Substrate supply, fine roots, and temperature control proteolytic enzyme activity in temperate forest soils

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Abstract. Temperature and substrate availability constrain the activity of the extracellular enzymes that decompose and release nutrients from soil organic matter (SOM). Proteolytic enzymes are the primary class of enzymes involved in the depolymerization of nitrogen (N) from proteinaceous components of SOM, and their activity affects the rate of N cycling in forest soils. The objectives of this study were to determine whether and how temperature and substrate availability affect the activity of proteolytic enzymes in temperate forest soils, and whether the activity of proteolytic enzymes and other enzymes involved in the acquisition of N (i.e., chitinolytic and ligninolytic enzymes) differs between trees species that form associations with either ectomycorrhizal or arbuscular mycorrhizal fungi. Temperature limitation of proteolytic enzyme activity was observed only early in the growing season when soil temperatures in the field were near 4°C. Substrate limitation to proteolytic activity persisted well into the growing season. Ligninolytic enzyme activity was higher in soils dominated by ectomycorrhizal associated tree species. In contrast, the activity of proteolytic and chitinolytic enzymes did not differ, but there were differences between mycorrhizal association in the control of roots on enzyme activity. Roots of ectomycorrhizal species but not those of arbuscular mycorrhizal species exerted significant control over proteolytic, chitinolytic, and ligninolytic enzyme activity; the absence of ectomycorrhizal fine roots reduced the activity of all three enzymes. These results suggest that climate warming in the absence of increases in substrate availability may have a modest effect on soil-N cycling, and that global changes that alter belowground carbon allocation by trees are likely to have a larger effect on nitrogen cycling in stands dominated by ectomycorrhizal fungi.

Key words: amino acids; microbial acclimation; mycorrhizal fungi; organic N cycling; proteolytic enzymes; temperate forests; temperature sensitivity of SOM decomposition.

INTRODUCTION

Nitrogen (N) limitation is widespread in the terrestrial biosphere (e.g., Vitousek and Howarth 1991, LeBauer and Treseder 2008). There is a rich and deep history of research on the terrestrial N cycle (e.g., Nadelhoffer et al. 1985, Aber et al. 1990, Reich et al. 1997), yet there remain significant uncertainties in our understanding of the processes that control N availability, particularly those that regulate the depolymerization of N from soil organic matter (SOM). Proteolytic enzymes are an important class of enzymes involved in the breakdown of protein, a large pool of organic N in SOM, into amino acids that can be used as a source of N by plants and microbes (Barraclough 1997, Schulten and Schnitzer 1998, Finzi and Berthrong 2005, Gallet-Budynek et al. 2009). The abundance and activity of proteolytic enzymes is a principal driver of the within-system cycle of soil N (Schimel and Bennett 2004), yet the mechanisms controlling the production and activity of proteolytic enzymes in most ecosystems remain largely unknown.

Following a simple model of enzyme kinetics (Fig. 1), the activity of proteolytic enzymes in soils should be a function of the interaction between three parameters: soil temperature, substrate concentration, and the enzyme pool size (Schimel and Weintraub 2003). Accordingly, factors that alter some combination of these three kinetic parameters should alter proteolytic enzyme activity and the availability of N in soil.

The most immediate effect of an increase in soil temperature on enzyme activity is to speed up the rate at which enzymes collide with and breakdown substrates. Over longer times scales however (e.g., growing season to years), the environmental conditions under which enzymes are produced and become active can override short-term temperature sensitivities. For example, extracellular enzymes produced in cold soils tend to exhibit greater temperature sensitivity than those produced in warm soils because they reach their peak activity at lower temperatures (Fenner et al. 2005, Koch et al. 2007). By contrast, the lower temperature sensitivity of enzymes produced under warm conditions has been linked to a decline in enzyme flexibility that reduces their
reaction rate with substrates (Siddiqui and Cavicchioli 2006, Bradford et al. 2010).

