

Phylogeny and Ecophysiology of Opportunistic “Snow Molds” from a Subalpine Forest Ecosystem

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Abstract Mats of coenocytic “snow molds” are commonly observed covering the soil and litter of alpine and subalpine areas immediately following snow melt. Here, we describe the phylogenetic placement, growth rates, and metabolic potential of cold-adapted fungi from under-snow mats in the subalpine forests of Colorado. SSU rDNA sequencing revealed that these fungi belong to the zygomycete orders Mucorales and Mortierellales. All of the isolates could grow at temperatures observed under the snow at our sites (0°C and –2°C) but were unable to grow at temperatures above 25°C and were unable to grow anaerobically. Growth rates for these fungi were very high at –2°C, approximately an order of magnitude faster than previously studied cold-tolerant fungi from Antarctic soils. Given the rapid aerobic growth of these fungi at low temperatures, we propose that they are uniquely adapted to take advantage of the flush of nutrient that occurs at the soil–snow interface beneath late winter snow packs. In addition, extracellular enzyme production was relatively high for the Mucorales, but quite low for the Mortierellales, perhaps indicating some niche separation between these fungi beneath the late winter snow pack.

Introduction

Recent research in alpine and subalpine ecosystems has revealed that microbes are abundant and active under late winter snow packs [13, 23]. The subnival environment is ideal for the growth of cold-loving and cold-tolerant

microbes due to prolonged periods of stable temperatures and abundant moisture [11]. In fact, it is very common to observe mats of “snow mold” covering plant litter and soil as snow banks recede in the spring at alpine and sub-alpine sites in the Rocky Mountains [22]. These fungal communities are ephemeral and rapidly disappear once the snow is gone.

We know very little about the types of fungi that function during the final months of snow cover in seasonally snow-covered environments. Environmental clone libraries of under-snow soils from alpine tundra and subalpine forests have revealed many fungal groups that may be unique to the under-snow environment and some that have never been cultured [15, 21]. These fungi may contribute substantially to biogeochemical fluxes beneath the snow and understanding their physiologies could lend important insight into how global biogeochemical cycles will change as global warming affects both the duration and depth of snow packs over much of the Earth. For example, biogeochemical studies of subalpine forest soils in Colorado have shown that microbial communities are very active under the late winter snow pack resulting in high levels of extracellular enzyme activity and CO₂ fluxes from snow-covered soils [16, 28]. At these same sites, we have consistently observed mats of snow mold covering the ground as the snow banks recede. Here, we describe initial studies of the phylogenetics and physiology of several fungi that were isolated at low temperatures from samples of the under-snow fungal mats.

Materials and Methods

Study Site

The study site is at 3,050 m above sea level 25 km west of Boulder, Colorado (40°1'58"N; 105°32'47"W) near the

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University of Colorado Mountain Research Station. The forest is dominated by *Pinus contorta* (lodgepole pine), *Abies lasiocarpa* (subalpine fir), and *Picea engelmannii* (Engelmann spruce). The soils are sandy inceptisols derived from granitic moraine and are covered by an organic horizon that ranges in depth from 0–6 cm. Detailed descriptions of the site can be found in Monson et al. [16] and Weintraub et al. [28]. The site is generally covered with snow pack to an average depth of approximately 1.5 m from October through May each year. Soil temperatures average 0.3°C at this site with a lengthy period in late winter and early spring when the temperature is very close to 0°C.

