



Research paper

Active summer carbon storage for winter persistence in trees at the cold alpine treeline

Mai-He Li^{1,2,3,†}, Yong Jiang^{3,†}, Ao Wang^{1,2}, Xiaobin Li⁴, Wanze Zhu⁵, Cai-Feng Yan⁶, Zhong Du⁷, Zheng Shi⁸, Jingpin Lei^{9,10}, Leonie Schönbeck², Peng He^{2,3}, Fei-Hai Yu¹ and Xue Wang^{1,2,3,11}

¹Zhejiang Provincial Key Laboratory of Plant Evolutionary Ecology and Conservation, Taizhou University, Taizhou 318000, China; ²Forest dynamics, Swiss Federal Research Institute WSL, Zuercherstrasse 111, CH-8903 Birmensdorf, Switzerland; ³Institute of Applied Ecology, Chinese Academy of Sciences, Shenyang 110016, China; ⁴State Key Laboratory for Microbial Metabolism and School of Life Sciences and Biotechnology, Shanghai Jiaotong University, Shanghai 200030, China; ⁵Institute of Mountain Hazard and Environment, Chinese Academy of Sciences, Chengdu 610041, China; ⁶Institute of Environment and Sustainable Development in Agriculture, Chinese Academy of Agricultural Sciences, Beijing 100081, China; ⁷College of Land and Resources, China West Normal University, Nanchong, Sichuan 637002, China; ⁸Research Institute of Forest Ecology, Environment and Protection, Chinese Academy of Forestry, Beijing 100091, China; ⁹Research Institute of Forestry, Chinese Academy of Forestry, Beijing 10091, China; ¹⁰Co-innovation Centre for Sustainable Forestry in Southern China, Nanjing Forestry University, Nanjing 210037, China; ¹¹Corresponding author (wangxueiae@163.com)

Received September 19, 2017; accepted February 22, 2018; handling Editor Peter Millard

The low-temperature limited alpine treeline is one of the most obvious boundaries in mountain landscapes. The question of whether resource limitation is the physiological mechanism for the formation of the alpine treeline is still waiting for conclusive evidence and answers. We therefore examined non-structural carbohydrates (NSC) and nitrogen (N) in treeline trees (TATs) and low-elevation trees (LETs) in both summer and winter in 11 alpine treeline cases ranging from subtropical monsoon to temperate continental climates across Eurasia. We found that tissue N concentration did not decrease with increasing elevation at the individual treeline level, but the mean root N concentration was lower in TATs than in LETs across treelines in summer. The TATs did not have lower tissue NSC concentrations than LETs in summer. However, the present study with multiple tree species across a large geographical scale, for the first time, revealed a common phenomenon that TATs had significantly lower NSC concentration in roots but not in the aboveground tissues than LETs in winter. Compared with LETs, TATs exhibited both a passive NSC storage in aboveground tissues in excess of carbon demand and an active starch storage in roots at the expense of growth reduction during the growing season. This starch accumulation disappeared in winter. Our results highlight some important aspects of the N and carbon physiology in relation to season in trees at their upper limits. Whether or to what extent the disadvantages of winter root NSC and summer root N level of TATs affect the growth of treeline trees and the alpine treeline formation needs to be further studied.

Keywords: alpine treeline, altitudinal gradient, carbon balance, nitrogen, non-structural carbohydrates, starch, sugars.

Introduction

The alpine treeline is one of the most obvious boundaries in mountain landscapes and is globally determined by a growing season soil (–10 cm) temperature of 6.5 ± 0.8 °C (Körner and Paulsen 2004). Recently, Li et al. (2017) found that a critical minimum air temperature of 0.7 ± 0.4 °C limited xylogenesis

and thus led to formation of the *Abies georgei* var. *smithii* treeline on Sygera Mountains on the southeastern Tibetan Plateau. Trees at their upper elevational limits, thus, were highly sensitive to global warming, exhibiting increased growth rate and stem density, changes in species composition and upward migration (Liang et al. 2011, 2016, Hagedorn et al. 2014, Qi et al.

[†]These authors contributed equally to this work.

2015). All these trees' processes are closely related to the availability of the non-structural carbohydrates (NSC) produced by photosynthesis (Kozłowski 1992) and nitrogen (N) storage (Millard and Thomson 1989, Millard and Proe 1992). However, what is the carbon- and N physiological mechanism for the formation of the alpine treeline landscape or pattern is still awaiting conclusive evidence and answers, as questioned very recently in the *Journal of Ecology* (Fajardo and Piper 2017).

The availability of NSC in a plant can be presented by the concentration and pool size (concentration \times biomass), reflecting a plant's carbon balance between the carbon supply and carbon demand (Woodruff and Meinzer 2011, Michelot et al. 2012). The major compounds of NSC are starch and some low molecular weight carbohydrates (soluble sugars). A lower level of plant tissue NSC is assumed to indicate carbon shortage for growth needed, whereas a high NSC level or an increase in NSC storage might occur when carbon supply exceeds carbon demand. Recent physioecological studies have thus tried to find out whether the increased growth rates of trees at their upper limits and whether the upward migration of the alpine treeline in response to past climate warming are caused by an increased carbon supply (Li et al. 2008a, Gruber et al. 2011, Wieser et al. 2014); a conclusive answer is, however, still lacking.

Many studies have investigated the carbon balance of trees at the alpine treeline (TATs = treeline trees) in different parts of the world (Körner 2003, Shi et al. 2006, Li et al. 2008b, Sveinbjörnsson et al. 2010, Fajardo et al. 2011, Gruber et al. 2011, Zhu et al. 2012a, 2012b; Yu et al. 2014) and found an increase in NSC with increasing elevation during the growing season (Hoch et al. 2002, Shi et al. 2008, Fajardo et al. 2011, Dang et al. 2015). However, NSC levels in trees are known to exhibit seasonal variations (Fischer and Holl 1992, Barbaroux and Breda 2002, Zhu et al. 2012a). Such a seasonal variation in tissue NSC level of plants reflects the biological rhythms including photosynthesis, growth dynamics, respiration and the trade-off between vegetative and reproductive growth, which all are associated with the seasonality of environmental factors (Wurth et al. 2005). In winter, except for the tropical alpine treeline, for example, the photosynthetic production and carbon-utilization of respiration in TATs may be negligible. The winter NSC storage is, however, very important for winter survival and re-growth in early spring of the next year (Zhu et al. 2012b), which may contribute to the alpine treeline formation (Li et al. 2008a, 2008b). Hence, investigations on winter NSC storage would be expected to yield better insights into the carbon balance in trees (Landhaeusser and Loeffers 2012).

However, the status of winter NSC in TATs or in trees at high elevations where it is normally inaccessible in winter due to deep snow cover has been rarely studied and is poorly understood. Li et al. (2008a, 2008b) investigated the tissue NSC of TATs and trees at lower elevations (LETs = low-elevation trees) in the Himalayas after the dormant season (i.e., at the early beginning of growing season) and proposed a winter carbon shortage in TATs.

To confirm such a winter carbon shortage, Zhu et al. (2012a) studied the monthly variation of tissue NSC in *Quercus aquifolioides* along an elevational gradient up to its upper limit in southwestern China, and found that the NSC concentrations in carbon-storage tissues decreased with increasing elevation in winter. Their results (Zhu et al. 2012a), for the first time, clearly indicated a winter carbon shortage in the storage tissues in TATs. As they studied only one species in only one treeline case, it is thus questioned whether their results are a common feature or not.

