Extracellular enzymes and soft rot decay: Are ascomycetes important degraders in fresh water?

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Allochthonous carbon constitutes a crucial proportion of the overall carbon budgets of freshwater ecosystems as it is a key source of energy for many organisms. However, this carbon often enters the systems in recalcitrant forms (*e.g.* lignocellulose) and must be degraded by microbes into simpler compounds before most heterotrophic organisms can utilize it. Twenty-seven ascomycete taxa isolated from submerged dead plant material in a variety of freshwater habitats were qualitatively tested *in vitro* for the production of extracellular plant-degrading enzymes important in the breakdown of cellulose, hemicellulose, lignin, starch, and pectin. Isolates were also assayed for chitinolytic activity and grown on balsa wood to test for soft rot decay capabilities. All species were able to degrade starch, hemicellulose, and cellobiose (the glucose dimer). Over half of the species produced enzymes important in the breakdown of cellulose, lignin, and pectin. After five weeks of growth, only one species produced chitinase. Eighteen species were capable of soft rot decay after eight weeks of growth. There were no discernable differences between tropical and temperate species in their capabilities to degrade substrates. Species isolated from lotic and lentic habitats also had similar results for all enzyme and soft rot assays. Herbaceous, lignicolous, and substrate-generalist species exhibited similar results for all enzyme assays, but varied in their ability to cause soft rot decay. Two-thirds of the lignicolous species and one of two substrate generalist species caused soft rot decay, yet neither of the two herbaceous species caused soft rot. The results of this study are combined with those from previous publications and indicate that freshwater ascomycete fungi are, in general, capable of breaking down lignocellulose and other complex plant compounds, and likely play a crucial role in the recycling of carbon in freshwater ecosystems.

Key words: Allochthonous carbon, ecosystem processes, freshwater fungi, functional ecology, nutrient cycling, species distributions, wood decay

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Introduction

Freshwater ecosystems receive large inputs of fixed carbon in the form of woody and herbaceous debris from the riparian zone (Webster *et al.*, 1999). This material is a crucial source of energy and carbon for heterotrophs (Wetzel, 1995), yet is not directly available to most freshwater invertebrates and bacteria because they lack the enzymes needed to degrade complex plant structural compounds, such as lignin and cellulose, into usable intermediate compounds (Dudley and Anderson, 1982; Bärlocher and Porter, 1986; Romaní *et al.*, 2006). Additionally, basidiomycetes, the primary degraders of lignocellulose in terrestrial habitats, are rare in freshwater (Harmon *et al.*, 1986; Wong *et al.*, 1998; Vijaykrishna and Hyde, 2006; Shearer *et al.*, 2007). Ascomycetes are thought to play an important role in the breakdown of plant material in freshwater systems because they colonize submerged plant substrates, produce degradative enzymes, and decay wood via soft rot (Zare-Maivan and Shearer, 1988a,b; Shearer, 1992; Gessner and Chauvet, 1997; Wong *et al.*, 1998; Vijaykrishna *et al.*, 2006). During soft rot decay, the S_2 layer of the cell wall is broken down, thereby softening the exterior layers of wood, which may then slough off or be grazed by inverte-

brates (Savory, 1954).

The importance of Ingoldian hyphomycetes (an ecological group of aquatic anamorphic ascomycetes) in the degradation of leaf litter in lotic habitats is well understood (Suberkropp and Klug, 1976; Bärlocher, 1982). These fungi produce cellulases and pectinases, enzymes crucial in the breakdown of leaf litter (Chamier, 1985). Ingoldian hyphomycetes have also been isolated from woody substrates in running waters (Shearer, 1992), and while less is known about their role in breaking down wood *in situ*, many produce lignin modifying enzymes (Fisher *et al.*, 1983; Abdel-Raheem and Ali, 2004). Meiosporic ascomycetes are also likely to play an important role in breaking down plant debris in freshwater ecosystems and have been isolated from submerged wood and herbaceous material in lotic and lentic habitats around the world (Shearer, 1993, 2001). However, until more recently, little was known about the ability of this group of fungi to degrade plant material. Zare-Maivan and Shearer (1988a,b) showed that temperate wood-inhabiting meiosporic ascomycetes could, *in vitro*, degrade lignocellulose and other carbon sources (*e.g.* plant starch, pectin, lipids, and proteins) as well as form soft rot cavities in wood. Since then, few studies have further examined the decay capabilities of these fungi, especially in an ecological context.

