

RESEARCH ARTICLE

Carbon Allocation into Different Fine-Root Classes of Young *Abies alba* Trees Is Affected More by Phenology than by Simulated Browsing

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Abstract

Abies alba (European silver fir) was used to investigate possible effects of simulated browsing on C allocation belowground by ¹³CO₂ pulse-labelling at spring, summer or autumn, and by harvesting the trees at the same time point of the labelling or at a later season for biomass and for ¹³C-allocation into the fine-root system. Before budburst in spring, the leader shoots and 50% of all lateral shoots of half of the investigated 5-year old *Abies alba* saplings were clipped to simulate browsing. At harvest, different fine-root classes were separated, and starch as an important storage compartment was analysed for concentrations. The phenology had a strong effect on the allocation of the ¹³C-label from shoots to roots. In spring, shoots did not supply the fine-roots with high amounts of the ¹³C-label, because the fine-roots contained less than 1% of the applied ¹³C. In summer and autumn, however, shoots allocated relatively high amounts of the ¹³C-label to the fine roots. The incorporation of the ¹³C-label as structural C or as starch into the roots is strongly dependent on the root type and the root diameter. In newly formed fine roots, 3–5% of the applied ¹³C was incorporated, whereas 1–3% in the ≤0.5 mm root class and 1–1.5% in the >0.5–1.0 mm root class were recorded. Highest ¹³C-enrichment in the starch was recorded in the newly formed fine roots in autumn. The clipping treatment had a significant positive effect on the amount of allocated ¹³C-label to the fine roots after the spring labelling, with high relative ¹³C-contents observed in the ≤0.5 mm and the >0.5–1.0 mm fine-root classes of clipped trees. No effects of the clipping were observed after summer and autumn labelling in the ¹³C-allocation patterns. Overall, our data imply that the season of C assimilation and, thus, the phenology of trees is the main determinant of the C allocation from shoots to roots and is clearly more important than browsing.

Introduction

European silver fir (*Abies alba* Mill.), a typical tree of montane and lower subalpine forests in the Alps, has a highly relevant protective function against landslide due to its deep rooting system [1], its high resistance against bark beetles (*Ips typographus* L.), and its general ease of natural regeneration [2]. It produces high quality wood that is used for furniture, construction timber and pulp. However, *A. alba* has been declining throughout its natural range since the 18th century due to forest management practices [2] and anthropogenic air pollution. Moreover, the high population number of game animals, e.g. deer, and, therefore, high grazing pressure on young *A. alba* trees is thought to prevent regeneration [3,4], especially due to browsing of the leader shoot, reducing *A. alba* height growth for several years [5].

Although stress responses of different conifers to either artificial or natural defoliation through clipping or herbivory have been studied extensively [6–10], information on belowground responses is scarce. Based on studies with non-woody plants, it has been suggested that herbivore-mediated shifts in belowground carbon (C) allocation patterns of plants are the result of a close coupling of above- and belowground processes in terrestrial ecosystems, induced by plant defense mechanisms [11,12]. The first studies on belowground responses of trees focussed on root growth, either directly influenced by mammalian browsing of shoots or indirectly by mammalian trampling [13,14]. Hester et al. [9] were the first studying the effect of simulated browsing on above- and belowground growth of different tree species in a pot-experiment and reported not only negative above- but also negative belowground responses due to simulated browsing by clipping. Recent studies also addressed the fate of assimilated C belowground and the belowground starch storage responses to browsing events [15–20], with only one study so far applying isotopically labelled C [21]. Although the ¹³C-isotope label in that latter study was measured only in the tree rings of the stems without observing any significant effects of the browsing, this study showed the potential of the ¹³C-isotope application for tracking recently fixed photoassimilates within the plants. Moreover, the ¹³C-isotope method provides the location of ¹³C-incorporation (e.g. root tips, coarse roots) as well as the form of the incorporated ¹³C-label (e.g. starch, cellulose) [22].

The main objectives of the present study were to investigate the effects of browsing, simulated through clipping, on the C allocation belowground at different times during the growing season, to distinguish C incorporation among different types of fine roots, and to investigate the changes in starch storage patterns in the fine roots due to clipping. Deduced from these objectives we tested the following hypotheses: (1) the time point (seasons) of the ¹³C-pulse-labelling and, thus, phenology has a strong effect on the allocation of the ¹³C-label from the shoot to the roots, (2) the incorporation of the ¹³C-label as structural C or as starch into the roots is strongly dependent on the root type and the root diameter, and (3) clipping strongly influences the amount of allocated ¹³C-label as well as the site of incorporation and the form of the incorporated ¹³C-label (structural C *versus* starch) within the root system. Therefore, 5-year-old *A. alba* saplings, both clipped and control trees, were pulse-labelled with ¹³CO₂, either in spring, summer, or autumn, and the assimilated ¹³C was traced into the fine-root systems, with specific emphasis on the incorporation of ¹³C into starch as the main storage carbohydrate.

Material and Methods

Experimental design and plant material

The experiment was carried out in 2008 in the tree nursery of the Swiss Federal Research Institute (WSL) in the Swiss lowlands (Birmensdorf, 47°22 N, 08°27 E, 550 m a.s.l.). According to

Palacio et al. [15], saplings of an approximate height of 30–50 cm height are considered to be one of the most vulnerable stages of a young tree in relation to large herbivore browsing (compare also [6,9]). Thus, in total, 68 five-year-old nursery-grown *A. alba* saplings (height ± 30 cm; provenance Beggingen, 47°47' N, 08°33' E, 650 m a.s.l.) were grown in a nursery soil with a pH of 7.2 and were fertilised once at the age of four years with organic NPK fertiliser (Unikorn II, Hauert, Switzerland; 20 g m⁻²).

