



# Seedling size and nutrient availability in the fall determine nitrogen resorption and storage compound allocation in *Quercus variabilis*

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## Abstract

Soil fertility and resorption of leaf compounds in the fall can influence resource buildup in plants. However, whether intraspecific differences in seedling size can affect nutrient reserve buildup is unknown. This study examined the effects of seedling size and fall fertilization on the uptake and resorption of nitrogen (N), as well as the allocation of non-structural carbohydrates (NSC) and N in cultivated *Quercus variabilis* Blume. After the formation of terminal buds ( $T_1$ ), seedlings were stratified into small (shoot height < 30 cm) and large seedlings. During the hardening period, seedlings were treated with three different rates of <sup>15</sup>N-enriched fertilizer (0, 12, or 24 mg N seedling<sup>-1</sup>) and monitored until leaf fall ( $T_2$ ). Small seedlings had lower N resorption efficiency and resorbed proportionally less N than large seedlings. Fall fertilization notably improved N and NSC reserves, without reducing N resorption efficiency. Large seedlings allocated proportionally less N to leaves than small seedlings although both sizes seedlings absorbed similar amounts of N from fall fertilization. The priority perennial organ for NSC allocation was roots, while N allocation was dependent on the phenological growth stage of the seedling. Roots were prioritized during the rapid growth phase, while stems were prioritized during the hardening period. Under same fertilizer regime during the growth phase, large seedlings tends to have lower N concentration and have higher resorption efficiency compare to small seedlings, fall fertilization can increase N storage in large seedlings and NSC levels in both seedling sizes, without affecting growth.

**Keywords** Oak · Plant size · Fall fertilization · Labeled nitrogen · Plant storage · Resource allocation

## Introduction

Survival and growth of seedlings after transplanting are critical for the success of a forest restoration program (Grossnickle 2012). Transplant performance depends on the morphological and physiological attributes of seedlings (Grossnickle and MacDonald 2018). For

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forest tree species, large seedlings frequently show higher rates of survival and growth as well as greater competitiveness than small seedlings (Villar-Salvador et al. 2012, 2015). The high overall transplanting performance of large seedlings has been attributed in part to the presence of larger nutrient reserves (Tsakalidimi et al. 2012; Villar-Salvador et al. 2012) that can be remobilized to support new root and shoot growth (Millard and Grelet 2010; Oliet et al. 2011); this is critical for seedling establishment and early survival (Villar-Salvador et al. 2012). In contrast, plants with low nutrient reserves can depend to a greater extent on the nutritional status of the soil and, hence, be more sensitive to changes in soil fertility (Wang et al. 2019).

Non-structural carbohydrates (NSC) and nitrogen (N) are the most important storage compounds in plants (Kozłowski 1992; Millard et al. 2007; Millard and Grelet 2010), providing crucial support for new organ growth, respiration, reproduction, and defense (Villar-Salvador et al. 2015). Seedling growth in the 1st year can be affected by genetic variability (Roach 1987), seed size (Westoby et al. 1996; Leishman et al. 2000; Shi et al. 2019) and nursery cultivation regime (Grossnickle 2012), yet few studies have addressed the effects of seedling size on the buildup of N and NSC reserves in plant species.

In deciduous trees, N and NSC storage before winter depends on effective resorption from senescent leaves (Aerts 1990; Brant and Chen 2015; Chapin 1980; Killingbeck 1996). Resorption is considered to be a nutrient conservation mechanism (He et al. 2020; Yuan et al. 2006) that can reduce plants' dependence on soil fertility. Although a number of studies have examined the effect of soil fertility on nutrient resorption (Aerts and Chapin 1999; Kobe et al. 2005; Vergutz et al., 2012), debate continues about the effects of nutrient availability on nutrient resorption. Differences in data availability and analytical methods have led to contrasting conclusions, making it difficult to separate the effects of nutritional status from other covariates such as climate and plant composition (Yuan and Chen 2015). Compared with observations along natural nutrient gradients, nutrient addition experiments can provide further insights into nutrient resorption (Chapin et al. 1986; Yuan and Chen 2015). Soil fertility, however, is not the only major factor to affect leaf nutrient levels: intraspecific differences in plant size and other characteristics can play a role (Lü et al. 2020). Under the same nutrient supply conditions, large plants can have lower tissue nutrient concentrations than small plants (Villar-Salvador et al. 2012); this could occur due to nutrient dilution, which is often linked to higher growth rates. Organ nutrient concentrations can subsequently affect resorption efficiencies. Therefore, plant size variation must be taken into account in order to separate the effects of soil fertility from the effects of plant characteristics. Plant size can also change the nutrient allocation patterns of storage organs due to differences in root-shoot ratios (Grossnickle 2012).

N fertilization can enhance the formation of plant NSC and N reserves (Islam et al. 2009). Fertilization during the active growth phase can extend the growing period and delay hardening of seedlings, thus reducing their cold resistance (Rikala and Repo 1997; Andivia et al. 2011; Salifu et al. 2009; Villar-Salvador et al. 2015). In contrast, fertilization in the fall can increase plant N content without hindering the hardening process or substantially affecting seedling size (Li et al. 2014; van den Driessche 1985; Zhu et al. 2013). Therefore, fall fertilization is considered to be more suitable for the buildup of N reserves. It has been widely adopted in nurseries to improve seedling quality and increase plant nutrient storage in seedlings with low nutritional status (Li et al. 2014). Few studies have addressed the effects of fall fertilization on N resorption and allocation to different storage organs (Sanz Pérez et al. 2007; Villar-Salvador et al. 2013).