Currently, most freshwater ascomycetes have been isolated from limited ecoclimatic ranges (Shearer, 2001; Shearer *et al.*, 2008). While it is likely that certain conditions (*e.g.* temperature), which vary across ecoclimates, affect the quantitative impacts that microbes have upon plant debris (Bucher *et al.*, 2004; M. Ardón, pers. comm.), it has been proposed that ecoclimatic distribution does not affect the basic ability of freshwater ascomycetes to degrade plant structural compounds (Yuen *et al.*, 1998, 1999). However, no study has directly addressed this hypothesis by concurrently testing both temperate and tropical species. Furthermore, earlier studies of freshwater ascomycete enzymatic capabilities (Zare-Maivan and Shearer, 1988b; Yuen *et al.*, 1998, 1999) followed different experimental protocols, making it somewhat difficult to compare their results in order to adequately assess the potential influence of ecoclimatic range on degradative capabilities (Pointing, 1999).

As a group, freshwater ascomycetes also appear to exhibit moderate habitat specificity, as most species have, thus far, been isolated from only lotic or lentic habitats, yet a few are known to occur in both habitat types (Shearer, 2001; Shearer *et al.*, 2008). While the two habitat types differ in many biological, chemical, and physical characteristics, recent evidence has shown that the energy budgets of both lotic (Wallace *et al.*, 1997) and lentic (Pace *et al.*, 2004) ecosystems rely heavily on subsidies of allochthonous carbon. It is therefore important to determine if ascomycetes from both habitat types are equally capable of playing the important ecological role as degraders of larger, more recalcitrant forms of carbon.

Differences in biochemical and physical characteristics, input rates, and residence times of herbaceous and woody substrates lead to their being broken down differently in fresh water (Shearer, 1992). Whereas leaves are fragmented quickly by microbes and invertebrate shredders (Bärlocher, 1982), wood must be superficially decayed (a slower process) by soft rot fungi before most invertebrate scrapers and borers can utilize it (Dudley and Anderson, 1982; Bärlocher and Porter, 1986; Gessner and Van Ryckegem, 2002). The majority of freshwater ascomycetes are known to only occur on woody or herbaceous debris, but not both (Shearer, 2001; Shearer *et al.*, 2008); however, the mechanisms underlying this pattern have yet to be fully explored. A recent study found no differences among the lignicolous, herbaceous, and substrate generalist ascomycetes tested for any enzyme, including those important in breaking down either leaves (*e.g.* pectinases) or wood (*e.g.* lignin-modifying enzymes) (Abdel-Raheem and Shearer, 2002), indicating that enzymatic capabilities are unlikely to limit substrate colonization or habitation. However, the relationship between *in situ* substrate specificity and the ability to decay wood has yet to be explored for freshwater ascomycetes.

In this investigation, ascomycete taxa isolated from submerged plant debris in lentic and lotic freshwater habitats in temperate and

tropical ecoclimates were assayed for the ability to degrade plant structural compounds as well as form soft rot cavities in wood. Results indicate that the fungi, in general, were able to produce the enzymes needed to degrade the structural compounds, as well as form soft rot. In addition, little variation due to the ecological distributions of these fungi was seen in their degradative capabilities. Data from previous similar studies were compiled with the results from these experiments and show that, in general, ascomycetes are very capable degraders of complex carbon compounds and therefore likely play a critical role in the recycling of carbon in freshwater systems.

Materials and methods

Seventeen meiosporic and ten mitosporic (two Ingoldian, one aero-aquatic, and seven non-specialized) freshwater ascomycetes and one positive control terrestrial basidiomycete (Table 1) were tested for production of a suite of extracellular, degradative enzymes and the ability to cause soft rot decay in wood. The ascomycetes were isolated from submerged plant debris according to the procedures of Shearer *et al.* (2004) and reference colonies are maintained at the University of Illinois at Urbana-Champaign: H, F, and R isolates in the collection of H.A. Raja; AF and E isolates in the collection of A. Ferrer; and CS and TC isolates in the collection of C.A. Shearer. All chemicals used were manufactured by Sigma-Aldrich Co. (St. Louis, MO, USA) unless otherwise noted.

Cultures for inoculum were maintained on Petri dishes (hereafter referred to as plates) containing 25 mL of a medium comprised of agar (1.6% w/v, Bacto Laboratories, Liverpool, Australia), peptone (0.125% w/v, Bacto Labs), yeast extract (0.125% w/v, Bacto Labs), and glucose (0.4% w/v, Acros Organics, Morris Plains, NJ) at room temperature under normal laboratory conditions. Unless otherwise noted, for all assays, circular plugs (5 mm diameter) were cut from the leading edge of the inoculum colony and placed onto plates containing 25 mL of the basal medium (1.6% w/v agar, 0.1% w/v peptone, 0.01% w/v yeast extract) supplemented with the test compound(s). Three replicates of each species were grown for up to 14 days at 25 C in darkness before being tested for enzyme activity. Sterile techniques were used throughout, uninoculated plates were kept as controls, and results were scored as either negative (-) or positive (+).

