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Fast replenishment of initial carbon stores after defoliation by the pine processionary moth and its relationship to the re-growth ability of trees

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Abstract Defoliation by herbivores may alter the source:sink balance of trees leading to transient decreases in carbon (C) stores. When C stores are replenished concurrently with re-growth both processes may compete, store formation proceeding at the expenses of growth. However, the interactions between both processes are not fully understood. We investigated the effects of defoliation by the pine processionary moth (PPM, *Thaumetopoea pityocampa* Dennis and Schiff.) on the non-structural carbohydrate (NSC) and nitrogen (N) stores and the growth of *Pinus nigra* Arnold trees. Short-term effects were evaluated immediately after a PPM outbreak and at the end of the first growing season in trees suffering a range of defoliation damage. Long-term effects were explored by a 17-year-long PPM defoliation experiment, with 11 years of repeated defoliation treatments followed by 6 years of recovery. Defoliation by PPM was followed by transient NSC decreases, but trees were able to exceed initial NSC pools and compensate growth in just one growing season. Such recovery was linked to increased foliage N. Repeated severe defoliations decreased growth and survival of trees in the long-term, but trees increased starch allocation to stems. Defoliation led to an accumulation of C storage compounds in *P. nigra* trees irrespective of their ability to re-grow. In trees included in the short-term experiment, the accumulation of stores proceeded concurrently with re-growth. However, the repeated severe defoliations included in our long-term experiment impaired the growth of trees, surplus C being accumulated as stores. These results indicate that, growth declines in pines defoliated by PPM are not due to C (source) limitation but may respond to the reduced sink strength of growing meristems due to defoliation, and thus, a decrease in C allocation to growth.

Keywords *Pinus nigra* · *Thaumetopoea pityocampa* · Insect herbivory · Non-structural carbohydrate · Nitrogen · Storage allocation

Introduction

Defoliation by herbivores reduces canopy leaf area causing a decrease in the net carbon (C) gain of trees by current photosynthesis and altering the balance between C sinks and sources (Trumble et al. 1993). This may lead to changes in C-allocation patterns, C demands of growing sinks being supplied temporally by C "stores" (Kozlowski 1992; Pinkard et al. 1998; Quentin et al. 2011), namely non-structural carbohydrates (NSC) and lipids, which can be mobilised in the future to support the growth or other plant functions (Chapin et al. 1990). This broad definition

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of stores includes accumulation, reserve formation and recycling (Chapin et al. 1990).

Depending on the severity, frequency and timing of damage, C stores may be decreased or even depleted, and the growth of trees may become C-source (photosynthetic) limited (Trumble et al. 1993). This situation seems to be more dramatic in evergreen conifer trees, which store most of their C and nitrogen (N) in the old foliage, and hence, may lose most of their C and N stores with defoliation (Li et al. 2002; Millard et al. 2001). Since the carboxylating enzyme Rubisco is one of the most important N-storage proteins in plants (Millard et al. 2007, 2010), the decrease in N stores after defoliation may also impair the C-uptake ability of evergreen conifers after damage.

Multiple studies have shown that C stores of evergreen trees decrease soon after defoliation (Ericsson et al. 1980; Li et al. 2002; Webb and Karchesy 1977). However, trees are able to compensate to some degree for loss of foliage by changing allocation patterns favouring foliage production, increasing the photosynthetic rate of surviving leaves and inducing changes in leaf morphology (Heichel and Turner 1976; Pinkard and Beadle 1998; Vanderklein and Reich 1999). Consequently, light defoliations do not normally cause a decrease in NSC pools (Kolb et al. 1992; Tschaplinski and Blake 1994; Van der Heyden and Stock 1995) and the few studies that have followed the evolution of NSC pools after moderate or severe defoliations indicate C (source) limitation is only transient and of short duration (Palacio et al. 2008, 2011). In evergreen conifers, trees seem to be able to replenish their initial NSC pools after severe defoliations rather soon (Li et al. 2002; Roitto et al. 2003), although long-term studies assessing their recovery are lacking.

The ability of plants to increase C-gain ability after defoliation has been suggested as a mechanism to support re-growth, and ultimately, tree tolerance to defoliation (Eyles et al. 2009). However, the observation that NSC pools are replenished concurrently with re-growth, i.e., throughout the course of a growing season (Li et al. 2002; Palacio et al. 2008, 2011), poses the question as to what point both processes interfere. According to the cost-benefit theory for plant storage (Chapin et al. 1990), the replenishment of C stores after defoliation should compete with growth, if true reserve formation is involved. Implicit in this idea is the assumption that plant growth is limited by C (source) availability and that C allocation to storage can be regarded as a cause for decreased plant growth. Alternatively, C-store accumulation would proceed when acquisition exceeds demands (Chapin et al. 1990), once growth has been impaired by defoliation. Identifying how growth recovery and C-store replenishment interact is crucial to understand plant responses to disturbance and stress. However, to our knowledge, no previous studies have directly assessed the relationship between both processes in defoliated trees.

The pine processionary moth (PPM), *Thaumetopoea pityocampa* (Dennis and Schiff.) is one of the most severe insect pests affecting Mediterranean evergreen conifers such as *Pinus* and *Cedrus* species (Masutti and Battisti 1990). PPM caterpillars can consume needles of any age during autumn and winter. However, they feed preferentially on the old needles of trees, and defoliations are most intense during winter when larvae are in the fourth and fifth instar (Démolin 1969). PPM larvae do not damage the buds, which burst in spring (Guyon 1986). When larvae are fully developed, they abandon the tree, marching in lines (hence their name) until they burrow in the ground where they turn into a chrysalis and then a moth, after a period that can range from a few months to several years (Battisti 1988). Such variability determines the population dynamics of this species, which like many other foliage feeding insects shows periodical outbreaks, with an average periodicity of 6 years (Battisti 1988), although the cycle is not regular and it can exhibit sharp variations (Gery and Miller 1985). Defoliation by PPM has been shown to decrease the growth and the reproductive abilities of pine species and may threat the survival of drought-stressed trees (Hódar et al. 2003; Kanat et al. 2005). C (source) limitation has been suggested as a cause of such growth declines (Hódar et al. 2003), although no studies have evaluated the impact of PPM damage on pine C stores.

The aim of this study was to evaluate the impact of PPM on the NSC and N stores of planted *Pinus nigra* Arnold trees and their ability to replenish such stores in the short- (1 year) and the long-term (i.e., 6 years, the time frame of an average PPM outbreak). We use mass-based concentrations of NSC (namely soluble sugars and starch) as estimators of store formation and use (Chapin et al. 1990). We are, however, aware that part of the NSC pools of plants may actually be sequestered, and hence, not readily available for plant growth (Millard and Grelet 2010). Consequently, C stores may be somewhat overestimated in our analysis. We consider just NSC since, although lipids are important C storage compounds for some conifers, NSC account for most of mobile C pools in pine (Li et al. 2002). We hypothesized that: (1) defoliation by the PPM will decrease NSC concentrations of trees causing a transient C limitation; (2) C stores will be soon replenished so that the initial C limitation will be overcome in the course of a growing season; (3) within the average time-frame of PPM outbreaks (6-year periodicity), C stores of trees will be fully recovered by the end of a cycle. In addition, we specifically assessed the relationship between initial NSC stores and growth on the recovery of NSC reserves of *P. nigra* trees after PPM outbreaks by using structural equation models (SEM). Since we expected trees not to be limited by C (source) availability, our hypothesis was that the replenishment of carbohydrate stores would not compete with the ability of trees to compensate growth after defoliation.