In the beginning of April 2008, the tree saplings were excavated with their root systems attached, using a root-cutting machine ('Beetroder', Baertschi Agrartechnik AG, Hüswil, Switzerland). The saplings were then replanted into pots (22.5 cm height, 26 cm in diameter) and assigned to two treatments, 35 plants in a browsing treatment and 33 plants in an untreated control. The browsing treatment was performed at the end of April, just prior to bud burst, when a maximum of browsing by wild ungulates can be assumed [23]. The browsing was carried out according to Häslar et al. [10], with clipping two-thirds of the previous year's leader shoots and one-third of the previous year's shoot growth at every second lateral branch (proportion of buds removed: 56 \pm 1.3%), using diagonal pliers to break the plant tissue in a comparable way to ungulate browsing.

All saplings were randomly arranged in four rows of 17 trees and shaded with wooden frames above the trees and shading nets at the sides. Thus, the trees received only about one-third of direct sunlight, simulating conditions as in the forest understory [10].

¹³CO₂ pulse-labelling

The ¹³CO₂ pulse-labelling was conducted with three randomly assembled groups of *A. alba* tree individuals. Each group was labelled during one physiologically significant phase, i.e., in spring (early May 2008, subsequent to sprouting; 28 trees), in summer (early July 2008, period of stem growth; 20 trees) and in autumn (end of September 2008, end of the growing season; 12 trees). In total, 60 trees were labelled with ¹³CO₂. In addition to these 60 trees, eight trees (four clipped and four controls) remained unlabelled and were used to calculate the respective ¹³C enrichment in the trees (Table 1).

At each labelling date, randomly chosen groups of four *A. alba* trees were lined up side by side, and the shoots of the four trees (two clipped and two control trees) were covered with one polyethylene bag (volume approx. 300 l, 100 μ m thickness) and pulse-labelled with ¹³CO₂. The polyethylene bags were then tightly wrapped at the stems, below the crowns, and sealed with string and adhesive tape. With a syringe, 480 ml of gaseous ¹³CO₂ (CIL Cambridge Isotope Laboratories, Switzerland; 280 mg ¹³C) were added to each polyethylene bag.

Table 1. Number of harvested and ¹³CO₂ pulse-labelled trees during the seasons in two years for two clipping treatments and three ¹³CO₂ labelling seasons. Treatments are 'Control' = unclipped trees, and 'Clipped' = clipped trees.

Treatment	¹³ CO ₂ labelling season	Harvesting season				Sum of trees
		Spring	Summer	Autumn	Spring+1 yr	
Control	Spring	4	4	4	1	13
	Summer	-	4	4	2	10
	Autumn	-	-	4	2	6
	Sum of trees	4	8	12	5	29
Clipped	Spring	4	4	4	3	15
	Summer	-	4	4	2	10
	Autumn	-	-	4	2	6
	Sum of trees	4	8	12	7	31

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This equalled 120 ml $^{13}\text{CO}_2$ (70 mg ^{13}C) for each tree individual. In spring, 7 polyethylen bags with total 28 trees were labelled, in summer 5 bags with 20 trees, and in autumn 3 bags with 12 trees.

The addition of the gas was performed following Kagawa et al. [24] and Endrulat et al. [22] to ensure efficient incorporation of the label. After closure of the bags, 2 x 40 ml $^{13}\text{CO}_2$ were injected into each bag, followed by another 2 x 40 ml after 15 minutes. The trees were left in the bags for 1 h, before bags were opened for another hour. This procedure was repeated three times within one day. The labelling always started around 11 a.m. and lasted until 4 p.m. (summertime) in the afternoon. Since we aimed that plants were exposed to full sunlight for all three labelling dates, we started close to noon because in autumn fog prevails in the mornings. To reduce water condensation and thus limitation of CO_2 incorporation rate, two electric fans per bag were used to ensure continuous circulation within the bag. The fans were equipped with 80 g silica gel in the front to additionally reduce humidity in the bags. Maximum temperatures at the labelling dates were 21.3°C (May), 23.8°C (July) and 14.5°C (September).

Tree measurements

Before and after the clipping treatment and immediately before each sapling was harvested, non-destructive morphological measurements were carried out for each tree. These measurements included sapling height, length of new leader shoot, number of buds, number of new shoots (terminals and laterals), and number of branches off the main stem. Furthermore, the origin of the new leader was recorded as originating from an apical, interwhorl or secondary whorl bud according to Häslér et al. [10] (S1 Table).

Sampling and harvest

Immediately after each of the last labelling exposures, 10 needles of every labelled tree were randomly sampled, milled and analyzed for the maximum ^{13}C label in each tree. The maximum ^{13}C contents in the needles were then used to calculate the relative ^{13}C contents in the roots. Whole trees were harvested at one to four different time points after the three ^{13}C -labelling dates: In spring, summer, and autumn 2008, and as well as in spring 2009, i.e., 1, 3, 5, and 12 months after a labelling event (Table 1). Of each harvest, four replicate saplings of both treatments (clipped and control) were harvested, except in the spring 2009, one year after the start (spring+1 yr), when a maximum of three replicates was harvested. At each harvest, two non-labelled control trees were sampled as well.

