Effects of mixture of branch order-based roots and nitrogen addition on root decay in a subtropical pine plantation

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Abstract Non-additive effects may occur when branching order-based fine roots decompose in mixture, due to their distinct chemical composition. However, these effects and how they respond to increased nitrogen (N) availability are poorly known. We conducted a 524-day field litterbag experiment to examine the potential non-additive effects of order-based fine root decomposition and their responses to different rates of N addition in a *Pinus elliottii* (slash pine) plantation in China. The intact fine roots of slash pine trees were hierarchically dissected and split into two groups (lower-order roots (the first and second order roots) and higher-order roots (the third to fifth order roots)). The decomposition of these two root groups was monitored either separately or in 1:1 mixture. The non-additive effects did not occur when the two root groups decomposed in mixture. However, high rate of N addition shifted the root group interactions from additive to synergistic effects through contrasting effects on individual root group (lower-order roots). High rate of N addition retarded the decomposition of lower-order roots more in isolation than in mixture, due to the decreased binding of acid-unhydrolyzable residue to inorganic N ions in mixture. Therefore, we suggest that although stoichiometric heterogeneity holds within the branching hierarchy of fine roots, the tight coupling of N with acid-unhydrolyzable residue of root tissues may limit N use by microdecomposers, and thereby causing the additive effect. Since the mixing effect of order-based root decomposition is

affected by N addition, understanding the chemical heterogeneity within fine root systems and their linkage with ambient N availability during decomposition either in isolation or in mixture may facilitate the accurate prediction of root decomposition responses to N loading.

Keywords Decomposition · Root branching order · Mixing effect · Acid-unhydrolyzable residue · Nitrogen addition

Introduction

Decomposition of fine roots represents a significant C flux in terrestrial ecosystems (Silver and Miya 2001). Similar to leaves, fine roots in natural ecosystems generally decompose in mixtures comprising roots from different plant species (Robinson et al. 1999; De Graaff et al. 2011; Shi et al. 2013), and/or in mixtures comprising different diameters of roots from the same species (De Graaff et al. 2013). As a result of litter mixing, the decomposition of roots from a plant species may be affected by the decomposition of roots from other plant species, with additive or non-additive (e.g., synergistic and antagonistic) effects. The non-additive effects are commonly found in leaf mixtures (Harguindeguy et al. 2008; Wang et al. 2009; Cuchietti et al. 2014), with different chemical composition (Gartner and Cardon 2004). It has been demonstrated that the synergistic effect may occur through the complementary resource use by microdecomposers (Vos et al. 2013). The antagonistic effect, however, may occur as high amounts of secondary compounds such as phenolics from low-quality litter can decrease the decomposition of high-quality litters (Hättenschwiler et al. 2005). Despite this, only one study has focused on the potential non-additive effects on the decomposition of root mixtures of a perennial grass species (De Graaff et al. 2013).

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There exists a huge morphological and chemical heterogeneity within fine root systems of woody tree species (Pregitzer et al. 2002), and thus in root-derived decomposing materials (Hishi 2007; Xiong et al. 2013; Wang et al. 2014). The branching fine root systems could be hierarchically categorized into non-lignified lower-order roots (e.g., the distal first and second orders) and lignified higher-order roots (e.g., the proximal third to fifth orders) structurally supportive of lower-order roots (Xia et al. 2010). Although the lower- and higher-order roots generally have contrasting life span (McCormack et al. 2013), they may decompose together when the long-lived higher-order roots die in parallel with concurrent death of short-lived lower-order roots. Lower-order roots typically possess thinner diameter and higher nutrient concentrations (e.g., N and P) (Li et al. 2010), yet they decompose more slowly than higher-order roots (Fan and Guo 2010; Goebel et al. 2011), due to high amounts of acid-unhydrolyzable residue (which includes lignin and phenolics) (Xiong et al. 2013). Given the contrasting morphological and chemical traits among order-based roots, the traditional diameter-based (e.g., <2 mm in diameter) root decomposition studies that mingle multiple orders of roots may bias the estimate of decomposition rate of fine roots due to occurrence of the non-additive effects.