General cellulase activity was assayed using the cellulose-azure method. Glass culture tubes containing a layer of basal medium supplemented with 1% w/v cellulose-azure on top of a layer of plain basal medium were inoculated and checked daily for three weeks for migration of the azure dye (released from the cellulose-azure via hydrolysis) into the clear lower layer (modified from Pointing, 1999). Tubes of positive isolates were observed for three more weeks for decolorization of the dye, an indirect indication of the production of lignin-modifying enzymes (Pointing, 1999). To detect production of endoglucanases, which hydrolyze ß-1,4-glycosyl bonds along the interior of the cellulose chain (Sinsabaugh and Liptak, 1997), species were tested for the ability to degrade 1% w/v low viscosity carboxymethylcellulose (CMC). Breakdown of CMC was determined by using a Congo Red/NaCl stain/de-stain system; cleared areas around the colony appeared yellow-orange against the red color of intact CMC (modified from Pointing, 1999). Production of ß-glucosidase, which breaks down cellobiose, the dimer of glucose (Sinsabaugh and Liptak, 1997), was assayed using the iron/esculin method. Plates containing ferric sulfate (0.02% w/v) and esculin (0.5% w/v) were inoculated and examined daily for darkening of the medium caused by a reaction of the coumarin (released from the esculin via ß-glucosidase) with the ferric sulfate (modified from Pointing, 1999).

To test for the ability to break down hemicelluloses, which bind to cellulose microfibrils (Sinsabaugh and Liptak, 1997), colonies were grown on the basal medium supplemented with birchwood xylan (0.5% w/v). Plates were flooded with an aqueous IKI solution for five minutes. A yellow zone surrounding the colony in the resulting brown medium indicated the breakdown of xylan (modified from Egger, 1986).

Production of the lignin modifying enzymes (LMEs) laccase and peroxidase, as well as tyrosinase, was assayed together using a medium containing agar (1.6% w/v), glucose (0.2% w/v), peptone (0.01% w/v), and yeast

Table 1. Fungal isolates tested for production of degradative enzymes and soft rot formation, their reproductive state, ecological characters, and isolate number.

Species Name	State	Zone[1]	Habitat	Subtype	Substrate	Isolate #
Aquapoterium pinicola Raja & Shearer	Meiotic	Warm Temperate	Lentic	Swamp	Herbaceous	F 47-1
Aquaticola sp. AF 173-1	Meiotic	Tropical	Lotic	Stream	Woody	AF 173-1
Arnium gigantosporum Raja & Shearer	Meiotic	Warm Temperate	Lentic	Lake	Woody	F 77-1
Arthrobotrys sp. H 071-1	Mitotic[M]	Warm Temperate	Lentic	Swamp	Woody	H 071-1
Brachiosphaera tropicalis Nawawi	Mitotic[H]	Tropical	Lotic	Stream	Woody	E 192-1
Conioscyphascus varius Réblová & Seifert	Meiotic	Tropical	Lotic	Stream	Woody	AF 169-1
Dactylaria tunicata Goh & K.D.Hyde	Mitotic[M]	Warm Temperate	Lotic	Stream	Woody	H 69-1
Desertella fumimontarum Raja & Shearer	Mitotic[M]	Warm Temperate	Lotic	Stream	Woody	H 013-1
Ellisembia sp. E 166-2	Mitotic[M]	Tropical	Lotic	Stream	Woody	E 166-2
Flabellospora multiradiata Nawawi	Mitotic[H]	Tropical	Lotic	Stream	Woody	E 204-1
Helicodendron giganteum Glen Bott	Mitotic[A]	Cool Temperate	Lentic	Pond	Woody	CS 988-1
Helicoma perelegans Thaxt. ex Linder	Mitotic[M]	Warm Temperate	Lotic	Stream	Woody	H 008-1
Jahnula bipileata Raja & Shearer	Meiotic	Warm Temperate	Lentic	Lake	Woody	F 49-1
Luttrellia guttulata A.Ferrer & Shearer	Meiotic	Tropical	Lotic	Stream	Woody	AF 181-1
Massarina ingoldiana Shearer & K.D.Hyde	Meiotic	Warm Temperate	Both	Lake/Stream	Both	F 13-1
Massarina sp I: F 12-1	Meiotic	Warm Temperate	Lentic	Lake/Swamp	Both	F 12-1
Massarina sp. II: R 056-1	Meiotic	Warm Temperate	Lotic	Stream	Woody	R 056-1
Massarina sp. III: AF 180-1	Meiotic	Tropical	Lotic	Stream	Woody	AF 180-1
Megalohypha aqua-dulces A.Ferrer & Shearer	Meiotic	Tropical	Lotic	Stream	Woody	AF 005-2
Ophiobolus shoemakeri Raja & Shearer	Meiotic	Warm Temperate	Lentic	Lake	Herbaceous	F 22-1
Porosphaerellopsis bipolaris K.M.Tsui & K.D.Hyde	Meiotic	Tropical	Lotic	Stream	Woody	AF 171-1
Pyrenomycete sp. AF 170-1	Meiotic	Tropical	Lotic	Stream	Woody	AF 170-1
Sporidesmium tropicale M.B.Ellis	Mitotic[M]	Tropical	Lentic	Lake	Woody	F 79-1
Submersisphaeria sp. F 044-1	Meiotic	Warm Temperate	Lotic	Stream	Woody	F 044-1
Torrentispora fibrosa K.D. Hyde *et al.*	Meiotic	Warm Temperate	Both	Lake/Stream	Woody	F 39-1
Xylomyces elegans Goh *et al.*	Mitotic[M]	Warm Temperate	Lotic	Stream	Woody	H 080-1
Zopfiella lundqvistii Shearer & J.L.Crane	Meiotic	Warm Temperate	Lentic	Swamp	Woody	F 17-1

