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Tree Physiology

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Journal:	*Tree Physiology*
Manuscript ID	TP-2019-416.R2
Manuscript Type:	Research Paper
Date Submitted by the Author:	n/a
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Keywords:	Carbon Allocation, Herbivory, Storage, Non-Structural Carbohydrates, carbon isotope labelling

No preferential C-allocation to storage over growth in clipped birch and oak saplings

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Abstract

Herbivory is one of the most globally distributed disturbances affecting C-cycling in trees, yet our understanding of how it alters tree C-allocation to different functions like storage, growth or rhizodeposition is still limited. Prioritized C-allocation to storage replenishment vs. growth could explain the fast recovery of C-storage pools frequently observed in growth-reduced defoliated trees. We performed continuous ^{13}C-labelling coupled to ~~a~~ clipping ~~experiment~~ to quantify the effects of simulated browsing on the growth, leaf morphology and relative allocation of stored *vs*. recently assimilated C to the growth (bulk biomass) and non-structural carbohydrate (NSC) stores (soluble sugars and starch) of the different organs of two tree species ~~with contrasting wood anatomy~~: diffuse-porous (*Betula pubescens*) and ring-porous (*Quercus petraea*). C-t~~T~~ransfers ~~of C~~ from plants to bulk and rhizosphere soil were also evaluated. Clipped birch and oak trees shifted their C-allocation patterns above-ground as a means to recover from defoliation. However, such increased allocation to current-year stems and leaves did not entail reductions in the allocation to the rhizosphere, which remained unchanged between clipped and control trees of both species. *B. pubescens* and *Q. petraea* showed differences in their vulnerability and recovery strategies to clipping, the ring-porous species being less affected in terms of growth and architecture by clipping than the diffuse-porous. These contrasting patterns could be partly explained by differences in their C cycling after clipping. Defoliated oaks showed a faster recovery of their canopy biomass, which was supported by increased allocation of ~~recently fixed~~new C, but associated with large decreases in their fine root biomass. Following clipping, both species recovered NSC pools to a larger extent than growth, but the allocation of ^{13}C-labelled photo-assimilates into storage compounds was not increased as compared to controls. Despite their different response to clipping, our results indicate no preventative allocation into storage occurred during the first year after clipping in either of the species.

Keywords: *Quercus petraea, Betula pubescens*, Carbon (C) allocation, $\delta^{13}C$ stable isotopes, non-structural carbohydrates, C-storage, below-ground allocation.

Introduction

Trees account for ca. 90% of the global biomass of carbon (C) (Körner 2003), and hence play a fundamental role in global C dynamics. C-allocation patterns in trees may shift depending on multiple factors, including age (Hartmann et al. 2018), phenology (~~Klein et al. 2016;~~ Palacio et al. 2018), environmental conditions like water and light availability or temperature (Domisch et al. 2001; Messier and Nikinmaa 2000; Weber et al. 2019) and disturbances (Canham et al. 1994; ~~Raitio et al. 1994;~~ Van der Heyden and Stock 1995). C-allocation within the tree biomass may determine tree vulnerability to environmental stress and disturbance (Canham et al. 1999; ~~McDowell 2011; Myers and Kitajima 2007;~~ Wiley and Helliker 2012). Further, C-allocation to different functions like storage, growth, reproduction or rhizodeposition may affect the amount of C cycled and sequestered by trees (Hartmann et al. 2018). Understanding the response of tree C-allocation patterns to different factors may be crucial to predict the response of trees to global change (Körner 2003; Wiley and Helliker 2012).

Herbivory is one of the most globally distributed disturbances affecting C-cycling patterns in trees (Clark et al. 2010). Defoliation by herbivores reduces canopy leaf area causing a decrease in the net C gain of trees by current photosynthesis and altering the balance between C sinks and sources (Trumble et al. 1993). This may lead to important changes in C-allocation patterns, which can influence the environment by changes in below-ground C inputs (Eyles et al. 2009; Pinkard and Beadle 1998). Depending on the severity of damage, C demands of growing sinks may be supplied temporarily from storage (Pinkard et al. 1998; Quentin et al. 2011; Van der Heyden and Stock 1995), namely non-structural carbohydrates (NSC) and lipids, some of which can be mobilised to support growth or other plant functions (Chapin et al. 1990). Accordingly, several studies have reported a decrease in starch pools after defoliation in deciduous (Canham et al. 1994; Kosola et al. 2001; Van der Heyden and Stock 1995) and evergreen tree species (Ericsson et al. 1985; Fierravanti et al. 2019~~; Webb and Karchesy 1977~~). However, trees are able to compensate to some degree for loss of foliage by changing allocation patterns (e.g. favouring foliage production), upregulating photosynthesis and changing leaf morphology (Fuenzalida et al. 2019; ~~Hoogesteger and Karlsson 1992; Pinkard et al. 2007;~~ Pinkard and Beadle 1998). Recovery from light defoliation is considered to rely mainly on current photo-assimilates produced by surviving foliage (Barry et al. 2011) and does not normally cause a significant decrease in NSC pools (~~Piper and Fajardo 2014;~~ Tschaplinski and Blake 1994; Van der Heyden and Stock 1995). In the case of moderate or severe defoliation, decreases in NSC concentrations tend to be transient and of short duration, becoming non-significant over the course of a growing season, while the effects on tree growth seem to be more long-lasting (Palacio et al. 2008; Piper et al. 2015; Puri et al. 2015). Sometimes defoliated trees show even higher NSC concentrations than undefoliated controls (Palacio et al. 2012; Piper et al. 2015~~; Ramirez et al. 2018~~).

The differential dynamics of growth vs. recovery of NSC pools in defoliated trees have been interpreted in relation to two, non-exclusive processes: 1) a C-sink limitation to growth due to reductions in the numbers of buds, limiting levels of non-C reserves, hormonal changes or allometric adjustments in response to reduced leaf area, leading to surplus-C being allocated to storage (Palacio et al. 2012; Palacio et al. 2008; Piper et al. 2015; Puri et al. 2015; Schmid et al. 2017); 2) a preventative prioritized C-allocation to storage over growth, ultimately leading to C-limitation (Piper et al. 2015; Puri et al. 2015; Wiley et al. 2017a; Wiley and Helliker 2012; Wiley et al. 2013). Preferential allocation of C to NSC over growth has recently been demonstrated in C-starved plants subjected to prolonged shading (Weber et al. 2019) or complete darkness

(Weber et al. 2018). Nevertheless, its occurrence in other potentially C-limiting conditions such as defoliation remains equivocal (Wiley et al. 2017a; ~~Wiley et al. ~~2013).

In addition to changes in C-allocation among tree organs, defoliation can induce shifts in C-transfers below-ground, influencing soil microbial communities (Bardgett and Wardle 2003~~; Pestaña and Santolamazza-Carbone 2011, ~~but see Barto and Rillig 2010) and nutrient cycling (Ayres et al. 2004). Approximately 50% of the C produced by woody plants is allocated below-ground, either directly to the roots, or as rhizodeposition of C exudates from roots to the surrounding soil (Giardina et al. 2005). Defoliation can reduce below-ground C-allocation by enhancing fine root mortality, particularly in trees (Bryant et al. 1993; Tuomi et al. 1990; Vanderklein and Reich 1999, but see Endrulat et al. 2016; Kosola et al. 2001). In contrast, in herbaceous plants herbivory can increase short-term allocation of C below-ground (Orians et al. 2011), as has also been found for *Populus* spp. (Babst et al. 2005). Defoliation has been demonstrated to elicit short-term increases in the flux of C to root exudates in grasses (Paterson et al. 2005), while the detection of effects in woody species remains elusive (Ayres et al. 2004; Frost and Hunter 2008). In general, there is a lack of information related to below-ground responses of woody plants to defoliation.

Differences in wood anatomy have also been assumed to entail differences in C-allocation dynamics (Barbaroux and Bréda 2002), with putative consequences on the response of trees to defoliation (Foster 2017). Ring-porous species complete part of earlywood growth (including large earlywood vessel formation) before bud burst in spring (Dougherty et al. 1979). This phenology is putatively a result of winter embolism of large-diameter vessels, and the need to produce a new set of xylem vessels prior to bud burst to supply newly emerging leaves with water (Lechowicz 1984). Contrastingly, diffuse-porous species have only small xylem vessels and winter embolism has relatively less impact on the hydraulic conductivity of the tree, so leaf expansion can proceed using xylem formed in the previous growing season~~establishedxylem~~, without the need to produce new radial stem growth (~~Essiamah and Eschrich 1985;~~ Lechowicz 1984~~-~~). The consequence for C-cycling is that ring-porous species show greater seasonal variations in NSC pools and concentrations, and a relatively greater dependence on the remobilization of stored NSC for earlywood growth in spring, than diffuse-porous species (Barbaroux and Bréda 2002; Barbaroux et al. 2003, but see Palacio et al. 2011). It has recently been suggested that these differences in C storage and allocation underline potential differences between ring-porous and diffuse-porous species in the vulnerability to spring defoliation, the former being more resistant to defoliation owing to their larger C-stores and advanced wood growth phenology (Foster 2017). Nevertheless, to our knowledge this possibility has never been explored experimentally.

We performed continuous ^{13}C-labelling coupled to a clipping experiment to quantify the effects of simulated browsing on the relative allocation of stored and recently assimilated C to growth (bulk biomass) and NSC (soluble sugars and starch) of the different organs of two tree species with contrasting wood anatomy: diffuse-porous downy birch (*Betula pubescens* Ehrh.) and ring-porous sessile oak (*Quercus petraea* [Matt.] Liebl.). Transfers from the plants to bulk (i.e. not in direct contact with tree roots) and rhizosphere soil were also evaluated. Clipping was selected ~~as a defoliating treatment~~ to mimic the effects of browsing: a major factor hampering the regeneration of native forests worldwide (~~Côté et al. 2004;~~ Gill 2006; Hester et al. 2004). The use of continuous ^{13}C-labelling at close-to-ambient concentrations was chosen as a quantitative mean to separate current from stored C-assimilates, estimate C-allocation to different organs and C-compounds over the course of the growing season and track allocation below-ground, without the potential drawbacks of pulse-chase labelling (see Paterson et al. 2009). We hypothesized that~~:~~ ~~(i)~~ clipping would lead to: (i) increased C-allocation above-ground *vs.* below-ground (i.e. roots and the rhizosphere); (ii) ~~clipped trees would show~~ increased allocation of new C into storage ~~relative to control trees~~; and (iii) ~~detrimental effects of clipping on~~reduced ~~tree~~ growth and C storage, ~~would be~~ more noticeable in birch than in oak, owing to the ring-porous wood anatomy and subsequent larger storage C-pools of the latter (Foster 2017).

Materials and methods

Experimental set up

The experimental set up was the same as described in Palacio et al. (2011). In brief, in 2007 we applied two clipping treatments: control (unclipped) and clipped (i.e. 66% shoots removal in two consecutive dates: July and September 2007) to two-year-old sessile oak (*Quercus petraea* [Matt.] Liebl.) and downy birch (*Betula pubescens* Ehrh.) saplings planted in pots (Fig. 1). In April 2008, before bud burst, five trees of each species and treatment were harvested to account for differences in biomass and NSC allocation in the short-term. At that same time, five extra trees of each species and treatment were moved into a polytunnel with altered $\delta^{13}C$ air composition to take part in a continuous $\delta^{13}C$-labelling experiment, while five control trees of each species were left at the greenhouse to serve as “ambient” trees. The aim of the continuous labelling was to separate newly fixed C from “old” C. In August 2008, trees from the C-labelling experiment were harvested to evaluate differences in new C allocation to bulk biomass and NSC (SS and starch) between clipped and control trees of both species one year after clipping (Fig. 1). “Ambient” trees were harvested in November 2008 to provide natural abundance $\delta^{13}C$ values of the different organs of both species to be used in calculations. Further details of these experimental procedures follow.

Clipping experiment

Trees of both study species were lifted from a nursery while dormant (5 April 2007) and planted in 44 l pots filled with gravel at the bottom for drainage and freely-drained soil derived from granite and granitic gneiss (Countesswells Association, Glentworth and Muir 1963)~~.~~. At planting, saplings were approximately 0.3-0.5 m high, a stage considered highly vulnerable to large herbivore browsing in nature (Gill 2006; Hester et al. 2000; Hester et al. 1996). After planting, saplings were moved into an unheated greenhouse and 20 trees of each species were randomly allocated into “clipped” and “control” treatments, leading to 10 replicates per species and treatment combination. Five extra trees per species were allocated to the “ambient” group, which did not receive clipping or ^{13}C-labelling. Trees were numbered and positioned in the greenhouse following a Latin square design. Between April and November 2007, soil was kept moist with tap water without exceeding field capacity and saplings received 0.5 l of a nutrient solution with 3.0 mol N m^{-3} as NH_4NO_3, 1.33 mol m^{-3} $Na_2HPO_4 \cdot 12 H_2O$ and 1 mol m^{-3} K_2SO_4 once per week, to remove any potential nutrient limitation to growth. A natural photoperiod was used and the greenhouse ventilated to provide temperatures close to ambient. To account for initial tree variability and avoid potential confounding effects on tree growth, morphological measurements (tree height, length, stem diameter, and number of short shoots and long shoots) were taken from every tree at planting and prior to each clipping and harvest.

Clipping was applied as in Palacio et al. (2011) by removing 66% of current-year shoots (2 out of every 3 current-year shoots) in early July and early September 2007, after the first and second flushes of shoot growth were finished (Fig. 1). This intensity of damage was selected to reproduce high densities of browsing animals

(Speed et al. 2011). In birch, clipping treatments were designed to reproduce browsing damage by red deer or sheep by removing current-year long-shoots (including stems, buds and leaves) up to the maximum stem diameters normally eaten by red deer or sheep (Shipley et al. 1999). In oaks, clipped shoots were selected to ensure a decrease in total tree leaf area of approx. 66% owing to the highly variable shoot length and fewer shoots of this species. While the use of clipping to simulate browsing has received some criticism (Baldwin 1990), woody plant responses to well-simulated damage do not differ significantly from responses to real herbivore damage (Bergman 2002; Hester et al. 2004).

Short-term effects of clipping on biomass and carbohydrate allocation

Twenty dormant saplings (five of each species and clipping treatment) were removed from their pots on 2 April 2008 and separated into: one and two-year-old stems (i.e. stems formed in 2007 and 2006, respectively), woody stems (>2 years), coarse roots (> 2 mm diameter) and fine roots (< 2mm diameter). Samples were freeze-dried and weighed (*ca.* 0.005 mg) and then milled to a fine powder in a ball mill (Retsch Mixer MM301, Leeds, UK).

Soluble sugars (SS) were extracted with 80% (v/v) ethanol and their concentration determined using the phenol-sulphuric method as modified by Buysse and Merckx (1993). Starch and complex sugars remaining in the undissolved pellet after ethanol extractions were reduced (i.e. enzymatically) to glucose and analyzed as described in Palacio et al. (2007).

Continuous ^{13}C labelling experiment

On 1 April 2008, five saplings from each species and clipping treatment combination were transferred to an aluminium and polythene tunnel, 2.4 m wide, 3.0 m long and 2.2 high (Super 8 Hobby Tunnel, Northernpolytunnels, Colne, UK) as described previously (Palacio et al., 2011; Fig. 1). At this time, trees were only just starting to break bud. The polytunnel was supplied with air having CO_2 with a depleted ^{13}C-signature (relative to atmospheric CO_2), in order to differentiate current (new) from previous (old) plant assimilates (Nogués et al. 2004; Paterson et al. 2009). This was achieved by partially scrubbing CO_2 from the air using a CO_2-scrubber unit (Texol, Dundee, UK) that reduced the CO_2 concentration to 74-103 µmol mol^{-1}. The scrubbed air was then mixed with CO_2 from a gas cylinder (BOC, Worsley, UK) with a $\delta^{13}C$-signature of -34.0 ‰, using Brooks 580s thermal mass flow controllers, interfaced with a Brooks control unit (both Flotech Solutions Ltd, Stockport, UK). Resulting CO_2 concentrations inside the polytunnel averaged 332 ppm and had an average $\delta^{13}C$ of -21.4‰. Temperature inside the polytunnel was checked regularly with a shielded thermometer. On the 6th of May 2008 we installed a shade mesh intercepting ~30% of the light on top of the polytunnel to reduce warming.

