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Simulated Atmospheric Nitrogen Deposition Alters Decomposition of Ephemeral Roots

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Abstract

Roots concentrated on the smallest distal branching orders have short life spans and thus dominate root mortality, and may contribute predominately to plant carbon and nutrient transfer into soil. Yet the effects of nitrogen (N) enrichment on decomposition of the finest root branching orders have not yet been examined. Resolving such N effects is critical for predicting the ecosystem consequences of increased anthropogenic N deposition. The first four root orders were separated into two classes: first- and second-order roots; third- and fourth-order roots. We studied the effects of N addition on decomposition of different root order classes in four temperate tree species over 4 years. Asymptotic decay models best fit the decomposition and allowed us to examine effects of N on initial versus later stages of decomposition separately. Very early in decomposition, N fertilization stimulated decomposition rates in higher-order roots, but had no effects on initial rates of decomposition in lower-order roots. In contrast, later in decomposition, N fertilization inhibited decomposition, ultimately resulting in a larger, slowly decomposing fraction in both lower-order and higher-order roots. Inhibitory effects of N addition on lignin-degrading enzyme activity might be an important mechanism explaining the negative effects of N on decomposition here. This study highlights the importance of long-term studies for understanding N effects on decomposition, and suggests that contrasting effects of N on different decomposition processes and carbon pools should be widely considered in biogeochemical models. Furthermore, the inhibitory effects of elevated atmospheric N deposition on decomposition of lower-order roots suggest that these roots may provide a critical mechanism of carbon and nutrient retention in soil because of their rapid input via root mortality.

Key words: fine roots; decomposition; litter quality; microbial enzymes; nitrogen; root order; temperate forests; lignin.

Introduction

Human activities such as fossil-fuel burning, application of artificial nitrogenous fertilizers and the cultivation of nitrogen (N)-fixing crops have increased atmospheric deposition of nitrogen by three- to five-fold over the past century (IPCC 2007), and thus has the potential to alter element cycling and carbon (C) storage on large scales. N deposition will likely continue to increase further

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(Galloway and others 2004; Lamarque and others 2005). As plants and microbes in most temperate ecosystems have historically evolved under N-limiting environment (Vitousek and Howarth 1991), the increased N deposition can be expected to affect the balance between ecosystem C inputs (that is, primary production) and losses (that is, decomposition) with implications for both ecosystem carbon sequestration and nutrient cycling.

The process of plant residue decomposition is of fundamental importance to ecosystems (Hobbie 1992, 1996), because it frequently determines ecosystem productivity by regulating the transfer of nutrients to the soil, and influences the amount of atmospheric CO_2 that can be sequestered in soils as soil organic matter (Berg and McClaugherty 2003; Prescott 2010). Although the role of chronic N addition in influencing litter decomposition has been studied extensively (Berg and others 1982; Berg 2000; Berg and Matzner 1997; Hobbie 2005, 2008; Keeler and others 2009; Hobbie and others 2012), the factors that regulate the process of decomposition in response to N availability are still uncertain. For example, some studies showed that inhibitory effects of N addition on decomposing litter can be explained by the negative effects of N on lignin-degrading enzyme activity (Carreiro and others 2000; Sinsabaugh and others 2002; Hobbie and others 2012). In contrast, other studies demonstrating inhibitory effects of N addition on decomposition observed no evidence for such enzymatic effects (Keeler and others 2009), or lignin degradation per se was unaffected by N fertilization (Hobbie 2008). These contrasting results suggest that the underlying mechanisms responsible for the inhibition effects of N on decomposition can vary among different ecosystems, and more work is needed to disentangle various controls of N effects on decomposition. Additionally, the magnitude and direction of the N effects may shift in long-term studies, stimulating decomposition during the early stages whereas depressing its later stages of decomposition when lignin degradation dominates (Berg and others 1982, 2010; Berg 1986; Berg and Matzner 1997; Hobbie and others 2012). Unfortunately, most experiments of N effects on decomposition do not last long enough to clarify whether such inhibitory effects exist during its late stages of decay.

Our current available knowledge of N effects on decomposition is almost exclusively based on studies of aboveground plant litter (for example, Berg and others 1982, 2010; Hobbie 2005, 2008; Aerts and others 2006; Janssens and others 2010; Hobbie and others 2012), yet belowground litter provides the dominant input of plant material into soil in many forest ecosystems (Gill and Jackson 2000; Ruess and others 2003). However, despite the fact that approximately one-third of global annual terrestrial net primary production (NPP) is allocated for fine root production (Jackson and others 1997), rarely have studies attempted to explore the influence of N enrichment on root decomposition, and no published empirical studies have focused on the finest, lowest-order roots that dominate turnover (Wells and Eissenstat 2001; Xia and others 2010). The increased N inputs from atmospheric deposition can be expected to influence the patterns of root decomposition by supplying decomposer microbes with this limiting nutrient. This supposition has been supported by indirect evidence that root litter frequently immobilizes N during the early stages of decomposition, suggesting that initial root substrate contains insufficient N to meet the growth and maintenance requirements of its microbial community (Parton and others 2007; Goebel and others 2011; Sun and others 2012, 2013). Additionally, many decomposition studies demonstrated that root decomposition rates were often positively correlated with initial root N concentrations (Berg 1984; Silver and Miya 2001; Fan and Guo 2010).