A = Aero-Aquatic; H = Hyphomycete; M = Miscellaneous; [1] = According to Bailey (1996).

extract (0.001% w/v). After inoculation and growth, four wells (5 mm diameter) were cut into the leading edge of each colony. As a control, one drop of 95% ethanol was placed into the first well. One drop of syringaldazine in 95% ethanol was placed into the second and third wells, and one drop of aqueous hydrogen peroxide was added to the syringaldazine solution in the third well. Development of a pink color around the second well within 25 minutes indicated laccase activity. A similar color surrounding the third well within 25 minutes indicated peroxidase activity, but only if darker than the laccase reaction. In the fourth well, two drops of p-cresol in an aqueous glycine solution were added. Plates were checked after 0.5, 3, and 24 hours for an orange-brown color surrounding the well indicating production of tyrosinase (modified from Pointing, 1999), which detoxifies the phenoxy radicals produced during lignin modification (Pointing, 1999).

To test for the ability to hydrolyze amylose, a main constituent of plant starch (Garroway and Evans, 1984), colonies were grown on the basal medium supplemented with soluble starch (0.2% w/v). Plates were flooded with an IKI solution for five minutes, and a yellow clearing zone against a blue background indicated amylase activity (modified from Gessner, 1980).

Pectin, a heterosaccharide found in plant cell walls, can be degraded by either pectic lyase, which uses a *trans*-elimination method to break bonds, or polygalacturonase, which hydrolyzes bonds (Sinsabaugh and Liptak, 1997). Production of these enzymes was assayed separately using the basal medium supplemented with apple pectin (0.5% w/v). The medium was buffered to pH 7 (phosphate-phosphate buffer system) for the pectic lyase test and pH 5 (phosphate-citrate buffer system) for the polygalacturonase test. After growth, plates were flooded with aqueous hexadecyltrimethylammonium bromide, which precipitates intact pectin. A clear zone around the colony in the resulting opaque medium therefore indicated enzymatic activity (modified from Hankin and Anagnostakis, 1975).

Chitin, a structural compound in invertebrate exoskeletons and fungal hyphae, is a potential source of nitrogen and carbon for fungi (Chamier, 1985). Chitin is a polymer of N-acetyl-D-glucosamine and, similar to cellulose, is broken down by both endo- and exo-bond-splitting enzymes (Fukamizo *et al.*, 1986). A method analogous to the cellulose-azure test was implemented to determine chitinase production with chitin azure in the overlay. After inoculation, tubes were checked daily for five weeks for migration of dye into the clear lower layer, indicative of hydrolysis of the chitin azure (modified from Untereiner and Malloch, 1999).

The ability to form soft rot cavities in the S_2 layer of woody tissue was determined by growing the fungi for eight weeks on plates containing pre-sterilized strips of balsa wood ($55 \times 2 \times 2$ mm; two per plate) pressed into the basal medium. After removing aerial mycelial growth, the surface of the wood was scraped using a dissection needle. Scrapings were mounted in lactic acid and viewed for the presence of soft rot cavities using a light microscope with polarized light, Nomarski interference, and phase optics (modified from Zare-Maivan and Shearer, 1988b).

Results

Results of assays for enzyme production and soft rot cavity formation by the 27 freshwater ascomycetes are shown in Table 2. The number of species positive for each test is included at the bottom of the table. No species tested positive for all assays. Two-thirds (18 of 27) were cellulolytic (based on the cellulose azure test) and two-thirds were positive for endoglucanase, although there was not complete correspondence between the two tests: *H. giganteum* tested positive for general cellulases but negative for endoglucanase while *Massarina* sp. I: F 12-1 had the opposite results. Therefore, 19 species in total degraded cellulose. All species were capable of hydrolyzing cellobiose via ß-glucosidase and hemicellulose via xylanase.

Production of one lignin-modifying enzyme (LME), laccase, was far more prevalent (15 species) than production of the other, peroxidase (three species). All three species that produced peroxidase, *A. gigantosporum*, *P. bipolaris*, and Pyrenomycete sp. AF 170-1,

Table 2. Results of assays for production of general cellulases (CL), endoglucanase (EN), ß-glucosidase (BG), xylanase (XY), laccase (LA), peroxidase (PE), tyrosinase (TY), amylase (AM), pectic lyase (PL), polygalacturonase (PO), chitinase (CH), and soft rot (SR).