Trees were distributed within the polytunnel following a Latin square design, which was changed in the middle of the experiment. They regularly received 0.5 l of the same nutrient solution described above. We took initial and final tree morphological measurements (as described above) at the beginning and at the end of the ^{13}C-labelling period. On 5th August 2008, four months after the beginning of the ^{13}C-labelling, trees inside the polytunnel were harvested for analysis (Fig. 1). At this time, leaf senescence was starting and most of the annual growth had been completed. Harvested trees were separated into: current-year (formed in 2008), one and two-year-old stems (formed in 2007 and 2006, respectively), woody stems (>2 years), coarse roots (> 2mm diameter) and fine roots (< 2mm diameter). A three-year-old branch was clipped off each tree to measure leaf area, individual leaf weight and specific leaf area according to the protocols in Cornelissen et al. (2003). Rhizosphere and bulk soil were harvested from each pot. Rhizosphere soil was collected by separating roots from the soil, gently shaking them and then submerging fine roots in distilled water. Samples were freeze-dried and weighed (ca. 0.005 mg). Samples were milled to a fine powder in a ball mill (Retsch Mixer MM301, Leeds, UK) and analysed for NSC as described above.

Ambient trees

In early November 2008, the five non-clipped, ”ambient”, saplings of each species were harvested from the greenhouse for analysis of the $\delta^{13}C$ at natural abundance in the bulk biomass of the same fractions considered for ^{13}C-labelled trees. Throughout 2008, growth conditions for these trees were similar to those in 2007 (see above). At the time of harvest, trees were shedding their leaves and radial growth had been completed.

C isotope analysis

The ^{13}C signature of samples was measured by continuous flow isotope ratio mass spectrometer (Thermo Finnigan Delta Plus$^{\text{advantage}}$) interfaced to an elemental analyser (Thermo FlashEA1112, Thermo Finnigan, Bremen, Germany). Data were expressed as $\delta^{13}C_{\text{V-PDB}}$:

$$\delta^{13}C\ (‰) = (R_S / R_{VPDB} - 1) \times 1000$$

where R_S and R_{VPDB} are the molecular abundance ratios of carbon isotopes ($^{13}C/^{12}C$), of the sample and international standard (Vienna Pee Dee Belemnite), respectively. Long-term precision for quality control standards (milled flour) was δ^{13}CV-PDB: -26.0 ± 0.24‰ (mean ± SD, n=187). $\delta^{13}C$ was measured in bulk plant biomass, bulk soil and rhizosphere soil. Measurements of $\delta^{13}C$ in SS and starch followed compound-specific analyses as detailed below.

$\delta^{13}C$ in NSC were measured following the procedure in Tcherkez et al. (2003). In brief, leaf powder was suspended with 1 mL of distilled water in an Eppendorf tube (Eppendorf Scientific, Hamburg, Germany). After centrifugation, starch was extracted from the pellet by HCl solubilization. Soluble proteins of the supernatant were heat denatured and precipitated and SS and organic acids of the protein-free extract were separated by HPLC. After lyophilization, 200 mg of purified starch were weighted into tin capsules (Courtage Analyze Service, Mont Saint-Aignan, France) for isotope analysis. Determinations of $\delta^{13}C$ in NSC were conducted at the *Centres Científics i Tecnològics* (CCiT) of the University of Barcelona using an elemental analyser (EA1108, Series 1, Carbo Erba Instrumentazione, Milan, Italy) coupled to an isotope ratio mass spectrometer (Delta C, Finnigan, Mat., Bremen, Germany) operating in continuous flow mode. Data were expressed as indicated above.

Calculations

The proportion of newly assimilated C in the bulk biomass, SS and starch of the different fractions of trees grown in ^{13}C-depleted conditions (F_{Cnew}) was calculated using the following equation (Nogués et al. 2004):

$$F_{Cnew} = \frac{\delta^{13}C_{Sample} - \delta^{13}C_{Ambient}}{\delta^{13}C_{Grass} - \delta^{13}C_{Ambient}}$$

Where $\delta^{13}C_{Sample}$ was the isotopic composition in the sample; $\delta^{13}C_{Ambient}$ was the natural baseline $\delta^{13}C$ value for the bulk biomass, SS or starch of a given fraction of each species calculated from fractions collected from ambient trees in November 2008 (in the case of the bulk biomass) or, in the case of SS and starch, by applying an enrichment of 1.71 and 2.02, respectively, owing to values reported in the literature (Badeck et al. 2005);

and $\delta^{13}C_{Grass}$ was the average of the $\delta^{13}C$ values obtained for *Lolium perenne* plants that were grown from seed within the polytunnel and hence represented C arising from current assimilation. We assumed that the discrimination against ^{13}C during photosynthesis would be the same in both the grasses and the trees, because the grasses were grown under the same conditions as the trees and had no water stress.

Statistical analyses

We used univariate general linear models (glm) to analyse for differences in biomass and NSC-allocation between species, fractions and treatments. Short-term effects of simulated browsing on biomass and NSC-allocation of trees harvested in April 2008 (before the ^{13}C-labelling experiment) and the effects of simulated browsing on tree growth, architecture, leaf morphology, biomass, NSC and % new C-allocation (in bulk biomass, SS and starch) and NSC concentrations in the year after clipping (trees harvested in August 2008) were evaluated by glms with species, fractions and treatments as fixed effects. Treatment effects were further tested within fractions and species using one-way ANOVAs. The initial length (i.e. distance from the base to the tip of the tree) of trees at the beginning of the experiment was included as a covariate in all analyses. Starch and NSC concentrations were angularly transformed to meet normality and homogeneity in variance assumptions. Analyses were done with SPSS Statistics 17.0.

Results

Short-term effects of clipping on biomass and NSC allocation

At the beginning of the labelling experiment (i.e. seven months after the application of treatments), trees subjected to two successive clipping events were significantly smaller than control trees and showed a significant reduction in the biomass of the youngest shoot cohort, i.e. that formed in 2007 and directly affected by clipping, but larger main stem biomass (particularly in birch, Table 1, Table S1 available as Supplementary Data at Tree Physiology Online). Trees were still dormant and leafless at this time, and no other significant differences were observed in the allocation to different tree fractions in either of the two species. Differences in allocation to different plant components were quantified between birch and oak trees: birch allocated significantly more biomass to above-ground fractions like young and main stems, while oak had significantly more biomass in coarse roots (Table 1, Table S1). However, no significant treatment x species interaction was found, indicating that clipping did not lead to a different response in biomass allocation between species in the short-term.

Clipped trees of both species harvested in April 2008, seven months after the application of treatments, showed significantly higher starch concentrations in young stems (Tables S1 and S2, available as Supplementary Data at Tree Physiology Online). However, NSC pools were decreased in the youngest shoot cohort, showing the significant effect of clipping on the biomass reduction of this cohort for both species (Tables S1 and S3, available as Supplementary Data at Tree Physiology Online). Starch and SS pools in main stems were significantly larger in clipped trees of both species (Tables S1 and S3, available as Supplementary Data at Tree Physiology Online). This was not due to increased NSC concentrations (Tables S1 and S2, available as Supplementary Data at Tree Physiology Online), but to higher allocation of biomass to main stems in clipped trees (Tables 1 and S1 available as Supplementary Data at Tree Physiology Online). Overall, birch trees had higher SS concentrations, while oaks showed up to three times higher starch concentrations, particularly in coarse roots (Tables S1 and S2 available as Supplementary Data at Tree Physiology Online).

Changes in growth, architecture, leaf morphology, biomass and NSC allocation one year after clipping

Measurements taken at the end of the first year after the application of clipping treatments (trees harvested in August 2008), indicated a lower compensating ability in birch than in oak (Table 2). Although the effect of clipping on tree height was not significant in the general model (F = 2.5, P = 0.134), significant treatment effects arose in birch when both species were analysed separately, with clipped trees being significantly shorter than controls (Table 2). Clipped *B. pubescens* trees also had fewer branches, terminal shoots and short shoots than controls, indicating that significant effects on the architecture of clipped trees remained measureable even one year after clipping. On the contrary, regrowth of *Q. petraea* saplings completely compensated for height and branching differences, with clipped trees showing only a marginally significant smaller number of terminal shoots than control trees after one year (Table 2). As regards the morphology of leaves, we observed no significant differences in individual leaf area and weight or the SLA of clipped and control trees of both species (Table 2).

There were no significant differences in biomass allocation to leaves and coarse roots of both species in trees harvested one year after clipping, but clipped trees of both birch and oak allocated significantly more biomass to current-year stems than control trees (Fig. 2). In oak, clipped trees showed more biomass allocation to the main stems and a sharp reduction in biomass allocation to fine roots, which were reduced by 45% in relation to control trees (Fig. 2). Such reduction in fine root biomass led to a significant decrease in the root:shoot ratio of clipped oaks, not observed in birch (Table 2). At the end of the first growing season after clipping, the significant reduction of shoots formed in 2007 observed at the beginning of the C-labelling experiment in both species (Table 1) was only noticeable in birch, and differences between treatments in oak were no longer significant (Fig. 2). There were no significant differences in total plant biomass between treatments, either in the general model ($F = 3.1$, $P = 0.098$) or when species were analysed separately ($F = 2.3$, $P = 0.169$ and $F = 1.3$, $P = 0.280$ in birch and oak, respectively).

Differences between control and clipped trees in NSC pools of different plant fractions mimicked results for biomass allocation. Higher SS (and also starch in the case of oak) pools were found in current-year stems of clipped trees of both species (Fig. 3). Contrastingly, NSC pools were lower in one-year old stems of clipped birch trees (Fig. 3). Clipped oak trees showed also a trend for higher net allocation of SS to main stems, while both starch and SS pools were significantly reduced in their fine roots (Fig. 3).

The differences observed in NSC pool allocation were not driven by changes in NSC concentrations, which remained similar for both SS and starch across treatments and fractions, except for SS concentrations in the leaves of clipped birch trees, which were lower than those of control trees (Table 3). In any case, the lower SS concentrations of the leaves of clipped birch trees did not result in significantly different SS pools between control and clipped birch trees (Fig. 3, Table 3).

Changes in the net allocation of newly fixed C to different plant fractions and the soil during the recovery of clipped birch and oak trees

The two species showed very different responses in their $\delta^{13}C$ isotopic composition after labelling and clipping (Table S4 available as Supplementary Data at Tree Physiology Online). In general, birch allocated significantly more new C to biomass than oak, pointing to a lower reliance on C-stores and a higher C-fixing ability of birch than oak (Fig. 4, Table 4). Across species and treatments, the fractions receiving proportionally more newly fixed C were rapidly growing ones, including, in descending order, leaves, current-year stems, young (one and two-year-old) stems and fine roots (Fig. 4). Despite being winter deciduous, the leaves of both species were mainly built on newly fixed C, which accounted for 80% of the bulk leaf biomass in birch and over 65% in oak (Fig. 4).

Clipped trees allocated significantly more newly-fixed C to the different fractions than controls (Table 4, Fig. 4), but results were very different depending on the species (Fig. 4, Table S5 available as Supplementary Data at Tree Physiology Online). This explains why all interaction terms in the general model were significant (Table 4). In general, clipped trees tended to allocate more new C into fast growing fractions like young stems and fine roots than control trees (Fig. 4, Table S5 available as Supplementary Data at Tree Physiology Online), but when both species were analysed separately, effects were only significant in oak (Fig. 4). In the year after clipping, clipped *Q. petraea* trees allocated significantly more new C than controls to all fractions but the main stems (Fig. 4). Contrastingly, birch trees showed no differences in the allocation of new C to different fractions between control and clipped trees, except for the main stems, where control trees received a larger proportion of new C (Fig. 4).

The rhizosphere soil collected underneath both study species showed more ^{13}C-depleted values as compared to the bulk soil ($F = 118.6$, $P < 0.001$; Fig. 5), indicating that plant roots significantly altered the δ^{13}C signature of the soil in direct contact with them. δ^{13}C values of the rhizosphere soil collected underneath birch trees were more depleted than those of oak, potentially indicating a larger amount of labelled-C transferred to the rhizosphere in this species ($F = 6.5$, $P = 0.016$; Fig. 5). However, no significant clipping effects were observed in the δ^{13}C signature of bulk and rhizosphere soil in both species (Fig. 5). This indicates the loss of newly-fixed C from roots was similar in clipped and control treatments of both species.

Differences in the allocation of new C to NSC between clipped and control trees

Birch allocated more new C to SS than oak in all fractions and also significantly more new C to starch, particularly in fast turnover fractions like leaves, young stems and fine-roots, while both species showed similar new C-allocation to starch in coarse roots and main stems (Table 4, Fig. 6, Table S6 available as Supplementary Data at Tree Physiology Online). In general, clipping had no significant effect on the allocation of newly fixed C to storage (both SS and starch) during the next season after clipping (Table 4). However, in the case of the allocation to starch the response varied depending on the species and the fraction and, consequently, the interaction terms between species and treatment and the full interaction term were significant (Table 4). Simplified models run separately per species and fraction indicated that, in birch, clipped trees allocated significantly less C to starch in leaves and main stems (the latter marginally significant only) and marginally higher C to SS in current-year stems than control ones (Fig. 6). Contrastingly, clipped oaks showed a trend for higher allocation of newly fixed C to starch in current-year and main stems (Fig. 6), but results were only marginally significant.

Discussion

In accordance with our first hypothesis, our results indicate that clipped birch and oak trees shifted their C-allocation patterns above-ground as a means to recover from defoliation. However, contrary to our expectations, such increased allocation to current-year stems and leaves did not entail reductions in the allocation to the rhizosphere. As initially expected, *B. pubescens* and *Q. petraea* showed differences in their vulnerability and recovery strategies to clipping, the ring-porous species being less vulnerable than the diffuse porous. These contrasting patterns could be explained by differences in their C cycling after clipping. Defoliated oaks showed a more efficient recovery of their canopy, which was supported by a larger allocation of new C into biomass, particularly aboveground. However, this was associated with large decreases in the fine root biomass of clipped oak trees. Although in both species clipped trees recovered NSC pools faster than growth, the allocation of ^{13}C-labelled photo-assimilates into starch and SS was not increased as compared to controls. This indicates that, contrary to our second hypothesis, no preferential allocation into NSC occurred during the first year after clipping.

Trees recovered from defoliation by increasing C allocation aboveground but maintaining allocation to the rhizosphere

Clipping led to a rapid reduction in tree growth (Palacio et al. 2011) and also in biomass and NSC pools in current-year stems (the fraction directly affected by clipping treatments). Over the course of the first year after clipping, trees managed to recover initial differences in main stem diameter, total plant biomass and, in the case of oaks, also tree height (Table 2). This was mainly a result of shifting allocation above-ground, with increased allocation to current-year stems (almost double to that of control trees in oak) and producing similar leaf biomass to controls (Fig. 2). In oak, these results were consistent for both biomass allocation as a whole and when the proportion of new C allocated to bulk biomass was considered (Fig. 4). The ability of trees to recover from defoliation by increased above-ground allocation is a well-known phenomenon (Eyles et al. 2009; Quentin et al. 2011). Such increased allocation may be achieved by a combination of shifts in architecture, leaf morphology and C-allocation patterns (Eyles et al. 2009).

