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Wood decay by *Chlorociboria aeruginascens* (Nyl.) Kanouse (Helotiales, Leotiaceae) and associated basidiomycete fungi

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ABSTRACT

Two isolates of *Chlorociboria aeruginascens* (Nyl.) Kanouse incubated axenically on aspen wood blocks resulted in 18% and 32% mass loss after 134 wks (2 yrs 8 mo). Aspen wood decayed by *C. aeruginascens* contained cavities in the S2 layer of the secondary cell wall, similar to Type I soft rot attack, as well as erosion troughs and overall thinning and erosion of wood cell walls, resembling decay by Type II soft rot fungi. Using a selective medium, basidiomycete fungi were isolated from 9 of 17 samples of wood from a variety of tree species colonized and stained by *Chlorociboria* sp. (putatively *C. aeruginascens*) in a mixed hardwood forest from northern North America. Basidiomycete fungi were identified by ITS sequencing and cultural tests and were shown to be predominantly species of common white rot fungi. In axenic decay tests with aspen blocks, basidiomycete isolates caused mass losses in the range of 10%–50% after 26 wks incubation. Decay tests in which a basidiomycete isolate was co-inoculated with an isolate of *C. aeruginascens* did not differ significantly ($p < 0.05$) in mass loss from those caused by the respective basidiomycete alone. Results suggest that decay of wood colonized by *C. aeruginascens* is principally caused by white rot fungi and is neither enhanced nor inhibited by the presence of *C. aeruginascens*.

1. Introduction

The ascomycete fungus *Chlorociboria aeruginascens* (Nyl.) Kanouse (Helotiales, Leotiaceae) is common in north temperate forests worldwide and produces intense green stain in dead and decayed wood (Ramamurthi et al., 1957; Dixon, 1975). Another closely related species, *Chlorociboria aeruginosa* (Pers. per Pers.: Fr.) Seaver ex Ram., Korf and Bat., is more southern in distribution (Dixon, 1975) and is considered somewhat rare (Breitenbach and Kränzlin, 1984). The fruiting bodies and green stain caused by *C. aeruginascens* are often found on and in wood in an advanced state of decay (pers. obs.). Although *C. aeruginascens* can be grown in culture, the extent that it can decay wood itself is not known. The fungus has been suspected of causing a soft rot (Blanchette et al., 1992), as this decay type is caused by several other ascomycete wood decay fungi (Nilsson et al., 1989). Blanchette et al. (1992) observed *Chlorociboria*-stained *Populus* wood degraded in the manner of Type 2 soft rot, however only field-collected samples were examined and degradation could have been caused by other fungi present. Although wood that is colonized by *Chlorociboria* sp. commonly has the stringy texture of white rot decay, this may be the result of cohabiting basidiomycete fungi since fruiting bodies of the latter are often found on wood that is stained green by *Chlorociboria* sp. (pers. obs.). Blanchette et al. (1992) also noted the presence of white rot fungal hyphae coexisting in wood cells of *Chlorociboria*-stained *Populus* wood.

Benomyl® (methyl [1-[(butylamino)carbonyl]-1H-benzimidazol-2-yl]carbamate) (Dupont) is a fungicide, no longer commercially available, that inhibits growth of ascomycetes and their mitosporic anamorphs but allows the growth of basidiomycete fungi (Edgington et al., 1971). When used in small amounts (e.g., 2–10 ppm) as a medium amendment in agar, benomyl® allows the growth of basidiomycete fungi from wood that is also colonized by *C. aeruginascens*. In this way the basidiomycete decay fungi that cohabit with *C. aeruginascens* in wood may be investigated. In this study, basidiomycete fungi were isolated from 17 samples of *Chlorociboria*-stained and partially decayed wood of various species using a benomyl®-amended culture medium. The isolated basidiomycete decay fungi were then tested for the ability to decay wood alone and in combination with an isolate of *C. aeruginascens* from

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aspen wood. Two isolates of *C. aeruginascens* were also used in axenic decay tests and to assess the type of decay using light microscopy.