Plant N is a major component of Rubisco and other photosynthetic enzymes and structures, which further regulates photosynthesis, and thus determines carbon supply for growth and carbon balance in a plant (Bond et al. 1999, Ripullone et al. 2003, Lewis et al. 2004). Nitrogen itself, as most ecosystems are N-limited (Vitousek and Howarth 1991, Elser et al. 2007, LeBauer and Treseder 2008), was found to play an important role in controlling the trees' distribution at the upper limits in northwest Alaska (Sullivan et al. 2015). Nitrogen-limitation can directly (Millard and Grelet 2010), and may also indirectly through carbon source–sink relations, determine the growth of trees at the alpine treeline (Fajardo and Piper 2017). For example, Thébault et al. (2014) suggested an N-limitation for the alpine treeline formation, although most works have indicated that plant N availability increases, or at least does not decrease, with elevation (Körner 1989, Li et al. 2008a). Previous studies suggested that increased leaf N concentration with elevation is a physiological adaptation of plants to lower temperature (Weih and Karlsson 2001, Oleksyn et al. 2002). Given those contrasting results, more studies with different plant species are needed to clarify the general pattern of N in trees/plants at the upper limits.

Given this large body of literature, a conclusive feature of the alpine treeline landscape pattern in relation to plant carbohydrates and N is still lacking. According to Körner (2003) and Millard and Grelet (2010), N allocation to storage in trees is seasonally (i.e., phenologically) programmed, and carbon allocation to NSC is a passive buffer, we, therefore, hypothesized that: (i) TATs do not have lower tissue N concentration than LETs across time, and thus, tissue N does not play a role in determining the alpine treeline pattern since N can be reallocated to the points where N is needed; and (ii) a root NSC shortage in winter is a common feature of the alpine treeline trees across scales, because the short growing season at the alpine treeline limits photosynthate production, and thus, a passive NSC storage in excess of carbon demand will not occur. We, therefore, carried out a large-scale investigation of NSC and N in TATs compared with LETs in both summer and winter, to test these hypotheses.

Materials and methods

Alpine treeline sites

We investigated eight alpine treelines in China and three treelines in the Swiss Alps. We tried to select the natural alpine treeline for

the present study. A natural treeline was defined as the climatic upper distribution of trees without visible disturbance and suppression. The 11 treelines selected comprised of three broad-leaved trees (one deciduous and two evergreen) and eight conifers (of which one was deciduous). The treelines ranged from subtropical monsoon (Zheduo Mt, Balang Mts, Xuebaoding Mts and Minshan Mts), dry-temperate continental climate (Qilian Mts, Tianshan Mts), to humid (the three Swiss treelines) and wet-continental climate (Changbai Mt) (Table 1). The elevational position of the treelines varied greatly across latitudes from 30°N to 47°N, and longitudes from 7°E to 130°E (Table 1, Figure 1). Given the large difference in elevation of the treeline position associated with the large geographic scale, irrespective of the length of the growing season, the growing season soil temperatures at the treeline position across the 11 treelines studied are the same, being 6.5 ± 0.8 °C (Körner and Paulsen 2004), which makes the present large-scale comparison reasonable.

At each treeline, three to six plots (10 m × 10 m) were selected along the upper limit of trees (upright individuals with a height of >2 m) and along a lower elevation in closed forest with the same tree species (Table 1). Tree tissues were sampled twice (summer, winter) in each plot (Table 1). Height and diameter at breast height of trees in each plot were measured during the summer sampling (Table 1). To minimize the difference in phenology of trees between the upper limit and the lower elevation at a treeline site, the 'summer' sampling occurred during the

warmest period (July–August) and the 'winter' sampling was carried out after snowing when the lower sample elevation at a treeline was also already covered by thin snow.

In each plot, 6–10 trees were randomly selected for tissue sampling for each sampling season. Current-year mature leaves, stem sapwood and fine roots (<0.5 cm in diameter) were collected from each sample tree in both summer and winter (date listed in Table 1). The winter sampling was carried out only for eight treelines (Table 1) where they were accessible in winter.

Analysis of NSC and N

The powdered leaf material (0.1 g) was put into a 10 ml centrifuge tube, and 5 ml of 80% ethanol were added. The mixture was incubated at 80 °C in a water bath-shaker for 30 min, and then centrifuged at 3500 rpm for 10 min. The pellets were extracted two more times with 80% ethanol. Supernatants were retained, combined and stored at 4 °C to determine the total soluble sugars (Li et al. 2008b).

The ethanol-insoluble pellet was used for the starch extraction. Ethanol was removed by evaporation. Starch in the residue was released in 2 ml distilled water for 15 min in a boiling water-bath. After being cooled to room temperature, 2 ml of 9.2 M HClO₄ were added. Starch was hydrolyzed for 15 min. 4 ml distilled water was added to the samples, which were then centrifuged at 4000 rpm for 10 min. The pellets were extracted one more time with 2 ml of 4.6 M HClO₄. Supernatants were

Table 1. Characteristics of the treeline (TL) sites.

Treeline No.	Treeline sites	Species	Longitude (E)	Latitude (N)	Elevation (m a.s.l.)	Height (m)	DBH (cm)	Sampling dates (day/month/year)	
China									
TL1	Mt Zheduo	<i>Quercus aquifolioides</i> Rehd. et Wils.	101°55'	30°01'	3950	2.21 ± 0.11	5.9 ± 0.4	18/07/2014	08/11/2014
					3000	2.95 ± 0.25	7.0 ± 0.7	18/07/2014	08/11/2014
TL2	Mt Balang	<i>Abies faxoniana</i> Rehd.	102°58'	30°51'	3670	7.5 ± 0.4	28.7 ± 2.1	16/07/2014	06/11/2014
					2860	14 ± 1.7	46.8 ± 12.5	16/07/2014	06/11/2014
TL3	Mt Balang	<i>Quercus aquifolioides</i> Rehd. et Wils.	102°45'	30°53'	3590	2.9 ± 0.2	7.3 ± 0.9	15/07/2014	05/11/2014
					2840	4.9 ± 0.1	9.1 ± 1.5	15/07/2014	05/11/2014
TL4	Mt Xuebaoding	<i>Picea purpurea</i> Mast.	103°39'	32°44'	3580	7.5 ± 3.7	25.2 ± 3.7	10/07/2014	
					3100	15.6 ± 5.1	31.3 ± 4.2	10/07/2014	
TL5	Mt Minshan	<i>Abies faxoniana</i> Rehd.	103°53'	32°55'	3500	12.5 ± 1.9	32.2 ± 6.59	07/07/2014	
					2840	15.4 ± 1.0	32.9 ± 2.95	06/07/2014	
TL6	Mt Qilian	<i>Picea crassifolia</i> Kom.	100°17'	38°34'	3250	11.2 ± 0.5	24.7 ± 1.0	06/08/2014	02/11/2014
					2540	13.7 ± 0.9	34.9 ± 1.8	06/08/2014	02/11/2014
TL7	Mt Changbai	<i>Betula ermanii</i> Cham.	128°03'	42°03'	2030	4.2 ± 0.6	6.9 ± 1.6	21/08/2014	18/10/2014
					1700	13.2 ± 1.7	38.2 ± 2.7	21/08/2014	18/10/2014
TL8	Mt Tianshan	<i>Picea schrenkiana</i> var. <i>tianshanica</i> (Rupr.) Cheng et L. K. Fu	87°07'	43°13'	2580	7.4 ± 4.5	19.68 ± 4.8	22/07/2014	
					2100	18.7 ± 5.2	33.44 ± 2.7	21/07/2014	
Switzerland									
TL9	Davos	<i>Pinus cembra</i> L.	9°56'	46°45'	2120	6.0 ± 2.6	10.3 ± 1.2	07/07/2015	25/10/2015
					1840	6.33 ± 1.5	11.5 ± 0.9	07/07/2015	25/10/2015
TL10	Verbier	<i>Picea abies</i> (L.) Karst.	6°58'	46°14'	2180	2.8 ± 0.8	4.9 ± 0.9	09/07/2015	28/10/2015
					1890	4.7 ± 1.1	8.0 ± 1.5	09/07/2015	28/10/2015
TL11	Chandolin	<i>Larix decidua</i> Mill.	7°34'	46°15'	2200	5.3 ± 1.1	9.1 ± 1.7	09/07/2015	28/10/2015
					1900	6.0 ± 1.0	11.7 ± 1.9	09/07/2015	28/10/2015

DBH = diameter at breast height.