The whole root systems were carefully washed and partitioned into various root categories using a vernier caliper according to Endrulat et al. [22] to keep track of the spatial distribution of the ^{13}C -label within the roots; new roots (newly formed white-coloured fine roots), fine roots of various size classes (≤ 0.5 mm in diameter, >0.5 –1 mm in diameter, >1.0 –1.5 in diameter mm, >1.5 –2 mm in diameter; brown-coloured), and coarse roots (>2 mm in diameter; brown-coloured).

Branches and needles as well as stems were weighed (fresh weight), frozen in liquid nitrogen, and then stored at -20°C until lyophilisation and biomass (dry weight) determination.

Starch extraction

Starch was extracted from two fine-root classes, from the newly formed fine roots and the fine roots with a diameter ≤ 0.5 mm, and in spring, summer, and autumn of 2008. Starch extraction followed a protocol of Smith and Zeeman [25], modified according to Regier et al. [26] and Endrulat et al. [22]. Approximately 60 mg (dry weight; DW) of lyophilized and milled *A. alba* fine roots were extracted with 80% ethanol, and boiled to gelatinise the starch. Starch was then

digested with α -amylase and amyloglucosidase (Roche, Switzerland) in water solution. After digestion, the starch amount was measured as released glucose with a spectrophotometer (Tecan, Switzerland) at 340 nm. For ^{13}C isotopic measurements, the samples were filtrated with centrifugal Filter Devices (Microcon YM-10; Millipore, Switzerland) in order to take out the enzymes according to Götlicher et al. [27], and then dried in tin capsules (Säntis Analytical AG, Switzerland) in a vacuum centrifuge (Heto Lab Equipment, Denmark).

$\delta^{13}\text{C}$ analyses and isotope calculations

Carbon isotope and total C concentration analyses in needles, bulk fine-roots and starch were carried out with an Elemental Analyser (EA-1110, Carlo Erba, Italy; Euro EA 3000, Hekatech, Germany) connected to a continuous flow mass spectrometer (Delta V Advantage, Thermo Fisher Scientific, Germany) at WSL. The measured isotope ratios are expressed in δ -notation in permil units (‰), using Vienna-PeeDee Belemnite (V-PDB) as standard. The precision for ^{13}C analysis was $\pm 0.2\text{‰}$.

The amounts of assimilated ^{13}C were calculated according to Philip and Simard [28] and Keel et al. [29]. $\delta^{13}\text{C}$ (‰) and C (%) of labelled samples and unlabelled controls were used to calculate the ^{13}C enrichment as described in Endrulat et al. [22]. The needle $\delta^{13}\text{C}$ values immediately after each last pulse-labelling exposure were used as maximum signal of each tree individual according to Keel et al. [29]. The ^{13}C enrichment (mg) of the needles was set to 100%, and the measured ^{13}C contents in all fine-root samples were expressed in percentage of the maximum needle signals, and referred to as relative ^{13}C content (%). This approach enables the comparison of all tree individuals due to the elimination of the signal strength variation caused by variable CO_2 uptake during the labelling.

For ^{13}C analyses in roots, three fine-root classes were analysed: newly formed fine roots, fine roots of ≤ 0.5 mm in diameter, and fine roots of >0.5 –1 mm in diameter. For ^{13}C labelled starch of fine roots, only the newly formed fine roots and the fine roots of ≤ 0.5 mm in diameter were analysed.

Statistical analyses

All statistical analyses were performed using STATVIEW 5.0 (SAS Institute Inc., Cary, NC, USA). The effects of the clipping treatment and the various harvesting seasons on plants and root properties were analysed by two-way analyses of variance (ANOVA) with using Fisher's protected least significant difference (PLSD). The data were tested for normal distribution and homogeneity of variance. Starch and relative ^{13}C content data were log-transformed in order to meet the requested criteria. One data set was not normally distributed, therefore, the non-parametric Mann-Whitney (for two groups) and Kruskal-Wallis tests (for more than two groups) were applied.

Results

Biomass partitioning

The clipping of the trees caused over the whole investigation period a significant decrease of aboveground biomass (-5.8%) as well as of the needles (-13.8%; Tables 2 and 3). Subsequently, this loss resulted in a significant increase of the percentage of belowground biomass (+11.9%; Table 3), as well as in a significant increase of the proportion of biomass in coarse (+8.6%) and fine roots (+16%) (Tables 2 and 3). The root/shoot ratio of clipped trees remained more or less on a constant level (0.56–0.63), whereas the ratio of control trees at the beginning in spring was low (0.41) and increased constantly until autumn (to 0.60; Table 2).

Table 2. Total biomass (g±SE), root/shoot ratio, and percentage of the various tree components of control (unclipped) and clipped trees for two clipping treatments and four harvesting seasons. Aboveground is the sum of stem, branches, and needles, belowground is the sum of coarse and fine roots. Different letters indicate significant differences among harvesting seasons within one treatment ($P<0.05$). * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ indicate significant differences between 'Control' and 'Clipped' trees.

