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Compound-Specific Carbon Isotopes and Concentrations of Carbohydrates and Organic Acids as Indicators of Tree Decline in Mountain Pine

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Abstract: We investigated seasonal variations in $\delta^{13}C$ values and concentrations of carbohydrates and organic acids in needles of declining and healthy mountain pine (*Pinus mugo* ssp. *uncinata* (DC.) Domin) trees from the Swiss National Park (SNP), using compound-specific isotopes analysis (CSIA). Our goal was to study the impact of climatic drivers on the individual compounds and understand the reasons of partial tree declines in relation to healthy mountain pine trees under seasonal weather patterns. We found that temperature is the main climatic driver determining the seasonal carbon dynamics at the needle level. Lower seasonal $\delta^{13}C$ variability and lower concentration levels of sucrose in needles suggest less photosynthetic activity and sink carbon demand in declining compared to healthy mountain pine trees. Higher concentration levels of hexose (glucose and fructose) can play a reserve function for surviving mechanisms of mountain pine trees. Seasonal patterns of organic acid (malate and citrate) suggest an increasing investment in maintenance and repair mechanisms. The seasonal course of carbohydrates and organic acids can therefore be considered an indicator for a modified carbon metabolism within the leaves and possibly within the other tree tissues, partially explaining the decline of mountain pine trees.

Keywords: tree needles; declining trees; water shortage; Compound-Specific Isotope Analysis (CSIA); climate

1. Introduction

Many forest ecosystems are declining globally due to increasing heat and drought-induced tree mortality [1–5]. Several studies have reported on declines in Scots pine (*Pinus sylvestris* L.) stands in dry inner alpine valleys [6–9]. In particular, declines of mountain pine (*Pinus mugo* ssp. *uncinata* (DC.) Domin) trees in Northern Germany, Poland, Pyrenees, Carpathians, Northern Apennines, Balkan Peninsula [10], and in the Swiss National Park (SNP) have been reported [11–14]. However, mechanisms of metabolic changes, interactions of different environmental factors, and causes leading

to the partial decline and mortality of mountain pine trees in the SNP under recent climatic changes are still unresolved.

Adaptive responses to soil water deficit, pathogens, and insects could change or limit carbohydrate reserves in trees [15]. In recent debates on the possible limitation of carbohydrate reserves, it was stated that limitation rarely occurs [16], possibly only for trees exposed to long lasting drought periods [17]. The transport of photosynthetically derived carbon from leaves to belowground biomass is often reduced under drought hindering optimal development of a functional root system, which can lead to a lack of essential nutrients and to higher mortality rates in the long-term [17,18]. A better understanding of the metabolic processes in declining trees is of great interest for predicting forest response to future warming.

The traditional $\delta^{13}C$ bulk isotope analysis of soluble carbohydrates has limitations in determining the response of plants to environmental variations because compounds with slow turnover rates, like pinitol, can strongly dampen the signal [19]. More detailed information can be derived from the individual carbohydrates (sucrose, glucose, and fructose), and sugar alcohols (e.g., pinitol), but also from compounds like organic acids (malate and citrate) that are involved in more "downstream metabolic processes" leading to systematic but varying ^{13}C-enrichment of sink organs (such as stem) in comparison to leaves [20]. Thus far, there have been few investigations analyzing $\delta^{13}C$ of individual carbohydrates and organic acids [19–26] using high-performance liquid chromatography (HPLC). However, even fewer investigations were applied in natural forest ecosystems to address questions of tree eco-physiology [19,22,23,26,27]. Therefore, further studies are needed to understand how environmental changes impact $\delta^{13}C$ values and concentrations of individual compounds at the leaf level in declining trees.

One of the most dominant non-structural carbohydrates in tree leaves is sucrose, the preferred main transport metabolite in plants [28], which facilitates the carbon transfer from sources to sinks and functions as a precursor for biomarkers such as tree-ring cellulose. Due to its importance, changes in $\delta^{13}C$ values and sucrose concentrations are assumed to show strong responses to changing weather conditions [19]. Other soluble carbohydrates, like glucose and fructose, are also synthesized in considerable amounts in almost all plant tissues, often reflecting a carbon pool supplying all ongoing processes in a tissue such as biosynthesis of compounds, maintenance of cell structure, and respiration. Increasing sucrose, glucose, and fructose concentrations can be observed under drought and may protect against frost and water-loss due to their osmotic functionality [28]. Low levels of non-structural carbohydrates could be indicative of low photosynthetic rates and demand by carbon sink, thus potentially inducing tree decline and mortality [4].