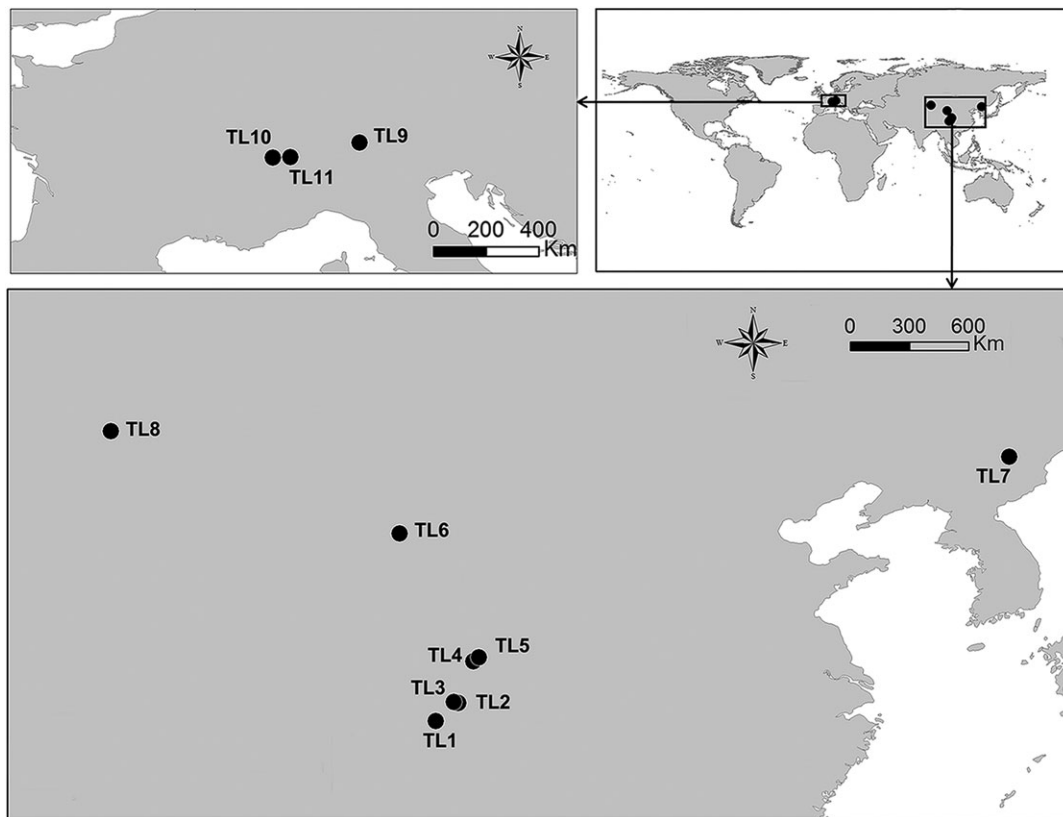


Figure 1. The geographic positions of the 11 investigated treelines (TLs). See Table 1 for detailed information.

retained, combined and filled to 25 ml to determine starch. The soluble sugar and starch concentrations were measured spectrophotometrically (ultraviolet-visible spectrophotometer 752 S, Cany Precision Instruments Co., Ltd, Shanghai, China) at 620 nm using the anthrone method, and the starch was calculated by multiplying the glucose concentrations by a conversion factor of 0.9. Glucose was used as the standard.

A carbon/N analyzer (vario MICRO cube, Elementar, Hanau, Germany) was used to analyze the total N.

Calculation

Tissue NSC was calculated as the sum of the concentration of soluble sugars plus the starch concentration for each tissue for each sampling season.

To understand the elevational variations of N and NSCs (sugars and starch) within each tissue type between TATs and LETs, an index (%) of 'TATs – LETs changes' in N and NSCs was defined and calculated for summer and winter separately (see Figures 4 and 5), using:

$$x = \frac{N \text{ (or NSCs) in TAT} - N \text{ (or NSCs) in LET}}{N \text{ (or NSCs) in LET}} \times 100\%$$

where N or NSCs is concentration of N, sugars or starch in a tissue type for the same sampling season; this index represents

the net changes in NSCs between TATs and LETs for the same season.

To understand the seasonal variations of sugars and starch within each tissue type, an index (%) of 'winter – summer changes' in NSCs was defined and calculated for TATs and LETs separately (see Figure 6), using:

$$x = \frac{\text{Winter NSCs} - \text{Summer NSCs}}{\text{Summer NSCs}} \times 100\%$$

where NSCs is concentration of sugars or starch in a tissue type within the same tree groups (TATs or LETs); this index represents the net changes in NSCs between seasons.

Data analysis

The normality of the distribution was checked by quantile–quantile plots before any statistical analyses. A mixed effects model was performed to test the effects of tissue type, elevation and their interactions on tissue NSC and N, with tissue type and elevation as fixed effects and treeline as a random effect to account for between-treeline variance in summer and winter, respectively. *T*-test was conducted to test the different responses of mobile carbohydrates and nutrients between high and low elevation, with the significance at $P < 0.05$. All statistical analyses were performed using R version 3.2.3 (<http://www.r-project.org>).

Results

Summer tissue NSC and N

In summer, different plant tissues had different elevational trends in NSC and N concentrations (both $P < 0.001$, Table 2), showing non-significant interactions between tissue type (T) and elevation (E) on NSC and N.

Tissue NSC and N concentrations significantly varied with tissue type (both $P < 0.001$, Table 2, Figure 2). Across all tree-lines, the tissue NSC and N concentrations followed a decreased order of leaves (16.3% for NSC, 1.3% for N), fine roots (9.5% for NSC, 0.5% for N) and stem sapwood (3.3% for NSC, 0.2% for N) (Figure 2a–f). In contrast, mean tissue NSC/N ratio was highest in stem sapwood (24), followed by fine roots (21) and leaves (14) (Figure 2g–i).

Elevation did not influence tissue N and NSC/N ratio (both $P > 0.05$, Table 2, Figure 2d–i) but significantly affected tissue NSC concentrations ($P = 0.018$, Table 2). Overall, tissue NSC levels in TATs were not lower than those in LETs (Figure 2a–c).

Winter tissue NSC and N

In winter, different plant tissues had different elevational trends in NSC concentrations, showing a significant T \times E interaction on NSC ($P < 0.001$, Table 2). Overall, TATs, compared with LETs, did not show any disadvantages in NSC concentrations in above-ground tissues (leaves, stem sapwood) (Figure 3a and b), but fine root NSC concentrations were significantly lower in TATs than in LETs (Figure 3c) in six out of eight treelines studied. Across the eight treelines, the mean root NSC concentration was 9.8% in TATs and 12.3% (+26%) in LETs.

Table 2. Effects of tissue and elevation on the concentrations of NSC (non-structural carbohydrates = soluble sugars + starch), N and NSC/N ratios of trees at 11 Eurasian alpine treelines and lower elevations across seasons, tested using a mixed effects model. The treeline was used as a random variable to account for between-treeline variance. The significant levels of $P < 0.05$ are shown in bold. *Tissue in winter only includes eight treelines.

	d.f.	NSC		N		NSC/N		Sugars		Starch		Sugars/starch	
		F	P	F	P	F	P	F	P	F	P	F	P
Summer													
Tissue (T)	2	225.72	<0.001	302	<0.001	7.94	<0.001	244.58	<0.001	51.69	<0.001	15.65	<0.001
Elevation (E)	1	5.67	0.018	3.14	0.077	0	0.507	0.56	0.453	6.31	0.013	22.38	<0.001
T \times E	2	1.16	0.316	1.387	0.252	0.79	0.456	1.29	0.278	0.95	0.388	4.79	0.009
Winter													
Tissue (T)*	2	67.98	<0.001	222.36	<0.001	10.46	<0.001	101.73	<0.001	9.94	<0.001	57.25	<0.001
Elevation (E)	1	3.1	0.08	0.47	0.495	0.29	0.59	3.29	0.071	0.76	0.385	0.7	0.404
T \times E	2	7.02	0.001	0.22	0.805	5.98	0.003	3.55	0.031	6.75	0.001	0.11	0.896