Despite the abundance of protein in soil organic matter, substrate limitation of proteolytic enzyme activity is common in forest soils (Schulten and Schnitzer 1998, Berthrong and Finzi 2006). Substrate limitation arises because of the physical protection of protein substrate in soil aggregates and the binding of proteins by organic molecules (Sollins et al. 2006, Rillig et al. 2007, McCarthy et al. 2008). Substrate limitation is likely to affect the temperature sensitivity of proteolytic enzymes (c.f., Davidson and Janssens 2006), though this interaction has not been widely studied.

Evidence of low enzyme activity despite optimum temperature and substrate conditions suggests that the standing pool size of enzymes may also be an important limit on enzyme activity (Wallenstein et al. 2009). The production of enzymes can be limited by the availability of carbon (C) and N for enzyme synthesis (Allison et al. 2009, Wallenstein et al. 2009) and microbial demands for the products of enzyme activity (Craine et al. 2007, Geisseler and Horwath 2008). Furthermore, once enzymes are produced, they can be rendered inactive by their binding to soil organic matter (Joanisse et al. 2007), though sorption reactions to clay minerals may actually stabilize and protect enzymes from degradation by preventing them from becoming substrates themselves (Allison 2006).

Temperate forest tree species of the northeastern U.S. differ in many of the factors that control the abundance and activity of proteolytic enzymes. These species differ in litter chemistry, affecting the availability of C and N for microbial growth, as well as in SOM chemistry (e.g., polyphenolic compounds) which leads to variation in protein binding by organic materials in the soil (Kraus et al. 2003, Talbot and Finzi 2008). Temperate forest trees also differ in mycorrhizal association. Most tree species in the northeast are colonized by ectomycorrhizal (ECM) fungi, which possess broad enzymatic capabilities allowing for the decomposition of labile and recalcitrant components of SOM and the transfer of N to host plants (Chalot and Brun 1998, Talbot et al. 2008). A smaller number of species are colonized by arbuscular mycorrhizal (AM) fungi, which do not appear to have as broad an enzymatic capability as ECM fungi (but see Hodge et al. 2001). In addition to differences in enzymatic capability, rates of root exudation in ECM tree species are higher than AM tree species (Smith 1976, Phillips and Fahey 2005) stimulating faster rates of C and N mineralization in the rhizosphere of ECM trees (Phillips and Fahey 2006). Higher rates of SOM decomposition must be mediated by greater enzyme production and activity in the rhizosphere, suggesting that ECM tree roots may provide soil microbes with at least some of the resources required in the synthesis of extracellular enzymes. However, the effect of tree roots per se has not been well established.

Proteolytic enzymes are not the only enzyme system involved in the release of N from SOM. Chitinolytic enzymes which release amino sugars and ligninolytic enzymes which can release N from recalcitrant sources may also have a substantial role in mobilizing N (Sinsabaugh 2010). Like proteolytic enzymes, chitinolytic and ligninolytic enzyme activities may be controlled by differences between tree species in the enzymatic capabilities of their fungal symbionts and rates of root exudation. Further, the greater recalcitrance of SOM in

Fig. 1. Conceptual model of proteolytic enzyme activity in soil. Activity is a function of the interaction among three kinetic parameters: temperature, substrate availability, and enzyme pool size. Increases or decreases in kinetic parameters due to variation in the biological, physical, and chemical properties of the soil environment lead to subsequent increases or decreases in proteolytic enzyme activity.
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Table 1. Litterfall characteristics, soil C:N ratios, amino acid concentrations, and inorganic N pools and cycling rates in the mineral soil and organic horizons for each tree species.