In addition, high amounts of sugar alcohols, such as pinitol, are generally found in conifer needles. Sugar alcohols have a very slow turnover compared to other plant sugars and are therefore not expected to reflect seasonal changes [19]. However, some studies suggested that pinitol plays a role in tolerance to low temperature and drought [19,28]. While $\delta^{13}C$ values and concentrations of individual sugars may respond to climatic changes, they are actually only an indirect indicator of specific metabolic responses. Organic acids, such as citrate and malate, can also function as carbon storage, however, they are known to support various other physiological functions in plant metabolism such as respiration, stomatal regulation, nutrient transport, pH adjustments in plant and soils, and root-soil interactions [20,25,29]. Thus, $\delta^{13}C$ values and concentrations of organic acids might therefore be potential indicators of drought stress [25].

Here, we present a first study of seasonal variations in $\delta^{13}C$ values and concentrations of individual carbohydrates (sucrose, glucose, fructose and sugar alcohol—pinitol) and organic acids (malate and citrate) in needles of declining and healthy mountain pine trees from the SNP.

We (1) investigated if $\delta^{13}C$ values and concentrations of individual compounds in declining mountain pine trees responded differently over the growing season to infer on potential physiological and metabolic changes; (2) compared the compound responses in declining with healthy mountain pine trees from the same site to better understand which specific compound in needles could be

indicative of tree decline; and (3) determined if potential environmental drivers (i.e., air temperature and vapor pressure deficit) are related to changes in δ^{13}C and concentrations in individual compounds.

2. Materials and Methods

### 2.1. Study Site

The study was carried out at the site Champlönch (46°40′48″ N, 10°10′20″ E; 1959–1964 m a.s.l.), which is located in the SNP. The SNP has an area of 174.2 km^2 and is the largest United Nations Educational, Scientific and Cultural Organization (UNESCO) biosphere reserve. The main tree species at the study site is mountain pine (*Pinus mugo* ssp. *uncinata*), which represent ca. 73% of the forests in the SNP, while European larch (*Larix decidua* Mill.) and Swiss stone pine (*Pinus cembra* L.) cover ca. 11%. The soil type is a rendzic leptosol. Shallow and rocky soils are found at the study site [30].

2.2. Climate Data

Our study site is characterized by a continental climate with average winter (December–February) temperatures of −9.2 °C, average summer (June–August) temperatures of 9.5 °C, and 910 mm annual precipitation according to the Buffalora weather station (46°39′ N, 10°16′ E, 1968 m a.s.l.) for the period from 1917 to 2014. The weather station is located ca. 11.3 km away from the study site. Average annual and spring temperatures increased by 0.5 °C and 1.0 °C, respectively, for the period 1990–2014 relative to 1917–1989, while average summer temperature increased by 0.6 °C and annual precipitation decreased by 88.9 mm.

Seasonal temperature (T$_{av}$—average, T$_{min}$—minimum, T$_{max}$—maximal) and vapor pressure deficit (VPD) was calculated with data from the Buffalora weather station. Sampling dates in 2014 were selected according to the phenology with similar weather conditions as in 2013 (Figure 1a,b). In 2013, the needle samples from healthy mountain pine trees were not collected on 24 May because the new needles had not yet emerged. The first sampling date in 2013 was 6 June, which was similar to the phenological conditions of 24 May, 2014 (Figure 1a,b).
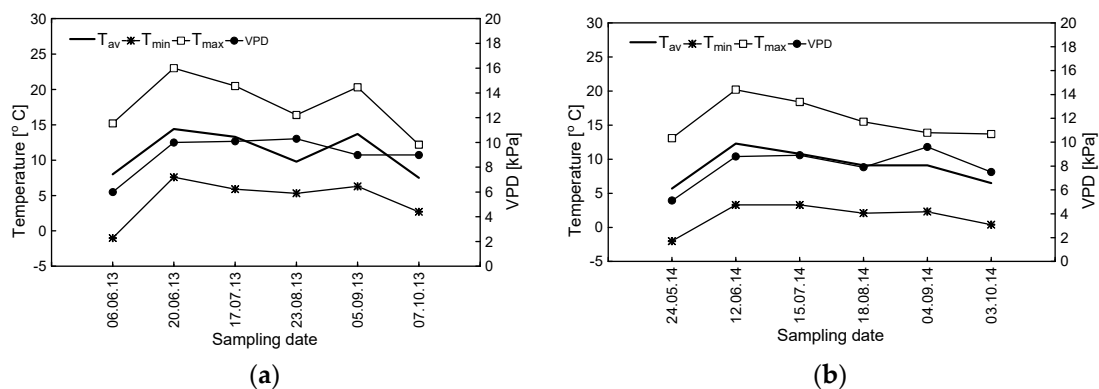
Figure 1. Variability of air temperatures: Average (T$_{av}$), minimum (T$_{min}$), maximum (T$_{max}$), and vapor pressure deficit (VPD) over the sampling season of 2013 (**a**) and 2014 (**b**).

