

Article

Whole-Tree Response of Non-Structural Carbohydrates, Carbon and Nitrogen Concentrations in Two Temperate Tree Species to 10-Year Nitrogen Fertilization

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Abstract: This study aimed to investigate the effects of long-term nitrogen fertilization on non-structural carbohydrates (NSC) and nitrogen (N) status and their interaction in mature trees at the whole-tree scale. Ten g N m⁻² yr⁻¹ of ammonium nitrate fertilizer were applied to 26-year-old *Larix gmelinii* Rupr. (larch) and *Fraxinus mandshurica* Rupr. (ash) trees in Northeastern China from 2002 to 2012. NSC, total carbon (C) and total N concentrations in different compartments were examined. For both species, concentrations of NSC and their components (soluble sugars and starch) tended to increase in aboveground organs but decrease in fine roots following N fertilization, with significant ($p < 0.05$) changes only observed in ash stems and larch roots. N fertilization increased N concentrations and decreased the C:N ratio in all organs, especially in foliage and roots, while the effects of fertilization on total C concentrations varied with tree species and organs. Concentrations of NSC (mainly reflected in soluble sugar) were generally negatively correlated with N concentration in fine roots but positively related to N concentration in aboveground woody organs in both control and fertilized treatments. However, fertilization strengthened this correlation in fine roots and weakened this relationship in aboveground organs. This study provides a decade-long insight into the effect of currently increasing N deposition on tree growth and function.

Keywords: non-structural carbohydrate; long-term nitrogen fertilization; fine root; whole-tree response; carbon to nitrogen ratio



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1. Introduction

Carbon (C) storage in trees is key for biogeochemical processes of terrestrial ecosystems [1,2]. At the tree level, C allocation is mediated by non-structural carbohydrates (NSC), which are the sum of starch and low-molecular soluble sugars (e.g., glucose, fructose and sucrose) [1,3]. NSC are the most important components in C storage in quantitative terms, because they are not only building blocks for the synthesis of important compounds (such as proteins and amino acids), but they can also serve as the energy source for metabolic activities such as growth, nutrient assimilation and other life maintaining processes [1,4,5]. In addition, NSC play a central role in tree resistance to environmental changes [6,7] because starch, the temporarily immobile forms of NSC, can be mobilized and re-allocated to metabolic processes when plants encounter C deficits (C demand exceeds concurrent C acquisition by photosynthesis) [4,8–10].

Given the increased input of anthropogenic nitrogen (N), N deposition has dramatically increased in terrestrial ecosystems, changing ecosystem structure and function [11–13]. For example, N deposition affects tree growth and mortality [14,15], and changes terrestrial plant diversity [16,17]. As the key substance in tree growth and physiology, the dynamics of NSC storage in trees associated with soil N availability have been extensively reported [18–21]. Most of the previous research focused on NSC concentrations in a single organ, such as roots or foliage, in response to N fertilization. These studies found that N fertilization induced an increase in NSC concentration in the foliage of trees such as *Quercus suber* [22], but a decline in NSC concentration in roots from *Populus tremuloides* [23], *Betula pendula* [24] and seven northern hardwood tree species [21]. There were also a few studies examining NSC response to N fertilization at the whole-tree level, but they showed largely divergent results [25,26]. Recently, a meta-analysis of 12 studies covering 22 tree species showed that NSC concentrations in response to N fertilization can differ among tree taxonomic groups, leaf habits and fertilization intensities, but the average concentrations across tree species generally increased in aboveground woody tissues but decreased in roots with N fertilization [27]. Collectively, there is currently substantial knowledge about N fertilization effects on NSC dynamics in young trees but less is known about such effects in mature trees, especially at the whole-tree scale. Given that NSC allocation is different during tree maturation due to differences in photosynthetic capacity and growth [10], extrapolating results from seedlings or saplings to mature trees is questionable [10,28]. By analyzing long-term N fertilization treatments on mature trees, we can better understand how NSC pools in forests will react to improved soil N availability, especially in view of the globally increasing atmospheric N deposition.

In addition to NSC pools, long term N fertilization also directly alters N storage in trees [29,30]. It has been widely recognized that NSC and N are functionally tightly linked [31–34]. This is not only because carbohydrates can be mobilized to support N-related metabolic processes [1,10,18], but also because there is a direct relation between N-supply and photosynthetic capacity, since a lot of N goes into the production of Rubisco and other enzymes that are involved in photosynthesis [35]. However, responses of N concentrations to long-term N fertilization in mature trees were different to those of NSC among several experimental studies since they generally revealed increasing tissue concentrations of N with fertilization. For example, in temperate forests, more than 10 years of N fertilization increased N concentrations in foliage in a mixed-oak forest [30] and increased N concentrations in roots in a northern hardwood forest [36]. Higher N concentrations were also observed in needles and roots of subtropical Chinese fir (*Cunninghamia lanceolata*) after eight years of N fertilization [37]. However, Kaakinen et al. [38] have shown that the response of NSC concentration in secondary xylem of stem wood to long-term N fertilization in mature trees of Norway spruce (*Picea abies* L. Karst.) varied between boreal and temperate sites in Finland. Few studies have examined the response of the relationship between carbohydrate and N allocations to N fertilization, but the inconsistent response patterns of NSC and N concentrations may imply that their linkage should be influenced not only by tree taxonomic groups (e.g., gymnosperm and angiosperm) and geographic regions (e.g., temperate and boreal zones) but also by the investigated tree organs. The small number of observations on NSC and N responses in mature trees to N fertilization limits our understanding of the linkage between NSC and N storage in trees, especially at the whole-tree scale.