The removal of apical buds by browsing, clipping or defoliating insects frequently leads to a decrease in apical dominance (due to changes in auxin fluxes, Teichmann and Muhr 2015) with subsequent increases in lateral branch growth (Haukioja et al. 1990; Wilson 1993). Clipped trees in our study showed a lower number of terminal shoots, but increased branching was not detected in either of the two study species. Clipped oaks showed similar lateral branch numbers to control trees, while clipped birch trees had less lateral branches than controls. However, birch trees showed a dramatic decrease in the number of short shoots produced after clipping (Table 2, Palacio et al. 2011), which may be a direct consequence of the decreased apical dominance after clipping (Haukioja et al. 1990). In addition, increases in the proportion of leaves per bud (Millard et al. 2001) or in the foliage to wood ratio (Mizumachi et al. 2004) have been reported as mechanisms to increase above-ground allocation in clipped trees. This was not the case in the trees included in this experiment, which showed similar leaf biomass to control trees but increased current-year stem biomass. It seems, therefore, that clipped trees in our experiment maximized the recovery of the canopy, increasing investment into new stems while keeping a similar allocation to foliage. In clipped birch trees, the decrease in short shoot number (likely in favour of long shoot development), may be a mechanism to recover canopy spread and renewal of bud numbers since, in this species, renewal buds are mostly borne in the long shoots (MacDonald et al. 1984).

Several previous studies have detected shifts in leaf morphology after defoliation to compensate losses in the C-assimilating capacity of the canopy, frequently leading to increased individual leaf area and weight and increased SLA (Fuenzalida et al 2019;

Millard et al. 2001; Piper and Fajardo 2015; Quentin et al. 2011; Trumble et al. 1993). We did not detect any significant differences in leaf morphology between clipped and control trees of either study species. Discrepancies with previous studies may be related to differences in the type of disturbance applied and in the duration of experiments. For example, Fuenzalida et al. (2019), Piper and Fajardo (2015) and Trumble et al. (1993) evaluated responses after defoliating insects or treatments simulating defoliation by arthropods, which may elicit a very different response by trees than clipping (Haukioja et al. 1990). Further, Millard et al. (2001) and Quentin et al. (2011) applied clipping in spring and measured the effects on leaf morphology at the end of the same growing season, while in our study effects on leaf morphology were recorded at the end of the next growing season after clipping, i.e. a much longer duration. Similarly, Eyles et al. (2009) carried out a shorter duration experiment and did not detect any significant effects on individual leaf area of *Eucalyptus globulus* five months after 40% defoliation.

Our results show that the increased above-ground allocation of clipped trees was largely supported by currently-fixed (new) C, particularly in oak (Fig. 4). Increases in photosynthetic rate have been repeatedly reported in defoliated trees (e.g. ~~Pinkard et al. 2007;~~ Pinkard et al. 1998; Vanderklein and Reich 1999). Although we did not measure photosynthetic rates in our study, clipped oak trees showed higher new-C-allocation to bulk biomass, compatible with increased C-fixing ability and with decreased respiratory losses (see below). Both in control and clipped trees of both species, new C was preferentially allocated to actively growing fractions, like leaves, young stems and fine roots.

Although clipping induced increased C-allocation above-ground, our results showed no significant effects of clipping on net deposition to soil in either of the two species analyzed. Frost and Hunter (2008) obtained similar results in red oak (Frost and

Hunter 2008) and suggested that rhizodeposition might be a tightly controlled process buffered against damage-induced shifts in C-allocation. Both species had a significant effect on the $\delta^{13}C$ isotopic composition of the soil, indicated by the depletion in ^{13}C detected in rhizosphere vs. bulk soils. Such an effect was larger in birch than oak, likely in relation to the larger C-fixing ability of the former. Consequently, trees were able to impose changes in the C dynamics of soils, but such effects were not modified by clipping.

B. pubescens *trees were more severely affected than oaks by clipping*.

In agreement with the predictions by Foster (2017), the ring-porous species, *Q. petraea*, was more efficient in recovering the biomass lost by clipping than the diffuse-porous, *B. pubescens*. Clipped birch trees showed lower height and altered architecture (reduced number of branches, terminal and lateral shoots) as compared to controls. Also, while biomass losses in stems formed in 2007 (the cohort directly affected by clipping treatments) were no longer significant at the end of the 2008 growing season in oaks, they were still noticeable in birch. Foster (2017) hypothesized that ring-porous species would be more resistant to spring defoliation than diffuse-porous ones owing to their earlier wood phenology and increased C-stores (Barbaroux and Bréda 2002; Barbaroux et al. 2003; Dougherty et al. 1979). We did not measure wood phenology in this study, but it seems likely that this might have had an effect on the differential responses of both species. In our experiment, clipping consisted of shoot removal in early July and early September 2007, after the first and second flushes of shoot growth were finished. If the differences in wood growth phenology between ring-porous and diffuse-porous adult trees can be applied to saplings, oaks would be expected to have started wood growth at least two weeks prior to the first defoliation event, while birch trees would be just starting (Foster 2017). Foster (2017) further hypothesized that such differences in damage due to wood growth phenology would entail a differential decrease in NSC stores, which would be more severely decreased in diffuse-porous trees and further exacerbated by their lower C-storage capacity. Our results do not confirm this prediction, since both species were equally able to recover NSC pools to the same level as controls on the same year of clipping (except for the shoot cohort directly affected by treatments). Similar fast recovery of NSC stores in defoliated trees has been previously reported (Palacio et al. 2008; Piper et al. 2015; Puri et al. 2015).

Instead, our results show that the differential recovery ability of birch and oak trees after clipping could be, at least partly, explained by the different effects of clipping on their C-cycling, including differences in C-allocation. In accordance with previous studies, oaks showed a larger reliance on storage than birch trees to support new growth (Barbaroux et al. 2003; but see Palacio et al. 2011). This increased ability to re-mobilise C-stores could have been crucial to support the re-growth of clipped oaks, at least initially. Several previous studies have reported a positive relationship between NSC storage and the re-growth ability of defoliated trees (Fierravanti et al. 2019; Kays and Canham 1991; Luostarinen and Kauppi 2005). However, clipped oak trees invested significantly less "old" C (and proportionally more "new" C) in their new growth than control trees (Fig. 4). Consequently, while clipped oaks recovered to a larger extent than clipped birch trees in our experiment, this was not linked to increased total NSC remobilisation as measured at the end of the growing season.

The increased allocation of new C in clipped oaks to support re-growth as compared to controls illustrates a shift in C cycling after damage resulting in significantly more new C being allocated to most fractions in clipped vs. control oaks, an effect not observed in birch. Such a change can be the result of increased C-fixation, but also of decreased losses by respiration or rhizosphere allocation. Differences in C allocation to the rhizosphere were not significant among treatments (see above). However, we cannot rule out the possibility that the increased allocation of new C assimilates in oak was (at least partly) due to reduced respiratory losses, particularly in relation to the drastic reduction in fine root biomass detected in clipped trees of this species. Accordingly, the increased investment into above-ground components in oak was associated with a reduced production of fine roots of 45% during the year after clipping. These changes were not observed in birch, which maintained similar biomass allocation below-ground between control and clipped trees. Increased fine root mortality is a frequently reported process in defoliated trees (Frost and Hunter 2008; Tuomi et al. 1990; Vanderklein and Reich 1999; but see Endrulat et al. 2016; Kosola et al. 2001). Tuomi et al. (1990) suggested that the reductions in fine root biomass after defoliation could vary largely depending on the relative root biomass, the degree of reserve depletion and the compensatory C gain of trees. All these three factors likely differed in the two study species, which could explain their contrasting response. Oaks have relatively high root:shoot ratios (Shaw 1974) as was the case also in this experiment (Table 1). This means an increased non-productive biomass to support during re-growth (Tuomi et al. 1990). The higher storage pool may not have been sufficient to recover above-ground losses and maintain a large root biomass in oak (Tuomi et al. 1990).

Clipping did not result in preferential C-allocation to storage in the long-term

Our study did not detect temporal differences in NSC concentrations in the different organs of clipped and control trees throughout the first year of recovery, but NSC concentrations of clipped trees of both species reached similar levels to those of control trees by the end of the first growing season. This indicates that decreases in NSC stores due to canopy re-growth, if any, were short-lived and fully compensated within less than one year. Similar results have been previously reported in the literature (e.g. Palacio et al. 2012; Puri et al. 2015; Wiley et al. 2013). In our experiment, the replenishment of stores was likely supported by an increased C-fixing ability in clipped trees, particularly in oak, as denoted by their higher new-C-allocation to bulk biomass.

Despite NSC concentrations of clipped trees were rapidly restored to even higher levels than control trees (Tables S2, S3), we did not detect a significant increase in new C-allocation to storage in clipped trees in the first year after clipping. The only significant effect of clipping on new C-allocation was a reduction in allocation to SS in leaves of clipped birches. Wiley et al. (2017b; 2013) suggested that the growth of defoliated trees would be largely limited by C-availability, first by the decrease in leaf area directly related to defoliation, and then by a prioritized allocation to storage over growth to secure tree survival under future potential defoliation events. They argued that the fact that NSC stores were replenished to control levels did not necessarily mean tree growth was not limited by C-availability, since prioritized allocation to storage over growth could still proceed (Wiley and Helliker 2012). Two recent experiments have experimentally demonstrated that NSC concentrations can be maintained to control levels over periods of C-limitation by preferential allocation of C into storage, calling for a cautious use of NSC concentrations to predict the C-status of trees (Weber et al. 2019; 2018). In both experiments, trees subjected to low or no illumination were progressively C-deprived, reaching minimum SS and starch ~~concentrations as low as 2% and 1%, respectively. Below these minimum~~ thresholds~~,~~ below which tree survival was impaired. In both cases, re-illumination resulted in a period of reduced growth and refilling of NSC stores up to a certain threshold. ~~Weber et al. (2019) reported a threshold of 25% and 30% replenishment of NSC concentrations before the growth rate~~

~~increased in *Acer* and *Quercus* saplings, respectively.~~ These results indicate that prioritized allocation to storage over growth does occur in C-starved trees (with very low NSC levels), and that such prioritization is arrested once a certain level of recovery of NSC is achieved.

In contrast to the experiments in Weber et al. (2019; 2018), our trees were not C-starved. We did not find the very significant depletion of NSC reported by these previous studies (Weber et al. 2019; 2018). Consequently, our trees probably did not prioritize allocation to NSC over growth. Starch and SS concentrations of defoliated trees are normally not depleted below the C-starvation thresholds detected by Weber et al. (2019; 2018), even after severe treatments (e.g. Kays and Canham 1991; Palacio et al. 2012; ~~Raitio et al. 1994;~~ Vanderklein and Reich 1999, but see Kosola et al. 2001; Puri et al. 2015). This seems to indicate C-limitation after defoliation in trees is normally short-lived and of low magnitude.

Conclusions

Our results show that clipping triggers a shift in biomass allocation aboveground favouring the recovery of the canopy both in oak and birch trees. However, such a shift does not entail a decrease in C-allocation to the rhizosphere, which seems to be a tightly regulated process. Future research on the mechanisms behind such tight regulation would greatly contribute to our understanding on the effects of defoliation on tree C-cycling and its impact on belowground processes. The observed differences in the recovery strategies of the two study species could have potential implications for their vulnerability under different browsing frequencies. The ring porous species, *Q. petraea*, showed a faster recovery of its canopy after clipping than the diffuse porous, *B. pubescens*. However, this came at the cost of a marked decrease in the fine root biomass of oak, which raises questions on the potential consequences for the nutrition of the tree and its vulnerability to sustained browsing over longer time periods. Finally, despite the different effect of clipping on the C-allocation of study species, none of them increased new C allocation to storage one year after damage. This indicates that clipping does not entail a sustained preventative allocation of C into storage in the long term.

Data and Materials Availability

Authors agree to make experimental data and materials available to third party academic researchers upon reasonable request.

Supplementary Data

The following supporting documents are provided as additional content:

- Table S1. Results of glms on the effect of clipping on the biomass (BM), and soluble sugars (SS) and starch pools and concentrations of the different fractions of *B. pubescens* and *Q. petraea* trees harvested at the beginning of the δ^{13}C-labelling experiment in April 2008.
- Table S2. Soluble sugars (SS) and starch concentrations (mg g^{-1}) in the different plant fractions of *B. pubescens* and *Q. petraea* trees harvested at the beginning of the δ^{13}C-labelling experiment in April 2008.
- Table S3. Soluble sugars (SS) and starch pools (as % of the total SS or starch pool in the plant) in the different plant fractions in *B. pubescens* and *Q. petraea* trees harvested at the beginning of the δ^{13}C-labelling experiment in April 2008.
- Table S4. Isotopic composition (δ^{13}C; ‰) of the different fractions of *B. pubescens* and *Q. petraea* saplings harvested in August 2008.

- Table S5. Results of glms on the effect of “Species” and “Treatment” on the allocation of new C to bulk biomass of the different fractions of *B. pubescens* and *Q. petraea* trees harvested in August 2008.

- Table S6. Results of glms on the effect of “Species” and “Treatment” on the allocation of new C to soluble sugars (SS) and starch in the different fractions of *B. pubescens* and *Q. petraea* trees harvested in August 2008.

Conflict of interest

None declared.

Funding

Ministerio de Ciencia, Innovación y Universidades, Spain (MEC-SEUI postdoctoral fellowship, a Ramón y Cajal fellowship (RYC-2013-14164) and project CGL2015-71360-P to SP). Scottish Government’s Rural and Environmental Research and Analysis Directorate to E.P., A.S., A.H. and P.M.

Acknowledgements

Thanks to Raul Castillo, Richard Gwatkin and Shona Osborne for their help in running the experiment, to Elena Lahoz and Elena Royo for carbohydrate analyses and to two anonymous Reviewers for useful comments on an earlier version of the manuscript.

Authors' Contributions

SP, PM, AH and EP designed the study; SP, EP and PM implemented continuous δ^{13}C-labelling. SP, EP, PM, MM, GL, SN and AAR run carbohydrate and stable isotope analyses. SP, PM and SN analysed data. All authors interpreted results. SP wrote the manuscript receiving revisions from all authors.

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Figure legends

Fig. 1. Experimental design with the indication of the main treatments (clipping – red arrows-, 13C-labelling), growth measurements (blue arrows) and harvests (black arrows) applied to *B. pubescens* and *Q. petraea* saplings.

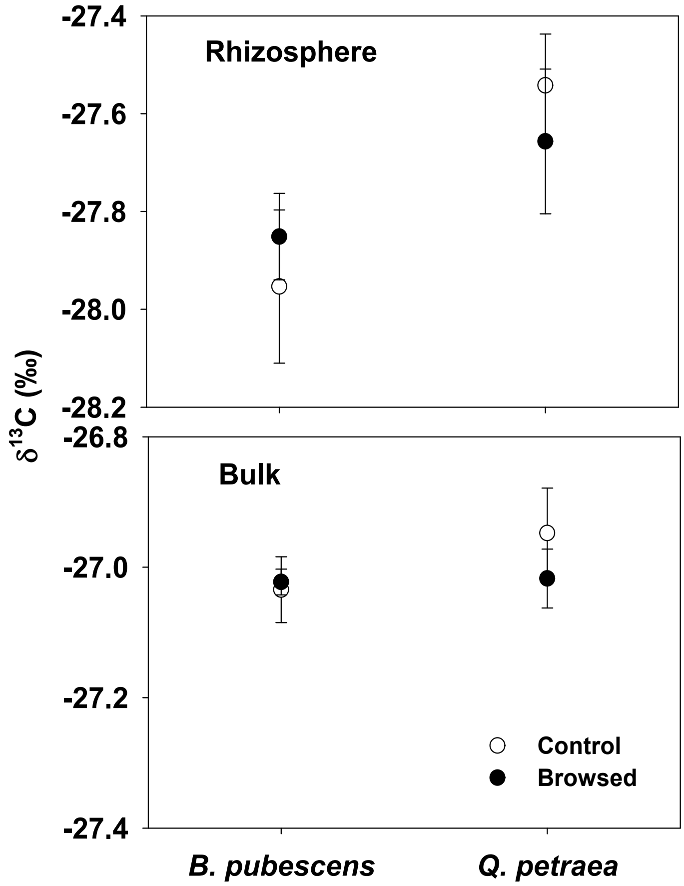
Fig. 2. Biomass allocation to different tree fractions in clipped and control trees of *B. pubescens* and *Q. petraea* harvested in August 2008 (ca. one year after clipping). Average values are shown for each fraction. L = Leaves, 0-St = current year stems, 1-St = one-year-old stems, 2-St = two-year-old stems, MS = main stems, CR = coarse roots, FR = fine roots. Significant differences between treatments within species are indicated by asterisks: * = $P < 0.10$, ** = $P < 0.05$.