2. Materials and methods

2.1. Isolation of fungi

Seventeen decayed wood samples from a variety of tree species presumed colonized by *Chlorociboria* sp. due to the deep green stain present were collected for fungal isolation (four sugar maple (*Acer saccharum*), seven quaking aspen (*Populus tremuloides*), three yellow birch (*Betula alleghaniensis*), one paper birch (*Betula papyrifera*), one *Amelanchier* sp. and one black spruce (*Picea mariana*) (Table 1). Fresh interior decayed wood was exposed using a knife or similar tool. Small pieces of green-stained wood (2–4 mm) were aseptically removed with a forceps or scalpel and plated onto 2% (w/v) malt extract (Difco) agar (1.5% w/v) (MEA) amended with 10 ppm benomyl® (Dupont) and 200 ppm streptomycin sulfate (Sigma). Pieces of green-stained wood were also plated onto unamended MEA to isolate *Chlorociboria* sp. Cultures were incubated at 22–24° C for 10–14 days and examined for fungal growth. To confirm that a fungus isolate was a basidiomycete, hyphae were examined microscopically (400X) for the presence of clamp connections; fungi lacking clamp connections but with hyphal and mat characters typical of basidiomycete fungi (lacking conidia, slow growth, compact, colorless or white mat) (Nakasone, 1990) were also retained. Isolates were tested with gum guiac reagent to indicate the presence of white rot decay enzymes (Marr, 1979).

In addition to the basidiomycetes isolated from green-stained wood, isolates of *C. aeruginascens* were obtained for wood decay testing: isolate ATCC 24028 (American Type Culture Collection, Beltsville, MD) isolated from stained wood (species not specified) by J.R. Dixon (date unknown) from New York; and isolate DR-468 isolated from decayed aspen (*P. tremuloides* Michx.), 29 July 2007, Houghton Co., Michigan by D. L. Richter. The latter isolate was identified as *C. aeruginascens* (Nyl.) Kan. ex Ram., Korf & Bat. subsp. *aeruginascens* based on the presence of fruiting bodies occurring on the stained wood bearing asci 40–60 μm long, with ascospores 6–8 x 1–2 μm (Dixon, 1975).

2.2. DNA sequencing and identification

Pure cultures of basidiomycete fungi were transferred to 2% malt agar slants and delivered to the Center for Forest Mycology Research in Madison, Wisconsin for DNA sequencing and identification, and where they are archived. DNA was extracted from fungal cultures using the technique of Lindner and Banik (2009) modified for use with 200 μL strip tubes as described by Lorch et al. (2013). The extracted DNA was amplified using the fungal specific primer pair ITS1F/ITS4 following the technique of Lindner and Banik (2009). Fungal identifications were based on the nearest BLAST match in GenBank using similarities of ≥97% to denote species identification. Similarities of less than 90% were tentatively assigned to higher taxa (order or family) that provided the best match.

2.3. Wood decay testing

Wood decay tests were conducted using AWPA E–10, *Standard Method of Testing Wood Preservatives by Laboratory Soil-Block Cultures* (AWPA, 2008). Briefly, 100 g ± 1 g dried (50 °C) forest topsoil (pH 5.8) was added to a square flint jar (5 × 5 × 13.5 cm); 30 ml ± 1 ml of distilled water was added to bring soil to approximately 90–100% water holding capacity; a plastic lid with a 5 mm diameter hole covered by a strip of adhesive first-aid tape was placed on the jar to allow respiration; an aspen strip (*P. tremuloides* Michx.) 0.5 × 3.2 × 2.0 cm was placed on top of the soil prior to autoclaving (120 °C); jars were autoclaved for 30 min.

