

Does Atmospheric NO_3^- Deposition Alter the Abundance and Activity of Ligninolytic Fungi in Forest Soils?

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ABSTRACT

Although field studies have demonstrated an ecosystem-specific effect of experimental atmospheric nitrogen (N) deposition on litter decomposition, a mechanistic understanding of how ligninolytic microbial communities respond to atmospheric deposition is lacking. Because high levels of inorganic N suppress lignin decomposition by some basidiomycetes, it is plausible that the abundance and activity of these key microorganisms underlies differential ecosystem responses of decomposition to atmospheric N deposition. We hypothesize that: (a) atmospheric N deposition will cause an ecosystem-specific reduction in basidiomycete activity and abundance with greatest decreases in ecosystems with lignin-rich forest litter and (b) the abundance of lignin degrading basidiomycetes will be positively correlated with ligninolytic enzyme activity. To test these hypotheses, we measured the effects of experimental N deposition on the potential activity of phenol oxidase enzymes, and the abundance of basidiomycete genes encoding laccase, a primary phenol oxidase enzyme, in three hardwood forests spanning a range of leaf litter lignin content. The black oak-white oak (BOWO) contains

high lignin litter, the sugar maple-basswood (SMBW) has low lignin litter, and the sugar maple-red oak (SMRO) is intermediate. An ecosystem by N deposition interaction significantly influenced phenol oxidase activity in the surface soil ($P = 0.05$), where phenol oxidase activity decreased with increasing experimental N deposition in the BOWO ecosystem. No consistent response to N deposition was evident for surface soil phenol oxidase activity within either the SMRO or SMBW ecosystem. This interaction did not influence laccase gene abundance. Instead, basidiomycete laccase gene abundance was reduced by experimental N deposition (main effect) in surface soil. There was only a weak correlation between basidiomycete laccase gene abundance and potential phenol oxidase enzyme activity, suggesting that the abundance of organisms possessing laccase genes may not control phenol oxidase activity in soil. Our results suggest that the regulation of laccase gene expression may mediate the decomposition response to atmospheric N deposition.

Key words: basidiomycete; laccase; phenol oxidase; atmospheric N deposition.

INTRODUCTION

Anticipating how atmospheric N deposition will alter soil C storage requires a mechanistic understanding of the underlying microbial dynamics controlling forest litter decomposition. Experi-

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mental evidence indicates that atmospheric N deposition can suppress as well as stimulate organic matter decomposition (Fog 1988; Knorr and others 2005), which could result in divergent changes in soil C storage. Much of this variability could arise from the abundance of particular soil microorganisms and the extent to which their activity is influenced by atmospheric N deposition. For example, lignin degradation, a rate-limiting step in the process of plant litter decomposition (Paul and Clark 1996; Yang and others 1980), is mediated by the enzymatic activity of a variety of litter-decomposing fungi, including ascomycetes and basidiomycetes. Although some bacteria and ascomycetes can degrade low-molecular weight fractions of lignin, basidiomycetes are the largest and most effective group of lignin degraders in soil (Hatakka 2001; Rabinovich and others 2004). Because basidiomycetes are the primary organisms involved in lignin degradation in forests (Bebber and others 2006; Frankland 1998), changes in their activity or abundance may influence decomposition. Unlike ascomycetes, the ligninolytic activity of some basidiomycetes can be repressed by high levels of inorganic N (Worrall and others 1997). Therefore, it is plausible that the extent to which atmospheric N deposition stimulates or suppresses decomposition could be related to basidiomycete abundance and activity in a particular soil.

Basidiomycetes and other decomposing microorganisms produce a suite of ligninolytic enzymes that have activity against a range of substrates; these extracellular enzymes include phenol oxidases, lignin peroxidases and Mn peroxidases (Hammel 1997). Measurement of enzymes in forest litter and soil have demonstrated that experimental N deposition can significantly reduce phenol oxidase activity, but not peroxidase activity (Carreiro and others 2000; Waldrop and others 2004b). The consistent decline in phenol oxidase activity with increased N deposition suggests that microbial communities producing phenol oxidases may be responsible for the variable relationship between lignin decay and atmospheric N deposition. Although polyphenol oxidases include laccase (EC 1.10.3.2), catechol oxidase (EC 1.10.3.1) and tyrosinase (EC 1.14.18.1), enzymes categorized as laccases are major contributors to phenol oxidase activity (Baldrian 2006), and are among the most widespread extracellular enzymes used by fungi in the degradation of lignin (Hatakka 2001; Luis and others 2004; Pointing and others 2005). Separating laccase enzyme activity from other polyphenol oxidases is challenging, because laccases oxidize a relatively wide range of phenolic

substrates (Hammel 1997). However, molecular approaches offer an alternative method for accurately determining the presence of fungi with the potential to produce laccase (Luis and others 2004). Therefore, examination of *in situ* laccase genes may provide a unique insight into the microbiological controls over lignin decay, a key process controlling the biogeochemical cycling of C in terrestrial ecosystems.

