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Temperature acclimation effects on growth, respiration and enzyme activities in an arctic and a temperate isolate of *Cenococcum geophilum* Fr.

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Abstract: Growth and respiration of ectomycorrhizal fungi associated with dwarf shrubs likely contribute significantly to carbon cycling in arctic tundra soils dominated by this vegetation type. Despite their importance little is known about how these fungi might respond to global warming. While previous studies have shown that some ectomycorrhizal fungi can tolerate or retain viability across a fairly wide range of temperatures little is known regarding their metabolic responses to temperature shifts. The present study was undertaken to examine the comparative physiological responses *in vitro* of arctic and temperate isolates of a common ectomycorrhizal fungus to shifts in growth temperature. Isolates of *Cenococcum geophilum* from Alaska and Maryland were grown at 12 and 20°C in liquid culture to study the process of temperature acclimation. Measurements on each isolate at the two growth temperatures included linear growth rates, dry weight accumulation, oxygen consumption and the specific activities of the soluble enzymes glucokinase, phosphoglucose isomerase, 6-phosphogluconate dehydrogenase and glucose-6-phosphate dehydrogenase. In addition the response to growth temperature of glucokinase kinetic parameters (Ea, Km and Vmax) were also evaluated. Increasing the growth temperature tended to decrease the length of the observed lag phase; however, the growth rates in dry weight at 20°C closely paralleled those at 20°C. Both isolates demonstrated lower respiration rates when grown at 20 versus 12°C. Thus for oxygen uptake each isolate demonstrated a phenotypic response known as ideal rate compensation. That is growth at 20°C resulted in oxygen uptake rates at 20°C that were similar to those

measured at 12°C for 12°C-grown mycelium. This strategy, if common in ectomycorrhizal fungi, would reduce the expected carbon demand placed on the host and decrease the amount of carbon dioxide released by respiration in response to anticipated increases in soil temperatures. Differences in overall respiration rates by 12 and 20°C grown mycelium could not be explained by changes in activities of soluble enzymes examined. Likewise few differences were observed in glucokinase kinetic parameters associated with growth temperature. Examination of soluble enzyme activity ratios as influenced by growth temperature suggests the potential exists to alter relative fluxes through primary metabolic pathways and warrants further investigation. Future studies of temperature acclimation should examine a wider range of ectomycorrhizal fungi and employ techniques such as DNA microarrays and metabolomics.

Key words: Arctic*, Cenococcum geophilum*, ectomycorrhizal fungi, enzyme activity, glycolysis, Km, oxygen uptake, pentose phosphate pathway, respiration, temperature acclimation, tundra.

Introduction: The ongoing input of large quantities of anthropogenic greenhouse gases has led to a consensus that the earth's climate is warming and will continue to do so in the foreseeable future (Steffen et al. 2007). Direct observations and models suggest the amount of surface warming will increase with increasing latitude (Nogués-Bravo et al. 2007, Overland et al. 2008). As high latitude tundra soils contain large stocks of carbon the question has been raised as to whether these soils will, under warmer conditions, continue to behave as sinks or becomes sources for greenhouse gases (Billings et al. 1982, Davidson and Janssens 2006). A crucial aspect of this question relates to the contribution of soil respiration in CO_2 efflux. Attempts to model soil respiration under global warming scenarios usually use instantaneous respiration measures and extrapolate based on fixed Q_{10} values (the ratio of two measured respiration rates separated by 10 degrees Celsius) (Tjoelker et al. 2001). Several potential errors can occur with this approach (Atkin and Tjoelker 2003, Atkin et al. 2005). For example organisms over time may adjust their basal metabolism, Q_{10}, or both to a new thermal regime, thus projections of unadjusted respiratory CO_2 release would be erroneous.

Acclimation to temperature involves adaptive alterations to metabolism, sometimes termed metabolic rate compensation (Hochachka and Somero 1973), which can take on a variety of patterns (Atkin and Tjoelker 2003). Full acclimation or ideal rate compensation occurs when an organism, after a suitable acclimation period, adjusts its respiratory rate such that the rate measured at the new temperature is essentially equivalent to that measured at its previous growth temperature (Atkin et al. 2000). In other words an organism grown at 10°C respires at an equivalent rate at 10°C as it would at 20°C when grown at 20°C. An acclimation pattern of this sort acts to lower the respiratory response to temperature increases compared to expectations based on short term measures or as projected by a simple Q_{10}. If soil organisms were to demonstrate full acclimation to changing soil temperatures over time this would mean that estimates of CO_2 release based on instantaneous temperature shifts and Q_{10}s would potentially overestimate soil carbon efflux.

Studies conducted to date suggest a range of acclimatory responses are possible for roots, whole soil or soil-associated taxa (Bååth and Wallender 2003, Kirschbaum 2004, Lange and Green 2005, Heinmeyer et al. 2007, Vicca et al. 2009). For example, Cooper (2004) found that roots of arctic *Ranunculus* species differed in acclimation potential when grown under controlled conditions. Some species, like those restricted to wet continually cold sites failed to acclimate, whereas others fully acclimated. The

latter strategy would potentially conserve carbon in soils that warm in summer. Recently Hartley et al. (2008) have suggested that plants tend to acclimate fully whereas soil microorganisms do not. To date little is known concerning the ability of ectomycorrhizal fungi to acclimate to temperature (López-Gutiérrez et al. 2008).