2.3. Selection of Trees and Sampling of Needles

We selected south (S)- and north (N)-facing aspects at the study site with healthy and declining mountain pine trees in May 2013. Averaged height of the healthy and declining mountain pine trees ranged within 13–15 m, while average diameter at 1.3 m tree height was ca. 28 cm for healthy and ca. 25 cm for declining mountain pine trees.

The S-aspect was located at 1964 m a.s.l. with a 35° slope, and is characterized as being sunny and dry. In contrast, the N-aspect was located at 1959 m a.s.l. with a 20° slope, and is characterized as being shady with moister soil, which is covered by denser ground vegetation compared to the

S-aspect. During sampling of healthy mountain pine trees in summer 2013 [26], we selected declining mountain pine trees (*Pinus mugo* ssp. *uncinata*) based on visual assessment of tree crowns (i.e., presence of reddish, brownish needle coloring) (Figure 2a). Needles (approx. 120 mg per sample) from eight declining mountain pine trees were sampled once per month from late May until October 2014. Needles (approximately 200 mg per sample) from eight healthy mountain pine trees were sampled biweekly from early June until October 2013. To be consistent between two data sets from different years, we provided data for healthy mountain pine trees in a similar manner, once per month (Figure 3b,d,f,h). Average age of the selected mountain pine trees was 150 to 200 years. However, it is worth noting that needles used for the analysis from the declining mountain pine trees were green without visible damage. After collection, all needle samples were heated in a microwave for 90 s at 250 W to stop enzymatic and metabolic activities [31]. Subsequently, the samples were delivered to the laboratory at the Paul Scherrer Institute, where they were dried for 24 h at 60 °C [31] and ground with a steel ball mill to a fine powder, which was used for all further analyses.
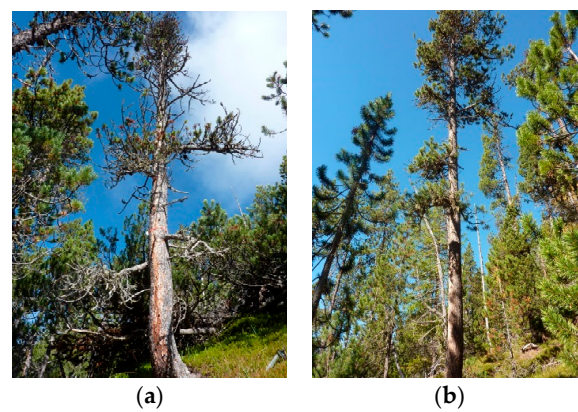

(a) (b)