Recent observations indicate that microbial community composition and activity are related to differences in litter biochemistry among forest ecosystems, wherein ecosystems with higher concentrations of lignin in leaf litter contain a higher relative abundance of basidiomycetes compared to ecosystems with lower leaf litter lignin concentrations (Blackwood and others 2007). In addition, the abundance of basidiomycete genes encoding the laccase enzyme can be five to ten times greater in forest litter with higher lignin content, relative to forest with lower lignin litter (Blackwood and others 2007). It is unclear if basidiomycetes laccase gene abundance is indicative of lignin degrading potential, because the relationship between basidiomycete laccase gene abundance and phenol oxidase activity in leaf litter was inconsistent over time and among ecosystems (Blackwood and others 2007). Therefore, the response of microbial communities in mineral soil may hold the key to understanding the variable influence of atmospheric N deposition on ligninolytic activity. The research presented here attempts to address these issues by investigating the effects of atmospheric N deposition on phenol oxidase activity and basidiomycete laccase gene abundance in both the forest floor and mineral soil of northern hardwood forests on four sampling dates.

Because basidiomycete abundance is greatest in ecosystems with lignin-rich litter, and because some basidiomycetes decrease phenol oxidase activity when N availability is high, it is plausible that atmospheric N deposition would exert an ecosystem-specific reduction in ligninolytic activity. Such a mechanism could explain the divergent responses of decomposition among ecosystems exposed to experimental atmospheric N deposition. However, several alternative mechanisms also may contribute to this response, including changes in the expression of extracellular lignin-degrading enzymes by basidiomycetes, or changes in the responsiveness of other litter decomposing microorganisms, such as ascomycetes or actinomycetes, to atmospheric N deposition. Here, we evaluated the hypotheses that: (a) atmospheric N deposition causes the greatest reductions in phenol oxidase

activity in lignin-rich litter in which the abundance of basidiomycetes is high, and (b) the abundance of basidiomycete laccase genes is positively related to the activity of phenol oxidase. To test these potential mechanisms, we quantified the effects of experimental N deposition on phenol oxidase activity and basidiomycete laccase gene abundance in three forest ecosystems, which span a range of leaf litter lignin content.

METHODS

Study Sites and Experimental Design

Our research was conducted in three forest ecosystems common in the Upper Great Lakes region (N 44°, W 85°): the black oak-white oak (BOWO; *Quercus velutina*–*Quercus alba*), sugar maple-red oak (SMRO; *Acer saccharum*–*Quercus rubra*), and sugar maple-basswood ecosystems (SMBW; *A. saccharum*–*Tilia americana*; Sinsabaugh and others 2004; Zak and Pregitzer 1990). In each ecosystem type, we studied three replicate stands separated by an average of 8 km; all stands are second-growth forest, approximately 90 years old. The SMBW and SMRO ecosystems are located on soils derived from sandy glacial till (typic haplorthods of the Kalkaska series), whereas the BOWO ecosystem is located on sandy glacial outwash (entic haplorthods of the Rubicon series); both parent materials are approximately 90% quartzitic sand. The three ecosystem types represent a range of overstory composition and litter lignin content (based on proximate carbon analysis *sensu* Ryan and others 1990). The BOWO ecosystem has significantly greater lignin content (34%) and has the lowest overstory productivity; SMRO is intermediate in lignin content (30%) with the highest productivity (2.90 Mg ha⁻¹ per year); SMBW is lowest in lignin content (26%) and intermediate in productivity (2.36 Mg ha⁻¹ per year; Blackwood and others 2007; Waldrop and others 2004a).