Fungal inocula were grown on MEA in 100 mm Petri dishes for 1–2 wks at 22–24 °C, at which time basidiomycete isolates covered the dish; *Chlorociboria* isolates were incubated 4–6 wks to cover the culture dish surface. After cooling, a piece of fungus-colonized agar approx. 1 × 2 × 0.5 cm was placed on the aspen strip, followed by an oven-dried (40 °C, 24 h), pre-weighed, aspen sapwood block (19 mm^3) which had been sterilized by autoclaving (120 °C). For the co-inoculated jars, pieces of inocula were laid side by side on the feeder strip and the aspen block placed on top. Lids were replaced tightly and jars were incubated for 26 wks at 20 °C ± 1 °C, 80% ± 4% RH. Five replicate blocks were set up for each basidiomycete fungus alone or co-inoculated test (N = 5).

For aspen blocks inoculated with isolates of *C. aeruginascens* alone, the procedure was the same except that 15 jars were set up for each isolate so that five blocks could be harvested at 52, 67, and 134 wks. As a comparison to placing blocks on top of the aspen strip, one set of five blocks inoculated with *C. aeruginascens* DR 468 was buried in the soil. In addition, five aspen blocks were buried in soil in uninoculated jars to determine mass loss due to leaching of extractives or other compounds (sterile controls). The latter two sets of blocks were removed at 67 wks.

Upon removal of blocks from jars, fungi were reisolated from one representative block per replicate set to confirm viability of the

Table 1
Fungal isolations from decayed wood samples colonized by *Chlorociboria* sp. Isolations were made on 2% malt extract agar (MEA) for non-basidiomycetes and MEA amended with 10 ppm benomyl® for basidiomycetes.

Wood species	2% MEA	2% MEA with benomyl®	Gum guiac[a]	Genbank accession number
Populus tremuloides	*Penicillium* sp.	Unidentified	+	
	unidentified black mold	Cantharellales		KP768316
Populus tremuloides	*Trichoderma* sp.	*Plicaturopsis crispa*	–	KP768310
	unidentified black mold			
Populus tremuloides	*Trichoderma* sp.	*Peniophorella praetermissa*	+	KP768311
	unidentified black mold			
Betula papyrifera	*Trichoderma* sp.	*Bjerkandera adusta*	+	KP768312
		Peniophorella praetermissa	+	KP768313
Populus tremuloides	*Trichoderma* sp.	Unidentified Cantharellales	+	KP768317
	Penicillium sp.			
Amelanchier sp.	Unidentified	*Gloeocystidiellum porosum*	+	KP768314
	molds (2)			
Picea mariana	Zygomycetes	*Peniophorella pubera*	+	KP768315

+ = positive for oxidase.
[a] Gum guiac drop test.

inoculated fungus. From decayed and/or stained wood a small portion (1–2 mm) was aseptically excised from the block and plated on MEA and incubated and examined as previously described. Blocks were oven-dried (40 °C, 24 h) and weighed, and mean percent mass loss was calculated for each replicate set of blocks. Means for axenic and paired culture of basidiomycetes and *Chlorociboria* sp. were compared with a paired sample t-test ($p < 0.05$). Wood exposed to *C. aeruginascens* was thin-sectioned on the transverse plane and examined at 1000X by light microscopy to examine cell wall degradation.

