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REGULAR ARTICLE

Nitrogen ion form and spatio-temporal variation in root distribution mediate nitrogen effects on lifespan of ectomycorrhizal roots

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Abstract
Background and Aims Absorptive roots active in soil resource uptake are often intimately associated with mycorrhizal fungi, yet it remains unclear how nitrogen (N) loading affects lifespan of absorptive roots associating with ectomycorrhizal (ECM) fungi.
Methods Through a three-year minirhizotron experiment, we investigated the responses of ECM lifespan to different rates of N addition and examined the roles of N ion form, rooting depth, seasonal root cohort, and ECM morphotype in mediating the N effects on ECM lifespan in a slash pine (*Pinus elliottii*) forest in subtropical China.
Results High rates of NH_4Cl significantly decreased foliar P concentrations and increased foliar N:P ratios, and mean ECM lifespan was negatively correlated to foliar P concentration. N additions generally increased the lifespan of most ectomycorrhizas, but the specific differences were context dependent. N rates and forms exerted significant positive effects on ECM lifespan with stronger effects occurring at high N rates and under ammonium N addition. N additions extended lifespan of ectomycorrhizas in shallower soil and born in spring and autumn, but shortened lifespan of ectomycorrhizas in deeper soil and born in summer and winter. N additions reduced lifespan of dichotomous ectomycorrhizas, but increased lifespan of coralloid ectomycorrhizas.
Conclusions The increased ECM lifespan in response to N additions may primarily be driven by the persistent and aggravated P limitation to plants. Our findings highlight the importance of environmental contexts in controlling ECM lifespan and the need to consider potential differences among mycorrhizal morphotypes when studying N—lifespan relationships of absorptive roots in the context of N deposition.

Keywords Absorptive roots · Ectomycorrhizas · Median lifespan · Morphotype · Nitrogen deposition

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Introduction

Fossil fuel combustion, nitrogen (N) fertilizer use, and the cultivation of N-fixing crops have increased the reactive N deposition by approximately four-fold over

the past century (IPCC 2013). This striking rise in N input has stimulated broad interest in investigating effects of soil N enrichment on fine-root dynamics (< 2 mm in diameter) (Pregitzer et al. 1993; Burton et al. 2000; Nadelhoffer 2000; Majdi and Andersson 2005). Fine roots represent a highly heterogeneous branching system, which can be partitioned into two functional modules (Xia et al. 2010). As the resource-acquiring module, the distal, non-woody roots generally have a shorter average life expectancy and represent the most dynamic portion of the fine-root system (McCormack et al. 2015). Species-specific studies of fine-root lifespan have shown that these absorptive roots (the distal two or three orders of roots *sensu* Pregitzer et al. 2002; Guo et al. 2008b) can be often replaced within one year (Withington et al. 2006; McCormack et al. 2012), and thus control a dominant flux of carbon (C) and nutrients from plants into soils (McCormack et al. 2015). Hence, understanding how their lifespan responds to increased N availability is critical for predicting the consequences of N loading to ecosystem C sequestration and nutrient cycling.

Although some studies have sought to link absorptive root lifespan with soil N availability, uncertainties remain due to the frequently inconsistent results observed (Bai et al. 2008; Guo et al. 2008a; Baldi et al. 2010; Adams et al. 2013). It is reported that root lifespan is reduced by N addition (Bai et al. 2008), due possibly to increased root N concentration and associated respiration rate (Burton et al. 2000). Roots are also found to live longer as soil N availability increases (Baldi et al. 2010; Adams et al. 2013). According to cost-benefit theory, root lifespan would be extended if the benefit (nutrients) from roots outweighs the C cost of keeping them alive (Eissenstat and Yanai 1997; Burton et al. 2000). Additionally, Hodge et al. (1999) showed that localized N enrichment had positive, negative, and neutral effects on root lifespan, depending on the level of N enrichment. These results jointly imply that moderate levels of N deposition serving as "nutrient supply" may facilitate root function, and thus extend root lifespan (Smithwick et al. 2013; McCormack and Guo 2014). However, as it exceeds a threshold, excess N serving as "nutrient stress" may shorten root lifespan via accumulating reactive N and oxygen species in cell tissues, which can induce respiratory stress (Smithwick et al. 2013).

Deposition of different N ions (e.g. NH_4^+ vs. NO_3^-) may cause divergent responses of root lifespan to N enrichment. It has been previously reported that plants (Nordin et al. 2001; Grassein et al. 2015) and mycorrhizal fungi (Finlay et al. 1989; Hawkins et al. 2000) preferentially utilize NH_4^+ (reduced N) over NO_3^- (oxidized N), due possibly to the lower energy expenditure of NH_4^+ assimilation (Recous et al. 1992). Moreover, excess NO_3^- in root tissues may not be completely reduced to amino acids, leading to accumulation of reactive N species (e.g. nitric oxide) (Delledonne et al. 2001). Hence, roots may avoid accumulation of reactive N by direct uptake of amino acids or NH_4^+ (Smithwick et al. 2013). To the best of our knowledge, only one study has examined the contrasting effects of organic and mineral N fertilization on root lifespan, showing extended root lifespan by fertilizing organic N whereas median lifespan of roots exposed to mineral N was similar to the non-fertilized roots (Baldi et al. 2010). However, it is still not clear whether atmospheric deposited NH_4^+ and NO_3^- would induce contrasting effects on absorptive root lifespan.

