifying specific fungal interactions, and decay stages.

Of the signature VOCs, emission of the monoterpene terpinolene correlated positively with iron reduction capacity demonstrated by the brown rot fungus *F. pinicola*, which is in line with the advancing growth and decomposition of spruce wood caused by the fungus. In parallel, the sesquiterpene α-humulene may be depicted as potentially brown rot decay signifying VOC upon fungal interactions. This was noticed by the positive correlation of increasing α-humulene emissions with accumulation of released glucose signifying efficient decomposition of

cellulose. However, this correlation could also be a consequence of more vigorous hyphal growth of *F. pinicola* (as measured by accumulation of ergosterol) on spruce sawdust.

Contrary to the terpenoid compounds, positive correlation of the amount of secreted proteins and increasing emissions of methyl 3-furoate (OVOC derivative of methyl 3-furan) in the white rot fungal (*P. radiata* and *T. abietinum*) single-species and two-species cultures on spruce wood was noticed in our study. Methyl furoates have been identified as MVOCs in fungal cultivations on Japanese beech wood (Konuma *et al.* 2015), but no clear relation with any of the compounds with either brown rot of white rot decay type could be recognized. In our study, NMDS orientation analysis moreover visualized the correspondence of methyl 3-furoate with white rot decay type, especially pointing the relation with fungal-produced manganese peroxidase activity, which is indicative of a strong oxidoreductive attack on lignin in wood (Hatakka and Hammel 2010; Floudas *et al.* 2012; Lundell *et al.* 2014; Kuuskeri *et al.* 2016). Thus, we were able to distinguish a few signature MVOCs to pinpoint the fungal decay types (either brown rot or white rot) on spruce wood, correlating with fungal activities and wood decomposition efficiency.

By measuring wood-decomposition enzyme activities, including CAZymes acting on wood cellulose and hemicelluloses, and lignin-modifiying oxidoreductases (Lundell *et al.* 2014; Rytioja *et al.* 2014b), we were able to observe distinctive physiological differences in the dynamic fungal communities for the cultivation period of three months. In our previous study on semi-solid wood cultivations including the same three fungal species and isolates, it was noticed that the dominant fungus in co-cultures resulted with the strongest clustering effect based on production of enzyme activities and oxalic acid (Mali *et al.* 2017). In our current study, similar biological processes and enzyme production patterns upon fungal interactions on solid wood substrate were noticed. Taken together, these two studies imply that fungal physiological responses upon interactions on natural substrates are conserved features, and connected to substrate decomposition trait and efficiency.

Notable is that the brown rot fungus *F. pinicola* had a limited capacity for protein secretion and CAZy enzyme activities in comparison to the protein (and diverse enzyme) secretion efficiency observed with the white rot species (*P. radiata* and *T. abietinum*). Our results are in accordance with a recent comparative brown rot–white rot fungal secretome-proteome study on wood (Presley *et al.* 2018). In accordance to our observation of production of xylanase activity by *F. pinicola* on spruce wood, hemicellulase activities and CAZYme secretion were likewise reported for the *Polyporales* brown rot species *Postia (Rhodonia) placenta* (Zhang *et al.* 2016; Presley *et al.* 2018) as well as the taxonomically more divergent brown rot species (*Serpula lacrymans* and *Gloeophyllum trabeum*), pointing to a two-step mechanism for brown rot decomposition of wood (Presley and Schilling 2017). In this mechanism, the initial strong oxidative phase upon wood colonization includes expression of oxidoreductases, and is followed by a later phase including hyphal secretion of hydrolytic CAZymes against wood polysaccharides. Recently, the importance of culture conditions on enzyme expression was also reported for *F. pinicola* (Wu *et al.* 2018).

Together with production of enzyme activities, the importance of non-enzymatic oxidative processes for the brown rot type of wood decay is well recognized (Xu and Goodell 2001; Shah, Mali and Lundell 2018). Fenton chemistry apparently is the degradative mechanism against lignocelluloses and wood polysaccharides adopted by the *Agaricomycetes* brown rot fungi (Eastwood *et al.* 2011; Floudas *et al.* 2012; Presley and Schilling 2017). Fenton chemistry requires acidic conditions, and is driven by cycling reduction of Fe^{3+} ions to Fe^{2+} in order to produce reactive oxygen species, mainly hydroxyl radicals (Xu and Goodell 2001). Oxidoreductase enzyme expression is connected to the early phase colonization of wood by brown rot fungi (Presley and Schilling 2017; Presley *et al.* 2018; Wu *et al.* 2018) and may thereby support generation of Fenton reactions. Furthermore, reduction of Fe^{3+} is an indication of on-going Fenton chemistry (Blanchette 1988; Eriksson, Blanchette and Ander 1990; Shah *et al.* 2013; Shah, Mali and Lundell 2018).