Treatment	Harvesting season	Trees (n)	Total biomass (g; ±SE)	Root / shoot ratio	Tree components (%)						
					Stem	Branches	Needles	Coarse roots	Fine roots	Above-ground	Below-ground
Control	Spring	5	48.9±7.0 ^c	0.41 ^b	17.3	14.2	39.4	17.2	11.9	70.9	29.1
	Summer	9	70.5±6.4 ^{bc}	0.44 ^b	18.7	14.5	36.5	18.8	11.4	69.8	30.2
	Autumn	13	81.6±6.4 ^b	0.60 ^a	16.4	14.4	31.6	19.5	18.0	62.5	37.5
	Spring+1 yr	6	104.8±10.5 ^a	0.54 ^a	13.4	11.8	40.3	18.2	16.3	65.5	34.5
	Mean		77.8±4.6	0.52	16.6	13.9	35.7	18.8	15.0	66.3	33.7
Clipped	Spring	5	41.2±8.5 ^b	0.59	18.1	12.4	32.8 ^{**}	19.7	16.9	63.4 [*]	36.6 [*]
	Summer	9	59.2±9.3 ^{ab}	0.57 ^{***}	21.4 [*]	14.2	28.4 ^{***}	20.8	15.3 [*]	64.0 ^{**}	36.0 ^{**}
	Autumn	13	72.6±4.8 ^a	0.63	18.4 [*]	15.6	27.7 ^{***}	22.2 [*]	16.2	61.7	38.4
	Spring+1 yr	8	67.6±8.4 ^{a*}	0.56	14.2	10.7	39.2	17.6	18.3	64.1	35.9
	Mean		63.5±4.0	0.59	18.2	13.7	31.2	20.0	16.5	63.1	36.9

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Both, clipping treatment and harvesting season, influenced the tree parameters significantly, with the one exception of the branches after the clipping treatment (Table 3). Interactions of the two factors, clipping and harvesting, occurred for the root/shoot ratio, the above- and belowground biomass, the needles, and the fine roots (Table 3). This fact indicates that the effects of clipping upon these tree parameters depend on the harvesting season.

Separating the fine roots into newly formed roots and into different diameter classes (Fig 1), we found that root biomass differed among various fine-root classes in all harvesting seasons with the exception of summer (Table 4). These significant effects were mainly caused by the small biomass of the newly formed fine roots (Fig 1). The clipping treatment caused a significant reduction of fine-root biomass in autumn and spring+1 yr (Table 4, Fig 1), but not in spring or summer (Table 4).

¹³C in needles and fine-roots

The mean recovery of the ¹³C isotope (ratio of incorporated ¹³C_{needles} to the amount of ¹³C added to one tree) after the pulse-labelling was 71% (±8.2%) for control and 55% (±6.2%) for

Table 3. Results of two-way ANOVA testing the effect of the clipping treatment (C) and the harvesting seasons (H) (spring, summer, autumn, spring+1 yr) on total biomass and root/shoot ratio as well as on the percentage of the various tree components. Significant effects ($P<0.05$) are given in bold.

Tree components	Clipping (C)			Harvesting season (H)			C x H		
	df	F	P	df	F	P	df	F	P
Total biomass	1	8.31	0.006	3	8.02	<0.001	3	1.47	0.231
Root/shoot ratio	1	14.57	<0.001	3	7.24	<0.001	3	2.78	0.049
Stem	1	5.34	0.024	3	15.09	<0.001	3	0.48	0.698
Branches	1	0.75	0.391	3	9.52	<0.001	3	1.45	0.237
Needles	1	47.35	<0.001	3	43.28	<0.001	3	4.76	0.005
Coarse roots	1	4.40	0.040	3	3.54	0.020	3	1.02	0.390
Fine roots	1	5.93	0.018	3	5.24	0.003	3	3.31	0.026
Aboveground	1	17.02	<0.001	3	8.28	<0.001	3	3.15	0.031
Belowground	1	17.02	<0.001	3	8.28	<0.001	3	3.15	0.031

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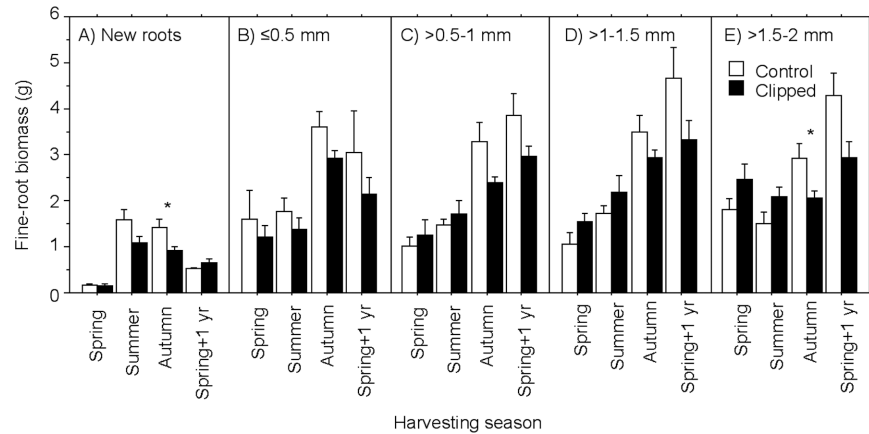


Fig 1. Mean biomass (g, ±SE; n = 5–13, see S1 Table) of fine-root classes of control and clipped trees: (A) new roots, (B) ≤0.5 mm, (C) >0.5–1.0 mm, (D) >1.0–1.5 mm, (E) >1.5–2.0 mm. Significant differences between control and clipped trees are given with * (P<0.05).