Ectomycorrhizal (ECM) fungi can in certain ecosystems constitute a significant portion of the fungal mycelium and hence carbon associated with roots (Antibus and Sinsabaugh 1993, Högberg et al. 2002) or soil (Wallender 2006). ECM shrubs are widespread and important in arctic and alpine tundra (Smith and Read 1997, Cripps and Eddington 2005, Tsuyuzaki et al. 2005, Watling 2005). Given the widespread occurrence of ECM hosts and associated mycelium these fungi could potentially be important as agents sequestrating carbon in biomass or returning photosynthetic carbon via respiration under changed climate conditions (Alberton et al. 2005). At present our knowledge is rather limited relative to both diversity and functioning of ectomycorrhizas and associated fungi in arctic and alpine systems (Gardes and Dahlberg 1996, Robinson 2001, Tibbett and Cairney 2007). The goal of the present study was to examine temperature acclimation and its physiological basis in *Cenococcum geophilum* Fr. through the comparison of isolates of arctic and temperate origin. *Cenococcum geophilum* was selected for study as it is a widespread and variable species (Shinohara et al. 1999). Isolates of *Cenococcum* show high genetic diversity and a capacity to adapt to a range of soil conditions (Panaccione et al. 2001, Conçalves et al. 2009). This species has also been found to be a common mycobiont in a number of arctic and alpine systems especially in association with *Salix* and *Dryas* species (Antibus and Linkins 1978, Harrington and Mitchell 2005, Mühlmann and Peintner 2008, Hrynkiewicz et al. 2009). The specific objectives of the present study were to compare arctic and temperate *C. geophilum* isolates in terms of (1) growth in solid and liquid media, (2) respiration at different growth and measurement temperatures and (3) production and kinetic properties of metabolic enzymes at different growth and assay temperatures.

Abbreviations: ECM: ectomycorrhizal; TES: N-tris(hydroxymethyl)methyl-2-amino-ethanesulfonic acid; BSA: bovine serum albumin; BME: β-mercaptoethanol; EDTA: ethylenediamine-tetraacetic acid; IU: International Units; GK: glucokinase; PGI: phosphoglucose isomerase; 6PGU: 6-phosphogluconate dehydrogenase; 6GPD: glucose-6-phosphate dehydrogenase; Ea: Arrhenius activation energy.

Materials and Methods:

Fungal isolates

Fungi used in the present study were *Cenococcum geophilum* Fr. (Virginia Tech-VT 715, isolated from roots of *Pinus virginiana* in Maryland by J.F. Worley) and *C. geophilum* (VT-1005, isolated from *Salix rotundifolia* mycorrhizae at Cape Simpson, Alaska (71.03° N 154.45° W). These isolates from this point on will be referred to as CgMD and CgAK. Both *C. geophilum* isolates were maintained on a modified Hagem's medium (Antibus 1980).

Linear growth studies

Linear growth studies were conducted in standard 90 mm Petri dishes containing 20 ml Hagem's agar using starters grown at the appropriate temperature (except 5°C Maryland isolate-starters grown at 10°C) for 28 days. Dishes were inoculated with mycelium taken from the growing margin of starters with a sterile 4 mm cork borer. Five replicate plates were inoculated in the center and incubated at 5, 10, 15, 20, and 25°C. Mycelial growth in plates was measured every 5 days by measuring from the edge of the agar plug to the growing margin in four separate directions for a total of 60 days. Linear growth rates were determined by linear

regression analysis and converting linear rates to areas assuming circular colony morphology.

Growth in liquid culture

Inocula for growth in liquid culture, respiration and enzyme studies were grown at 12 and 20°C 125 ml flasks containing 25 ml of the appropriate liquid medium. Starter flasks were inoculated with two 4 mm plugs taken from the growing margin of cultures on solid medium. After 21 to 25 days growth, mycelial mats were dispersed in 25 ml of medium with a sterile Eberbach semimicrohomogenizer and 1 ml aliquots of this suspension were used as inocula. Both isolates were grown in 25 ml of modified Hagem's in 125 ml flasks in static culture at 12 and 20°C for the periods indicated in each experiment. Mycelia were harvested by vacuum filtration onto pre-dried and weighed discs of Whatman No.1 filter paper, washed with approximately 200 ml of distilled water, dried at 60°C for 48 h, cooled in desiccators and weighed. Dry weights were corrected for the dry mass in the original inoculum. Isolates were harvested at weekly intervals with four replicates per harvest. The pH was determined on cultural filtrate and residual glucose remaining in growth media at various sampling times was determined using a modified glucose oxidase procedure (Phillips and Elevitch 1968).

Oxygen uptake

Mats of mycelium 28-35 days old were aseptically transferred to sterile reaction flasks containing 3.0 ml of 0.1 M phosphate buffer pH 5.8. Oxygen uptake was then measured with a Gilson Differential Respirometer (Linkins and Antibus 1978). Comparative temperature profiles were obtained by initiating measurements at low temperatures and sequentially raising the sample temperature, and by starting at high temperatures and sequentially lowering the sample temperature. Parallel studies were conducted in which mycelial mats were transferred to respiration flasks containing sterile growth medium, to test whether mycelial carbohydrate status might alter the results. Mycelial dry weights were obtained as described previously. Values of Ea for oxygen uptake were obtained from Arrhenius plots as described by Segel (1976).

