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Research paper

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

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The low-temperature limited alpine treeline is one of the most obvious boundaries in mountain landscapes. The question of

2015). All these trees' processes are closely related to the availability of the non-structural carbohydrates (NSC) produced by photosynthesis (Kozlowski 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 × 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. 2008*a*, 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. 2008*b*, Sveinbjörnsson et al. 2010, Fajardo et al. 2011, Gruber et al. 2011, Zhu et al. 2012*a*, 2012*b*; 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. 2012*a*). 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. 2012*b*), which may contribute to the alpine treeline formation (Li et al. 2008*a*, 2008*b*). Hence, investigations on winter NSC storage would be expected to yield better insights into the carbon balance in trees (Landhaeusser and Lieffers 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. (2008*a*, 2008*b*) 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. (2012*a*) 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. 2012*a*), 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

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

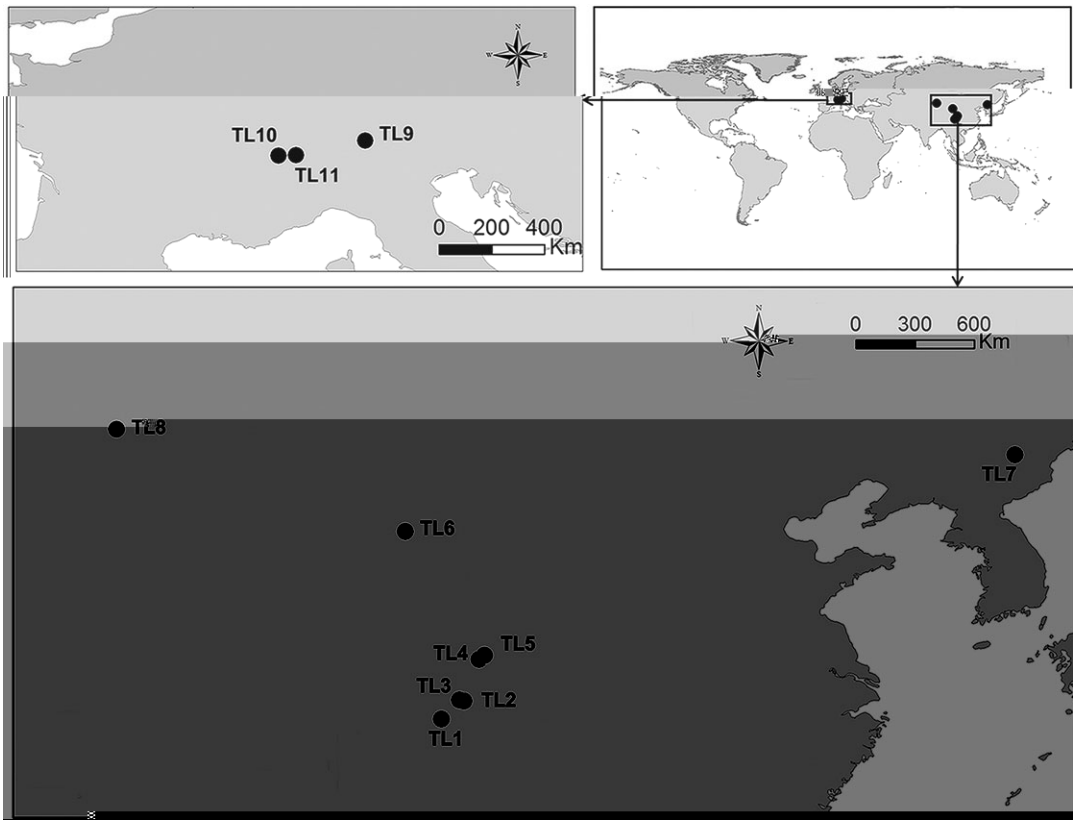

Figure 1. The geographic positions of the 11 investigated treelines (TLs). See Table 1 for detailed information.