3. Results

From the 17 samples of decayed wood with deep green stain presumed to be caused by *Chlorociboria aeruginascens*, nine samples yielded basidiomycete fungal isolates on MEA amended with benomyl® (Table 1). One sample of paper birch yielded two different basidiomycete isolates. All samples of decayed wood yielded a range of hyphomycetes and zygomycetes that predominated on MEA lacking benomyl®; only two samples of decayed wood yielded a culture of *Chlorociboria* (one from sugar maple and one from quaking aspen) indicating the difficulty of isolating *Chlorociboria* due likely to the presence of faster-growing mold fungi in the decayed wood and possible competition from the basidiomycete decay fungi present. However, no basidiomycetes were isolated from the two samples from which *Chlorociboria* sp. was isolated. Benomyl® did not inhibit the growth of zygomycete fungi, which may have prevented the isolation of some basidiomycete fungi. Two of the basidiomycete isolates failed to survive after several transfers to fresh media and could not be studied further. The remaining eight basidiomycete isolates were subjected to DNA sequencing of the ITS region for possible identification (Table 1). Six of the basidiomycete isolates were identified to species with a 98–99% match with ITS sequence in Genbank, while two isolates were identified to order. Basidiomycetes identified to species are common white rot fungi in north temperate forests. This was consistent with isolates giving a positive reaction to gum guiac indicating the presence of white rot decay enzymes. Only one basidiomycete isolate gave a negative reaction to gum guiac (Table 1).

After 26 weeks of incubation, basidiomycete isolates from *Chlorociboria*-stained wood were able to decay aspen blocks in soil block bottle tests, resulting in mass losses from mean 9% (Isolate 01) to 49% (Isolate 05) (Fig. 1). The lowest mass loss (9%) was caused by unidentified Cantharellales isolate 01, isolated from aspen wood, while the greatest mass loss (48.7%) was caused by isolate 05, *Peniophorella praetermissa*, also isolated from aspen wood. There were no significant differences between means of mass loss caused by a basidiomycete alone compared with the wood blocks co-inoculated with *C. aeruginascens* DR 468 ($p < 0.05$).

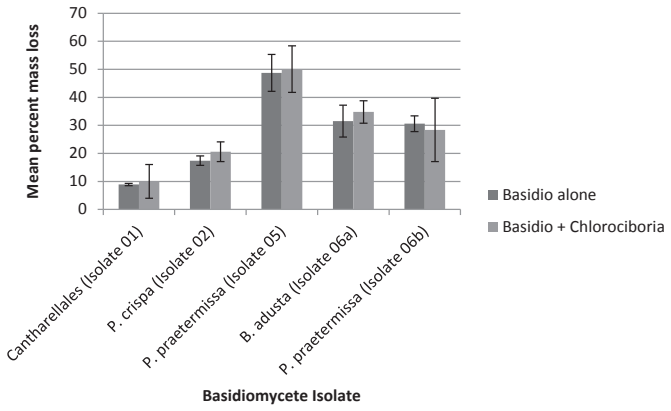

Fig. 1. Decay of aspen blocks incubated after 26 wks, 20 °C with five basidiomycete fungi isolated from *Chlorociboria*-stained wood. Isolates were incubated alone or co-inoculated with *C. aeruginascens* DR 468. No significant differences occurred in mass loss of blocks incubated with the basidiomycete alone or incubated with *C. aeruginascens* ($p < 0.05$).

The basidiomycete isolates were successfully reisolated from decayed blocks inoculated alone and co-inoculated with *C. aeruginascens,* indicating they were still viable in the blocks after 26 wks of incubation. However, *C. aeruginascens* was not reisolated from any of the representative blocks in the co-inoculated jars. All co-inoculated blocks showed minimal green staining in the aspen wood where the block was placed on the inoculum, except for the blocks inoculated with *Bjerkandra adusta* (isolate 06a) which had no green stain. Blocks inoculated with basidiomycete isolate 01 (unidentified Cantharellales) and *C. aeruginascens* showed the greatest amount of green stain in the aspen wood. In general it appeared that the basidiomycete isolates inhibited the growth of *C. aeruginascens* when compared with blocks inoculated with *C. aeruginascens* alone.