In each stand, three 10-m × 30-m plots were established, and N deposition treatments were initiated in 2001 and continue to the present. One plot was randomly assigned to each of three N deposition treatments: ambient, ambient plus 30 kg N ha⁻¹ per year and ambient plus 80 kg N ha⁻¹ per year. Ambient atmospheric N deposition in this region is approximately 11.7 kg ha⁻¹ per year (MacDonald and others 1993). Nitrogen additions were applied in six monthly increments during the growing season, which are broadcast across the plots as dry NaNO₃ pellets. Nitrogen deposition rates as high as 60 kg N ha⁻¹ per year have been

measured in major industrial and urban areas, particularly in the eastern US and western Europe (Galloway and others 1995). Our experimental sites were amended with NO₃⁻, because it is the dominant form of atmospheric N deposition in our study plots (Burton and others 1991). Microbial abundance and activity can be spatially and temporally variable, making it difficult to detect treatment effects at the landscape scale. For this reason, we collected samples on four dates from three replicate forest stands in each ecosystem type to enhance our ability to understand the microbial responses to substrate biochemistry and experimental N deposition.

Forest litter and surface soil samples (0–10 cm deep; 20 cm diameter) were collected in July, August, September, and October 2005. Five random samples of forest litter (Oi) and surface soil (Oe, Oa, A horizons at BOWO and SMRO; Oa, A horizons at SMBW) were collected within each plot. Five samples from each depth (forest litter and 0–10 cm) were composited within each plot, resulting in two composite samples per plot. Samples were transported on ice to the laboratory, where they were homogenized and partitioned to determine gravimetric moisture, phenol oxidase activity and laccase gene abundance. Gravimetric soil moisture content was determined by drying subsamples overnight at 105°C. Subsamples were stored at –20°C for enzyme analysis and –80°C for functional gene analysis.

Phenol Oxidase Activity

In each plot, extracellular phenol oxidase activity was assayed by mixing 0.5 g of composited forest litter or 1.0 g of composited surface soil in 50 mM sodium acetate buffer (Saiya-Cork and others 2002). Enzyme activity was measured using clear polystyrene 96-well microtiter plates (Fisher Scientific, Pittsburg, Pennsylvania, USA) with L-3, 4-dihydroxyphenylalanine as the substrate (L-DOPA, 10 mM, Sigma-Aldrich, St. Louis, MO; Saiya-Cork and others 2002). For each sample, we included sixteen replicate assay wells, eight replicate blank wells and eight replicate control wells. Assay wells received 200 µl of sample and 50 µl of L-DOPA, whereas blanks received 200 µl of sample and 50 µl of buffer. Substrate control wells received 200 µl of buffer and 50 µl of L-DOPA. Samples were incubated at 23°C in the dark for 24 h, and L-DOPA oxidation was measured spectrophotometrically at 460 nm (Bio-Tek Instruments, Winooski, Vermont, USA). Rates of phenol oxidase activity are expressed as µmol h⁻¹ per gram.

Laccase Gene Abundance

For each plot, genomic DNA was extracted from approximately 0.18 g of composited litter and 0.5 g of composited surface soil using the Power-Soil DNA Isolation kit (MoBio Laboratories, Carlsbad, California, USA) following the manufacturer's instructions. To facilitate lysis of fungal hyphae after addition of solution C1, samples were incubated at 70°C for 5 min, vortexed for 10 min, and incubated for an additional 5 min at 70°C.

The abundance of basidiomycete laccase genes was measured by quantitative PCR (QPCR) using the primer pair Cu1F and Cu2R (Blackwood and others 2007; Luis and others 2004). QPCR reactions were conducted in a 25 μ l volume with a final concentration of 0.5 μ M of each primer (Integrated DNA Technologies). Each PCR included 0.375 μ l of 1 \times ROX reference dye, 12.5 μ l of 1 \times SybrGreen Master Mix (Stratagene, La Jolla, California, USA), and 2.5 μ l of diluted genomic DNA. Samples were diluted (1:50–1:400) to achieve a strong PCR product with no background smear of DNA in an agarose gel stained with ethidium bromide. Preliminary experiments revealed no PCR inhibition in samples diluted in this manner. Quantitative PCR was conducted using a MX3000 thermocycler (Stratagene, La Jolla, California, USA) with an initial cycle of denaturation (4 min at 94°C) followed by 40 cycles with denaturation (1 min at 94°C), annealing (2 min at 54°C), and elongation (5 min at 72°C), and a final elongation (10 min at 72°C). To create a qPCR standard, Cu1F–Cu2R PCR product that was previously obtained from each ecosystem type was combined and the concentration of DNA in this mixture was determined using a PicoGreen assay following the manufacturer instructions (Invitrogen, Carlsbad, California, USA). A standard curve was created using serial dilutions of the Cu1F–Cu2R PCR product mixture (from 6.14×10^{-4} to 6.14×10^{-10} ng μ l⁻¹). The standard curve related the number of PCR cycles required to reach a fluorescence threshold of basidiomycete laccase genes (ng μ l⁻¹) present in the PCR template. Two independent QPCR reactions were performed for each sample, and each run included two sets of standards and no-template controls. Laccase DNA concentration (ng DNA g⁻¹ sample) was converted to number of gene copies g⁻¹ using the relative abundance of different PCR amplicon lengths (determined from LH-PCR profiles described below) and by assuming a molecular weight of 660 g mol⁻¹ per basepair.