The present study was conducted in northeastern China on two commercially important tree species from different tree taxonomic groups: Dahurian larch (*Larix gmelinii* Rupr.) and Manchurian ash (*Fraxinus mandschurica* Rupr.). After 10 years of N fertilization, we sampled the foliage, current-year and one-year-old twigs, different stem samples (e.g., bark, pith and the 10 most recent annual growth rings) and coarse and fine roots from 26-year-old monoculture plantations of both species. For each sampled tissue, concentrations of NSC (and its components, i.e., soluble sugars and starch), total C and total N were measured. The aim of this study was to reveal how NSC and N and their interrelation in mature trees

respond to long-term N fertilization at the whole-tree level. We hypothesized that (1) NSC concentration should increase in aboveground organs but decrease in roots in mature ash and larch in response to long-term N fertilization; (2) N concentration should increase at the whole-tree scale for both species following N fertilization; (3) based on the variation patterns of NSC and N concentrations, for a given tree organ, N concentrations should be positively related to NSC concentrations in the aboveground organs but negatively related to NSC concentrations in roots in either control or fertilized treatments.

2. Materials and Methods

2.1. Study Site

The study was conducted at the Maoershan Experimental Station (127°30′–127°34′ E, 45°21′–45°25′ N) of Northeast Forestry University, Heilongjiang, China. This region is characterized by a cold temperate and a continental monsoon climate. Annual mean temperature is 2.8 °C and the mean daily air temperatures for January (coldest month) and July (warmest month), respectively, range from −23 °C to 20.9 °C. Annual average precipitation in this region is 600–800 mm, 80% of which occurs during the peak seasons from June to August [39]. The plantation is on a southwest-facing slope with an altitudinal range from 280 to 330 m and 20 degrees of slope from bottom to top. Soil is Hap-Boric Luvisol [40]. More details about the soil are given by Wang et al. [41].

2.2. Experimental Design

This experiment is a subset of a larger experiment designed to test the effects of long-term N fertilization on stand and tree performance [42,43]. The experiment was carried out on two monoculture plantations of Dahurian larch (*Larix gmelinii* Rupr.) and Manchurian ash (*Fraxinus mandschurica* Rupr.). Both plantations were established in 1986 by planting two-year-old seedlings using a 1.5 × 2.0 m planting grid. The area of each plantation was over five hectares. In 2002, larch and ash trees had average trunk diameters at breast height of 11.7 and 9.1 cm, respectively (mean values across all trees at the plots). For each species, six plots (20 m × 30 m) were established in May 2002 in a randomized complete block design. From the six plots in each species' plantation, three were randomly chosen as N fertilization plots, and the residual plots were considered as unfertilized controls. From 2002 to 2012, each N-fertilized plot received 10 g N m^{−2} yr^{−1} of ammonium nitrate (NH₄⁺-NO₃[−]) fertilizer. The determination of fertilizer quantity is based on the natural temporal patterns of N mineralization in the study site (see Jia et al. [43] for details). From May to September, 15%, 21%, 28%, 21% and 15% of the fertilizer was applied monthly, respectively. Details of stand and soil characteristics for each plantation are shown in Table 1.

Table 1. Stand and soil characteristics for 0–20 cm soil depth in *Fraxinus mandschurica* and *Larix gmelinii* (mean ± 1 standard error).

	<i>Fraxinus mandschurica</i>		<i>Larix gmelinii</i>	
	Control	Fertilization	Control	Fertilization
Stand DBH (cm)	12.51	12.74	15.41	15.51
Soil pH	6.03 ± 0.10 a	5.63 ± 0.05 b	5.68 ± 0.06 a	5.42 ± 0.08 b
Total available inorganic N concentration (mg/kg) in soil	19.5 ± 1.7 b	32.5 ± 4.5 a	15.2 ± 1.4 b	37 ± 4.9 a
Total C concentration (mg/g) in soil	74.5 ± 6.5 a	77.8 ± 2.8 a	69.8 ± 5.4 a	76.1 ± 4.2 a
Total N concentration (mg/g) in soil	8.5 ± 1.1 a	8.9 ± 0.4 a	8.2 ± 0.7 a	8.6 ± 0.3 a
Total P concentration (mg/g) in soil	0.529 ± 0.015 a	0.555 ± 0.051 a	0.490 ± 0.056 a	0.540 ± 0.019 a

Note: For each species, different lowercase letters represent significant differences ($p < 0.05$) between the control and N fertilization treatments. DBH Represents Diameter at Breast High.

2.3. Aboveground and Belowground Tissue Sampling

Previous studies showed that NSC pools of trees at the end of the growing season might serve as a good indicator of C surplus condition in the current year [44]. In 2012, at the end of September, about one week before leaf fall, six trees with average diameter,

i.e., one tree per plot, were selected for sampling for each species. Tree density in ash and larch stands at the time of sampling was 3187 and 2267 plants/ha, respectively. For each selected individual tree, three branches (with leaves or needles) from the lower, middle and upper parts of the crown, respectively, were cut using a pruner and then combined together into a mixed sample [20]. Each branch was separated into foliage and current-year and one-year-old twigs, identified by stem scars. For each individual tree, stems were cored to the pith at breast height with an increment borer (5.15-mm diameter, Haglöf Sweden AB, Sweden) from four directions (North, South, East and West). Bark and pith in the stem cores were cut off, respectively. The 10 outermost rings (formed since N fertilization initiation) were divided, with every two adjoining rings cut off as an independent sample under a compound microscope. For each individually sampled tree, these independent stem samples from the four directions were merged according to their horizontal positions in a stem core. Hence, seven groups of stem samples per tree were ultimately obtained, including bark, pith and growth rings formed in the last 1–2, 3–4, 5–6, 7–8 and 9–10 years. Additionally, large roots (diameter > 3 cm) and all their attached intact root segments from the individual tree were very carefully harvested from 0–20 cm soil depth. Three soil samples from the same plot were randomly sampled to the depth of 20 cm and combined into a Ziploc bag as a composite sample for subsequent chemical analyses. After the collection, all plant and soil samples were put into a cooler filled with ice bags and taken to the laboratory within 2 h.