Our study used seedlings of *Quercus variabilis* Blume, a broadleaf deciduous tree that is dominant in many Chinese forests. This species is extensively used for forestation

(Zhang and Lu 2002). NSC and N reserves in oak species make up approximately 20–50% of the plant's NSC requirement and 50–98% of the N requirement for new growth in spring (Cerasoli et al. 2004; Maillard et al. 2001; Uscola et al. 2015; Wang et al. 2019). Oak seedlings typically show heterogeneous growth rates in the 1st year (Kormanik et al. 1998). Therefore, we speculated that the process of building up reserves in *Q. variabilis* would strongly depend on seedling size. In this study, we tested three hypotheses about the effects of seedling size and fall fertilization on N resorption and resource allocation:

- (1) Under the same nutrient supply conditions, intraspecific size heterogeneity can affect N resorption efficiency and N allocation. Large seedlings have lower N concentrations that can be alleviated by an increase in N resorption efficiency and/or an increase in N uptake after fall fertilization.
- (2) Differences in nutritional status mediated by seedling size can affect the efficiency of N uptake and allocation after fall fertilization. Fall fertilization increases plant N concentrations during the hardening period, which in turn reduces leaf N resorption, and these effects are stronger in small seedlings.
- (3) Large and small seedlings differ in N and NSC allocation to different plant organs.

## Materials and methods

### Acorn collection and seedling culture

In September 2012, acorns were collected from open pollinated *Quercus variabilis* Blume trees in the Sizuolou Forest Farm in Beijing, China (117° 148' E, 40° 282' N; elevation 316–467 m). After removing damaged or small acorns, the remaining/selected acorns ( $n=2000$ ) were stored in partially sealed polyethylene bags (wall thickness 100  $\mu\text{m}$ ) at 2 °C and 60% relative humidity in a constant temperature refrigerator (Kormanik et al. 1998). After about 6 months of cold storage, 640 acorns were removed from the refrigerator and accelerated the germination wet sand for 3 to 5 days. Then the germinated acorns were sown into individual containers (on March 28, 2013) (one acorn per container; diameter 6.4 cm, height 35.6 cm) that had been filled with a 3:1 mixture of peat (Pindstrup Seeding, pH 6.0, Screening 0–6 mm) and vermiculite (5 mm diameter, Xinyang Jinhualan Mining, Henan, China). The containers were then randomly assigned to 32 trays with 20 seedlings per tray (density 174.4 seedlings per  $\text{m}^2$ ). The trays were randomly arranged on a large rolling bench in a greenhouse at the Chinese Academy of Forestry Sciences in Beijing (40° 40' N, 116° 14' E). A black shade screen (40% light reduction) was placed outside the greenhouse, resulting in an average daily photosynthetic flux density of 600  $\mu\text{mol m}^{-2} \text{s}^{-1}$  inside the greenhouse.

### Growing season fertilization

Growing season fertilization began at 2 weeks after germination (starting April 12, 2013). Based on the methodology described in Timmer and Armstrong (1987), nitrogen was applied at an exponential rate (from 88.4 to 250 ppm) for 16 weeks (until August 25, 2013). The relative addition rate was 0.069 mg N per week and the cumulative rate of N addition by the end of the growing season was 50 mg N seedling<sup>-1</sup>. This was consistent

with the recommended dose of N required for maintaining sufficient nutrient levels in *Q. variabilis* (Li et al. 2014).

Nitrogen was supplied as  $\text{NH}_4\text{NO}_3$  (Shanghai Research Institute of Chemical Industry, Shanghai, China) and elemental P and K were supplied as  $\text{KH}_2\text{PO}_4$  (Guangdong Guanghua Sci-Tech, Guangzhou, China). At the end of the growing season fertilization period, each seedling had received 21.3 mg P and 26.6 mg K (50:26:33 for N:P:K). Microelements were supplied as EDTA (0.252 mg seedling<sup>-1</sup>; Foshan Xilong Chemical, Guangzhou, China) and DTPA (0.078 mg seedling<sup>-1</sup>; Jinke Fine Chemical Institute, Tianjin, China). Once a week, fertilizer solution (20 ml) was individually injected into media in each seedling container using a syringe. All seedlings were watered to field capacity on the morning of the day before fertilizing and again four days after to avoid fertility dilution. During this period, the average temperature was 25/18 °C, (day/night) and the mean relative humidity was 80% and a black shade screen was fixed outside of the green house resulting in an average daily light level inside the greenhouse of 820  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  for a duration of 11–14 h sunshine. All trays were randomly arranged and rotated weekly to minimize edge effects.

### Fall fertilization treatments using <sup>15</sup>N-labeled fertilizer

After the formation of terminal buds (by September 5, 2013), seedlings were stratified into two groups based on their shoot height: small seedlings (height < 30 cm) and large seedlings (> 30 cm). In total, we had 300 seedlings in each group distributed across 15 trays. A shoot height of 30 cm is about the average height of the population (Fig. S1).