Mounting evidence suggests that variation in root tissue chemistry, anatomy, and longevity is functionally linked more to root orders than to root diameter (Pregitzer and others 2002; Guo and others 2008a, b; Valenzuela-Estrada and others 2008). There is a complex branching structure within fine root systems, with the smallest distal branching orders (for example, first and second) used principally for water and nutrient acquisition functions and the higher-order mother roots (for example, third, fourth, and higher) performing mainly transport of water and nutrients, and structural support and storage functions (Guo and others 2008a; Valenzuela-Estrada and others 2008, 2009). Lower-order roots consistently have markedly smaller diameters, higher N and P concentrations, higher mycorrhizal colonization levels, and shorter life spans than the roots of higher orders do (Guo and others 2008a; McCormack and others 2012; Kong and others 2014). Furthermore, the first- and second-order roots typically do not undergo secondary development in all temperate tree species examined so far (Guo and others 2008a; Valenzuela-Estrada and others 2008), and thus these lower-order roots typically have lower lignin concentrations than higher-order roots do (Goebel and others 2011). Taken together, the lower-order roots that most frequently die (Eissenstat and

others 2000; Comas and others 2000; Xia and others 2010) and that are chemically distinct from the higher-order roots may have contrasting responses to nitrogen enrichment from these higher-order roots over the course of decomposition. Although most previous root decomposition studies used the single diameter class classification (for example, <2.0 mm in diameter), this diameter range likely encompasses at least four branch orders (Pregitzer and others 2002; Guo and others 2004, 2008a).

The main objective of this study was to examine the effects of N addition on decomposition of lower-order (orders 1 and 2) versus higher-order (orders 3 and 4) roots. We are not aware of any studies that have assessed the effects of N fertilization on decomposition of the finest root branching orders. On the basis of these differences in function among root orders and our current available knowledge of N effects on aboveground litter decomposition, we first hypothesized that N fertilization will accelerate the initial decomposition rates of both lower-order and higher-order roots. Second, we hypothesized that during the later stages of decay, N fertilization will inhibit decomposition rates of third- and fourth-order roots because of N inhibition of oxidative enzyme activity, but will stimulate decomposition rates of first- and second-order roots because secondary (wood) development does not occur in these lower-order roots.

Methods

Site Description

The decomposition experiment was established in four temperate forest types at the Laoshan Forest Research Station of the Northeast Forestry University in Heilongjiang Province, northeastern China (127°30′–127°34′E, 45°20′–45°25′N). Local weather conditions are influenced by the continental monsoon climate with a strong monsoon, windy spring, a warm and humid summer, and a dry and cold winter. The mean annual precipitation is 730 mm, most of which falls in July and August. The average annual air temperature is 2.8°C, with a mean monthly maximum of 20.9°C and a mean monthly minimum of −19.6°C. Soils at the site are Hap-Boric Luvisols (dark brown forest soil in Chinese Soil Taxonomic System) that exceed 50 cm in depth with high organic matter content and nitrogen content (Gong and others 1999). We assessed root decomposition of the four temperate tree species grown within four temperate forest types. They were aspen–birch forest (dominated by *Populus davidiana* Dode and *Betula platyphylla* Suk.), hardwood forest (dominated by *Fraxinus mandshurica* Rupr., *Juglans mandshurica* Maxim., and *Phellodendron amurense* Rupr.), Korean pine (*Pinus koraiensis* Sieb. et Zucc.) plantation, and Dahurian larch (*Larix gmelinii* Rupr.) plantation. The site characteristics of the four forest types in this study are summarized in Table 1. The four tree species we studied were *Betula platyphylla*, *Fraxinus mandshurica*, *Larix gmelinii*, and *Pinus koraiensis*. These species differed markedly in initial root C:N ratios, Ca concentrations, and lignin concentrations, which have been linked to the rates of root decay (Silver and Miya 2001). Of the four species, *Fraxinus mandshurica* formed arbuscular mycorrhizal (AM) fungi and the other three species were associated with ectomycorrhizal (EM) associations (Guo and others 2008a).

Decomposition Experiment

Within each forest type, we established ten 3×3 m^2 plots. Five plots were left as control plots, and five were randomly chosen to receive 10 g N m^{-2} y^{-1} as NH_4NO_3 in solution (3 l solution per plot per application), beginning in May 2009. Control plots received an equivalent volume as water without N. We applied fertilizer by spraying NH_4NO_3 solution or water over each plot in six equal applications during the growing season between May and October.

We excavated root branching segments in 2009 from the litter layer and the upper 15 cm mineral soil layer and then carefully separated lateral root branches from soil, ensuring that the finest lateral roots were still intact and remained attached to somewhat coarser roots. The sampled roots were transported and frozen in cold storage until later processing and analysis. Following the approach of Pregitzer and others (2002), the first four root orders were identified, and the small adhering soil particles were carefully removed using magnifying glasses and tweezers. The mean root diameter (mm) from first-order to fifth-order roots was determined (see Table 2). For each species, first- and second-order roots were combined into one class, and third- and fourth-order roots were combined into a second class. Therefore, we had two different root classes: first- and second-order roots, [1 + 2]; third- and fourth-order roots, [3 + 4]. In the present study, we opted to use fresh roots in our decomposition analysis because there is no perfect solution for collecting sufficient dead roots that have not already begun to decay, as described by Hobbie and others (2010), despite the fact that fine roots may gradually lose some functions as

Table 1. Site Characteristics of the Four Forest Types

Forest type	Location (slope)	T_{10} (°C)	W_{10} (gH_2Og^{-1} soil)	Age (year)	Density (trees ha^{-1})	DBH (cm)	Dominant tree species
Larch plantation	Toe slope (3°C)	11.6	0.396	40	1682	18.7	*Larix gmelinii* Rupr.
Pine plantation	Mid slope (10°C)	10.8	0.458	46	2904	16.1	*Pinus koraiensis* Sieb. et Zucc.
Aspen–birch forest	Mid slope (12°C)	12.9	0.552	58	2381	12.9	*Populus davidiana* Dode, *Betula platyphylla* Suk.
Hardwood forest	Toe slope (5°C)	12.3	0.579	49	2017	15.6	*Fraxinus mandshurica* Rupr., *Juglans mandshurica* Maxim., *Phellodendron amurense* Rupr.