After 52 wks, 67 wks, and 134 wks *C. aeruginascens* ATCC 24028 caused mean percent mass losses to aspen blocks of 4.6%, 7.0% and 18.2%, respectively (Fig. 2.). At these intervals *C. aeruginascens* DR 468 resulted in mean percent mass losses of 14%, 10% and 32% respectively (Table 1). Aspen blocks that were set up as sterile controls after 67 wks underwent no mass loss. The set of aspen blocks that were buried in the soil and inoculated with *C. aeruginascens* DR 468 had a mean percent mass loss of 21% after 67 wks, which is more than twice as much as the corresponding blocks placed on top of the soil. These results may indicate a higher moisture requirement for *C. aeruginascens* to cause decay, as is typical for many soft rot fungi (Zabel and Morrell, 1992).

After 134 wks of incubation, isolates of *C. aeruginascens* were successfully reisolated from one representative aspen block from each replicate set of blocks demonstrating viability of the fungi after considerable time in soil jars (2 yrs 7.5 mo). Aspen blocks colonized with *C. aeruginascens* ATCC 24028 after 134 wks were stained bright green throughout, while blocks colonized with *C. aeruginascens* DR 468 were variably stained, and in some cases green stain extended only partially into the block (Fig. 3). The general appearance of the aspen blocks exposed to either isolate of *C. aeruginascens* after 134 wks resembled decay caused by soft rot (Figs. 4 – 6). A few holes were present in the S2 layer of the wood cell wall (Fig. 4), indicative of an incipient state of Type I soft rot, but no diamond shaped cavities were observed in longitudinal section. Erosion troughs were observed directly beneath the fungal hyphae

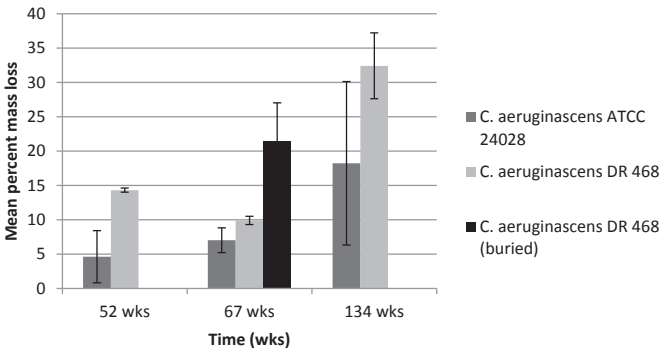

Fig. 2. Decay of aspen blocks incubated (20 °C) with two isolates of *C. aeruginascens* after 52, 67 and 134 wks. For the 67 wks series, one set of blocks was buried in the soil; in all other tests blocks were incubated above the soil.

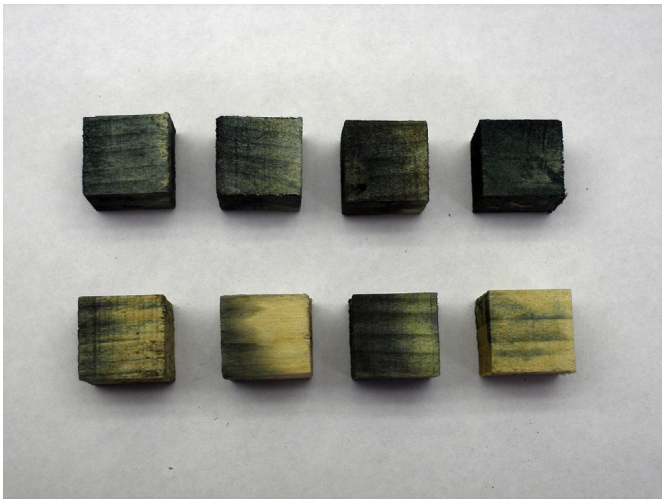

Fig. 3. Nineteen mm cube aspen wood blocks after 134 wks exposure to *C. aeruginascens*. Top row *C. aeruginascens* ATCC 24028. Bottom row *C. aeruginascens* DR 468.

