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Effect of fungal pretreatment of wood on successional decay by several inky cap mushroom species

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ABSTRACT

To explore the effect of fungal pretreatments on secondary inky cap decay, field degraded red maple sapwood blocks were exposed to Coprinoid fungi in pure culture decay tests. No significant decay in field degraded blocks was produced by the *Coprinopsis* or *Coprinus* isolates tested, but most *Coprinellus* isolates produced additional degradation in wood blocks previously decayed by 4–10 years of field exposure, with the greatest weight losses observed in blocks subjected to 10 years of field decay. The brown rot *Gloeophyllum trabeum* and white rot *Trametes versicolor* were used to pre-treat freshly cut red maple and poplar blocks to explore the effect of laboratory fungal pretreatment on subsequent inky cap decay. Brown rot pretreatment significantly increased subsequent decay of both wood types by most of the inky cap isolates tested. White rot pretreatments did not affect subsequent decay by *Coprinopsis* or *Coprinus* isolates but significantly increased decay by the *Coprinellus radians* isolates tested. Inky caps, particularly *Coprinellus* species, are characterized as late stage decay fungi known to degrade wood that has been chemically and physically modified by other degradative processes.

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1. Introduction

Inky caps are a mushroom forming group of fungi once entirely contained in the family Coprinaceae and the genus *Coprinus*. Phylogenetic data now suggests that some members belong to the family Psathyrellaceae. The genus *Coprinus*, recognized to be polyphyletic, has been divided into four unique genera; *Coprinus*, *Coprinellus*, *Coprinopsis* and *Parasola* (Hopple and Vilgalys 1994; Redhead et al. 2001). Traditionally, the inky caps have been known as the basidiomycetes most commonly found on dung (Richardson 2001) and prevalent in soils rich in organic matter. Observational reports show many inky cap fungi also grow on forest debris such as fallen woody materials and leaf litter (Rayner and Boddy 1988; Heilmann-Clausen and Boddy 2005). Limited studies addressing inky cap decay physiology were completed by Fries in the mid-1950's (1955, 1956), yet little is known today about the wood decay mechanisms and degradative ecology of these fungi. The early work of Fries (1955) and more recent physiological investigations (Oliver 2008) suggest that the inky caps growing on woody materials are different species than those growing on soil and dung. These differences are likely reflected in the recent phylogenetic reorganization of this group.

Through field observations of wood decay, Peiris et al. (2008) recognized that some species of inky caps are able to decay wood in late stages of decomposition and subsequently hypothesized that these fungi will colonize and efficiently decay only highly modified woody substrates. Heilmann-Clausen and Boddy (2005) proposed that the growth of inky caps on wood is limited by natural competition within the diverse community of decay fungi. As weak competitors, the inky caps are relegated to late stage colonization of woody substrates depleted in available sugars. Investigations of fungal succession are limited however as the field of fungal ecology is still in its infancy.

Late stage colonizers are of particular interest as they play a crucial role in the transformation of wood to soil. After a review of published manuscripts Pregitzer and Euskirchen (2004) estimated the mean pool of coarse woody debris in the temperate forest at 42.0 (±45.8, SD) $MgCha^{-1}$, comprising roughly 18% of the ecosystem's carbon pool. Fungi are responsible for recycling the bulk of this material, which maintains soil volume and preserves forest health. Because it is difficult to characterize wood in the later stages of decay, the chemistry of degraded wood and its specific effect on ecological systems is poorly understood.

This research focused on the effects of field and laboratory decay pretreatments on inky cap fungi colonization and degradation of woody substrates in an effort to characterize the role of inky cap fungi in decay sequences. The selected experimental design excluded competition to facilitate study of the substrate effects on

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growth. Experimental results from this physiology study could support recent phylogenetic changes to the inky cap group, enhance our understanding of coprinoid ecology and fungal succession and may serve to focus future investigations on the development of innovative biorefining technologies.

2. Experimental methods

2.1. Test isolates

Coprinopsis cinereus FGSC 9003 was obtained from the Fungal Genetics Stock Center at the University of Missouri. The USDA-FS Forest Products Laboratory in Madison, WI supplied isolates *Coprinopsis atramentarius* FP-101910-T, *Coprinus comatus* FP-101592-T and FP-101691-T, *Coprinellus micaceus* FP-101781-T and ME-798, *Coprinellus radians* ME-352 and ME-209 and *Trametes versicolor* MAD-697. *C. comatus* ATCC 12640, *Coprinellus micaceus* ATCC 20122, *Gloeophyllum trabeum* ATCC 11539 and *Phanerochaete chrysosporium* ATCC 24725 were purchased from the American Type Culture Collection (Manassas, VA). Cultures were maintained on potato dextrose agar (PDA) (Difco™) at 15 °C.