Fig. 3. Differences between clipped (grey bars) and control (black bars) *B. pubescens* and *Q. petraea* saplings in the allocation of SS and starch pools to different plant fractions one year after clipping (trees harvested in August 2008). Asterisks denote significant differences between treatments within a given species at $\alpha = 0.10$ (*) and $\alpha = 0.05$ (**).

Fig. 4. Differences between clipped (grey bars) and control (black bars) *B. pubescens* and *Q. petraea* saplings in the allocation of newly fixed C to the different plant organs one year after clipping (trees harvested in August 2008). Asterisks denote significant differences between treatments within a given species at $\alpha = 0.05$.

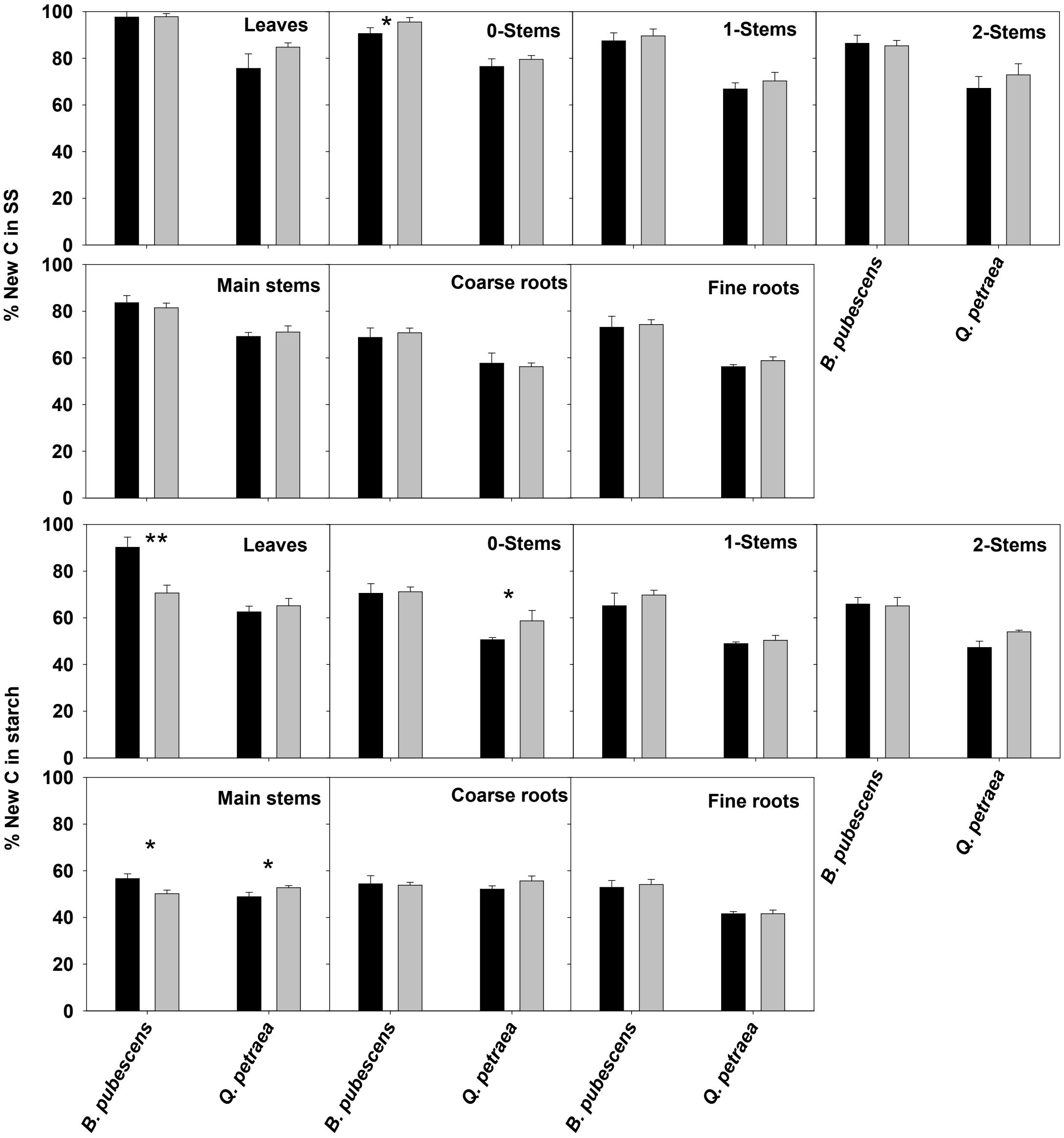
Fig 5. Isotopic composition (δ^{13}C; ‰) of the rhizosphere and bulk soil collected underneath control (white dots) and clipped (black dots) *B. pubescens* and *Q. petraea* trees harvested in August 2008 (ca. one year after clipping). No significant differences between treatments were detected at $\alpha = 0.05$.

Fig 6. Differences between control (black bars) and clipped (grey bars) *B. pubescens* and *Q. petraea* saplings in the allocation of newly fixed C to SS and starch to different plant fractions on the year after clipping (trees harvested in August 2008). Asterisks denote significant differences between treatments within a given species at $\alpha = 0.10$ (*), and $\alpha = 0.05$ (**).


Rhizosphere
Bulk
$\delta^{13}C$ (‰)
-27.4
-27.6
-27.8
-28.0
-28.2
-26.8
-27.0
-27.2
-27.4
○ Control
● Browsed
B. pubescens
Q. petraea

984

985


% New C in SS
% New C in starch
Leaves
0-Stems
1-Stems
2-Stems
Main stems
Coarse roots
Fine roots
0
20
40
60
80
100
*
**
B. pubescens
Q. petraea

986

987

No preferential C-allocation to storage over growth in clipped birch and oak saplings

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Abstract

Herbivory is one of the most globally distributed disturbances affecting C-cycling in trees, yet our understanding of how it alters tree C-allocation to different functions like storage, growth or rhizodeposition is still limited. Prioritized C-allocation to storage replenishment vs. growth could explain the fast recovery of C-storage pools frequently observed in growth-reduced defoliated trees. We performed continuous ^{13}C-labelling coupled to clipping to quantify the effects of simulated browsing on the growth, leaf morphology and relative allocation of stored *vs*. recently assimilated C to the growth (bulk biomass) and non-structural carbohydrate (NSC) stores (soluble sugars and starch) of the different organs of two tree species: diffuse-porous (*Betula pubescens*) and ring-porous (*Quercus petraea*). C-transfers from plants to bulk and rhizosphere soil were also evaluated. Clipped birch and oak trees shifted their C-allocation patterns above-ground as a means to recover from defoliation. However, such increased allocation to current-year stems and leaves did not entail reductions in the allocation to the rhizosphere, which remained unchanged between clipped and control trees of both species. *B. pubescens* and *Q. petraea* showed differences in their vulnerability and recovery strategies to clipping, the ring-porous species being less affected in terms of growth and architecture by clipping than the diffuse-porous. These contrasting patterns could be partly explained by differences in their C cycling after clipping. Defoliated oaks showed a faster recovery of their canopy biomass, which was supported by increased allocation of new C, but associated with large decreases in their fine root biomass. Following clipping, both species recovered NSC pools to a larger extent than growth, but the allocation of ^{13}C-labelled photo-assimilates into storage compounds was not increased as compared to controls. Despite their different response to clipping, our results indicate no preventative allocation into storage occurred during the first year after clipping in either of the species.

Keywords: *Quercus petraea, Betula pubescens*, Carbon (C) allocation, δ^{13}C stable isotopes, non-structural carbohydrates, C-storage, below-ground allocation.

Introduction

Trees account for ca. 90% of the global biomass of carbon (C) (Körner 2003), and hence play a fundamental role in global C dynamics. C-allocation patterns in trees may shift depending on multiple factors, including age (Hartmann et al. 2018), phenology (Palacio et al. 2018), environmental conditions like water and light availability or temperature (Domisch et al. 2001; Messier and Nikinmaa 2000; Weber et al. 2019) and disturbances (Canham et al. 1994; Van der Heyden and Stock 1995). C-allocation within the tree biomass may determine tree vulnerability to environmental stress and disturbance (Canham et al. 1999; Wiley and Helliker 2012). Further, C-allocation to different functions like storage, growth, reproduction or rhizodeposition may affect the amount of C cycled and sequestered by trees (Hartmann et al. 2018). Understanding the response of tree C-allocation patterns to different factors may be crucial to predict the response of trees to global change (Körner 2003; Wiley and Helliker 2012).

Herbivory is one of the most globally distributed disturbances affecting C-cycling patterns in trees (Clark et al. 2010). Defoliation by herbivores reduces canopy leaf area causing a decrease in the net C gain of trees by current photosynthesis and altering the balance between C sinks and sources (Trumble et al. 1993). This may lead to important changes in C-allocation patterns, which can influence the environment by changes in below-ground C inputs (Eyles et al. 2009; Pinkard and Beadle 1998). Depending on the severity of damage, C demands of growing sinks may be supplied temporarily from storage (Pinkard et al. 1998; Quentin et al. 2011; Van der Heyden and Stock 1995), namely non-structural carbohydrates (NSC) and lipids, some of which can be mobilised to support growth or other plant functions (Chapin et al. 1990). Accordingly, several studies have reported a decrease in starch pools after defoliation in deciduous (Canham et al. 1994; Kosola et al. 2001; Van der Heyden and Stock 1995) and evergreen tree species (Ericsson et al. 1985; Fierravanti et al. 2019). However, trees are able to compensate to some degree for loss of foliage by changing allocation patterns (e.g. favouring foliage production), upregulating photosynthesis and changing leaf morphology (Fuenzalida et al. 2019; Pinkard and Beadle 1998). Recovery from light defoliation is considered to rely mainly on current photo-assimilates produced by surviving foliage (Barry et al. 2011) and does not normally cause a significant decrease in NSC pools (Tschaplinski and Blake 1994; Van der Heyden and Stock 1995). In the case of moderate or severe defoliation, decreases in NSC concentrations tend to be transient and of short duration, becoming non-significant over the course of a growing season, while the effects on tree growth seem to be more long-lasting (Palacio et al. 2008; Piper et al. 2015; Puri et al. 2015). Sometimes defoliated trees show even higher NSC concentrations than undefoliated controls (Palacio et al. 2012; Piper et al. 2015).

The differential dynamics of growth vs. recovery of NSC pools in defoliated trees have been interpreted in relation to two, non-exclusive processes: 1) a C-sink limitation to growth due to reductions in the numbers of buds, limiting levels of non-C reserves, hormonal changes or allometric adjustments in response to reduced leaf area, leading to surplus-C being allocated to storage (Palacio et al. 2012; Palacio et al. 2008; Piper et al. 2015; Puri et al. 2015; Schmid et al. 2017); 2) a preventative prioritized C-allocation to storage over growth, ultimately leading to C-limitation (Piper et al. 2015; Puri et al. 2015; Wiley et al. 2017a; Wiley and Helliker 2012; Wiley et al. 2013). Preferential allocation of C to NSC over growth has recently been demonstrated in C-starved plants subjected to prolonged shading (Weber et al. 2019) or complete darkness (Weber et al. 2018). Nevertheless, its occurrence in other potentially C-limiting conditions such as defoliation remains equivocal (Wiley et al. 2017a; 2013).

In addition to changes in C-allocation among tree organs, defoliation can induce shifts in C-transfers below-ground, influencing soil microbial communities (Bardgett and Wardle 2003 but see Barto and Rillig 2010) and nutrient cycling (Ayres et al. 2004). Approximately 50% of the C produced by woody plants is allocated below-ground, either directly to the roots, or as rhizodeposition of C exudates from roots to the surrounding soil (Giardina et al. 2005). Defoliation can reduce below-ground C-allocation by enhancing fine root mortality, particularly in trees (Bryant et al. 1993; Tuomi et al. 1990; Vanderklein and Reich 1999, but see Endrulat et al. 2016; Kosola et al. 2001). In contrast, in herbaceous plants herbivory can increase short-term allocation of C below-ground (Orians et al. 2011), as has also been found for *Populus* spp. (Babst et al. 2005). Defoliation has been demonstrated to elicit short-term increases in the flux of C to root exudates in grasses (Paterson et al. 2005), while the detection of effects in woody species remains elusive (Ayres et al. 2004; Frost and Hunter 2008). In general, there is a lack of information related to below-ground responses of woody plants to defoliation.

Differences in wood anatomy have also been assumed to entail differences in C-allocation dynamics (Barbaroux and Bréda 2002), with putative consequences on the response of trees to defoliation (Foster 2017). Ring-porous species complete part of earlywood growth (including large earlywood vessel formation) before bud burst in spring (Dougherty et al. 1979). This phenology is putatively a result of winter embolism of large-diameter vessels, and the need to produce a new set of xylem vessels prior to bud burst to supply newly emerging leaves with water (Lechowicz 1984). Contrastingly, diffuse-porous species have only small xylem vessels and winter embolism has relatively less impact on the hydraulic conductivity of the tree, so leaf expansion can proceed using xylem formed in the previous growing season, without the need to produce new radial stem growth (Lechowicz 1984). The consequence for C-cycling is that ring-porous species show greater seasonal variations in NSC pools and concentrations, and a relatively greater dependence on the remobilization of stored NSC for earlywood growth in spring, than diffuse-porous species (Barbaroux and Bréda 2002; Barbaroux et al. 2003, but see Palacio et al. 2011). It has recently been suggested that these differences in C storage and allocation underline potential differences between ring-porous and diffuse-porous species in the vulnerability to spring defoliation, the former being more resistant to defoliation owing to their larger C-stores and advanced wood growth phenology (Foster 2017). Nevertheless, to our knowledge this possibility has never been explored experimentally.

We performed continuous ^{13}C-labelling coupled to a clipping experiment to quantify the effects of simulated browsing on the relative allocation of stored and recently assimilated C to growth (bulk biomass) and NSC (soluble sugars and starch) of the different organs of two tree species with contrasting wood anatomy: diffuse-porous downy birch (*Betula pubescens* Ehrh.) and ring-porous sessile oak (*Quercus petraea* [Matt.] Liebl.). Transfers from the plants to bulk (i.e. not in direct contact with tree roots) and rhizosphere soil were also evaluated. Clipping was selected to mimic the effects of browsing: a major factor hampering the regeneration of native forests worldwide (Gill 2006; Hester et al. 2004). The use of continuous ^{13}C-labelling at close-to-ambient concentrations was chosen as a quantitative mean to separate current from stored C-assimilates, estimate C-allocation to different organs and C-compounds over the course of the growing season and track allocation below-ground, without the potential drawbacks of pulse-chase labelling (see Paterson et al. 2009). We hypothesized that clipping would lead to: (i) increased C-allocation above-ground *vs.* below-ground (i.e. roots and the rhizosphere); (ii) increased allocation of new C into storage; and (iii) reduced growth and C storage, more noticeable in birch than in oak, owing to the ring-porous wood anatomy and subsequent larger storage C-pools of the latter (Foster 2017).