Composition of Laccase-containing Basidiomycetes

We used length heterogeneity PCR (LH-PCR) to assess differences in the composition of laccase-containing basidiomycetes among ecosystems and N deposition treatments. For LH-PCR analysis, basidiomycete laccase genes were amplified from purified DNA in a Robocycler 96 (Stratagene, La Jolla, California, USA), using the above conditions with the Cu1F primer labeled with a fluorescent dye (FAM) at its 5' end. PCR product from two independent reactions was composited, and LH-PCR was performed by electrophoresis and detection of PCR product on a sequencing apparatus. Briefly, 1 μ l of diluted FAM-labeled PCR product was mixed with 12 μ l of Hi-Di formamide, and 0.02 μ l of GeneScan-500 LIZ size standard. The samples were then denatured and subjected to electrophoresis on a 3730xl DNA Analyzer (Applied Biosystems, Foster City, California, USA). Electropherograms were analyzed using the software GeneMarker (SoftGenetics, State College, Pennsylvania, USA) and GelCompar II (Applied Maths, Austin, Texas, USA). PCR amplicons of different sizes represented laccase operational taxonomic units (OTUs; for example, different genes or species; Blackwood and others 2007), and the areas under the peaks represented abundance of the fragments. Relative peak areas were calculated by dividing an individual peak area by the total peak area (sum of the areas of all of the peaks). Fragments comprising over 1% of the total abundance were included in our analysis.

Statistical Analyses

We used a linear, mixed-effects model with repeated measures to test the effects of ecosystem type, N deposition treatment, sampling date, and their interactions on phenol oxidase activity and basidiomycete laccase gene abundance (Proc Mixed, SAS 9.1, SAS institute, Cary, North Carolina, USA). In all statistical tests, ecosystems were represented by replicate stands ($n = 3$); experimental N treatments were subplots within stands. Significance for all analyses was accepted at $\alpha = 0.05$. We tested our first hypothesis using the interaction between ecosystem and N deposition treatment. Soil water content was included as a covariate in our analysis of phenol oxidase activity, because soil water content is known to exert a positive effect on phenol oxidase activity (DeForest and others 2005). Tukey's tests were used for post hoc comparisons among main effect and interac-

tion means. Our second hypothesis was tested by performing the analysis of phenol oxidase activity using the mixed model described above with basidiomycete laccase gene abundance added as a covariate. Community similarities based on laccase LH-PCRs were analyzed by performing redundancy analysis (RDA) of Hellinger distances between profiles (CANOCO 4.0; Microcomputer Power, Ithaca, New York, USA).

RESULTS

Phenol Oxidase Activity

An experimental N deposition by ecosystem interaction significantly influenced phenol oxidase activity in the surface soil (Figure 1B; $P = 0.05$), but not in the forest litter (Figure 1A; $P = 0.16$). In surface soil, phenol oxidase activity decreased with increasing experimental N deposition in the BOWO ecosystem. No consistent response to N deposition was evident for surface soil phenol oxidase activity within either the SMRO or SMBW ecosystem. Sample date had no significant main effect nor did it interact with ecosystem type or N deposition treatment to influence phenol oxidase activity (Figure 2A, B); soil moisture was not a significant covariate in our analysis of phenol oxidase activity.

Rates of phenol oxidase activity differed among ecosystems (main effect), with significantly greater activity in the litter of BOWO (15-fold) and SMRO (28-fold) compared to the SMBW ecosystem ($P = 0.01$). Phenol oxidase activity was also greater in the surface soil of BOWO (20-fold) and SMRO (12-fold) compared to SMBW, but those differences were not statistically significant. As a main effect, experimental N deposition did not significantly affect enzyme activity in surface soil ($P = 0.09$), a result likely arising from a significant ecosystem by N deposition interaction. The main effect of experimental N deposition on phenol oxidase activity in litter also was not significant ($P = 0.17$). On average, phenol oxidase activity was an order of magnitude greater in the litter compared to the surface soil (Figure 1A, B).