2.4. Chemical Analyses of Plant and Soil Materials

In the lab, root samples were washed with de-ionized water to free them from adhering soil particles. Living and dead root segments were distinguished depending on their morphology, color and elasticity. Based on the root order system used in Pregitzer et al. [45], the first five root orders from the living root segments (with root tips assigned the first order root) were separated in culture dishes filled with de-ionized water and ice. All separated tissues were pulse-heated in a microwave oven (5 min at 800 W, with a glass of water inside to avoid overheating) [5] to de-nature enzymes. Afterwards, all samples were oven-dried at 65 °C until mass constancy.

The dried plant samples were ground to fine powder for chemical analyses. Total soluble sugar and starch concentrations were measured by the modified phenol-sulfuric acid method [46]. In detail, dried sample powder (40 mg) was extracted in 10 mL of 80% ethanol overnight. Then the mixed solutions were centrifuged at 2200–5000 rpm for 15 min, and supernatants were collected for soluble sugar concentration analysis. The residue was prepared for the analysis of starch concentrations by drying at 100 °C for 3 h, then hydrolyzing with 3 mL 3% solutions of hydrochloric acid in a boiling water bath for 0.5 h and finally filtering of the supernatant. For each of these two processes, 2 mL of the fixed volume supernatant was placed in a 25 mL colorimetric tube, then 1 mL of 5% phenol reagent and 5 mL of concentrated sulfuric acid were added quickly. After being shaken well, the mixed solution was left for 30 min at room temperature before absorbance measurement. The soluble sugar and starch concentrations were measured by reading the absorbance at 490 nm using a UV-VIS spectrophotometer (model UV-PC01, Shimadzu Corp., Kyoto, Japan). Total NSC concentrations were calculated as the sum of soluble sugars and starch concentrations on a dry matter basis. A sample of 20 mg plant powder was used to determine the total N and total C concentrations with a Macro Elemental Analyzer (vario MACRO, Frankfurt, Germany). All tissue N and C concentrations were expressed on a dry mass basis. C:N ratio was calculated as the total C concentration divided by total N concentration for each tissue [45].

Soil samples were naturally dried in a cool, ventilated place, and then all impurities such as fine root segments and stone were removed by hand. Soil samples were ground to powder and passed through a 2 mm sieve. Total soil N and C concentrations were determined by an elemental analyzer (Vario Macro, Frankfurt, Germany). Total soil phosphorus concentration, ammonium and nitrate concentrations were analyzed with a flow-injection

autoanalyzer (AA3, Norderstedt, Germany). Soil pH was measured with an acidometer (MT-5000, Shanghai, China) [41]. The soil characteristics for each single species plantation on control and fertilized plots are given in Table 1.

2.5. Statistical Analyses

Total available inorganic N concentrations in the soil were calculated as the sum of NH_4^+ -N and NO_3^- -N. For each treatment per tree species, each tree per plot was considered as a sample unit, thus data of three trees from three plots were used as replications. For each species, mean NSC, soluble sugar, starch, total C, total N concentrations and C:N ratio of branch, stem and root were calculated across the involved compartments. One-way factorial analyses of variance (ANOVA) ($p = 0.05$) were conducted to test the influence of N fertilization on NSC, soluble sugar, starch, total C and total N concentrations as well as the C:N ratio at the species level. For each species, the influence of fertilization and species and their interaction on chemical traits were tested by a two-way ANOVA for each organ (foliage, branch, stem and root). Pearson's correlation was used to determine the relationships between N concentration and each of NSC, soluble sugar and starch concentrations for each species, treatments and tissues. A preliminary analysis showed that the relationships between N and NSC (or components) were not significant in foliage and lateral roots due to the limited replication per species and treatment ($n = 3$). Using the data of the samples as described above, we only present these relationships in aboveground woody organs ($n = 24$), including current-year and one-year-old twigs, pith and five groups of growth rings, and the first five orders of fine roots ($n = 15$). Variations of NSC, soluble sugars, starch, total C and total N concentrations in tissues induced by N fertilization are expressed in % increase or decrease relative to the corresponding tissues from trees at the control plots. All statistical analyses were performed using SPSS software (2010, V. 19.0, SPSS Inc., Cary, NC, USA).

3. Results

3.1. NSC, Soluble Sugars and Starch

Significant differences in NSC concentrations between ash and larch were found in roots, branches and stems (Table 2). Independent of the fertilization treatment, larch had significantly higher NSC concentrations in roots but lower NSC concentrations in branches and stems than ash (Figure 1). By comparison, NSC concentrations in foliage were similar between ash and larch. Soluble sugars and starch also varied between species and among organs (Figure 1, Table 2). Following N fertilization, NSC, starch and sugar concentrations were generally reduced in roots and increased in branches (except larch) and stems averaged within each compartment (stem, roots etc.), with significant responses of NSC and starch concentrations only in ash stem and larch root (see inserted plots in Figure 1). For aboveground compartments, except for significant decreases of soluble sugar concentration in the bark and starch concentration in the pith for larch, NSC concentrations in other compartments generally increased in both species in response to N fertilization. Significant increases of NSC (and sugars and starch separately) were found in some of the analyzed growth rings in both species, as well as in foliage and current-year twigs for ash (Figure 1). In root compartments, except for increase of NSC concentrations in large roots, fine root NSC concentrations generally decreased following N fertilization, with significant responses occurring only in the first four root orders of larch and the second root order of ash (Figure 1). Although NSC, soluble sugar and starch concentrations responded differently between ash and larch following fertilization, there were no highly significant interactions between species and fertilization for these traits (Table 2).