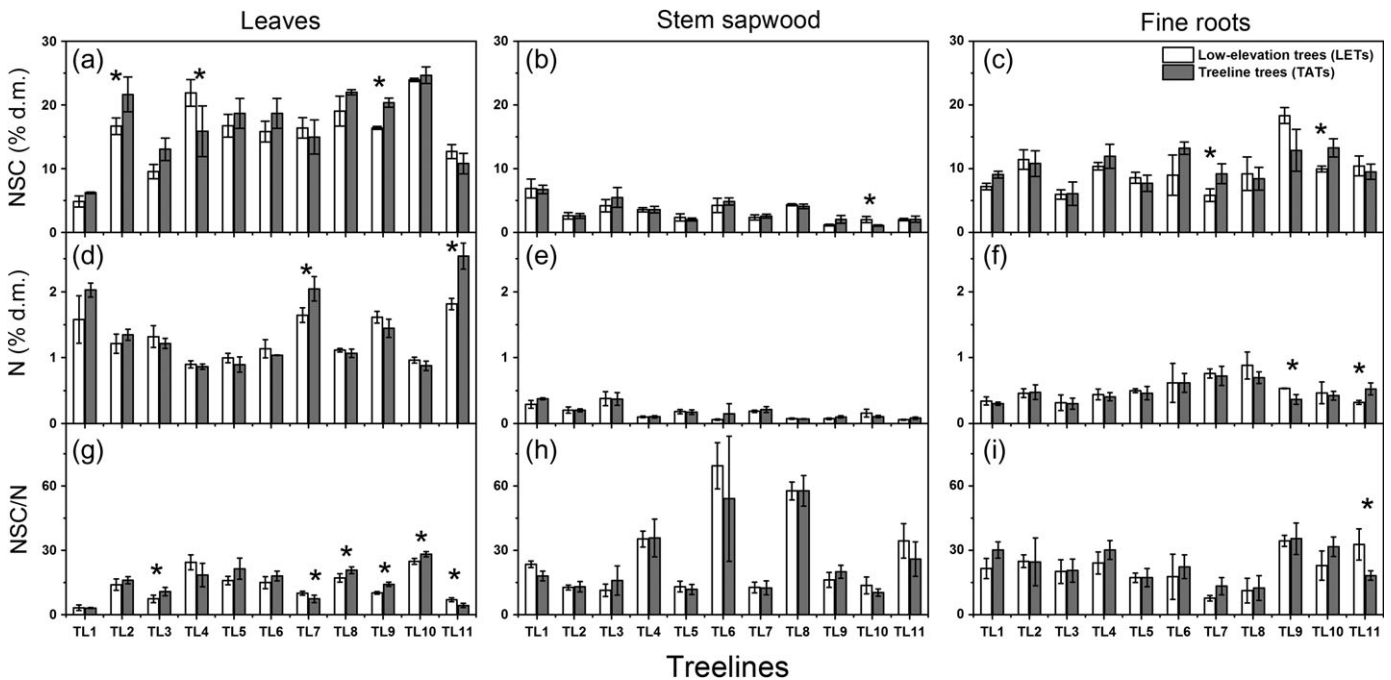


Figure 2. Non-structural carbohydrates (NSC) and N concentrations (% dry matter) and NSC/N ratios in leaves, stem sapwood and fine roots of trees at 11 Eurasian alpine treelines (TLs) and lower elevations in summer. The X-axis represents the 11 TLs given in Table 1. An asterisk indicates significant difference (T -test $P < 0.05$) between LETs and TATs for a given TL.

Unlike tissue NSC, different tissues had similar elevational trends in winter N concentrations because no $T \times E$ interaction on N was found (Table 2). Not elevation ($P > 0.05$) but tissue type ($P < 0.001$) had significant effects on tissue N concentrations (Table 2, Figure 3d–f). Across the eight treelines, leaves had the highest N (1.3%) concentrations, followed by fine roots (0.5%) and stem sapwood (0.4%) (Figure 3d–f). The mean tissue N across the eight treelines investigated also in winter showed that TATs stored more N in stem sapwood and roots but less in leaves than LETs in winter (Figure 4b), whereas the former had higher N concentrations in leaves and stem sapwood but lower concentration in roots than the latter in summer (Figure 4a), although the elevational effect on tissue N was not significant at the individual treeline level (Table 2).

Tissue NSC/N ratio was significantly affected by tissue type ($P < 0.001$, Table 2), and this tissue type effect was significantly influenced by elevation ($P = 0.003$ for a $T \times E$ interaction, Table 2). Fine roots (24) had the highest NSC/N ratio, then followed by stem sapwood (15) and leaves (12) (Figure 3g–i).

Active summer NSC storage in treeline trees

In summer, starch accumulated in TATs, showing that the starch concentrations were higher in TATs than LETs for all tissue types, and sugar concentrations were higher in leaves and stem sapwood but it was lower in fine roots in TATs than in LETs (Figure 5a).

In winter, the starch accumulation in TATs disappeared showing that the aboveground tissues (leaves, stem sapwood) of

TATs had higher sugar concentrations but lower starch concentrations than those in LETs (Figure 5b). Both sugars and starch in fine roots showed lower concentrations in TATs than in LETs (Figure 5b).

Summer starch accumulation in TATs converted to sugars, showing that TATs had higher sugar concentrations but lower starch concentrations in winter than in summer (Figure 6a), whereas LETs had higher concentrations of both sugars and starch in winter than in summer (Figure 6b).

Discussion

Tissue N–elevation relations of trees across seasons

The present large-scale study found that elevation had no effects on N levels in tree tissues across Eurasian treelines for both summer (Figure 2d–f) and winter (Figure 3d–f). In line with this finding, other large-scale investigations covering multiple species belonging to different functional groups and/or families also did not find any decreasing trends in mass-based leaf N concentrations with increasing elevation (He et al. 2006, Yan et al. 2013, Hong et al. 2014, Wang et al. 2015). A worldwide comparison by Kömer (1989) showed that plants, irrespective of life form, at higher elevations did not have less mass-based leaf N concentrations than plants at lower elevations. These multiple scale studies suggest that plants at high elevations exhibit N-physiological adaptation strategies to compensate for lower efficiency of physiological processes in low temperature environments (Reich and Oleksyn 2004).