Fall fertilization treatments were administered every week between September 8 and October 6, 2013. Prior to fall fertilization, seedlings were transplanted and filled with new medium to minimize the influence of residual fertilizer. Five trays per seedling size were chosen and randomly assigned to one of the three fall fertilization treatments: 0 mg N seedling<sup>-1</sup> (control), 12 mg N seedling<sup>-1</sup> (low N), and 24 mg N seedling<sup>-1</sup> (high N) (Li et al. 2014; Wang et al. 2019). The fertilizer was labeled with <sup>15</sup>N in the form of <sup>15</sup>NH<sub>4</sub><sup>15</sup>NO<sub>3</sub> at 10.2 atom% enrichment (Shanghai Research Institute of Chemical Industry, Shanghai, China). Seedlings received the same amount of P, K, and chelated micronutrients; the cumulative supplementation rates (mg seedling<sup>-1</sup>) were 9.82 for P, 12.29 for K, 1.51 for EDTA and 0.47 for DTPA. Seedlings were fertilized with mixed fertilizer solution (20 ml solution included <sup>15</sup>NH<sub>4</sub><sup>15</sup>NO<sub>3</sub>,  $\text{KH}_2\text{PO}_4$ , EDTA and DTPA) weekly.

For all treatments, irrigation was conducted every five days. Seedlings were watered to field capacity on the morning of staggered the fertilization day. The average temperature during this period was 22/15 °C (day/night) and the mean relative humidity was 55%. The average daily light level inside the greenhouse of 818  $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$  for a duration of 11–13 h sunshine. When the daily average outdoor temperatures were < 15 °C (after November 1, 2013), seedlings were moved outside the greenhouse and enclosed in individual open-topped white netting bags that allowed collection of senescent leaves and the seedlings were irrigated every 10–14 days during this period (Nov. 1, 2013 to Dec. 31, 2013).

### Morphological and nutritional measurements

Leaf net photosynthesis rate can be used to evaluate carbon assimilation during the hardening period. Using a portable infrared gas analyzer (LI-6400, LI-COR, NE, USA), we measured the leaf net photosynthesis rate of five seedlings from each seedling size and

fall fertilization treatment. Measurements were made every two weeks (September 11 and 25, October 9 and 23, 2013) between 09:00 and 11:00 am (25 °C, 100.5 kPa, 50% relative humidity, 600  $\mu\text{mol m}^{-2} \text{s}^{-1}$  PPFD, 395 ppm  $\text{CO}_2$  concentration).

Morphological and nutritional measurements were recorded at two sampling points: after the formation of terminal buds and before hardening ( $T_1$ ; September 5, 2013), and after complete leaf abscission at the end of hardening ( $T_2$ ; December 31, 2013). At each timepoint, we randomly harvested 25 seedlings per treatment. The growth medium was gently washed away from the roots, and the seedlings were separated into leaves (abscised leaves at  $T_2$ ), stems, and roots. All organs were dried in an oven at 65 °C for 48 h before weighing. Individual organs from all five seedlings taken from the same tray were pooled to form a composite sample, which was ground (Kewei Instrument Factory, Cangzhou, China) until it could be passed through a 0.25-mm sieve. The samples were then sent to the Institute of Quality Standards and Testing Technology for Agro-Products (Chinese Academy of Agricultural Sciences, Beijing, China) for further analysis. Total N concentration and  $^{15}\text{N}$  abundance were calculated using a semi-micro Kjeldahl distillation unit (UDK-152, Velp Scientifica, USA), coupled to a gas isotope ratio mass spectrometer (Finnigan MAT 251, Thermo Fisher Scientific, USA). The concentration of soluble sugars (SS) was determined using hydroalcoholic extraction and anthrone colorimetry, while starch concentration was analyzed using acid hydrolysis and anthrone colorimetry (Hansen and Moller, 1975). The absorbance spectrum of soluble sugars and starch was measured using a UV–visible spectrophotometer (Agilent 8453, Waldbronn, Germany). The total N or NSC content (mg) of each organ in the plant was calculated by multiplying organ mass by the corresponding N or NSC concentration.

## Calculations and statistical analyses

The amount of N contributed by fall fertilization treatment to the total N concentration ( $X$ ) in a specific organ at the end of fall fertilization was calculated using isotopic dilution equations in two different steps (Deléens et al. 1994). It can be expected that a fraction of labeled N found in an organ comes from currently assimilated  $^{15}\text{N}$  derives from fall fertilization ( $X$ ) and  $^{15}\text{N}$  in the seedlings before fall fertilization ( $Y$ ), respectively.  $X + Y = 1$ , where  $X$  can be calculated in each organ from:

$$A\%_{\text{organ}} = X(A\%_{\text{fall fertilization}}) + Y(A\%_{\text{before fall fertilization}}) \quad (1)$$

$$X = \frac{A\%_{\text{organ}} - A\%_{\text{before fall fertilization}}}{A\%_{\text{fall fertilization}} - A\%_{\text{before fall fertilization}}} \quad (2)$$

where  $A\%_{\text{organ}}$  is the abundance of  $^{15}\text{N}$  in a specific organ of the seedling after fall fertilization,  $A\%_{\text{before fall fertilization}}$  is the abundance of  $^{15}\text{N}$  in the unlabeled seedling, and  $A\%_{\text{fall fertilization}}$  is the amount of N administered during fall fertilization treatment.

Resorption efficiency was calculated using the following formula to avoid underestimation of NR due to mass loss occurs during senescence (van Heerwaarden et al. 2003; Vergutz et al., 2012):

$$\text{Resorption efficiency} = \left(1 - \frac{N_{\text{sen}}}{N_{\text{gr}}}\text{MLCF}\right) \times 100\% \quad (3)$$

where  $N_{gr}$  and  $N_{sen}$  are the N concentrations on a mass basis in green and senesced leaves, and MLCF is the mass loss correction factor, specifically the ratio of the dry mass of senesced leaves and the dry mass of green leaves (Vergutz et al., 2012).