Species Name	CL	EN	BG	XY	LA	PE	TY	AM	PL	PO	CH	SR
Aquapoterium pinicola	+	+	+	+	-	-	-	+	-	+	-	-
Aquaticola sp. AF 173-1	-	-	+	+	-	-	-	+	-	-	-	+
Arnium gigantosporum	-	-	+	+	+	+	-	+	-	-	-	+
Arthrobotrys sp. H 071-1	+	+	+	+	-	-	-	+	+	+	-	-
Brachiosphaera tropicalis	+	+	+	+	+	-	+	+	+	+	-	+
Conioscyphascus varius	+	+	+	+	-	-	-	+	+	+	-	+
Dactylaria tunicata	-	-	+	+	-	-	-	+	-	-	-	+
Desertella fumimontarum	+	+	+	+	-	-	-	+	+	+	-	-
Ellisembia sp. E 166-2	-	-	+	+	-	-	-	+	-	-	-	+
Flabellospora multiradiata	+*	+	+	+	+	-	+	+	+	+	-	+
Helicodendron giganteum	+*	-	+	+	+	-	-	+	+	+	-	-
Helicoma perelegans	+	+	+	+	+	-	-	+	-	-	-	+
Jahnula bipileata	+	+	+	+	+	-	+	+	-	+	-	+
Luttrellia guttulata	-	-	+	+	-	-	-	+	+	+	-	+
Massarina ingoldiana	+	+	+	+	+	-	+	+	+	+	-	+
Massarina sp I: F 12-1	-	+	+	+	+	-	-	+	-	+	-	-
Massarina sp. II: R 056-1	+	+	+	+	+	-	-	+	-	-	-	+
Massarina sp. III: AF 180-1	+	+	+	+	-	-	-	+	-	-	-	-
Megalohypha aqua-dulces	+*	+	+	+	+	-	-	+	+	+	-	-
Ophiobolus shoemakeri	+*	+	+	+	+	-	-	+	+	+	-	-
Porosphaerellopsis bipolaris	+	+	+	+	+	+	-	+	-	-	+	+
Pyrenomycete sp. AF 170-1	-	-	+	+	+	+	-	+	-	-	-	+
Sporidesmium tropicale	-	-	+	+	-	-	-	+	-	-	-	-
Submersisphaeria sp. F 044-1	-	-	+	+	+	-	-	+	-	-	-	+
Torrentispora fibrosa	+	+	+	+	+	-	-	+	-	+	-	+
Xylomyces elegans	+	+	+	+	-	-	+	+	+	+	-	+
Zopfiella lundqvistii	+	+	+	+	-	-	-	+	-	-	-	+
Total Number of Positive Species	**18**	**18**	**27**	**27**	**15**	**3**	**5**	**27**	**11**	**15**	**1**	**18**

+ = positive reaction; - = negative reaction; * = decolorization of azure dye following migration.

also produced laccase. The four species that decolorized the azure dye in the cellulose azure test produced laccase but not peroxidase. However, the other eleven species which produced laccase did not decolorize the azure dye, indicating a lack of congruence between those methods. Five species, four of which produced laccase (but not peroxidase), tested positive for the detoxifying enzyme tyrosinase. *Xylomyces elegans* also produced tyrosinase, but did not test positive for laccase or peroxidase.

All 27 species produced amylase, and are therefore capable of degrading plant starch. Eighteen species were capable of breaking down pectin: eleven produced both pectic lyase and polygalacturonase, three produced only pectic lyase, and four produced only polygalacturonase. After five weeks of growth, only one species, *P. bipolaris*, tested positive for chitinase. Eighteen species produced soft rot cavities in balsa wood after eight weeks (Figs 1-6). Cavities were typically elongate with tapered ends, but varied in length, width, and density among species, similar to previous reports (*e.g.* Zare-Maivan and Shearer, 1988b).