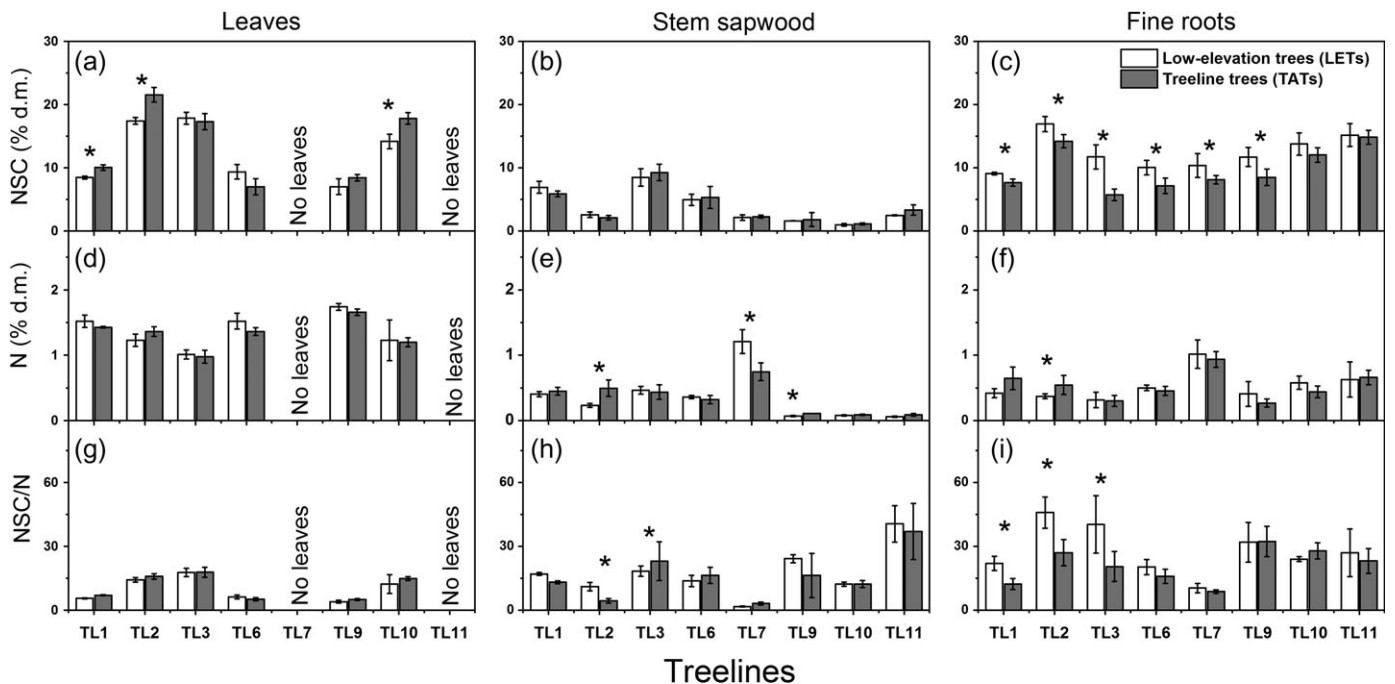


Figure 3. Non-structural carbohydrates (NSC) and N concentrations (% dry matter) and NSC/N ratios in leaves, stem sapwood and fine roots of trees at eight Eurasian alpine treelines (TLs) and lower elevations in winter. The X-axis represents the eight TLs given in Table 1. An asterisk indicates significant difference (T -test $P < 0.05$) between LETs and TATs for a given TL.

As the majority of studies indicated that plants do not decrease their tissue N concentrations with increasing elevation, there are also, however, local- and regional-scale studies that revealed lower N concentrations in plants at higher elevations than at lower elevations (Karlsson and Nordell 1996, Thébault et al. 2014, Sullivan et al. 2015), suggesting an N-limitation for plants at their upper limits. The explanation for N limitation could be slower soil N mineralization (Loomis et al. 2006, Thébault et al. 2014), lower rate of litter decomposition (Loomis et al. 2006) and less nutrient uptake (Karlsson and Nordell 1996) under low temperature. The exact reasons for such inconsistent results are unclear, and we may only speculate that such results of local-scale studies may be associated with very local properties of environmental factors or very specific species. For example, in the present study there was also an exception showing a significantly lower N concentration in stem sapwood in TATs than in LETs with *Betula ermanii* in TL7 in winter (Figure 3e).

Higher root N but lower leaf N concentration in TATs than in LETs at the end of season (Figure 4b) may indicate that the former reallocated much more N from leaves to storage in roots to reduce N loss caused by leaf senescence, abscission and browsing in winter (Millard and Grelet 2010). Lower root N but higher leaf N concentration in TATs than in LETs in summer (Figure 4a) suggests that TATs have tried to remobilize much more the N stored in roots to leaves for new growth, in order to grow somewhat more within the very short growing season at the alpine treeline. These findings supported the statement of Millard and Grelet (2010), who stated that N allocation to storage and remobilization for reuse in trees are seasonally programmed, and the annual growth of many tree species relies heavily on the internal cycling of N, by which N is stored over-winter and subsequently remobilized in spring for new growth (Millard and Thomson 1989, Millard and Proe 1992). However, whether the lower growing season root N in TATs indeed limits the growth of treeline trees and thus determines the alpine treeline formation need to be further studied.

Tree tissue NSC–elevation relation with time

In the 11 treeline cases studied, summer tissue NSC concentrations were not lower in TATs compared with those in LETs (Figure 2a–c), with the exception of leaf NSC in *Picea purpurea* (TL4, Figure 2a). Consistent with our results, tissue NSC of trees has been widely reported to increase, at least to not decrease, with increasing elevation up to the alpine treeline during the growing season (Hoch et al. 2002, Shi et al. 2008, Fajardo et al. 2011, 2013, Gruber et al. 2011, Hoch and Körner 2012, Zhu et al. 2012a, Yu et al. 2014). However, the present study found that overall there was a lower root NSC in TATs than in LETs in winter (Figure 3c). The difference in winter root NSC concentrations did not reach a significant level of $P < 0.05$ only for two out of the eight treelines investigated (Figure 3c); these two non-significant results may be caused by non-climatic treeline since the

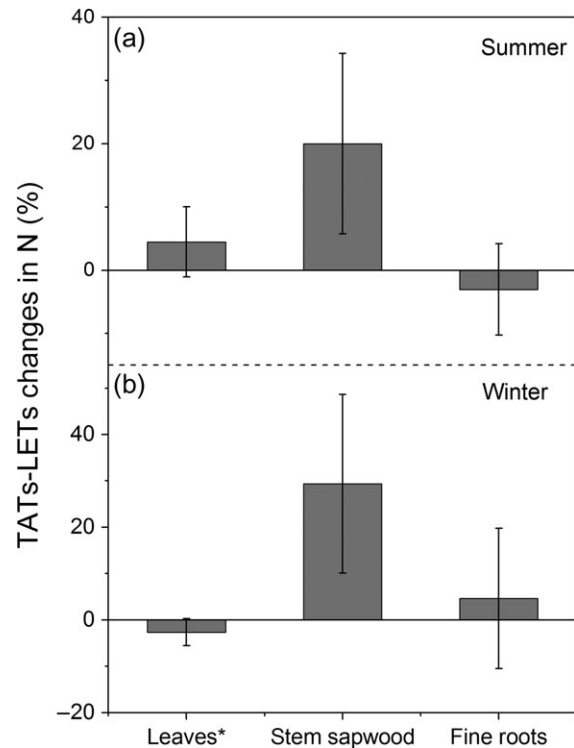


Figure 4. 'TATs – LETs changes' in N (%) for summer and winter, respectively, calculated with $x = \frac{N \text{ in TAT} - N \text{ in LET}}{N \text{ in LET}} \times 100\%$. *Leaves in winter include six evergreen treelines.

two treelines (TL10 Verbier and TL11 Chandolin in Valais, Switzerland) are located on ski mountains where the treeline might have been suppressed to a lower elevation. Through an investigation of the monthly variation of tissue NSC in *Q. aquifolioides* along an elevational gradient up to its upper limit in southwestern China, Zhu et al. (2012a) clearly indicated a NSC shortage in roots at high elevations in winter months. Genet et al. (2011) found that root NSC concentrations were significantly lower in *A. georgei* var. *smithii* trees at the alpine treeline (4330 m above sea level (a.s.l.)) than at 3480 m a.s.l. on Mt Sergyemla, the Tibetan Plateau. Recently, Dang et al. (2015) clearly indicated that NSC concentrations in stem wood and roots, measured in both early season and late season, significantly decreased with increasing elevation in *Abies fargesii* trees from closed forest to the alpine treeline (see Figures 3 and 4 in Dang et al. (2015)).

There are several possible reasons why winter roots have lower NSC concentrations in TATs compared with LETs. Previous studies suggested that thick snow cover at high elevations in winter would enhance the root carbon depletion by stimulating root respiration (Groffman et al. 2001, Scott-Denton et al. 2006). Thus the roots at high elevations in deep winter may deplete more carbon for respiration than those at lower elevations with thin or without snow cover, in the case of the warming effect by snow cover exceeding the elevational effects on soil temperature decrease. Another possibility is that the trees at low elevations may accumulate more NSC storage in all tissues

including roots at the end of growing season because of longer growing season and thus longer time of photosynthesis.