Fungal Isolations

Fungal samples (hyphal pieces) were aseptically collected from extensive fungal mats observed in the spring of 2005 at our subalpine forest sites in Colorado. Samples for this study were collected at the edges of rapidly receding snow banks on June 8, 2005. These fungal mats disappeared within days of the soil becoming snow free. Fungal samples were aseptically transported to the laboratory and kept at –20°C until used. Within 24 h of collection, hyphal fragments were inoculated directly onto forest litter (DF) agar plates and incubated at 3.8°C in the dark. The DF media used for the initial fungal isolations was composed of: 4.0 g of forest floor litter; 0.5 g of KCl; 1.0 g of KH₂PO₄; 1.0 g of (NH₄)₂SO₄; 2.0 g of NaNO₃; 0.5 g of MgSO₄ 7H₂O; and 20 g of agar (per liter of water). Trace minerals consisted of 50 mg of CaCl₂, 10 mg of FeSO₄, 10 mg of CuSO₄, 5 mg of MnSO₄, 1 mg of ZnSO₄, and 1 ml of soil extract solution (per liter of water) prepared as described by Meyer et al. [14]. After autoclaving, chlortetracycline and streptomycin (50 mg/l for both antibiotics) were added to inhibit bacterial growth. Isolates were subcultured several times to assure single, uncontaminated fungal colonies. We chose three isolates for physiological studies because they exhibited the most rapid growth rate at low temperatures and because they represented two phylogenetically diverse groups of the Zygomycota (see below). The fungal colonies of all of the isolates exhibited white, coenocytic mat-like mycelia (for photos see <http://amo.colorado.edu/photo-sno-1.html>) that quickly cover the entire plate (e.g. in about 10 days at 3.8°C for isolates 316 and 319). These mycelial mats resemble the mycelial mats observed at the site as snow banks recede.

Growth Rates

To quantify the effects of temperature on growth rate, we measured rates of radial growth on agar plates as described by Kerry [10]. The media used for growth experiments was

composed of the same ingredients as above except that forest floor litter was replaced with 5.0 g of inulin and 0.5 g yeast extract, and the concentration of MgSO₄ 7H₂O was increased to 12.3 g per liter. Fungal colonies used as inoculum for the growth experiments were grown at 3.8°C. Uniform “plugs” of inoculum were obtained by using sterile 6 mm diameter AcuPunch Biopsy Punches (Acuderm Inc., Ft. Lauderdale, FL, USA). All inoculum plugs were taken at the same distance from the center of the master plate to insure that the fungi used for all temperature treatments and replicates were at the same metabolic state at the beginning of the experiment. Three plates were inoculated for each isolate at each temperature tested (–2, 0, 4, 15, 21, 25, and 28°C), with the inoculum placed in the center of each plate. Radial growth was measured by marking the bottom of the plate in four locations at each time interval. Radial growth rates were determined using linear regression of plots of radial growth versus time. Curves for determining growth rates were used if they contained at least six time points per curve with an *R* value greater than 0.99 (*R*²>0.98).

To determine if growth of cultures followed Arrhenius-type temperature responses (i.e. growth rate being exponentially proportional to temperature) at low temperatures and growth inhibition (due to enzyme inhibition at temperatures above the optimum) at high temperatures, we used a simplified form of the integrated equation as described by Schoolfield et al. [24]:

$$r(T) = r_0 T/T_0 \exp[E_1(1/T_0 - 1/T)] / [1 + \exp[E_2(1/T_m - 1/T)]] \quad (1)$$

where *r*(*T*) is the radial growth rate at temperature *T* and *r*₀ is the radial growth rate at the temperature optimum (*T*₀), *E*₁ is the enthalpy of activation of the limiting enzyme divided by the universal gas constant (8.314 J K^{–1} mol^{–1}), *E*₂ is equal to the change in enthalpy of high temperature inactivation of the limiting enzyme divided by the universal gas constant and *T*_m is the temperature at which the growth-limiting enzyme is functioning at half-maximal activity. Initial estimates (starting values) for nonlinear regression analyses were obtained by using linear plots of the natural log of rate vs. 1/°K as described by Heitzer et al. [6] and Davidson et al. [4].