<table>
<thead>
<tr>
<th>Common soil name</th>
<th>Latin name</th>
<th>Litterfall C:N ratio</th>
<th>Bulk litterfall (g/m²)</th>
<th>Target litter (%</th>
<th>Soil pH</th>
<th>Soil C:N ratio</th>
<th>Amino acid concentration (µg AA-N/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mineral soil</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sugar maple</td>
<td>Acer saccharum</td>
<td>42.64a (1.24)</td>
<td>427.8g (19.7)</td>
<td>77</td>
<td>4.80a (0.05)a</td>
<td>12.32a (0.69)</td>
<td>1.49a (0.20)</td>
</tr>
<tr>
<td>White ash</td>
<td>Fraxinus americana</td>
<td>44.70a (1.74)</td>
<td>377.2g (19.1)</td>
<td>56</td>
<td>5.10b (0.10)b</td>
<td>13.35a (0.42)</td>
<td>1.80a (0.25)</td>
</tr>
<tr>
<td>Eastern hemlock</td>
<td>Tsuga canadensis</td>
<td>65.95a (1.85)</td>
<td>206.9g (15.8)</td>
<td>67</td>
<td>3.88b (0.08)b</td>
<td>25.60a (0.94)</td>
<td>2.26b (0.51)</td>
</tr>
<tr>
<td>American beech</td>
<td>Fagus grandifolia</td>
<td>52.02a (1.29)</td>
<td>273.5g (4.9)</td>
<td>56</td>
<td>4.44a (0.07)b</td>
<td>19.97a (1.17)</td>
<td>1.93a (0.24)</td>
</tr>
</tbody>
</table>

Organic horizon

<table>
<thead>
<tr>
<th>Target tree</th>
<th>Soil pH</th>
<th>Soil C:N ratio</th>
<th>Amino acid concentration (µg AA-N/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eastern hemlock</td>
<td>3.49a (0.01)</td>
<td>29.91 a (1.98)</td>
<td>19.54a (1.82)</td>
</tr>
<tr>
<td>American beech</td>
<td>3.87a (0.11)</td>
<td>23.86b (0.94)</td>
<td>26.07b (1.63)</td>
</tr>
</tbody>
</table>

Notes: Each value is the mean (with SE in parentheses). Soil N cycling rates and pools are the means of samples taken in April, June, and August of 2008. Superscript letters within a column denote significant differences among species for a given horizon at P < 0.05. Target litter (%) is the mean percentage for each species of the total bulk litterfall mass that is composed of that species’ litter. Abbreviations: net N min., net nitrogen mineralization; net nitrif., net nitrification; AA N, amino acid nitrogen.

The standing basal area was composed of the target tree species, (2) the fresh litterfall layer was dominated by the target species, and (3) the core 5 m of the plot contained only the target tree species (Lovett et al. 2004).

Significant organic horizons were present only in the beech and hemlock plots. The lack of organic horizon in the white ash plots may be due to the presence of earthworms, which are not present in the other plots.

Upon plot establishment, we measured background characteristics of each plot (Table 1). All trees with diameter at breast height (dbh) > 5 cm were measured and identified to species. Litterfall from each plot was collected in October and November of 2007. Litterfall was sorted to species, dried at 60°C, and weighed. The target tree species litter from each plot was ground and analyzed for %C and %N on an elemental analyzer (NC2500; CE Elantech, Lakewood, New Jersey, USA).

Soils collected from the top 15 cm of the mineral soil horizon from each plot and the organic horizon from the hemlock and beech plots were analyzed for %C and %N, and pH.

To meet our research objectives, we performed seasonal monitoring of soil enzyme activities in conjunction with two experiments. The first experiment examined the response of proteolytic enzyme activity to manipulations of temperature and substrate availability at three key seasonal time points. The second experiment investigated the role of roots in promoting enzyme activities by using in-growth treatments to either include or exclude fine roots in the field.

Soil sampling protocol

Soil samples for the first experiment examining temperature and substrate limitation to proteolytic enzyme activity were collected three times in April, June, and August of 2008. For the seasonal monitoring of enzyme activity, we collected soils in April, June, and August of 2008 and 2009. We choose the April, June, and August time points because they span the majority of the growing season including leaf out, peak photosynthesis, and leaf area index, and the start of the
seasonal decline in C uptake at the Harvard Forest, respectively (Urbanski et al. 2007). At each sampling point, we collected a 20 × 20 cm sample of the Oe and Oe horizon in the hemlock and beech plots. Directly beneath this location we removed the top 15 cm of mineral soil using a 5 cm diameter soil bulk-density sampler in each plot. After collection fine roots were removed from the soils, which were then subsequently sieved through a 2-mm mesh for mineral soils and an 8-mm mesh for organic horizons. Subsamples of each soil were immediately frozen at −80°C for later assays of extracellular enzyme activity. Initial pools of amino acid and inorganic N were extracted within 18 hours of sample collection. Experimental manipulations and assays of proteolytic rates began within 48 hours of sample collection.