Materials and methods

Species and study site

Pinus nigra subsp. *nigra* Arn. trees were sampled in a plantation located near Mora de Rubielos, Teruel, eastern Spain (40°12′N, 0°43′W, 1,150 m a.s.l.). The trees were planted between 1968 and 1971 and form a relatively open stand. Average diameter at breast height (DBH, measured at 1.3 m) and total tree height were 8.0 ± 0.4 cm and 4.3 ± 0.1 m, respectively, at the beginning of the study (1992). The studied *P. nigra* trees usually bear up to 3- to 4-year-old needles. Climate in this area is Mediterranean with a pronounced summer drought. The mean annual temperature in the study area is 12 °C and the total annual rainfall is 436 mm based on monthly climatic data from the nearby "Mora de Rubielos" climatic station, located ca. 6 km from the sampling site (40°15′N, 0°45′W, 1,040 m a.s.l., period 1992–2010). During the study period, the precipitation recorded from April to June (mean = 144 mm) was low (49–117 mm) in 1993, 1994, 2001 and 2005.

The natural vegetation is dominated by *Quercus ilex* L. subsp. *ballota* (Desf.) Samp., *Quercus coccifera* L. and *Juniperus* species. The soils are basic, nutrient-poor and developed on clays. Based on historical records of PPM incidence in the area performed since the 1970s by the Spain and Aragón Forest Services, no previous severe defoliation had affected the studied stand. A more detailed description of the study site can be found in Hernández-Alonso et al. (2005).

Experimental design

We combined observations after a natural PPM outbreak occurring in winter 2009–2010, with a 17-year-long defoliation experiment conducted between 1992 and 2009, to assess the short- and long-term effects, respectively, of PPM defoliations on the C stores and growth of trees and their subsequent recovery. Short-term effects inform us about the mechanisms behind the recovery of trees while long-term data illustrate the expected response of trees in the longer term under extreme defoliation scenarios.

Short-term effects: natural PPM outbreak in winter 2009–2010

In winter 2009–2010, a natural PPM outbreak affected the studied stand, leading to severe defoliation (over 80 % of the whole canopy) of some individuals. In April 2011, we randomly selected trees within the stand not included in other defoliation experiments and that showed a wide range of crown defoliation and related PPM incidence. We visually assessed the degree of needle loss (defoliation) of each sampled tree by comparing them with five randomly selected reference trees showing no damage. These trees were used as reference "control" trees to correct for observer bias in estimates of defoliation. Since percentage estimates of crown defoliation vary among observers, all defoliation estimates were made by the same person (JJC). The mean diameter of sampled trees measured at 1.3 m was 16.97 ± 0.32 cm (mean ± SE), whereas the mean age, estimated at 1.3 m by counting rings in radial cores, was 36 ± 1 years. The mean distance between sampled trees ranged between 5 and 10 m.

Long-term effects: defoliation experiment

In 1992, we randomly selected 20 trees within the study stand and allocated them to two different defoliation treatments: 100 % defoliation and control (undefoliated). Although frequent complete defoliations by PPM are rare in nature, we aimed at simulating extreme defoliation scenarios to detect mid- to long-term responses in growth to severe needle loss and to discern the time required by trees to recover growth levels similar to those observed before defoliations started. All trees were tagged and their height and DBH measured with tapes and ladders to account for initial (i.e., not due to treatments) tree variability in size. Treatments were applied for 11 years, between 1993 and 2003, by transposing PPM nests to trees included in the "defoliation" treatment. Defoliation damage was checked regularly throughout autumn and winter. If larvae hatched from transplanted nests were not enough to cause a complete defoliation of trees, more nests were transplanted from nearby affected trees. If still, canopy defoliation was not complete, needles of undefoliated branches were gradually clipped with scissors, avoiding clipping complete branches, and buds were left intact. This procedure aimed at achieving gradual but complete (and hence comparable) canopy defoliation, mimicking natural (yet extremely severe) PPM outbreaks.

Treatments were applied in autumn and winter for two consecutive years followed by a year of recovery. Consequently, defoliations were applied in 1993–1994, 1996–1997, 1999–2000 and 2002–2003, and trees were released from defoliation in years 1995, 1998 and 2001. From 2004, trees were left to recover until November 2009 (6 years later), when they were felled and harvested for analysis. Three of the initial ten trees included in the defoliation treatment died during the experiment, and consequently, our sample size was reduced to $n = 7$ for defoliated trees.

Growth measurements

Short-term effects of PPM on growth were assessed by measuring the needle production, branch length and radial growth (earlywood (EW), latewood (LW) and total current-year wood ring width) of trees in September 2011 (i.e., one growing season after the PPM attack). Primary growth was estimated by randomly collecting three current-year branches from the upper third, southern-oriented and light-exposed side of the crowns of each tree. All sampled branches were healthy and had grown in 2011, and thus, contained needles formed in that year. We measured the length of the shoot formed in 2011 (i.e., lateral branch growth) and averaged the individual branch values per tree. Then, we measured the average needle biomass production in 2011 of each tree by drying (in the oven at 60 °C for 72 h) and weighing all the needles formed in 2011 of the different branches harvested.

Secondary growth was measured in two radial cores per tree, taken at 1.3 m (breast height) using a Pressler increment borer. Wood samples were carefully cut and visually cross-dated to measure the width of the 2011 tree ring. Once dated, we measured the earlywood and latewood widths to the nearest 0.01 mm using a binocular scope and a LINTAB measuring device (Rinntech, Heidelberg, Germany). Total tree-ring width was calculated as the sum of the earlywood and latewood widths. Values of the two radii were averaged to obtain annual estimates of radial growth per tree.

The growth of trees included in the long-term experiment was monitored annually by measuring the height and DBH of each tree in November when both types of growth have been completed. Secondary growth was measured as in the trees included in the short-term experiment, by collecting two cores per tree at 1.3 m. Wood cores were subsequently sanded and visually cross-dated, and the accuracy of visual cross-dating checked with the program COFECHA (Holmes 1983).