Extraction of soluble enzymes

Mycelia from 20 flasks of each isolate were harvested after 28-32 days of growth at 20°C and 35-40 days at 12°C. Preliminary experiments indicated that cultures at the two growth temperatures were at developmentally similar stages at these sampling dates based on growth rate, media pH and glucose levels. Mycelia were harvested by vacuum filtration, washed with 200 ml of distilled water, and resuspended in 150 ml of 25 mM TES (pH 7.5) containing 0.1% BSA, 0.1 mM EDTA and 0.01 mM BME. The mycelial suspension was divided into 50 ml samples and homogenized over an ice bath with a Brinkman Polytron at full power for two 15 s bursts. Homogenizations were separated by a 30 s cooling period. The homogenate was filtered through two layers of Miracloth and centrifuged at 4,000 x g for 15 min at 4°C. The resulting supernatant solution was centrifuged at 14,500 x g for 15 min. Final supernatant solutions were pooled and brought to 45% saturation with crystalline NH_4SO_4. This 45% fraction was stirred for 15 min at 4°C and centrifuged at 30,000 x g for 15 min. The resulting pellet was resuspended in 20 ml of 25 mM TES (pH 7.5) and stored on ice until further use. The supernatant solution from the 45% NH_4SO_4 fraction was brought to 90% saturation with crystalline NH_4SO_4, stirred for 15 min, and centrifuged at 30,000 x g for 15 min. The resultant pellet was resuspended in 20 ml of 25 mM TES (pH 7.5). The 45% and 90% fractions were dialyzed and concentrated using a UM-20 Amicon ultrafiltration system (Amicon Corp., Lexington, MA) to 10 ml of 25 mM TES final volume.

Soluble enzyme activities

Specific activities were determined by following the reduction of pyridine nucleotide (NADP) at

340 nm in 25 mM TES (pH 7.5), according to Wedding and Harley (1976). Assays were conducted in 0.5 ml cuvettes at 25.0°C using a Gilford Model 250 recording spectrophotometer equipped with a Thermoset attachment. The assay mixture was equilibrated at 25.0°C for 2 min prior to the addition of substrate. The soluble enzymes assayed and the procedures used are detailed below.

Glucokinase (D-hexose 6-phosphotransferase, E.C. 2.7.1.1). Assays contained 0.1 ml of 25 mM ATP in 50 mM $MgCl_2$, 0.05 ml of 5 IU G6PD, 0.05 ml of 1.5 mM NADP, 0.05 ml of 20 mM D-glucose and 0.25 ml of sample extract in 25 mM TES (pH 7.5).

Phosphoglucose isomerase (D-glucose-6-phosphate ketol isomerase, E.C. 5.3.1.9). Assays contained 0.1 ml of 50 mM $MgCl_2$, 0.05 ml of 5 IU G6PD, 0.05 ml of 1.5 mM NADP, 0.05 ml of 25 mM fructose-6-phosphate and 0.25 ml of sample extract in 25 mM TES (pH 7.5).

6-Phosphogluconate dehydrogenase (6-phospho-D-gluconate: NADP 2-oxido-reductasese, E.C. 1.1.1.44). Assays contained 0.05 ml of 50 mM $MgCl_2$, 0.05 ml of H_2O, 0.05 ml of 1.5 mM NADP, 0.05 ml of 25 mM 6-phosphogluconate and 0.30 ml of sample extract in 25 mM TES (pH 7.5).

Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP oxidoreductase, E.C. 1.1.1.49). Assays contained 0.05 ml of 50 mM $MgCl_2$, 0.05 ml of H_2O, 0.05 ml of 1.5 mM NADP, 0.05 ml of 100 mM glucose-6-phosphate and 0.30 ml of sample extract in 25 mM TES (pH 7.5).

All assays were conducted with three replicates plus a blank (0.05 ml of H_2O instead of substrate). Blank reduction of NADP occurred only in the glucokinase assay. Responses were linear over the time frames employed here. Specific activities were determined from initial velocities according to Segel (1976), with a molar extinction coefficient for NADP of 6.22×10^3. Protein determinations on all samples were made using the Bio-Rad Laboratories (Richmond, CA) modification of the method of Bradford (1976), with a bovine gamma globulin standard. Activity data for each enzyme and isolate at the two growth temperatures were analyzed by analysis of variance and compared by t-tests using SPSS programs (Nie et al. 1975). In cases where heterogeneity of variance could not be corrected by log transformation of data, nonparametric comparisons were utilized.

Enzyme kinetic analysis

Procedures for extraction and partial purification of enzymes are given in detail above. All assays were performed using proteins precipitated in 90% NH_4SO_4, followed by dialysis and concentration using ultrafiltration. Prior to enzyme assays, extracts were brought to constant protein concentrations in 25 mM TES (pH 7.5). GK and 6PGU were assayed as described above, except that substrate concentrations were varied. GK activity was measured at five or six substrate concentrations between final concentrations of 0.04 to 2.0 mM glucose. Preliminary experiments indicated the Km of 6PGU to be approximately 0.1 mM for 6-phosphogluconate, activity was measured at 0.1 mM (nonsaturating) and 1.0 mM (saturating) concentrations of substrate.

Assays were conducted in 0.5 ml cuvettes using a Gilford Model 250 spectrophotometer equipped with a Thermoset attachment. Assay mixtures were equilibrated to assay temperature for 2 min prior to substrate addition. Initial reaction velocity (vi) was measured in duplicate over a range from 2.5 to 25.0°C at each [s]. Km and Vmax values at each temperature were determined from Woolf-Augustinson-Hofstee plots of vi versus vi/[s] (Segel 1976) using linear regression analysis. Arrhenius plots were constructed using logarithms of Vmax plotted against the reciprocal of the absolute temperature. Values of Ea were obtained from

Arrhenius plots (Segel 1976). Protein determinations were made as described above.

Results:

Temperature and growth

The results for growth on solid media for 60 days are shown in Figure 1. Optimal growth for CgMD was 20°C and dropped off quickly below this temperature. CgAK grew well at lower temperatures and grew at a relatively constant rate from 12 to 20°C. In liquid culture CgMD and CgAK demonstrated prolonged lag phases of up to 21 days at 12°C after which point growth accelerated to nearly match rates at 20°C (data not shown). Growth rates calculated by running linear regressions of dry weight between days 28 and 70 were as follows (units of mg dry mass per day): CgMD 20°C = 1.1 (r^2=0.84), CgMD 12°C = 1.1 (r^2=0.99), CgAK 20°C = 1.6 (r^2=0.90) and CgAK 12°C =0.93 (r^2=0.94). Growth at 12°C of CgAK in liquid culture, in contrast to observations on solid media, did not equal the rate obtained at 20°C. Likewise, whereas CgMD grew significantly faster than CgAK at 20°C on agar, CgAK grew faster than CgMD at 20°C in liquid culture. Widden and Parkinson (1978) demonstrated a similar effect of culture medium form in the growth rate comparisons across temperature in arctic microfungi. Changes in pH and glucose concentrations in liquid media paralleled changes in dry mass (data not shown).