Table 2. ANOVA results of tree species and N fertilization on non-structural carbohydrate (NSC), starch, soluble sugar, total carbon (C), total nitrogen (N) concentrations and C:N ratio in organs of foliage, branch, stem and root.

Source of		NSC Concentration		Starch Concentration		Soluble Sugar Concentration		Total C Concentration		Total N Concentration		C:N Ratio	
Variation	df	F	p	F	p	F	p	F	p	F	p	F	p
Foliage													
Species (Sp)	1	0.755	0.410	10.326	0.012	3.070	0.118	170.365	p < 0.01	0.392	0.549	6.566	0.034
Fertilization (Fert)	1	5.547	0.046	1.537	0.250	3.623	0.093	8.100	0.022	16.887	0.003	14.934	0.005
Sp × Fert	1	1.907	0.205	3.410	0.102	0.014	0.909	4.502	0.067	4.222	0.074	2.849	0.130
Branch													
Species (Sp)	1	20.742	p < 0.01	109.928	p < 0.01	2.436	0.134	248.563	p < 0.01	8.302	p < 0.01	4.967	0.037
Fertilization (Fert)	1	0.233	0.634	0.444	0.513	0.024	0.879	0.547	0.468	0.979	0.334	0.717	0.407
Sp × Fert	1	1.232	0.280	1.481	0.238	0.339	0.567	0.491	0.491	0.042	0.840	0.085	0.774
Stem													
Species (Sp)	1	31.269	p < 0.01	22.029	p < 0.01	12.349	0.001	150.148	p < 0.01	10.098	0.002	18.190	p < 0.01
Fertilization (Fert)	1	14.269	p < 0.01	17.640	p < 0.01	0.530	0.469	2.990	0.088	0.696	0.407	1.688	0.198
Sp × Fert	1	5.730	0.019	3.490	0.066	3.732	0.058	2.640	0.109	0.004	0.949	0.038	0.847
Root													
Species (Sp)	1	10.997	0.001	4.528	0.037	1.317	0.255	3.754	0.057	4.304	0.042	4.149	0.046
Fertilization (Fert)	1	14.408	p < 0.01	10.060	0.002	0.381	0.539	0.767	0.384	2.301	0.134	2.663	0.107
Sp × Fert	1	1.460	0.231	1.475	0.229	<0.01	0.992	1.519	0.222	0.004	0.951	0.662	0.419

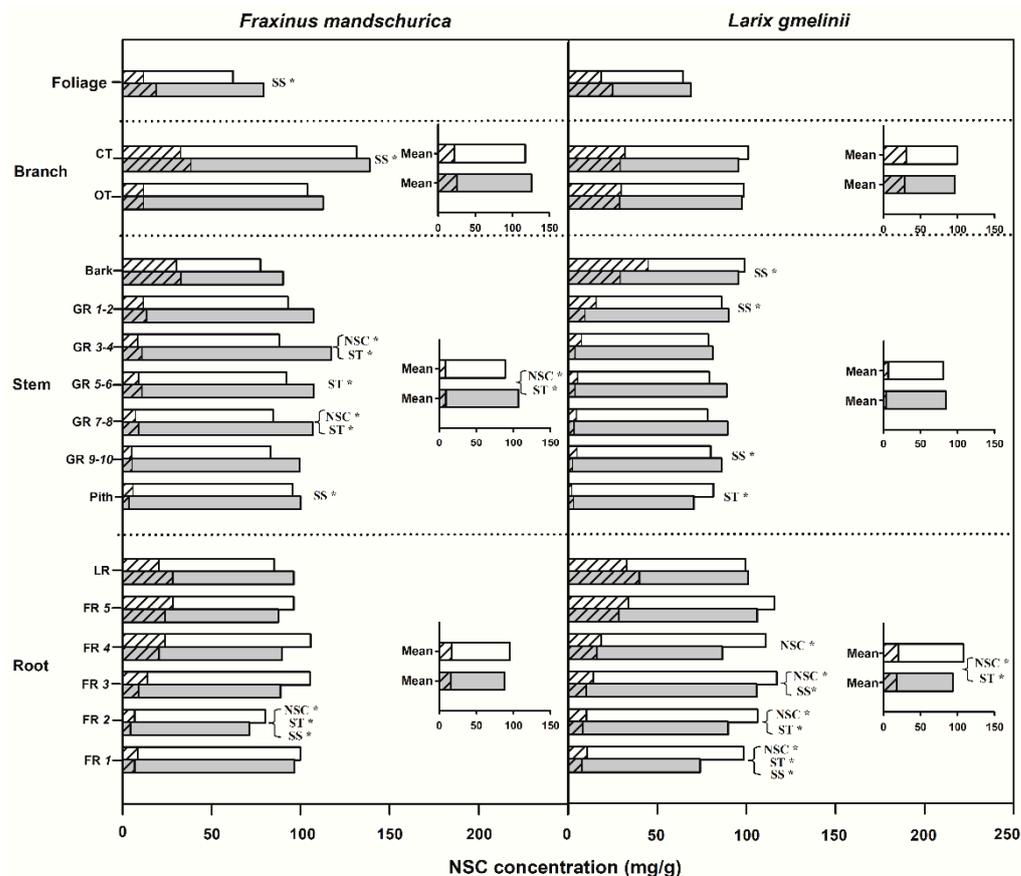


Figure 1. Non-structural carbohydrates (NSC), soluble sugar (SS) and starch (ST) concentrations for compartments from organs (foliage, branch, stem and roots) of *Fraxinus manschurica* and *Larix gmelinii* plantations in control and N fertilization plots. Inset: Mean values of NSC, SS and ST concentrations within an organ across compartments in control and N fertilization treatments. Bars in white and in grey represent the control and N fertilization, respectively. For the same bar, the left shading part and the right blank part represent SS and ST concentrations, respectively. CT, current-year twig; OT, one-year-old twig; GR, growth ring; LR, large root; FR, fine root branching order. “*” behind “NSC”, “SS” or “ST” within a compartment indicates significant ($p < 0.05$) differences of this chemical trait between control and N fertilization treatments.