Accordingly, iron reduction was observed in the spruce wood cultures dominated by the brown rot fungus *F. pinicola*. Furthermore, iron reduction correlated positively with emission of the terpinolene VOC together with production of oxalic acid in high concentrations. The ability to secrete oxalic acid is characteristic to brown rot fungi with accumulating concentrations and thereby, increasing acidity of the substrate and growth medium (Mäkelä *et al.* 2002; Mali *et al.* 2017). It is suggested that by generating an oxalic acid diffusion gradient in the solid wood substrate, the brown rot fungus is regulating Fenton chemistry zones leading to protection of the hyphae from destruction by the Fenton-generated reactive oxygen species such as hydroxyl radicals (Schilling and Jellison 2005; Zhang *et al.* 2016; Presley *et al.* 2018). Extracellular oxalic acid is bound to cellulose in its anionic form as oxalate, which furthermore regulates the pH gradient and subsequently promotes wood cell wall destruction (Shimada *et al.* 1997; Xu and Goodell 2001; Schilling and Jellison 2005).

In our study, most of the brown rot fungal secreted oxalic acid in the cultures of *F. pinicola* was in fact detected in its anionic form—as oxalate chelates—attached to the solid wood lignocellulose during the last months of cultivation, whereas in the brown rot—white rot co-cultures (FpTa and FpPr), water soluble oxalic acid was observed throughout the cultivation period. These findings indicate the importance of oxalic acid as an additional regulator in the brown rot—white rot hyphal interactions.

Fungal growth and metabolism on wood upon decay processes include both respiration and fermentation (microaerophilic to anaerobic regions in decaying wood), leading to the release of substantial amounts of CO_2 (Mattila, Kuuskeri and Lundell 2017; Mattila *et al.* 2018). In our current study, most of the CO_2 production was estimated to occur during the second cultivation month in the cultures including *F. pinicola* coinciding with the most rapid mass loss of spruce wood, both signifying the efficient and destructive brown rot decay. Another sign of efficient decomposition of wood is the presence of water-dissolved sugars; glucose mainly from cellulose, and pentoses like xylose from hemicellulose. Accumulation of released dissolved glucose is a clear indication of fungal decomposition of wood cellulose and hemicellulose components (Mattila, Kuuskeri and Lundell 2017). Our finding of the relative increase in carbon content in spruce wood is explained by the rapid mass loss (biodegradation of solid wood). A large study conducted in temperate forests showed that carbon concentration in dead spruce wood increases as the wood decay proceeds (Köster *et al.* 2015).

CONCLUSIONS

Our findings indicate the impact and functional significance of saprotrophic fungal species-species interactions on their growth and biodegradative actions against wood as their substrate and living environment. Moreover, fungal biodegradation mechanisms and wood-decay traits (here brown rot versus white rot

decay types) have an influence on the profiles of VOCs released from wood upon fungal decomposition and advancing decay. On Norway spruce wood as growth substrate, emission of three of the four signature VOCs correlated with the fungal decay type. The multitude of compounds released from decaying dead wood and woody debris need more detailed studies for better understanding of the impact of fungal decomposition on emitted VOCs in boreal forest ecosystems. Evidently, the dominant fungal species—likewise the brown rot fungus *F. pinicola* in our study—in hyphal interactions is acting as the driving force in advancement of wood decay. Our results pinpoint the dominance of brown rot decay type in rapid decomposition of spruce wood cellulose leading to the release of excess glucose, which may enhance co-existence in the same habitat (decaying dead wood) of other microbes and fungi with less robust wood decay mechanism. However, white rot fungi like *P. radiata* demonstrate tolerance and ability to produce an effective set of lignin-active oxidoreductases even in the presence of the brown rot fungus *F. pinicola*.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSEC online.

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Conflict of interest. None declared.

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