To assess whether a particular treatment favored the storage of N, SS, or starch in specific organs in the seedlings, we quantified and compared the content of these compounds in the leaves, stems, and roots of seedlings at  $T_1$  and  $T_2$ . We assumed that if a seedling showed no preference of organs in terms of storage, the sink strength for storage compounds of a particular organ would be directly proportional to its mass. Therefore, if an organ is a priority site for storage, it would contain a higher amount of nutrients than predicted by its mass. Predicted nutrient content (mg) of a particular organ ( $i$ ) was calculated as:

$$\text{Predicted nutrient content of organ } (i) = \frac{\text{mass of organ}}{\text{mass of plant}} \times \text{nutrient content of plant} \quad (4)$$

Using data from individual seedlings, we analyzed the morphological characteristics of the plant and leaf net photosynthesis rates (25 seedlings per treatment). For N and NSC concentration, treatment means were derived from a composite sample for each replicate and treatment (5 seedlings per replicate, 5 replicates, in total 25 seedlings per treatment).

## Statistical analyses

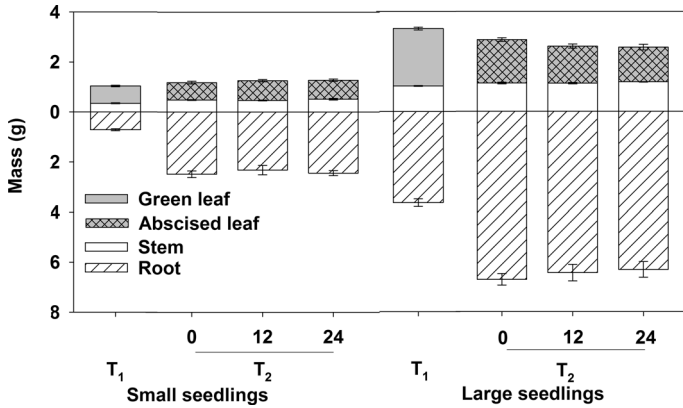
The effects of seedling size on morphological features and on N and NSC concentrations at  $T_1$  were analyzed using an independent samples t-test. The effects of seedling size, fall fertilization and their interaction on morphological features and on N and NSC concentrations at  $T_2$  were assessed using two-way ANOVA. The data met the requirements for normality and variance homogeneity, and no transformations were necessary. Separation of means was ranked based on Duncan's multiple range test at  $P=0.05$ . All statistical analyses were performed in SPSS 16.0 (IBM, Chicago, IL, USA).

## Results

### Seedling growth and leaf net photosynthesis rates

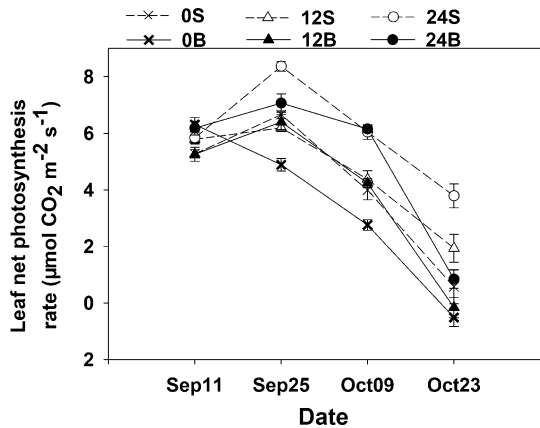
Before the hardening period, at  $T_1$ , roots, stems and leaves mass of large seedlings were 414%, 191% and 233%, respectively, larger than small seedlings ( $P < 0.001$ ; Fig. 1). From  $T_1$  to  $T_2$ , the mass of large seedlings increased more than 60% while the mass of small seedlings increased more than 170%. This increase was mainly due to root growth, which increased 78% in large seedlings and >230% in small seedlings. At  $T_2$ , roots accounted for a large proportion (>83%) of plant mass (roots + stems) in large and small seedlings. Fall fertilization had no effect on the mass of specific organs (root mass  $P=0.938$ ; stem mass  $P=0.368$ ).

The leaf net photosynthesis rate of non-fertilized seedlings (controls) decreased over time (Fig. 2). In seedlings receiving fall fertilization, the leaf net photosynthesis rate reached the maximum value about halfway through the hardening period (on September 25, 2013), after which it decreased rapidly ( $P < 0.001$ ). Overall, the leaf net photosynthesis rate of large seedlings decreased faster than that of small seedlings ( $P=0.047$ ).



**Fig. 1** Mass of specific organs (mean  $\pm$  SE) in one-year-old *Quercus variabilis* seedlings measured at T<sub>1</sub> (before hardening) and T<sub>2</sub> (after abscission at the end of hardening). Seedlings were classified as small (height < 30 cm) or large at T<sub>1</sub> and monitored until T<sub>2</sub> to examine the effect of three fall fertilization treatments (0, 12, or 24 mg N seedling<sup>-1</sup>). (n = 25)

**Fig. 2** Leaf net photosynthesis rate (mean  $\pm$  SE) of one-year-old *Quercus variabilis* seedlings during the hardening period. Seedlings were classified as small (S) small (height < 30 cm) or large (L) just before the hardening period and monitored until the end of hardening to examine the effect of three fall fertilization treatments (0, 12, or 24 mg N seedling<sup>-1</sup>). (n = 25)



### N and NSC concentration

Despite being exposed to the same conditions during the rapid growth period, at T<sub>1</sub>, the small seedlings had larger N concentrations in the roots, stems, and entire plant than large seedlings (Table 1). At T<sub>2</sub>, N concentrations in small seedlings were only about 30–40% larger than those in large seedlings. Additionally, fall fertilized seedlings had larger root N concentration values than non-fertilized ones. While stem N concentrations in small seedlings increased independently of fall fertilization rates, large seedlings showed an increase in stem N concentration only under the highest fall fertilization rate (24 mg N); this indicates an interaction between seedling size and fall fertilization.