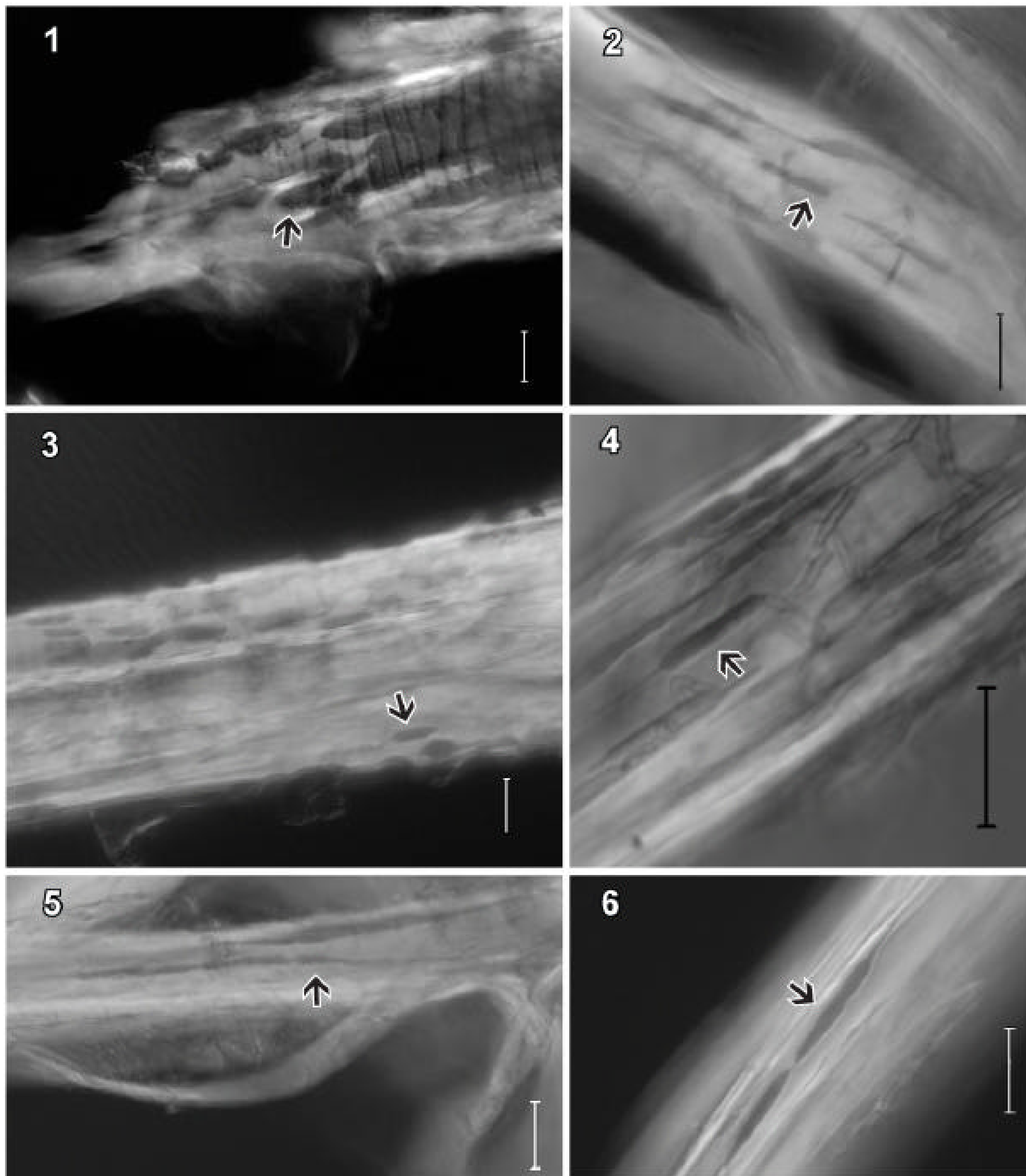
To assess the potential variation in degradative capabilities among these fungi based on ecological distributions, species were compared with respect to only one distributional characteristic at a time. Due to small sample sizes, data were visually assessed for trends in lieu of statistical analyses. Enzymes which were produced by all species (ß-glucosidase, xylanase, and amylase) or by only one species (chitinase) were left out of comparisons. Within the species that occur in lotic habitats on woody debris, there were no major differences between those isolated from tropical and temperate ecoclimates in their ability to produce plant-degrading enzymes or soft rot cavities (Table 3). For species from both ecoclimates, the percentage positive was similarly high (*i.e.* $\geq$ 50%) for general cellulases, endoglucanase, laccase, and soft rot cavity formation; moderate (*i.e.* 20-50%) for pectinases; and low (*i.e.* $\leq$ 20%) for peroxidase and tyrosinase.

Habitat distribution (*i.e.* lotic or lentic) also did not appear to affect the capability of freshwater ascomycetes to break down plant structural compounds (Table 3). When compared based on habitat distribution, the percentage of temperate lignicolous species testing positive was similarly high for general cellulases, endoglucanase, laccase and soft rot; low for peroxidase and tyrosinase; and moderate for pectinases. There were no differences in enzymatic production between the lignicolous and herbaceous species isolated from temperate lentic systems, as well (Table 3). General cellulases, endoglucanase, and laccase were all commonly produced; pectinases were produced by most species; and peroxidase and tyrosinase were produced by only a few species. However, while four of the six lignicolous species formed soft rot cavities, neither of the two herbaceous species did.