Human-induced anthropogenic N deposition has doubled the reactive N input into soils (Galloway et al. 2008), which may affect decomposition of mixed litters (Vivanco and Austin 2011). For instance, N addition could shift the interactions between component species in mixture from additive effect either to synergistic effect, as a result of positive response of individual component species within mixture (Vivanco and Austin 2011), or to antagonistic effect, due to the elimination of N limitation to the microbial decomposers in low-quality litters (Lin et al. 2013). Additionally, N addition could also shift the interactions from non-additive effects (antagonistic effect) to additive effect via reducing variations in litter quality and increasing detritivore abundance (Rosemond et al. 2010). However, these observations and corresponding mechanisms were built up primarily based on the empirical studies on leaf decomposition. As far as we know, information on the mixing effect of order-based fine root decomposition and its response to N loading is lacking.

Pinus elliottii (slash pine), native to North America, has been widely planted as one of important coniferous tree species for vegetation restoration in subtropical China, where N deposition rates are increasing (Liu et al. 2013). To test the mixing effect of order-based root decomposition and its responses to increased N availability, we conducted a field experiment of the decomposition of lower-order roots, higher-order roots, and a 1:1 mixture (mass basis) of the two root groups of *P. elliottii* at ambient and N-fertilized conditions for 524 days. We used the decay rate, acid-unhydrolyzable residue concentration, and N release to characterize the

decomposition patterns and responses to N addition of each root group. We hypothesized that (i) lower- and higher-order roots would decompose at a lower rate in isolation than in mixture (i.e., presence of the synergistic effect), due to the morphological and initial chemical differences between the two root groups; (ii) N addition would affect the interaction (mixing) effect between these two order-based root groups, since the complementary use of N by microbial decomposers during the decomposition would be reduced by the exogenous N addition; and (iii) the exogenous N effects on the decomposition of order-based root mixture would be N-rate-dependent, with the higher effects occurring under higher N rates.

Materials and methods

Study site and experimental design

This study was carried out at our ongoing N-addition manipulative experiment site, which is situated at the Qianyanzhou (QYZ) Experimental Station of Red Soil and Hilly Land, Chinese Academy of Sciences (CAS), Jiangxi province, south-eastern China (26° 44' 29.1" N, 115° 03' 29.2" E, 102 m a.s.l.). The region is characterized by subtropical humid monsoon climate. The mean annual air temperature and mean annual precipitation are 17.9 °C and 1475 mm, respectively (Wen et al. 2010). Soils of the region are weathered from red sandstone and mudstone and classified as Typic Dystrudepts Udepts Inceptisols according to the USDA system (Wang et al. 2012). The organic matter content was 58.3 ± 7.5 g kg⁻¹ for 0–5 cm soil layer and 24.4 ± 2.1 g kg⁻¹ for 5–10 cm soil layer. The total N and P contents were, respectively, 1.7 ± 0.3 g kg⁻¹ and 217 ± 60 mg kg⁻¹ for 0–5 cm soil layer and 1.0 ± 0.1 g kg⁻¹ and 174 ± 20 mg kg⁻¹ for 5–10 cm soil layer. The original vegetation on the gently undulating terrain was evergreen broad-leaved forests, but it was heavily destroyed due to logging and land conversion for agricultural production prior to the 1980s. The vegetation was restored around 1985 by planting slash pine (*Pinus elliottii*), Masson pine (*Pinus massoniana*), and Chinese fir (*Cunninghamia lanceolata*). The understory vegetation is dominated by *Woodwardia japonica*, *Loropetalum chinense*, and *Dicranopteris dichotoma* (Wang et al. 2012). The ambient wet N deposition rate at the site is about 10 kg N ha⁻¹ year⁻¹ with approximately 68 % being ammonium-N (Zhan et al. 2014). More detailed site information is available in Kou et al. (2015a).

In November 2011, we laid out nine plots (20 × 20 m, slope angle <15°) in a randomized complete block design. The neighboring plots were isolated by a buffer zone (at least 10 m) and subjected to three N addition rates: control (ambient N deposition), +40 (low N), and +120 (high N) kg N ha⁻¹ year⁻¹ (NH₄Cl) above the ambient atmospheric N

deposition rate. Each treatment was replicated three times. Fertilizers were weighed and fully dissolved in 30 L tap water, and then evenly sprayed onto N-addition zone on non-rainy days. The control plots were supplied with equivalent amount of tap water. The N addition initiated on 1 May 2012 and proceeded at a month interval to simulate chronic atmospheric N deposition that would occur over time.