Plant harvest

Only needles and main stem wood were considered in the analyses. These fractions (particularly needles) account for the largest proportion of total NSC pools in pine trees and, consequently, are most affected by defoliations (Li et al. 2002). For the short-term experiment, needle and stem-wood samples were obtained from marked trees in April 2011, prior to bud burst, and September 2011, at the end of the growing season, when trees had completed both their primary and radial growth. All samples were collected between 1000 and 1300 h to avoid diurnal variability in carbohydrate concentrations. Current-year needles were collected from three current-year branches from the upper third, southern-oriented and light-exposed side of the crowns of each tree. In September 2011, these were the same branches used for primary growth measurements (see above). Stem-wood samples were obtained from two radial cores per tree, taken at 1.3 m using a Pressler increment borer. After collection in the field, needle and wood samples were taken to the laboratory in a portable cooler. Needles were dried in the oven at 60 °C for 72 h. Portions of current-year tree rings were separated from each core using a razor blade. Wood samples were subsequently frozen and stored at −20 °C until freeze dried. All dried samples were weighted and milled to a fine powder in a ball mill (Retsch Mixer MM301, Leeds, UK) prior to chemical analyses.

Plant samples for the long-term experiment were collected from the trees felled at the end of the experiment in November 2009, when primary and secondary growth had terminated and trees were dormant. From each tree, we collected two slices of the stem at 1.3 m height, plus needle samples of the youngest four cohorts from sun-exposed top branches. All samples were collected between 1000 and 1300 h, kept in a cooler until transported to the lab, and then frozen at −20 °C until processing. Since trees were relatively young, most of the stem wood was sapwood. Samples of stem sapwood were hence collected from different positions within each slice and divided in two sections for chemical analyses: an outer section, including the youngest rings formed between 2004 and 2009, and an inner section with the oldest rings (formed between 1996 and 2003). All samples were freeze dried, weighted and milled to a fine powder in a ball mill (Retsch Mixer MM301, Leeds, UK) prior to chemical analyses.

Chemical analyses

Total C and N mass-based concentrations were analyzed with an elemental analyzer (Elementar VarioMAX N/CM, Hanau, Germany). Soluble sugars (SS) were extracted with 80 % (v/v) ethanol and their concentration determined colorimetrically, using the phenol–sulphuric method of Dubois et al. (1956) as modified by Buysse and Merckx (1993). Starch and complex sugars remaining in the undissolved pellet after ethanol extractions were enzymatically reduced to glucose and analyzed as described in Palacio et al. (2007). Non-structural carbohydrates measured after ethanol extraction are referred to as soluble sugars and carbohydrates measured after enzymatic digestion are referred to as starch. Both are expressed in glucose equivalents. The sum of SS and starch is referred to as total non-structural carbohydrates (NSC). NSC pools of current-year needles included in the short-term experiment and collected in September 2011 were calculated as the product between the average current-year needle biomass per branch and the NSC concentrations of current-year needles.

Statistical analyses

Short-term effects of PPM defoliation on the SS, starch, NSC and N concentrations and the C:N ratio of needles and sapwood of *P. nigra* trees in April and September 2011 and on the starch, NSC and N pools of needles in September 2011 were analyzed by univariate general linear models (GLMs, least-squares fit) with "defoliation" as a fixed factor and the "DBH of the tree in April" as a covariate to account for tree individual variability in size. The same GLM was used to evaluate the short-term effects of PPM defoliation on the growth of trees, i.e., current-year needle biomass per branch, lateral branch length, and earlywood (EW), latewood (LW) and total current-year wood ring width as measured in September 2011 in the same trees.

Long-term effects of defoliation by PPM on the SS, starch, NSC and N concentrations and the C:N ratio of needles and sapwood of *P. nigra* trees were analyzed by GLMs with "defoliation treatment" (defoliated/control) and "cohort" as fixed factors and the "DBH of trees in 1992", the year prior to the first application of treatments, as a covariate. Four different cohorts of needles were considered, while in stems, sapwood was divided in two segments: 1996–2003 and 2004–2009. Long-term defoliation effects on the growth of trees were evaluated by GLMs with "defoliation treatment" as a fixed factor and the "DBH of trees in 1992" plus the "height of trees in 1992" as covariates. Response variables were the DBH, height increment and total current-year wood ring width of trees on the first year after the application of treatments (1993), the last year of the application of treatments (2003), the year of harvest (2009), and the average DBH, height increments and annual wood ring width values for the period of defoliations (1993–2003) and recovery (2003–2009). When variables were not normally distributed, they were angularly transformed [$\mathrm{arctg}(x^{0.5})$] to meet the requirements of GLMs. All GLM and regression analyses were run in JMP 8.0 (SAS Institute Inc., Cary, USA, 1989–2007).

The relationship between store replenishment and regrowth ability of trees was studied using data from the short-term experiment. We followed two different methods: correlations and structural equation modeling (SEM). In the first approach, the difference between April and September NSC concentrations in leaves and stem sapwood were correlated against the different growth indicators (needle biomass productivity, branch length growth and wood ring width increment) by Pearson correlation analyses. Correlations were run in SPSS 15.0. (SPSS for Windows, Chicago, USA, 2009).

Secondly, SEM was used to evaluate the more complex relationships between defoliation by PPM, N and NSC concentrations in April, the difference between April and September NSC concentrations (as an indicator of store replenishment) and growth. SEM is a robust tool to unravel multivariate relationships between plant traits, as it enables disentangling direct and indirect effects among variables (Shipley 2004). We first built a theoretical model including the logical relationships between our variables based on previous knowledge. In our model defoliation was directly related to NSC and N concentrations in April, owing to the well-documented immediate effects of defoliation on N and NSC stores (Ericsson et al. 1980; Tschaplinski and Blake 1994; Webb and Karchesy 1977). We also considered defoliation to be directly related to the growth attained at the end of summer, to account for other effects of defoliation on plant growth different to the impact on NSC and N availability. NSC concentrations in April were logically related not only to store replenishment in September but also to growth, since pine trees are known to invest both stored NSC and new photoassimilates into new growth (von Felten et al. 2007). The concentration of N in April was also directly related to growth and the difference between April and September NSC concentrations, owing to the close relationship between total N and Rubisco concentrations in leaves, and hence, the carboxylation potential of trees (Millard et al. 2007). Finally, growth attained throughout the growing season was related to the difference between April and September NSC concentrations as a way to explore the relationship between NSC accumulation (store replenishment) and growth after defoliation.

We fitted our theoretical model using SEM to six different data sets, depending on the origin of NSC concentrations (needles or stem sapwood) and the variable used as a proxy for tree growth (needle biomass production, branch length growth or current-year wood ring width increment). SEM estimation followed the maximum likelihood method (Arbuckle 2009). The goodness of fit of each model was evaluated by a combination of statistics and fit indexes including: the Chi-square and its probability level, the root mean-square error of approximation (RMSEA), the root mean-square residual (RMR), the Goodness-of-Fit Index (GFI), the Adjusted Goodness-of-Fit Index (AGFI), the Normed Fit Index (NFI) and the Akaike Information Criterion (AIC). Non-significant Chi-square statistics indicate an acceptable model fit, while values close to zero for the RMSEA and RMR indexes and values close to one for the probability level, GFI, AGFI, and NFI statistics indicate consistency between the theoretical and evaluated models. Finally, the AIC is an index used to compare different models: the most parsimonious models showing the lowest AIC values. Since different indices follow different approximations and hence reflect different aspects of model fit, the use of a combination of statistics and indexes provides a robust tool to evaluate the fit of models generated by SEM (Jöreskog 1993). SEMs were performed in Amos 18.0.