Materials and methods

Experimental set up

The experimental set up was the same as described in Palacio et al. (2011). In brief, in 2007 we applied two clipping treatments: control (unclipped) and clipped (i.e. 66% shoots removal in two consecutive dates: July and September 2007) to two-year-old sessile oak (*Quercus petraea* [Matt.] Liebl.) and downy birch (*Betula pubescens* Ehrh.) saplings planted in pots (Fig. 1). In April 2008, before bud burst, five trees of each species and treatment were harvested to account for differences in biomass and NSC allocation in the short-term. At that same time, five extra trees of each species and treatment were moved into a polytunnel with altered $\delta^{13}C$ air composition to take part in a continuous $\delta^{13}C$-labelling experiment, while five control trees of each species were left at the greenhouse to serve as “ambient” trees. The aim of the continuous labelling was to separate newly fixed C from “old” C. In August 2008, trees from the C-labelling experiment were harvested to evaluate differences in new C allocation to bulk biomass and NSC (SS and starch) between clipped and control trees of both species one year after clipping (Fig. 1). “Ambient” trees were harvested in November 2008 to provide natural abundance $\delta^{13}C$ values of the different organs of both species to be used in calculations. Further details of these experimental procedures follow.

Clipping experiment

Trees of both study species were lifted from a nursery while dormant (5 April 2007) and planted in 44 l pots filled with gravel at the bottom for drainage and freely-drained soil derived from granite and granitic gneiss (Countesswells Association, Glentworth and Muir 1963). At planting, saplings were approximately 0.3-0.5 m high, a stage considered highly vulnerable to large herbivore browsing in nature (Gill 2006; Hester et al. 2000; Hester et al. 1996). After planting, saplings were moved into an unheated greenhouse and 20 trees of each species were randomly allocated into "clipped" and "control" treatments, leading to 10 replicates per species and treatment combination. Five extra trees per species were allocated to the "ambient" group, which did not receive clipping or ^{13}C-labelling. Trees were numbered and positioned in the greenhouse following a Latin square design. Between April and November 2007, soil was kept moist with tap water without exceeding field capacity and saplings received 0.5 l of a nutrient solution with 3.0 mol N m^{-3} as NH_4NO_3, 1.33 mol m^{-3} $Na_2HPO_4 \bullet 12 H_2O$ and 1 mol m^{-3} K_2SO_4 once per week, to remove any potential nutrient limitation to growth. A natural photoperiod was used and the greenhouse ventilated to provide temperatures close to ambient. To account for initial tree variability and avoid potential confounding effects on tree growth, morphological measurements (tree height, length, stem diameter, and number of short shoots and long shoots) were taken from every tree at planting and prior to each clipping and harvest.

Clipping was applied as in Palacio et al. (2011) by removing 66% of current-year shoots (2 out of every 3 current-year shoots) in early July and early September 2007, after the first and second flushes of shoot growth were finished (Fig. 1). This intensity of damage was selected to reproduce high densities of browsing animals (Speed et al. 2011). In birch, clipping treatments were designed to reproduce browsing damage by red deer or sheep by removing current-year long-shoots (including stems, buds and leaves) up to the maximum stem diameters normally eaten by red deer or sheep (Shipley et al. 1999). In oaks, clipped shoots were selected to ensure a decrease in total tree leaf area of approx. 66% owing to the highly variable shoot length and fewer shoots of this species. While the use of clipping to simulate browsing has received some criticism (Baldwin 1990), woody plant responses to well-simulated damage do not differ significantly from responses to real herbivore damage (Bergman 2002; Hester et al. 2004).

Short-term effects of clipping on biomass and carbohydrate allocation

Twenty dormant saplings (five of each species and clipping treatment) were removed from their pots on 2 April 2008 and separated into: one and two-year-old stems (i.e. stems formed in 2007 and 2006, respectively), woody stems (>2 years), coarse roots (> 2 mm diameter) and fine roots (< 2mm diameter). Samples were freeze-dried and weighed (*ca.* 0.005 mg) and then milled to a fine powder in a ball mill (Retsch Mixer MM301, Leeds, UK).

Soluble sugars (SS) were extracted with 80% (v/v) ethanol and their concentration determined using the phenol-sulphuric method as modified by Buysse and Merckx (1993). Starch and complex sugars remaining in the undissolved pellet after ethanol extractions were reduced (i.e. enzymatically) to glucose and analyzed as described in Palacio et al. (2007).

Continuous ^{13}C labelling experiment

On 1 April 2008, five saplings from each species and clipping treatment combination were transferred to an aluminium and polythene tunnel, 2.4 m wide, 3.0 m long and 2.2 high (Super 8 Hobby Tunnel, Northernpolytunnels, Colne, UK) as described previously

(Palacio et al., 2011; Fig. 1). At this time, trees were only just starting to break bud. The polytunnel was supplied with air having CO_2 with a depleted ^{13}C-signature (relative to atmospheric CO_2), in order to differentiate current (new) from previous (old) plant assimilates (Nogués et al. 2004; Paterson et al. 2009). This was achieved by partially scrubbing CO_2 from the air using a CO_2-scrubber unit (Texol, Dundee, UK) that reduced the CO_2 concentration to 74-103 µmol mol^{-1}. The scrubbed air was then mixed with CO_2 from a gas cylinder (BOC, Worsley, UK) with a $\delta^{13}C$-signature of -34.0 ‰, using Brooks 580s thermal mass flow controllers, interfaced with a Brooks control unit (both Flotech Solutions Ltd, Stockport, UK). Resulting CO_2 concentrations inside the polytunnel averaged 332 ppm and had an average $\delta^{13}C$ of -21.4‰. Temperature inside the polytunnel was checked regularly with a shielded thermometer. On the 6th of May 2008 we installed a shade mesh intercepting ~30% of the light on top of the polytunnel to reduce warming.

Trees were distributed within the polytunnel following a Latin square design, which was changed in the middle of the experiment. They regularly received 0.5 l of the same nutrient solution described above. We took initial and final tree morphological measurements (as described above) at the beginning and at the end of the ^{13}C-labelling period. On 5th August 2008, four months after the beginning of the ^{13}C-labelling, trees inside the polytunnel were harvested for analysis (Fig. 1). At this time, leaf senescence was starting and most of the annual growth had been completed. Harvested trees were separated into: current-year (formed in 2008), one and two-year-old stems (formed in 2007 and 2006, respectively), woody stems (>2 years), coarse roots (> 2mm diameter) and fine roots (< 2mm diameter). A three-year-old branch was clipped off each tree to measure leaf area, individual leaf weight and specific leaf area according to the protocols in Cornelissen et al. (2003). Rhizosphere and bulk soil were harvested from each pot. Rhizosphere soil was collected by separating roots from the soil, gently shaking them and then submerging fine roots in distilled water. Samples were freeze-dried and weighed (ca. 0.005 mg). Samples were milled to a fine powder in a ball mill (Retsch Mixer MM301, Leeds, UK) and analysed for NSC as described above.

Ambient trees

In early November 2008, the five non-clipped, ”ambient”, saplings of each species were harvested from the greenhouse for analysis of the $\delta^{13}C$ at natural abundance in the bulk biomass of the same fractions considered for ^{13}C-labelled trees. Throughout 2008, growth conditions for these trees were similar to those in 2007 (see above). At the time of harvest, trees were shedding their leaves and radial growth had been completed.

C isotope analysis

The ^{13}C signature of samples was measured by continuous flow isotope ratio mass spectrometer (Thermo Finnigan Delta Plus^{advantage}) interfaced to an elemental analyser (Thermo FlashEA1112, Thermo Finnigan, Bremen, Germany). Data were expressed as $\delta^{13}C_{V\text{-}PDB}$:

$$\delta^{13}C\ (‰) = (R_S / R_{VPDB} - 1) \times 1000$$

where R_S and R_{VPDB} are the molecular abundance ratios of carbon isotopes ($^{13}C/^{12}C$), of the sample and international standard (Vienna Pee Dee Belemnite), respectively. Long-term precision for quality control standards (milled flour) was δ^{13}CV-PDB: -26.0 ± 0.24‰ (mean ± SD, n=187). $\delta^{13}C$ was measured in bulk plant biomass, bulk soil and rhizosphere soil. Measurements of $\delta^{13}C$ in SS and starch followed compound-specific analyses as detailed below.

$\delta^{13}C$ in NSC were measured following the procedure in Tcherkez et al. (2003). In brief, leaf powder was suspended with 1 mL of distilled water in an Eppendorf tube (Eppendorf Scientific, Hamburg, Germany). After centrifugation, starch was extracted from the pellet by HCl solubilization. Soluble proteins of the supernatant were heat denatured and precipitated and SS and organic acids of the protein-free extract were separated by HPLC. After lyophilization, 200 mg of purified starch were weighted into tin capsules (Courtage Analyze Service, Mont Saint-Aignan, France) for isotope analysis. Determinations of $\delta^{13}C$ in NSC were conducted at the *Centres Científics i Tecnològics* (CCiT) of the University of Barcelona using an elemental analyser (EA1108, Series 1, Carbo Erba Instrumentazione, Milan, Italy) coupled to an isotope ratio mass spectrometer (Delta C, Finnigan, Mat., Bremen, Germany) operating in continuous flow mode. Data were expressed as indicated above.

Calculations

The proportion of newly assimilated C in the bulk biomass, SS and starch of the different fractions of trees grown in ^{13}C-depleted conditions (F_{Cnew}) was calculated using the following equation (Nogués et al. 2004):

$$F_{Cnew} = \frac{\delta^{13}C_{Sample} - \delta^{13}C_{Ambient}}{\delta^{13}C_{Grass} - \delta^{13}C_{Ambient}}$$

Where $\delta^{13}C_{Sample}$ was the isotopic composition in the sample; $\delta^{13}C_{Ambient}$ was the natural baseline $\delta^{13}C$ value for the bulk biomass, SS or starch of a given fraction of each species calculated from fractions collected from ambient trees in November 2008 (in the case of the bulk biomass) or, in the case of SS and starch, by applying an enrichment of 1.71 and 2.02, respectively, owing to values reported in the literature (Badeck et al. 2005); and $\delta^{13}C_{Grass}$ was the average of the $\delta^{13}C$ values obtained for *Lolium perenne* plants that were grown from seed within the polytunnel and hence represented C arising from current assimilation. We assumed that the discrimination against ^{13}C during photosynthesis would be the same in both the grasses and the trees, because the grasses were grown under the same conditions as the trees and had no water stress.

Statistical analyses

We used univariate general linear models (glm) to analyse for differences in biomass and NSC-allocation between species, fractions and treatments. Short-term effects of simulated browsing on biomass and NSC-allocation of trees harvested in April 2008 (before the ^{13}C-labelling experiment) and the effects of simulated browsing on tree growth, architecture, leaf morphology, biomass, NSC and % new C-allocation (in bulk biomass, SS and starch) and NSC concentrations in the year after clipping (trees harvested in August 2008) were evaluated by glms with species, fractions and treatments as fixed effects. Treatment effects were further tested within fractions and species using one-way ANOVAs. The initial length (i.e. distance from the base to the tip of the tree) of trees at the beginning of the experiment was included as a covariate in all analyses. Starch and NSC concentrations were angularly transformed to meet normality and homogeneity in variance assumptions. Analyses were done with SPSS Statistics 17.0.

Results

Short-term effects of clipping on biomass and NSC allocation

At the beginning of the labelling experiment (i.e. seven months after the application of treatments), trees subjected to two successive clipping events were significantly smaller than control trees and showed a significant reduction in the biomass of the youngest shoot cohort, i.e. that formed in 2007 and directly affected by clipping, but larger main stem biomass (particularly in birch, Table 1, Table S1 available as Supplementary Data at Tree Physiology Online). Trees were still dormant and leafless at this time, and no other significant differences were observed in the allocation to different tree fractions in either of the two species. Differences in allocation to different plant components were quantified between birch and oak trees: birch allocated significantly more biomass to above-ground fractions like young and main stems, while oak had significantly more biomass in coarse roots (Table 1, Table S1). However, no significant treatment x species interaction was found, indicating that clipping did not lead to a different response in biomass allocation between species in the short-term.

Clipped trees of both species harvested in April 2008, seven months after the application of treatments, showed significantly higher starch concentrations in young stems (Tables S1 and S2, available as Supplementary Data at Tree Physiology Online). However, NSC pools were decreased in the youngest shoot cohort, showing the significant effect of clipping on the biomass reduction of this cohort for both species (Tables S1 and S3, available as Supplementary Data at Tree Physiology Online). Starch and SS pools in main stems were significantly larger in clipped trees of both species (Tables S1 and S3, available as Supplementary Data at Tree Physiology Online). This was not due to increased NSC concentrations (Tables S1 and S2, available as Supplementary Data at Tree Physiology Online), but to higher allocation of biomass to main stems in clipped trees (Tables 1 and S1 available as Supplementary Data at Tree Physiology Online). Overall, birch trees had higher SS concentrations, while oaks showed up to three times higher starch concentrations, particularly in coarse roots (Tables S1 and S2 available as Supplementary Data at Tree Physiology Online).

Changes in growth, architecture, leaf morphology, biomass and NSC allocation one year after clipping

Measurements taken at the end of the first year after the application of clipping treatments (trees harvested in August 2008), indicated a lower compensating ability in birch than in oak (Table 2). Although the effect of clipping on tree height was not significant in the general model (F = 2.5, P = 0.134), significant treatment effects arose in birch when both species were analysed separately, with clipped trees being significantly shorter than controls (Table 2). Clipped *B. pubescens* trees also had fewer branches, terminal shoots and short shoots than controls, indicating that significant effects on the architecture of clipped trees remained measureable even one year after clipping. On the contrary, regrowth of *Q. petraea* saplings completely compensated for height and branching differences, with clipped trees showing only a marginally significant smaller number of terminal shoots than control trees after one year (Table 2). As regards the morphology of leaves, we observed no significant differences in individual leaf area and weight or the SLA of clipped and control trees of both species (Table 2).

There were no significant differences in biomass allocation to leaves and coarse roots of both species in trees harvested one year after clipping, but clipped trees of both birch and oak allocated significantly more biomass to current-year stems than control trees (Fig. 2). In oak, clipped trees showed more biomass allocation to the main stems and a sharp reduction in biomass allocation to fine roots, which were reduced by 45% in relation to control trees (Fig. 2). Such reduction in fine root biomass led to a significant decrease in the root:shoot ratio of clipped oaks, not observed in birch (Table 2). At the end of the first growing season after clipping, the significant reduction of shoots formed in 2007 observed at the beginning of the C-labelling experiment in both species (Table

1) was only noticeable in birch, and differences between treatments in oak were no longer significant (Fig. 2). There were no significant differences in total plant biomass between treatments, either in the general model (F = 3.1, P = 0.098) or when species were analysed separately (F = 2.3, P = 0.169 and F = 1.3, P = 0.280 in birch and oak, respectively).

Differences between control and clipped trees in NSC pools of different plant fractions mimicked results for biomass allocation. Higher SS (and also starch in the case of oak) pools were found in current-year stems of clipped trees of both species (Fig. 3). Contrastingly, NSC pools were lower in one-year old stems of clipped birch trees (Fig. 3). Clipped oak trees showed also a trend for higher net allocation of SS to main stems, while both starch and SS pools were significantly reduced in their fine roots (Fig. 3).

The differences observed in NSC pool allocation were not driven by changes in NSC concentrations, which remained similar for both SS and starch across treatments and fractions, except for SS concentrations in the leaves of clipped birch trees, which were lower than those of control trees (Table 3). In any case, the lower SS concentrations of the leaves of clipped birch trees did not result in significantly different SS pools between control and clipped birch trees (Fig. 3, Table 3).