The aboveground plant parts exposed to the cold atmosphere at high elevations need to have high NSC concentrations to avoid intra- and intercellular ice formation (El Zein et al. 2011, Charrier et al. 2013). We thus may speculate that, to be able to protect tissue structure from freezing injury in cold environment, plants at high elevations need to reallocate mobile sugars from belowground tissues to aboveground tissues via diffusion and remobilization, to increase the NSC concentration in aboveground tissues, if the stem is not freezing (R. Zweifel, personal communication) and if soil water and temperature conditions allow xylem sap to flow. This may then result in decreased root NSC concentration with increasing elevation. Further, trees, especially evergreen species having over-wintering leaves, could try to store resources in aboveground tissues rather than in belowground tissues to reduce the costs of transport in fall and in next spring (Chapin et al. 1990), which may, again, result in lower root NSC concentrations of trees at high elevations.

Active and passive carbohydrate storage in trees at high elevations

Compared with LETs, TATs did not have less carbon supply for growth during the growing season (Figure 2a–c). However, the fact is that tree growth decreases with increasing elevation and tree growth ceases at a certain elevation, forming the alpine treeline (Li et al. 2003, Li and Yang 2004). The short growing season of ~3 months at the alpine treeline may result in small annual height increment but surely does not lead to height growth cessation. In mid-summer, the aboveground tissues in TATs had higher concentrations of both sugars and starch than in LETs (Figure 5a); this starch accumulation might be a passive storage, because the sugar levels exceeded the growth demand needed by TATs. However, for that growing time roots in TATs had lower sugar levels but much higher starch concentrations than those in LETs (Figure 5a), which may be an active storage process of NSC (Wiley and Helliker 2012) in TATs for safe over-wintering in the cold environment. In other words, roots of TATs tried already in mid-summer to increase their reversible storage (i.e., starch) at the expense of growth reduction, since the lower sugar concentrations in TATs' roots (Figure 5a) may imply less NSC available for root growth and thus small root growth rate in TATs compared with LETs (Li et al. 2003, Li and Yang 2004). Recently, whether NSC storage is a result of a passive process resulting from photosynthate production in excess of carbon demand or of an active process with priority over growth is debated (Wiley and Helliker 2012, Palacio et al. 2014). Our study provided direct evidence for both an active storage (roots in Figure 5a) and a passive storage (aboveground tissues in Figure 5a) of stressed trees and proved the previous findings, that starch is accumulated during the growing seasons to ensure continuous starch-to-sugar conversion during the cold period (Sauter 1988, Sauter and van Cleve 1991).

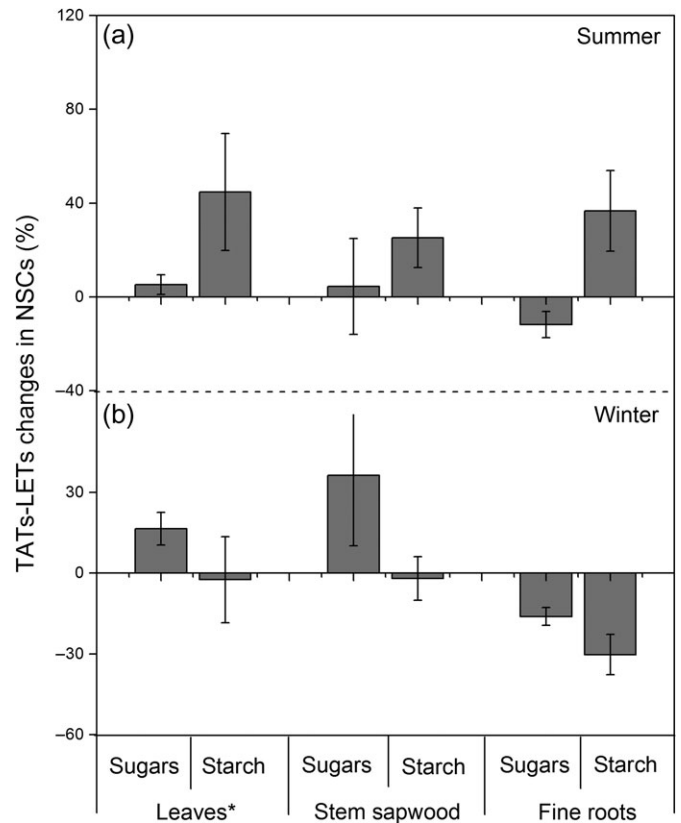


Figure 5. 'TATs – LETs changes' in NSC (%) for summer (11 treelines) and winter (8 treelines), respectively, calculated with $x = \frac{\text{NSCs in TAT} - \text{NSCs in LET}}{\text{NSCs in LET}} \times 100\%$. *Leaves in winter include six evergreen treelines.

Some months later, the greater summer starch concentrations in TATs compared with LETs (Figure 5a) disappeared, showing that tissues of TATs had lower starch concentrations than those in LETs (Figure 5b; see also Figure 3c). This is a result of the continuous starch-to-sugar conversion, because TATs need to have higher sugar concentrations (see Figure 5b) and thus higher osmotic pressure to reduce or/and avoid intra- and intercellular ice formation for safe over-wintering in the cold environment (Gail 1926, Duca 2015). The same reasons have then led to higher sugar concentrations but lower starch levels of TATs in winter compared with summer (Figure 6a).

However, the same starch–sugar conversion process, or at least the same conversion magnitude, seemed not to be necessary for LETs to safely over-winter at lower elevations, showing that LETs had higher concentrations of both starch and sugars in winter than in summer (Figure 6b). Classical, very early studies (Lidforss 1896, Miyake 1902) have already found that almost all the starch disappeared and the soluble sugars increased in over-wintering leaves of different trees grown in cold environments, but greater or less amounts of starch were still found in over-wintering leaves of trees in somewhat warm conditions. A recent study (Simard et al. 2013) indicated also that starch disappeared in winter, while mobile sugars

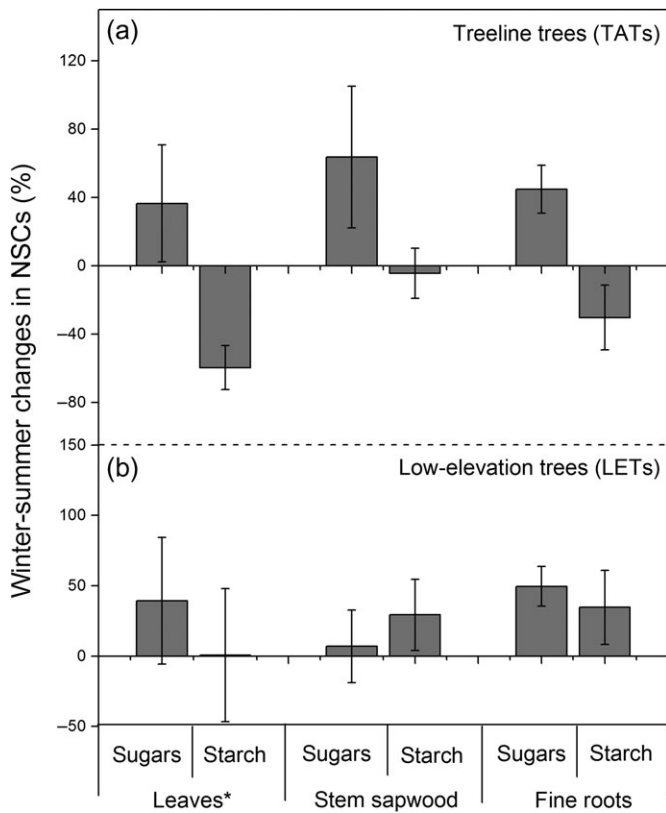


Figure 6. 'Winter – summer changes' in NSC (%) for treeline trees and low-elevation trees, respectively, calculated with $x = \frac{\text{Winter NSCs} - \text{Summer NSCs}}{\text{Summer NSCs}} \times 100\%$. *Leaves in winter include six evergreen treelines.

increased in *Picea abies* and *Larix decidua* trees grown at high elevations in the Alps.