DNA Extraction, Sequencing, and Phylogenetics

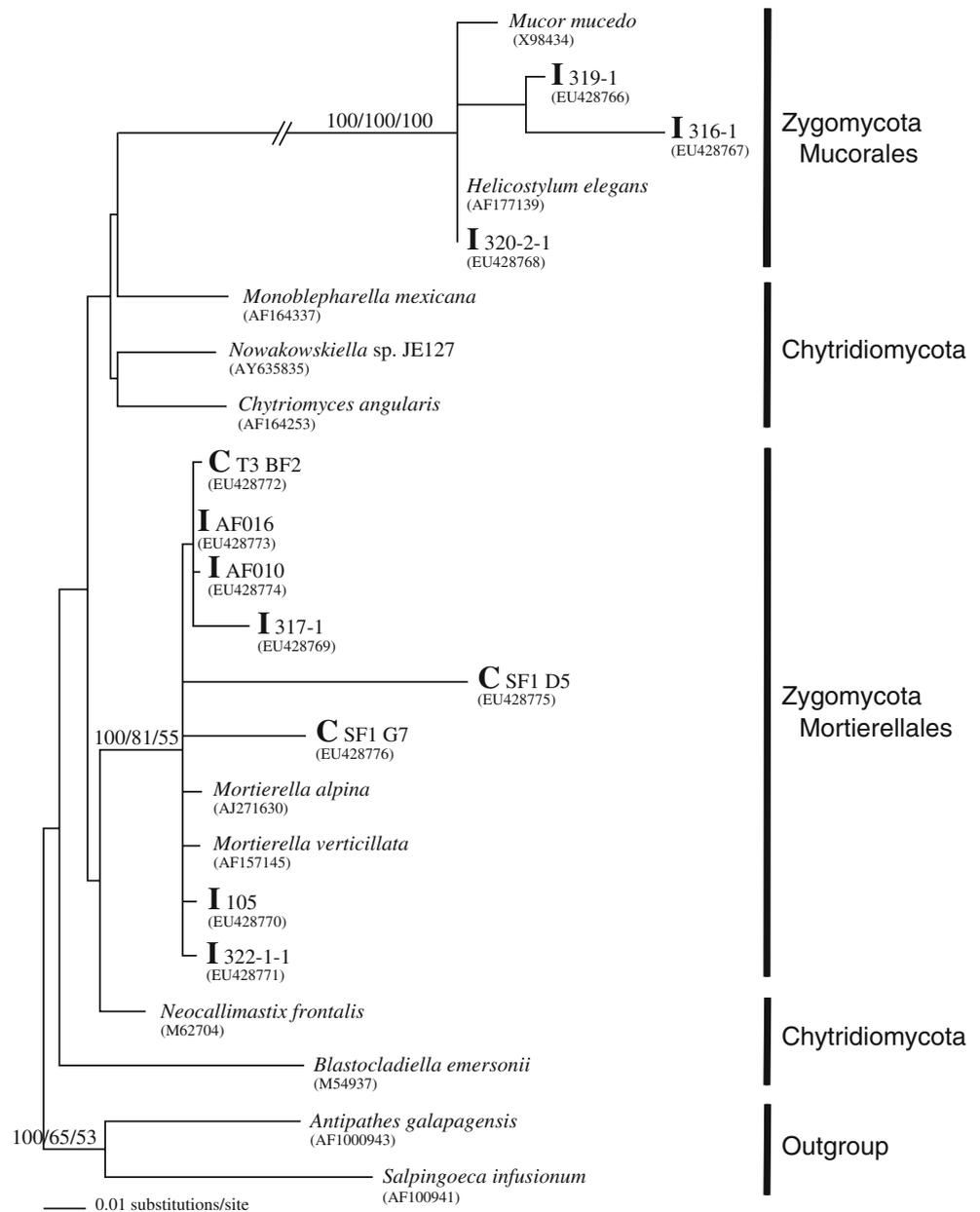
Biomass for sequencing isolates was grown in liquid media described above except that agar was not included. After cultures reached stationary phase the biomass was harvested by centrifugation, lyophilized, and stored at –80°C. DNA was extracted using the Omega bio-tek E.Z.N.A. Fungal DNA Kit (D3390). The Small Subunit of ribosomal

DNA was amplified using standard polymerase chain reaction (PCR) with generic fungal primers (e.g. NS1, NS4, and ITS4). PCR product was cleaned-up using shrimp alkaline phosphatase and exonuclease-1 enzymes per the manufacturer's instructions (USB Corporation, Cleveland, OH, USA). Sequencing of the SSU rDNA gene was done by Functional Biosciences, Madison WI, USA.