Responses of absorptive root lifespan to increased N availability generally exhibit spatio-temporal variability (Bai et al. 2008; Baldi et al. 2010; Adams et al. 2013) and are likely mediated by associations with mycorrhizal fungi (Majdi and Nylund 1996; Rygiewicz et al. 1997; Pritchard et al. 2014). Previous studies have found contrasting N effects on lifespan of absorptive roots inhabiting different soil depths (Majdi and Nylund 1996; Adams et al. 2013) or born in varying seasons (Bai et al. 2008). In contrast, effects of mycorrhizas on absorptive root lifespan have not been as well documented as spatio-temporal effects. It has been estimated that mycorrhizal fungi can colonize approximately 50 % of absorptive roots across 14 arbuscular tree species (Liu et al. 2015) and 85 % of absorptive roots across three ectomycorrhizal (ECM) tree species (Kubisch et al. 2015), though colonization rates are often variable across species as well as soil fertility and climate (Treseder 2004; Soudzilovskaia et al. 2015). Colonization by different species of mycorrhizal fungi may also have different effects on the lifespan of absorptive roots due to contrasting maintenance cost of the mycorrhizas or as a result of differential resistance to environmental stresses. For example, ectomycorrhizas formed by *Cenococcum geophilum* were observed to live substantially longer than those formed by other co-occurring species (Fernandez et al. 2013).

In this study, we focused on absorptive roots associated with ECM fungi (i.e. ectomycorrhizas) to examine their lifespan responses to varying rates and forms of N

addition in a slash pine (*Pinus elliottii*) plantation in southern China. Our previous work indicates that plants in this plantation are co-limited by N and phosphorus (P) (Kou et al. 2015b), based on the foliar N:P ratios (15 species belonging to five plant functional types) as suggested by Güsewell (2004). We hypothesized that: (1) low rates of N would prolong ECM lifespan as nutrient limitations are alleviated whereas high rates of N would shorten ECM lifespan as N levels buildup to levels inducing root tissue stress; (2) responses of ECM lifespan to N addition would depend on N ion form, since plants generally have preference to different forms of N; and (3) N effects on ECM lifespan would be influenced by spatio-temporal factors with ectomycorrhizas born in varying depths and seasons presenting varying sensitivity to N.

Materials and methods

Site descriptions and experimental design

This study was part of the chronic N amendment experiment established in November 2011 in a 28-year-old slash pine (*Pinus elliottii*) plantation at the Qianyanzhou (QYZ) Ecological Station, Chinese Academy of Sciences (CAS), Jiangxi province, southeastern China (26°44′29″ N, 115°03′29″ E, 102 m a.s.l.). A typical subtropical monsoon climate covers this region with a mean annual temperature of 17.9 °C and mean annual precipitation of 1475 mm (Wen et al. 2010). Stand density at the study site was ca. 833 stems ha^{-1}, and the mean tree height and mean diameter at breast height were 17.5 m and 20.9 cm, respectively (Kou et al. 2015b). The soil weathered from red sandstone and mud stone is classified as Typic Dystrudepts Udepts Inceptisols (Wang et al. 2012). More details about soil and vegetation can be found in Kou et al. (2015b). The ambient wet N deposition rate at the site is about 33 kg N ha^{-1} yr^{-1} with approximately 32 % from ammonium-N, 26 % from nitrate-N, and 42 % from dissolved organic N plus total particulate N (Zhu et al. 2015).

The simulated N deposition experiment is a randomized complete block design with three replicates for each treatment. Each block was divided into five 20 × 20 m plots (slope angle <15°), which were at least 10 m apart. Within each block, one plot at random served as control (no N addition), and two N fertilizers (NH_4Cl vs. $NaNO_3$) at two levels (40 vs. 120 kg N ha^{-1} yr^{-1}) were randomly assigned and applied onto the remaining four plots. No control plots were located immediately below one of the fertilization plots in this study. Hence, any potential runoff or lateral flow would not transport added fertilizer from experimental plots into control plots. The fertilization activity was initiated on 1 May 2012 and annual application of fertilizers was divided into monthly increments (i.e. 12 equal applications per year). For each application, the two forms of fertilizers were applied in aqueous solution and 30 L of solution was evenly sprayed onto N-fertilization zone on non-rainy days. Each time, the control plots were supplied with 30 L of tap water.

Minirhizotron installation and image collection

ECM lifespan was estimated using the minirhizotron technique (CI-600, CID Inc., Camas, WA, USA). Specifically, a total of 75 minirhizotron tubes (6.4 cm in diameter and 100 cm in length) were installed in April 2012. Five tubes were buried at the center and four quartering quadrats of each 20 × 20 m plot. All tubes were buried at an angle of 45° off the vertical to a tube depth of 80 cm (equals to a vertical soil depth of ca. 57 cm). The bottom end of each tube was sealed with a plug to prevent water infiltration. The portion of each tube exposing aboveground was first wrapped with black tape to isolate sunlight and then with white tape to minimize heat exchange. The top end of each tube was capped using a white-tape-wrapped cap and a ziplock bag to reduce water vapor. A reference line along the axial direction was drawn at external surface of each tube extending aboveground to mark a permanent start-scanning position for all sampling dates.

Root images were taken consecutively at a month interval from November 2012 to August 2015, except for December 2012, February 2013 and 2014 (the dormant season) and August 2013 (due to equipment failure), which resulted in a total of 29 sessions over the three-year observation period. Root images (21.6 × 19.6 cm, 300 dpi) were captured at four tube panes/depths (0–20 cm, 20–40 cm, 40–60 cm, and 60–80 cm) with a rotating scanner, laptop, and

associated software, which resulted in a total of 8700 images during the whole observation period.