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clipped trees. However, mean δ¹³C values in needles directly after the last labelling exposure varied significantly among trees of the same labelling bag (P<0.019 for all bags) and among the three labelling dates (P<0.001). Clipped and unclipped control trees did not differ significantly in their foliar δ¹³C values (P = 0.391), and no significant interaction between treatment and labelling date was found (P = 0.078), indicating similar conditions for all labelling dates and no difference in uptake between the two treatments.

Fine roots of the same diameter class of clipped and control trees had variable values in terms of ¹³C enrichment, expressed as relative ¹³C content (%), and the ¹³C enrichment differed strongly among seasons of labelling (Fig 2). Spring labelled trees had the lowest relative ¹³C contents (<1%) in the fine roots in comparison to summer and autumn labelled fine roots (Fig 2). Highest relative ¹³C contents (3–5%) were observed only in newly formed roots of the summer or the autumn harvests immediately after the ¹³CO₂-labelling (Fig 2). Medium high ¹³C contents (1–3%) were recorded in fine roots of ≤0.5 mm and of >0.5–1.0 mm after the summer and the autumn labellings in all harvesting seasons including spring+1 yr (Fig 2).

Clipping the trees had significantly positive effects on the relative ¹³C content of the roots of the ≤0.5 mm and >0.5–1.0 mm root classes when the trees were labelled in spring (Fig 2), whereas clipping had significantly negative effects on the >0.5–1.0 mm root class when trees were labelled in autumn (Table 5). The harvesting season had a significantly positive effect on new roots and the ≤0.5 mm root class after summer labelling, with much higher relative ¹³C contents after the summer and autumn labelling (Table 5, Fig 2).

Table 4. Results of two-way ANOVA testing the effect of the clipping treatment (C) and the various fine-root classes (R; new roots, size classes ≤0.5 mm, >0.5–1.0 mm, >1.0–1.5 mm, and >1.5–2.0 mm) on fine-root biomass at various harvesting seasons. Significant effects (P<0.05) are given in bold (autumn data were tested with non-parametric tests due to non-normal distribution).

Harvesting season	Clipping (C)			Fine-root classes (R)			C x R		
	df	F	P	df	F	P	df	F	P
Spring	1	1.00	0.324	4	11.41	<0.001	4	0.98	0.429
Summer	1	0.26	0.609	4	1.90	0.1181	4	2.14	0.083
Autumn	1	-	0.007	4	-	<0.001	-	-	-
Spring+1 yr	1	9.77	0.003	4	19.10	<0.001	4	0.94	0.446

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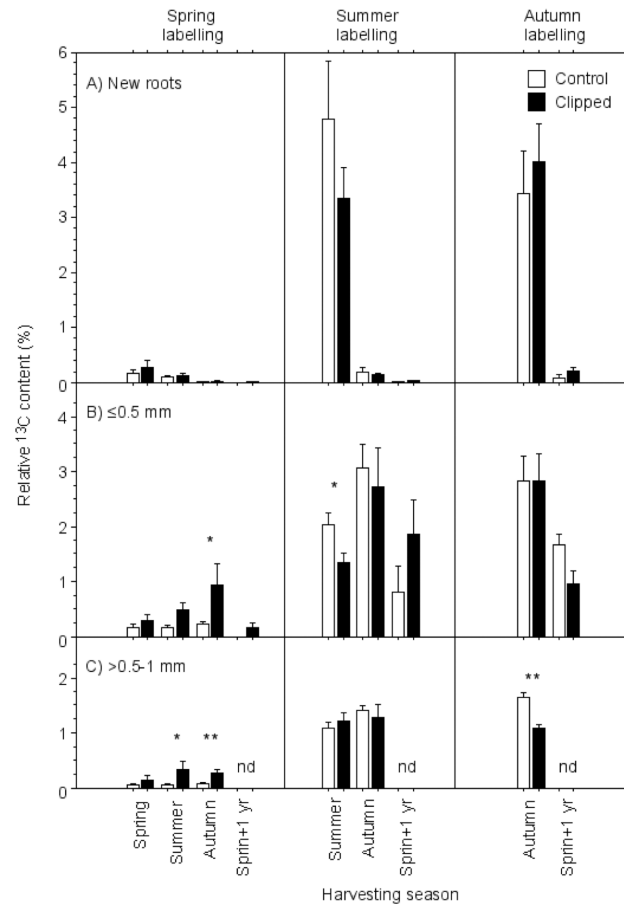


Fig 2. Means of relative ¹³C contents (%; ±SE; n = 4 for spring, summer, and autumn, n = 1–3 for spring+1 yr, see Table 1) recovered from bulk fine-roots of (A) new fine roots, (B) fine roots of class ≤0.5 mm, and (C) fine roots of class >0.5–1.0 mm for different seasons of ¹³CO₂ pulse-labelling (spring, summer, autumn) and for different harvesting seasons. Relative ¹³C contents are expressed in percentage of the maximum needle signals. Significant differences between control and clipped trees are given with * (P<0.05) and ** (P<0.01).

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Table 5. Results of two-way ANOVA testing the effect of the clipping treatment (C) and the harvesting seasons (H) (spring, summer, autumn) on relative ¹³C contents (%) of three fine-root classes. Significant effects (P<0.05) are given in bold. (-: data not available).