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:

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

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 treelines, 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 × E interaction on NSC ($P < 0.001$, Table 2). Overall, TATs, compared with LETs, did not show any disadvantages in NSC concentrations in aboveground 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 × 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 × 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

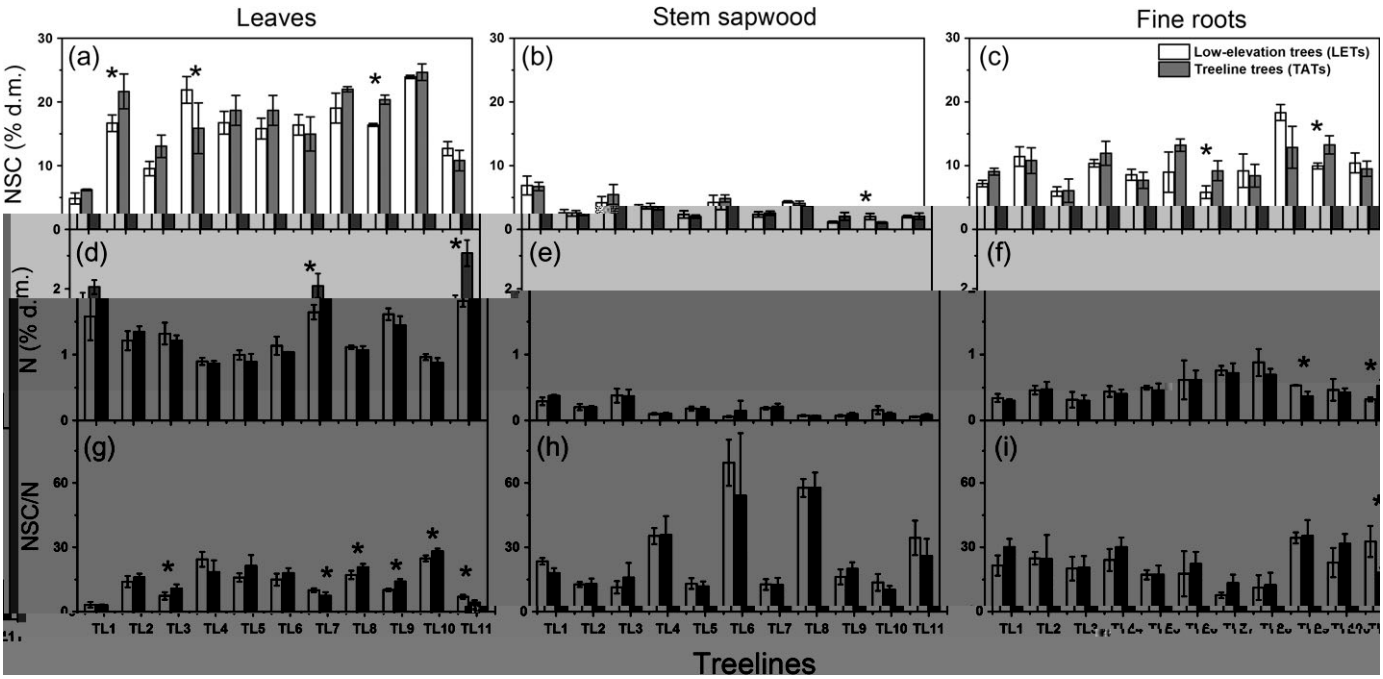

Figure 2. Non-structural carbohydrates (NSC) and N concentrations (% dry matter) and NSC/N ratios in leaves, stem sapwood and fine roots of trees at 11 Eurasian alpine treelines (TLs) and lower elevations in summer. The *X*-axis represents the 11 TLs given in Table 1. An asterisk indicates significant 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 × 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 × 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 Körner (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).