Root-derived material preparation

Intact fine roots were dug out from the 0–10 cm mineral soil layer under a non-disturbed *P. elliotii* plantation forest and brought to laboratory within 2 h in mid-May 2013. Soil and organic matter particles adhering to roots were gently rinsed off with running water. Root orders of the clean live roots were identified using fine forceps following the protocol of Pregitzer et al. (2002). In this study, only the first five order roots were reserved and partitioned into two groups (decomposing substrates), based on the similarity in tissue chemistry (Xia et al. 2010). Most distal two order roots (the first and second orders) were combined as lower-order roots and the proximal three order roots (the third to fifth orders) were grouped into higher-order roots. Diameter, specific root length, and root tissue density of these two root groups were determined in September 2013 (Kou et al. 2015a). The classified roots were first air-dried and stored for about 1 week, and then oven-dried to a constant mass at 40 °C.

Litterbag arrangement, harvest, and root litter chemical analyses

Nylon litterbags (10×10 cm, 0.1 mm meshed) were filled with 2.003 ± 0.002 g (dry weight) branching order-based roots as decomposing substrates. The litterbags consisted of either lower-order roots or higher-order roots or a mixture in equal proportions of the two order-based root groups (1.001 ± 0.001 for lower-order roots and 1.003 ± 0.002 for higher-order roots). Although the small mesh size would unavoidably limit the entrance of some decomposer organisms (e.g., microarthropods) into litterbags, yet it prevented the physical loss of lower-order roots. On 23 May 2013, one subplot (2.5×3.0 m) was established at the center of each plot. All the litterbags were buried at an angle of approximately 30° to the vertical direction and fixed on the ground using nylon strings and steel nails. An additional set of three replicates of each root group was used for initial chemical analyses.

The litterbags were collected after 69 (31 July 2013), 190 (29 November 2013), 281 (28 February 2014), 371 (29 May 2014), and 524 (29 October 2014) days of decomposition. On each harvest date, one litterbag of each type of decomposing substrates was collected from each subplot. Any litterbags broken by soil animals or contaminated by pine oil or root exudates, another one would be sampled for residual mass determination

and chemical analysis. The laboratory processing of the harvested litterbags was situation-dependent. When the litterbags were dry, they were immediately cleaned by gently brushing away the soil outside. However, when the litterbags were wet, they were first oven-dried, and then their clay particles attached were carefully removed prior to opening the litterbags. Additionally, the in-growing roots and sometimes tiny arthropods were also removed from the litterbags by tweezers. The retrieved root litter was oven-dried to a constant weight at 40 °C to determine the residual mass. To identify the N addition effects on the decomposition of individual root group, lower- and higher-order roots in mixture were separated based on root color (dark-brown for lower-order roots vs. yellowish-brown for higher-order roots), size (ca. 3 mm for lower-order roots vs. >25 mm for higher-order roots in length (data not shown); 0.33 mm for lower-order roots vs. 0.77 mm for higher-order roots in diameter), and texture (smooth for lower-order roots vs. rough for higher-order roots). More specifically, the larger-size higher-order roots were first picked out, and then dark-brown lower-order roots were separated from the remaining smaller-size, rough higher-order roots using forceps. In this study, the separation of root mixtures could only be achieved at the first four harvest dates due to the severe fragmentation of higher-order roots at the final harvest date (524 days).

Both the chemical composition and C fraction (extractives, acid-unhydrolyzable residue, and acid-hydrolyzable residue) of lower- and higher-order roots were determined. All root samples were ground with a Restch MM400 mixer mill (Retsch GmbH, Haan, Germany) to determine the content of C and N using a Vario MACRO cube elemental analyzer (Elementar, Germany). Carbon fraction was determined by the sulfuric acid digestion method combined with chloroform-methanol extraction (Ryan et al. 1990; Xiong et al. 2013; Kou et al. 2015b). Specifically, a milled powder subsample (200–300 mg) was first extracted with chloroform-methanol (2:1 v/v, 15 ml) for 2 h and then filtered. The oven-dried residue was digested with sulfuric acid (72 %, 15 ml) for 3 h and then centrifuged (2470×g, 8 min). Supernatants in centrifuge tubes (50 ml) were repeatedly replaced with deionized water (approximately ten times) until the pH was 7. The residue was filtered through a cellulose acetate membrane (0.45 μm) with a vacuum pump and oven-dried to a constant weight (*W*). Ash content of sample and of the residue (*W*) was determined by combusting samples (40–50 mg) in muffle furnace at 550 °C for 4 h. The acid-unhydrolyzable residue concentration was calculated as follows: acid-unhydrolyzable residue concentration (%) = $(W - \text{ash-in-}W) \times 100 / (\text{subsample} - \text{ash-in-subsample})$. All initial root chemical parameters and residual mass over time were expressed on an ash-free, dry mass basis.