Results

Short-term effects of PPM defoliation on N and NSC concentrations

The mean defoliation of sampled trees was 42 ± 5 %. The defoliation intensity was a good surrogate of PPM incidence, because canopy defoliation and the number of PPM nests counted per tree (mean 3 ± 1 nests per tree) were strongly related ($r = 0.68$, $P < 0.001$).

Defoliation by PPM had a strong immediate effect on the NSC of *P. nigra* trees in the needles and a weak impact on the NSC accumulated in stem sapwood and the needle N concentrations. Trees harvested soon after the end of PPM defoliation but before bud break, in April 2011, showed decreased carbohydrate concentrations (including SS, starch and NSC) in both the needles and stems as defoliation increased (Table 1; Fig. 1). On the contrary, N concentrations in old needles increased significantly with defoliation, and hence trees with higher needle loss showed significantly lower C:N ratios in their needles (Fig. 1; Table 1).

Recovery of NSC stores and growth after one growing season

At the end of the growing season (September 2011), trees included in the short-term analysis showed significantly higher NSC concentrations and pools in current-year needles with increasing defoliation (Table 1; Fig. 2). This indicates trees not only replenished their C stores in just one growing season but also the refilling of stores was proportional to defoliation intensity, more defoliated trees showing increased NSC concentrations and pools at the end of summer (Fig. 2). Patterns for N were also highly significant, N concentrations and pools increasing and C:N ratios decreasing with damage (Table 1; Fig. 2). In stems, defoliation effects were not significant for any of the chemical variables studied (Table 1). Similarly, GLMs showed there was a significant positive effect of PPM defoliation on branch growth ($F = 11.153$, $df = 1$, $P = 0.002$) and foliage production ($F = 17.369$, $df = 1$, $P < 0.001$), trees with higher defoliation damage showed increased branch growth and higher current-year needle biomass (Fig. 2). This also explains the recovery of needle NSC and N pools. Defoliation effects were not significant for any indicator of secondary growth (EW width: $P = 0.579$, LW width: $P = 0.311$, tree ring width: $P = 0.464$, Fig. 2).

Long-term effects of PPM defoliations on NSC and N concentrations and growth

There were significant differences in the NSC and N concentrations and the C:N ratio of the different cohorts of tree rings and needles analyzed (Table 2). In sapwood, the outermost rings, i.e., the youngest ones, showed higher concentrations and sharper variations of NSC and N than older rings. This indicates NSC and N were preferentially stored and remobilized from younger rings within the sapwood of *P. nigra* trees. Contrastingly, older needles stored more NSC than younger cohorts (Table 2). For N, old cohorts of needles of defoliated trees showed lower concentrations than younger ones, while in control trees the trend was reversed (Table 2).

Differences between treatments showed that defoliated trees accumulated more NSC in their sapwood and more N in both their sapwood and needles than the control trees, consequently, the C:N ratio of defoliated trees was also lower (Table 2). Starch needle concentrations seemed to follow an opposite trend, decreasing in defoliated trees as compared to controls, although effects were weak due to the low replication ($n = 3$) and not significant for needle SS and NSC concentrations (Table 2). Taken together, these results seem to indicate a shift from needle to stem accumulation of NSC in response to long-term defoliation treatments. Increased NSC (in sapwood) and N status of defoliated trees 6 years after the last defoliation event indicates that these trees may reach the next PPM population outbreak (which have an average periodicity of 6 years) with more C and N stores than non-defoliated trees (Table 2). The effects on NSC in sapwood stood for trees subjected to similar treatments but with 9 years of recovery (data not shown).

The analysis of long-term effects on growth showed defoliation treatments significantly decreased height and diameter growth of trees and the width of rings formed after PPM defoliations (Table 3). These effects were already significant on the first year after defoliations started (1993, $F = 41.90$, $df = 1$, $P < 0.001$ for tree ring width), and they remained until the end of the recovery period (2009), 6 years after the completion of treatments, except for tree ring width which did not show significant differences in 2009 (Table 3). Long-term defoliation by PPM had also an important effect on the survival of trees, and 30 % of the ten initial individuals died. These data will be considered elsewhere (Camarero and R. Hernández, unpublished results).

Relationship between stores replenishment and growth after defoliation

The six models obtained with SEM, built from the different data sets available, provided a reasonably good fit to our theoretical model (Table 4). However, of all models explored, the model which included the NSC data of sapwood and the width of the current-year tree ring as a surrogate of tree growth (number 6) showed the best fit

Table 1 Statistics assessing the effects of defoliation on the NSC and N concentrations and pools of trees harvested in April 2011 (just after PPM defoliation) and in September 2011 (one growing season after PPM defoliation)

Variables	April 2011				September 2011			
	Needles		Stems		Needles		Stems	
	F	*P* value	*F*	*P* value	*F*	*P* value	*F*	*P* value
Concentrations								
SS	7.14	**0.010**	0.56	0.457	3.89	0.055	2.03	0.161
Starch	42.76	**<0.001**	5.60	**0.022**	4.68	**0.036**	3.39	0.071
NSC	42.51	**<0.001**	5.58	**0.022**	5.53	**0.023**	1.03	0.316
Total N	8.09	**0.007**	–	–	22.24	**<0.001**	–	–
C:N	8.27	**0.006**	–	–	25.03	**<0.001**	–	–
Pools								
SS	–	–	–	–	19.70	**<0.001**	–	–
Starch	–	–	–	–	21.81	**<0.001**	–	–
NSC	–	–	–	–	23.16	**<0.001**	–	–
Total N	–	–	–	–	21.47	**<0.001**	–	–

Differences due to defoliation intensity in starch, soluble sugars (SS), total non-structural carbohydrate (NSC) and nitrogen (N) concentrations and the C:N ratios were assessed in needles and stems by GLMs. The model comprised “defoliation” as a fixed factor and the DBH at the beginning of the experiment as a covariate. Only “defoliation” effects are shown. Significant effects ($P < 0.05$) are shown in bold, $df = 1$ in all cases