At T<sub>1</sub>, large seedlings had larger root and plant NSC concentrations than small seedlings. Although seedling size did not have a significant effect on plant NSC concentrations, large seedlings had 20% higher stem NSC concentrations than small seedlings (Table 1). At T<sub>2</sub>, small seedlings had slightly larger root NSC concentrations than large



**Table 1** Concentration of total nitrogen (N) and total non-structural carbohydrates (NSC) in the stem, root, and entire plant (the sum of leaf, stem and root at  $T_1$ , the sum of stem and root at  $T_2$ ) in one-year-old *Quercus variabilis* seedlings, measured at  $T_1$  (before hardening) and  $T_2$  (after leaf abscission at the end of the hardening period)

Time of sampling	Seedling size and treatment	N concentration			NSC concentration		
		Stem	Root	Plant	Stem	Root	Plant
$T_1$	Small	1.53 ± 0.03	2.42 ± 0.09	2.42 ± 0.05	9.57 ± 0.15	15.72 ± 0.24	13.29 ± 0.13
	Large	0.65 ± 0.02	0.92 ± 0.02	1.23 ± 0.02	10.02 ± 0.22	21.19 ± 0.34	18.51 ± 0.17
t-values		28.0***	16.1***	23.5**	- 1.7	- 13.2***	24.1***
$T_2$	Small-0	0.93 ± 0.03	1.1 ± 0.06	1.07 ± 0.05	12.27 ± 0.13	23.29 ± 0.24	21.48 ± 0.27
	Small-12	1.23 ± 0.03	1.12 ± 0.02	1.13 ± 0.01	12.81 ± 0.29	24.75 ± 0.64	22.72 ± 0.57
	Small-24	1.19 ± 0.04	1.24 ± 0.01	1.23 ± 0.01	15.28 ± 0.28	24.56 ± 0.12	22.95 ± 0.16
	Large-0	0.65 ± 0.03	0.88 ± 0.02	0.85 ± 0.02	16.94 ± 0.18	22.19 ± 0.46	21.43 ± 0.39
	Large-12	0.73 ± 0.03	0.96 ± 0.02	0.93 ± 0.02	17.49 ± 0.4	22.51 ± 0.83	21.76 ± 0.78
	Large-24	0.94 ± 0.04	0.94 ± 0.03	0.94 ± 0.03	18.27 ± 0.74	23.62 ± 0.73	22.79 ± 0.69
	F values						
	Size	155.3***	74.9***	120***	165***	9.6**	0.8
	FF	34.8***	4.9*	11.2***	16.7***	3	3.7*
	Size × FF	8.4**	2.7	1.5	3.1	0.8	0.5

Seedlings were classified as small (height < 30 cm) or large at  $T_1$  and monitored until  $T_2$  to examine the effect of three fall fertilization (FF) treatments (0, 12, or 24 mg N seedling<sup>-1</sup>), as well as the interaction between seedling size and fall fertilization. Values are mean ± SE. \*, 0.01 <  $P$  ≤ 0.05; \*\*, 0.001 <  $P$  ≤ 0.01; \*\*\*,  $P$  ≤ 0.001. (n = 5)

seedlings. Stem and plant NSC concentrations increased with fall fertilization, whereas there was no effect on root NSC concentration.

### **N and NSC allocation in seedling organs before hardening ( $T_1$ )**

Large seedlings had a higher content of N, SS, and starch in all organs than small seedlings did ( $P$  < 0.001; Fig. 3). The allocation of N and NSC was dependent on seedling size. More than 47% of N, 48% of SS and 36% of starch were allocated to leaves. Large seedlings increased the allocation of N, SS, and starch to leaves at the expense of stems, and they increased the allocation of N and starch to leaves at the expense of roots. In contrast, small seedlings increased the allocation of N to the leaves at the expense of stems, they increased allocation of SS to stems at the expense of roots, and they allocated starch to different organs based on organ mass.

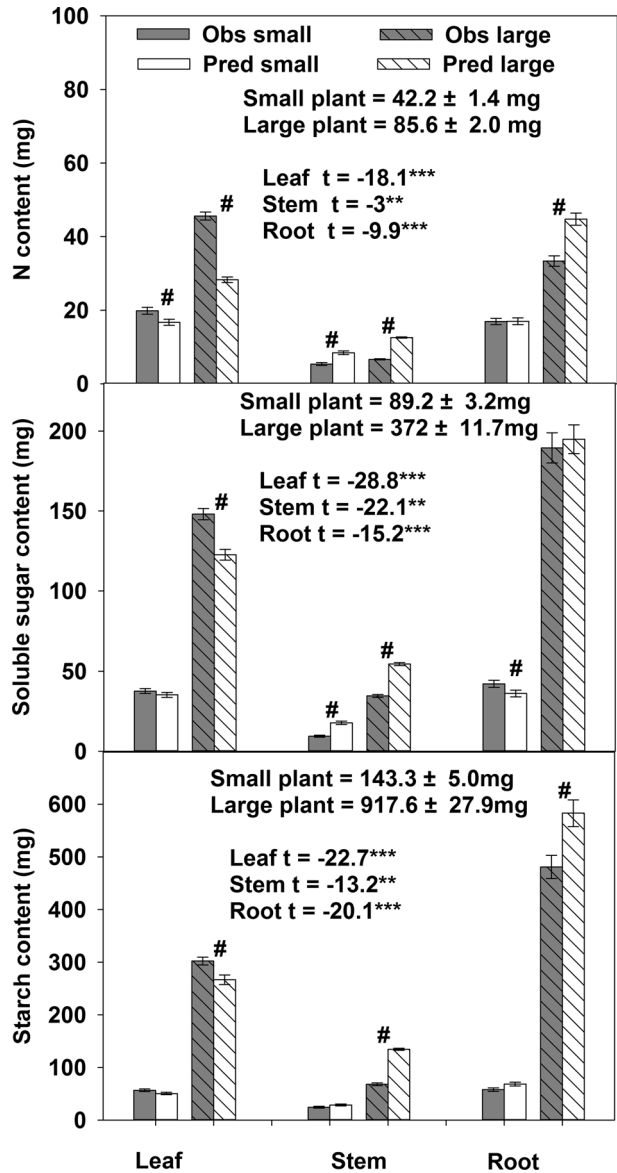
### **Allocation of <sup>15</sup>N-labeled N and NSC in seedling organs after leaf abscission ( $T_2$ )**