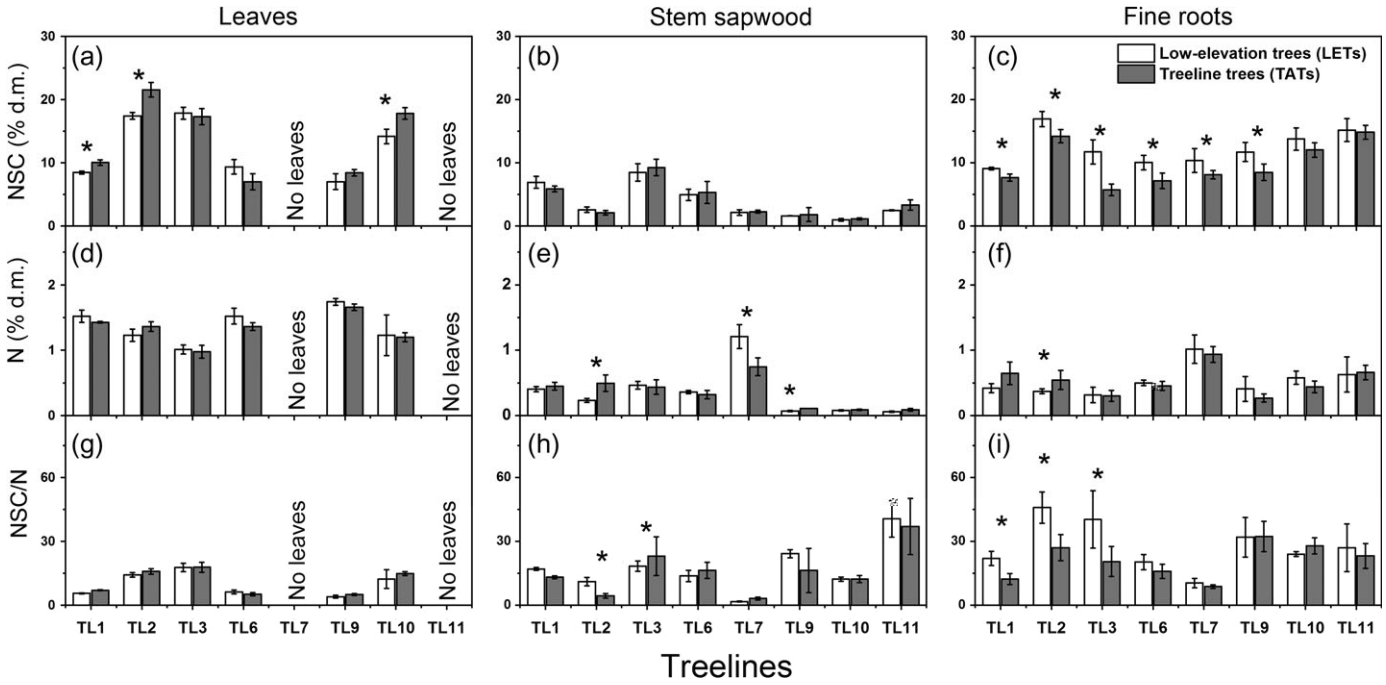

Figure 3. Non-structural carbohydrates (NSC) and N concentrations (% dry matter) and NSC/N ratios in leaves, stem sapwood and fine roots of trees at eight Eurasian alpine treelines (TLs) and lower elevations in winter. The *X*-axis representLs)eightfound8(gesp38.4(in)-228.5(s.)-221.5(Table)TJET/GS1QBT8.50

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. 2012*a*, 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

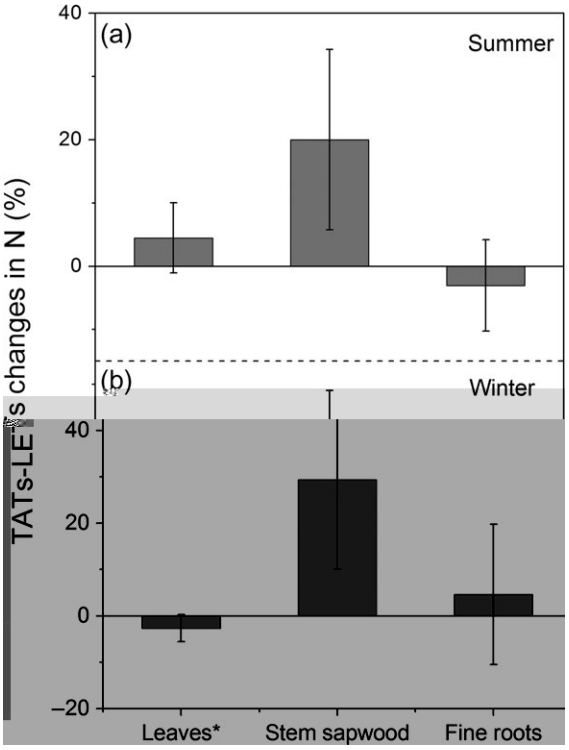

Figure 4. 'TATs – LETs changes' in N (%) for summer and winter, respectively, calculated with $x = \frac{\text{N in TAT} - \text{N in LET}}{\text{N in LET}} \times 100\%$. *Leaves in winter include six evergreen treelines.

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. (2012*a*) 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 2d), 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).

Some months later, the gre7(r)-229(1(ra)30(tT)-314(su)15.8(mm)1

that tissues of TATs had lower starch concentrations than those in LETs (Figure 2d).

5b; see also Figure 3c). This is asult of the con-

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 × 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.

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