Median lifespan of ectomycorrhizas and cohort estimates

Normally, there should be a time interval (0.25–1 years) between tube installation and image collection to ensure tight tube-soil contact (Baddeley and Watson 2005; Hansson et al. 2013). The tight contact may be indicated that soil structure present in images from several consecutive sessions is relatively consistent in appearance. Hence, we previewed images from the 29 sampling sessions and excluded first session (November 2012) because the soil structure was apparently different from subsequent sessions. Moreover, roots occurred on the first recorded session (January 2013) were not used, due to uncertainties about their birth dates. Therefore, from March 2013, the appearance of new roots and their subsequent death dates were recorded. Root death was indicated when a root turned black (Majdi and Andersson 2005) and/or shriveled to approximately half the original diameter (McCormack et al. 2012). If the observation of a root became obscured due to changing soil structure or if a root was still considered to be alive at the end of the observation period, then that root was identified as censored in subsequent analyses (i.e. time to root death could not be directly determined).

Root images were analyzed using WinRHIZO Tron MF 2009a software (Regent Instrument Inc., Quebec, Canada). A total of 15,616 ectomycorrhizas (including all the 1st and 2nd order roots associated with ECM fungi) were observed across 15 plots over the three years. Specifically, we tracked 1924, 2840, 3626, 4685, and 2541 ectomycorrhizas in the control, low NH_4Cl, high NH_4Cl, low $NaNO_3$, and high $NaNO_3$ plots, respectively (Table S1). The birth date, death date, and depth location of each ectomycorrhiza was recorded. Ectomycorrhizas were then grouped into different cohorts where each cohort was defined as all roots emerging on the same observation date and within the same tube depth increment. Finally, as a preliminary assessment of the potential for differential effects on root lifespan imparted by different fungal species, we classified each ectomycorrhiza into one of two dominant ectomycorrhizal morphotypes. Different pine tree species often produce several distinct and contrasting ECM morphotypes including monopodial, dichotomous, and coralloid structures. In this study, the monopodial morphotype was not commonly observed and therefore our analysis only focused on coralloid and dichotomous morphotypes (Fig. S1). However, some species have potential to produce multiple morphotypes in different contexts (Agerer 1991) and as such interpretation of broad morphotypes should be considered conservatively.

Needle and root sampling and tissue chemistry analysis

We sampled green needles of *P. elliottii* at the end of September in 2013 and 2014. Specifically, three healthy mature individual trees within the center of each plot were randomly selected (with similar DBH and heights to the average levels), and one representative twig from sun-exposed, first-order branch per tree was collected from the upper two-thirds of the crown using a tall tree trimmer with a bamboo pole. The fully-expanded, current-year needles of each cohort were immediately detached from the twigs. The sampled needles from the three individual trees in each plot were pooled and homogenized to yield one composite sample (approximately 300 g).

Intact roots of *P. elliottii* in each plot were sampled twice (April and September) each year in 2013 and 2014. Specifically, the intact roots were excavated from the top soil (0–10 cm) and transported to the laboratory within two hours using ice and a cooler. Absorptive roots were reserved for tissue chemistry analysis. All root samples were oven-dried (60 °C, for 48 h) to a constant weight and finely ground using a Restch MM400 mixer mill (Retsch GmbH, Haan, Germany). Concentrations of total C and N in foliage and roots were determined using a Vario MAX elemental analyzer (Elementar, Germany). Concentrations of total P in foliage were determined by inductively coupled plasma-optical emission spectrometry (ICP-OES, Perkin Elmer Optima 5300 DV) after digestion of the samples in concentrated HNO_3.

Statistical analysis

Effects of N addition on median lifespan of ectomycorrhizas were estimated using Kaplan-Meier method (Kaplan and Meier 1958). Log-rank tests were used to determine the difference among treatments. Cox proportional hazards tests (Cox 1972)

were used to examine effects of N rate (0, 40, and 120 kg N ha^{-1} yr^{-1}), N form (ammonium- and nitrate-based N), rooting depth (vertical depth of roots in soil, 0–28 and 29–57 cm), season of birth (spring, March to May; summer, June to August; autumn, September to November; or winter, December to February), and ECM morphotype (dichotomous and coralloid ectomycorrhizas) on ECM lifespan.

One-way ANOVA was used to test treatment effects on foliage (N and P concentrations and N:P ratios in September 2013 and 2014), root (N concentrations and N:C ratios in April and September 2013 and 2014) chemistry, and the average number of ectomycorrhizas within cohort. Regression analysis was carried out to determine the relationships between the number of ectomycorrhizas within cohort and lifespan (mean lifespan of ectomycorrhizas within each cohort), between foliar P concentration and lifespan (mean lifespan of ectomycorrhizas living in shallower soil depth and born in September 2013 and 2014), and between root N concentration and lifespan (mean lifespan of ectomycorrhizas living in shallower soil depth and born in April and September 2013 and 2014). Results were considered statistically significant at $P < 0.05$. All statistical analyses were performed using the SPSS software version 18.0.

Results

N effects on median lifespan of ectomycorrhizas

ECM lifespan was significantly extended by 13.7 %, from 248 (control) to 282 days on average by N addition ($P < 0.001$, Fig. 1; Table S1). The N rate and form had significant effects on ECM lifespan ($P < 0.001$, Table 1). Compared with low N rates, ECM lifespan responded more strongly to high N rates ($P < 0.001$, Table 1; Fig. 1), with an increase of 9.5 %, from 275 (low N) to 301 days (high N). Ammonium N addition exerted stronger effects on the lifespan than nitrate N ($P < 0.001$, Table 1; Fig. 1), with an increase of 9.8 %, from 275 (NO_3^--N) to 302 days (NH_4^+-N). Across all treatments, ectomycorrhizas had longer lifespan with coralloid than with dichotomous morphotypes ($P < 0.001$, Table 1), but the two morphotypes had contrasting responses to N additions. Specifically, dichotomous ectomycorrhizas had longer lifespan in the control (303 days) than in the N-added (252 days on average) plots (Fig. S2a). In contrast, coralloid ectomycorrhizas had longer lifespan in the N-addition (305 days on average) than in the control (213 days) plots (Fig. S2b).