Figure 2. Declining(**a**) and healthy (**b**) mountain pine trees from the study site at the Swiss National Park.
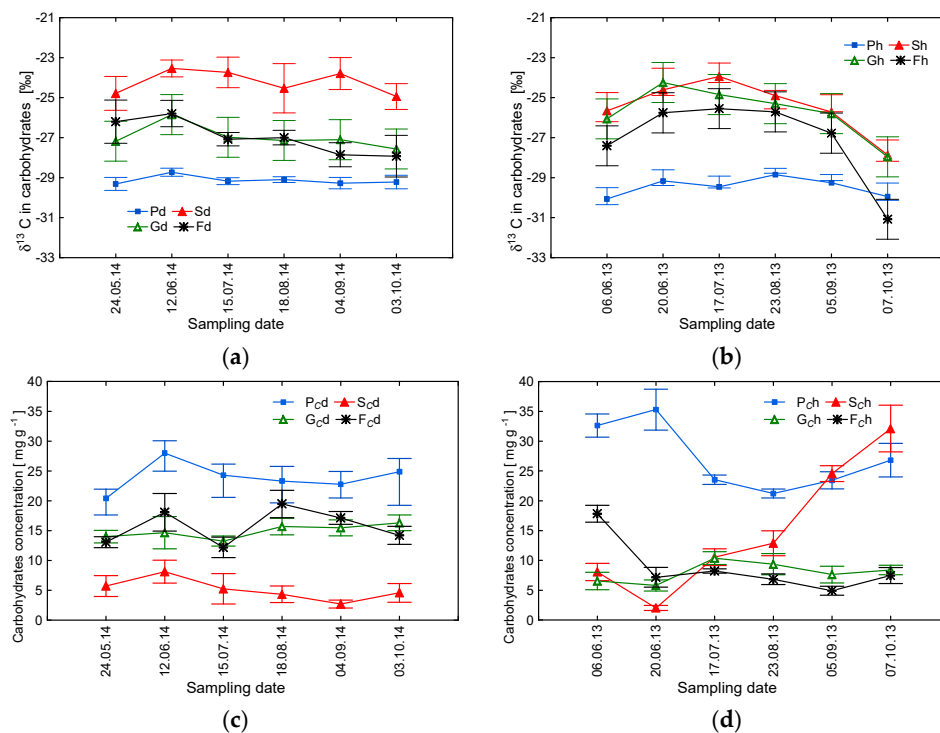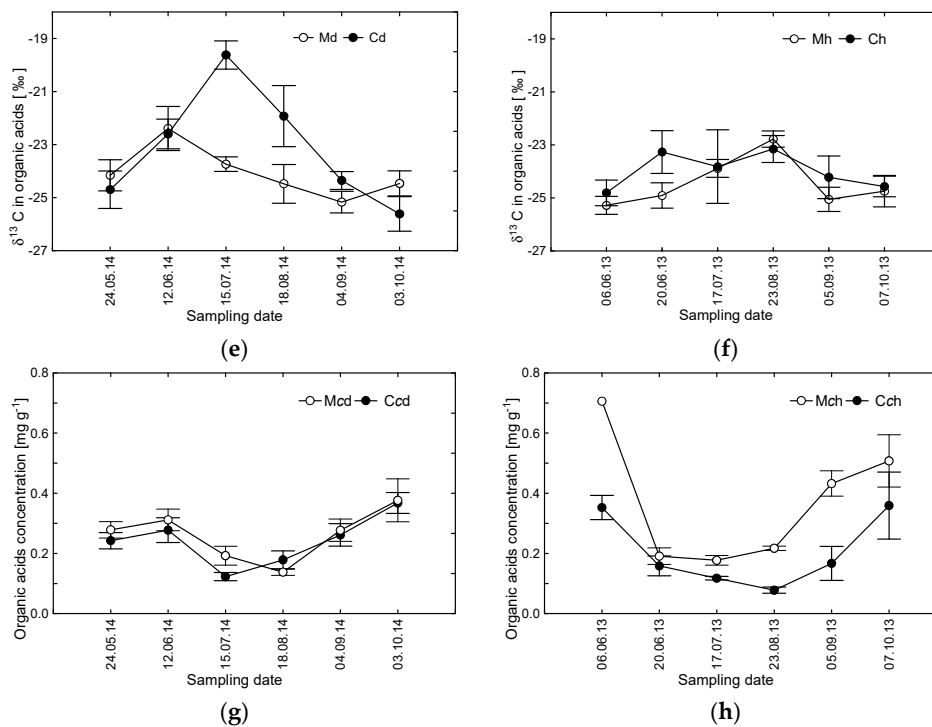

(a) (b)

(c) (d)

Figure 3. *Cont.*

Figure 3. (**a**,**b**) δ^{13}C values of pinitol (P), sucrose (S), glucose (G), and fructose (F); (**c**,**d**) concentrations (Pc, Sc, Gc, Fc); (**e**,**g**) δ^{13}C values of organic acids (malate, M; citrate, C); (**f**,**h**) concentrations (Mc, Cc) in needles of declining (**d**) and healthy (**h**) mountain pine trees. Error bars are based on ± 1 SE (standard error).