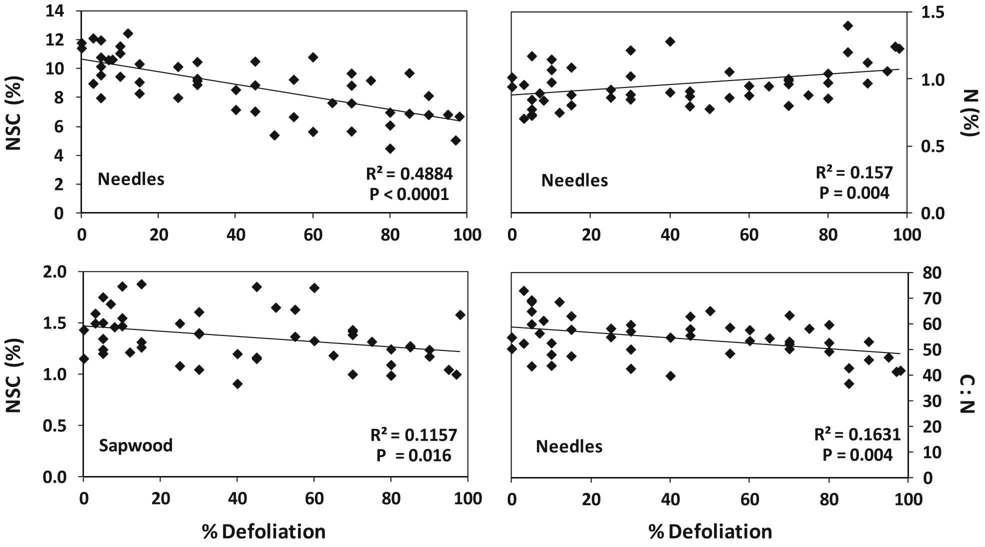

Fig. 1 Relationship between the percentage of canopy defoliation by the PPM and the NSC concentrations of the needles and stems and the N concentrations and C:N ratios of the current-year needles of *P. nigra* trees included in the short-term experiment and measured in April 2011. Results of linear regression analyses are shown separately in each *chart*

(Table 4), and hence was the one selected for representation (Fig. 3). Nevertheless, provided there was considerable and meaningful variation in the significance of the different relationships of the models obtained with different data sets (Table 5; Fig. 3), all models will be considered in the discussion of results.

According to all SEMs obtained and in agreement with the results of GLMs, defoliation had a significant immediate positive effect on foliage N concentrations and a negative one on sapwood and needle NSC concentrations (Table 5; Fig. 3). Not surprisingly, NSC concentrations in spring were negatively related to the replenishment of NSC stores at the end of the growth season (measured as the difference between September and April NSC concentrations) in all models (Table 5; Fig. 3). However, they were unrelated to any measurement of growth included in the analyses (Table 5; Fig. 3). Foliage N concentrations in April were unrelated to the replenishment of sapwood NSC (Fig. 3), but they were strongly and positively related to the increase in needle NSC concentrations throughout the growing season (see data sets 1–3 in Table 5). Similarly, N concentrations did not show any effect on stem wood growth (Fig. 3), but they were significantly and positively related to needle biomass production and branch length increment (Table 5).

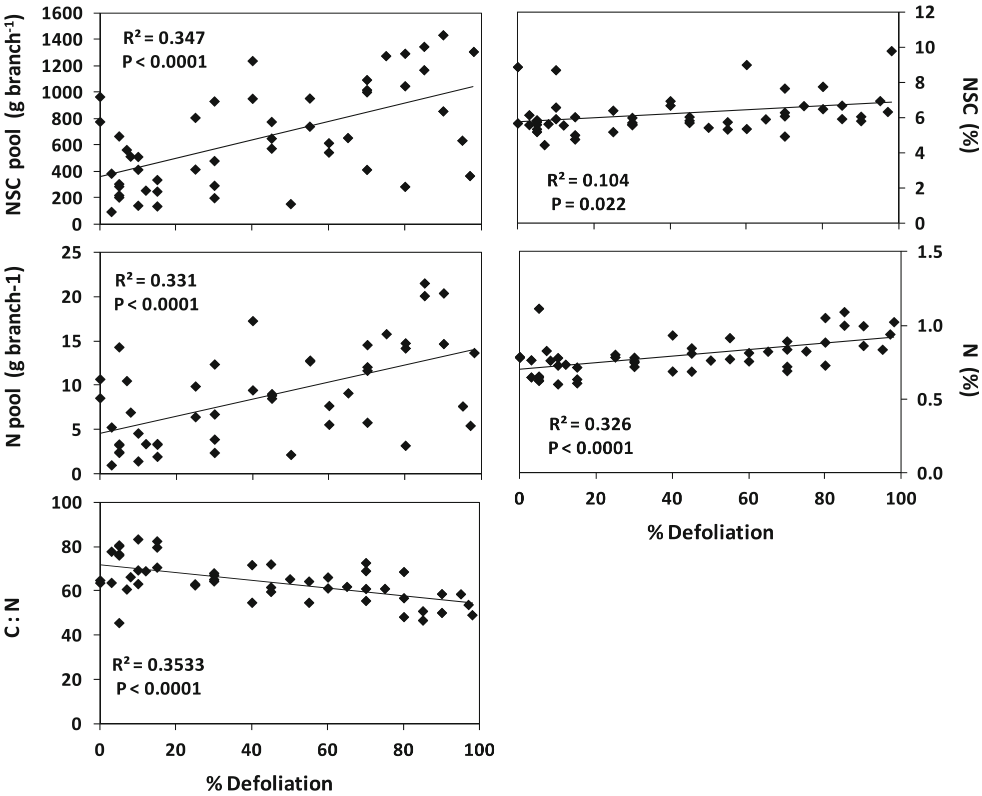

Fig. 2 Relationship between the percentage of canopy defoliation by the PPM in April 2011 and the NSC and N concentrations and pools and the C:N ratio of the current-year needles of *P. nigra* trees included in the short-term experiment and measured in September 2011. Regression lines are shown only when significant ($P < 0.05$)

Table 2 Soluble sugars (SS), starch, total non-structural carbohydrate (NSC), nitrogen (N) concentrations and C:N ratios in the older (1996–2003 rings) and younger (2003–2009 rings) sections of the sapwood and the three youngest cohorts of needles of *P. nigra* trees defoliated by the PPM in the long-term experiment, plus summary statistics of GLMs analyzing the differences between treatments and cohorts

Fraction	Control		Defoliated		Statistics	
Sapwood	1996–2003 rings	2003–2009 rings	1996–2003 rings	2003–2009 rings	Treat.	Cohort
SS (%)	0.35 (0.03)	0.57 (0.02)	0.44 (0.04)	0.79 (0.08)	**14.02 (<0.001)**	**52.01 (<0.001)**
Starch (%)	0.67 (0.04)	0.73 (0.03)	0.65 (0.02)	0.83 (0.07)	0.90 (0.350)	**7.27 (0.011)**
NSC (%)	1.02 (0.07)	1.30 (0.05)	1.09 (0.05)	1.62 (0.12)	**6.48 (0.016)**	**32.726 (<0.001)**
Total N (%)	0.02 (0.002)	0.03 (0.003)	0.04 (0.002)	0.05 (0.004)	**41.84 (<0.001)**	**11.15 (0.002)**
C:N	2,233.19 (176.10)	1,806.59 (164.95)	1,385.78 (67.52)	1,073.68 (78.13)	**31.76 (<0.001)**	**7.01 (0.013)**