Laccase Gene Abundance and Composition

We found no significant interaction between ecosystem type and experimental N deposition on basidiomycete laccase abundance in the litter or surface soil (Figure 1C, D). However, as a main effect, experimental N deposition significantly reduced basidiomycete laccase gene abundance in the

surface soil ($P = 0.05$), with greatest mean abundance under ambient N deposition ($2.9 \times 10^6 \pm 0.6 \times 10^6$ copies/g; mean \pm SE) and lower abundance under ambient plus 30 kg ha⁻¹ per year ($1.7 \times 10^6 \pm 0.4 \times 10^6$ copies/g) and ambient plus 80 kg ha⁻¹ per year treatments ($2.0 \times 10^6 \pm 0.4 \times 10^6$ copies/g). Experimental N deposition (main effect) had no significant effect on laccase gene abundance in litter. The abundance of basidiomycete laccase genes differed significantly among ecosystems (main effect) in both litter and surface soil. For example, laccase gene abundance was almost two times greater in the litter of SMRO ($7.99 \times 10^6 \pm 1.46 \times 10^6$ copies/g) and BOWO ($7.38 \times 10^6 \pm 1.31 \times 10^6$ copies/g), compared to SMBW ($3.86 \times 10^6 \pm 0.94 \times 10^6$ copies/g; $P = 0.09$). In the surface soil, laccase gene abundance was 1.5 times greater in BOWO ($2.98 \times 10^6 \pm 0.61 \times 10^6$ copies/g) compared to SMRO ($1.61 \times 10^6 \pm 0.33 \times 10^6$ copies/g) and SMBW ($2.05 \times 10^6 \pm 0.48 \times 10^6$ copies/g; $P = 0.03$). On average, laccase gene abundance was 2.5 times greater in the forest litter compared to the surface soil.

Collection date, as a main effect, significantly influenced laccase gene abundance in the litter ($P = 0.02$) and surface soil ($P < 0.0001$). Mean basidiomycete laccase gene abundance in litter was four fold greater in July, August and October compared to September (Figure 2C). In the surface soil, mean abundance was at least four times greater in August compared to July, September and October (Figure 2D). Ecosystem by collection date interaction on basidiomycete laccase gene abundance was significant in the surface soil and moderately significant in the litter ($P = 0.04$, $P = 0.08$, respectively). Surface soil laccase gene abundance was greatest in August, with greater abundance in SMRO ($10.20 \times 10^6 \pm 6.0 \times 10^6$ copies/g) and BOWO ($8.33 \times 10^6 \pm 1.2 \times 10^6$ copies/g) ecosystems compared to SMBW ($5.12 \times 10^6 \pm 1.3 \times 10^6$ copies/g).

We found no significant correlation between basidiomycete laccase abundance and phenol oxidase activity after accounting for the effects of ecosystem, experimental N treatment, horizon, and collection date. Without taking these effects into account, there was a small but significant correlation between the basidiomycete laccase gene abundance and phenol oxidase activity ($r^2 = 0.18$, $P = 0.006$).

Canonical ordination revealed that basidiomycete laccase LH-PCR profiles were distinct among ecosystems and between litter and surface soils within ecosystems, with no overlap in standard error bars (Figure 3). Pooling litter and surface soil,