2.4. Extraction and Purification of Sugars and Organic Acids

From the milled sample, 60 mg of powder was placed in Eppendorf tubes, 1.5 mL of Milli-Q water was added, and then vortexed until the powder was fully suspended. The tubes were placed in a water bath at 85 °C for 30 min according to the protocol [19,31]. The samples were allowed to cool down for 30 min followed by centrifugation at 10,000× *g* for 2 min.

For sugar purification, we used Thermo Scientific™ Dionex™ OnGuard II CRTG 1CC cartridges (Thermo Fisher Scientific, Waltham, MA, USA): Cation exchange cartridge "H" to remove amino acids, anion exchange cartridge "A" (HCO_3^-) to remove organic acids, and cartridge "P" to remove phenolic compounds present in the plant water extracts [19,32]. After sugar purification, we eluted organic acids from the cartridge "A" with 30 mL 1 M HCl solution (modified after Reference [20]). Purified samples were freeze-dried, dissolved in deionized water, and stored in the freezer until use.

2.5. Compound-Specific δ^{13}C Isotope Analysis

Compound-specific δ^{13}C isotope analysis (CSIA) was performed on-line using a High-Performance Liquid Chromatography (HPLC) coupled via LC Isolink interface to a Delta V Advantage IRMS (both Thermo Fisher Scientific, Bremen, Germany). Soluble carbohydrates were separated on a Dionex CarboPac™ PA 20 3 × 150 mm column (Dionex, Olten, Switzerland) at 22 °C, using a 2 mM NaOH solution as the mobile phase with a flow rate of 250 μL min^{-1} [19,32]. Organic acids were separated on an Allure Organic Acids 4.6 × 300 mm column (Restek, Bellefonte, PA, USA) at 8 °C, using a 100 mM KH_2PO_4 (pH 3) solution as the mobile phase with a flow rate of 500 μL min^{-1}. Evaluation of δ^{13}C values and concentrations of individual compounds was performed according to [32].

2.6. Statistical Analysis

Pearson correlation analyses between $\delta^{13}C$ values and concentrations of individual needle compounds versus climatic data from the Buffalora weather station were carried out using the STATISTICA 13.3 (TIBCO Software Inc., Palo Alto, CA, USA). We applied unpaired *t*-test analysis to reveal whether $\delta^{13}C$ values and concentrations of carbohydrates and organic acids differ between aspects and between declining and healthy mountain pine trees.

3. Results and Discussion

3.1. $\delta^{13}C$ Values and Concentrations of Carbohydrates and Organic Acids as a Potential Indicator of Tree Decline

The $\delta^{13}C$ values and concentrations in needles of declining mountain pine trees did not significantly differ at the lowest *p*-values between compounds ($p > 0.08$) between N- and S-facing aspects for the individual compounds. Therefore we merged the data from both aspects for each individual compound separately ($n = 8$) (Figure 3a,c,e,g). $\delta^{13}C$ of individual soluble carbohydrates in needles of declining mountain pine trees (Figure 3a) showed less clear seasonal patterns compared to healthy mountain pine trees (Figure 3b). A strong depletion of sucrose, glucose, and fructose in needles of healthy mountain pine trees by the end of the growing season was detected (Figure 3b). This drop in the $\delta^{13}C$ could be caused by rain and cold night temperature on the day before. We found that $\delta^{13}C$ values of sucrose in declining mountain pine trees were less negative compared to $\delta^{13}C$ values of hexoses (i.e., glucose and fructose), and pinitol by 3 and 5‰ (Figure 3a). This finding was in contrast to the healthy trees, where $\delta^{13}C$ values of sucrose were similar to $\delta^{13}C$ values of hexoses and varied no more than 1–2‰ between each other during the season (Figure 3b). $\delta^{13}C$ of pinitol showed lower seasonal variability compared to other carbohydrates (Figure 3a), which was not the case for healthy mountain pine trees (Figure 3b). A decrease of hexose (fructose and glucose) towards the end of the growing season was detected for both declining (Figure 3a) and healthy (Figure 3b) mountain pine trees. The latter showed much stronger depletion, which may be a response of the healthy mountain pine trees to the development of the cold conditions or of the declining pine trees to desiccation tolerance [26,28]. The ^{13}C-enrichment in sucrose compared to other carbohydrates for declining mountain pine trees might be explained by the decreasing pool size, i.e., the preferential use of ^{13}C-depleted sucrose for biosynthetic and respiratory processes, but might also be a result of physiological adaptations (Figure 3a). Stomatal conductance might have been lower in the declining than in the healthy mountain pine trees given the need to use a more conservative water-use strategy [30].