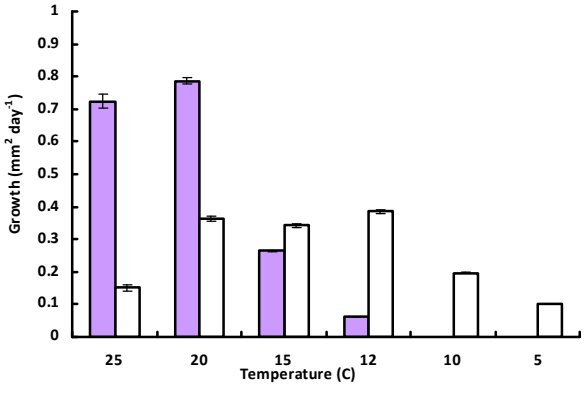

Figure.1. The effect of growth temperature on *Cenococcum geophilum* from Maryland (filled bars) or Alaska (empty bars). Values are mean areas determined from five replicate plates; vertical bars indicate standard errors of means.

The pH of media fell to 3.0 at both growth temperatures in CgMD and 2.5 at both temperatures in CgAK. Carbon was not likely limiting to growth as media contained nearly 60% of the original glucose at 56 days. I did not attempt to determine whether carbon use efficiency differed with growth temperature as the base medium did include asparagine as an additional growth factor.

The effect of temperature on oxygen uptake

The rates of oxygen uptake per unit dry weight were determined for each isolate using mycelia grown at 12 and 20°C (Fig. 2). Figure 2A records the response of oxygen uptake to temperature by the CgMD. Oxygen uptake rates were lower at all temperatures for mycelium grown at 20°C than for that grown at 12°C. When respiration at 20° C for 20°C-grown mycelium is compared to respiration at 12°C for 12°C-grown mycelium the two rates are roughly equivalent (Fig. 2A-dashed line). Although the shape of the temperature response differed slightly in CgAK (Fig. 2B) oxygen uptake rate was consistently lower in 20°C-grown tissue versus that grown at 12°C. Again oxygen consumption at 20°C for 20°C-grown cells is equivalent to or slightly lower than observed at 12°C for 12°C-grown cells. These observations remained valid whether sample respiration was first examined at lower temperatures and gradually increased or at higher temperatures and gradually decreased. Similar results were also obtained when oxygen uptake by mycelial slurries was measured with an oxygen electrode (data not shown).

Apparent activation energy (Ea) calculated from Arrhenius plots is commonly used to characterize the response of various processes such as, growth, respiration, ion uptake and enzyme activity to temperature. Values of Ea determined from oxygen uptake studies for isolates grown at 12 and 20°C are presented in Table 1. Although

Table 1. The influence of growth temperature (°C) and reaction medium on isolate Ea values (kilojoules) for oxygen uptake.

		Growth temperature	
Isolate	Reaction medium	12	20
C. geophilum (Maryland)	buffer	52.4	37.4*
	Hagem's	52.9	44.7
C. geophilum (Alaska)	buffer	64.6	45.0
	Hagem's	51.9	57.7

*Values for 20 and 12°C significantly different by Mann-Whitney U-test ($p \leq 0.05$).

Table 2. The influence of growth temperature on enzyme specific activities (nmoles min^{-1} mg^{-1} protein) measured at 25°C.

		Growth temperature	
Isolate	Enzyme&	12	20
C. geophilum (Maryland)	GK	11.9 ± 4.7	17.5 ± 7.0*
	PGI	98.4 ± 37.8	158.4 ± 36.9*
	6PGU	7.9 ± 3.3	11.5 ± 3.5
	G6PD	37.6 ± 28.4	17.0 ± 4.0
C. geophilum (Alaska)	GK	19.0 ± 10.1	22.5 ± 9.9
	PGI	94.0 ± 41.2	137.3 ± 117.5
	6PGU	14.4 ± 6.3	16.4 ± 9.2
	G6PD	39.5 ± 17.9	40.9 ± 16.1

& GK = glucokinase; PGI = phosphoglucose isomerase; 6PGU= 6-phosphogluconate dehydrogenase; G6PD= glucose-6-phosphate dehydrogenase. Mean ± standard deviation.

* Indicates values for 20 and 12°C are significantly different by Mann-Whitney U-test ($p \leq 0.05$).

Table 3. Influence of growth temperature on soluble enzyme specific activity ratios of *Cenococcum geophilum* isolates measured at 25°C.

		Growth temperature	
Isolate	Ratio	12	20
C. geophilum (Maryland)	PGI:G6PD	5.8	4.2
	PGI:6PGU	12.5	13.8
C. geophilum (Alaska)	PGI:G6PD	2.4	3.4
	PGI:6PGU	2.5	2.9

the plots are not presented, the response of oxygen uptake was linear over the range examined. In general, Ea values are lower for 20°C-grown mycelium than for 12° C-grown mycelium, although the difference is statistically significant only for CgMD measured in buffer. Were these curves to be used to calculate Q_{10}s, one would observe lower values for the 20°C-grown mycelium. Comparison of Ea values obtained with buffer or fresh culture medium (Table 1) indicates that high levels of external carbohydrates or nutrients did not meaningfully alter the pattern of results.