Sequences from isolates and clones were compiled using Sequencher 4.1 (Gene Codes Corp, Ann Arbor, MI, USA). We chose three isolates for physiological studies based on their rapid growth rates at low temperatures, the length of sequence obtained, and their phylogenetic diversity (see Fig. 1). The sequence lengths for these isolates are as

follows: isolate 316-1 (1,560 base pairs); isolate 317-1 (1,892 base pairs); and isolate 319-1 (800 base pairs). Isolate sequences were aligned in ARB (<http://www.arb-home.de>) with selected Zygomycota and Chytridiomycota guide sequences compiled from recent molecular studies of fungi [9, 26]. Alignments were exported from ARB using a mask that excludes ambiguously aligned positions. We employed MrModeltest 2.2 [17] to determine the best-fit model and parameters of nucleotide substitution for each alignment, and this model was used for our phylogenetic analysis. Bootstrapping using distance and maximum parsimony (1,000 resamplings) were performed in PAUP* (version 4.0b8a, Sinauer Associates, Inc., Sunderland, MA,

Figure 1 Bayesian phylogenetic tree based on 18S rDNA sequences showing the relationship of the isolates 316-1, 317-1, and 319-1 to other isolates and sequences from under-snow environmental clone libraries of the same gene [15]. Isolates are represented by an initial letter "I"; environmental clones by an initial letter "C". GenBank accession numbers are given in parentheses. Support at selected nodes is Bayesian posterior probability, neighbor joining distance bootstraps, and maximum parsimony bootstraps (i.e. Bayes-PP/NJ-BS/MP-BS)



USA). Bayesian analysis and posterior probabilities were determined by Markov chain Monte Carlo methods implemented using MRBAYES [7, 19]. A total of 1,370,000 generations were run and burn-in was clearly accomplished after 100,000 generations. Trees prior to burn-in were omitted. Consensus trees were generated and branch lengths estimated with PAUP* 4.0b10 using maximum likelihood and the model parameter values for the substitution model generated in MrModeltest 2.2.

Enzyme Assays

The activities of ten extracellular enzymes were assayed for each of the fungal isolates. Weintraub et al. [28] quantified activities of these enzymes on a year-round basis at our study sites and we endeavored to measure enzyme activities of the isolates using conditions as close as possible to those used by Weintraub et al. [28]. Thus, fungal isolates were grown at 14°C until they had reached stationary phase (approximately 18 days as determined by previous experience) in an Inulin based liquid media (identical to the DF media except Inulin 5 gm/l was substituted for the forest litter and no agar was added). The samples were then stored at 4°C for 5 days. Samples were filtered to remove fungal biomass and the filtrate was analyzed for enzyme activity using the procedures outlined by Saiya-Cork et al. [20]. Sterile media was used for control wells. Assays were conducted in 96-well plates with measurements on each isolate conducted in 16 replicate wells for all assays.

All of the enzyme assays except phenol oxidase, peroxidase, and urease were fluorimetric. The phenol oxidase and peroxidase assays were colorimetric, using L-3,4-dihydroxyphenylalanine (L-DOPA) as the substrate. The colorimetric urease assay used urea as the substrate. The assay plates were incubated in the dark at 14°C for 18 h. Both fluorescence and absorbance were measured using a Synergy HT Multi-Detection Microplate Reader (Biotek, Winooski, Vermont). After correcting for quenching and for the negative controls, enzyme activities were expressed as nanomole reaction product per hour milliliter per media ($\text{nmol h}^{-1}\text{ml}^{-1}$).