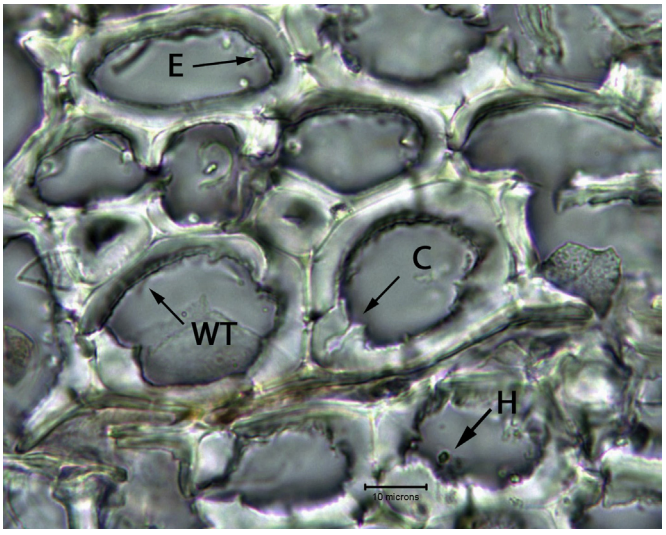

Fig. 4. Transverse section of aspen wood block after 134 wks colonization by *C. aeruginascens* showing fungal hyphae (H), and general erosion (E) and wall thinning (WT) of vessel cell walls. A Type I soft rot cavity (C) is evident in the S2 layer. Dry mass loss of this specimen was 25%.

and there was frequent overall erosion of the S3 and S2 layers (Figs. 5 and 6), indicative of Type II soft rot (Zabel and Morrell, 1992; Worrall et al., 1997; Schwarze, 2007). The surfaces of the samples did not show the fine surface checking that is characteristic of advanced soft rot decay in the field, and the wood appeared sound macroscopically.

4. Discussion

This study demonstrated that viable basidiomycete decay fungi are present in wood that is naturally colonized and stained by the ascomycete *Chlorociboria*, putatively *C. aeruginascens.* Basidiomycetes that were identified to species by ITS sequencing are known to be common white rot fungi in north temperate forests. Basidiomycete isolate 02 was identified as *Plicaturopsis crispa* with a 99% match to ITS sequence in Genbank. This fungus is very common as a colonizer of dead branches and trunks of hardwoods, causing a

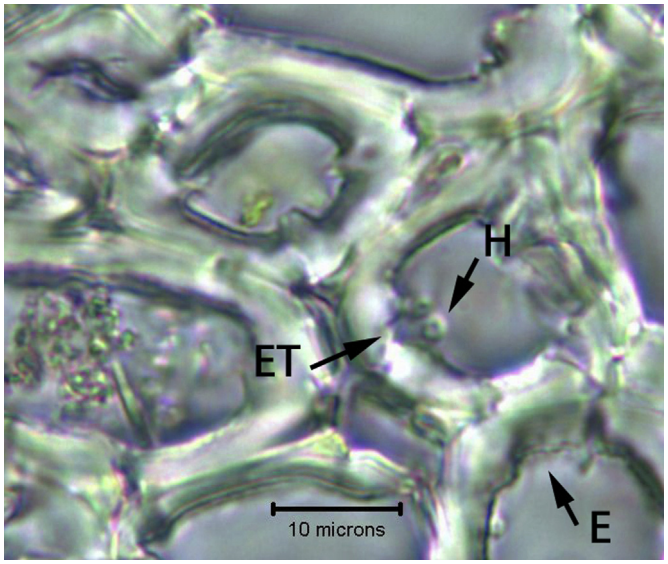

Fig. 5. Transverse section from the same sample block as in Fig. 4 showing fungal hyphae (H), an erosion trough beneath the hyphae (ET), and general erosion of the S3 cell layer (E).