Labelling season	Fine-root class	Clipping (C)			Harvesting season (H)			C x H		
		df	F	P	df	F	P	df	F	P
Spring	New roots	1	0.05	0.833	2	5.75	0.012	2	0.30	0.747
	≤0.5 mm	1	9.08	0.008	2	2.91	0.080	2	0.71	0.504
	>0.5–1.0 mm	1	14.16	0.001	2	3.78	0.043	2	0.09	0.914
Summer	New roots	1	1.00	0.337	1	222.34	<0.001	1	0.36	0.559
	≤0.5 mm	1	2.09	0.172	1	8.99	0.010	1	0.74	0.405
	>0.5–1.0 mm	1	0.05	0.836	1	1.00	0.338	1	0.79	0.393
Autumn	New roots	1	0.31	0.598	-	-	-	-	-	-
	≤0.5 mm	1	<0.01	0.994	-	-	-	-	-	-
	>0.5–1.0 mm	1	25.76	0.002	-	-	-	-	-	-

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Table 6. Results of two-way ANOVA of the effect of the clipping treatment (C) and the harvesting seasons (H) (spring, summer, autumn) on the starch concentration and on the relative ¹³C content of the starch of two fine-root classes. Significant effects (*P*<0.05) are given in bold. (-: data not available).

Starch	Labelling season	Fine-root class	Clipping (C)			Harvesting season (H)			C x H		
			df	F	P	df	F	P	df	F	P
Concentration	-	New roots	1	0.67	0.418	2	25.05	<0.001	2	0.93	0.403
		≤0.5 mm	1	<0.01	0.973	2	6.01	0.005	2	0.32	0.731
Relative ¹³ C content	Spring	New roots	1	2.81	0.116	2	11.87	0.001	2	0.48	0.632
		≤0.5 mm	1	7.13	0.016	2	2.39	0.121	2	0.76	0.483
	Summer	New roots	1	2.58	0.134	1	61.16	<0.001	1	0.84	0.377
		≤0.5 mm	1	0.09	0.771	1	0.67	0.434	1	2.00	0.188
	Autumn	New roots	1	3.00	0.134	-	-	-	-	-	-
		≤0.5 mm	1	0.19	0.668	-	-	-	-	-	-

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Starch concentrations and recovered ¹³C label in starch

Highest starch concentrations (20–24 mg g⁻¹) were found in newly grown fine roots in autumn (S2 Table). The clipping treatment did not significantly influence the starch concentrations in the new roots and the ≤0.5 mm fine root class, whereas the harvesting season influenced them in both root classes significantly (Table 6).

Starch in the fine roots of spring ¹³CO₂-labelled trees was only a little labelled with ¹³C, with mean relative ¹³C contents in starch below 0.01% (Fig 3). In contrast, plants labelled in summer allocated more label into the roots than those labelled in spring: mean relative ¹³C contents of new fine roots were up to 0.06% at the summer harvest (Fig 3). Highest starch ¹³C enrichments were found after the autumn labelling, with up to 0.3% in new roots and 0.1% in the ≤0.5 mm root class (Fig 3). Clipping had no significant effect on the relative ¹³C content of fine roots, except for the ≤0.5 mm root class after spring labelling (Table 6). In contrast, harvesting season had significant effects on ¹³C in new roots for the spring labelling as well as for the summer labelling (Table 6).

Discussion

The aim of the present study was to investigate the effects of browsing, simulated through clipping, and of different seasons on the C allocation to the root systems of young *Abies alba* trees. The simulated browsing reduced the aboveground biomass immediately, since a large proportion of the branches have been cut off. The aboveground biomass was reduced mainly due to the clipped branches and due to the proportion of buds removed, and as a consequence, due to the lower number of new shoots produced in the clipped trees. This reduction is in accordance with other studies, e.g. the study of Hester et al. [9], reporting *Pinus sylvestris* trees having lost a similar amount of buds (58%) due to browsing, whereas the amount was lower in deciduous trees (43% in *Betula pendula* and 29% in *Sorbus aucuparia*, respectively). Nevertheless, we found compensatory growth of aboveground parts, e.g. shoots and needles, one year after clipping, as also reported by others [7,9,30]. More than half of the clipped trees built multiple stem forms, similar to results of Häsler et al. [10]. Clipping also affected belowground growth. Evidence of a short-term root growth due to the clipping of shoots was an up to 46% increased biomass in the fine-roots of the classes >0.5–1.0 mm, >1.0–1.5 mm, and >1.5–2.0 mm at spring and summer harvests. Babst et al. [31] as well found increased export of newly assimilated ¹¹C (*t*_{1/2}: 20.4 min) to roots in *Populus tremuloides* following jasmonic acid application (simulating a mechanical damage to leaves after feeding by insects). However, after a longer

time period, as in our study after half a year or longer, simulated browsing usually results in a significantly reduced fine-root biomass of treated trees compared to control trees. Such below-ground reductions were found as well in other tree species, such as *Quercus* spp., *Betula pubescens*, *Fagus sylvatica*, and *Abies alba*, after herbivore damage or defoliation [15,20,21,32,33]. Only a few studies, however, observed no belowground changes [18,34].