**Seasonal monitoring**

The 2 mol/L KCl extractable pool sizes of amino acids, NH₄⁺ and NO₃⁻ were determined for each soil sample for every sampling date in 2008. Ten and 30 g of organic horizon and mineral soil sample, respectively, were extracted in 100 mL of 2 mol/L KCl. In addition, rates of net N mineralization and net nitrification were measured by quantifying the change in the 2 mol/L KCl extractable pool sizes of NH₄⁺ and NO₃⁻ after a 28-day soil incubation in the lab (Finzi et al. 1998). The concentration of amino acids for all samples was quantified using the o-phthalaldialdehyde and β-mercaptoethanol (OPAME) method (Jones et al. 2002). Concentrations of amino acid N were determined by comparing the fluorescence of the samples relative to a standard curve composed of glycine. The concentration of NH₄⁺ and NO₃⁻ for all samples was quantified using a flow injection autoanalyzer (Lachat Quickchem 8000; Hach Company, Loveland, Colorado, USA).

For every soil at each sample date in 2008 and 2009, we also assayed the potential activities of the chitinolytic enzyme, n-acetyl-glucosaminidase (NAG), and the lignonolytic enzyme, phenol oxidase. All the assays were run using a pH 5.0 sodium acetate buffer at 23°C. NAG activity was determined using a fluorometric microplate assay, while phenol oxidase activity was determined using a colorometric microplate assay (Saiya-Cork et al. 2002).

**Temperature and substrate limitation to proteolytic activity**

In 2008, we performed an experiment to determine how temperature and substrate availability affect proteolytic enzyme activity throughout the growing season. In this factorial experiment, proteolytic rates were assayed at two temperatures (field temperature at time of sampling and 23°C) and two substrate levels (ambient or elevated protein in the form of casein) in soils from each plot and both soil horizons (n = 144). This experiment was performed for every sample date in 2008. The temperature of the soil in the field in April, June, and August were 4°C, 15°C, and 18°C, respectively (K. Savage, personal communication). Within 12 hours of soil sample collection, four replicate 2–3 g subsamples of each soil were placed in their proper incubation temperature and allowed to equilibrate in closed 50-mL centrifuge tubes for 12 hours. We choose 12 hours as an equilibration time to minimize the time between the assay and the field sampling and to ensure that all of the samples were at the correct assay temperature.

After equilibration, we assayed proteolysis following a method modified from Watanabe and Hayano (1995) and Lipson et al. (1999). To perform the assay under ambient protein conditions, initial and incubated subsamples received 10 mL of a 0.5-mmol/L sodium acetate buffer (pH 5.0) with a small volume of toluene (400 µL) added to inhibit microbial uptake. The assay of proteolysis under elevated conditions was performed similarly, except the sodium acetate buffer contained 0.6% casein. After the reagent addition, the initial samples were immediately terminated and the incubated subsamples were placed back into their respective assay temperature for 4 hours. We choose 4 hours as an incubation time because previous work has shown proteolytic enzyme activity to be linear over this period in temperate forest soils (Finzi and Berthrong 2005, Rothstein 2009). Enzyme activity in all the initial and incubated subsamples was terminated through the addition of 3 mL of a trichloroacetic acid solution. Proteolytic rates for each experimental treatment were calculated from the difference between amino acid concentrations in the incubated and initial subsamples of each treatment assayed using the OPAME method described above (Jones et al. 2002).