3.2. Total Carbon, Total Nitrogen and C:N Ratio

There were significant interspecific differences in C and N concentrations and C:N ratios for most organs, except for N concentrations in foliage and C concentrations in roots (Table 2). Larch foliage, branches, stems and roots generally had higher C concentrations and C:N ratios but lower N concentrations than ash, regardless of the N-treatment (Figures 2–4). N fertilization showed significant influence on C (except for larch) and N concentrations and C:N ratio in foliage for both species, but had no effect on branch, stem and root when averaged across individual tissues (see inserted plots in Figures 2–4, Table 2). There were also no significant interactions between species and fertilization for any of these traits (Table 2). The influence of N fertilization on C concentrations varied with organs for each species (Figure 2). In the aboveground organs, a significant increase of the total C concentration was only found in ash foliage and the last 3 to 10-year-old growth rings. C concentrations in the belowground organs also responded differently between ash and larch since they significantly decreased in coarse roots (lateral and fifth order roots) and increased in the first four fine root orders in larch. In ash, C concentrations decreased significantly in lateral roots and the third to fifth fine root orders but remained unchanged in the first two fine root orders (Figure 2). By comparison, N concentrations consistently increased with N fertilization in all organs of ash and larch (Figure 3), with

increments ranging from 7% (foliage) to 13% (roots) in ash and from 6% (branch) to 24% (foliage) in larch. For the branches, stems and root compartments, strong increases of N concentrations were found in fine and lateral roots for both species, as well as in the bark of larch and current-year twigs and 9–10-year-old growth rings of ash (Figure 3). Following N fertilization, C:N ratios in each compartment decreased in both species, and had similar significant trends as N concentrations, suggesting that there was a consistent increase of N with fertilization but less (to no) effect on the C content (Figures 3 and 4).