We found high $\delta^{13}C$ of sucrose (Figure 3a) and low sucrose concentration (Figure 3c) in needles of the declining mountain pine trees at a compound-specific level. $\delta^{13}C$ increase could be a result of reduced stomatal conductance, supporting the classical model for carbon isotope fractionation [33]. Thereafter, the concentrations showed a decreasing trend. This could be explained by a reduction of carbon supply as a result of needle senescence with a concomitant lower C-demand for tree growth. The opposite pattern for sucrose concentration was found in needles of healthy mountain pine trees, which showed an increasing trend towards the end of the growing season (Figure 3d). Pinitol concentration in needles of healthy mountain pine trees (Figure 3d) is also higher than that in declining mountain pine trees (Figure 3b), which can be explained by higher resistance of healthy compared to declining mountain pine trees towards the frost events [19]. Sudachkova et al. [34] showed that the need for reserve compounds in conifers was stronger at the beginning and at the end of the growing period, when the processes of xylogenesis had ended and when the carbon reserve pools are replenished, while glucose is converted into the starch compound.

In most cases, stomatal limitation is a response to drought whereas increased photosynthesis, often due to enhanced photosynthetically active radiation, under abundant water availability leads to carbon isotope enrichment [33]. Low sucrose concentration could be partially explained by receding export of carbohydrates from the leaves to the phloem for production of reserves under

dry conditions [35]. Low carbohydrate concentrations may sometimes occur as a result of limited carbon assimilation and stomatal control under drought [36]. Severe water stress could lead to a continuous or transitory increase of leaf respiration that could also partly cause a decrease in sucrose concentration [34]. Fresh assimilates in declining trees are more likely used for maintenance and respiration than for storage or biomass production (i.e., growth) [21,30]. Lower transport rates into the phloem under water shortage [15] might function as a metabolic feedback and decrease sucrose synthesis [28].

Furthermore, we measured $\delta^{13}C$ values and concentrations of organic acids in needles of declining (Figure 3e,g) and healthy (Figure 3f,h) mountain pine trees. Organic acids were clearly more ^{13}C-enriched in needles of declining mountain pine trees compared to carbohydrates and were more variable, e.g., 2.7‰ difference between hexose (Figure 3a) and malate, up to 4‰ with citrate (Figure 3e), and almost 4‰ more enriched compared to healthy mountain pine trees (Figure 3f).

The seasonal pattern of $\delta^{13}C$ variability in organic acids is coherent with $\delta^{13}C$ variability in carbohydrates (r = 0.84; $p < 0.05$), however, with an offset up to 4.8‰ over the season. In particular, $\delta^{13}C$ of citrate showed the highest seasonal variability of all compounds, with maximum values in mid-summer (Figure 3e) indicating −17.2‰ from the average mean of −23.2‰ during the season. This $\delta^{13}C$ increase could be a result of reduced stomatal conductance and enhanced assimilation. The increase in $\delta^{13}C$ values of citrate is accompanied by a decrease in citrate concentration (Figure 3g) in needles of declining mountain pine trees. Thus, a preferential use of lighter citrate isotopologes may explain the ^{13}C-enrichment of citrate. The seasonal pattern of organic acid concentrations in needles of healthy mountain pine trees (Figure 3h) is rather similar to that of carbohydrates (Figure 3d). Increase of carbohydrate concentrations in needles of healthy mountain pine trees at the beginning and at the end of the season can be an indicator for stored forms of fixed carbon, which seems to be higher in healthy compared to declining mountain pine trees.

3.2. Averaged Seasonal Patterns

Based on a *t*-test, we found significant differences ($p \leq 0.05$) in seasonally averaged $\delta^{13}C$ values of sucrose in needles of declining compared with healthy mountain pine trees (Figure 4a). However, there are no significant differences in $\delta^{13}C$ values of other individual carbohydrates ($p \geq 0.071$) or organic acids compounds ($p > 0.39$) (Figure 4b) in needles of declining and healthy mountain pine trees. We found significant differences in averaged concentration of sucrose ($p = 0.017$), glucose ($p = 0.0001$), and fructose ($p = 0.001$) compounds (Figure 4c) in needles of declining compared to healthy mountain pine trees. Variability of sucrose concentration is large compared to glucose and fructose concentrations. We assume that this is a result of differences in assimilation rates and sucrose synthesis between declining and healthy mountain pine trees, indicating that declining trees are less vital than healthy trees and that their metabolic activity to produce carbon reserves is reduced.