We assayed the activity of proteolytic enzymes as a class rather than specific proteolytic enzymes (e.g., leucine-aminopeptidase, glycine-aminopeptidase) because the Watanabe and Hayano (1995) method allowed us to measure proteolytic rates under ambient and elevated protein substrate conditions. Assays for individual enzymes are conducted under saturating substrate conditions and therefore could not be used to address the role of substrate limitation in the depolymerization of N (Saiya-Cork et al. 2002). In addition, this method...
integrates the activity of a larger suite of proteolytic enzymes in soils, which may have more relevance ecologically than more specific enzyme assays. We acknowledge the loss of plant–microbe interactions and soil disturbance as methodological artifacts when employing this assay of proteolytic enzyme activity (c.f., Hofmockel et al. 2010). However, these assumptions are inherent to any assay of soil enzyme activity (Wallenstein and Weintraub 2008).

**In-growth experiment**

We conducted a growing season long field experiment to investigate the role of roots on proteolysis and extracellular enzyme activity. This experiment used mineral soil in-growth cores and organic horizon in-growth bags (see Plate 1). Organic horizon bags (5 × 10 × 3 cm; Wallander et al. 2001) and PVC core frames for mineral soil (5 cm diameter × 15 cm deep) were constructed using either 1-mm fiberglass mesh or 50-μm nylon mesh (Sefar Industries, Depew, New York, USA; Langley et al. 2006). The 50-μm nylon mesh excludes roots but allows fungal hyphae and heterotrophic microbes to explore the soil (herein “microbes”), while the 1mm mesh allows both roots and fungal hyphae ingrowth (“roots + microbes”).

The in-growth cores were filled with soil collected from each plot in April of 2008. The organic and mineral soil horizons were sieved as previously described. From these samples we created a single, bulk sample of organic or mineral soil horizon for each species. The homogenized soil was then placed back into the in-growth treatments to approximate bulk density (Hendricks et al. 2006). In May of 2008, one core from each treatment was placed into each plot and soil horizon (n = 48 mineral soil cores; n = 24 organic horizon bags). In-growth bags were installed at the interface between the organic horizon and mineral soil horizon layers. In-growth cores were installed into the soil by first removing the organic horizon, then placing the core in the top 15 cm of the mineral soil horizon and finally replacing the organic horizon. The cores/bags were harvested during the first week of October 2008 and then assayed for proteolytic, NAG, and phenol oxidase enzyme activity.

**Statistical analysis**

We used three-way nested ANOVA to test for the effects of mycorrhizal association, tree species nested within mycorrhizal association, and sample date on the pools and fluxes of amino acid and inorganic N, NAG activity, and phenol oxidase activity. The temperature × substrate limitation experiment was also tested by four-way ANOVA with the effects of temperature, substrate availability, mycorrhizal association, and tree species nested within mycorrhizal association as independent variables. Finally, differences in enzyme activity in the in-growth experiment were tested by three-way ANOVA with mycorrhizal association, tree species nested within mycorrhizal association, and ingrowth treatment as independent variables.

Data were analyzed in SAS (2003) with the GLM procedure. Data were assessed for normality and homogeneity of variance and log-transformed to meet model assumptions when needed. For the analysis of the seasonal enzyme activity, when the interaction with year was not significant, we streamlined the data presentation by averaging across years. Statistical tests were performed separately by horizon, given the unequal design and known ecological differences in the role of these horizons. Tukey’s studentized range test was used for all post hoc comparisons of mean differences among species or experimental treatments.

**RESULTS**

**Seasonal monitoring**

The pool size and cycling rates of inorganic N were significantly higher in the plots dominated by the AM-associated trees, maple and ash, than by the ECM-associated trees, hemlock and beech (Table 1). Concentrations of amino acids and rates of proteolysis did not differ between tree species or mycorrhizal association (Table 1). The organic soil horizons in the hemlock and beech plots had significantly higher pool sizes and cycling rates of inorganic and organic N than the mineral soils (Table 1). Rates of phenol oxidase activity were significantly higher (P < 0.0001) in the mineral soils of the ECM trees than in the AM trees (Fig. 2a). NAG activity did not differ between tree species (Fig. 2b). Seasonally, both phenol oxidase and NAG activity declined in the mineral soil as the growing season progressed (Fig. 2). Phenol oxidase activity was significantly higher in April and June than in August for the ECM soils (P < 0.05); whereas in the AM soils the seasonal decline was not significant. Across all species, NAG activity was significantly higher in April than in June and August (P < 0.05). Both enzyme activities were significantly greater in the organic horizons than in the mineral soils of the ECM trees (P < 0.0001, Table 2).