2.2. Genetic identification

Isolate identifications were confirmed in our laboratory by amplifying the non-coding rDNA region using the primer pairs ITS_1-F and ITS_4-B and PCR techniques. Amplified regions were sequenced and checked by BLAST searching sequences previously uploaded in the NCBI-Genbank.

2.3. Soil block jar decay tests

Soil block jar decay tests were modified from the AWPA E10 Standard (AWPA 2006). One cup of moist substrate (1:1:1- potting mix: peat moss: vermiculite) was lightly packed into a clean wide mouth pint Mason Jar™. Two 1/3 pieces of birch tongue depressors were laid side by side on top of the substrate as feeder strips for the fungi. An inverted dome lid and ring were used to loosely cap the jar, which was covered with foil and steam sterilized at 121 °C for 1 h. Sterilization was repeated 24 h later. After sterilization the feeder strips were inoculated with 4–1 cm diameter plugs of mycelium taken from the actively growing edge of a 2 week old Petri dish culture incubated at 25 °C. Feeder strips were colonized for 2 weeks before wood samples were added. Wood blocks were oven dried (24–48 h at 90 °C), weighed, sterilized (30 min at 121 °C), added aseptically to the jars and incubated at 25 °C for specific time periods. Following incubation the blocks were harvested, oven dried and post-decay weight recorded. The mass loss (weight difference) in the blocks is attributed to fungal decay.

2.4. Degradation of field decayed blocks

Nineteen millimeter (19 mm) cubed sapwood blocks were cut from archived red maple bole samples representing 0, 2, 4, 6, 8 and 10 years of field decay under the canopy in the Penobscot Experimental Forest in Bradley, ME. After determining dry weight, blocks were steam sterilized in an autoclave at 121 °C for 30 min. and aseptically placed into modified soil block jar decay tests (AWPA 2006) inoculated with inky cap isolates. Three replicates per fungal isolate were used for each field decay period. After 8 weeks of incubation at 25 °C, blocks were harvested, oven dried, and weighed to calculate percent weight loss.

2.5. Laboratory pretreatment: inky cap growth on pre-decayed hardwood

Soil block jar decay assays were inoculated with the brown rot fungus *G. trabeum* ATCC 11539 or the white rot fungus *T. versicolor* MAD-697 as previously described. Non-inoculated controls were also prepared. Red maple and poplar wood blocks (19 mm^3) were oven dried, weighed and steam sterilized before aseptic placement in the jars. After 8 weeks of decay the blocks were harvested, oven dried, weighed and steam sterilized (121 °C for 30 min). The original soil block jars were sterilized following harvest and when cool inoculated with the inky cap fungi to simulate successional decay. Four replicates of each isolate were used for treatments and controls. After 8 weeks of decay the blocks were harvested, dried as above, and weighed to determine mass loss.

2.6. Laboratory pretreatment: inky cap response to lengthening pre-decay

Soil block jar pretreatments of pine and maple wood blocks by *G. trabeum* ATCC 11539 and *P. chrysosporium* ATCC 24725 were conducted for 2, 4, 8 and 16 weeks. The pre-treated blocks were subsequently exposed to *C. micaceus* and *C. radians* to measure the effects of brown and white rot treatments on inky cap decay.

2.7. HPLC wood sugar analysis

The field decayed blocks and blocks pretreated with brown rot/white rot were analyzed for wood sugars using a Shimadzu HPLC with a RID 10A refractive index detector (Marlborough, MA) fitted with a Bio-RAD Aminex HPX-87P (lead ion) column (Hercules, CA) heated to 80 °C, using deionized distilled water as a mobile phase, a 20 μl injection volume and a flow rate of 0.6 ml min^{-1}. Prior to HPLC analysis, blocks were ground in a Wiley mill through a 40-mesh screen. Wood powders were hydrolyzed with 4% H_2SO_4 to depolymerize polysaccharide oligomers to monomers, then neutralized with calcium hydroxide, and filtered through a Whatman GD/X 0.2 μm PES filter prior to the analysis.