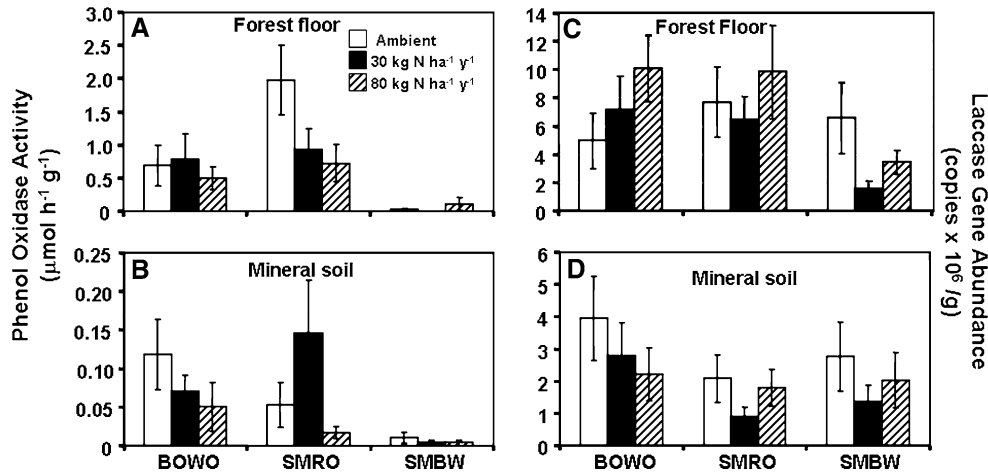


Figure 1. Ecosystem by experimental N deposition interactions for **A** forest litter phenol oxidase activity, **B** surface soil phenol oxidase activity, **C** forest litter laccase gene abundance and **D** surface soil laccase gene abundance. N treatment includes ambient (open bars), ambient plus 30 kg N ha⁻¹ per year (solid bars) and ambient plus 80 kg N ha⁻¹ per year (hatched bars). Bars represent means (\pm SE), $n = 12$.

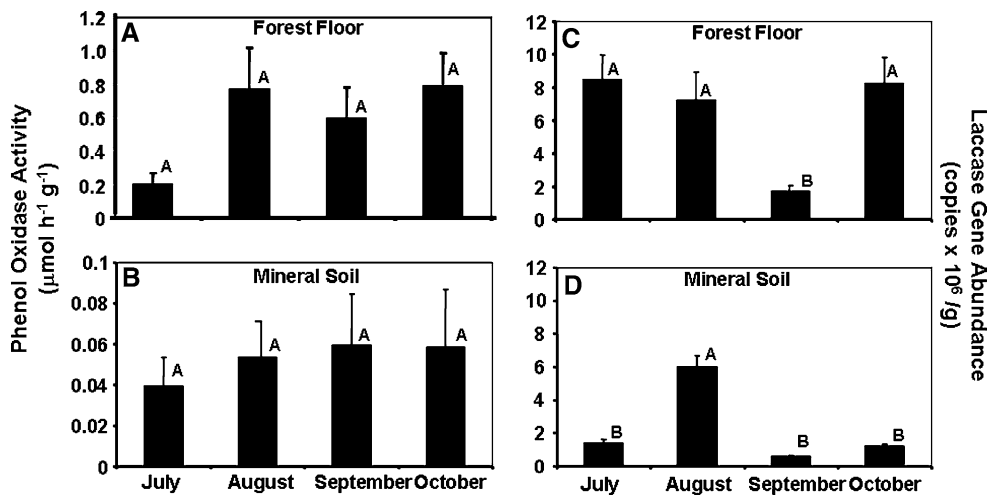


Figure 2. Phenol oxidase enzyme activity in the **A** forest litter and **B** surface soil and laccase gene abundance in the **C** forest litter and **D** surface soil in July, August, September, and October 2005. Symbols represent mean \pm SE; $n = 27$.

soil layer accounted for 5.1% ($P = 0.001$) and ecosystem type accounted for 5.5% of the variance ($P = 0.001$) in LH-PCR profiles. For the surface soil, RDA analysis revealed that ecosystem had a significant effect on basidiomycete laccase LH-PCR profiles ($P = 0.001$), contributing 18.5% of the variance. Nitrogen deposition treatment accounted for 3.8% of the variance in the length heterogeneity ($P = 0.08$) and date accounted for an additional 5.8% of the variance ($P = 0.03$). For litter, RDA indicated ecosystem accounted for 7.8% of the variance ($P = 0.001$); experimental N deposition had no significant effect ($P = 0.56$). Date composed 10.4% of the variance ($P = 0.001$), whereas soil water content had no effect on community composition. Experimental factors therefore accounted for a total of 28.1% (surface soil)

and 18.2% (litter) of the total variability in basidiomycete laccase LH-PCR profiles.