T_{10} and W_{10} stand for the average soil temperature and gravimetric water content at 10 cm depth measured during the growing seasons of 2011 and 2013. DBH stands for mean tree diameter at breast height.

Table 2. Mean Root Diameter (mm) from First-order to Fifth-order Roots of the Four Temperate Tree Species

Tree species	Root diameter				
	First-order	Second-order	Third-order	Fourth-order	Fifth-order
Pinus koraiensis	0.29 (0.01)	0.29 (0.02)	0.53 (0.03)	1.59 (0.12)	2.46 (0.28)
Larix gmelinii	0.26 (0.02)	0.28 (0.01)	0.46 (0.05)	0.85 (0.09)	1.71 (0.12)
Fraxinus mandshurica	0.34 (0.01)	0.45 (0.01)	0.55 (0.02)	0.89 (0.07)	1.38 (0.09)
Betula platyphylla	0.21 (0.01)	0.26 (0.03)	0.27 (0.06)	0.43 (0.09)	0.95 (0.06)

Values are means (with SE in parentheses). Root orders were identified by the morphometric approach, as refined by Pregitzer and others (2002).

they age (Eissenstat and Volder 2005) and are associated with saprotrophic fungi when still living (Resendes and others 2008; Li and others 2015).

All root order samples were oven-dried at 65°C to constant mass. Approximately 260 mg of root material of each root class were then sealed into decomposition bags (nylon, 10 × 10 cm^2, mesh size 120 μm). This mesh size of litterbags would prevent earthworms and other microarthropods from entering, which have been observed to play an important role in litter decomposition in many forest ecosystems (Hobbie and others 2006; Hättenschwiler and Bracht 2010), so our study focuses mainly on microbially mediated decomposition. Besides that, the use of mesh litter bags separated roots from soil and rhizosphere communities and thus may underestimate rates of fine root decomposition and nutrient cycling (Dornbush and others 2002; Fisk and others 2011; Li and others 2015), especially for the finest root branching orders (Sun and others 2012).

Litterbags of each root class were horizontally deployed at a soil depth of 10 cm (A horizon) in May 2010, and sequentially harvested in July and October 2010 and in October 2011–2013. Three litterbags for each root class were collected at each harvest. Upon harvest, attached soil and other debris were carefully removed from the litterbags, and the remaining root order segments were again oven-dried at 65°C to constant mass and weighed. We never found new roots of the four tree species growing into the bags, but we did find occasionally that some grass roots grew into the litterbags, which were easily removed using tweezers based on their distinct morphology, color, and architecture. Because the amount of harvested substrates from litterbags was far from being sufficient for quantifying the activities of microbial enzymes, we buried three additional decomposition bags of each root class in May 2010. These additional bags were filled with approximately 3.8 g of oven-dried sample material, and were retrieved in October 2011. Subsamples of harvested substrates for enzyme assays were frozen prior to analysis.

At the start of the litterbag experiment, oven-dried subsamples of litter material were ground and analyzed for initial substrate chemistry. Total C was analyzed by a multi N/C 3100 analyzer and HT1300 Solids Module (Analytik Jena AG, Jena, Germany). Substrate initial nutrients such as K, Ca, and Mg

were determined using a novAA 350 atomic absorption spectrometer analyzer (Analytik Jena AG, Jena, Germany) following digestion in 1 mol/L HCl (Bao 2000). After subsamples were digested in a solution of H_2SO_4 (98%)–$HClO_4$ (72%), total N concentration in the digested solution was determined by the Semimicro-Kjeldahl method and P was analyzed using the vanadomolybdate yellow color method (Watanabe and others 1998). Ash content was determined via loss on ignition at 450°C for 8 h in a muffle furnace. The estimated ash content varied from 4.6 to 30.1% with an average of 10.2%. This so-called ''ash'' included intrinsic ashes within the substrate, adhered soil particles, and other matter not removed during root cleaning, and was used to correct mass remaining. Analysis of substrate C fractions for litter samples followed the methods of McClaugherty and others (1984) and Ryan and others (1990). Briefly, root C-fraction concentrations including ''extractives'' (removed using a two-stage extraction in dichloromethane and boiling water, respectively), ''acid-hydrolyzable'' structural components (removed using a two-stage digestion in 72 and 2.5% sulfuric acid, respectively), and ''acid-unhydrolyzable'' structural components (that is, the residual of the two-stage sulfuric acid digestion minus ash mass) were determined by the forest products serial digestion method (Ryan and others 1990). Analysis of total non-structural carbohydrate (TNC) concentrations of litter samples followed the technique of Seifter and others (1950).

In late July 2013, we measured the pH in the root litter layer to assess whether treatments (control and N-fertilized) were affecting root decomposition by altering pH. Within each plot, five random soil samples of the litterbag horizon were collected, and combined in the field into one composite sample. In the lab, subsamples were coarsely chopped and equilibrated with deionized water (1:10) before pH determination. The pH was measured using an acidity meter (Sartorius PT-21, Shanghai, China).