Needles	Control			Defoliated			Statistics	
	0-yr	1-yr	2-yr	0-yr	1-yr	2-yr	Treat.	Cohort
SS (%)	4.66 (0.31)	5.13 (0.43)	5.41 (0.45)	5.24 (0.19)	5.28 (0.25)	5.56 (0.28)	0.05 (0.825)	2.10 (0.161)
Starch (%)	3.92 (0.25)	4.53 (0.52)	4.31 (0.43)	4.08 (0.13)	3.77 (0.21)	4.00 (0.17)	**5.63 (0.026)**	1.59 (0.221)
NSC (%)	8.56 (0.54)	9.66 (0.93)	9.72 (0.85)	9.31 (0.11)	9.05 (0.05)	9.56 (0.14)	1.40 (0.249)	2.44 (0.132)
Total N (%)	0.81 (0.03)	0.83 (0.07)	0.89 (0.08)	1.04 (0.06)	0.92 (0.05)	0.86 (0.04)	**5.53 (0.027)**	**6.88 (<0.001)**
C:N	64.18 (2.62)	63.70 (5.61)	60.40 (5.86)	51.09 (3.56)	57.93 (3.44)	63.02 (2.33)	4.03 (0.056)	**22.94 (<0.001)**

Only fixed factor effects are shown (their interaction was not significant in any case). Values are means, while standard errors are indicated in parentheses. For statistics, values are *F* ratios, while *P* values are indicated in parentheses, $df = 1$ in all cases. Significant ($P < 0.05$) factor effects are indicated in bold. See "Materials and methods" for further details. Sapwood: control, $n = 10$; defoliated, $n = 8$. Needles: $n = 3$

0-yr, current-year needles (from 2009); 1-yr, 1-year-old needles (from 2008); 2-yr, 2-year-old needles (from 2007); Treat., treatment

Direct effects of defoliation on growth were not significant for any growth indicator measured, and only positive indirect effects were recorded for leaf biomass production and branch length growth, through the aforementioned impact on N concentrations (Table 5). For wood growth, defoliation had no significant effect whatsoever, which

Table 3 Radial, height and tree ring width growth of control and defoliated *P. nigra* trees included in the long-term PPM defoliation experiment along with the results of statistical tests (GLMs) on treatment effects

Variables	Control		Defoliated		Statistics	
	Mean	SE	Mean	SE	*F*	*P* value
Radial growth (cm)						
DBH end treatment period	14.4	0.7	9.4	1.1	**48.01**	**<0.001**
Treatment period	6.8	0.7	0.9	0.2	**48.01**	**<0.001**
Recovery period	2.2	0.2	1.3	0.3	**7.95**	**0.014**
DBH final harvest	16.5	0.8	10.8	1.3	**39.23**	**<0.001**
Height growth (m)						
End treatment period	0.4	0.02	0.02	0.004	**145.38**	**<0.001**
Treatment period	2.8	0.2	0.4	0.05	**98.71**	**<0.001**
Recovery period	1.5	0.1	0.6	0.1	**59.76**	**<0.001**
Final harvest	0.4	0.02	0.3	0.05	**5.47**	**0.035**
Tree-ring width (mm)						
End treatment period	3.2	0.4	0.03	0.02	**24.46**	**<0.001**
Treatment period	2.3	0.1	0.2	0.04	**30.47**	**<0.001**
Recovery period	2.2	0.1	1.2	0.1	**9.44**	**0.009**
Final harvest	1.8	0.2	1.5	0.2	0.92	0.211

GLMs (least squares) were fit with “treatment” (control/defoliated) as a fixed factor and the size of trees at the beginning of the experiment (DBH and height of trees in 1992) as covariates. Treatments were applied between 1996 and 2003 (treatment period) while trees were left to recover between 2004 and 2009 (recovery period). Trees were finally harvested in 2009, when the experiment terminated. Growth data are shown for the last year of the treatment period (2003, “DBH end treatment period”), the final harvest and also as the average radial increment during treatment and recovery periods. Significant effects ($P < 0.05$) are shown in bold, $df = 1$ in all cases. See “Materials and methods” for further details on the design and analysis

Table 4 Input variables for non-structural carbohydrates (NSC) and growth included in the different data sets used to construct the structural equation models evaluated and summary of statistical indices indicating their goodness of fit

Data set	NSC[a]	Growth	Chi-square[§]	*P*	RMR	GFI	AGFI	NFI	AIC
1	Needle	Needle BM	0.326	0.850	0.190	0.997	0.980	0.998	26.326
2	Needle	Branch len.	0.412	0.814	0.281	0.997	0.975	0.997	26.412
3	Needle	Ring width	0.268	0.875	0.083	0.998	0.984	**0.998**	26.268
4	Stem	Needle BM	0.364	0.833	0.077	0.997	0.978	0.995	26.364
5	Stem	Branch len.	0.924	0.630	0.136	0.992	0.944	0.988	26.924
6	Stem	Ring width	0.151	**0.927**	**0.017**	**0.999**	**0.991**	0.997	**26.151**

All models conformed to the same theoretical model shown in Fig. 4. RMSEA index rendered values lower than 0.001 for all models. The best model for each index is highlighted in bold. Overall, data set 6 is the one that produced the model with the best fit

Needle BM, current-year needle biomass production; Branch Len., branch length growth; Ring width, total current-year tree-ring width

[a] Indicates origin of the NSC data included in the models

[§] All Chi-squares were not significant at $\alpha = 0.05$

agrees with our results of GLMs for the short-term experiments (Fig. 2).

The growth attained at the end of summer by defoliated trees was unrelated to NSC refilling when foliage production and tree ring width were used as indicators of growth (Table 5; Fig. 3). Similarly, when the difference in the NSC status of April and September was directly related to growth parameters, correlations rendered no significant results ($P > 0.05$; data not shown), indicating both processes were unrelated. However, when branch length growth was used as a proxy for tree growth in SEMs, it was significantly and negatively related to the level of NSC accumulated in both needles and sapwood throughout the growing season (Table 5). Taken together, these results indicate NSC accumulation proceeded even when trees were re-growing, although branches that grew more in length had less surplus of NSC for accumulation.