Results

All of the isolates shown in Fig. 1 are rapidly growing, coenocytic white molds that belong to the phylum Zygomycota. Pictures of these isolates can be viewed at (<http://amo.colorado.edu/photo-sno-1.html>). We chose three isolates for in depth physiological studies based on their rapid growth at low temperatures and their phylogenetic diversity. Isolates 316-1 and 319-1 are closely related to one another and are in the order Mucorales, whereas isolate

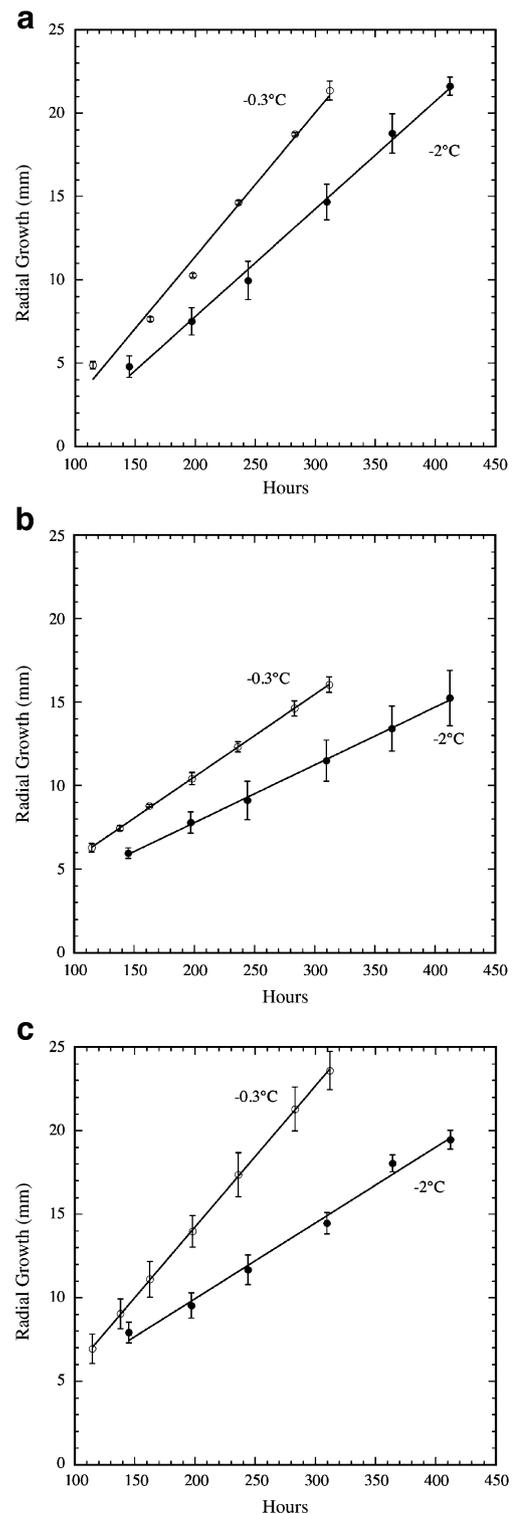


Figure 2 Growth of isolates 316-1 (A) and 317-1 (B) and 319-1 (C) at temperatures below 0°C. Lines are linear regressions and error bars represent standard deviations of the mean for three replicate curves at each temperature for each isolate

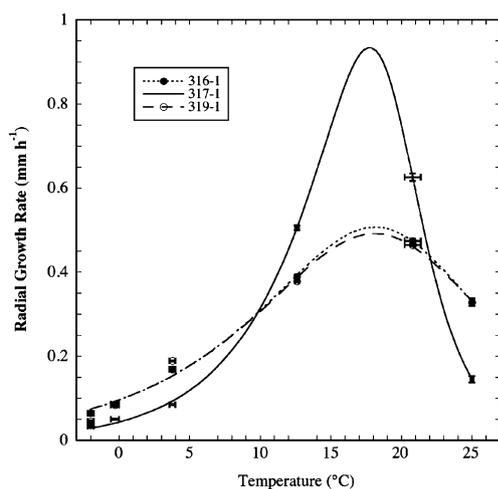


Figure 3 Temperature response curves for the growth of the three isolates at various temperatures. The rates plotted are the means of three replicate experiments at each temperature and error bars (both x and y errors) are one standard deviation of the mean. Curve fits are nonlinear regression fits of the Schoolfield equation (Eq. 1) to each data set. R^2 values for curve fits are 0.997, 0.999, and 0.984 for isolates 316-1, 317-1, and 319-1, respectively

317-1 is quite different phylogenetically and falls in the order Mortierellales (Fig. 1). Traditional morphological classification of these fungi is not possible at the present time because they have not sporulated in culture.