DISCUSSION

Experimental N deposition altered phenol oxidase activity in the surface soil in an ecosystem-specific manner consistent with our hypothesis, wherein experimental N deposition decreased phenol oxidase activity to a greater extent in BOWO (higher-lignin litter) compared to SMBW (lower-lignin litter). However, experimental N deposition had no consistent effect on phenol oxidase activity in surface soils from the SMRO site (intermediate lignin content), with highest activity occurring in the ambient +30 kg N ha⁻¹ per year treatment. Experimental N treatment did not affect basidio-

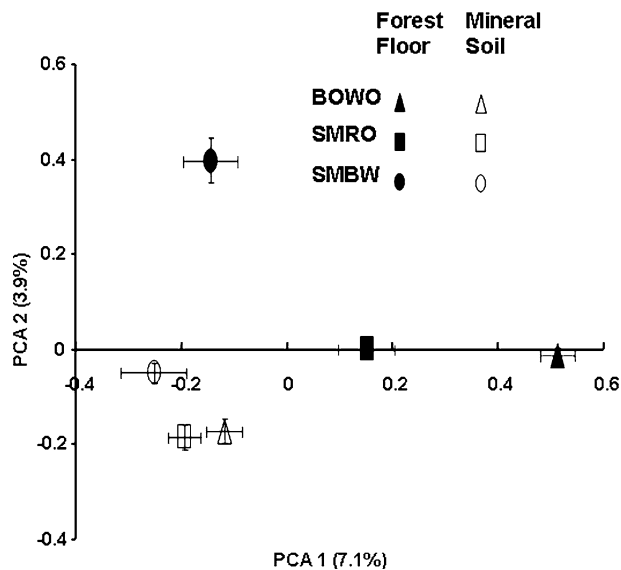


Figure 3. Canonical ordination plots showing ecosystem and horizon differences in LH-PCR profiles for forest litter and surface soil in BOWO, SMRO and SMBW ecosystems. Symbols represent mean \pm SE; $n = 36$.

mycete laccase gene abundance in an ecosystem specific manner. Instead, N deposition significantly decreased laccase gene abundance in the surface soil (main effect; 2.9×10^6 copies/g in ambient plots vs. $1.7\text{--}1.9 \times 10^6$ copies/g in N deposition plots). Despite this response, we found no relationship between phenol oxidase activity and laccase gene abundance, suggesting that basidiomycete gene abundance cannot explain the divergent responses of decomposition in ecosystems receiving experimental N deposition (Waldrop and others 2004b; Knorr and others 2005).

It is possible that laccase-containing basidiomycetes in the litter were less sensitive to experimental N deposition, resulting in no significant N treatment effects. Laboratory experiments testing the effects of inorganic N concentrations on pine and birch sapwood decay by various basidiomycete species demonstrated that greater inorganic N availability caused decay to increase in some cases, decrease in others, and in some instances not change at all (Worrall and others 1997). These results suggest that responses to inorganic N availability are both species- and substrate-specific. In our experiment, microbial communities in the decomposing leaf material of surface soils were more responsive to N treatment effects than those in forest litter. Our results are consistent with other field studies demonstrating that microbial communities in the forest litter and mineral soil have variable responses to experimental N deposition

(Blackwood and others 2007; Gallo and others 2004). The effects of experimental N deposition on basidiomycetes laccase LH-PCR profiles were marginally significant in the surface soil ($P = 0.08$), but not the forest litter ($P = 0.56$). Together, our LH-PCR and phenol oxidase activity results suggest that surface soil basidiomycete communities are distinct from the forest litter, and may be more sensitive to experimental N deposition.

The fact that we observed only marginal effects of experimental N deposition on LH-PCR profiles of laccase-containing basidiomycetes suggests that changes in phenol oxidase activity induced by experimental N treatment are caused by physiological adjustments of the extant community, rather than a change in community composition. However, our results indicate that the abundance of laccase-containing basidiomycetes is not necessarily driving phenol oxidase activity, at least not under the conditions of our experiment. It is possible we amplified laccases that encode for other physiological functions as discussed in Xiao and others (2004); however there is some evidence indicating lignin oxidation is the most likely function for basidiomycete laccase in soil (Blackwood and others 2007). It is also possible that some laccase genes involved in decomposition do not oxidize L-DOPA, the substrate used in our enzyme assay (Nagai and others 2003). Furthermore, some ectomycorrhizal fungi have laccase-encoding genes (Chen and others 2003) and may not have the same response to atmospheric N deposition as saprotrophic basidiomycetes. A decline in ectomycorrhizal basidiomycete abundance under increased N deposition (Lilleskov and others 2002) could confound laccase-specific results, and contribute to the variable response in laccase gene abundance and phenol oxidase activity. Ectomycorrhizal fungi were not a significant proportion of the laccase containing basidiomycete population in forest floor samples examined at our study sites (Blackwood and others 2007). It is likely that ectomycorrhizal fungi comprise a larger proportion of the mineral soil and contribute to the variable relationship between laccase gene abundance and phenol oxidase activity.