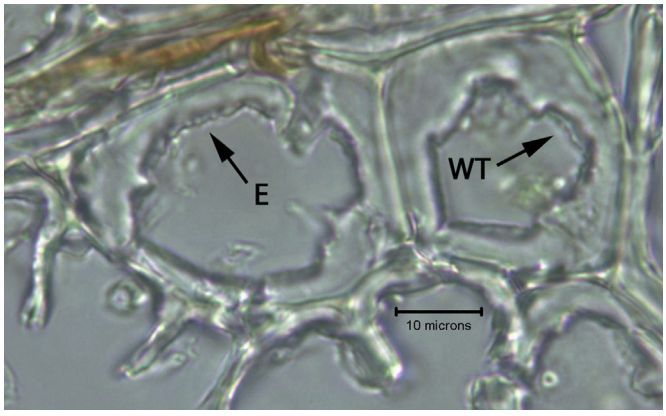

Fig. 6. Transverse section from the same sample block as in Fig. 4 showing wall thinning (WT) and general erosion of the S3 cell layer (E).

white rot (Ginns and Lefebvre, 1993; Brazee et al., 2014). Of the ten unique basidiomycetes isolated from *Chlorociboria*-colonized wood, this was the only isolate that gave a negative reaction to the gum guiac drop test. Although a negative reaction is usually associated with brown rot fungi, Gilbertson et al. (1975) demonstrated that many important white rot fungi also give a negative reaction in gum guiac. Additionally, clamps were not detected in the culture of isolate 02 despite being previously described for *P. crispa* (Nakasone, 1990), however it is often difficult to observe this morphological feature.

All other basidiomycete isolates tested positive with gum guiac indicating the presence of white rot fungi (Table 1). Two isolates from *P. tremuloides* (01 and 07) with identical ITS sequences could not be identified to species or genus using ITS sequencing. They were most closely related (with 83% match) to an unidentified species within the Cantharellales, an order that includes many morphologically dissimilar genera including white rot corticioid fungi, chanterelles, tooth and clavarioid fungi (Moncalvo et al., 2006). Their positive reaction with gum guiac indicates that they have the ability to cause white rot. The other basidiomycetes were

all identified as common white rot fungi. *Gloecystidiellum porosum* (99% match to ITS sequence in Genbank) is widely distributed across North America on decorticated wood, twigs and branches of hardwoods and confers (Nakasone, 1982); this culture was associated with a species of *Amelanchier*. *Peniophorella pubera* (99% match with ITS sequence in Genbank), found on the black spruce sample, is common on decayed, decorticated wood of deciduous trees but is sometimes associated with conifers (Eriksson and Ryvarden, 1975). A single sample of decayed paper birch yielded two different basidiomycete fungi. Isolate 06a was identified as *Bjerkandera adusta* (99% match with ITS sequence in Genbank). Fruiting bodies of this fungus were also present on the surface of this specimen. The other isolate, 06b, identified as *P. praetermissa* (98% match with ITS sequence in Genbank), is a corticioid white rot fungus commonly found on hardwoods (Nakasone, 1990). This fungus has been shown to be a large species-complex that has been difficult to resolve (Hallenberg et al., 2007). It is apparently not host specific and was also found on *P. tremuloides* (05).

Isolation of these basidiomycete decay fungi from *Chlorociboria*–stained wood provides insights into what is actually growing in and decaying the wood, not the overall fungal community in the wood. Isolation gives a much better indication of the decay fungi present since most are culturable. More extensive DNA sequencing analysis would be necessary to characterize the host of ascomycetes and endophytes also present in the wood, which was beyond the scope of this study.

The amount of decay caused by a particular fungus in a wood sample that is exposed to natural inoculum is likely dependent upon the timing of its entry into the wood. For the isolations made in this study, it is not known whether the *Chlorociboria*-stained wood samples were first colonized by *Chlorociboria* and then colonized by a basidiomycete decay fungus, or if a basidiomycete colonized the wood first. Only two of the 17 isolation attempts from *Chlorociboria*-stained wood yielded *Chlorociboria* sp, and in these cases a basidiomycete was not isolated. Conversely, basidiomycete fungi were isolated from nine of the 17 samples of *Chlorociboria*-stained wood, and none of these nine samples yielded an isolate of *Chlorociboria* sp. Thus, this study could not determine whether both fungi are active in the naturally exposed wood at the same time. On the contrary, our laboratory decay study with co-inoculated pure cultures suggests that certain basidiomycete fungi can out-compete *Chlorociboria* sp. and render the latter nonviable after 26 wks of co-incubation.