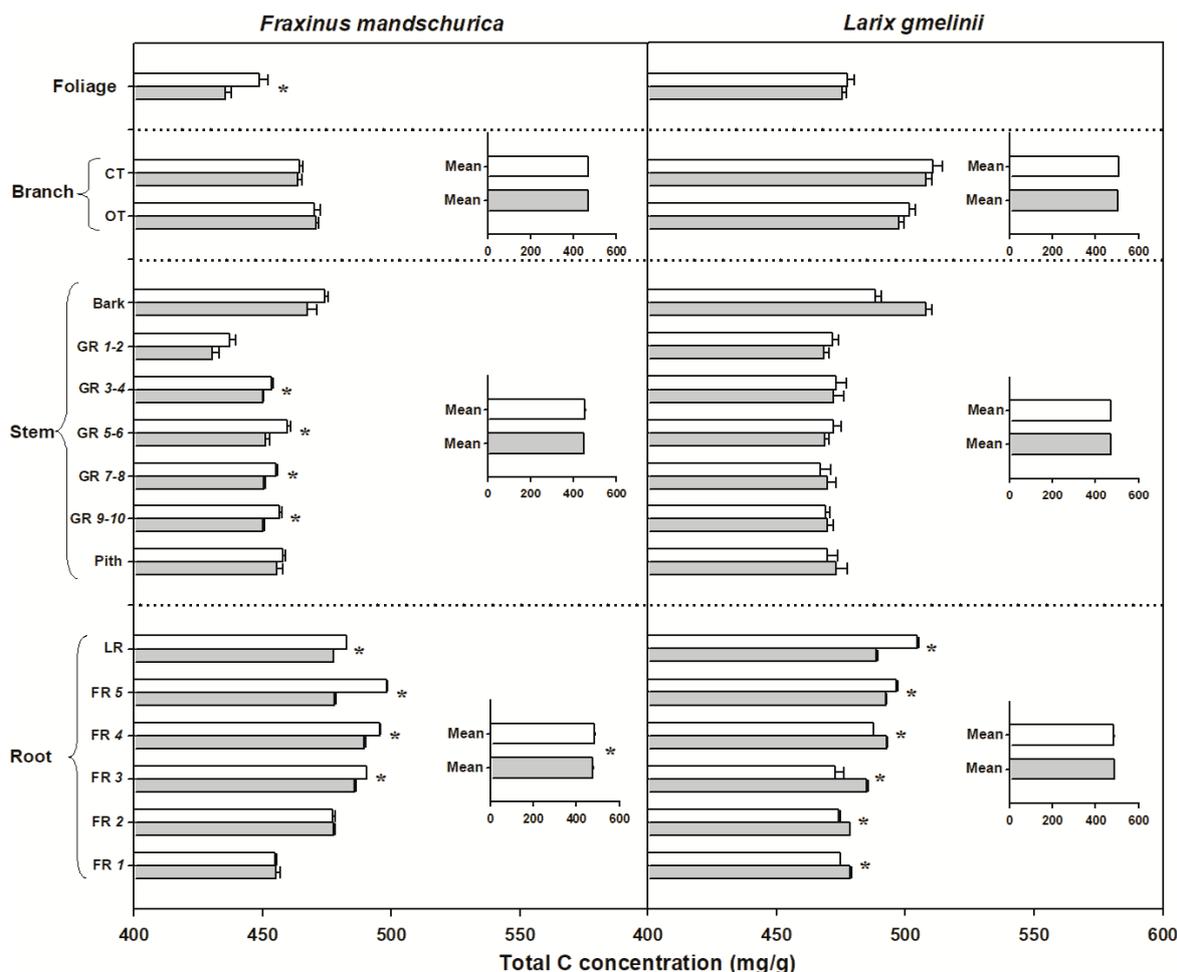


Figure 2. Total carbon (C) concentrations for compartments from organs (foliage, branch, stem and roots) in *Fraxinus manschurica* and *Larix gmelinii* in control (white bar) and N fertilization (grey bar) plots. Inset: Mean values of C concentration within an organ across compartments in control and N fertilization treatments. CT, current-year twig; OT, one-year-old twig; GR, growth ring; LR, large root; FR, fine root branching order. “*” within a compartment indicates significant ($p < 0.05$) differences between control and N fertilization treatments.

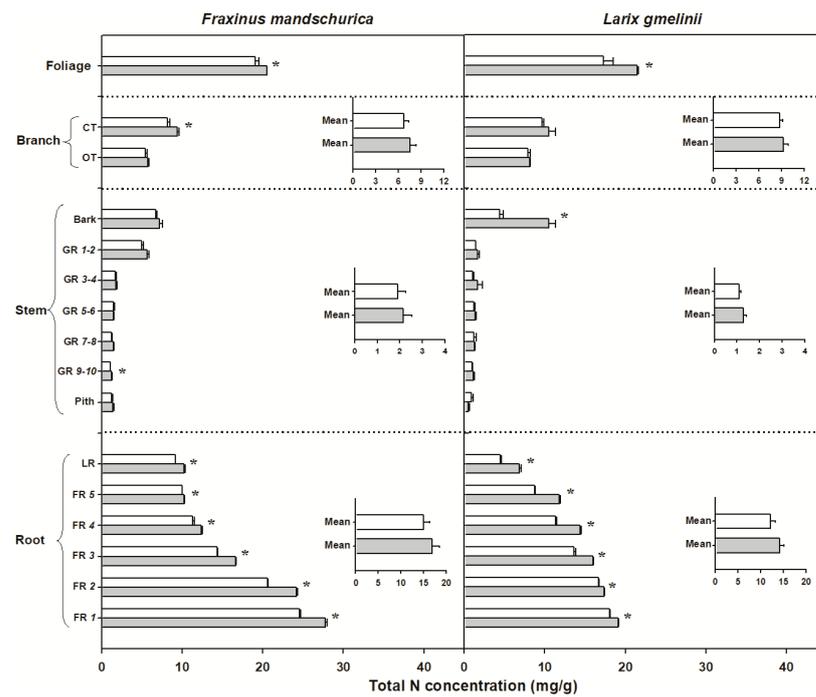


Figure 3. Total nitrogen (N) concentrations for compartments from organs (foliage, branch, stem and roots) in *Fraxinus mandschurica* and *Larix gmelinii* in control (white bar) and N fertilization (grey bar) plots. Inset: Mean values of N concentration within an organ across compartments in control and N fertilization treatments. CT, current-year twig; OT, one-year-old twig; GR, growth ring; LR, large root; FR, fine root branching order. “*” within a compartment indicates significant ($p < 0.05$) differences between control and N fertilization treatments.

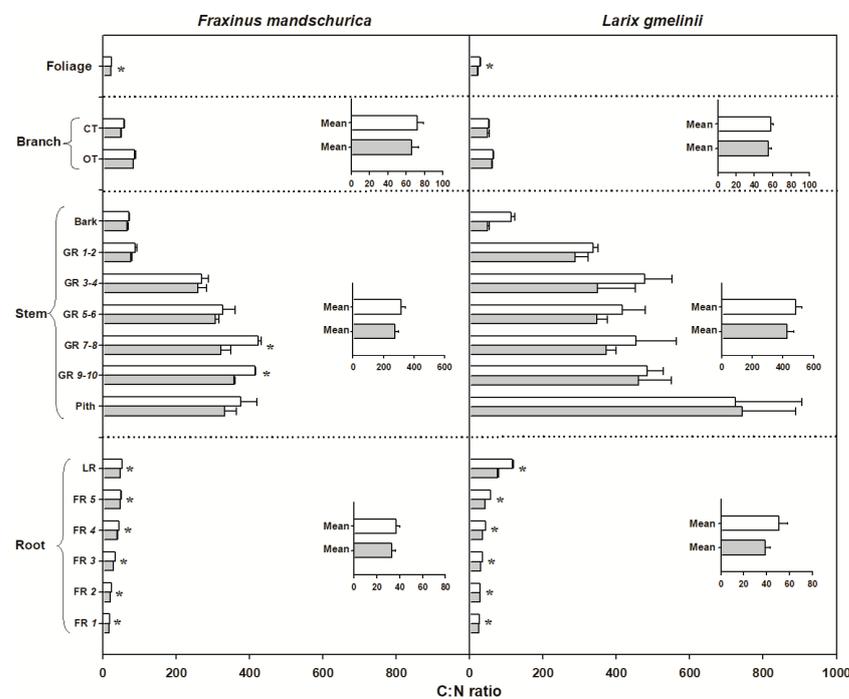


Figure 4. C:N ratio for compartments from organs (foliage, branch, stem and roots) in *Fraxinus mandschurica* and *Larix gmelinii* in control (white bar) and N fertilization (grey bar) plots. Inset: Mean values of C:N ratio within an organ across compartments in control and N fertilization treatments. CT, current-year twig; OT, one-year-old twig; GR, growth ring; LR, large root; FR, fine root branching order. “*” within a compartment indicates significant ($p < 0.05$) differences between control and N fertilization treatments.