Spatio-temporal factors influencing N effects on median lifespan of ectomycorrhizas

Ectomycorrhizas lived longer in deeper (306 days, Fig. 2b; Table S1) than shallower soil depths (217 days, Fig. 2a; Table S1) in the control plots. Rooting depth had significant effects in regulating N effects on ECM lifespan ($P < 0.001$, Table 1). N addition extended ECM lifespan on average by 72 days in the shallower soil depth, while shortened lifespan on average by 25 days in the deeper soil depth ($P < 0.001$, Fig. 2; Table S1). In the control plots, ectomycorrhizas born in varying seasons had different lifespans with 272 days in spring (Fig. 3a; Table S1), 334 days in summer (Fig. 3b; Table S1), 187 days in autumn (Fig. 3c; Table S1), and 334 days in winter (Fig. 3d; Table S1), respectively. Birth season also had significant effects on N-mediated lifespan changes ($P < 0.001$, Table 1). N addition generally extended ECM lifespan on average by 32 days in spring and 80 days in autumn, while shortened lifespan on average by 22 days in summer and 144 days in winter ($P < 0.001$, Fig. 3; Table S1).

N effects on ECM cohort and cohort size-lifespan relationship

N addition did not affect the number of cohorts produced ($P > 0.05$, Fig. 4a), but high rates of NH_4Cl and low rates of $NaNO_3$ significantly increased the average number of ectomycorrhizas within a single cohort ($P = 0.001$, Fig. 4b) by 81.8 % and 60.0 %, respectively. No significant relationship was found between mean ECM lifespan and average number of ectomycorrhizas within cohorts across all treatments ($P > 0.05$, Fig. S3b).

N effects on foliage and root chemistry and tissue chemistry-lifespan relationship

Root N concentrations and N:C ratios were not affected by N additions in April 2013 and 2014 ($P > 0.05$, Fig. S4). High N rates significantly increased root tissue N concentrations and N:C ratios in September 2013 ($P < 0.05$, Fig. 5a and c). Adding N (except for low rate of $NaNO_3$) significantly increased root N concentrations and N:C ratios in September 2014 ($P < 0.05$, Fig. 5b and

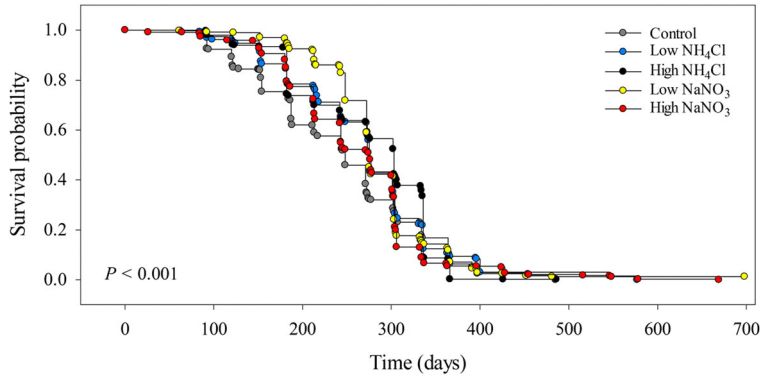

Fig. 1 Survival probability curves of ectomycorrhizas of slash pine under different treatment conditions. *P* values indicate significance of N effects on median lifespan (days) of ectomycorrhizas

d). N addition (low N rates) significantly increased foliar N concentrations in September 2013, but not in September 2014 (Fig. S5). High rates of NH_4Cl significantly decreased foliar P concentrations ($P < 0.05$, Fig. 5e and f) and increased foliar N:P ratios ($P < 0.05$, Fig. 5g and h) in September 2013 and 2014. Mean ECM lifespan was negatively correlated to foliar P concentration (in September 2013 and 2014) across all the treatments ($r^2 = 0.404$, $P = 0.048$, Fig. 6). No significant relationship was observed between mean ECM lifespan and root N concentration (in April and September 2013 and 2014) across all the treatments ($P > 0.05$, Fig. S3a).

Discussion

N effects on ECM lifespan

In contrast to the first hypothesis that ECM lifespan would be extended by low rates of N, but reduced by high rates of N, ECM lifespan was extended by both low and high rates of N additions ($P < 0.001$, Fig. 1; Table S1). It has been previously established that there is a positive relationship between plant tissue N concentration and respiration rate at a given temperature (Burton et al. 2002; Reich et al. 2008). We therefore expected that high rates of N would shorten ECM

Table 1 Summary of Cox proportional hazards fit for effects of categorical covariates (i.e. N rate, N form, rooting depth, season of birth, and ECM morphotype) on ECM lifespan of slash pine. Risk ratios for covariates are mortality risk relative to a reference level (shown in bold), where the ratio > 1.0 indicates a greater mortality risk than the reference level, and the ratio < 1.0 indicates a lower mortality risk than the reference level. Upper and lower 95 % refer to confidence intervals for risk ratio. Results were considered statistically significant at $P < 0.05$