Discussion

The finding that all of the freshwater ascomycetes tested here were capable of breaking down cellobiose, starch, and xylan agrees with results from prior studies. Over 80% of the species previously tested using similar procedures were capable of hydrolyzing these substrates, irrespective of ecological distribution (Zare-Maivan and Shearer, 1988b; Yuen *et al.*, 1998; Abdel-Raheem and Shearer, 2002; Bucher *et al.*, 2004). It can therefore be concluded that the vast majority of freshwater ascomycetes, regardless of ecoclimate, habitat, and substrate distributions, are capable of breaking down cellobiose, hemicellulose, and plant starch, three ecologically important carbon compounds found in plant-based debris.

This is the first reported study of the enzyme production and soft rot cavity formation abilities of freshwater ascomycetes that included a significant number of both temperate and tropical species and no differentiation in degradative capabilities was found based upon ecoclimatic distribution. Our results are in accord with previous enzymatic studies which focused on tropical lotic (Bucher *et al.*, 2004) or temperate lignicolous species (Abdel-Raheem and Shearer, 2002) and followed similar protocols to those used here. The approximately 80% of species from each ecoclimate that were capable of soft rot decay in this study is also very comparable to the 82% of temperate lignicolous ascomycetes which formed soft rot in the only other study



Figs 1-6. . Soft rot cavities formed by six freshwater ascomycetes when grown on balsa wood for eight weeks. **1.** *Dactylaria tunicata*. **2.** *Zopfiella lundqvistii*. **3.** *Luttrellia guttulata*. **4.** *Arnium gigantosporum*. **5.** *Helicoma perelegans*. **6.** *Conioscyphascus varius*. Scale bars = 20 µm.

that used similar methods to those used here (Zare-Maivan and Shearer, 1988b). These results support the hypothesis (Yuen *et al.*, 1998, 1999) that the ecoclimatic distribution of isolates has no effect on the basic capability of freshwater ascomycetes to produce plant-degrading enzymes or form soft rot cavities. Therefore, it is likely that these fungi perform qualitatively similar roles in the degradation of plant material in freshwater systems irrespective of ecoclimate. However, it is probable that species in different ecoclimates do not have quantitatively similar impacts upon plant debris *in situ*. Rather, decay rates are likely to be higher nearer the equator (Bucher *et al.*, 2004), conceivably caused by increased metabolic

Table 3. The number of species in each ecological classification used for comparisons and the percentage of these species positive for each test. Column headings as in Table 2.

Ecological Classification	No. Species	%							
		CL	EN	LA	PE	TY	PL	PO	SR
Temperate (Lotic, Lignicolous)	6	67	67	50	0	17	33	33	83
Tropical (Lotic, Lignicolous)	10	60	60	50	20	20	50	50	80
Lentic (Temperate, Lignicolous)	5	80	60	60	20	20	40	60	60
Lotic (Temperate, Lignicolous)	6	67	67	50	0	17	33	33	83
Lignicolous (Temperate, Lentic)	5	80	60	60	20	20	40	60	60
Herbaceous (Temperate, Lentic)	2	100	100	50	0	0	50	100	0

activity due to elevated temperatures (M. Ardón, pers. comm.).

Given that some freshwater ascomycetes are habitat-specific (Shearer, 2001; Shearer *et al.*, 2008) and that allochthonous carbon constitutes a crucial energy source for both lotic and lentic habitats (Wetzel, 1995; Wallace *et al.*, 1997; Pace *et al.*, 2004), it is important that species from both habitat types were able to degrade large carbon-based compounds and decay wood. It is therefore plausible that these fungi play similarly important roles in decomposition in both habitat types by breaking down complex carbon compounds into simpler compounds which may then be used by other heterotrophs. Only one previous study of freshwater ascomycete enzyme production included habitat data (Bucher *et al.*, 2004) and all species tested were from tropical streams. However, using the site collection data available on-line in the Freshwater Ascomycete Database (Shearer *et al.*, 2008), it is evident that the temperate species tested by Abdel-Raheem and Shearer (2002) included some isolated from only lentic or lotic habitats as well as some habitat generalists. When analyzed based on habitat distribution, their data are in agreement with the lack of difference seen here between lentic and lotic temperate species in degradative capabilities. No previous study of freshwater ascomycete decay capabilities included any species from tropical lentic habitats. Here, one such species, *S. tropicale*, was examined and produced only those enzymes which all species produced (amylase, ß-glucosidase, and xylanase) and failed to form soft cavities. However, more topical lentic ascomycetes need to be assayed to obtain a better understanding of the degradative capabilities of species distributed as such.