Changes in the net allocation of newly fixed C to different plant fractions and the soil during the recovery of clipped birch and oak trees

The two species showed very different responses in their δ^{13}C isotopic composition after labelling and clipping (Table S4 available as Supplementary Data at Tree Physiology Online). In general, birch allocated significantly more new C to biomass than oak, pointing to a lower reliance on C-stores and a higher C-fixing ability of birch than oak (Fig. 4, Table 4). Across species and treatments, the fractions receiving proportionally more newly fixed C were rapidly growing ones, including, in descending order, leaves, current-year stems, young (one and two-year-old) stems and fine roots (Fig. 4). Despite being winter deciduous, the leaves of both species were mainly built on newly fixed C, which accounted for 80% of the bulk leaf biomass in birch and over 65% in oak (Fig. 4).

Clipped trees allocated significantly more newly-fixed C to the different fractions than controls (Table 4, Fig. 4), but results were very different depending on the species (Fig. 4, Table S5 available as Supplementary Data at Tree Physiology Online). This explains why all interaction terms in the general model were significant (Table 4). In general, clipped trees tended to allocate more new C into fast growing fractions like young stems and fine roots than control trees (Fig. 4, Table S5 available as Supplementary Data at Tree Physiology Online), but when both species were analysed separately, effects were only significant in oak (Fig. 4). In the year after clipping, clipped *Q. petraea* trees allocated significantly more new C than controls to all fractions but the main stems (Fig. 4). Contrastingly, birch trees showed no differences in the allocation of new C to different fractions between control and clipped trees, except for the main stems, where control trees received a larger proportion of new C (Fig. 4).

The rhizosphere soil collected underneath both study species showed more ^{13}C-depleted values as compared to the bulk soil ($F = 118.6$, $P < 0.001$; Fig. 5), indicating that plant roots significantly altered the δ^{13}C signature of the soil in direct contact with them. δ^{13}C values of the rhizosphere soil collected underneath birch trees were more depleted than those of oak, potentially indicating a larger amount of labelled-C transferred to the rhizosphere in this species ($F = 6.5$, $P = 0.016$; Fig. 5). However, no significant clipping effects were observed in the δ^{13}C signature of bulk and rhizosphere soil in both species (Fig. 5). This indicates the loss of newly-fixed C from roots was similar in clipped and control treatments of both species.

Differences in the allocation of new C to NSC between clipped and control trees

Birch allocated more new C to SS than oak in all fractions and also significantly more new C to starch, particularly in fast turnover fractions like leaves, young stems and fine-roots, while both species showed similar new C-allocation to starch in coarse roots and main stems (Table 4, Fig. 6, Table S6 available as Supplementary Data at Tree Physiology Online). In general, clipping had no significant effect on the allocation of newly fixed C to storage (both SS and starch) during the next season after clipping (Table 4). However, in the case of the allocation to starch the response varied depending on the species and the fraction and, consequently, the interaction terms between species and treatment and the full interaction term were significant (Table 4). Simplified models run separately per species and fraction indicated that, in birch, clipped trees allocated significantly less C to starch in leaves and main stems (the latter marginally significant only) and marginally higher C to SS in current-year stems than control ones (Fig. 6). Contrastingly, clipped oaks showed a trend for higher allocation of newly fixed C to starch in current-year and main stems (Fig. 6), but results were only marginally significant.

Discussion

In accordance with our first hypothesis, our results indicate that clipped birch and oak trees shifted their C-allocation patterns above-ground as a means to recover from defoliation. However, contrary to our expectations, such increased allocation to current-year stems and leaves did not entail reductions in the allocation to the rhizosphere. As initially expected, *B. pubescens* and *Q. petraea* showed differences in their vulnerability and recovery strategies to clipping, the ring-porous species being less vulnerable than the diffuse porous. These contrasting patterns could be explained by differences in their C cycling after clipping. Defoliated oaks showed a more efficient recovery of their canopy, which was supported by a larger allocation of new C into biomass, particularly aboveground. However, this was associated with large decreases in the fine root biomass of clipped oak trees. Although in both species clipped trees recovered NSC pools faster than growth, the allocation of ^{13}C-labelled photo-assimilates into starch and SS was not increased as compared to controls. This indicates that, contrary to our second hypothesis, no preferential allocation into NSC occurred during the first year after clipping.

Trees recovered from defoliation by increasing C allocation aboveground but maintaining allocation to the rhizosphere

Clipping led to a rapid reduction in tree growth (Palacio et al. 2011) and also in biomass and NSC pools in current-year stems (the fraction directly affected by clipping treatments). Over the course of the first year after clipping, trees managed to recover initial differences in main stem diameter, total plant biomass and, in the case of oaks, also tree height (Table 2). This was mainly a result of shifting allocation above-ground, with increased allocation to current-year stems (almost double to that of control trees in oak) and producing similar leaf biomass to controls (Fig. 2). In oak, these results were consistent for both biomass allocation as a whole and when the proportion of new C allocated to bulk biomass was considered (Fig. 4). The ability of trees to recover from defoliation by increased above-ground allocation is a well-known phenomenon (Eyles et al. 2009; Quentin et al. 2011). Such increased allocation may be achieved by a combination of shifts in architecture, leaf morphology and C-allocation patterns (Eyles et al. 2009).

The removal of apical buds by browsing, clipping or defoliating insects frequently leads to a decrease in apical dominance (due to changes in auxin fluxes, Teichmann and Muhr 2015) with subsequent increases in lateral branch growth (Haukioja et al. 1990; Wilson 1993). Clipped trees in our study showed a lower number of terminal shoots, but increased branching was not detected in either of the two study species. Clipped oaks showed similar lateral branch numbers to control trees, while clipped birch trees had less lateral branches than controls. However, birch trees showed a dramatic decrease in the number of short shoots produced after clipping (Table 2, Palacio et al. 2011), which may be a direct consequence of the decreased apical dominance after clipping (Haukioja et al. 1990). In addition, increases in the proportion of leaves per bud (Millard et al. 2001) or in the foliage to wood ratio (Mizumachi et al. 2004) have been reported as mechanisms to increase above-ground allocation in clipped trees. This was not the case in the trees included in this experiment, which showed similar leaf biomass to control trees but increased current-year stem biomass. It seems, therefore, that clipped trees in our experiment maximized the recovery of the canopy, increasing investment into new stems while keeping a similar allocation to foliage. In clipped birch trees, the decrease in short shoot number (likely in favour of long shoot development), may be a mechanism to recover canopy spread and renewal of bud numbers since, in this species, renewal buds are mostly borne in the long shoots (MacDonald et al. 1984).

Several previous studies have detected shifts in leaf morphology after defoliation to compensate losses in the C-assimilating capacity of the canopy, frequently leading to increased individual leaf area and weight and increased SLA (Fuenzalida et al 2019;

Millard et al. 2001; Piper and Fajardo 2015; Quentin et al. 2011; Trumble et al. 1993). We did not detect any significant differences in leaf morphology between clipped and control trees of either study species. Discrepancies with previous studies may be related to differences in the type of disturbance applied and in the duration of experiments. For example, Fuenzalida et al. (2019), Piper and Fajardo (2015) and Trumble et al. (1993) evaluated responses after defoliating insects or treatments simulating defoliation by arthropods, which may elicit a very different response by trees than clipping (Haukioja et al. 1990). Further, Millard et al. (2001) and Quentin et al. (2011) applied clipping in spring and measured the effects on leaf morphology at the end of the same growing season, while in our study effects on leaf morphology were recorded at the end of the next growing season after clipping, i.e. a much longer duration. Similarly, Eyles et al. (2009) carried out a shorter duration experiment and did not detect any significant effects on individual leaf area of *Eucalyptus globulus* five months after 40% defoliation.

Our results show that the increased above-ground allocation of clipped trees was largely supported by currently-fixed (new) C, particularly in oak (Fig. 4). Increases in photosynthetic rate have been repeatedly reported in defoliated trees (e.g. Pinkard et al. 1998; Vanderklein and Reich 1999). Although we did not measure photosynthetic rates in our study, clipped oak trees showed higher new-C-allocation to bulk biomass, compatible with increased C-fixing ability and with decreased respiratory losses (see below). Both in control and clipped trees of both species, new C was preferentially allocated to actively growing fractions, like leaves, young stems and fine roots.

Although clipping induced increased C-allocation above-ground, our results showed no significant effects of clipping on net deposition to soil in either of the two species analyzed. Frost and Hunter (2008) obtained similar results in red oak (Frost and Hunter 2008) and suggested that rhizodeposition might be a tightly controlled process buffered against damage-induced shifts in C-allocation. Both species had a significant effect on the $\delta^{13}C$ isotopic composition of the soil, indicated by the depletion in ^{13}C detected in rhizosphere vs. bulk soils. Such an effect was larger in birch than oak, likely in relation to the larger C-fixing ability of the former. Consequently, trees were able to impose changes in the C dynamics of soils, but such effects were not modified by clipping.

B. pubescens *trees were more severely affected than oaks by clipping.*

In agreement with the predictions by Foster (2017), the ring-porous species, *Q. petraea*, was more efficient in recovering the biomass lost by clipping than the diffuse-porous, *B. pubescens*. Clipped birch trees showed lower height and altered architecture (reduced number of branches, terminal and lateral shoots) as compared to controls. Also, while biomass losses in stems formed in 2007 (the cohort directly affected by clipping treatments) were no longer significant at the end of the 2008 growing season in oaks, they were still noticeable in birch. Foster (2017) hypothesized that ring-porous species would be more resistant to spring defoliation than diffuse-porous ones owing to their earlier wood phenology and increased C-stores (Barbaroux and Bréda 2002; Barbaroux et al. 2003; Dougherty et al. 1979). We did not measure wood phenology in this study, but it seems likely that this might have had an effect on the differential responses of both species. In our experiment, clipping consisted of shoot removal in early July and early September 2007, after the first and second flushes of shoot growth were finished. If the differences in wood growth phenology between ring-porous and diffuse-porous adult trees can be applied to saplings, oaks would be expected to have started wood growth at least two weeks prior to the first defoliation event, while birch trees would be just starting (Foster 2017). Foster (2017) further hypothesized that such differences in damage due to wood growth phenology would entail a differential decrease in NSC stores, which would be more severely decreased in diffuse-porous trees and further exacerbated by their lower C-storage capacity. Our results do not confirm this prediction, since both species were equally able to recover NSC pools to the same level as controls on the same year of clipping (except for the shoot cohort directly affected by treatments). Similar fast recovery of NSC stores in defoliated trees has been previously reported (Palacio et al. 2008; Piper et al. 2015; Puri et al. 2015).

Instead, our results show that the differential recovery ability of birch and oak trees after clipping could be, at least partly, explained by the different effects of clipping on their C-cycling, including differences in C-allocation. In accordance with previous studies, oaks showed a larger reliance on storage than birch trees to support new growth (Barbaroux et al. 2003; but see Palacio et al. 2011). This increased ability to re-mobilise C-stores could have been crucial to support the re-growth of clipped oaks, at least initially. Several previous studies have reported a positive relationship between NSC storage and the re-growth ability of defoliated trees (Fierravanti et al. 2019; Kays and Canham 1991; Luostarinen and Kauppi 2005). However, clipped oak trees invested significantly less “old” C (and proportionally more “new” C) in their new growth than control trees (Fig. 4). Consequently, while clipped oaks recovered to a larger extent than clipped birch trees in our experiment, this was not linked to increased total NSC remobilisation as measured at the end of the growing season.

The increased allocation of new C in clipped oaks to support re-growth as compared to controls illustrates a shift in C cycling after damage resulting in significantly more new C being allocated to most fractions in clipped vs. control oaks, an effect not observed in birch. Such a change can be the result of increased C-fixation, but also of decreased losses by respiration or rhizosphere allocation. Differences in C allocation to the rhizosphere were not significant among treatments (see above). However, we cannot rule out the possibility that the increased allocation of new C assimilates in oak was (at least partly) due to reduced respiratory losses, particularly in relation to the drastic reduction in fine root biomass detected in clipped trees of this species. Accordingly, the increased investment into above-ground components in oak was associated with a reduced production of fine roots of 45% during the year after clipping. These changes were not observed in birch, which maintained similar biomass allocation below-ground between control and clipped trees. Increased fine root mortality is a frequently reported process in defoliated trees (Frost and Hunter 2008; Tuomi et al. 1990; Vanderklein and Reich 1999; but see Endrulat et al. 2016; Kosola et al. 2001). Tuomi et al. (1990) suggested that the reductions in fine root biomass after defoliation could vary largely depending on the relative root biomass, the degree of reserve depletion and the compensatory C gain of trees. All these three factors likely differed in the two study species, which could explain their contrasting response. Oaks have relatively high root:shoot ratios (Shaw 1974) as was the case also in this experiment (Table 1). This means an increased non-productive biomass to support during re-growth (Tuomi et al. 1990). The higher storage pool may not have been sufficient to recover above-ground losses and maintain a large root biomass in oak (Tuomi et al. 1990).

Clipping did not result in preferential C-allocation to storage in the long-term

Our study did not detect temporal differences in NSC concentrations in the different organs of clipped and control trees throughout the first year of recovery, but NSC concentrations of clipped trees of both species reached similar levels to those of control trees by the end of the first growing season. This indicates that decreases in NSC stores due to canopy re-growth, if any, were short-lived and fully compensated within less than one year. Similar results have been previously reported in the literature (e.g. Palacio et al. 2012; Puri et al. 2015; Wiley et al. 2013). In our experiment, the replenishment of stores was likely supported by an increased C-fixing ability in clipped trees, particularly in oak, as denoted by their higher new-C-allocation to bulk biomass.

Despite NSC concentrations of clipped trees were rapidly restored to even higher levels than control trees (Tables S2, S3), we did not detect a significant increase in new C-allocation to storage in clipped trees in the first year after clipping. The only significant effect of clipping on new C-allocation was a reduction in allocation to SS in leaves of clipped birches. Wiley et al. (2017b; 2013) suggested that the growth of defoliated trees would be largely limited by C-availability, first by the decrease in leaf area directly related to defoliation, and then by a prioritized allocation to storage over growth to secure tree survival under future potential defoliation events. They argued that the fact that NSC stores were replenished to control levels did not necessarily mean tree growth was not limited by C-availability, since prioritized allocation to storage over growth could still proceed (Wiley and Helliker 2012). Two recent experiments have experimentally demonstrated that NSC concentrations can be maintained to control levels over periods of C-limitation by preferential allocation of C into storage, calling for a cautious use of NSC concentrations to predict the C-status of trees (Weber et al. 2019; 2018). In both experiments, trees subjected to low or no illumination were progressively C-deprived, reaching minimum SS and starch thresholds below which tree survival was impaired. In both cases, re-illumination resulted in a period of reduced growth and refilling of NSC stores up to a certain threshold. These results indicate that prioritized allocation to storage over growth does occur in C-starved trees (with very low NSC levels), and that such prioritization is arrested once a certain level of recovery of NSC is achieved.

In contrast to the experiments in Weber et al. (2019; 2018), our trees were not C-starved. We did not find the very significant depletion of NSC reported by these previous studies (Weber et al. 2019; 2018). Consequently, our trees probably did not prioritize allocation to NSC over growth. Starch and SS concentrations of defoliated trees are normally not depleted below the C-starvation thresholds detected by Weber et al. (2019; 2018), even after severe treatments (e.g. Kays and Canham 1991; Palacio et al. 2012; Vanderklein and Reich 1999, but see Kosola et al. 2001; Puri et al. 2015). This seems to indicate C-limitation after defoliation in trees is normally short-lived and of low magnitude.

Conclusions

Our results show that clipping triggers a shift in biomass allocation aboveground favouring the recovery of the canopy both in oak and birch trees. However, such a shift does not entail a decrease in C-allocation to the rhizosphere, which seems to be a tightly regulated process. Future research on the mechanisms behind such tight regulation would greatly contribute to our understanding on the effects of defoliation on tree C-cycling and its impact on belowground processes. The observed differences in the recovery strategies of the two study species could have potential implications for their vulnerability under different browsing frequencies. The ring porous species, *Q. petraea*, showed a faster recovery of its canopy after clipping than the diffuse porous, *B. pubescens*. However, this came at the cost of a marked decrease in the fine root biomass of oak, which raises questions on the potential consequences for the nutrition of the tree and its vulnerability to sustained browsing over longer time periods. Finally, despite the different effect of clipping on the C-allocation of study species, none of them increased new C allocation to storage one year after damage. This indicates that clipping does not entail a sustained preventative allocation of C into storage in the long term.