Statistical analysis

Root decomposition rate was fitted to a first-order negative exponential decay equation (Olson 1963): $X_t = X_0 e^{-kt}$, where

X_t is the residual mass at time t , X_0 is the initial mass, e is the base of natural logarithm, and k is the decay rate constant. The mixing effect of order-based roots was assessed by comparing the observed against expected values (residual mass and k) using Student's t test. In this study, the “observed” represents the residual mass or k of lower- and higher-order roots when they decomposed in mixture, and the “expected” refers to the average of lower- and higher-order roots when they decomposed in isolation. The mixing effect is synergistic when the observed is higher than the expected, and on the opposite, the mixing effect is antagonistic.

One-way ANOVA was used to test the interspecific differences of morphological traits and initial tissue chemistry parameters among root-derived decomposing substrates. Repeated measures two-way ANOVA were used to test the effects of N treatments (CK, low N, and high N) and decomposition conditions (“isolation” vs. “mixture” for each root group or “observed” vs. “expected” for the whole mixture) on residual mass (% of initial mass), residual acid-unhydrolyzable residue (% of initial mass), and residual N (% of initial mass) of roots. Except on the final harvest date (524 days), when higher-order roots in the litterbags were severely fragmented, on other harvest dates (69, 190, 281, and 371 days), parameters above for each root group in mixture were measured separately to assess the separate effect of the mixing decomposition on each root group. For these parameters of each root group, the significant differences between “isolation” and “mixture” at different N-addition treatment conditions were further examined by Student's t test. Data were log-transformed where necessary to improve normality and reduce heteroscedasticity. Significant differences between means were compared using Tukey's HSD test. Linear regression analysis was carried out to determine the relationships between expected residual mass and observed residual mass with respect to different N addition rates. All statistical analyses were performed using the SPSS software version 18.0.

Results

Root morphological traits and initial tissue chemistry

Lower-order roots had smaller mean diameter, higher specific root length (SRL), but lower root tissue density (RTD) than higher-order roots. In addition, lower-order roots had higher extractives and acid-unhydrolyzable residue and N concentrations, but lower acid-hydrolyzable residue concentration relative to higher-order roots. In terms of their ratios, lower-order roots had lower C/N, acid-unhydrolyzable residue/N, and acid-hydrolyzable residue/N ratios, but higher acid-unhydrolyzable residue/acid-hydrolyzable residue ratios compared with higher-order roots (Table 1). Above parameters

Table 1 Morphological traits and initial chemical composition of order-based roots. Values are means with SE in parentheses. Different superscript letters within each column represent significant differences ($P < 0.05$) among decomposing roots. SRL represents specific root length, RTD root tissue density, AUR acid-unhydrolyzable residue, and AHR acid-hydrolyzable residue

Root type	Morphological traits				C fractions and N concentrations				Key ratios			
	Diameter (mm)	SRL (m g ⁻¹)	RTD (g cm ⁻³)	Extractives (%)	AUR (%)	AHR (%)	N (mg g ⁻¹)	C/N	AUR/N	AHR/N	AUR/AHR	
Lower-order roots (orders 1–2)	0.33 ^c (0.02)	44.32 ^a (3.26)	0.27 ^c (0.01)	12.14 ^a (0.79)	50.41 ^a (1.22)	37.45 ^c (0.88)	12.96 ^a (0.07)	36.34 ^c (0.21)	38.92 ^a (1.10)	28.90 ^c (0.52)	1.35 ^a (0.06)	
Higher-order roots (orders 3–5)	0.77 ^a (0.05)	11.89 ^c (1.04)	0.33 ^a (0.01)	11.12 ^a (0.76)	36.28 ^c (0.54)	52.60 ^a (0.23)	9.11 ^c (0.11)	50.46 ^a (1.44)	39.86 ^a (0.96)	57.77 ^a (0.91)	0.69 ^c (0.01)	
1:1 mixture (orders 1–2, 3–5)	0.55 ^b (0.02)	28.10 ^b (1.70)	0.30 ^b (0.01)	10.92 ^a (0.71)	42.51 ^b (0.65)	46.58 ^b (0.20)	10.79 ^b (0.06)	43.56 ^b (0.23)	39.39 ^a (0.41)	43.17 ^b (0.22)	0.91 ^b (0.01)	

Root morphological traits were determined in September 2013 (Kou et al. 2015a). C fractions, N concentrations, and key ratios for lower- and higher-order roots were derived from Kou et al. (2015b). For 1:1 mixture, morphological traits were calculated as the mean values of the two root groups and other parameters were calculated from the mixture

lied in the intermediate values for the mixture of lower- and higher-order roots, except for the extractives (Table 1).