Enzyme Assays

Using root samples of the additional litterbags retrieved in October 2011, we quantified the activity of five microbial enzymes that degrade lignin and cellulose according to the methods described in Sinsabaugh and others (1992) and Saiya-Cork and others (2002): three enzymes involved in cellulose degradation, β-1,4-glucosidase (β-G), cellobiohydrolase (CBH), and endocellulase (Endo); two enzymes involved in the degradation of polyphenols such as lignin and other highly recalcitrant C compounds, phenol oxidase (PhenOx), and peroxidase (Perox). Enzyme activity assays were performed by blending 0.5 g of root litter in 125 ml of acetate buffer (50 mmol/l, pH 5.0). Activities of β-1,4-glucosidase and cellobiohydrolase were assayed using the substrates (p-nitrophenyl-β-D-glucopyranoside and p-nitrophenyl-β-D-cellobioside, respectively) bound to the chromogen p-nitrophenol (pNP) and adding them to the litter slurry, incubating the mixture in the dark at 25°C for 1–4 h depending on the activity, and spectrophotometrically measuring the optical density (OD) of pNP at 410 nm (Sinsabaugh and Linkins 1990). Phenol oxidase and peroxidase activities were measured spectrophotometrically using L-3,4-dihydroxyphenylalanine (L-DOPA), and DOPA + H_2O_2 as substrates, respectively. And the OD of the oxidized reaction product was determined at 460 nm. Activity of endocellulase was measured viscometrically using carboxymethylcellulose as a substrate (Almin and Eriksson 1967). Enzyme activities for the pNP and L-DOPA assays are expressed as mmol substrate converted per hour per g litter dry mass (μmol h^{-1} g^{-1}). Activity of endocellulase was expressed as viscometric units per gram of ash-free dry mass litter per hour.

Data Analyses

We fit the proportion of initial mass remaining data against time to three alternative models (sensu Weider and Lang 1982) and quantified the best fit among them: a single-exponential decay model, $X = e^{-kt}$; a double-exponential decay model, $X = Ce^{-k_1 t} + (1 - C)e^{-k_2 t}$; and an asymptotic decay model, $X = A + (1 - A)e^{-k_a t}$, where X is the proportion of initial biomass remaining at time t. Note that the three models constrain the proportion of initial mass remaining data at time zero to be 1. In the single-exponential model, k is the decay constant. In the double-exponential model, C is the fraction of the initial mass that decays with decay rate k_1, whereas the remaining fraction ($1 - C$) decays with rate k_2. In the asymptotic model, A is the fraction of the initial mass with a decay rate of zero (the asymptote) whereas the remaining fraction ($1 - A$) decays with the decay rate k_a. Despite the fact that the decay rate would never actually equal zero, the asymptotic model hypothesized that there is a fraction of substrate that decays so slowly that the decay rate is almost zero. Thus, A can be assumed to represent the cumulative proportional mass loss when decay rates are very nearly zero,

which was widely modeled in previous litter decomposition studies (for example, Berg and Ekbohm 1991; Berg and Tamm 1991, 1994; Berg and others 1996, 2010; Berg 2000; Hobbie and others 2010, 2012).

Two-way analysis of variance (ANOVA) was used to assess the influence of root order and tree species on initial tissue chemistry concentrations or ratios. Decomposition model parameters and extracellular enzyme activity of decomposing substrates were compared among N-fertilized treatments, root order, and tree species using three-way ANOVA. To determine whether N-fertilized treatments were influencing decomposition by altering soil pH, decomposition model parameters of each root class within replicate plots were also compared using ANOVA, with soil pH included as a covariate. Linear regression was also used to determine how strongly the differential microbial enzyme responses to N enrichment were linked to variation in cumulative mass loss. All statistical analyses were carried out using the SPSS software (2001, ver. 13.0, SPSS Inc., Chicago, IL).

Results

Initial Substrate Chemistry

As hypothesized, the substrate initial chemical constituents differed among the roots of different order classes prior to their placement in decomposition litterbags (Table 3). Substrate initial nutrient concentrations were generally higher in [1 + 2] roots than [3 + 4] roots, except Ca concentration. For example, initial N concentrations in [1 + 2] roots were almost twice that of [3 + 4] roots across the four species (Table 4; species × order interaction, $P < 0.0001$). Patterns of C:N ratios among roots of different order showed an opposite trend to that of N concentrations (Table 4, order effect $P < 0.0001$ and species × order effect, $P < 0.0001$). However, in contrast to our expectations, initial concentrations of the acid-unhydrolyzable fraction (AUF) were consistently higher in [1 + 2] roots than in [3 + 4] roots among four species (Table 4; order effect, $P < 0.0001$ and species × order effect, $P < 0.0001$). Additionally, [1 + 2] roots generally had lower concentrations of the acid-hydrolyzable fraction (AHF) and total non-structural carbohydrates (TNC) than [3 + 4] roots. Of the C-fraction concentrations measured, only extractives were not affected by root order (Table 4; order effect, $P > 0.05$ and species × order effect, $P > 0.05$).

Table 3. Initial Root Chemistry Parameters (Means with SE) at the Start of the Litterbag Experiment in the Four Temperate Tree Species