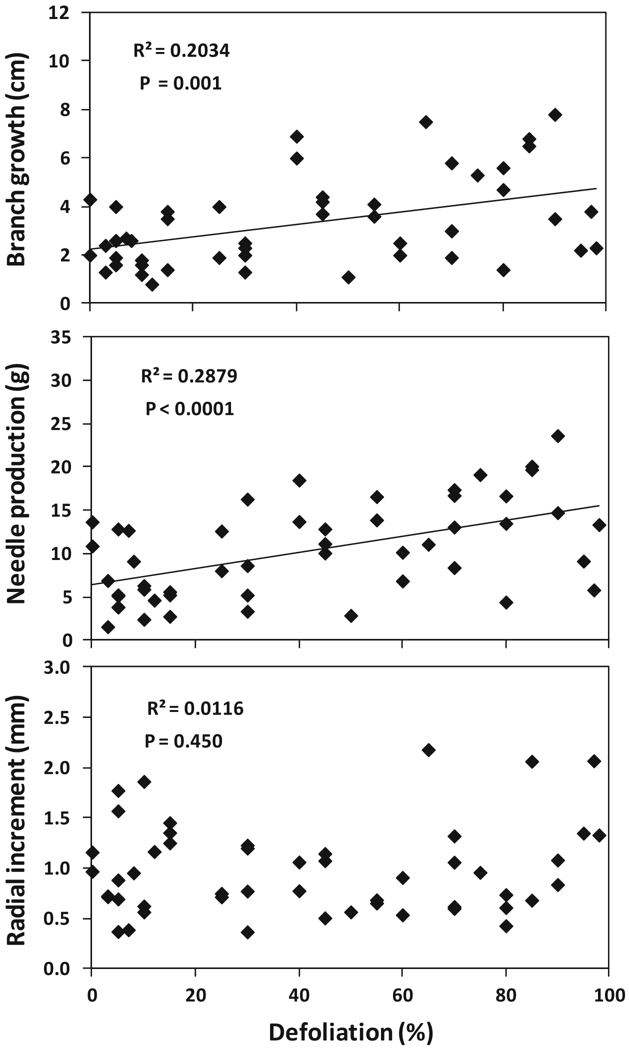

Fig. 3 Relationship between the percentage of canopy defoliation by the PPM in April 2011 and the branch growth, foliage production (biomass of current-year needles) and radial increment (tree ring width of the current year) of *P. nigra* trees included in the short-term experiment and measured in September 2011. Regression lines are shown only when significant ($P < 0.05$)

Discussion

Transient C limitation after PPM defoliation

Defoliation by PPM decreased the NSC concentrations of trees leading to a short-term C (source) limitation. The observed decrease in SS, starch and NSC concentrations proportional to defoliation in April indicates trees had already started remobilizing C stores from their old needles to recover from damage before bud burst. This does not seem to be the case for N, which showed an opposite trend, although increases were very moderate (see Fig. 2). However, in accordance with our hypotheses, such C limitation was only transient and *P. nigra* trees were able to replenish their NSC stores in just one growing season. Similarly, trees subjected to extreme repeated defoliation events were able to compensate C losses in the course of an average PPM outbreak cycle. This may enhance their chances to survive subsequent defoliations. Altogether, these results seem to indicate reported (Hódar et al. 2003; Kanat et al. 2005) and observed (Table 2) declines in tree growth and/or reproduction after PPM attacks are not caused by a C (source) limitation.

The fast recovery of NSC concentrations in our experiment responded to a shift in biomass allocation to foliage, as indicated by the significantly greater biomass of current-year needles in more severely defoliated trees (Fig. 3). However, compensatory increases in the photosynthesis of trees may have also contributed to the recovery of NSC concentrations (Eyles et al. 2011; Pinkard and Beadle 1998; Pinkard et al. 1998; Vanderklein and Reich 1999). According to our results, increased foliage N (hence Rubisco) concentrations could be partly responsible for the fast recovery of the NSC status through an up-regulation of the photosynthetic ability of trees (Hoogesteger and Karlsson 1992). Although we did not measure the photosynthetic rate of trees in this study, the SEM analysis indicated a strong positive effect of needle N concentrations in April on foliage NSC replenishment in September (Table 5). In addition, needle N concentration increased with defoliation immediately after damage (Fig. 1) and the situation persisted at the end of the growth season (Fig. 2) and even 6 years after the attacks (Table 2). Similarly, Hódar et al. (2004) found increased N concentrations in the needles of pine trees defoliated by the PPM.

Increased sink strength after defoliation (Stitt et al. 1991) could have also contributed to up-regulate photosynthesis. Pinkard et al. (2007) found compensatory increases in the photosynthetic rate of defoliated *Eucalyptus globulus* trees were positively related to the level of damage, which they attributed to a regulation of photosynthetic responses by increased sink strength. Similarly, we found the recovery of NSC pools and concentrations of trees to be proportional to damage (Fig. 2). Recent advances indicate leaf starch and sucrose concentrations can act as regulators of photosynthesis, accumulation of carbohydrates in leaves leading to a down-regulation of the photosynthetic rate (Kasai 2008).

Interestingly, we found no relationship between the NSC status of trees soon after defoliation and their ability to re-grow (Table 5; Fig. 4). This may indicate the re-growth of defoliated *P. nigra* trees was primarily supplied by current assimilation. Our results for the short-term experiment show needle NSC concentrations were more sensitive to defoliation than stem sapwood ones. In addition, previous studies indicate early twig growth of pine trees largely depends on current-year photosynthates (Hansen and Beck

Table 5 Input variables and standardized path coefficients (*P* values, in parentheses) of the different predictor → predictand relationships included in the structural equation models ($n = 50$ in all cases) built with varying combinations of the different data sets available (see input variables)

	Data 1	Data 2	Data 3	Data 4	Data 5
Input variables					
NSC[a]	Needles	Needles	Needles	Sapwood	Sapwood
Growth[b]	Needle BM	Branch length	Ring width	Needle BM	Branch length
Relationships					
Defoliation → NSC (April)	**−0.699 (<0.001)**	**−0.699 (<0.001)**	**−0.699 (<0.001)**	**−0.340 (0.011)**	**−0.340 (0.011)**
Defoliation → N (April)	**0.396 (0.003)**	**0.396 (0.003)**	**0.396 (0.003)**	**0.396 (0.003)**	**0.396 (0.003)**
NSC (April) → growth	0.009 (0.959)	0.017 (0.917)	0.023 (0.906)	−0.187 (0.135)	−0.211 (0.089)
Defoliation → growth	0.286 (0.104)	0.333 (0.058)	0.043 (0.832)	0.221 (0.102)	0.254 (0.058)
N (April) → growth	**0.366 (0.005)**	**0.329 (0.012)**	0.203 (0.182)	**0.357 (0.005)**	**0.319 (0.012)**
Growth → NSC (Sept.–April)	−0.104 (0.079)	**−0.117 (.045)**	−0.048 (0.370)	−0.171 (0.139)	**−0.303 (0.006)**
NSC (April) → NSC (Sept.–April)	**−0.907 (<0.001)**	**−0.912 (<0.001)**	**−0.890 (<0.001)**	**−0.763 (<0.001)**	**−0.806 (<0.001)**
N (April) → NSC (Sept.–April)	**0.365 (0.005)**	**0.167 (0.004)**	**0.130 (0.019)**	−0.016 (0.887)	0.033 (0.751)

Note these models were not considered in the main SEM analysis represented in Fig. 4, since they showed a poorer fit than the selected model number 6 (see Table 4 for indexes of goodness of fit). All models conformed to the same theoretical model shown in Fig. 4. Significant effects (at $\alpha = 0.05$) are indicated in bold

NSC (April) and NSC (Sept.–April): non-structural carbohydrate concentrations measured in April and difference between April and September 2011 NSC concentrations, respectively; N (April): nitrogen concentrations measured in April 2011

[a] Indicates origin of the NSC data included in the models

[b] Abbreviations as in Table 4

1990, 1994; von Felten et al. 2007). Defoliation may increase the dependence of twig growth on current photosynthesis in pine. Indeed, in our study, foliage N concentrations in April were significantly and positively related to leaf biomass production and branch length increment (Table 5), suggesting current photosynthesis was more important than stores in leaf biomass and primary growth recovery of *P. nigra* trees. The lack of a similar response in radial growth (Table 5; Fig. 4) could be attributed to the observation that wood formation in pines is supported by a mixture of stored and current-year C (von Felten et al. 2007).