Total averaged $\delta^{13}C$ values for pinitol, sucrose, glucose, and fructose (PSGF) and carbohydrate concentrations did not differ significantly between healthy and declining mountain pine trees ($p \geq 0.76$). Therefore, only analysis of individual compounds can provide detailed information about carbon dynamic at the leaf level.

High hexose (glucose and fructose) concentrations in needles of declining compared with healthy mountain pine trees (Figure 4c) might indicate osmotic regulation to reduce water loss. Particularly, hexose can be more efficient in this case than sucrose to store extra sugars and to form a pool of reserve carbohydrates [37]. Thus, our results emphasize the role of sucrose in tree decline, and the role of hexose as a compound for carbohydrate reserves.

Averaged citrate concentrations differ significantly ($p = 0.0092$) between declining and healthy mountain pine trees. Citrate may also act as an additional carbon pool (due to reduction of sucrose), supporting maintenance of plant metabolism and respiration [21,29]. However, citrate might also be used as a carbon transport metabolite, which is transported to sinks, such as roots, where it facilitates the transport of nutrients, such as iron [29,38], towards needles.
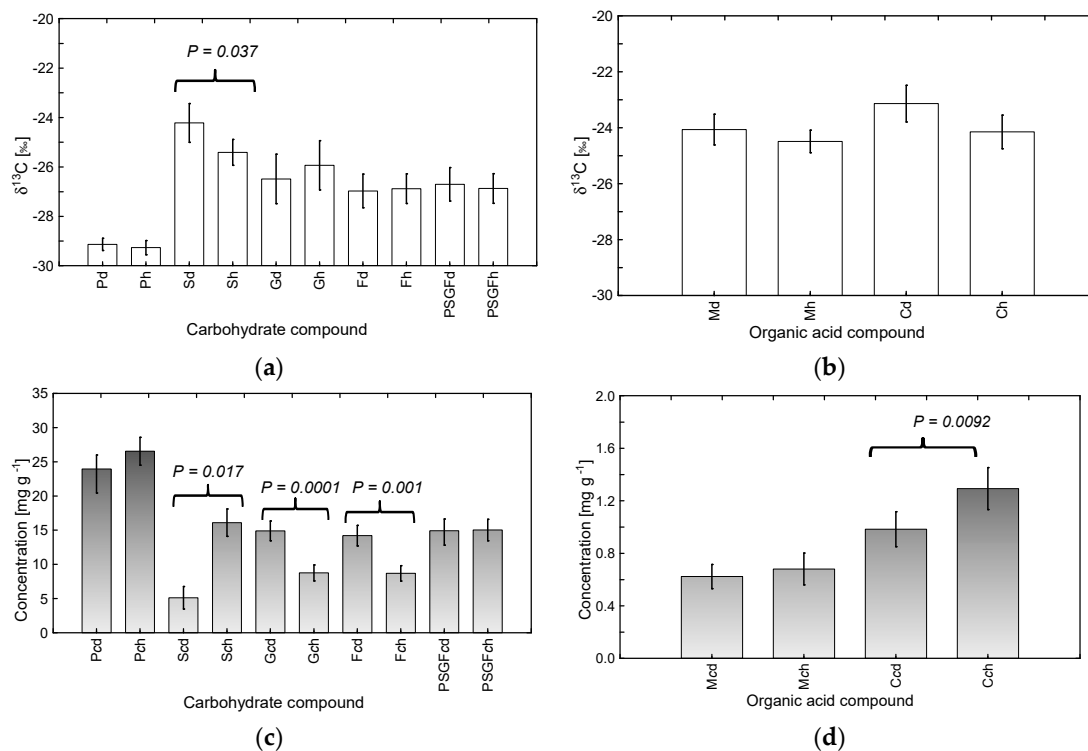
Figure 4. Averaged seasonal δ^{13}C values (**a**) of carbohydrates: pinitol (P), sucrose (S), glucose (G), and fructose (F); and (**b**) organic acids: malate (M) and citrate (C); and their concentrations (**c**,**d**), and average of total carbohydrates (PSGF) in needles of declining (d) and healthy (h) mountain pine trees. Error bars are based on $\pm$ 1 SE (standard error). Statistically significant differences in δ^{13}C values and concentrations of carbohydrates and organic acids in needles of healthy and declining mountain pine trees are marked on the top as *P*-values.