Tree species	Root order	Initial root nutrients (mg element g^{-1} root)					Initial C-fraction (mg compound g^{-1})			TNC (mg g^{-1})	C:N
		N	P	K	Ca	Mg	Extractives	AHF	AUF		
Pinus koraiensis	Order 1–2	21.6 (0.9)	1.9 (0.4)	4.4 (0.5)	8.2 (1.6)	2.6 (0.1)	190.2 (17.4)	283.7 (20.7)	528.5 (40.1)	101.7 (10.9)	29.6 (1.4)
	Order 3–4	12.3 (0.5)	1.4 (0.4)	2.9 (0.1)	9.7 (0.8)	1.9 (0.2)	223.9 (25.1)	378.5 (28.1)	398.6 (32.9)	150.2 (18.4)	48.2 (1.9)
Larix gmelinii	Order 1–2	25.5 (2.3)	2.8 (0.2)	5.8 (0.2)	7.7 (1.1)	3.0 (0.2)	179.8 (13.8)	307.2 (16.9)	513.4 (38.7)	98.3 (9.6)	23.6 (1.8)
	Order 3–4	13.9 (1.2)	1.9 (0.2)	4.5 (0.3)	4.6 (0.5)	2.7 (0.3)	169.6 (15.2)	394.3 (20.1)	435.8 (37.5)	138.0 (14.1)	45.2 (3.1)
Fraxinus mandshurica	Order 1–2	31.9 (1.8)	2.5 (0.1)	4.0 (0.1)	6.2 (0.5)	1.9 (0.5)	331.6 (26.9)	268.5 (15.5)	401.7 (29.6)	195.8 (20.6)	14.4 (2.1)
	Order 3–4	18.3 (1.3)	1.7 (0.2)	4.9 (0.3)	8.1 (0.5)	1.9 (0.2)	327.0 (20.2)	383.1 (21.3)	289.3 (26.4)	279.1 (23.5)	23.7 (2.4)
Betula platyphylla	Order 1–2	28.1 (1.6)	2.2 (0.1)	5.3 (0.6)	9.8 (0.8)	2.4 (0.5)	280.3 (26.6)	322.8 (17.6)	397.5 (34.1)	164.9 (18.3)	18.7 (2.0)
	Order 3–4	16.7 (1.1)	1.4 (0.2)	3.7 (0.3)	9.7 (0.7)	2.0 (0.1)	302.4 (30.4)	396.3 (18.2)	302.1 (28.2)	214.6 (21.4)	34.8 (3.1)

Values are means (with SE in parentheses). For each species, first- and second-order roots were combined into one class, and third- and fourth-order roots were combined into a second class. Initial C-fraction is expressed per gram on an ash-free dry mass basis.
AHF = acid-hydrolyzable fraction; AUF = acid-unhydrolyzable fraction; TNC = total non-structural carbohydrate.

Table 4. Results from an Analysis of Variance (ANOVA) of Root Order on Initial Tissue Chemistry in the Four Temperate Tree Species of Initial Chemical Concentrations or Ratios

Factor	N	P	K	Ca	Mg	Extractives	AHF	AUF	TNC	C:N
Root order	***	***	**	***	**	ns	***	***	***	***
Species	***	ns	*	ns	*	*	*	**	**	***
Root order × species	***	**	***	***	**	ns	**	***	***	***

Abbreviations are as in Table 2.
$*P \le 0.05$; $**P \le 0.01$; $***P \le 0.001$; ns, not significant ($P > 0.05$).

Decomposition Models

In the great majority of cases, asymptotic models of root decomposition for each root class provided a better fit (mean $R^2 = 0.69$, median $R^2 = 0.72$, range = 0.49–0.91) than single-exponential decomposition models (mean $R^2 = 0.57$, median $R^2 = 0.54$, range = 0.28–0.73). This occurred because the four tree species we studied experienced initially rapid rates of root mass loss (k_a, range = 0.49–0.78 y^{-1}) followed by rates of decomposition that were very nearly zero with 0.09–0.36 of the initial mass remaining (Figure 1; Online Appendix). Additionally, asymptotes (A) obtained by fitting the asymptotic decay models were tightly negatively correlated with decay constants (k) calculated by the single-exponential decay models ($r = -0.82$, $P < 0.0001$). In other words, faster exponential decay rates typically corresponded to lower fractions of initial mass remaining whose decay rates approached zero. A double-exponential decay model was never the single best fit to the proportion of initial mass remaining data. Consequently, we fit individual replicates using asymptotic decay models, and present parameter values from this model.

Furthermore, despite their lower C:N ratio and smaller diameters, lower-order [1 + 2] roots decomposed slower than higher-order [3 + 4] roots over a 4-year observation period in the four species, as reflected by significant period, order class, or order × period effects (Figure 1). The fastest decay rate (y^{-1}) determined by single-exponential decay models was observed in *Fraxinus mandshurica* with decreasing decay rates in the order of *Fraxinus mandshurica* > *Betula platyphylla* > *Larix gmelinii* > *Pinus koraiensis*.

N Fertilization Effects on Decomposition Among Different Root Orders

As hypothesized, for higher-order [3 + 4] roots among the four species, nitrogen addition generally stimulated initial rates of decomposition (Tukey's HSD, $P < 0.01$; Figure 1). However, in contrast to our expectations, N enrichment had no significant effects on the initial decomposition rates in lower-order [1 + 2] roots ($P = 0.29$).

As expected, for higher-order [3 + 4] roots, additions of N increased the fraction of substrate whose decomposition rates approached zero (A) compared to the control litter (Tukey's HSD $P < 0.05$ in all cases; Figure 1). However, in contrast to our expectations, N fertilization also ultimately resulted in greater A (that is, a larger fraction of slowly decomposing substrate) compared to control treatments (Tukey's HSD $P < 0.05$ in all cases; Figure 1) in [1 + 2] roots across the four species.

N Fertilization Effects on Extracellular Enzyme Activity

The responses of the five microbial enzymes that degrade either cellulose or lignin may provide an important mechanism explaining decreased decay rates caused by N enrichment during the later stages of decomposition. After 2 years, addition of N generally stimulated the activity of microbial cellulose (β-1,4-glucosidase, cellobiohydrolase, and endocellulase) (Figure 2), but decreased the activity of ligninolytic enzyme in this experiment (Figure 3). The integrated activities of the five microbial C-acquiring enzymes (sum of the activity of β-1,4-glucosidase, endocellulase, cellobiohydrolase, phenol oxidase, and peroxidase, with the estimate value calculated by the duration of the interval) was positively related to cumulative mass loss at this harvest for each root class ($P < 0.01$ in all cases).