2.8. Analysis of nitrogen

Nitrogen content was measured in field decayed blocks by dry combustion at 1050–1350 °C in an oxygen atmosphere. Released gases carried by helium were cleaned of oxygen via passage through steel wool, and moisture was removed using a condenser. A copper catalyst bound residual oxygen and converted nitrous oxide to N_2 gas. CO_2 was scrubbed from the combustion gas and N_2, was measured by thermal conductivity and reported based on sample weight, atmospheric pressure, and calibrated standards. Analyses were performed by the Soil Analytical Laboratory at the University of Maine (Orono, Maine).

2.9. Statistics

The statistical program R 2.5.1 was used to analyze results with controls compared to treatments by Analysis of Variance (ANOVA). Tukey HSD post-hoc analysis was used for multiple comparisons of means. All error bars are standard deviation of the mean. A significance value of $P = 0.05$ was used for all analyses.

3. Results

3.1. Genetic identification

The NCBI-BLAST search results from the amplified ITS regions of each isolate matched cultural identification with the exception of

isolate *C. micaceus* ATCC 20122. BLAST results for this isolate varied at the species level, with 97% congruency with *C. flocculosus*, 92% congruency with *C. radians*, and 91% shared base pairs with *C. micaceus.*

3.2. Degradation of field decayed blocks

Of the isolates tested, only the *C. radians* isolates caused substantial weight loss in the field-decayed red maple blocks with significant losses following field decay periods of 4 years or more, and yielding the largest percentage weight losses in blocks decayed in the field for 10 years (Fig. 1).

HPLC analysis of the wood sugars in the field decayed blocks prior to treatment with inky cap isolates detected extractable glucose and xylose levels of ~3 mg/ml and ~1.5 mg/ml respectively for all decay periods, including the un-decayed controls. Other free wood sugars were not detected (HPLC data not shown). Total % nitrogen in the field-decayed wood was measured and although values increased with the length of decay (from 0.063 ± 0.003 at time zero to 0.109 ± 0.054 after ten years), these increases were not statistically significant.

3.3. Laboratory pretreatment: inky cap growth on pre-decayed hardwood

The pretreatments produced average % weight losses of 36.14 (±6.45) in red maple and 41.12 (±6.13) in poplar (*G. trabeum*), and 16.12 (±4.17) in red maple and 17.64 (±5.68) in poplar (*T. versicolor*).

For both hardwoods, the inky caps produced significantly greater weight losses following 8 weeks of pretreatment with the brown rot fungus as compared to losses following the white rot treatment. On poplar, all inky cap isolates produced greater average weight losses following pre-treatment by the brown rot *G. trabeum* as compared to the untreated controls. These were losses significantly different from losses in the controls except in blocks decayed by *C. comatus* FP-101592-T, and *C. micaceus* isolates FP-101781-T and ATCC 20122. Significant weight losses following a *T. versicolor* pretreatment were observed only with *C. radians* isolates (Fig. 2).

With red maple, a greater number of inky cap isolates achieved significant weight losses following brown rot pretreatment, with all isolates except *C. micaceus* ATCC 20122 producing greater losses following the *G. trabeum* pretreatment as compared to losses in the controls. As observed in the poplar assays, the *C. radians* isolates were the only fungi to significantly decay red maple following *T. versicolor* pretreatments (Fig. 3).

3.4. Laboratory pretreatment: inky cap response to lengthening pre-decay

Longer pretreatments tended to increase successional decay by *Coprinellus* isolates, particularly with pine blocks pretreated with *G. trabeum*, as seen in Fig. 4.

4. Discussion and conclusions

This study of successional decay of field degraded red maple blocks and red maple, poplar and pine blocks pre-treated in the laboratory by a brown and white rot fungus supports the hypothesis of Peiris et al. (2008) that the chemistry of partially degraded woody substrates affects successional colonization and can facilitate decay by inky cap fungi.

While the *Coprinus* and *Coprinopsis* isolates tested marginally degraded red maple blocks exposed to resident microbial communities in field test plots for up to ten years, *Coprinellus* isolates, with the exception of *C. micaceus* ATCC 20122, decayed the field-degraded red maple to varying degrees, producing average secondary weight losses of 2–24%.