All three isolates could grow at the lowest temperature tested (-2.0°C) and were unable to grow at temperatures above 25°C . Plots of growth (radial extension) versus time for all three isolates at temperatures below 0°C are shown in Fig. 2. Growth rates for all isolates were estimated from these types of plots by using linear regression and the rates were plotted against temperature (Fig. 3). Equation 1 fit the data for all three isolates indicating Arrhenius (i.e. growth rate being exponentially proportional to temperature) behavior at low temperatures and enzyme inhibition at temperatures above the approximate optimal temperature ($\sim 18^{\circ}\text{C}$) for all isolates (Fig. 3).

Enzymatic activity of the isolates followed phylogenetic placement with the two Mucorales isolates (316-1 and 319-1) differing in pattern of activity from the Mortierellales

isolate (317-1). Isolate 317-1 was the only isolate to show peroxidase activity, but showed no ability to degrade *N*-acetylglucosamine polymers (Table 1). None of the isolates showed detectable extracellular urease, phenol oxidase, phosphatase, or peptidase activity.

Discussion

The three isolates were chosen for study because their morphology matched that of commonly observed snowmolds at our subalpine sites [22] and because they fell into clades that had already been identified as being found in under-snow clone libraries, but not in libraries produced from the same soils during the summer [15, 21]. These fungi are also similar to fungi that have consistently been found in cold Arctic and Antarctic soils [1, 2, 10].

The goal of our studies was to ascertain if these fungi could grow at reasonable rates at low temperatures commonly observed beneath the late winter snow pack. Soil temperatures at our subalpine forest field sites range from about -2°C to just above 0°C during the last 2 months of snow cover [16, 28]. Our isolates grew well at these temperatures in the lab (Figs. 2 and 3) and the rates observed are also very fast compared to Antarctic fungal isolates that have been studied at these same temperature ranges. Hughes et al. [8] found radial growth rates of from 0.001 to 0.003 mm h^{-1} for five Antarctic fungi at -2°C . Surprisingly, our snow mold isolates grew more than an order of magnitude faster, exhibiting radial growth rates of from 0.035 (± 0.005) to 0.065 mm h^{-1} (± 0.0007). To our knowledge, these are the fastest recorded growth rates for filamentous fungi at -2°C , but more work is needed to determine if differences in growth media could account for some of the difference between our results and studies such as Hughes et al. [8].

These growth rates are also fast enough to account for the development of the visible snow mold mats that cover the ground under the snow pack in the spring. Even a single locus of inoculum (e.g. a single spore) could expand to an area of over 15,000 mm^2 over a 2-month period if it grew at

Table 1 Enzyme activity (and standard error) for the three isolates

Isolate	Enzyme					
	NAG	CB	AG	BG	BX	Perox
316-1	52 (2)	2.7 (0.1)	24.6 (0.4)	5 (0.2)	0.9 (0.1)	0
319-1	85 (2)	38.0 (1.1)	29.6 (0.7)	90 (2.6)	1.0 (0.1)	0
317-1	0	0.6 (0.1)	1.5 (0.1)	0.5 (0.1)	0.3 (0.4)	41 (15)

Units are $\text{nmol ml}^{-1} \text{h}^{-1}$ for all enzymes. None of the isolates showed detectable urease, phosphatase, phenol oxidase, or peptidase activity. Enzyme abbreviations: NAG (β -1,4-*N*-acetyl-glucosaminidase), CB (β -D-1,4-cellobiosidase), AG (α -1,4-glucosidase), BG (β -1,4-glucosidase), BX (β -1,4-Xylosidase), Perox (Peroxidase)

an average rate of 0.065 mm h^{-1} for that period. Of course, there are probably many loci of inoculum in these seasonally snow-covered soils, and so even the very large mats of several square meters that we observe could reasonably be formed by these fungi.