**Temperature and substrate limitation to proteolytic activity**

Proteolytic enzyme activity responded significantly to increases in temperature and substrate availability across the growing season (Fig. 3). In April when the soils were cold, there was an interactive effect of increasing temperature and substrate availability on proteolytic enzyme activity (P < 0.002, Fig. 3a). As the soils warmed in June, proteolytic enzyme activity was no longer temperature sensitive but remained substrate limited (P < 0.0001, Fig. 3b). By August, there was little effect of temperature or substrate limitation on proteolytic rates in the soil with the exception of a weak stimulation by temperature (P < 0.03) in white ash (Fig. 3c).
To account for the difference in the amount of warming in April, June, and August, we calculated the percent change in proteolytic rates between the field and elevated temperature treatments for each 1°C increase under ambient protein conditions (Fig. 4). In all species, the largest increase in proteolytic rates per 1°C increase occurred in April, followed by a steep decline in the temperature sensitivity in June and August (Fig. 4). There was no relationship between proteolytic enzyme activity and soil moisture ($R^2$, 0.01; data not shown).

A qualitatively similar pattern of temperature-by-substrate limitation was observed in the organic horizon of the beech and hemlock soils throughout the growing season (Table 2). In April, there was an interactive effect of temperature and substrate limitation on proteolytic rates ($P < 0.05$, Table 2). This interaction was not significant in June though proteolytic rates remained temperature ($P < 0.003$) and substrate limited ($P < 0.008$, Table 2). By August, there was no temperature limitation, but the organic horizons remained substrate limited ($P < 0.03$, Table 2).

Table 2. Mean (±SE) seasonal extracellular enzyme activity and the response of proteolysis to temperature and substrate manipulations in the organic horizon of the beech and hemlock stands.

<table>
<thead>
<tr>
<th>Species</th>
<th>Month</th>
<th>Seasonal monitoring</th>
<th>Temperature ($T$) and substrate limitation to proteolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Phenol oxidase*</td>
<td>NAG</td>
</tr>
<tr>
<td>Eastern hemlock</td>
<td>April</td>
<td>1.97* (0.30)</td>
<td>0.43 (0.05)</td>
</tr>
<tr>
<td></td>
<td>June</td>
<td>1.62* (0.33)</td>
<td>0.47 (0.07)</td>
</tr>
<tr>
<td>American beech</td>
<td>August</td>
<td>1.14* (0.22)</td>
<td>0.47 (0.06)</td>
</tr>
<tr>
<td></td>
<td>April</td>
<td>4.05* (0.45)</td>
<td>0.48 (0.08)</td>
</tr>
<tr>
<td></td>
<td>June</td>
<td>3.16* (0.51)</td>
<td>0.60 (0.09)</td>
</tr>
<tr>
<td></td>
<td>August</td>
<td>2.81* (0.34)</td>
<td>0.63 (0.07)</td>
</tr>
</tbody>
</table>

Notes: Different lowercase letters indicate significant differences ($P < 0.05$) between months. Asterisks indicate parameters with significant differences ($P < 0.05$) between species. Phenol oxidase and n-acetyl-glucosaminidase (NAG) activity is expressed as $\mu$mol[g dry soil]$^{-1}$h$^{-1}$. Proteolytic data are expressed in units of $\mu$g AA-N[g dry soil]$^{-1}$h$^{-1}$, where AA stands for amino acid. Field temperature in April, June, and August 2008 was 4°C, 15°C, and 18°C, respectively. For Temperature and substrate limitation to proteolysis, the column titles denote the temperature ($T$) and protein substrate conditions of the experiment. Proteolytic rates were assayed at two temperatures (Field $T$ at time of sampling or 23°C) with or without added protein substrate (+ protein). Field $T$ in April, June, and August 2008 was 4°C, 15°C, and 18°C, respectively.
In-growth experiment