Data and Materials Availability

Authors agree to make experimental data and materials available to third party academic researchers upon reasonable request.

Supplementary Data

The following supporting documents are provided as additional content:

- Table S1. Results of glms on the effect of clipping on the biomass (BM), and soluble sugars (SS) and starch pools and concentrations of the different fractions of *B. pubescens* and *Q. petraea* trees harvested at the beginning of the δ^{13}C-labelling experiment in April 2008.
- Table S2. Soluble sugars (SS) and starch concentrations (mg g^{-1}) in the different plant fractions of *B. pubescens* and *Q. petraea* trees harvested at the beginning of the δ^{13}C-labelling experiment in April 2008.
- Table S3. Soluble sugars (SS) and starch pools (as % of the total SS or starch pool in the plant) in the different plant fractions in *B. pubescens* and *Q. petraea* trees harvested at the beginning of the δ^{13}C-labelling experiment in April 2008.
- Table S4. Isotopic composition (δ^{13}C; ‰) of the different fractions of *B. pubescens* and *Q. petraea* saplings harvested in August 2008.
- Table S5. Results of glms on the effect of “Species” and “Treatment” on the allocation of new C to bulk biomass of the different fractions of *B. pubescens* and *Q. petraea* trees harvested in August 2008.

- Table S6. Results of glms on the effect of “Species” and “Treatment” on the allocation of new C to soluble sugars (SS) and starch in the different fractions of *B. pubescens* and *Q. petraea* trees harvested in August 2008.

Conflict of interest

None declared.

Funding

Ministerio de Ciencia, Innovación y Universidades, Spain (MEC-SEUI postdoctoral fellowship, a Ramón y Cajal fellowship (RYC-2013-14164) and project CGL2015-71360-P to SP). Scottish Government’s Rural and Environmental Research and Analysis Directorate to E.P., A.S., A.H. and P.M.

Acknowledgements

Thanks to Raul Castillo, Richard Gwatkin and Shona Osborne for their help in running the experiment, to Elena Lahoz and Elena Royo for carbohydrate analyses and to two anonymous Reviewers for useful comments on an earlier version of the manuscript.

Authors' Contributions

SP, PM, AH and EP designed the study; SP, EP and PM implemented continuous δ^{13}C-labelling. SP, EP, PM, MM, GL, SN and AAR run carbohydrate and stable isotope analyses. SP, PM and SN analysed data. All authors interpreted results. SP wrote the manuscript receiving revisions from all authors.

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Figure legends

Fig. 1. Experimental design with the indication of the main treatments (clipping – red arrows-, 13C-labelling), growth measurements (blue arrows) and harvests (black arrows) applied to *B. pubescens* and *Q. petraea* saplings.

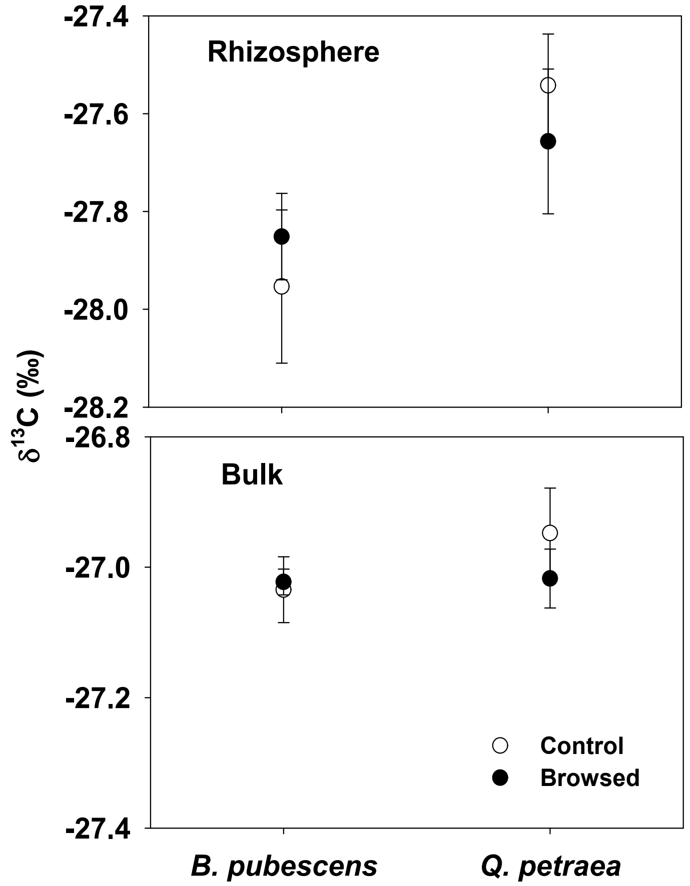
Fig. 2. Biomass allocation to different tree fractions in clipped and control trees of *B. pubescens* and *Q. petraea* harvested in August 2008 (ca. one year after clipping). Average values are shown for each fraction. L = Leaves, 0-St = current year stems, 1-St = one-year-old stems, 2-St = two-year-old stems, MS = main stems, CR = coarse roots, FR = fine roots. Significant differences between treatments within species are indicated by asterisks: * = P < 0.10, ** = P < 0.05.

Fig. 3. Differences between clipped (grey bars) and control (black bars) *B. pubescens* and *Q. petraea* saplings in the allocation of SS and starch pools to different plant fractions one year after clipping (trees harvested in August 2008). Asterisks denote significant differences between treatments within a given species at α = 0.10 (*) and α = 0.05 (**).

Fig. 4. Differences between clipped (grey bars) and control (black bars) *B. pubescens* and *Q. petraea* saplings in the allocation of newly fixed C to the different plant organs one year after clipping (trees harvested in August 2008). Asterisks denote significant differences between treatments within a given species at α = 0.05.

Fig 5. Isotopic composition ($\delta^{13}C$; ‰) of the rhizosphere and bulk soil collected underneath control (white dots) and clipped (black dots) *B. pubescens* and *Q. petraea* trees harvested in August 2008 (ca. one year after clipping). No significant differences between treatments were detected at α = 0.05.

Fig 6. Differences between control (black bars) and clipped (grey bars) *B. pubescens* and *Q. petraea* saplings in the allocation of newly fixed C to SS and starch to different plant fractions on the year after clipping (trees harvested in August 2008). Asterisks denote significant differences between treatments within a given species at $\alpha = 0.10$ (*), and $\alpha = 0.05$ (**).


Rhizosphere
Bulk
δ13C (‰)
-27.4
-27.6
-27.8
-28.0
-28.2
-26.8
-27.0
-27.2
-27.4
○ Control
● Browsed
B. pubescens
Q. petraea

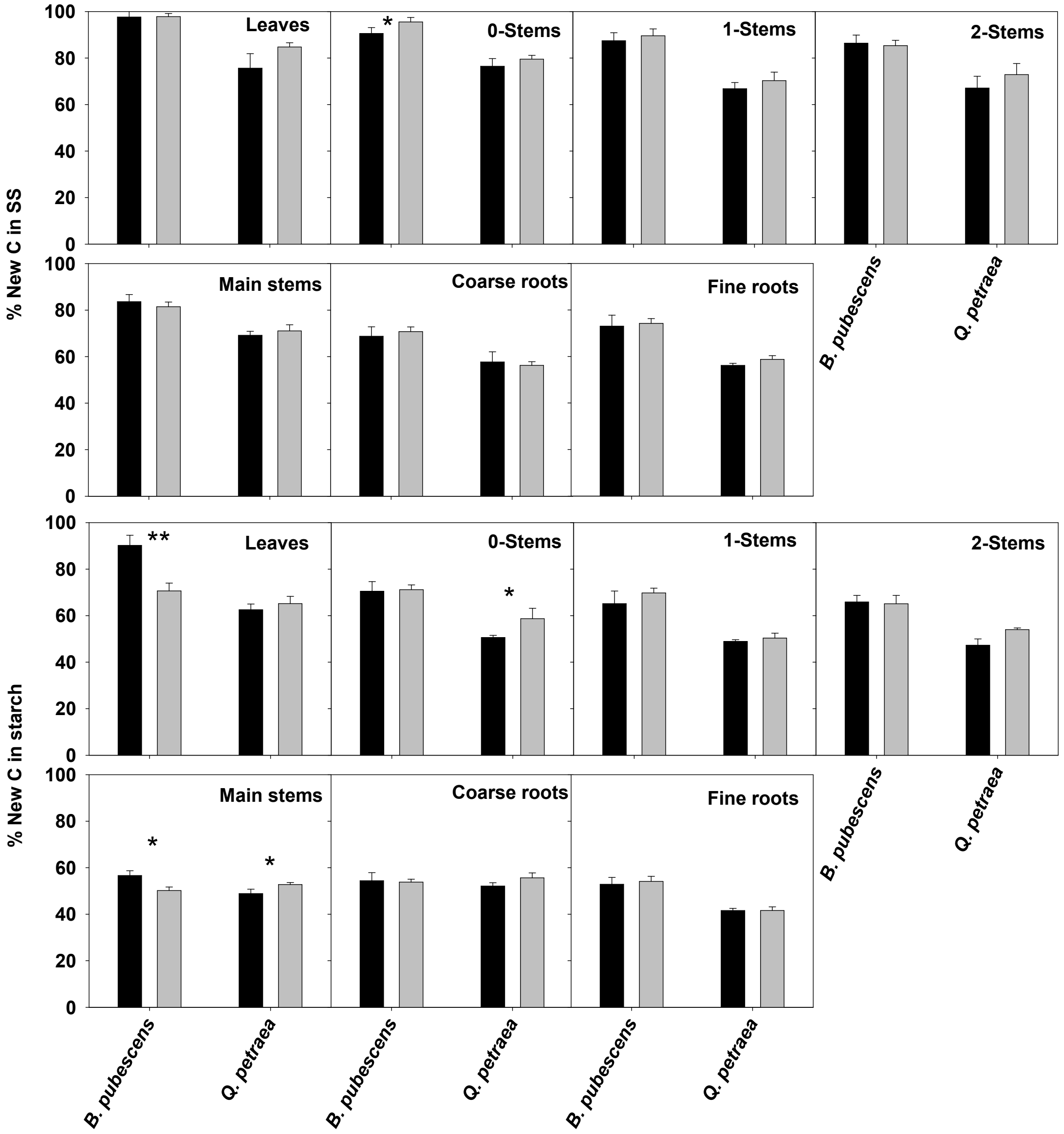

% New C in SS
% New C in starch
Leaves
0-Stems
1-Stems
2-Stems
Main stems
Coarse roots
Fine roots
*
**
*
*
*
0
20
40
60
80
100
B. pubescens
Q. petraea

Table 1. Biomass allocation to the different plant fractions in *B. pubescens* and *Q. petraea* trees harvested at the beginning of the δ^{13}C-labelling experiment in April 2008 (i.e. at the beginning of the first growing season after clipping). Data are means and SE (in parentheses). Significant differences between treatments within species are indicated by asterisks: * = $P < 0.10$, ** = $P < 0.05$, $N = 5$.

BM allocation (% total plant biomass)	***B. pubescens***		***Q. petraea***	
	Clipped	**Control**	**Clipped**	**Control**
One-year-old stems	2.4 (0.3)	9.8 (1.6)**	1.3 (0.3)	4.8 (0.7)**
Two-year-old stems	12.5 (2.8)	14.3 (1.8)	11.1 (2.0)	11.8 (1.5)
Main stems	30.3 (1.8)	23.8 (2.0)*	21.7 (1.5)	18.6 (1.3)
Coarse roots	25.0 (1.9)	24.8 (2.0)	39.6 (3.0)	36.8 (1.6)
Fine roots	16.0 (1.3)	16.6 (3.7)	10.0 (1.2)	13.8 (0.9)

Table 2. Results for architectural: tree height (cm), canopy area (m2), basal stem diameter (mm), number of branches, number of terminal shoots, number of lateral shoots, number of long shoots, number of short shoots; and morphological: individual leaf area (LA; cm^2), individual leaf weight (g), and specific leaf area (SLA, cm^2 g^{-1}) variables measured in clipped and control saplings of *B. pubescens* and *Q. petraea* before harvest in August 2008 (ca. one year after clipping). Significant differences between treatments within species are highlighted in bold. Asterisks indicate the degree of significance: * = $P < 0.10$; ** = $P < 0.05$; *** = $P < 0.001$, $N = 5$.

Measurements	***B. pubescens***		***Q. petraea***	
	Clipped	**Control**	**Clipped**	**Control**
Tree height (cm)	**130.9 (8.7)**	**161.8 (11.3)** **	124.8 (6.9)	128.6 (17.4)
Canopy area (m^2)	2.9 (0.4)	2.5 (0.2)	2.3 (0.4)	2.6 (0.3)
Stem diam (mm)	15.5 (0.3)	15.3 (0.5)	14.3 (0.5)	14.9 (0.5)
No. branches	**12.4 (0.7)**	**25.8 (1.7)** ***	7.4 (1.4)	13.2 (2.9)
No. terminal shoots	**15.4 (1.6)**	**38.2 (7.5)** **	**6.0 (0.8)**	**12.2 (2.8)** *
No. lateral shoots	47.4 (6.9)	61.6 (17.4)	15.4 (1.9)	20.8 (5.0)
No. long shoots	62.8 (7.9)	99.8 (23.9)	21.4 (1.4)	33.0 (6.8)
No. short shoots	**20.6 (3.1)**	**237.0 (59.0)** **	0.2 (0.2)	1.4 (0.9)
Root:Shoot ratio	0.52 (0.05)	0.44 (0.07)	**0.57 (0.06)**	**0.81 (0.04)** **
Ind. LA (cm^2)	10.8 (0.8)	9.2 (0.4)	41.2 (5.4)	40.6 (4.9)
Ind. leaf weight (g)	0.04 (0.00)	0.03 (0.00)	0.18 (0.03)	0.19 (0.02)
SLA (cm^2 g^{-1})	250.1 (11.0)	273.9 (20.8)	231.2 (10.0)	219.9 (10.5)

Table 3. Soluble sugar (SS) and starch concentrations in the different fractions of clipped (B) and control (C) *B. pubescens* and *Q. petraea* trees harvested in August 2008 (ca. one year after clipping). L = Leaves, 0-St = current year stems, 1-St = one-year-old stems, 2-St = two-year-old stems, MS = main stems, CR = coarse roots, FR = fine roots. Data are means and SE (in parentheses). Significant differences between treatments within a given species at α = 0.05 are indicated by asterisks.

	B. pubescens				***Q. petraea***			
	SS (mg g^{-1})		**Starch (mg g^{-1})**		**SS (mg g^{-1})**		**Starch (mg g^{-1})**	
	B	**C**	**B**	**C**	**B**	**C**	**B**	**C**
L	65.2	79.1*	69.2	68.0	56.7	50.8	55.0	57.7
	(3.6)	(4.0)	(6.8)	(3.9)	(4.8)	(3.5)	(9.6)	(5.1)
0-St	37.9	38.9	40.3	42.9	20.8	21.1	70.2	81.2
	(1.7)	(1.4)	(2.6)	(3.7)	(1.1)	(0.9)	(8.8)	(15.9)
1-St	33.4	37.9	36.3	44.9	22.4	22.2	68.2	68.3
	(3.9)	(2.5)	(2.1)	(6.3)	(2.2)	(1.0)	(10.0)	(10.4)
2-St	30.9	33.0	43.5	51.6	21.3	19.6	73.6	90.9
	(2.4)	(2.0)	(3.1)	(9.3)	(2.2)	(2.3)	(13.4)	(10.9)
MS	23.8	26.3	54.8	43.6	20.0	16.5	107.0	121.3
	(2.1)	(1.3)	(6.4)	(3.0)	(2.0)	(0.7)	(10.1)	(14.0)
CR	28.1	30.3	182.2	147.6	27.9	24.3	270.3	266.8
	(2.5)	(2.7)	(14.4)	(12.7)	(3.3)	(1.1)	(6.4)	(9.9)
FR	23.4	25.3	85.8	75.0	22.0	19.2	65.7	66.7
	(1.7)	(1.6)	(5.2)	(8.5)	(1.5)	(1.1)	(5.8)	(5.6)

Table 4. Summary statistics of glm analysis showing the effects of the different factors included in the full model on the allocation of new C to bulk biomass, SS and starch to the different fractions of *B. pubescens* and *Q. petraea* saplings subjected to different clipping treatments and harvested in August 2008 (ca. one year after clipping). F-rations along with P-values (in parentheses) are shown. Significant differences at α = 0.05 are highlighted in bold.