We believe the differences in laccase gene abundance and phenol oxidase activity are more likely due to variation in gene expression. Further studies are required to determine the relationship between the presence of functional genes and their expression, as well as the function of their enzymes in the environment. Because laccase gene abundance was not influenced by an interaction between ecosystem and experimental N deposition

treatment, it is possible that ecosystem specific changes in phenol oxidase activity are caused by changes in laccase expression in the surface soil, rather than by a change in the abundance of organisms containing the laccase gene. For example, laboratory experiments using the basidiomycete *Trametes trogii* have demonstrated that high inorganic N in growth media resulted in no transcription and no subsequent enzyme activity of the laccase-encoding gene (Colao and others 2003). Therefore, the presence of the laccase-encoding gene alone may not reflect physiological adjustments to experimental N deposition. One field study found less than 30% of the laccase genes present in soil were expressed as mRNA at a given point in time (Luis and others 2005). Congruent with this observation, 29% of forest litter samples and 20% of surface soil samples had no phenol oxidase activity in the BOWO ecosystem, but basidiomycete laccase genes were present in detectable quantities. By contrast, in the SMBW ecosystem, 80% of the forest litter samples and 86% of the surface soil samples had no phenol oxidase activity, even though we observed the presence of laccase-containing basidiomycete genes. In combination, these observations suggest that transcription of laccase may be up- or down-regulated in response to environmental conditions, independent of gene abundance. Such a response may explain why atmospheric N deposition can exert ecosystem-specific effects on decomposition.

Changes in gene abundance and transcription may be temporally disconnected, making it difficult to correlate gene abundance with enzyme activity. Although the abundance of laccase-containing basidiomycetes was highly variable through time, concurrent seasonal changes in enzyme activity did not occur. For example, July forest litter samples had a relatively high mean abundance of laccase-containing basidiomycetes (Figure 2C) and a low mean rate of phenol oxidase activity (Figure 2A). Active growth of laccase-containing basidiomycete communities may not coincide with laccase gene expression, resulting in high gene abundance and low enzyme activity on the July sampling date. In contrast, September forest litter samples have a low abundance of laccase-containing basidiomycetes (Figure 2C), but high rates of phenol oxidase activity (Figure 2A). In this case, the steady rate of enzyme activity may be due to persistence of functional enzymes (Burns 1982) that are independent of the current or recent abundance of laccase containing basidiomycetes. Additionally,

the ecosystem-specific responses to experimental N deposition of phenol oxidase activity may be driven by phenol oxidase enzymes or microorganisms not measured by our analyses. The range of substrates that laccase can oxidize is broad, and includes many compounds that can be oxidized by other enzymes (Baldrian 2006). Therefore, organisms producing enzymes such as catechol oxidase or tyrosinase may be contributing to the ecosystem specific N treatment effects on phenol oxidase activity. Furthermore, other laccase producing organisms that are not affected by N treatment, such as ascomycetes, could also contribute to soil phenol oxidase activity (Worrall and others 1997).

Field studies have documented ecosystem-specific decomposition responses to atmospheric N deposition, wherein surface soil organic matter content can increase and decrease. However, a direct link between changes in microbial abundance or physiology and enzyme activity is lacking. Decreases in phenol oxidase activity under high levels of N deposition may be due to several mechanisms including changes in the composition, abundance or activity of laccase producing basidiomycetes. Although laccase is a major contributor to phenol oxidase activity (Baldrian 2006), our evidence is consistent with Blackwood and other (2007) and suggests that neither the composition nor the abundance of laccase-containing basidiomycetes is driving the previously reported phenol oxidase response to atmospheric N deposition (Carreiro and others 2000; Waldrop and others 2004b). Although we did detect an N deposition main effect on basidiomycetes laccase gene abundance in the mineral soil, this response did not correspond to ecosystem specific changes in phenol oxidase activity under experimental N deposition. The regulation of laccase gene expression, rather than abundance, may mediate the decomposition response to atmospheric N deposition. Alternatively, it is possible that other litter decomposing microorganisms, such as ascomycetes or actinomycetes, are driving ecosystem specific responses to decomposition. Testing each of these alternative hypotheses is necessary to understand the microbial dynamics controlling N deposition effects on decomposition. Based on our results, it appears that the compositional differences in basidiomycete laccase gene abundance among ecosystems and the extent to which N deposition alters their activity are more important to regulating the production of phenol oxidase and subsequent responses of organic matter decomposition than is the abundance of the laccase gene.

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