Covariate		Cox regression coefficient	SE	Wald	*P*-value	Risk ratio	95 % CI	
							Lower	Upper
N rate	**Unfertilized**							
	Low N (N40)	-0.175	0.029	36.745	$P < 0.001$	0.839	0.793	0.888
	High N (N120)	-0.184	0.031	35.289	$P < 0.001$	0.832	0.783	0.884
N form	**Nitrate**							
	Ammonium	-0.119	0.019	37.674	$P < 0.001$	0.888	0.854	0.922
Rooting depth	**0–28 cm**							
	29–57 cm	-0.352	0.018	383.688	$P < 0.001$	0.703	0.679	0.729
Season of birth	**Winter**							
	Spring	-0.829	0.040	421.445	$P < 0.001$	0.437	0.403	0.472
	Summer	-1.039	0.035	899.160	$P < 0.001$	0.354	0.331	0.379
	Autumn	-0.389	0.033	137.538	$P < 0.001$	0.678	0.635	0.724
ECM morphotype	**Dichotomous**							
	Coralloid	-0.104	0.018	31.403	$P < 0.001$	0.902	0.869	0.935

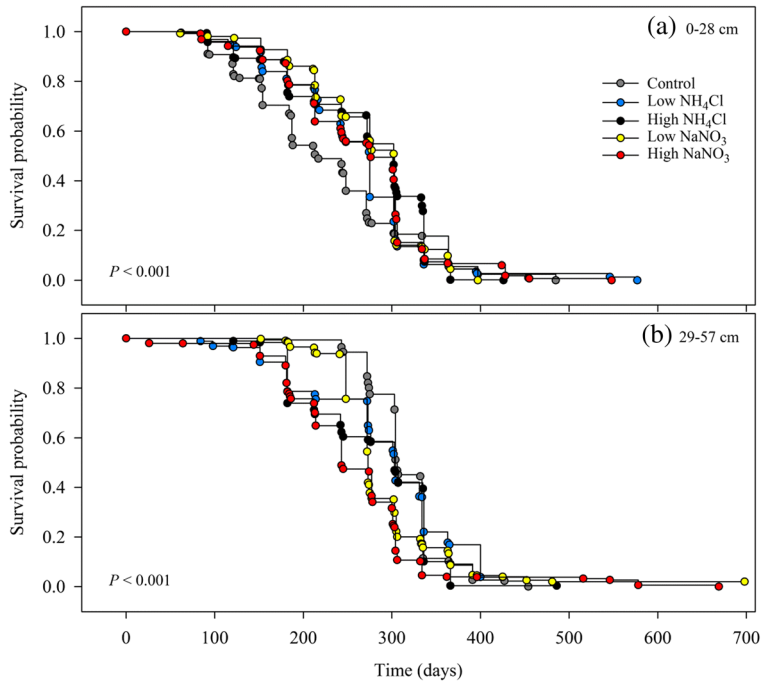

Fig. 2 Survival probability curves of ectomycorrhizas of slash pine in shallower (0–28 cm, (**a**) and deeper (29–57 cm, (**b**) soil profiles under different treatment conditions. *P* values indicate significance of N effects on median lifespan (days) of ectomycorrhizas

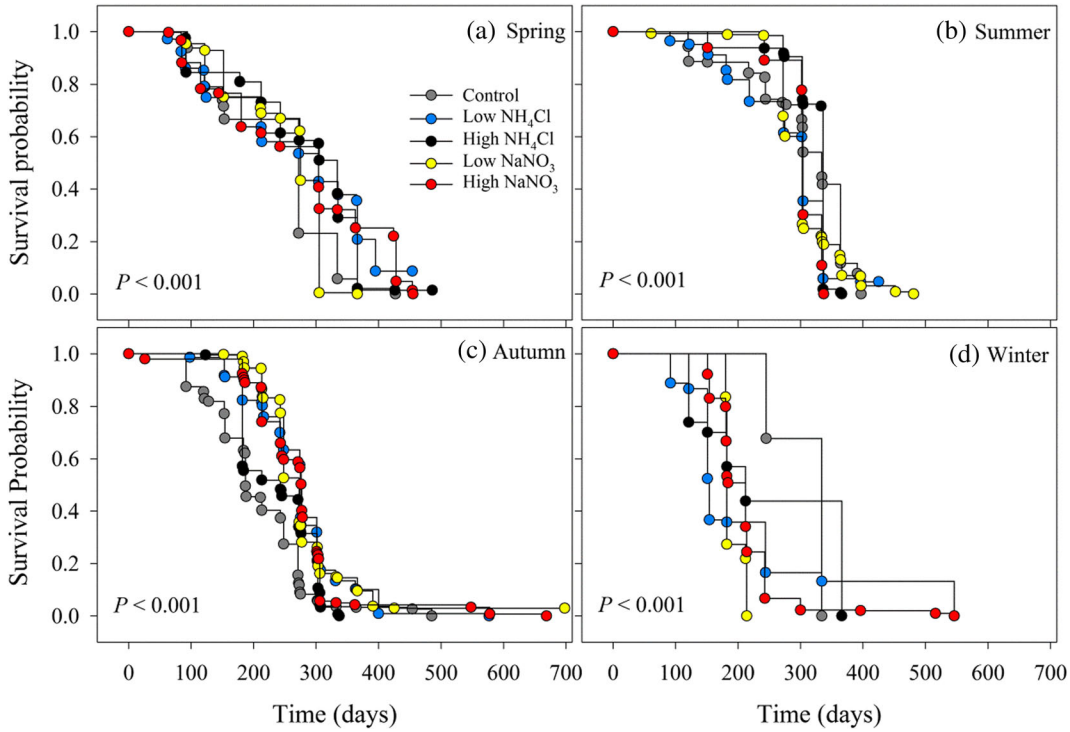

Fig. 3 Survival probability curves of ectomycorrhizas of slash pine in spring (March to May, (**a**), summer (June to August, (**b**), autumn (September to November, (**c**), and winter (December to February, (**d**) under different treatment conditions. *P* values indicate significance of N effects on median lifespan (days) of ectomycorrhizas