Another goal of this study was to determine if our isolates expressed extracellular enzyme activity as has been measured at our sites in the winter. We measured the same suite of enzymes that Weintraub et al. [28] measured in under-snow soils and found that the two Mucorales isolates showed much higher activity of carbohydrate degrading enzymes than the Mortierellales isolate (317-1), whereas isolate 317-1 showed some peroxidase activity. Thus, it is possible that these fungi contribute to the patterns of enzyme activity that Weintraub et al. [28] observed in the field, with the Mortierellales contributing to the unexpectedly high peroxidase activity under the snow and the Mucorales isolates contributing to under-snow peaks in NAG, CB, AG, BG, and BX activity. However, none of the isolates have the capacity to contribute to the very high peptidase activity that Weintraub et al. [28] recorded in under-snow soils at our sites. Much more work would be needed to directly attribute an ecological function to any of our isolates, but our growth and enzyme-activity measurements demonstrate the potential for these fungi to contribute to high levels of under-snow microbial activity at our sites.

A Proposed Niche for Zygomycetous Snow Molds

Given the unusually fast growth of our isolates at low temperatures we propose that these fungi are fast growing, ruderal or r-selected species that exploit the unique high nutrient conditions that prevail during the final months of snow cover at our field sites. As soils thaw beneath the insulating blanket of late winter snow, nutrients are released from decomposing plant materials [3, 12] and from plant roots [25]. We propose that fast growing snow molds participate in plant litter decomposition and can uniquely exploit the high availability of nutrients by growing rapidly at the soil-snow interface where they receive nutrients from the soil below them and water and O_2 from the snow pack above. We already know that many soil organisms are also very active in the soil column during this period [16, 28] and it is very likely that soils become saturated and anoxic during the final stages of snowmelt. All three of our isolates are strict aerobes (Wilson and Gebauer, unpublished data), which could further explain their mat-like growth on the soil surface where they can access atmospheric O_2 even when soils below them are anoxic.

Evidence from other ecosystems supports a ruderal niche for cold-tolerant zygomycetes. Wynn-Williams [29] asserted that fungi in the *Mortierella* and *Mucor* groups are important transient mineralizers of dissolved organic

carbon (DOC) in many cold Antarctic soils and Kerry [10] found that several *Mortierella* spp. exhibited the highest growth rates at 4°C of the over 20 sub-Antarctic fungal species she tested. Tokumasu [27] observed that many *Mortierella* spp. are secondary colonists of fallen pine needles especially during the colder parts of the year, indicating that they take advantage of nutrients released by slower-growing fungi that begin the needle breakdown process.

The enzyme activity measurements support the hypothesis that our *Mortierella* snow fungus (317-1) is mineralizing readily available dissolved organic substrates rather than breaking down soil litter polymers. Cold-tolerant *Mortierella* spp. have also been characterized as being “saccharophilic” [5] or “sugar fungi” [18, 29] meaning that they grow rapidly in response to sugars released into the soil solution. Pugh and Allsop [18] speculated that *Mortierella* are so abundant in vegetated Antarctic areas “because of the nutrients which are made available by the breakdown of plant cells during freeze–thaw cycles, and possibly during normal plant exudation”. Their ability to rapidly grow on sugars may further explain why they are so abundant at our forested sites in Colorado. Recently Scott-Denton et al. [25] demonstrated that Lodgepole Pines (*P. contorta*) release large amounts of sugar (especially sucrose) from their roots in the late winter at our sites. These readily available sugars add to the already rich soup of nutrients that we believe fuels the rapid growth of snow molds at the snow–soil interface.

In summary, the fungi described herein have the potential to play an important role in low-temperature carbon metabolism and respiration as transiently abundant decomposers in alpine forest soils. The overall role of Zygomycetes in alpine forest respiration and the turnover of carbon in forest litter needs additional study, especially with regard to how much their rapid growth at low temperatures contributes to the exponential patterns of CO_2 flux that Monson et al. [16] have observed during the late winter at our sites.

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