Soft rot fungi are often associated with wood that is either too wet or too dry for the growth of basidiomycete wood decay fungi (Blanchette et al., 2004). A likely scenario is that decaying wood on the forest floor becomes water-soaked and is no longer able to support the growth of basidiomycetous fungi, allowing its colonization by species of *Chlorociboria*. As indicated by laboratory isolations on selective media, viable basidiomycete decay fungi are common in wood colonized by the green stain ascomycete fungus *Chlorociboria* sp. From results of the decay tests where an associated basidiomycete isolate was simultaneously co-inoculated with *C. aeruginascens*, the presence of *C. aeruginascens* did not inhibit or stimulate decay by the basidiomycete. In contrast, *C. aeruginascens* itself appeared to be inhibited by the basidiomycete, as indicated by the minimal amount of green staining in the decayed blocks at the end of the test and by our inability to reisolate *C. aeruginascens* from the co-inoculated blocks. White rot enzymes are known to have a bleaching effect on wood, which may also have contributed to lightening the color of the co-inoculated aspen blocks by the end of the experiment.

Both isolates of *C. aeruginascens* were able to cause limited mass loss in the aspen blocks when incubated alone for 134 wks (DR 468–32%; ATCC 24028–18%). These results demonstrate variation in decay efficacy by isolates of *C. aeruginascens*. Although Robinson and Laks (2010) state that *Chlorociboria* caused no mass loss in four wood species after 24 weeks incubation, their study was designed to examine pigment production; data on wood decay were not presented. Furthermore, their incubations were conducted in vermiculite, which may be unsuitable for *Chlorociboria* to colonize and decay wood. Additionally, soft rots degrade wood very slowly and 24 weeks may not be long enough to produce detectable mass loss. In our study, one isolate of *C. aeruginascens* (DR 468) caused over twice as much decay to aspen blocks buried in the soil compared to blocks that were place above ground in the soil jars after 67 wks of incubation. The ability to cause greater mass loss to wood blocks in soil where blocks undergo consistently higher moisture conditions during incubation is another characteristic of soft rot fungi (Zabel and Morrell, 1992). The gum guiac drop test to detect oxidase enzymes could not be used on cultures of *Chlorociboria* sp. due to the green color of the mycelium.

Although soft rot and white rot decay cannot always be distinguished microscopically (Nilsson et al., 1989), the general appearance of the wood blocks decayed by *C. aeruginascens*, including holes in the S2 layer, erosion troughs and an overall erosion of vessel cell walls is further indicative of soft rot decay. Other ascomycetes, including *Bisporella citrina* (Helotiales) and *Bulgaria inquinans* (Leotiales), have been associated with low levels of soft rot decay, resulting in 4% and 2% mass loss, respectively, in birch wood after 12 weeks of incubation (Worrall et al., 1997). Given the extensive incubation time required for mass loss to develop and the minimal amount of damage to the cell walls, we conclude that *C. aeruginascens* is a weak soft rotter that causes primarily Type II attack, confirming the conclusions of Blanchette et al. (1992).

In conclusion, it appears that *Chlorociboria*-stained wood is predominantly degraded by white rot fungi. It is presumed that *C. aeruginascens* is a late colonizer of decayed wood, probably due to its requirement for high moisture content. Isolates of *C. aeruginascens* can produce mass loss on their own, but only over long time periods. Wood decayed by *C. aeruginascens* has properties of both Type-I and Type-II soft rot decay.

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