Effects of growth temperature on soluble enzyme activities

Table 2 presents mean total (45% + 90% fractions) specific activities for the enzymes glucokinase (GK), phosphoglucose isomerase (PGI), 6-phosphogluconate dehydrogenase (6PGU) and glucose-6-phosphate dehydrogenase (6GPD) from the fungi grown at 12 and 20°C. Data presented are means calculated for three to five separate experimental extractions. Growth at 12°C tended to lower mean specific activities of enzymes relative to growth at 20°C. Variability was observed from experiment to experiment, likely due to differential growth, extraction and precipitation of proteins, resulting in few statistically significant differences. Activities of both GK and PGI were significantly decreased for CgMD at 12°C (Table 2). Overall the extent of reduction of enzyme activities was greater in CgMD than CgAK; the average reduction was 39.2% in CgMD as opposed to 15.7% in CgAK. Table 3 presents the activity ratios of an enzyme associated strictly with the Embden-Meyerhof/ glycolytic pathway (PGI) with that of two associated with the pentose phosphate pathway (G6PD and 6PGU). In three out of four possible comparisons the ratios decrease with growth at 12°C suggesting the potential for a shift in relative activities of these pathways.

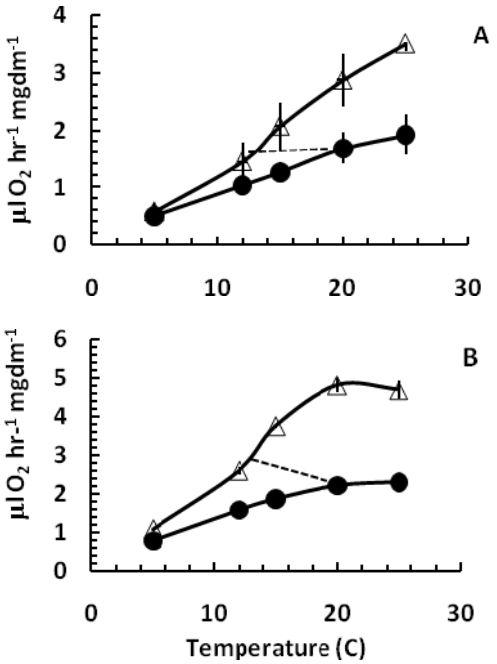

Figure 2. Oxygen uptake response curves for mycelium grown at mycelium grown at 12°C (Δ) or 20°C (●) for isolates of *Cenococcum geophilum* from Maryland (A) or Alaska (B). Vertical lines represent standard deviations.

Effects of growth temperature on enzyme kinetics

Arrhenius plots of Vmax for GK of isolates grown at 12 and 20°C are presented in Figure 3. The response of Vmax was linear over the range examined (2.5 to 25.0°C). The slopes of lines at the two growth temperatures were not significantly different ($p>0.05$) meaning that Ea was not affected by growth at the two temperatures examined. Ea values were similar in the two isolates. The responses of estimated GK Km values are shown in Figure 4. Km demonstrated a significant linear ($p<0.05$) decline with assay temperature at both growth temperatures in CgAK (Fig. 4B), a pattern known as positive thermal modulation (Hochachka and Somero 1973). A similar pattern was observed in CgMD when grown at 12°C but CgMD Km was unresponsive to temperature with growth at 20°C (Fig. 4A). Pairwise comparisons of Km for 12 and 20°C grown extractions did not reveal a significant effect on

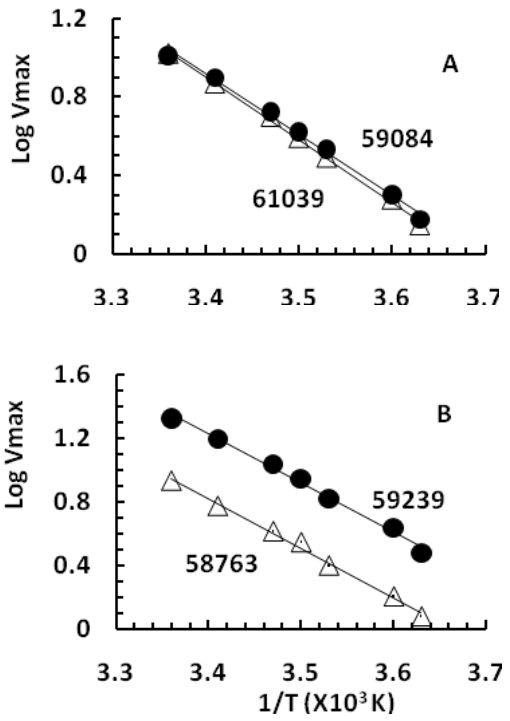

Figure 3. Arrhenius plots of Vmax for glucokinase activity from mycelium grown at 12°C (Δ) or 20°C (●) for isolates of *Cenococcum geophilum* from Maryland (A) or Alaska (B). Numbers next to lines represent Ea values in kilojoules. Each point is the mean of three to five experiments. Units are nmoles $NADP^+$ reduced $min^{-1}mg^{-1}$ protein.

GK Km at specific assay temperatures. Ratios of Vmax to Km for GK in the two isolates at the two growth temperatures are shown in Figure 5. Ratios declined with assay temperature in both isolates, in CgAK (Fig. 5B) growth at 20°C resulted in higher overall ratios.

Response to temperature curves for 6PGU activity determined at high and low substrate concentrations are shown for isolates at both growth temperatures in Figure 6. As expected temperature dependence of activity is greater at saturating than nonsaturating substrate concentrations, reflecting the potential role of substrate levels in controlling metabolic responses (Q_{10} for example) to temperature. Activity of 6PGU in CgMD was decreased by growth at 12° C. Activities determined at 25°C under saturating conditions are consistent with activity estimates for 6PGU in Table 2.