The exclusion of roots from the mineral soil horizon led to a significant reduction in proteolytic, NAG, and phenol oxidase activity in the two ECM tree species \((P < 0.05, \text{Fig. 5})\). In the two AM species, the exclusion of roots had no effect on enzyme activity. The exclusion of roots in the organic horizon similarly decreased proteolytic \((P < 0.06)\), NAG (not significant), and phenol oxidase activity \((P < 0.04, \text{Fig. 5})\).

**DISCUSSION**

The similarity in the temperature and substrate limitation of proteolytic enzymes across species and mycorrhizal associations (Fig. 3), suggests that there are common kinetic constraints on proteolytic enzyme activity. Proteolytic enzyme activity was temperature sensitive only early in the growing season when the soils were cold. Enzymes produced under cold conditions tend to have lower temperature optima and greater physical flexibility, which is thought to make early enzymes more temperature sensitive than those produced under warm conditions (Siddiqui and Cavicchioli 2006, Koch et al. 2007). The significant interaction between temperature and substrate limitation (Fig. 3) shows that low concentrations of protein substrate also limit proteolytic enzyme activity early in the growing season. The persistence of substrate limitation in to June in the mineral soil and August in the organic horizon

![Figure 3](image1.png)

**Fig. 3.** Mean (±SE) proteolytic enzyme activity in the top 15 cm of mineral soil for each tree species in response to increases in temperature and additions of protein substrate in (a) April, (b) June, and (c) August of 2008. AA-N is amino acid nitrogen.

![Figure 4](image2.png)

**Fig. 4.** Mean (±SE) percentage change in proteolytic rates between the field and elevated temperature treatments for each 1°C increase under ambient protein conditions in the top 15 cm of mineral soil for each tree species in April, June, and August of 2008.
along with evidence that substrate limitation is widespread among biomes (Hofmockel et al. 2010) suggests that the availability of protein is likely to constrain the temperature response of a key component of the N cycle to climate warming (Davidson and Janssens 2006).

Soil microbes alter their activity in response to shifting nutrient and temperature regimes, and this may explain why neither temperature nor substrate availability limited proteolytic activity late in the growing season (Figs. 3 and 4). Several different examples help make this case. For example, low N availability led to a reduction in the synthesis of enzymes by microbes in warm, arctic soils (Wallenstein et al. 2009). In the same forests studied here, increasing soil temperature across the growing season resulted in declines in microbial respiration as a consequence of thermal or microbial community adaptation (Bradford et al. 2008). And, field rates of root exudation have been shown to decline throughout the growing season (Weintraub et al. 2007, Phillips et al. 2008), potentially limiting the supply of resources for enzyme synthesis by soil microbes. The decline in temperature and substrate limitation was not related to seasonal changes in soil moisture but coincides with important phenological changes including the

![Graphs showing mean (+ SE) response of proteolytic, NAG, and phenol oxidase activity to the two in-growth treatments in the top 15 cm of mineral soil for each tree species, and in the organic horizons of hemlock and beech. The contribution of roots to enzyme activity was calculated using the equation: Roots = (activity in the Roots + Microbes treatment) – (activity in the Microbes treatment). See Methods for additional description of the in-growth cores.]

Fig. 5. Mean (± SE) response of (a) proteolytic, (b) NAG, and (c) phenol oxidase activity to the two in-growth treatments in the top 15 cm of mineral soil for each tree species, and in the organic horizons of hemlock and beech. The contribution of roots to enzyme activity was calculated using the equation: Roots = (activity in the Roots + Microbes treatment) – (activity in the Microbes treatment). See Methods for additional description of the in-growth cores.
depletion of carbohydrate stores in roots (Kozlowski 1992) and a decrease in root production (Hendrick and Pregitzer 1996). Consistent with the response of proteolytic enzymes, we also found declines in NAG and phenol oxidase activity over the course of the growing season (Figs. 2 and 3).