Defoliation effects on growth

We observed no significant short-term effects of defoliation on stem wood growth and even significant positive effects on leaf biomass production and branch length growth (Fig. 3). These results seem to contradict previous reports of decreased radial and height growth on pine trees after PPM attacks (Hódar et al. 2003; Kanat et al. 2005) and our own results for the long-term defoliation experiment (Table 2). The explanation to these apparent discrepancies may lay in the different approaches followed. While most previous studies, and also our long-term experience, compared undefoliated trees with severely defoliated ones (Kanat et al. 2005), trees in our short-term experiment were selected over a range of damage from 0 to 100 % defoliation, so that the number of trees with very severe damage (above 80 %) was low. Hódar et al. (2003) analyzed the impact of PPM defoliation intensity on the growth rate of the leader shoots of *P. sylvestris* and found that increased intensities of defoliation led to progressive decreases in shoot growth rate. Although no direct statistical comparison between control and defoliated trees subjected to different intensities of damage was performed, their results indicate that only those trees subjected to high defoliation intensities show markedly reduced growth as compared to control trees (see Fig. 1 in Hódar et al. 2003). Consequently, when comparing control and defoliated trees, only severe defoliation treatments seem to produce significant different abilities in trees to compensate defoliation damage. For pine trees, removal of up to 80 % of leaves has been shown to have no significant impact on the lateral branch growth of trees (Handa et al. 2005). Consequently, most of our observations in the short-term experiment may have fallen below the level of damage for compensation growth to be detected, leading to the observed results.

In any case, the results from our short-term experiment indicate that *P. nigra* trees are able to recover efficiently from moderate (and punctual) PPM defoliations, and illustrate some of the mechanisms used to achieve such recovery (see above). Contrastingly, our long-term experiment provides information about the potential impacts of severe and repeated PPM defoliations on *P. nigra* growth

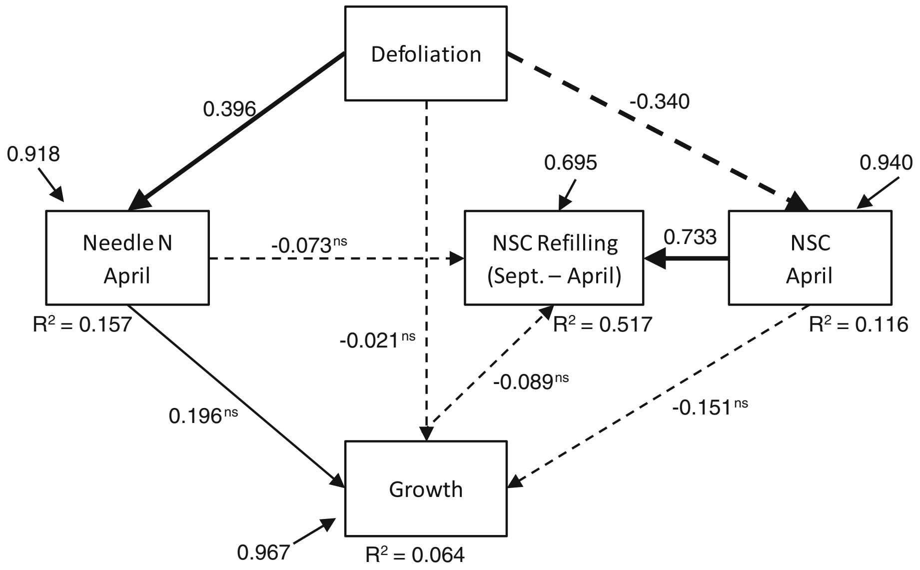

Fig. 4 Structural equation model showing the relationships between the non-structural carbohydrate (NSC) and nitrogen (N) concentrations and the growth of *P. nigra* trees included in the short-term experiment of PPM defoliation. Data used for NSC and N concentrations belong to sapwood and needles, respectively; while *growth* represents current-year tree ring width ($n = 50$). These variables were selected following model 6, the one with the best goodness of fit (see Table 4). Results for models considering NSC in needles and branch growth processes are summarized in Table 5. *Solid* and *dashed arrows* indicate positive and negative effects, respectively. *Arrow widths* are proportional to the magnitude of standardized path coefficients. *ns* indicates paths were non-significant at $\alpha = 0.05$. Unexplained variance, i.e., error terms, of each observed variable are indicated by *arrows* located near response variables. The proportion of explained variance (R^2) is interpreted similarly to a regression analysis

and NSC stores. Our results demonstrate that although repeated severe defoliation by PPM can have a strong impact on the growth and survival of *P. nigra* trees, 66 % of the trees were able to survive the extreme treatments imposed and they did not show C starvation.

Interactions between NSC accumulation and growth after defoliation

Defoliation led to increased accumulation of C storage compounds in *P. nigra* trees in both the short- and the long-term while re-growth was only maintained under moderate damage. Correlation analyses and SEM indicated that, when damage was moderate, as in our short-term experiment, C-store accumulation and re-growth after defoliation were generally unrelated. The only component of growth that seemed to have a negative impact on NSC accumulation was branch elongation, with branches that grew more showing smaller surpluses of NSC for accumulation (Table 5). Indeed, in our short-term experiment, trees with higher defoliation levels showed both higher NSC pools and concentrations and re-growth than trees with less damage (Figs. 2, 3). These results are in agreement with our third hypothesis.

However, in trees subjected to extreme and recurrent defoliations (such as our long-term treatments), 33 % of trees died, and for those trees that survived, growth was not recovered to the same extent as NSC concentrations. The increase in stem NSC concentrations indicates these trees were not limited by C availability (Hoch et al. 2003; Körner 2003). What, then, limited their growth? We suggest repeated extreme defoliations reduced the sink strength of growing meristems (sink limitation), so that the surplus of fixed carbohydrates not invested in growth accumulated as storage compounds. In line with this suggestion, indole-3-acetic acid (IAA), which favours assimilate import into sink organs (Darussalam et al. 1998), has been shown to decrease near the cambial region of pruned *P. sylvestris* trees (Sundberg et al. 1993), and this has been related to reduced radial growth after pruning (Thomas et al. 2006).

In conclusion, our results show *P. nigra* trees respond to defoliation by accumulating carbohydrate storage compounds. Defoliation by PPM caused only a transient C (source) limitation in *P. nigra* trees, and after just one growing season, trees were even able to over-compensate initial NSC losses. In addition, within the average periodicity of PPM outbreaks, trees are able to fully recover their initial NSC concentrations. When defoliation is moderate, we suggest the sink strength of growing meristems is not decreased and the compensatory increase in the photosynthetic ability of trees enables them to recover both growth and initial NSC status. However, when damage is extreme and recurrent, the sink strength of growth processes may be reduced leading to decreased tree growth and surplus sugars being accumulated as NSC.

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