Discussion: Temperature plays a central role

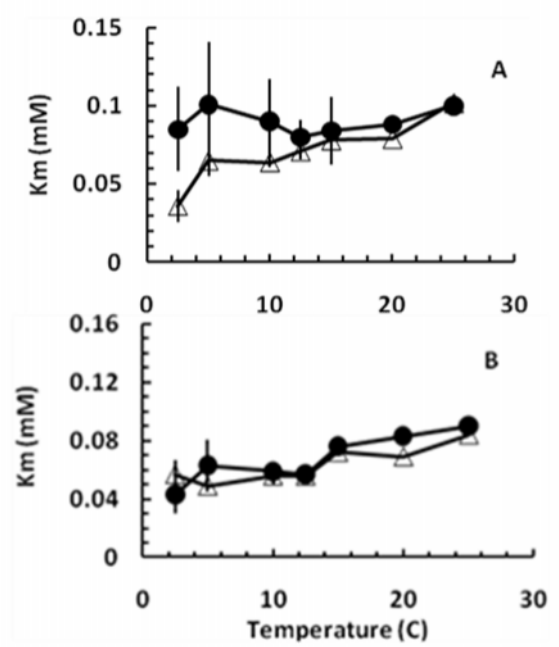

Figure 4. The effect of measurement temperature on Km values for glucokinase from mycelium grown at 12°C (Δ) or 20°C (●) for isolates of *Cenococcum geophilum* from Maryland (A) or Alaska (B). Each point represents the mean of three to five experiments; bars represent standard deviation.

in controlling all aspects of growth and development in ectothermic organisms (Clarke 2003). Fungi as ectotherms face two major challenges in dealing with environmental temperature fluctuation (Hochachka and Somero 2002), they must maintain structural integrity of macromolecules and macromolecular structures (proteins and membranes) and must maintain adequate energy generation such that processes like ion movement, macromolecule synthesis and replacement can continue. There is evidence that a range of ECM fungi can maintain structural integrity and grow after being exposed *in vitro* to a range of subzero temperatures (France et al. 1979, Cripps and Grimme unpublished). In fact they may possess abilities to survive subfreezing temperatures that exceed those of their

associated roots (Lehto et al. 2008). Potential mechanisms for dealing with freezing have been identified in arctic and temperate *Hebeloma*

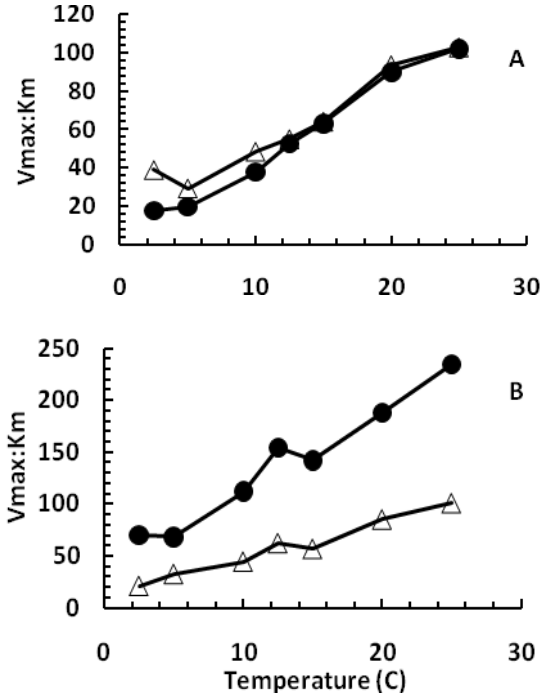

Figure 5. The effect of measurement temperature on Vmax to Km ratios for glucokinase from mycelium grown at 12°C (Δ) or 20°C (●) for isolates of *Cenococcum geophilum* from Maryland (A) or Alaska (B). Each point represents the mean of three to five experiments; bars represent standard deviation. Units are nmoles $NADP^+$ reduced $min^{-1}mg^{-1}$ protein mM^{-1} substrate.

spp. (Tibbett et al. 2002) and the field has been reviewed by Tibbett and Cairney (2007). Here, I am not concerned with temperature tolerance by *C. geophilum*, but instead have sought to understand how temperature impacts growth and aspects related to carbon metabolism in arctic and temperate isolates.

A number of the studies dealing with the effects of temperature on the growth of ECM fungi have employed the technique of harvesting the fungi once after a predetermined period of growth at each temperature (Mikola 1948, Theodorou and Bowen 1971). While the results from such studies provide valuable information on the relative rates of growth of fungi at different temperatures, they do not provide information about the maximal growth rates possible at such

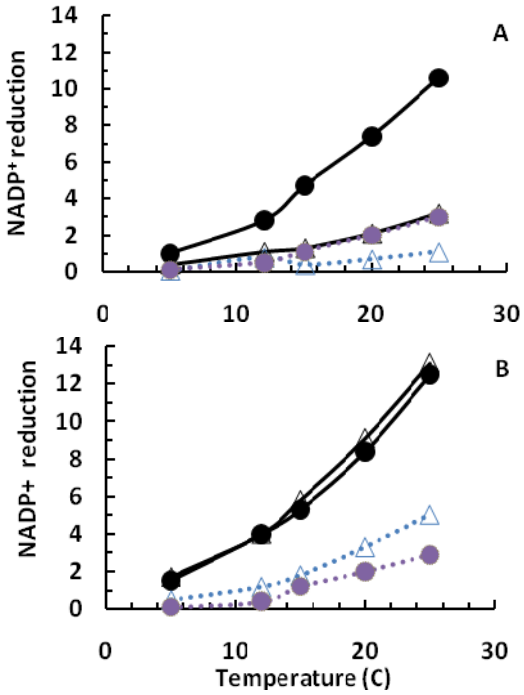

Figure 6. Response to measurement temperature curves for 6-phosphogluconate dehydrogenase activity grown at 12°C (Δ) or 20°C (●) for isolates of *Cenococcum geophilum* from Maryland (A) or Alaska (B). Measurements were made with substrate concentrations of either 1.0 mM (solid line) or 0.1 mM (dashed line). Units are nmoles $NADP^+$ reduced min^{-1} mg $protein^{-1}$.