Model term	**Bulk BM**	**SS**	**Starch**
Initial length	**20.4 (< 0.001)**	**12.3 (0.001)**	0.2 (0.641)
Species	**170.7 (< 0.001)**	**212.1 (< 0.001)**	**105.2 (< 0.001)**
Treatment	**12.0 (0.001)**	1.9 (0.175)	0.1 (0.814)
Fraction	**155.6 (< 0.001)**	**43.2 (< 0.001)**	**33.8 (< 0.001)**
Species * Treatment	**17.6 (< 0.001)**	1.5 (0.225)	**10.0 (0.002)**
Species * Fraction	**2.8 (0.013)**	1.2 (0.314)	**6.6 (< 0.001)**
Treatment * Fraction	**2.5 (0.027)**	0.3 (0.957)	**2.7 (0.017)**
Species * Treat. * Fraction	0.8 (0.562)	0.9 (0.505)	**2.5 (0.025)**

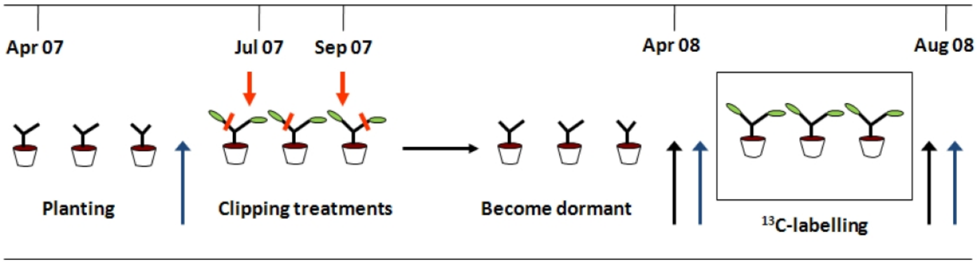


Figure 1

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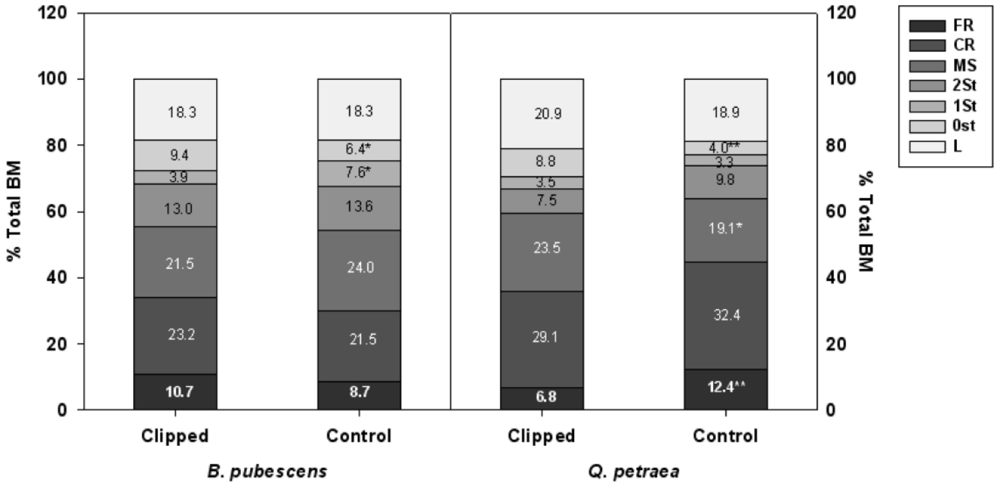

Figure 2

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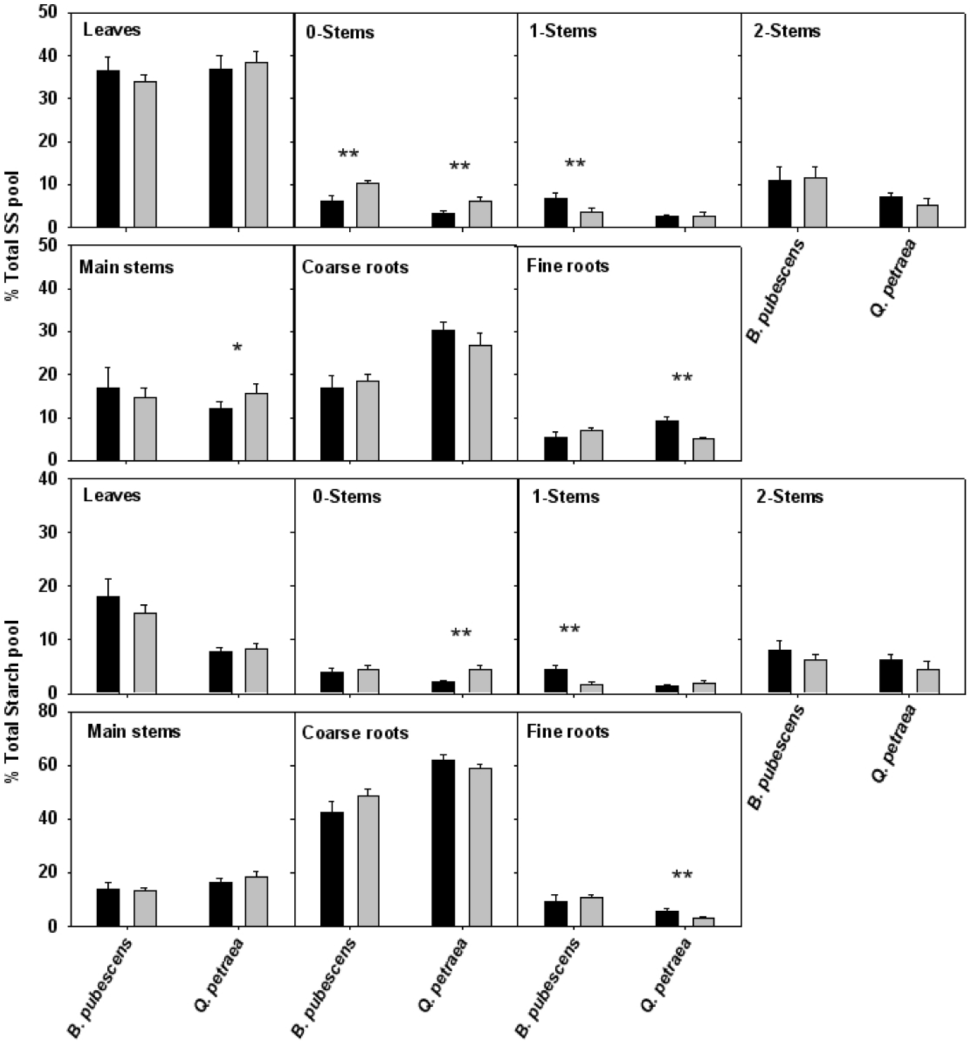

Figure 3

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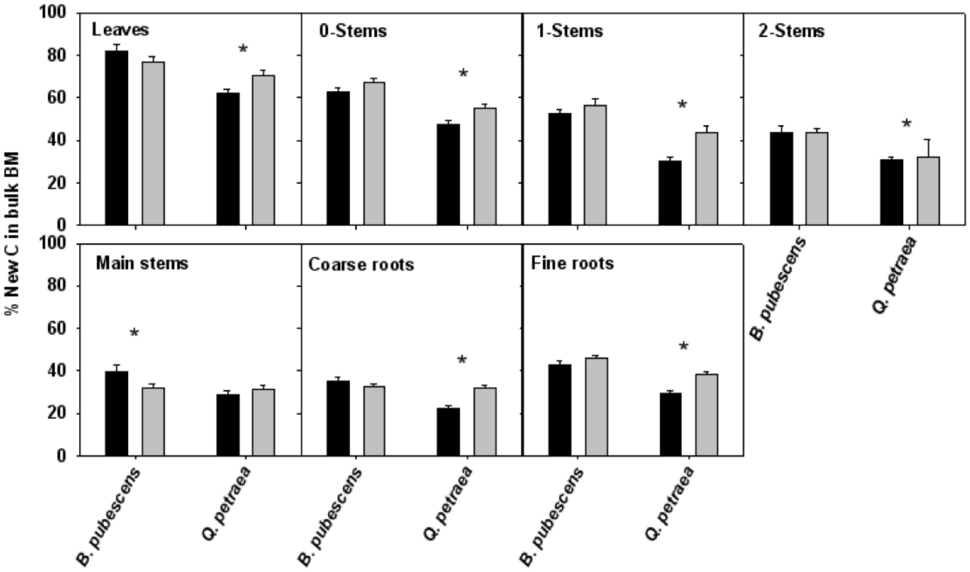

Figure 4

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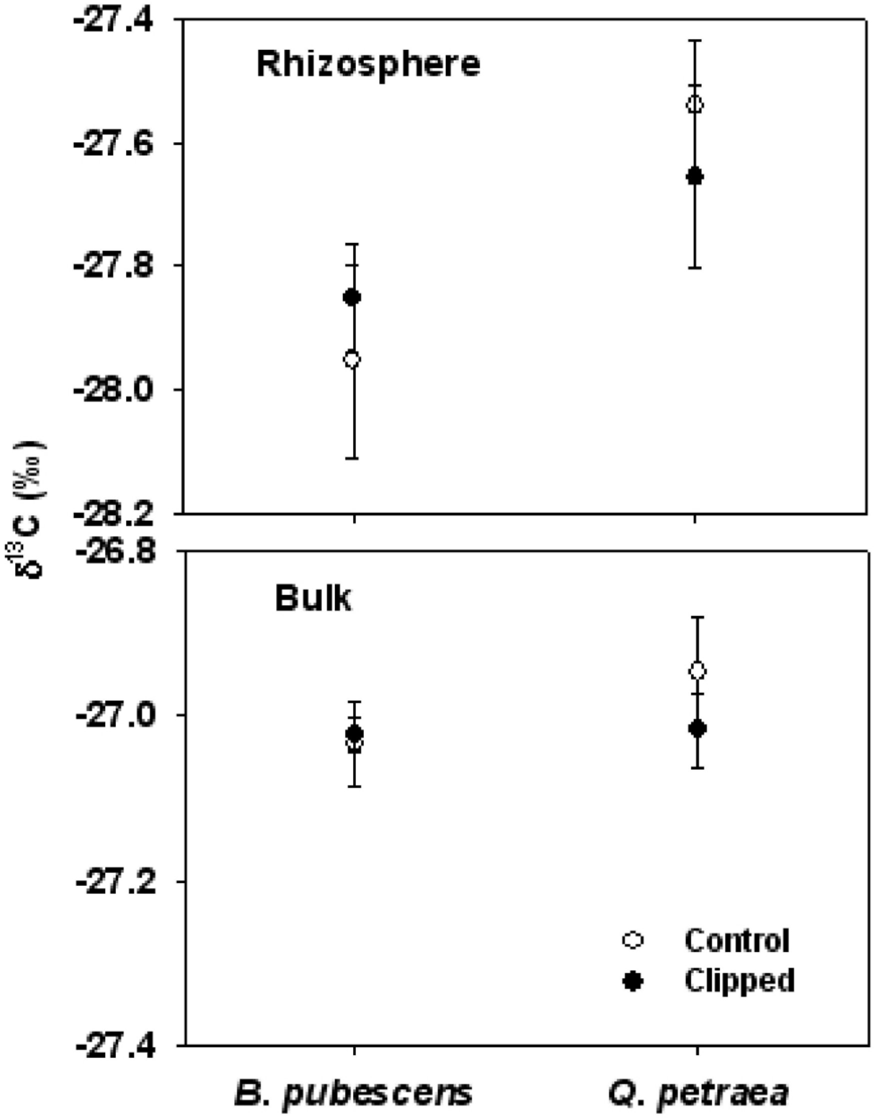

Figure 5

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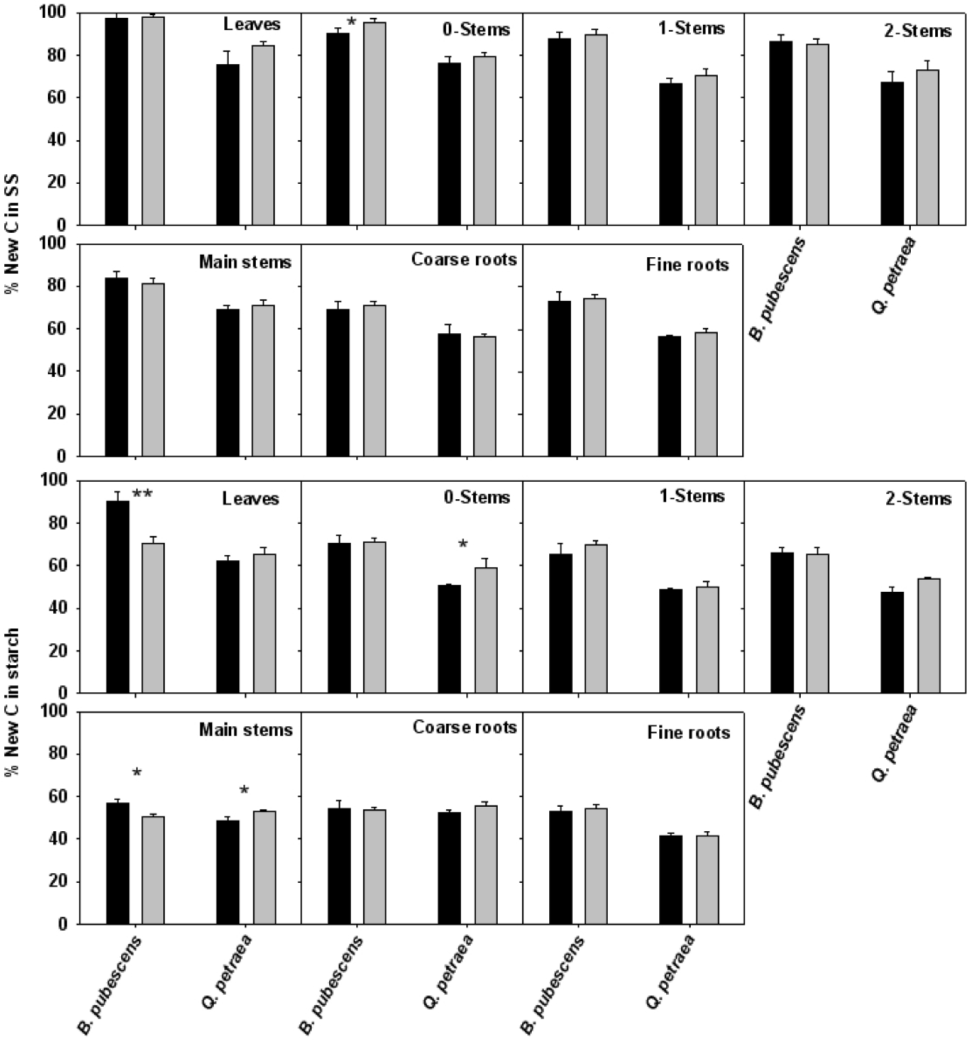

Figure 6

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