Mixing effect of order-based root decay and its responses to N addition

Decay rate constants (k) did not differ significantly between the “observed” (the 1:1 mixture) and “expected” (the average of lower- and higher-order roots decomposing separately) at ambient conditions ($P>0.05$, Fig. 1), indicating that the additive effects occurred in the decomposition of order-based root

mixture. Different rates of N addition exerted differential effects on the mixture decomposition of lower- and higher-order roots ($P=0.002$, Table 2; Fig. 1). The high N rate significantly stimulated the decomposition of order-based root mixture (Fig. 1c), indicating a shift from the additive effect to the synergistic effect. However, the low N rate did not eliminate the additive effect of root mixture (Fig. 1b). The observed residual mass significantly correlated with the expected residual mass under both ambient and N addition conditions (R^2 ranges from 0.935 to 0.949, $P<0.0001$, Fig. 2).

Decay of individual root group and its responses to N addition

During the first 371 days, decomposition of lower-order roots did not differ significantly when in isolation or in mixture both at ambient (Fig. 3a) and low N rate (Fig. 3b), but lower-order roots decomposed faster in mixture than in isolation at high N rate ($P<0.001$, Fig. 3c). Regardless of N addition, no significant difference was observed for higher-order roots when decomposed separately or in mixture (Fig. 3d–f).

Acid-unhydrolyzable residue concentrations and N release patterns of root groups

At high N rate, the residual acid-unhydrolyzable residue (% of initial mass) was significantly higher in lower-order roots decomposing separately than in mixture ($P<0.001$, Fig. 4c), but there were no differences at ambient (Fig. 4a) and low N rate (Fig. 4b). For higher-order roots, the residual acid-unhydrolyzable residue did not differ significantly when decomposed separately or in mixture at all N rates (Fig. 4d–f). Lower-order roots generally experienced net N release when decomposed separately or in mixture at ambient (Fig. 5a) and low N rate (Fig. 5b), but showed N immobilization when decomposed separately at high N rate ($P=0.019$, Fig. 5c). Higher-order root decomposition experienced N immobilization at all N rates (Fig. 5d–f). Higher-order roots showed higher N immobilization when decomposed separately than in mixture at both low- ($P=0.002$, Fig. 5e) and high-N ($P=0.010$, Fig. 5f) rates.

Discussion

Additive effect of order-based root decay

In contrast to our first hypothesis, decomposition of lower- and higher-order root mixture did not present synergistic effects (Fig. 1a). Previous studies have shown that non-additive effects can occur as roots of multiple plant species were mixed, which may be due to their different chemical composition (Robinson et al. 1999; De Graaff et al. 2011). We

Fig. 1 Decomposition (residual mass over time and decay constant k) of order-based root mixtures of *P. Elliottii* under ambient (a), low (b), and high (c) N addition rates. Data are expressed as means $\pm$ standard error ($n=3$, $P<0.05$). The observed decomposition (lower- and higher-order roots when decomposed in mixture) is indicated by gray-filled circles, and the expected decomposition (lower- and higher-order roots when decomposed in isolation) is indicated by black-filled circles

Table 2 Results of the repeated measures two-way ANOVA for residual mass, residual acid-unhydrolyzable residue, and residual N, with different N addition rates (CK, low N, and high N) and

decomposition conditions (“isolation” vs. “mixture” for each root group, or “observed” vs. “expected” for the whole mixture) as factors