The majority of the species tested here were lignicolous, but two were isolated from herbaceous material. The data for the temperate lentic ascomycetes here match those seen previously when freshwater ascomycetes were more comprehensively compared based on *in situ* substrate specificity (Abdel-Raheem and Shearer, 2002). Due to the very low sample size of herbaceous species, it is difficult to compare percentages of species that produced each enzyme based upon substrate specificity. However, one of the two species isolated from only herbaceous material tested positive for laccase, which is the major LME of some aggressive terrestrial white-rot fungi that can completely degrade lignin (Eggert *et al.*, 1996). Conversely, species that are only known to occur on woody debris produced pectinase enzymes. These results are in agreement with those of Abdel-Raheem and Shearer (2002) and indicate that enzymatic capability alone is not likely to limit substrate distribution and that species found on either substrate can degrade a wide range of plant compounds. However, while four of the six lignicolous species produced soft rot cavities, neither herbaceous species was capable of decaying wood. Additionally, one of the species that has been reported from both woody and herbaceous substrates (*M. ingoldiana*) produced soft rot, while the other (*Massarina* sp. I: F 12-1) did not. More herbaceous freshwater ascomycetes need to be tested for the ability to decay wood to determine if any are capable of soft rot formation or if there is a link between wood decay ability and *in situ* substrate specificity.

The absence of a positive reaction should not be regarded as a complete inability of a species to produce the enzyme or rot being tested. Both the test medium (Zare-Maivan and Shearer, 1988b; Leung and Pointing, 2002) and protocol (Abdel-Raheem and Shearer, 2002) used have been shown to affect results of such tests. Because not all possible media and

protocol combinations were used here (due to logistical constraints), it is possible that more species are capable of producing an enzyme than just those reported positive. Particular attention should be paid to testing freshwater ascomycetes for the ability to break down chitin. Only one species in this study, *P. bipolaris*, tested positive for chitinase. *Arthrobotrys* sp. H 071-1, a known nematophage, did not test positive for chitinase, an enzyme needed to degrade the prey's cuticle (Huang *et al.*, 2004). Similar results have been reported previously, but with caution (Zare-Maivan and Shearer, 1988b), since it is likely that chitinase enzymes are inducible (Chamier, 1985) and it is therefore plausible that different results would be obtained under different culture conditions. Many fungi, especially those which grow on woody debris, are nitrogen-limited (Barron, 2003) and, in particular, soft rot-causing fungi have been shown to supplement their diet to obtain nitrogen (Filley *et al.*, 2001). In freshwater systems, substantial nitrogen is available as chitin in invertebrate exoskeletons and dead fungal mycelia (Dudley and Anderson, 1982; Chamier, 1985). The chitin-azure method employed here to test for chitinase has only been used once previously (Untereiner and Malloch, 1999) and never before to specifically test aquatic ascomycetes. Therefore, this method should be explored further with varying culture conditions before freshwater ascomycetes are dismissed as generally incapable of degrading chitin.

It has been well documented that Ingoldian hyphomycetes produce plant-degrading enzymes (Chamier, 1985) and break down leaf litter in streams (Suberkropp and Klug, 1976; Bärlocher, 1982). However, less is known about the ability of lentic, as well as lignicolous lotic, meiosporic ascomycetes to degrade dead plant material in freshwater systems. Including this study, over 150 freshwater ascomycete species have now been qualitatively tested in the laboratory for the ability to produce extracellular, degradative enzymes using methods similar to those employed here (Zare-Maivan and Shearer, 1988b; Abdel-Raheem, 1997; Yuen *et al.*, 1998, 1999; Abdel-Raheem and Shearer, 2002; Abdel-Raheem and Ali, 2004; Bucher *et al.*, 2004). Results from these studies are summarized in Table 4 and indicate that freshwater ascomycetes, in general, have the enzymatic capabilities necessary to break down lignocellulose, plant starch, and pectin, as well as degrade wood via soft rot. Therefore, these fungi should be considered as likely key players in the process of carbon recycling in freshwater systems.

Table 4. Summary of results of enzyme and rot assays of freshwater ascomycetes from this study and other published similar studies (see text for citations).

Substrate	Species Tested	No. Species Positive	% Species Positive
Cellulose	164	134	82
Hemicellulose	129	121	94
Lignin	175	110	63
Starch	109	99	91
Pectin	74	49	66
Wood (Soft Rot)	57	45	79

Recent ecosystem-wide studies have elucidated the importance of allochthonous carbon, such as lignocellulose, to the energy budget of both lentic (*e.g.* Pace *et al.*, 2004) and lotic (*e.g.* Wallace *et al.*, 1997) habitats indicating that the mechanisms responsible for the turnover of this material are crucial to the functioning of both types of habitats. However, such studies do not typically address the initial stages of degradation of allochthonous carbon-based compounds. Here it is shown that, regardless of their native ecoclimate, habitat, and substrate, ascomycetes isolated from freshwater have the capability to turn over this crucial source of energy for heterotrophs in freshwater systems (Wetzel, 1995), and thus fill this critical niche. However, the relationship between *in vitro* breakdown of isolated plant substrates by fungi and the *in situ* effects these fungi have on plant debris and the subsequent importance of this pathway of carbon turnover to the functioning of freshwater ecosystems are yet to be determined and therefore offer exciting avenues for future research. Molecular and stable isotope field methods must be worked out for these fungi in order to provide researchers with the tools necessary to address these questions so critical to freshwater ecology.

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