temperatures. From the present study, it is clear that a temperate isolate of *C. geophilum*, after a marked lag phase when grown at 12°C, is capable of a growth rate during linear growth equaling that observed at 20°C. Such results would not be apparent using a single harvest technique. Hacskaylo et al. (1965) obtained results similar to the present findings using ECM fungi grown over a range from 2 to 29°C. Most of their isolates exhibited maximal growth rates which were nearly temperature independent over a range from 13 to 29°C. In several of their isolates, prolonged lag phases were also observed at lower growth temperatures. The results of my study and those of Hacskaylo et al.

(1965) indicate that certain ECM fungi have the genetic potential to maintain a relatively constant maximal growth rate in pure culture over a range of temperatures. Cline and Reid (1987) also studied the growth of *C. geophilum* isolates from a wide range of environments in liquid culture. They used a range of temperatures from 16 to 32°C and found individual isolates with optimal growth at 16, 21 and 28°C and indicated that some isolates might grow below 16°C if tested. On solid media we found clear differences between CgMD and CgAK. CgAK grew well at 5°C and actually performed best at intermediate temperatures; CgMD grew very poorly below 10°C even though this isolate had been maintained for years as a stock culture at approximately 4°C. The growth of CgAK is not surprising given that soils on the North Slope of Alaska are underlain with permafrost and subsurface soil temperatures seldom exceed 5°C (Zak and Kling 2006).
The ability of an organism acclimated at different temperatures to maintain metabolic function at similar levels at these temperatures has been termed metabolic rate compensation (Hochachka and Somero, 1973). Rate compensation must act to maintain relative homeostasis despite changes in the average kinetic energies of molecules involved in reactions. A large body of information has accumulated concerning the effects of acclimation temperature on respiration (Prasad et al. 1979, Graham and Patterson 1982, Atkin and Tjoelker 2003, Lange and Green 2005, Tjoelker et al. 2009). However, very few studies have dealt with ECM fungi (Tibbett and Cairney 2007).

In a pair of recent studies López-Gutiérrez et al. (2008) and Malcolm et al. (2008) examined acclimation of respiration (CO_2 release) to temperature in ECM fungi from Alaska and Pennsylvania. When congeneric isolates were compared, those from Alaska had higher respiration rates than more southern counterparts over a range from 11 to 23°C. Although I measured oxygen uptake, the results agree in that CgAK demonstrated the higher rate than CgMD. In their study *C. geophilum* respired at a much lower rate than Basidiomycetes.

Their Pennsylvania isolate of *C. geophilum* acclimated quickly; being one of only three fungi of twelve tested showing acclimation to temperature shift after 7 days. The authors point out that species differences in basal respiration rates, Q_{10}s and ability to acclimate will be important in determining ECM carbon release as determined by ECM fungal community composition. My oxygen release data supports their findings in showing that both CgMD and CgAK when grown at 20°C, exhibited oxygen uptake rates at 20°C that were similar to rates at 12°C for mycelium grown at 12°C. Therefore, these isolates demonstrated what has been termed ideal compensation for oxygen uptake (Hochachka and Somero 1973); a phenotypic change in metabolic rate in response to growth temperature. Carbon dioxide release will follow the same pattern assuming carbon source or respiratory quotients do not change with growth temperature. This capacity to hold respiration within a relatively narrow range at the organism's prevailing environmental temperature is likely an evolutionary stable strategy (Clarke 2003).

Oxygen uptake by CgMD and CgAK isolates demonstrated linear Arrhenius plots; a pattern similar to that observed for field-collected ECM *Salix rotundifolia* roots studied at Barrow, Alaska (Linkins and Antibus 1978). The findings indicate a lack of abrupt shifts in metabolism associated with changes in rate controlling factors, such as a phase shift in membrane state, over a range from 2.5 to 25°C. Tundra soils in northern Alaska have been shown to demonstrate linear Arrhenius plots for CO_2 release down to 0°C at which point an abrupt shift or break occurs (Mikan et al. 2002).

In ectothermic organisms, metabolic rate compensation to temperature is accomplished via the regulation of rates of enzymatic activity (Hochachka and Somero, 1973, Somero 1986). Some potential factors that were examined here include: changes in specific activities of enzymes produced, changes in metabolic pathways used or changes to enzyme molecules reflected through altered kinetic properties (Ea, Vmax, Km or Vmax:Km).

An increased production of metabolic enzymes concomitant with lowered growth temperature has been suggested as a mechanism explaining rate compensation. This makes sense if more catalytic protein is needed to overcome the reduced average energy of substrate (with less required at higher temperatures). Estimates of enzymatic activity for organisms grown at different temperatures have revealed two general strategies (Hochachka and Somero 1973). Lowered growth temperatures will often result in increases in activities of enzymes involved in catabolic reactions. In other cases acclimation at different temperatures differentially affects enzymes of various metabolic pathways leading to changes in metabolic patterns. A decrease in total mean specific activities was observed for *C. geophilum* with a reduction of growth temperature to 12°C. The observed reduction was much smaller in the arctic isolate CgAK than in the temperate isolate. For these *Cenococcum* strains I saw no evidence that increased enzyme concentration would account for the observed metabolic rate compensation.