Root type	Source of variation		Residual mass		Residual AUR		Residual N	
			F	P	F	P	F	P
Lower-order roots	Between subjects	Treatment	17.306	<0.001	16.462	<0.001	11.207	0.002
		Condition	16.901	0.001	21.978	0.001	5.038	0.044
		Treatment×condition	5.566	0.019	3.215	0.076	0.195	0.825
	Within subjects	Time	168.564	<0.001	4.951	0.006	16.576	<0.001
		Time×treatment	1.650	0.181	1.111	0.375	0.593	0.733
		Time×condition	0.546	0.662	1.908	0.146	4.248	0.011
		Time×treatment×condition	0.301	0.930	0.310	0.928	0.635	0.702
Higher-order roots	Between subjects	Treatment	14.118	0.001	8.925	0.004	5.148	0.024
		Condition	0.027	0.873	1.068	0.322	10.372	0.007
		Treatment×condition	0.968	0.408	0.780	0.480	0.330	0.725
	Within subjects	Time	515.485	<0.001	115.663	<0.001	0.689	0.565
		Time×treatment	3.710	0.006	0.939	0.479	0.345	0.908
		Time×condition	4.458	0.009	1.854	0.155	0.176	0.912
		Time×treatment×condition	1.820	0.123	0.364	0.897	0.571	0.751
The whole	Between subjects	Treatment	11.082	0.002	13.492	0.001	7.157	0.009
		Condition	9.327	0.010	0.026	0.874	9.283	0.010
		Treatment×condition	3.707	0.056	0.126	0.883	0.242	0.789
	Within subjects	Time	600.167	<0.001	87.289	<0.001	3.342	0.030
		Time×treatment	3.252	0.005	0.396	0.917	0.538	0.776
		Time×condition	0.392	0.813	22.680	<0.001	0.431	0.732
		Time×treatment×condition	1.635	0.140	0.265	0.974	0.562	0.758

CK, low N, and high N represent control (no N addition), 40 kg N (NH₄Cl) ha⁻¹ year⁻¹, and 120 kg N (NH₄Cl) ha⁻¹ year⁻¹, respectively

therefore expected that mixing order-based root groups from a single species would also present non-additive effects if they have contrasting tissue chemistry. However, the “observed”

Fig. 2 Relationships between observed residual mass (% of initial mass, $n=15$) and expected residual mass (% of initial mass, $n=15$) under ambient and N addition conditions: *light-gray circles* indicate control; *medium-gray triangles*, low N rate; *dark-gray squares*, high N rate. *Blue dashed line* represents 1:1 line

decomposition rate (i.e., 1:1 mixture) did not differ (k -values, Fig. 1a), but significantly correlated (residual mass, Fig. 2) with the “expected” decomposition rate (i.e., the average of lower- and higher-order roots in isolation decomposition), suggesting that the additive effect occurred when roots with different orders decomposed in mixture. This may imply that the occurrence of synergistic effect depends on plant species (Robinson et al. 1999; Cong et al. 2015). A recent study also shows that the mixture decomposition of different diameters (0–0.5, 0.5–1, and 1–2.5 mm) of roots from a single grass species did not display the synergistic effect (De Graaff et al. 2013). Therefore, regardless of the branch order- or diameter-based root mixture, the synergistic effect commonly observed in decomposition of root mixtures among species (Robinson et al. 1999; De Graaff et al. 2011) may not hold within species.

The synergistic effect is generally driven by the chemical heterogeneity of mixed litters via the priming effects (Wardle et al. 1997; Chapman and Koch 2007) or niche complementarity among microdecomposer groups (Hector et al. 1999). Lower-order roots are the primary plant structures for nutrient uptake within the branching fine root systems (Xia et al. 2010) and are commonly associated with mycorrhizal fungi (Guo

Fig. 3 Residual mass (% of initial mass) of order-based fine root decomposition in isolation or in mixture under ambient (a, d), low (b, e), and high (c, f) N addition rates during the first 371-day decomposition. a–c Lower-order roots; d–f higher-order roots. Gray-

filled circles indicate decomposition of root groups in isolation, and black-filled circles indicate decomposition of root groups in mixture. Data are expressed as means±standard error ($n=3$, $P<0.05$)

et al. 2008), and thus they have higher N and P concentrations than higher-order roots (Pregitzer et al. 2002; Li et al. 2010). However, no interaction between the two order-based root

groups actually occurred as they decomposed in mixture (Fig. 3a, d), indicating that stoichiometric dissimilarity may not always drive the synergistic effect (Hättenschwiler and