Tree roots of ECM species had a large, positive effect on extracellular enzyme activity. Tree roots of AM species had little to no effect (Fig. 5). A number of processes may explain why the presence of roots promotes enzyme activity in ECM soils. The production of root exudates and rates of C and N mineralization in the rhizosphere are higher in ECM than in AM tree species (Smith 1976, Phillips and Fahey 2005, 2006), suggesting that ECM tree roots subsidize microbial enzyme production. Mycorrhizal and non-mycorrhizal plants are also capable of producing proteolytic enzymes (Paungfoo-Lonhienne et al. 2008), raising the possibility that the ECM tree species studied here release more proteolytic enzymes directly into the soil. There may also have been vigorous colonization of new, fine roots by ECM fungi and this may have stimulated enzyme activity in the “roots + microbes” in-growth cores. Finally, the ECM fungal taxa present in these soils may not possess long distance foraging capability and instead concentrate their activity near roots (Agerer 2001). Regardless of the specific mechanism, ECM tree roots exert important control over enzyme activity in the soil by modifying microbial activity.

The similarity in the rates of NAG activity in the mineral soils and organic horizons of the ECM tree species in the in-growth experiment (Fig. 5) suggests an experimental artifact of the treatments. This similarity is in stark contrast to the consistent, order-of-magnitude greater NAG activity observed in the field in the organic horizons than in the mineral soils (Table 2, Fig. 2). The soils that were used in the in-growth cores and bags were sieved and homogenized by species prior to the field incubation. This may have resulted in a large flush of chitin substrate into these soils as a result of severing fungal hyphae. Alternatively, roots from the organic horizon may have proliferated into the in-growth cores and stimulated greater NAG activity. The artifact associated with the in-growth cores does not, however, impact our conclusions because (1) they affected only NAG activity and not other enzymes, and (2) our conclusions depend on relative not absolute differences between treatments.

Based on the observation that AM roots exert little effect over enzyme activity, a non-root process must support enzyme activity in soils dominated by AM tree species. Litter inputs by AM tree species in temperate forests have a narrow C:N ratio, labile chemistry, and result in narrow C:N ratio soils (Table 1; Finzi et al. 1998, Lovett et al. 2004) that typically have high rates of C and N mineralization (Ollinger et al. 2002). Further, AM soils displayed lower activities of phenol oxidase in the bulk soil than ECM soils (Fig. 2). Thus, it may be that the decomposition of labile SOM requires fewer active enzymes or provides the resources required to support enzyme activity in AM soils. By contrast, the litter chemistry of ECM trees is generally recalcitrant leading to slower rates of decomposition and N cycling. In ECM soils, it appears that ECM-root inputs are subsidizing the decomposition of more recalcitrant organic matter, which typically incurs a larger resource cost (i.e., more enzyme synthesis, Sinsabaugh 2010).

Much of the research on the response of SOM decomposition to increasing temperatures has focused on C mineralization and microbial respiration with a rather limited set of studies examining N cycling responses (e.g., Rustad et al. 2001, Koch et al. 2007). This study links some of the processes thought to control the response of soil C mineralization to increasing temperature (e.g., thermal acclimation and substrate depletion; Bradford et al. 2008, 2010) with those that mobilize N from SOM (e.g., Melillo et al. 2002). We show that proteolytic enzyme activity is both tempera-
tured and substrate limited, with seasonal declines in both limitations mirroring seasonal declines in microbial respiration in the same forest (Bradford et al. 2008). Further, we show an important linkage between belowground plant-C flux (e.g., plant roots), SOM decomposition (e.g., phenol oxidase activity) and the depolymerization of N from SOM (e.g., NAG and proteolytic enzyme activity) in stands dominated by ECM trees. Given the widespread occurrence of N limitation in terrestrial ecosystems and the importance of N supply to primary production (Vitousek and Howarth 1991), future research should couple the C- and N-cycle responses to increasing soil temperatures to better understand temperate forest responses to climate change.

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