3.3. Relationship between N and NSC

Within each species, significant correlations were found between N and both NSC and soluble sugar concentrations in fine roots and aboveground woody organs (i.e., branch and stem) from the control plots, except for fine roots of ash (Table 3). However, the correlations showed an opposite direction between above- and belowground organs, which were positive among aboveground woody compartments but negative among fine root compartments. In comparison, N and starch concentrations were generally poorly correlated, except for aboveground woody organs from the control plots and fine roots from the N fertilized plots, with most correlation coefficients (r) less than 0.4. The weak correlation between N and starch resulted in smaller Pearson's correlation coefficients for the correlation between N and NSC concentrations than those between N and soluble sugar concentrations.

Table 3. Pearson's correlations between N concentration and NSC, soluble sugar and starch concentrations under control (C) and N fertilization (Fert) treatments in *Fraxinus manschurica* and *Larix gmelinii*. For each treatment per species, 24 and 15 replications were used for correlation analysis of aboveground woody organs and fine roots, respectively.

	N Concentration			
	Aboveground Woody Organs		Fine Roots	
	C	Fert	C	Fert
<i>Fraxinus manschurica</i>				
Soluble sugar concentration	0.866 **	0.843 **	−0.860 **	−0.880 **
Starch concentration	0.589 **	0.054	0.394	0.560 *
NSC concentration	0.809 **	0.622 **	−0.330	−0.072
<i>Larix gmelinii</i>				
Soluble sugar concentration	0.938 **	0.955 **	−0.879 **	−0.934 **
Starch concentration	−0.210	−0.400	0.240	−0.134
NSC concentration	0.595 **	0.418 *	−0.587 *	−0.656 **

*: $p < 0.05$, **: $p < 0.01$.

The correlation between N and both NSC and components under N fertilization showed similar significant trends to those of the control (Table 3). The strongest relationship was also exhibited between N and soluble sugar concentrations. Pearson's correlation coefficients for the relationships between N and both NSC and soluble sugar concentrations were generally slightly increased in fine roots in both species (with no significant correlations between N and NSC concentrations in ash) but decreased in aboveground woody organs of ash following N fertilization.

4. Discussion

4.1. Effects of N Fertilization on NSC, Soluble Sugar and Starch Concentrations

NSC, as a key substrate for tree functional processes, are affected by environmental changes, such as soil N availability [27]. However, the response of NSC concentrations in mature trees to long-term N fertilization has been rarely examined, especially at the whole-tree level. Our results on mature trees of ash and larch revealed a general increase of NSC concentrations in aboveground organs but a decrease of NSC concentrations in fine roots after 10 years of N fertilization, largely supporting our first hypothesis. Kaakinen et al. [38] reported similar NSC responses to long-term N fertilization in secondary xylem of stem wood of mature Norway spruce from southern Finland. Normally, mature trees cannot be simply compared to seedlings or saplings. For example, they differ in their carbohydrate allocation and storage pools [10], and their growth and photosynthetic capacity do not scale isometrically with tree size. In addition, mature trees generally have a more constrained growth period and earlier leaf senescence but a disproportionately greater leaf N content than seedlings or saplings [10,47–49]. However, when comparing the mature trees of our study to seedlings or saplings of other reports, their overall responses of NSC concentrations to N fertilization were similar. For example, a meta-analysis covering 22 tree species of

mainly seedlings or saplings, showed that NSC in roots decreased with N fertilization, but within aboveground woody tissues, NSC increased in the reported cases [27].

At the level of the whole-tree, the general opposite change of NSC concentrations in aboveground woody organs and fine roots following N fertilization is likely driven by different C allocation patterns between the above- and belowground tissues [27,37]. NSC allocation in trees should theoretically follow an optimal partitioning, which means that plants may allocate additional NSC to organs that have a relatively higher metabolic activity [27,50,51]. Under sufficient supply of water and soil nutrients, trees generally allocate more C to the growth of aboveground relative to belowground organs [27,52], which can be further enhanced in response to additional N-fertilization [53], because trees need relatively fewer roots, and less C export to root symbionts to satisfy their N demand [37,54,55].

It has been suggested that NSC tissue concentrations in trees are changing in response to imbalances between C source- (acquisition by photosynthesis) and C sink-activities (demand for growth and respiration processes) [7,10,56]. In the current study, NSC concentrations increased slightly following N fertilization in aboveground organs. At the same time, N fertilization also led to a small increase of stem radial growth in ash (1.8%) and larch (0.6%). Although we measured NSC concentrations only at a single time point (end of season), the higher concentrations at fertilized plots, might suggest that the photosynthetic C supply could easily compensate the slight increase in C demand with N-fertilization. According to high tissue NSC concentrations in control and N-fertilized plots, it is reasonable to assume that mature trees of both investigated species were likely not C limited [56] regardless of the N fertilization treatment.

For belowground organs, a consistently significant decrease of NSC concentrations with N fertilization occurred only in fine roots of larch. Generally, short-term negative C source-sink imbalances can decrease NSC concentrations in plant tissues [57]. However, when the C source-sink imbalance persists over longer time periods, trees often recover their NSC concentrations to levels prior to the C source-sink change [58]. Therefore, low NSC concentration in fine roots of our study might not be caused by long-term C source-sink imbalances but are more likely due to either a change in root anatomy/morphology [41] or a shift in stoichiometry (e.g., an increase in N concentrations). For example, the stronger decrease of NSC in fine roots of larch compared to ash following fertilization was accompanied by a stronger increase of N concentrations in larch than in ash.