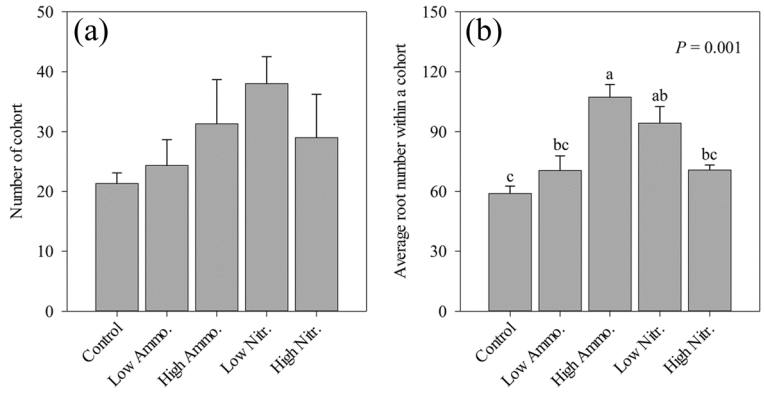

Fig. 4 Effects of N addition on number of root cohort (**a**) and average root number within a cohort (**b**). Ammo. and Nitr. represent ammonium- (NH_4Cl) and nitrate-based ($NaNO_3$) N additions, respectively. Values are expressed as means ± standard error ($n = 3$). Different lowercase letters indicate significant differences among treatments ($P < 0.05$)

lifespan due to an excess of reactive N and oxygen species in root cell tissues which can induce respiratory stress (Smithwick et al. 2013). Although respiration was not measured in this study, the potential respiratory stress may not occur as evidenced by increased ECM lifespan at high N rates. Recent research shows that the respiration-N relationship does not hold if N availability is altered significantly (Burton et al. 2012). For absorptive roots, much of the N is tightly bound in acid-unhydrolyzable residue such as phenolics and lignin (Xiong et al. 2013; Kou et al. 2015a). The storage of excess N in high-molecular weight compounds may therefore result in the lack of higher respiration rates (Bauer et al. 2004). Alternatively, even if there was increased respiratory stress, the plant may have allocated more resources into the production of compounds/molecules that scavenge free radicals and may help avoid some of the damage of increased respirations. The neutral and positive responses of ECM lifespan to chronic N addition observed here is consistent with previous studies, which also reported neutral to positive effects of N addition on the lifespan of ectomycorrhizas in a pine plantation (Pritchard et al.

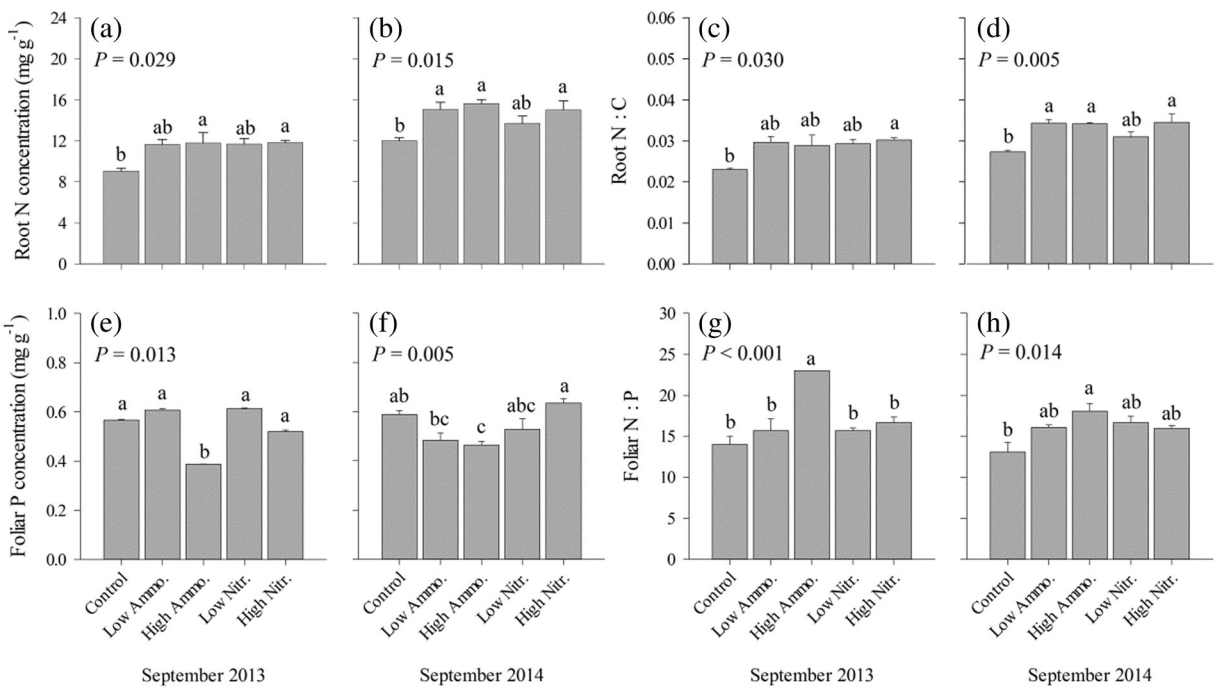

Fig. 5 Effects of N addition on root N concentration (**a** and **b**), root N:C ratio (**c** and **d**), foliar P concentration (**e** and **f**), and foliar N:P ratio (**g** and **h**) in September 2013 and 2014. Ammo. and Nitr. represent ammonium- (NH_4Cl) and nitrate-based ($NaNO_3$) N additions, respectively. Values are expressed as means ± standard error ($n = 3$). Different lowercase letters indicate significant differences among treatments ($P < 0.05$)