Starch concentrations in the fine roots of our study, in contrast to the biomass, did not show any changes due to the clipping treatment. Other studies made the same observation, that a 50% defoliation does not affect the amount of root starch [15,17,18,20,21,35]. Only a severe defoliation (100%), however, is able to diminish root starch significantly as shown by Piper et al. [18] with *Nothofagus* spp. or Jacquet et al. [17] with *Pinus pinaster*. Kosola et al. [35] additionally found that starch accumulation after defoliation occurred mainly in August, but not during the other measured seasons (early summer, late autumn). Overall, the above mentioned reports on root biomass and root starch showed that the severity of defoliation and the environmental circumstances (e.g. season of measurement) matter strongly.

Pulse-labelling with ^{13}C offers a straightforward method to track recently fixed photoassimilates from the shoots to the roots (e.g. [22,27,36,37]). Concerning our three hypotheses (1–3) postulated earlier, our data supported (1) and (2), but (3) only partly:

1. According to our first hypothesis, the seasons of the ^{13}C -pulse-labelling and, thus, phenology had a strong effect on the allocation of the ^{13}C -label from the shoot to the roots. Shoots labelled in spring did not supply the fine-roots with high amounts of the ^{13}C -label, because the fine-roots had a relatively low ^{13}C -content (<1%). Labelling in summer and autumn, however, revealed that relatively high amounts of the ^{13}C -label (1–5%) were allocated to the fine roots. Thus, it can be assumed that photoassimilates from spring mainly remain in the shoots to support shoot growth, whereas photoassimilates from summer and autumn are allocated in larger portions to the fine roots (compare also [38]). Our findings correspond with reports by Ericsson et al. [39] and by Hansen et al. [40], using a ^{14}C -label, who observed that spring assimilates were used mainly for aboveground growth, whereas autumn assimilates were allocated mainly to belowground parts as storage. Keel et al. [41] found similarly that ^{13}C -labels from June were transported to the roots to a lesser amount compared to August. Moreover, Kuptz et al. [42] stated upon their labelling results that 'during spring, only negligible amounts of new photosynthates enter the transfer pool', but 'during early summer, new photosynthates enter the transfer pool, directly supplying growth and being further transported to roots and soils, and during late summer, new photosynthates enter both the transfer and storage pools, supplying both growth and maintenance' [42]. Our isotopic data fully support these findings of Kuptz et al. [42]. Subsequently, highest ^{13}C -enrichments in the starch in the fine roots were recorded in our study in autumn after autumn labelling.
2. As proposed in our second hypothesis, the incorporation of the ^{13}C -label into the roots was strongly dependent on the root type and the root diameter. Labelling in summer and autumn showed that relatively high amounts of the ^{13}C -label (1–5%) were allocated to the fine roots, with 3–5% in newly formed fine roots, 1–3% in the ≤ 0.5 mm root class, and 1–1.5% in the > 0.5 –1.0 mm root class. Comparably, the absolute highest ^{13}C -values in the starch were measured in the newly formed fine roots after the autumn labelling. Keel et al. [41], recording ^{13}C -labels in starch likewise, observed highest values in the finest root class.
3. In accordance to our third hypothesis, clipping strongly influenced the amount of allocated ^{13}C -label as well as the site of incorporation and the form of the incorporated ^{13}C -label. Clipping had a significantly positive effects on the ^{13}C -allocation to fine roots after spring labelling, with significantly higher relative ^{13}C -contents observed in the ≤ 0.5 mm and the