Addition of externally supplied N significantly decreased the pH in the root litter layer compared to the control treatment ($P < 0.001$). Soil pH in the root litter layer was unrelated to k, k_a, or A of each root class in the results of ANCOVA.

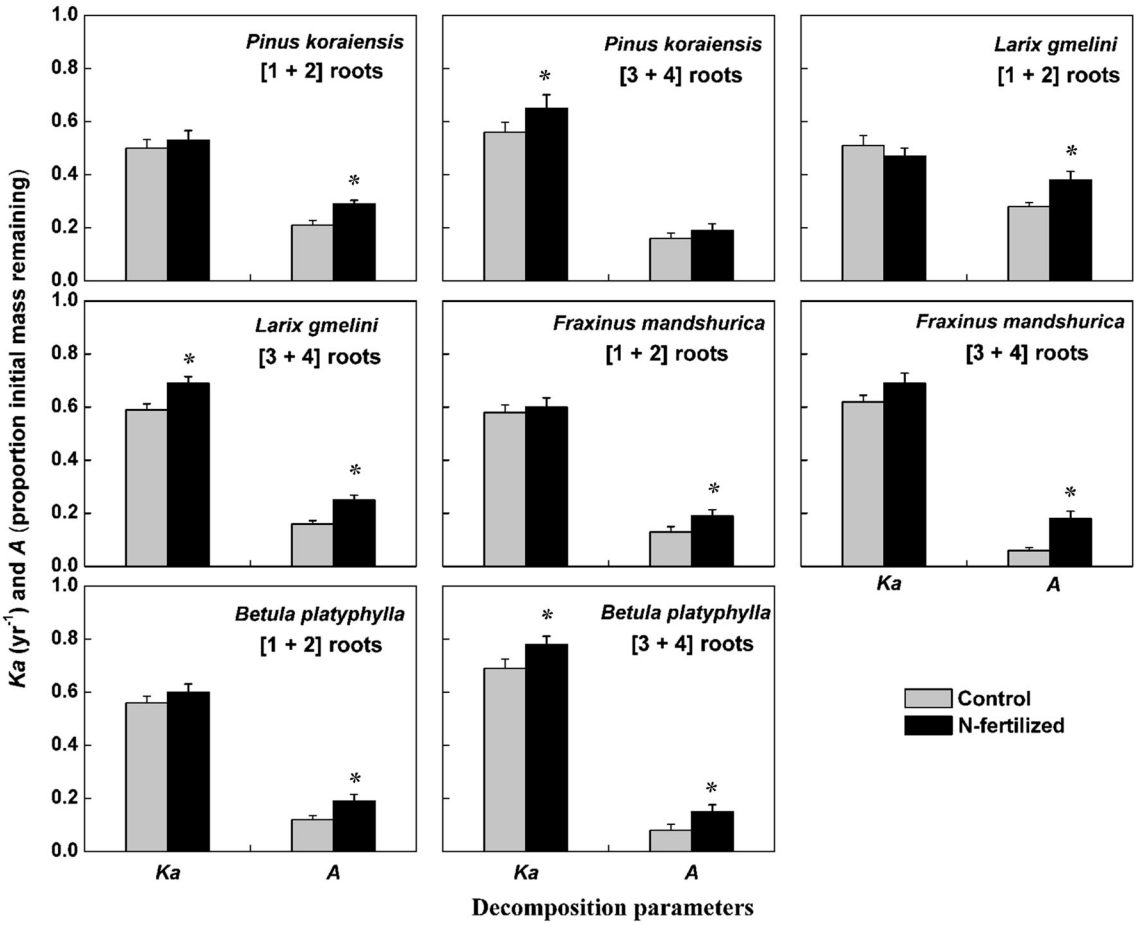

Figure 1. Decomposition parameters obtained from fitting decomposition data over 4 years to asymptotic decomposition models to show differences among treatments, tree species, and root classes: k_a, asymptotic model decomposition rate constant; A, fraction of the initial mass remaining at which decomposition rate approaches zero (that is, asymptote). Values are means + SE. An *asterisk* indicates significant Bonferroni-corrected pairwise comparisons within a decomposition parameter of a particular treatment with the control treatment (experiment-wise alpha = 0.05).

Discussion

Rarely have studies of root mortality and decomposition accounted for potentially important variation in functional heterogeneity within the fine root system (<2 mm in diameter). We believe this is the first report of N fertilization effects on decomposition of the finest root branching orders that dominate root turnover. Across substrates, asymptotic decomposition models best described decomposition. Fitting decomposition data to such models can be particularly informative in elucidating mechanisms associated with N effects on different decomposition processes and carbon pools (Berg and Ekbohm 1991; Berg and others 1996; Berg 2000; Hobbie and others 2012), while very few studies of N effects on decomposition are sufficiently long to allow estimation of the asymptotic mass remaining (Knorr and others 2005). Over a 4-year observation period, we conclude that N fertilization accelerated initial rates of decomposition in higher-order [3 + 4] roots, but had no effects on initial rates in lower-order [1 + 2] roots. However, later in decomposition, externally supplied N increased the fraction of very slowly decomposing litter (A) of both [1 + 2] and [3 + 4] roots, a result that was robust across the four forest types. Below we explore these contrasting effects of N on initial versus later stages of decomposition in more depth, linking patterns of decomposition to fine root function and structure, and addressing underlying mechanisms that underlie N effects and implications for ecosystem carbon storage.