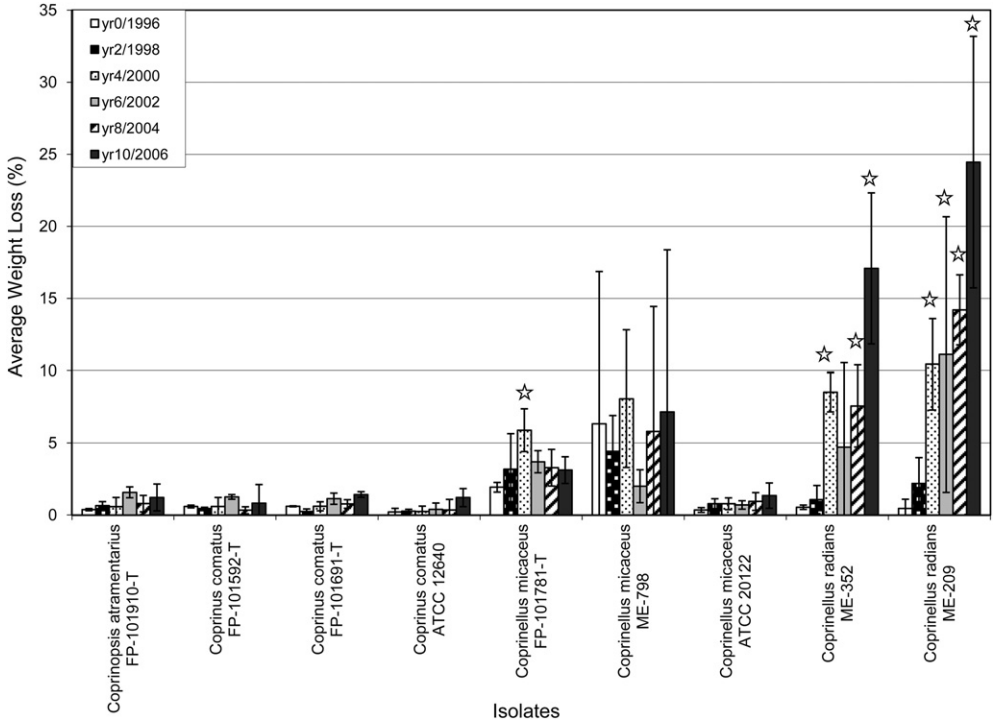

Fig. 1. Weight losses in field decayed red maple sapwood blocks degraded by inky caps. Sapwood blocks represent 0 to 10 years of field decay. Collection years are noted in the figure key. Stars (☆) denote significantly greater weight loss as compared to time zero blocks.

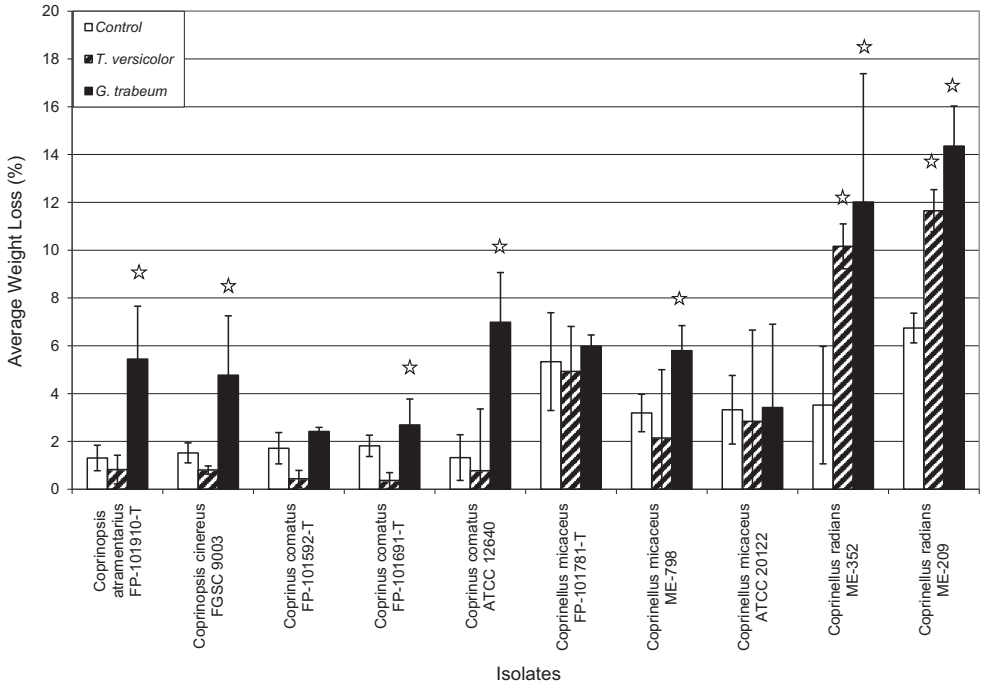

Fig. 2. Average weight losses following inky cap decay of poplar blocks pretreated with the fungi *G. trabeum* (GT) or *T. versicolor* (TV). Stars (☆) denote significantly greater weight loss compared to the control.