Conclusion

Our results strongly support our second hypothesis that the alpine TATs showed a common feature of root winter NSC disadvantage compared with LETs. The alpine TATs exhibited both a passive NSC storage in aboveground tissues and an active storage in roots during the growing season (Figure 5a). This active storage supports the trees to survive over the cold winter at high elevations. In line with our first hypothesis, tissue N concentration did not decrease with increasing elevation at the individual treeline level, the mean value across treelines showed, however, that TATs had lower root N concentrations than LETs in summer (Figure 4a). Whether or to what extent these disadvantages of winter root NSC and summer root N affect the growth of treeline trees and the alpine treeline formation needs to be further studied, just as Fajardo and Piper (2017) stated very recently that 'the current global explanation for treeline formation (C-sink-limitation driven by low temperature) must be revisited'. To obtain a more precise feature of the carbon- or nutrient-physiological mechanisms for the

global alpine treeline formation, a future re-assessment needs to take into account not only the concentration of tissue NSC and nutrients, but also their pool size (concentration \times biomass), as well as their stoichiometry (Wang et al. 2018). The season-dependent root NSC-elevation relation (Figure 2c vs Figure 3c) implies a temperature-controlled root NSC balance, which suggests that global warming will enhance the root carbon supply in treeline trees and further lead to upward shift of the alpine treeline landscape boundary.

Funding

We sincerely thank Dr Peter Millard and three anonymous referees for their insightful comments on our paper. This work was supported by the National Natural Science Foundation of China (41371076, 31570501), the Strategic Priority Research Program of the Chinese Academy of Sciences (XDB15010302) and the Fundamental Research Funds for the Central Nonprofit Research Institution of CAF (CAFYBB2014ZD001), and is also partly supported by Sino-Swiss Science and Technology Cooperation (SSSTC) program (EG 06-032015).

References

- Barbaroux C, Breda N (2002) Contrasting distribution and seasonal dynamics of carbohydrate reserves in stem wood of adult ring-porous sessile oak and diffuse-porous beech trees. *Tree Physiol* 22:1201–1210.
- Bond BJ, Farnsworth BT, Coulombe RA, Winner WE (1999) Foliage physiology and biochemistry in response to light gradients in conifers with varying shade tolerance. *Oecologia* 120:183–192.
- Chapin FS, Schulze E-D, Mooney HA (1990) The ecology and economics of storage in plants. *Ann Rev Ecol Syst* 21:423–447.
- Charrier G, Cochard H, Ameziane T (2013) Evaluation of the impact of frost resistances on potential altitudinal limit of trees. *Tree Physiol* 33:891–902.
- Dang HS, Zhang KR, Zhang QF, Xu YM, Leegood R (2015) Temporal variations of mobile carbohydrates in *Abies fargesii* at the upper tree limits. *Plant Biol* 17:106–113.
- Duca M (2015) Physiology of plant resistance to unfavorable environmental factors. In: 'Plant physiology'. Springer, Dordrecht, The Netherlands, pp. 271–308.
- El Zein R, Maillard P, Breda N, Marchand J, Montpied P, Gerant D (2011) Seasonal changes of C and N non-structural compounds in the stem sapwood of adult sessile oak and beech trees. *Tree Physiol* 31:843–854.
- Elser JJ, Bracken MES, Cleland EE et al. (2007) Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems. *Ecol Lett* 10:1135–1142.
- Fajardo A, Piper FI (2017) An assessment of carbon and nutrient limitations in the formation of the southern Andes tree line. *J Ecol* 105:517–527.
- Fajardo A, Piper FI, Cavieres LA (2011) Distinguishing local from global climate influences in the variation of carbon status with altitude in a tree line species. *Glob Ecol Biogeogr* 20:307–318.
- Fajardo A, Piper FI, Hoch G (2013) Similar variation in carbon storage between deciduous and evergreen treeline species across elevational gradients. *Ann Bot* 112:623–631.
- Fischer C, Holl W (1992) Food reserves of scots pine (*Pinus sylvestris* L.) II. Seasonal changes and radial distribution of carbohydrate and fat reserves in pine wood. *Trees* 6:147–155.