Seedling size had no effect on the content of labeled N in stems, roots, or abscised leaves ( $P$  = 0.086), whereas fall fertilization had a significant positive effect ( $P$  < 0.001; Fig. 4). Compared to small seedlings, fall fertilization of large seedlings increased labeled N in the perennial organs (stem and root) at the expense of abscised leaves.

Labeled N was allocated to roots in proportion to root mass, as indicated by the similarity between observed and predicted values (Fig. S2). Large and small seedlings allocated



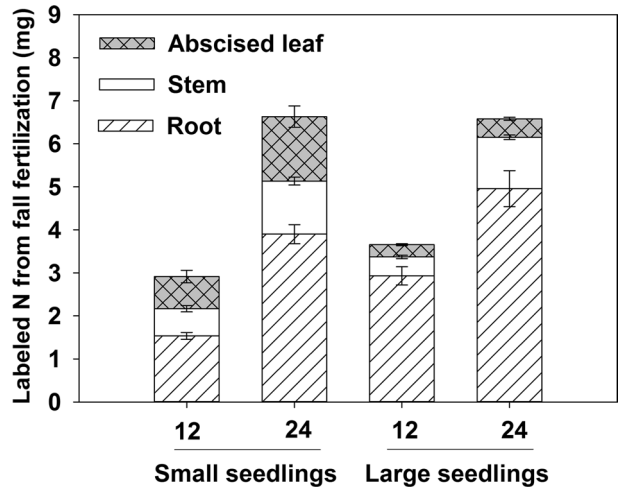
**Fig. 3** Observed (Obs) and predicted (Pred) values of nitrogen, soluble sugar, and starch content (mean  $\pm$  SE) in different organs of one-year-old *Quercus variabilis* seedlings at T<sub>1</sub> (before hardening). Seedlings were classified as small (height < 30 cm) or large. For organ-level comparisons, # indicates significant differences between the observed and predicted values. The results of t-tests are also shown: \*\*, 0.001 < P  $\leq$  0.01; \*\*\*, P  $\leq$  0.001. (n = 5)



labeled N differently depending on fall fertilization (interaction between size and fall fertilization;  $P = 0.008$ ). The labeled N in roots increased with fertilization, and small seedlings had more than two times the amount of labeled N than large seedlings. The amount of labeled N in stems increased with fall fertilization, and this increase was greater in large seedlings.

After leaf abscission (T<sub>2</sub>), unlabeled N and NSC (either SS or starch) were allocated mainly to the roots; large seedlings had higher concentrations of unlabeled N and NSC than small seedlings, and fall fertilization had no effect on this allocation (Fig. S2). Small

**Fig. 4** Labeled N content in different organs (mean  $\pm$  SE) after fall fertilization in one-year-old *Quercus variabilis* seedlings at the end of the hardening period. Seedlings were classified as small (height < 30 cm) or large before the hardening period and monitored until the end of the hardening period to examine the effects of fall fertilization treatments (12 or 24 mg N seedling<sup>-1</sup>). ( $n = 5$ )

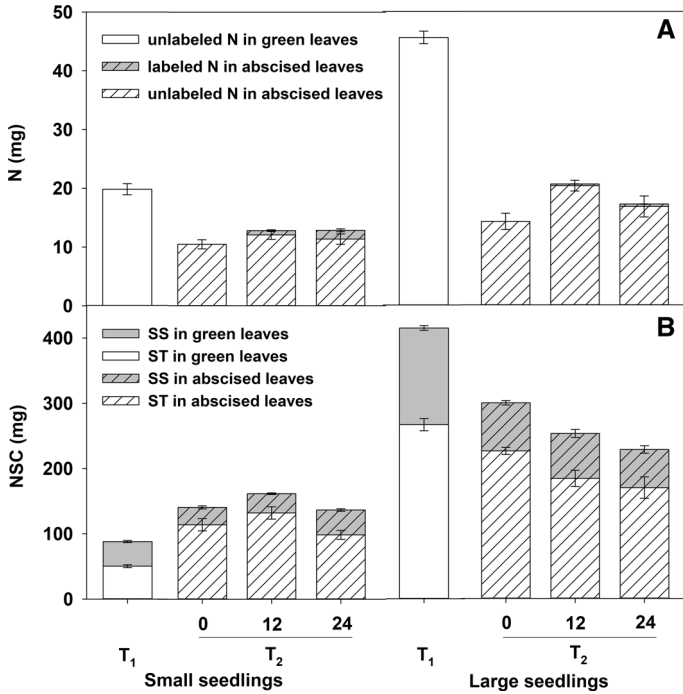


seedlings, especially the controls, showed lower unlabeled N content in stems at  $T_2$  than at  $T_1$ . The opposite trend was observed in large seedlings, where the highest increase in unlabeled N was observed in the seedlings fertilized with 24 mg N. This allocation was proportional to stem mass in small seedlings, but lower than predicted in large seedlings, except under the highest fall fertilization rate. Unlabeled N in the stem increased with fall fertilization rates and the values were higher than predicted, except in the case of large seedlings fertilized at the low N rate (12 mg).