Secondary weight losses in blocks decayed by *C. micaceus* ATCC 20122 were substantially less than losses produced by other isolates of the same species. When considered with discrepancies in the GenBank BLAST search results for the amplified ITS region of this isolate, these weight losses suggest that the culture had been misidentified in the ATCC collection. The physiological responses of all other isolates tested in this study support recent taxonomic changes and assignment of new Coprinoid genera. Data such as that collected in this study is critical to better understanding fungal biodiversity and will be used to support or refute taxonomic reorganization.

Laboratory pretreatments applied to maple and poplar blocks for 8 weeks supported increased inky cap decay with brown rotted wood, facilitating the greatest inky cap weight losses. Particularly, *C. radians* isolates ME-352 and ME-209 were capable of secondary

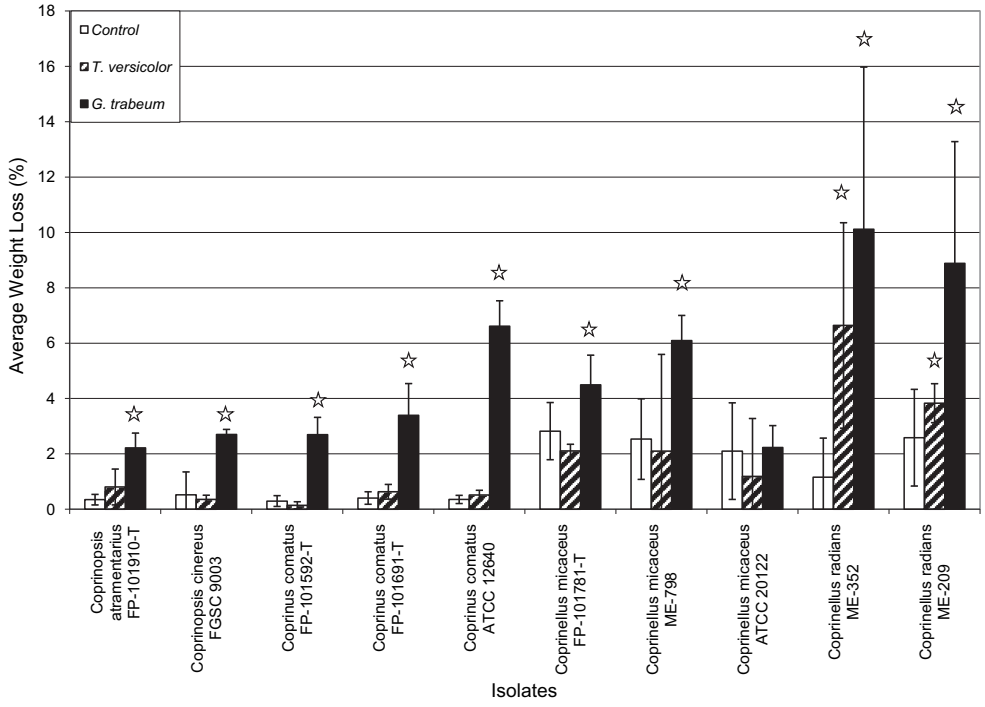

Fig. 3. Average weight losses following inky cap decay of red maple wood blocks pretreated with the fungi *G. trabeum* (GT) or *T. versicolor* (TV). Stars (☆) denote significantly greater weight loss compared to the control.