We note that the nature of the N treatment is different in this study than in most foliar litter decomposition studies, because the litterbags are at 10 cm depth and much of the added N will not

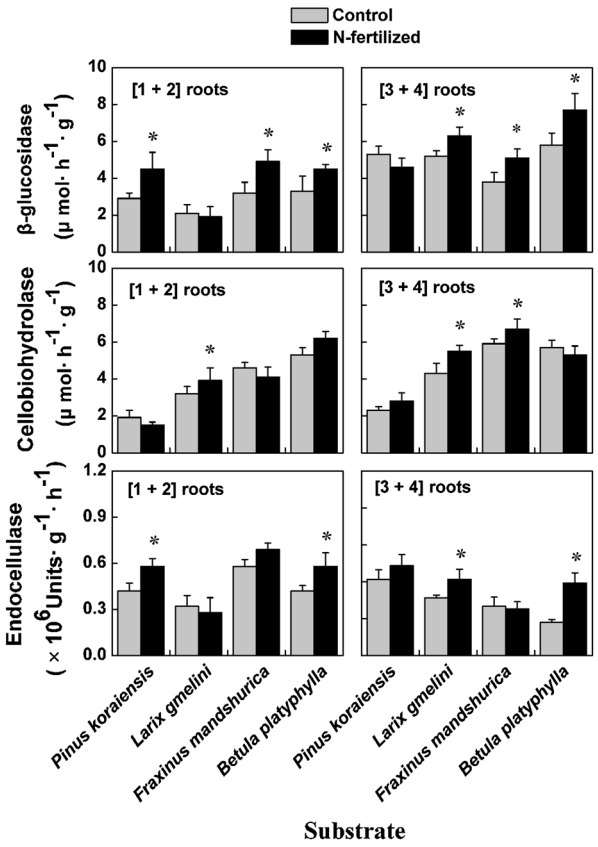

Figure 2. Activities of three microbial enzymes involved in cellulose decomposition (β-glucosidase, cellobiohydrolase, and endocellulase) during the second year of the experiment among treatments. An *asterisk* indicates that a particular treatment differed significantly from the control treatment (experiment-wise alpha = 0.05). Values are means + SE. Data are based on three additional decomposition bags retrieved in October 2011 per treatment for each root order class.

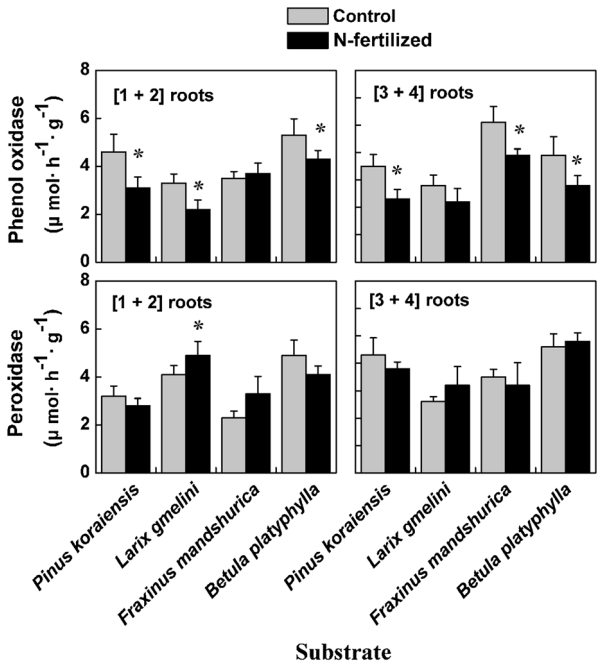

Figure 3. Activities of two microbial enzymes involved in lignin decomposition (phenol oxidase and peroxidase) during the second year of the experiment among treatments. An *asterisk* indicates that a particular treatment differed significantly from the control treatment (experiment-wise alpha = 0.05). Values are means + SE. Data are based on three additional decomposition bags retrieved in October 2011 per treatment for each root order class.

penetrate that deeply immediately, being strongly immobilized in the surface organic horizons. Moreover, the N environment in the litterbags may become progressively more enriched through time as the fertilizer penetrates downward through the soil.

Very early in decomposition, N fertilization stimulated decomposition rates of higher-order roots likely because of the large discrepancy between decomposer and litter C:N ratio when N is most likely to limit the rate of carbon use and thus mass loss in early stages of decomposition. This result is largely consistent with past syntheses of N fertilization effects on leaf litter decomposition (for example, Berg and Ekbohm 1991; Berg 2000; Hobbie 2008; Hobbie and others 2012). However, in contrast to our expectations, decomposition in lower-order roots demonstrated no readily apparent difference between unfertilized and N-fertilized plots. Such contrasting effects of N addition on the early stages of decomposition in [1 + 2] versus [3 + 4] roots suggested the joint control of substrate's initial C quality and N concentrations on root decomposition deserves further investigation. Litter decomposition is a process in which microbes mineralize substrate C and N to meet the balance of C and N in their biomass for their growth and maintenance requirements (Aerts 1997; Hobbie and Vitousek 2000; Ågren and others 2001, 2013). When litter contains relatively low concentrations of labile C (for example, TNC and cellulose) and high concentrations of N, such as in [1 + 2] roots, microbial decomposition can be expected to be limited by low substrate C quality (or ''C limitation'' sensu (Berg and McClaugherty 2003; Moorehead and Sinsabaugh 2006). Consequently, N fertilization effects on decomposition of lower-order roots were largely neutral during the early stage of decomposition. In contrast, decomposition of [3 + 4] roots that contains relatively high C quality (for example, TNC and cellulose) and low N concentrations might be less constrained by C

limitation, and thus resulting in more rapid biomass loss in N-fertilized plots compared to control plots.