Fig. 4 Residual acid-unhydrolyzable residue (% of initial mass) of order-based fine root decomposition in isolation or in mixture under ambient (a, d), low (b, e), and high (c, f) N addition rates during the first 371-day decomposition. a–c Lower-order roots; d–f higher-order roots. Gray-

filled circles indicate decomposition of root groups in isolation, and black-filled circles indicate decomposition of root groups in mixture. Data are expressed as means±standard error ($n=3$, $P<0.05$)

Fig. 5 Residual N (% of initial mass) of order-based fine root decomposition in isolation or in mixture under ambient (a, d), low (b, e), and high (c, f) N addition rates during the first 371-day decomposition. a–c Lower-order roots; d–f higher-order roots. Gray-

filled circles indicate decomposition of root groups in isolation, and black-filled circles indicate decomposition of root groups in mixture. Data are expressed as means±standard error ($n=3$, $P<0.05$)

Jørgensen 2010). Although lower-order roots possessed higher N concentration, they had similar acid-unhydrolyzable residue/N ratios to higher-order roots (Table 1). This implies that the tissue N concentration increases in proportion to acid-unhydrolyzable residue of order-based roots and much of the N in lower-order roots is tightly bound in acid-unhydrolyzable residue (Xiong et al. 2013). Hence, the complementary resource (e.g., N) use by microbial decomposers (Vos et al. 2013) or priming effects of high-quality litters on low-quality litters (Wardle et al. 1997) may, to some extent, be hindered due to the limited N release. Alternatively, the high amount of polyphenols (e.g., chitin and tannin, collectively referred to as acid-unhydrolyzable residue) in lower-order roots typically exerts negative effects on decomposition (Langley et al. 2006) and thus may offset the positive effects of N transfer (if any) on decomposition of root mixture.

Responses of mixing effect of order-based root decomposition to N addition

Exogenous N addition shifted the additive effect of order-based root mixture decomposition to synergistic effect ($P=0.002$, Table 2; Fig. 1c), which partly supports our second hypothesis that N addition could affect the interaction among order-based root mixture decomposition. This finding confirms a previous study that N addition transformed neutral effects of leaf mixtures to positive non-additive effects on

mass loss during decomposition (Vivanco and Austin 2011). The phenomenon has been only observed in few studies on mixture decomposition of leaf litter (Rosemond et al. 2010; Vivanco and Austin 2011; Lin et al. 2013) and depends on different mechanisms. Nitrogen addition can shift the interactions of decomposing component species from antagonistic effect to additive effect, via reducing competition among different microbial decomposers (Rosemond et al. 2010). In addition, N addition could also transform the decomposing species interactions from additive effect to antagonistic effect, due to ameliorated N limitation to the microbial decomposers acting on low-quality component species (Lin et al. 2013).

The modification of N addition on litter species interactions during decomposition may be induced by individual species responses (Vivanco and Austin 2011). We separated lower-order roots from higher-order roots in mixture (except the final harvest date) and found that N addition exerted neutral effects on higher-order roots irrespective of decomposition conditions (“isolation” or “mixture”) (Fig. 3e, f), but stronger retarding effects on lower-order root decomposition when they decomposed in isolation than in mixture ($P<0.001$, Fig. 3c). This indicates that the shift of the mixing effect was mainly driven by responses of lower-order roots. It has been reported that the acid-unhydrolyzable residue (i.e., lignin and phenolics) in decomposing litters can bind to inorganic N ions to form recalcitrant compounds (new acid-unhydrolyzable residue), and thus decreases decomposition rate (Fog 1988;

Berg and Matzner 1997). We observed that N addition increased the residual acid-unhydrolyzable residue (% of initial dry mass) of lower-order roots when they decomposed separately, but not when they decomposed in mixture (Fig. 4c), which could be interpreted as evidence for the differential N effects on lower-order roots under different decomposition conditions ($P=0.019$, Table 2). With respect to the weak N effects on lower-order roots in mixture, we speculate that higher-order roots in mixture may reduce the affinity between inorganic N ions and acid-unhydrolyzable residue via physically (larger diameter of higher-order roots) impeding the access of inorganic N ions to lower-order roots and/or chemically (higher C/N ratio of higher-order roots) increasing the use of inorganic N ions by microorganisms acting on higher-order roots.