Both seedling size and fall fertilization led to an increase in NSC content from  $T_1$  to  $T_2$ . At  $T_2$ , large seedlings had higher SS and starch content in stems than small seedlings. Fall fertilization led to an increase in SS content in large seedlings (Fig. S2) and to an increase in starch content in large and small seedlings. At  $T_2$ , SS content was smaller than predicted based on stem mass for large and small seedlings across all fall fertilization treatments. The allocation of starch to stems was as predicted in large seedlings, but lower than predicted in small seedlings.

### Leaf N resorption and N and NSC content in abscised leaves

Abscised leaves from large seedlings had significantly higher unlabeled N content than small seedlings ( $P < 0.001$ ). Fall fertilization increased content of unlabeled N ( $P = 0.006$ ) and labeled N ( $P = 0.048$ ) in abscised leaves from large and small seedlings. Large seedlings resorbed significantly more N than small seedlings ( $P < 0.001$ ; Fig. 5A). Fall fertilization had no effect on N resorption efficiency, and small seedlings resorbed significantly less N than large seedlings ( $P < 0.001$ ). Additionally, SS and starch content in abscised leaves was higher for large seedlings than for small ones ( $P < 0.001$ ; Fig. 5B). Fall fertilization reduced starch content in abscised leaves of large and small seedlings ( $P = 0.035$ ).



**Fig. 5** **A** Total nitrogen (N) and **B** total non-structural carbohydrate content (NSC) (mean ± SE) in green (non-senescent) leaves before hardening (T<sub>1</sub>) and in abscised leaves at the end of the hardening period (T<sub>2</sub>) in one-year-old *Quercus variabilis* seedlings. Seedlings were classified as small (height < 30 cm) or large before hardening (T<sub>1</sub>) and monitored until the end of the hardening period (T<sub>2</sub>) to examine the effect of three fall fertilization treatments (0, 12, or 24 mg N seedling<sup>-1</sup>). The amount of unlabeled N resorbed from the leaves into the plant was calculated as the difference in N content in the leaves between T<sub>1</sub> and T<sub>2</sub>. Resorbed labeled N was estimated from the amount of unlabeled N resorption and labeled N content in abscised leaves (see Methods). SS and ST in panel B are the content of soluble sugar and starch respectively

## Discussion

### Intraspecific differences in N resorption and hardening process

Consistent with part of our first hypothesis, we found that large seedlings resorbed larger amounts of N than small seedlings. Contrary to our second hypothesis, we found that fall fertilization did not affect N resorption efficiency. Our data suggest that the amount of N resorbed from leaves depends on the N content of the leaves before hardening (N source): the higher this initial amount, the greater the amount of N resorbed. This would be consistent with the source-driven hypothesis of internal N remobilization (Millard 1996; Millard and Grelet 2010), which suggests that remobilization and resorption involve the same mechanisms.

Our results suggest that sink strength can also contribute to size-related differences in N resorption efficiency. First, in large seedlings, which had high resorption efficiency, also had greater mass increment during hardening ( $2.97 \pm 0.2$  vs  $1.84 \pm 0.09$  g, Fig. 1). Second, large seedlings contained less N than that predicted from mass of roots and stems mass, but only in the stems of small seedlings (Fig. 3). This suggests that large seedlings have

capacity for N reserves in roots and stems, whereas small seedlings have such capacity only in stems. Furthermore, although large seedlings had higher N content, N concentration in perennial organs at the end of the growing period was two times higher in small than large seedlings. These results suggest that large seedlings, which have higher N resorption efficiency during the hardening phase, have greater sink strength in perennial organs than small ones before hardening ( $T_1$ ). Similar findings were reported in a study about internal nitrogen cycling of Mediterranean *Quercus* species (Silla and Escudero 2003).

Our results also indicate that higher N concentrations before hardening can lead to lower N resorption efficiencies. N resorption efficiency differences may be explained by differential allocation at different N levels into soluble or insoluble proteins. In the case of low internal N concentration, N is typically stored as soluble proteins that facilitate hydrolysis and N resorption. Therefore, a reduction in internal nutrient availability can enhance N resorption efficiency (Pugnaire and Chapin 1993). A meta-analysis from global data showed that across plants of the same species, N resorption efficiency tends to decline with an increase in plant N concentration (Yuan and Chen 2015). However, that analysis relied mostly on data about plants in their native habitats, potentially confounding species and environmental effects (Aerts and Chapin 1999; Kobe et al. 2005; Vergutz et al., 2012; Yuan and Chen 2015). Few studies of N resorption have included non-leaf plant tissues in order to take into account sink strength. We have extended previous work by analyzing data on the N content in leaves (source), as well as N concentration in the roots and stems (sink). We were able to demonstrate that, after excluding the influence of soil fertility, both source and sink influence N resorption.