Across substrates, the negative effects of externally supplied N on late-stage decomposition seem to be closely linked to dynamics in microbial enzyme activities. After 2 years of decomposition, N fertilization treatments were generally associated with lower activity of the lignin-degrading enzyme phenol oxidase, consistent with a larger, slowly decomposing fraction. This pattern has been predicted by theory (Manzoni and others 2008) and is similar to that observed for leaf litter in related studies (Sinsabaugh and others 2002; Hobbie and others 2012). Activity of microbial enzymes that degrade lignin and cellulose, two major compounds in plant tissues, were differentially influenced by the increased N availability to microbial communities. Cellulases, which are produced by many bacterial and fungal species, were increased by fertilization treatment in all of the decomposing litters. However, phenol oxidase, an important ligninolytic enzyme produced only by white-rot fungi in the Basidiomycota and Xylariaceae Ascomycota (Dix and Webster 1995), was greatly inhibited by N additions in almost all substrates. The negative effects of N addition on phenol oxidase activities might result from either decreased white-rot production of this enzyme, and/or from reduced competitiveness and thereby inhibited abundance of white-rot fungi relative to other fungi in N fertilization treatments (Carreiro and others 2000). This possibility that elevated atmospheric N deposition might decrease the abundance or change the activity of white-rot fungi should be investigated in future studies, especially in forest ecosystems where Basidiomycota dominates (Sinsabaugh and others 1992, 2005; Sinsabaugh 2010). Taken together, the results presented here provided further evidence that ligninolytic enzyme suppression can be an important mechanism explaining decreased decay rates of plant litter in N-fertilized experiments and support the inclusion of such effects in simulation models (Sinsabaugh and others 2008).

In contrast to our hypothesis, inhibitory effects of N fertilization on decomposition also occurred in lower-order roots. One possible explanation is that, in addition to the true molecular lignins, lower-order roots have been demonstrated to have other highly inhibiting C compounds such as suberin and tannin protein complexes, that is, lower-order roots usually corresponded to higher concentrations of the acid-unhydrolyzable fraction (Hendricks and others 2000; Guo and others 2004). In addition, lower-order roots are generally heavily colonized by mycorrhizal fungi (Guo and others 2008a; Valenzuela-Estrada and others 2008; Kong and others 2014), which produce the compound melanin and increase defense-related, secondary antifungal metabolites in these roots, both likely increase the formation of additional recalcitrant organic compounds in N-fertilized plots compared to control plots and thereby suppresses litter decay (Fog 1988; Davidson and others 2003).

Another factor that may contribute to the inhibition effects of N on decomposition is that during decomposition, carbon constituents in litter (for example, polyphenols, carbohydrates) may react with plant tissue N to form additional compounds that are resistant to decay (Nömmik and Vahtras 1982; Fog 1988; but see Knicker and others 1997). These resistant compounds can be increased by N fertilization, leading to slower decay rates in N-fertilized plots compared to control plots (Stevenson 1994; Berg and Matzner 1997). This interpretation here is in agreement with previous studies that the decomposing litter in N-fertilized plots sometimes had lower levels of A (a smaller slow fraction) than litter in control plots (Berg and Ekbohm 1991; Berg 2000; Hobbie and others 2012). An alternative explanation for the inhibition effects of added N on decomposition is that N altered the composition and/or physiology of the decomposer community in ways that inhibited its ability to decay (Bardgett and others 1999; Compton and others 2004; Frey and others 2004). For example, a modeling study showed that an N-induced decrease in microbial C:N stoichiometry by N fertilization could conceivably result in reduced demand for carbon and thereby decrease decomposition rates (Ågren and others 2001).

An additional factor that may contribute to negative effects of N on root decomposition is the decreased flux of the labile C from roots to soil in the form of exudates, secretions, and sloughed cells under N-fertilized plots (Sun, *unpublished data*), which has been demonstrated to be closely related with litter decomposition (Kuzyakov and others 2007; Phillips and Fahey 2007).

Although root litter is a major soil carbon and nutrient input in many forest ecosystems, studies have rarely considered how elevated atmospheric N deposition in these forested ecosystems effect root decomposition, especially of the finest, lowest-order roots. The results of this research have at least two important implications at the forest-stand level. First, given the fact that these lower-order roots have short life spans (Wells and Eissenstat 2001; Xia and others 2010), and these lower-order

roots, once dead, may surprisingly decompose at a slower rate than higher-order roots (Fan and Guo 2010; Goebel and others 2011; Xiong and others 2012), the negative effects of elevated atmospheric N deposition on decomposition in the lower-order roots implies that these lower-order roots may provide a critical mechanism of C and nutrient retention in the soil. Second, the contrasting effects of N additions on initial versus later stages of decomposition should be more widely considered in biogeochemical models; however, we are aware of only one study (Gerber and others 2010). Considering such studies would inform modeling efforts that aim to examine the effects of elevated atmospheric N deposition on terrestrial carbon storage, as suggested by Hobbie and others (2012).

In summary, using a branch order approach, we first studied the response of root decomposition of different root branching orders and its microbial enzymes to nitrogen deposition. By examining early versus late stages of root decomposition separately using an asymptotic model, we demonstrated that effects of N addition shifted over the course of decomposition, with N stimulating or having no effect the initial stages of decomposition, but ultimately resulting in a larger fraction of slowly decomposing litter (A) in both lower-order and higher-order roots. The results presented here highlight the importance of long-term studies for understanding effects of N additions on fine root decomposition. Furthermore, more studies of N effects on fine root decomposition should be conducted in a wider range of tree species (arbuscular mycorrhizal vs. ectomycorrhizal) and sites (grasslands vs. forests) and should be coupled with measurements aimed at elucidating underlying mechanisms responsible for the effects of N addition on decomposition, especially for the smallest distal branching orders that dominate root turnover.

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