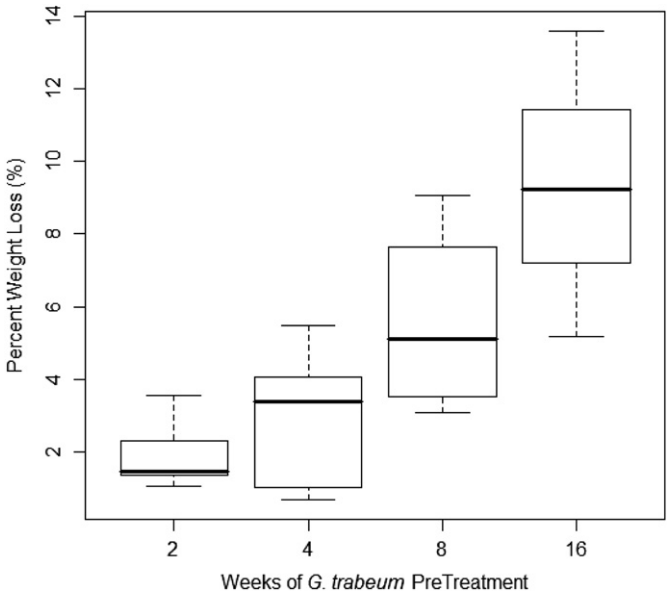

Fig. 4. Additional weight losses associated with *C. micaceus* FP-101781 decay of pine wood blocks following 2, 4, 8 and 16 weeks of *Gloeophyllum trabeum* pretreatment.

decay. In laboratory decay tests using the same parameters as this study and an assortment of sound woods, the same *C. radians* isolates failed to achieve weight losses equal to those recorded in this study (Oliver 2008). When variable length *G. trabeum* and *P. chrysosporium* pretreatments were applied, *Coprinellus* decay was positively correlated with increased length of pretreatment, particularly on pine decayed by the brown rot *G. trabeum*. These findings further support the hypothesis that some inky caps colonize only previously degraded woody substrates (Heilmann-Clausen and Boddy 2005; Peiris et al. 2008).

The HPLC sugar analysis conducted as part of this study are difficult to interpret but it is likely that increased mass losses following successional colonization by inky caps are related to greater weight losses achieved during pre-treatment. *G. trabeum* pretreatment yielded initial weight losses comparable to losses reported previously by Richter et al. (2005) and Shi et al. (2007) and approximately 20% greater than the losses in blocks pre-treated with *T. versicolor* (data not shown), which were less than previously reported (Celimene et al. 1999; Schirp and Wolcott 2005). A brown rot fungus degrading hardwoods to a greater extent than a white rot fungus is not normally reported. Typically, white rot fungi predominately attack hardwoods (Schmidt 2006) while brown rot fungi attack softwoods (Goodell 2003).

The brown rot *G. trabeum* typically degrades hemicellulose and cellulose with minimal demethylation and oxidation of the lignin matrix. Hemicellulose glucomannans are removed preferentially to xylan which is removed preferentially to cellulose, with complete depolymerization of the holocellulose before the degradative products are utilized (Filley et al. 2002). The free radical mechanisms employed by *G. trabeum* facilitate wood degradation at a distance from the invading hyphae where liberated wood saccharides cannot be absorbed (Goodell et al. 1997; Daniel 2003).

In white rot, wood degradation occurs primarily via enzymatic systems. As the lignin matrix undergoes side chain cleavage, demethoxylation, demethylation, and mineralization, the holocellulose is metabolized primarily by exo-enzymes. Liberated monosaccharides regulate wood polysaccharide enzyme expression ensuring that the holocellulose is repeatedly depolymerized to meet metabolic demands and support continued enzyme production.

Fries (1955) hypothesized that inky caps prefer simple sugars, such as the monomers and oligomers of partially degraded polysaccharides, but not absorbed, by brown rot fungi. This hypothesis may explain the significant weight losses observed in wood pretreated with *G. trabeum* and subsequently colonized and decayed by inky caps that scavenge carbohydrates late in decay when competition is reduced. Brown rot residues and late stage field degraded woody materials tend to be rich in lignin and saccharide depleted.

The inky caps, as efficient peroxidase producers (Anh et al. 2007), are known to degrade lignin. Perhaps these fungi, particularly the *Coprinellus* species, have evolved to thrive in substrates that limit other competition. Future work should focus on characterizing fully the role of inky caps in progressive decay. Evidence that some inky cap fungi can decompose wood will facilitate characterization of their role in larger decay systems and generate interest in their degradative capabilities.

Understanding lignin chemistry and the natural processes that degrade woody matrices will become increasingly important as we seek to access wood saccharides for biofuel conversion and develop innovative applications for lignin residues without disturbing the health and sustainability of our forests and forest soils.

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