- Gail FW (1926) Osmotic pressure of cell sap and its possible relation to winter killing and leaf fall. *Bot Gaz* 81:434–445.
- Genet M, Li M, Luo T, Fourcaud T, Clement-Vidal A, Stokes A (2011) Linking carbon supply to root cell-wall chemistry and mechanics at high altitudes in *Abies georgei*. *Ann Bot* 107:311–320.
- Groffman PM, Driscoll CT, Fahey TJ, Hardy JP, Fitzhugh RD, Tierney GL (2001) Colder soils in a warmer world: a snow manipulation study in a northern hardwood forest ecosystem. *Biogeochemistry* 56:135–150.
- Gruber A, Pirkebner D, Oberhuber W, Wieser G (2011) Spatial and seasonal variations in mobile carbohydrates in *Pinus cembra* in the timberline ecotone of the Central Austrian Alps. *Eur J Forest Res* 130:173–179.
- Hagedorn F, Shiyatov SG, Mazepa VS et al. (2014) Treeline advances along the Urals mountain range – driven by improved winter conditions? *Glob Chang Biol* 20:3530–3543.
- He JS, Wang ZH, Wang XP, Schmid B, Zuo WY, Zhou M, Zheng CY, Wang MF, Fang JY (2006) A test of the generality of leaf trait relationships on the Tibetan Plateau. *New Phytol* 170:835–848.
- Hoch G, Körner C (2012) Global patterns of mobile carbon stores in trees at the high-elevation tree line. *Glob Ecol Biogeogr* 21:861–871.
- Hoch G, Popp M, Körner C (2002) Altitudinal increase of mobile carbon pools in *Pinus cembra* suggests sink limitation of growth at the Swiss treeline. *Oikos* 98:361–374.
- Hong J, Wang X, Wu J (2014) Stoichiometry of root and leaf nitrogen and phosphorus in a dry alpine steppe on the northern Tibetan plateau. *PLoS One* 9:e109052.
- Karlsson PS, Nordell KO (1996) Effects of soil temperature on the nitrogen economy and growth of mountain birch seedlings near its presumed low temperature distribution limit. *Ecoscience* 32:183–189.
- Körner C (1989) The nutritional status of plants from high altitudes – a worldwide comparison. *Oecologia* 81:379–391.
- Körner C (2003) Carbon limitation in trees. *J Ecol* 91:4–17.
- Körner C, Paulsen J (2004) A world-wide study of high altitude treeline temperatures. *J Biogeogr* 31:713–732.
- Kozlowski TT (1992) Carbohydrate sources and sinks in woody plants. *Bot Rev* 58:107–222.
- Landhaeusser SM, Loeffers VJ (2012) Defoliation increases risk of carbon starvation in root systems of mature aspen. *Trees* 26:653–661.
- LeBauer DS, Treseder KK (2008) Nitrogen limitation of net primary productivity in terrestrial ecosystems is globally distributed. *Ecology* 89:371–379.
- Lewis JD, Lucash M, Olszyk DM, Tingey DT (2004) Relationships between needle nitrogen concentration and photosynthetic responses of Douglas-fir seedlings to elevated CO₂ and temperature. *New Phytol* 162:355–364.
- Li M, Yang J, Krauchi N (2003) Growth responses of *Picea abies* and *Larix decidua* to elevation in subalpine areas of Tyrol, Austria. *Can J For Res* 33:653–662.
- Li MH, Yang J (2004) Effects of microsite on growth of *Pinus cembra* in the subalpine zone of the Austrian Alps. *Ann For Sci* 61:319–325.
- Li MH, Xiao WF, Shi P, Wang SG, Zhong YD, Liu XL, Wang XD, Cai XH, Shi ZM (2008a) Nitrogen and carbon source-sink relationships in trees at the Himalayan treelines compared with lower elevations. *Plant Cell Environ* 31:1377–1387.
- Li MH, Xiao WF, Wang SG, Cheng GW, Cherubini P, Cai XH, Liu XL, Wang XD, Zhu WZ (2008b) Mobile carbohydrates in Himalayan treeline trees I. Evidence for carbon gain limitation but not for growth limitation. *Tree Physiol* 28:1287–1296.
- Li X, Liang E, Gricar J, Rossi S, Cufar K, Ellison AM (2017) Critical minimum temperature limits xylogenesis and maintains treelines on the southeastern Tibetan Plateau. *Sci Bull* 62:804–812.
- Liang EY, Wang YF, Eckstein D, Luo TX (2011) Little change in the fir tree-line position on the southeastern Tibetan Plateau after 200 years of warming. *New Phytol* 190:760–769.
- Liang EY, Wang YF, Piao SL et al. (2016) Species interactions slow warming-induced upward shifts of treelines on the Tibetan Plateau. *Proc Natl Acad Sci USA* 113:4380–4385.
- Lidforss B (1896) Zur Physiologie und Biologie der wintergrünen Flora. *Botanisches Centralblatt* 17:33–44.
- Loomis PF, Ruess RW, Sveinbjörnsson B, Kielland K (2006) Nitrogen cycling at treeline: latitudinal and elevational patterns across a boreal landscape. *Ecoscience* 13:544–556.
- Michelot A, Simard S, Rathgeber C, Dufrene E, Damesin C (2012) Comparing the intra-annual wood formation of three European species (*Fagus sylvatica*, *Quercus petraea* and *Pinus sylvestris*) as related to leaf phenology and non-structural carbohydrate dynamics. *Tree Physiol* 32:1033–1045.
- Millard P, Grelet G (2010) Nitrogen storage and remobilization by trees: ecophysiological relevance in a changing world. *Tree Physiol* 30:1083–1095.
- Millard P, Proe MF (1992) Storage and internal cycling of nitrogen in relation to seasonal growth of *Sitka spruce*. *Tree Physiol* 10:33–43.
- Millard P, Thomson CM (1989) The effect of the autumn senescence of leaves on the internal cycling of nitrogen for the spring growth of apple trees. *J Exp Bot* 40:1285–1289.
- Miyake K (1902) On the starch of evergreen leaves and its relation to photosynthesis during the winter. *Bot Gaz* 33:321–340.
- Oleksyn J, Reich PB, Zytowski R, Karolewski P, Tjoelker MG (2002) Needle nutrients in geographically diverse *Pinus sylvestris* L. populations. *Ann For Sci* 59:1–18.
- Palacio S, Hoch G, Sala A, Koerner C, Millard P (2014) Does carbon storage limit tree growth? *New Phytol* 201:1096–1100.
- Qi Z, Liu H, Wu X, Hao Q (2015) Climate-driven speedup of alpine tree-line forest growth in the Tianshan Mountains, Northwestern China. *Glob Chang Biol* 21:816–826.
- Reich PB, Oleksyn J (2004) Global patterns of plant leaf N and P in relation to temperature and latitude. *Proc Natl Acad Sci USA* 101:11001–11006.
- Ripullone F, Grassi G, Lauteri M, Borghetti M (2003) Photosynthesis-nitrogen relationships: interpretation of different patterns between *Pseudotsuga menziesii* and *Populus × euroamericana* in a mini-stand experiment. *Tree Physiol* 23:137–144.
- Sauter JJ (1988) Temperature-induced changes in starch and sugars in the stem of *Populus × canadensis* «robusta». *J Plant Physiol* 132:608–612.
- Sauter JJ, van Cleve B (1991) Biochemical and ultrastructural results during starch-sugar-conversion in ray parenchyma cells of *Populus* during cold adaptation. *J Plant Physiol* 139:19–26.
- Scott-Denton LE, Rosenstiel TN, Monson RK (2006) Differential controls by climate and substrate over the heterotrophic and rhizospheric components of soil respiration. *Glob Chang Biol* 12:205–216.
- Shi P, Körner C, Hoch G (2006) End of season carbon supply status of woody species near the treeline in western China. *Basic Appl Ecol* 7:370–377.
- Shi P, Körner C, Hoch G (2008) A test of the growth-limitation theory for alpine tree line formation in evergreen and deciduous taxa of the eastern Himalayas. *Funct Ecol* 22:213–220.
- Simard S, Giovannelli A, Treydte K, Traversi ML, King GM, Frank D, Fonti P (2013) Intra-annual dynamics of non-structural carbohydrates in the cambium of mature conifer trees reflects radial growth demands. *Tree Physiol* 33:913–923.
- Sullivan PF, Ellison SBZ, McNowen RW, Brownlee AH, Sveinbjörnsson B (2015) Evidence of soil nutrient availability as the proximate constraint on growth of treeline trees in northwest Alaska. *Ecology* 96:716–727.
- Sveinbjörnsson B, Smith M, Traustason T, Ruess RW, Sullivan PF (2010) Variation in carbohydrate source-sink relations of forest and treeline white spruce in southern, interior and northern Alaska. *Oecologia* 163:833–843.

- Thébault A, Clément J-C, Ibanez S, Roy J, Geremia RA, Pérez CA, Buttler A, Estienne Y, Lavorel S (2014) Nitrogen limitation and microbial diversity at the treeline. *Oikos* 123:729–740.
- Vitousek PM, Howarth RW (1991) Nitrogen limitation on land and in the sea-how can it occur. *Biogeochemistry* 13:87–115.
- Wang A, Wang X, Tognetti R, Lei JP, Pan HL, Liu XL, Jiang Y, Wang XY, Yu FH, Li MH (2018) Elevation alters carbon and nutrient concentrations and stoichiometry in *Quercus aquifolioides* in southwestern China. *Sci Total Environ* 622–623:1463–1475.
- Wang Z, Xia C, Yu D, Wu Z (2015) Low-temperature induced leaf elements accumulation in aquatic macrophytes across Tibetan Plateau. *Ecol Eng* 75:1–8.
- Weih M, Karlsson PS (2001) Growth response of Mountain birch to air and soil temperature: is increasing leaf-nitrogen content an acclimation to lower air temperature? *New Phytol* 150:147–155.
- Wieser G, Gruber A, Oberhuber W (2014) Sap flow characteristics and whole-tree water use of *Pinus cembra* across the treeline ecotone of the central Tyrolean Alps. *Eur J For Res* 133:287–295.
- Wiley E, Helliker B (2012) A re-evaluation of carbon storage in trees lends greater support for carbon limitation to growth. *New Phytol* 195:285–289.
- Woodruff DR, Meinzer FC (2011) Water stress, shoot growth and storage of non-structural carbohydrates along a tree height gradient in a tall conifer. *Plant Cell Environ* 34:1920–1930.
- Wurth MKR, Peláez-Riedl S, Wright SJ, Korner C (2005) Non-structural carbohydrate pools in a tropical forest. *Oecologia* 143:11–24.
- Yan C, Han S, Zhou Y, Zheng X, Yu D, Zheng J, Dai G, Li M-H (2013) Needle $\delta^{13}\text{C}$ and mobile carbohydrates in *Pinus koraiensis* in relation to decreased temperature and increased moisture along an elevational gradient in NE China. *Trees* 27:389–399.
- Yu D, Wang Q, Liu J, Zhou W, Qi L, Wang X, Zhou L, Dai L (2014) Formation mechanisms of the alpine Erman's birch (*Betula ermanii*) treeline on Changbai Mountain in Northeast China. *Trees* 28:935–947.
- Zhu WZ, Cao M, Wang SG, Xiao WF, Li MH (2012a) Seasonal dynamics of mobile carbon supply in *Quercus aquifolioides* at the upper elevational limit. *PLoS One* 7:e34213.
- Zhu WZ, Xiang JS, Wang SG, Li MH (2012b) Resprouting ability and mobile carbohydrate reserves in an oak shrubland decline with increasing elevation on the eastern edge of the Qinghai-Tibet Plateau. *For Ecol Manage* 278:118–126.