4.2. Effects of N Fertilization on N Concentrations and C:N Ratio

In comparison to the non-significant response of aboveground woody organs in our study, foliage and fine root N concentrations tended to respond strongest to long-term N fertilization in mature ash and larch trees (Figure 3), which is in accordance with our second hypothesis. Simultaneous increases of N concentrations in both foliage and fine roots were also observed in temperate and subtropical forests under long-term N fertilization [30,36,37]. This result indicates that long-term N fertilization mainly has a strong influence on the N-accumulation in ephemeral organs such as foliage and fine roots rather than in long-lived woody organs (i.e., stem and branch). Metabolic activities of both foliage and fine roots should be thus facilitated by N fertilization [42,59,60], supporting the theory of functional equilibrium to some extent [61].

C:N ratio is also an indicator for the lifespan of a plant organ. In general, higher C:N ratios are found in organs with longer lifespans [62,63]. Our observations (Figure 4) and those of Li et al. [53] found that C:N ratio largely declined in both foliage and fine roots with long-term N fertilization. The decrease in C:N ratio caused by long-term N fertilization was found to be consistent with the decline of foliage lifespan under 6–11 years of N fertilization in a tropical forest [64]. However, we did not measure the lifespan of foliage or fine roots in the deciduous species observed in our study. Hence, we do not have direct evidence for the assumption that long term N fertilization could shorten the lifespan of ephemeral organs of mature deciduous temperate trees as well.

Total C of plant tissues can be divided between structural (the sum of cell-wall polysaccharides and lignin) and nonstructural C (the sum of organic compounds that are not bound in cell walls, incl. NSC) [65]. In our study, long-term N fertilization induced significant increases of total C in fine roots of larch but decreases of total C in coarse roots of both species and in older stem growth rings of ash. NSC concentrations showed generally an opposite trend to total C concentration in the above-mentioned compartments. Based on the variations of total C and NSC concentrations, the sum of other non-structural compounds and structural carbohydrates, which we did not measure separately, should thus have increased in fine roots but decreased in coarse roots and stem wood following N fertilization. Given that non-structural compounds other than NSC normally resemble only a small proportion of the total C, we assume that the structural carbohydrates (i.e., cellulose, hemicellulose and lignin) were increased in woody tissues but declined in fine roots (only larch) with N fertilization. Previous studies also reported that xylem anatomy, cellulose, hemicellulose and lignin content in trees changed with N fertilization [19,53,66], thereby changing tree resistance to insect and disease infestations [25].

4.3. Correlation of NSC Concentrations and N Concentration

Tissue N concentrations have been previously reported to be closely related to tissue NSC concentrations in trees [18,67]. For example, Cheng et al. [18] found a negative relationship between NSC concentration and N concentration following two months of N fertilization on a whole-tree basis in *Malus domestica* Borkh. As with above- and below-ground organs in our study, the correlation of N and NSC showed an opposite relationship among each involved compartment, with strong negative correlations in fine roots but positive relationships in aboveground woody organs (i.e., branches and stems, Table 3). These results support our third hypothesis. These opposite relationships are likely led by the different allocation patterns of N and NSC among above- and belowground compartments.

Within the complex lateral root branching system, individual roots have different functions depending on their position [45,68,69]. Generally, the first three order roots are typically absorptive roots for the uptake of water and nutrients [41,70,71], and therefore possess a greater effective absorbing surface due to root hairs and high mycorrhizal colonization, as well as an active metabolism for nutrient assimilation and respiration [45,59,68,69,72]. As a result, root tips possess greater concentrations of proteins than higher order roots [73]. In addition, root tips are generally not lignified, therefore they contain much fewer structural tissues that reduce the N concentration per dry mass. Thus, less proteins but higher lignin content generally leads to decreasing tissue N concentrations with increasing root order [19,73,74]. According to the function of NSC to fuel N assimilation, the consumption of NSC should decline with root order, resulting in a generally opposite pattern of NSC and N allocations among fine root compartments and thus a strong negative relationship between NSC and N for each species (Table 3). While for aboveground woody organs, newer tissues have both greater NSC and N concentrations than older ones due to a priority for storage or greater percentage of living cells in the former [75,76]. This may lead to the positive correlation of N and NSC among aboveground woody organs in both control and N fertilized treatments. Overall, the correlation of N and NSC concentrations among aboveground woody organs and among fine roots highlighted that NSC and N allocations are highly influenced by tissue position.

5. Conclusions

Improved N supply by long-term fertilization generally increased NSC concentrations in aboveground organs but decreased them in roots, suggesting that NSC allocation patterns were different between the above- and belowground organs. N fertilization appears to facilitate N accumulation at the whole-tree level due to a general increase of N concentration in each compartment, which benefits metabolism, especially in ephemeral tissues. In addition, compared with C concentrations, which largely varied with tree species and compartments, C:N ratios consistently declined in all compartments (especially in fine

roots and foliage) following N fertilization. Different allocation patterns of NSC and N concentrations among aboveground woody organs and among fine root orders resulted in an opposite direction of correlations between N and NSC (mainly soluble sugar) regardless of the fertilized treatments. Following N fertilization, the relationship between N and NSC concentrations was generally strengthened in fine roots but weakened in aboveground woody organs. Our results highlight that long-term N fertilization influences the NSC and N status and their interrelation in mature trees, while the size and direction of the effect depending on tree species and organ type, implying complexity in predicting the responses of plant growth and physiology to N deposition.

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