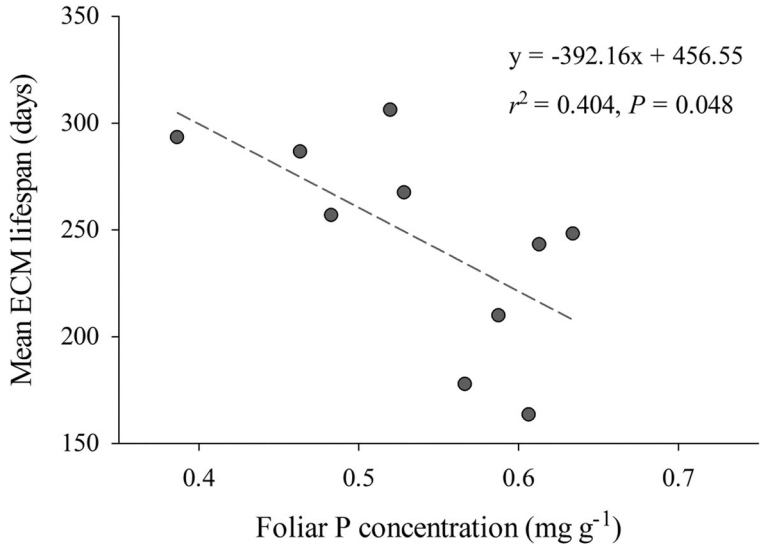

Fig. 6 Relationships between foliar P concentration and mean lifespan (days) of ectomycorrhizas living in shallower soil depth and born in September 2013 and 2014 (n = 10)

2014). However, it is interesting to note that the different morphotypes observed in our study expressed contrasting responses to N addition with lifespan of coralloid ectomycorrhizas increasing with fertilization while lifespan of dichotomous ectomycorrhizas decreased (Fig. S2). This suggests that the effects of N additions may not necessarily be the same in magnitude or even direction across all ectomycorrhizal species.

The prolonged lifespan of ectomycorrhizas by N addition might be related to plants' need for P. To seek stoichiometric balance, plants acquire not only N, but also other mineral elements such as P (Elser et al. 2000). Mounting evidence has shown that excess N input can induce or exacerbate P limitation to plants (Aber et al. 1989; Braun et al. 2010; Wang et al. 2014; See et al. 2015). In this study, we observed stimulated root proliferation as indicated by increased average number of ectomycorrhizas within a cohort (P = 0.001, Fig. 4b), reduced foliar P concentrations, and increased foliar N:P ratios (P < 0.05, Fig. 5e-h) in response to N addition. Collectively, these plastic responses of absorptive roots and foliage may indicate alleviated N limitation, but persistent and exacerbated P limitation to plants. Furthermore, we found a significant negative relationship between mean ECM lifespan and foliar P concentration (Fig. 6), suggesting that changes in ECM lifespan were primarily driven by P-limitation. Cost-benefit theory assumes that a root would live longer if the benefit it provides outweighs the cost it consumes (Eissenstat and Yanai 1997; Burton et al. 2000). Hence, these ectomycorrhizas were provided with greater maintenance resources, due possibly to the fact that they were still functionally efficient at acquiring P.

Contrasting effects of N ion form on ECM lifespan

Different forms of N exerted contrasting effects on ECM lifespan, with longer lifespan observed with ammonium N than nitrate N additions (P < 0.001, Table 1; Fig. 1). This finding supports the second hypothesis that responses of ECM lifespan to N addition depends on N ion forms. Compared with NO_3^-, NH_4^+ has greater capacity to accelerate soil acidification due to its higher potential to induce proton production at the root level (Matson et al. 1999), which can aggravate P limitation via binding PO_4^{3-} to soil metal ions, e.g. Al^{3+} and Fe^{3+} (Bünemann et al. 2011). As discussed above, P limitation could be one of the driving mechanisms underlying the increased ECM lifespan in N-treated plots. Hence, the differential potentials of the two forms of N ions to acidic soil may contribute to their contrasting effects on ECM lifespan. Additionally, it has been documented that ectomycorrhizal fungi have preferences to NH_4^+ over NO_3^- (Finlay et al. 1989). The increased metabolic expense of NO_3^- acquisition may then be associated with more rapid root aging. Plant uptake of different N ions may therefore give rise to differential lifespan responses.

A laboratory ^{15}N-labeling study on the soil at this forest shows that NO_3^- input stimulated autotrophic nitrification and NO_3^- accumulation in soil, due possibly to NO_3^- addition-induced reductions in microbial assimilation of NH_4^+ (Gao et al. 2016). Despite this, we did not observe

reduced ECM lifespan as reported by Bai et al. (2008) showing a negative relationship between root longevity and soil NO_3^- concentration. Compared to NH_4^+, NO_3^- has higher mobility and is more likely to be influenced by in-situ environmental factors (e.g. leaching losses). Hence, the effects of NO_3^- addition (or accumulation) on lifespan of ectomycorrhizas may be weakened, especially under heavy rainfall conditions. An alternative interpretation is that the negative effects of NO_3^- accumulation on ectomycorrhizas were, to some extent, offset by the positive effects of P limitation. It should be noted that, to simulate different forms of N ion deposition (NH_4^+ vs. NO_3^-), Na^+ and Cl^- were synchronously introduced. This may, to some extent, contribute to the N form effects, due to the potential impact of exogenous salt ions on mycorrhizal functioning (Evelin et al. 2009) and microbial activity (Megda et al. 2014).