We observed that large and small seedlings took up the same amount of N in whole seedling levels during the hardening phase. After fall fertilization, however, large seedlings allocated a lower proportion of labeled N to the leaves and a higher proportion to the perennial organs (Fig. S1). The lower resorption efficiency in small seedlings may lead to higher allocation of labelled N to abscised leaves after fall fertilization. It is also possible that large and small seedlings allocate N differently during hardening. Large seedlings may tend to allocate labelled N directly to the perennial organs because they have higher sink strength than small ones, while small seedlings allocate it directly to the leaves and later resorb it from them. This has been observed in *Larix olgensis* Henry seedlings, where a part of the fall fertilized N was allocated to the leaves, from which it translocated to the roots and stems (Zhu et al. 2013).

### Intraspecific variability in N and NSC allocation patterns

Consistent with our third hypothesis, our findings indicate that seedling size plays an important role in the allocation of storage compounds. Despite having originated from the same parent tree and being grown in the same conditions, large and small seedlings in our study allocated N and NSC differently. Although large seedlings had twice the N content as small ones, their N concentration was two times lower due to the dilution effect. For *Quercus* species, the recommended N concentration is around 1.5% (Uscola et al. 2015). Small seedlings, but not large ones, typically reached optimal N concentrations before hardening. Not even large seedlings growing rapidly under exponential fertilization achieved optimal N loading.

We found that seedlings of both sizes allocated N preferentially to leaves, but large and small seedlings achieved this differently, consistent with our third hypothesis. Small

seedlings allocated N to leaves at the expense of stems; large seedlings, at the expense of stems and roots (Fig. 3). Small seedlings increased NSC content in roots at the expense of stems.

Previous studies have shown that roots are the priority organ for storage compounds in certain oak species (Cerasoli et al. 2004; Uscola et al. 2015; Villar-salvador et al. 2015). Similarly, our findings show that roots are the priority perennial organ for NSC allocation, but N allocation changes depending on the growth phase. N was allocated to roots during rapid growth but to stems during hardening. For oak species, hardening phase is an important period for seedling mass accumulation, especially for root (Zhang and Lu 2002). However, continued growth during the hardening process can also lead to nutrient dilution which may have a negative effect on subsequent field performance (Boivin et al. 2004). Similar to previous studies (Rikala et al. 2004; Islam et al. 2009), the results of this study find that fall fertilization during hardening period might compensate for N and NSC dilution of perennial organs as they continue to add biomass during the fall (Tab. 1).

In large seedlings, the amount of NSC in leaves was lower in fall-fertilized plants than in control plants, which could be due to higher NSC resorption (Fig. 5B). This implies greater C sink strength in fall-fertilized plants, since C levels depend much more on the sink than the source (Brüggemann et al. 2011; Paul and Foyer 2001). Fall fertilization may be associated with a stronger C sink because photosynthesis also increased with fall fertilization; the greater C sink strength may also reflect higher N uptake as a result of fertilization, would increase metabolism and therefore demand for C. Fall fertilization was associated with greater photosynthesis during hardening, and this effect was stronger for small seedlings than for large ones (Fig. 2). Unfortunately, we were unable to calculate NSC resorption efficiency under our experimental conditions, so future work is needed to explore why NSC resorption varies with fall fertilization.

In this study, we found that large and small seedlings resorbed N with different efficiency even when grown under the same conditions. This variation likely reflects physical differences and nutrient allocation between larger and smaller seedlings. Further studies are required to understand whether the interaction between seedling size and fall fertilization can lead to similar nutrient resorption responses in other tree species.

The results of the present study have ecological and practical implications for forest restoration plantations. We found that under exponential fertilization during the growth phase, small seedlings were able to reach optimal N concentrations, while large, faster-growing seedlings were not. An increase in fertilization rate can help increase nutrient loading in large seedlings, but this could lead to toxicity in small seedlings. Therefore fall fertilization may be the best method to simultaneously optimize the nutritional status of large and small seedlings. Fall fertilization also increased NSC concentrations in both seedling sizes, without affecting growth. This finding may be especially relevant in colder regions, since an increase in tissue NSC concentration can enhance seedling frost tolerance (Morin et al. 2007; Villar-Salvador et al. 2015). For plants of the same species and same age, many researches find that large seedlings have higher afforesting survival rate than small seedlings (South et al. 2005, Pinto et al. 2012, Villar-Salvador et al. 2012, Andivia E et al. 2021) through higher growth and resource mobilization (Villar-Salvador et al. 2012), which are important processes for seedling establishment, avoid stress and reduce competition (Grossnickle 2005; Rosner and Rose 2006). According to the different adaptability of seedlings sizes to the different sites, choose the corresponding cultivation methods could be one of the effective way to define and refine the proper type of seedling for reforestation and restoration.

## Conclusions

Our study demonstrates that N resorption in *Q. variabilis* seedlings is affected by the N content in leaves (source) and the N concentration in roots and stems (sink). By comparing large and small seedlings subjected to three fall fertilization treatments, we were able to confirm and extend previous results on N and NSC allocation. We showed that the amount of N resorbed from leaves depended on the original N content in the leaves (N source). Before hardening, large seedlings had higher N content in leaves than small seedlings, so they resorbed more N. Sink strength was also an important driver of size-related differences in N resorption efficiency. Compared to small seedlings, large seedlings that resorbed N more efficiently had lower N concentrations in the perennial organs at the end of the growth phase, and their perennial organs gained more mass during the hardening phase. Furthermore, resorption efficiency was not affected by fall fertilization. Large and small seedlings took up similar amounts of N from fall fertilization, but large seedlings allocated less N to leaves than small seedlings did. Large and small seedlings allocated NSC preferentially to roots, whereas they allocated N preferentially to roots during growth but to stems during hardening.

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