Studies of animals and plants (Hochachka and Somero 1973, Dwelle and Stallknecht, 1978) suggest that lowered acclimation temperatures can increase the activity of the pentose phosphate pathway relative to the glycolytic pathway. Christophersen (1967) observed an increase in the activities of enzymes of the pentose phosphate pathway when yeasts were grown at 20°C as opposed to 37°C. If the specific activities of pentose phosphate pathway enzymes are enhanced relative to those of the glycolytic pathway, then the activity ratios of the latter enzymes to those of the pentose phosphate pathway should decrease with a decrease in temperature. Consistent with this idea the PGI:G6PD and PGI:6PGU ratios did decrease in three of four comparisons of *C. geophilum* and two of two in *Hebeloma* (Antibus 1980). The data suggest the potential exists for increased carbon flow through the pentose phosphate pathway relative to the glycolytic pathway at the lower temperature. These observations need independent corroboration though methods that actually measure pathway flux as obtained using radiorespirometry, substrate concentration measures or metabolomic analysis (Ding et al. 2009).

The utilization of activity estimates as a means of determining quantitative changes in enzyme concentrations has a number of drawbacks. Activity estimates derived on a protein basis are subject to changes in the ratio of enzymatic to nonenzymatic protein that might occur with a shift in growth temperature. Certain qualitative changes, such as changes in enzyme catalytic efficiency with growth temperature, might also affect activity estimates (Hochachka and Somero 1973). In the past attempts have been made to relate activation energy values of key enzymes with acclimation temperature (LaPointe et al. 1989). Some consider a decrease in Ea with decreased growth temperature adaptive in that it reduces the temperature dependence of enzyme-dependent processes. Alternatively an increase in Ea occurring with a decrease in growth temperature could be adaptive in that a slight increase in temperature can affect a large increase in reaction rate.

The present findings revealed no phenotypic change in Ea of GK for either isolate in the range of growth temperatures used here. Similar values were observed for the two isolates even though the isolates originated in climates with

very different thermal regimes. These findings are in agreement with studies of wall surface-bound acid phosphomonesterases in the same isolates (Antibus et al. 1986). Yet there is need to examine a wider range of growth temperatures in this regard and more enzymes. For example it has been shown that arctic *Hebelomas* produced cold active proteases and phosphatases after growth at or below 6°C (Tibbett et al. 1998, 1999).

The relationship between Km and temperature during acclimation has been examined in a range of organisms (Rainey et al. 1987, LaPointe et al. 1989). Km is considered an estimate of the substrate binding affinity of enzymes. In animals Kms for specific enzymes have been observed to vary with latitude in an adaptive fashion (Hochachka and Somero 2002). Another common observation is an increase in Km with increasing assay temperature, a property termed positive thermal modulation. If one assumes cell substrate concentrations in the range of Km then changes in Km with temperature might buffer against kinetic effects of temperature shifts on substrate activity. In my study Kms of GK in most instances increased with increasing assay temperature. I did not observe significant phenotypic differences associated with altered growth temperature. These findings would support the opinion of Hochachka and Somero (1973, 2002) that maintenance of enzyme regulatory function would likely select against wholesale reduction of Km as a mechanism for compensating to lowered temperatures. Still the dynamic impact of substrate concentrations on enzyme reaction rates was shown here with 6PGU. Here acclimation at 12°C by CgMD was accompanied by a decreased activity of this enzyme at all assay temperatures at high substrate concentrations, whereas CgAK 6PGU was not. Preliminary studies with native polyacrylamide gel electrophoresis of extracts revealed that these two isolates produced different isozymes of 6PGU when grown at 20°C.

Whereas Vmax is often regarded to reflect quantity of enzyme and Km its substrate affinity some feel that Vmax to Km ratios reflect enzyme catalytic efficiency (LaPointe et al. 1989). Enzyme efficiency could be a strategy to overcome the kinetic effects of lowered temperature and this has been related to a lowering in the Vmax:Km. CgAK showed a large reduction in Vmax:Km, brought about largely by a reduction in Vmax, with growth at 12°C a pattern consistent with cold acclimation in some plants (LaPointe et al. 1989). No response to growth temperature was observed in CgMD. Further studies using pure enzyme would be necessary in order to measure a true efficiency, the actual rate of substrate turnover per enzyme molecule.

Consistent with the observations of Malcolm et al. (2008) we have found that *C. geophilum* isolated from arctic tundra soil dominated by permafrost respired at a higher rate than a temperate isolate. The specific activities of the arctic isolate were less depressed by the lower growth temperature yet both isolates demonstrated a clear pattern of ideal metabolic rate compensation for oxygen uptake. Metabolic rate compensation was not obviously linked to enzyme kinetic differences. No differences were observed between isolates for Ea or Km of GK. The GK Vmax:Km appeared more sensitive to growth at 12° in the arctic isolate and differences in the acclimation response of 6PGU were observed as well, however, much remains to be learned about the mechanisms of temperature acclimation in these fungi.

Here we've shown that isolates of an ECM fungus from arctic and temperate sites can acclimate by maintaining oxygen uptake within a narrow range in pure culture, this process potentially would reduce fungal carbon demand and CO2 release as soil warms. More work is clearly needed into the temperature responses of arctic and alpine mycorrhizal fungi. This is

justified by the potential contribution of ECM fungi to soil carbon cycling and related changes that might occur with fungal community species shifts upon soil warming (Alberton et al. 2005, Malcolm et al. 2008). Obstacles to progress include the remoteness of field sites and difficulties in growing many fungi in common/important families, Russulaceae and Cortinariaceae in the case of arctic and alpine willows. We also do not know if results based on pure culture studies reflect in a real way what occurs in symbiosis. Future studies should draw upon the advances in the fields of DNA microarray analysis and metabolomics to add to our understanding of these fungi.

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