N-rate effect on interactions of order-based root decay

As predicted, high rate ($120 \text{ kg N ha}^{-1} \text{ year}^{-1}$) rather than low rate ($40 \text{ kg N ha}^{-1} \text{ year}^{-1}$) of NH_4Cl addition affected the interactions among order-based root mixture decomposition (Fig. 1b, c). Given the individual responses of root groups in mixture to N addition (Fig. 3c), lower-order roots could be used as a proxy to interpret the differential responses of root mixture to different N rates. It has been shown that the binding of acid-unhydrolyzable residue to N ions under field conditions can be stimulated by increasing ^{15}N -labeled ammonium rates (Axelsson and Berg 1988). In a previous study, we observed that relative to low N rate, high N rate exerted stronger retarding effects on decomposition of lower-order roots in isolation via promoting the binding of acid-unhydrolyzable residue to inorganic N ions (Kou et al. 2015b). However, the residual acid-unhydrolyzable residue of lower-order roots in mixture did not increase by increasing the N rate, suggesting no promoted binding of acid-unhydrolyzable residue to inorganic N ions by N addition. Additionally, we found significant interactive effects of “N treatment” by “decomposition condition” on residual mass of lower-order roots ($P=0.019$, Table 2), which in parallel contributes to explaining the N-rate effect on mixture decomposition. Due to the smaller size of lower-order roots, we speculate that the weakened binding effects could be ascribed to the low amount of lower-order roots in mixture (half of that in isolation decomposition), which may reduce the potential of lower-order roots to bind the free N ions.

The N-rate effect could alternatively be explained by the neighbor effects of higher-order roots on lower-order roots. Due to the relatively lower quality (Pregitzer et al. 2002; Fan and Guo 2010), higher-order roots in mixture may serve as a competitor via inorganic N immobilization, thus decreasing the N interactions with the acid-unhydrolyzable residue of lower-order roots. It has been demonstrated that relative to low rate of N addition ($54 \text{ kg N ha}^{-1} \text{ year}^{-1}$), high rate of N

addition ($170 \text{ kg N ha}^{-1} \text{ year}^{-1}$) can increase the N immobilization in both above- and below-ground litters (Norris et al. 2013). We found that the N addition at the high rate, to some extent, increased the N immobilization in higher-order roots as compared to the low rate (Fig. 5f), which may suggest high demand of microbial N during decomposition of higher-order roots. Hence, the presence of N-rate effect is probably due to the fact that the decomposition of lower-order roots in isolation would be retarded by the increase of N rate, but the retarding effect is buffered during the decomposition of mixture by the presence of higher-order roots.

The current decomposition experiment has several limitations, which need to be noticed. Specifically, like other root decomposition studies (Xiong et al. 2013; Zhao et al. 2014), litterbags with small-sized mesh (0.1 mm) were employed in this study. Although this mesh size may successfully avoid the physical loss of lower-order roots, it actually impedes the access of soil fauna into litterbags. Soil fauna plays an important role in litter decomposition and determines the magnitude and direction of litter diversity effects (Hättenschwiler and Gasser 2005). Hence, the lack of involvement of soil fauna may, to some extent, affect the pattern of the mixing effect. Secondly, we applied NH_4Cl as fertilizers to simulate the dominant NH_4^+ deposition in this region (Zhan et al. 2014). Despite that the effects of NH_4^+ on decomposition were captured, we could not rule out the possibility that external chloride ions also might contribute to the decomposition process. Considering the toxicity of chloride ions to microorganisms (Zahran 1997; Megda et al. 2014), it may affect decomposition via inhibiting the growth and activities of microorganisms. Hence, future studies should take into account the potential effects of salinity stress (e.g., Cl^-) on decomposition. Finally, this study used only one pine tree species and one mixing ratio (1:1). Given the complexity of the natural decomposition conditions, further studies are required to investigate mixture decomposition of order-based roots with different mixing ratios and among multiple species, and mechanistically reveal their responses to N loading.

Conclusions

To the best of our knowledge, this is the first report on order-based root mixture decomposition and its response to increased N availability. We found that (1) additive effects occurred during order-based root mixture decomposition of *P. elliotii*; (2) like leaf mixture decomposition, N addition affected the decomposition of root groups (lower-order roots) in order-based root mixture; and (3) the effect depended on the N addition rate. Our results indicate that the tight bound of N in acid-unhydrolyzable residue may hinder the priming effects or the complementary use of N by microbial decomposers, and thus result in the additive effect. The mixing effect

and its dependence upon N availability during decomposition of order-based roots with different chemical composition have implications for the accurate prediction of root decomposition and its responses to increased N loading.

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