Spatio-temporal factors mediate N effects on ECM lifespan

Lifespan of ectomycorrhizas differed among the two soil layers and among seasonal cohorts (Figs. 2 and 3, Table 1). Ectomycorrhizas lived significantly longer in deeper soil depth ($P < 0.001$, Table 1), which is consistent with the observations in effects of rooting depth on absorptive root lifespan (Guo et al. 2008a; Adams et al. 2013) and with previous observations of ectomycorrhizas in pine ecosystems (Pritchard et al. 2014). Generally, roots in the deeper layers experience less fluctuations in temperature and moisture (Eissenstat and Yanai 1997; Wells et al. 2002) and weak feeding by herbivory and infection by pathogen (Baddeley and Watson 2005), which could reduce stress-related mortality of ectomycorrhizas. Additionally, ectomycorrhizas born in summer and winter had longer lifespan than those born in spring and autumn (Fig. 3, Table 1). This result contrasts with previous studies showing longer lifespan for roots born in the later season and shorter lifespan for roots born in the earlier season (Bai et al. 2008; Adams et al. 2013) as well as longer lifespan in spring and winter and shorter lifespan in summer and autumn (Guo et al. 2008a). This discrepancy may be due to differences in climate zones (e.g. temperate vs. subtropical zone), life-forms (e.g. herbaceous vs. woody plants), division of seasons (e.g. April to June for spring vs. March to May for spring), or due to differences in the individual plant and fungal species involved which may vary in their phenological patterns (Koide et al. 2007; Kauserud et al. 2012; McCormack et al. 2014).

N addition extended lifespan of ectomycorrhizas located in shallower soil layers, but shortened lifespan of those in deeper soil depths ($P < 0.001$, Fig. 2; Table S1). Earlier research has documented a similar pattern that N additions reduced longevity of mycorrhizal short roots located in deeper soil depths (Majdi and Nylund 1996), while others have observed increases with N fertilization in deeper soils (Pritchard et al. 2014). Also, N addition extended lifespan of ectomycorrhizas born in spring and autumn, but shortened lifespan of those born in summer and winter ($P < 0.001$, Fig. 3; Table S1). This result agrees with the observations in Bai et al. (2008) that N addition extended spring- and autumn-born roots, but reduced summer-born roots. Taken together, the highly variable responses of ectomycorrhizas at different depths and with seasons of birth confirm the fact that absorptive root lifespan does respond to soil N enrichment, but with a large spatial and temporal variability.

The spatial variability in ECM lifespan responses to N addition may be explained by the contrasting frequency of dichotomous and coralloid ectomycorrhizas in the control and fertilized plots. We found that N addition increased the total number of dichotomous ectomycorrhizas on average by 2.5 folds and decreased that of coralloid ectomycorrhizas on average by1.9 folds in shallower soils, while increased the total number of both dichotomous (4.8 folds) and coralloid ectomycorrhizas (5.9 folds) in deeper soils (Fig. S6). Considering the longer median lifespan of dichotomous than coralloid ectomycorrhizas at ambient conditions, the opposite responses observed between shallower and deeper soils could be largely ascribed to differences in the fungal community being acted upon.

The contrasting N effect occurring in different seasons may be related to changes in chemical composition and physiological activity of roots across the year (López et al. 2001). Evidence has shown that the lignin-phenol yield in absorptive roots is highly season-dependent with higher contents in summer (Wang et al. 2015), and localized N enrichment can decrease phenolic content in roots (Adams and Eissenstat 2015), which may increase the risks of herbivore attack. Hence, the reduced ECM lifespan by N addition in summer could be attributed to changes in phenolic contents in roots. It has been suggested that spring-born roots are primarily associated with resource-acquisition, while autumn-born roots are associated with

resource-storage and lateral root production (Bai et al. 2008). To adapt to the P limitation induced by exogenous N addition, plants may therefore extend root lifespan to increase P capture in these two seasons. Furthermore, it should be noted that the number of roots produced in winter was significantly smaller in control (n = 41, 11.6 % of the average root number across four N-treated plots; Table S1) than in N-treated plots, which resulted in reduced statistical power in estimating median ECM lifespan (Adams et al. 2013).

Conclusions

Absorptive roots, the fast-cycling, shorter-lived module of the fine-root system, are frequently associated with mycorrhizal fungi. The joint root—ectomycorrhizal fungal structures, ectomycorrhizas are likely to be sensitive to soil N enrichment. However, few studies have directly examined N effects on lifespan of ectomycorrhizas in relation to different related factors including N ion forms and ECM morphotype. Using a three-year minirhizotron experiment, we observed that ECM lifespan did respond to N addition, but that the responses differed spatially and temporally, and depended on rates and ion forms of input N. The lifespan extension may occur in response to the persistent P limitation as the benefit of increased P acquisition from a mycorrhizal root would outweigh the potential cost associated with increased lifespan (Eissenstat and Yanai 1997; Burton et al. 2000). However, positive responses to fertilization may not necessarily be consistent across all fungal species. Our findings emphasize the importance of environmental (i.e. edaphic) contexts in controlling lifespan of ectomycorrhizas and suggest a need for future studies to explicitly consider potential differences among mycorrhizal morphotypes in these different contexts of N supply rates and N forms when modeling N—lifespan relationships of absorptive roots.

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Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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