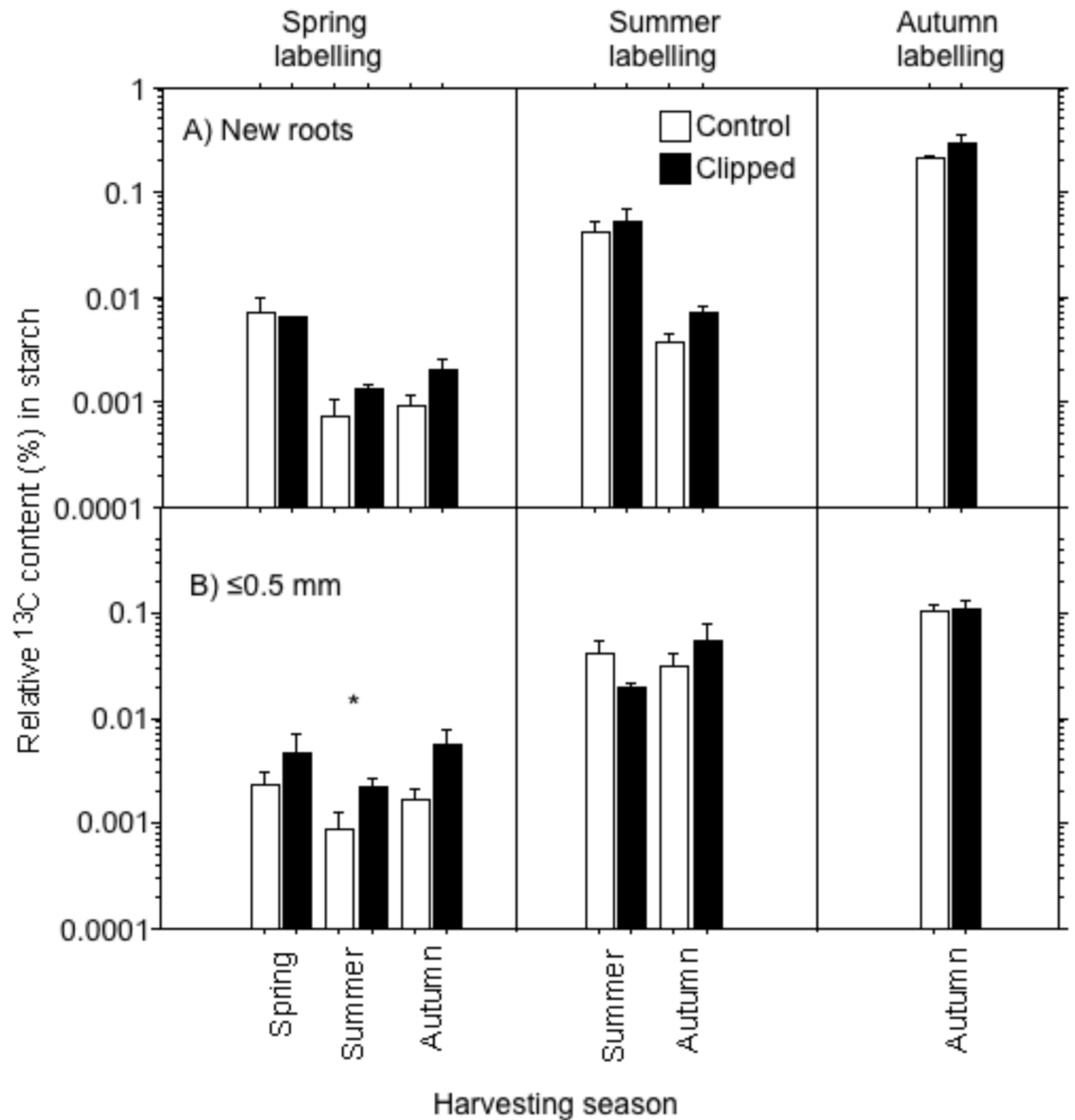


Fig 3. Means of relative ¹³C content (% ± SE, n = 4) recovered from starch of (A) new fine roots and (B) of fine roots of class ≤0.5 mm for different seasons of ¹³CO₂ pulse-labelling (spring, summer, autumn) and for different harvesting seasons (spring, summer, autumn) in controls and in clipped trees (logarithmic display). Relative ¹³C contents are expressed in percentage of the maximum needle signals. Significant differences between control and clipped trees are given with * (P<0.05).

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>0.5–1.0 mm fine-root classes of clipped trees than those of control trees. Similarly, significantly higher relative ¹³C-contents of starch were observed in the ≤0.5 mm fine-root class of clipped trees after spring labelling. After summer and autumn labelling, however, mainly no effects in the ¹³C-allocation to the fine roots were observed, assuming that clipping had not a strong effect on the ¹³C-allocation pattern.

Our study showed that clipping resulted in a reduction of the belowground biomass but not in an increase of the starch concentration in the roots. Thus, we conclude that no trade-off between growth and storage occurred. A trade-off would occur only if a C limitation would be

present [43–45]. A sustained severe defoliation would lead to such a C limitation, with increased storage at the expense of growth enhancing the survival chance [45]. In general, tree growth can be limited by the availability of C within the plant (C limitation) or by the tree's ability to use the available C it has because of e.g. nutrient shortage or environmental conditions (sink limitation) [44]. A growing body of literature suggests that current tree growth is sink limited under most conditions, with much of the evidence deduced from trees' high amounts of nonstructural carbon (NSC) [44]. These conclusions rely on the assumption that C storage is passive and that NSC does not accumulate when growth is C limited. However, storage may be an active process, occurring at the expense of growth [44].

The processes behind the source-to-sink transport within plants are complex. Plants respond to herbivore feeding by increasing the production of the phytohormone jasmonic acid [46]. A systemic jasmonic acid induction of defense responses also occurs from roots to shoots and *vice versa*, thereby affecting the performance of herbivores. However, jasmonic acid also causes the re-allocation of primary metabolites between roots and shoots, and it controls several developmental processes such as root growth or senescence [46]. The molecular processes behind this source-to-sink transport most likely are phloem sucrose transport and invertases in sink organs, as the latter increase sucrose unloading by converting sucrose to hexoses [47,48].

In conclusion, our data imply that season of C assimilation and, thus, the seasonal phenology of trees is a main driver of C allocation from shoots to roots, and, as seen in our experiment, clearly more decisive than simulated browsing. However, within the fine-root system, the C allocation pattern varied greatly. Fine roots which are highly physiologically active, e.g. newly grown fine roots, received the highest amounts of newly assimilated C, but only after summer and autumn labelling. This indicates that newly formed fine roots are highly active only in the second half of the vegetation period in exploring the soil and storing C.

Supporting Information

S1 Table. Number of harvested trees. Number of harvested trees with the varying origins of new leader shoots for two clipping treatments and for four harvesting seasons. Treatments are 'Control' = unclipped trees, and 'Clipped' = clipped trees.
(DOCX)

S2 Table. Means of starch concentrations. Means of starch concentrations (mg g^{-1} dry weight, $\pm\text{SE}$) in new fine roots and in fine roots of class ≤ 0.5 mm for two clipping treatments and for three harvesting seasons. Treatments are 'Control' = unclipped trees, and 'Clipped' = clipped trees.
(DOCX)

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Author Contributions

Conceived and designed the experiments: TE NB IB. Performed the experiments: TE IB. Analyzed the data: TE IB. Contributed reagents/materials/analysis tools: TE IB. Wrote the paper: TE NB IB.

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