3.3. Correlations between δ^{13}C and Concentrations of Individual Compounds in Declining Trees with Climate Data

The correlation analysis showed significant relationships ($p < 0.05$) between δ^{13}C of sucrose in needles of declining mountain pine trees and average air temperature (T_{av}) (r = 0.90, p = 0.014; Figure 5a). Minimum air temperature (T_{min}) only influenced δ^{13}C in sucrose (r = 0.82, p = 0.048) (Figure 5b). T_{min} could lead to a reduction in stomatal conductance and cause relatively low intercellular versus ambient CO_2 changes [39], thus affecting δ^{13}C of the carbohydrates. Only the δ^{13}C in sucrose in needles of declining pine trees correlated positively with VPD (r = 0.89, p = 0.01) (Figure 5d), which may be a result of drought due to increases in temperature and evaporation.

δ^{13}C in pinitol, sucrose, glucose, total carbohydrates, and organic acids (malate and citrate) in needles of declining mountain pine trees correlated significantly ($p < 0.05$) with T_{max} (r = 0.85, p = 0.035; 0.81, p = 0.049; 0.83, p = 0.040, 0.84, p = 0.034 and 0.84, p = 0.034), respectively (Figure 5c). δ^{13}C of pinitol in needles of healthy mountain pine trees showed significant correlations with T_{min} (r = 0.81; p = 0.049), while glucose positively correlated with T_{max} (r = 0.88, p = 0.021) and total carbohydrate concentration (r = 0.82; p = 0.046). Sugar alcohols, like pinitol, are potentially involved in cryoprotection, playing a role in low temperature (frost) and drought resistance, and may also be important to endure drought stress [28]. Yet, impact of T_{max} on glucose and organic acids can be an indicator for protection against water loss under developing drought conditions. δ^{13}C of carbohydrates in needles can indicate the impact of water availability, because higher δ^{13}C occurs under water limitation. Therefore, water deficit and elevated temperature can give rise to significant changes in leaf biochemistry.
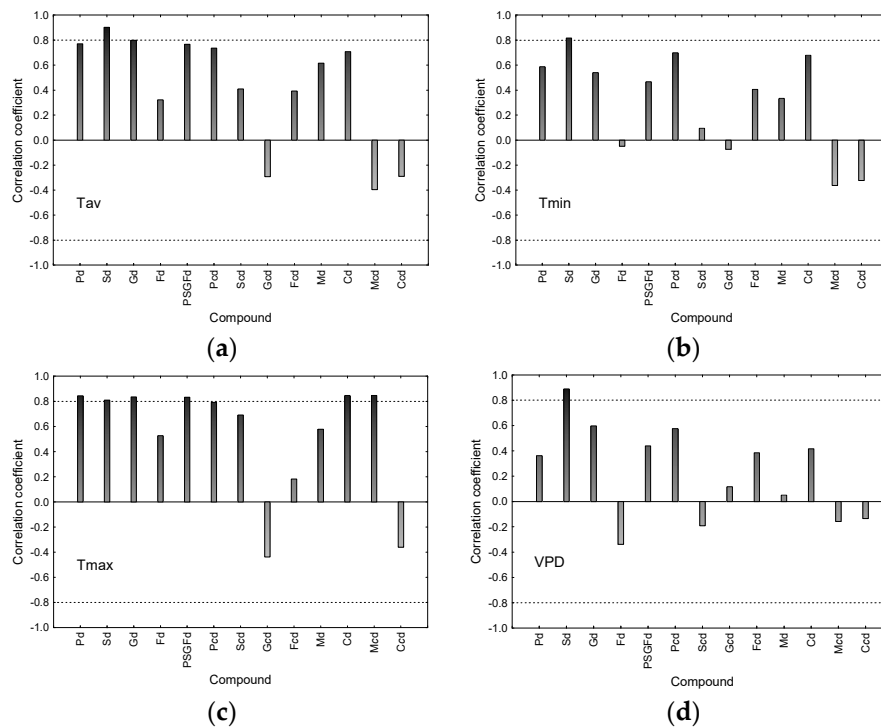
Figure 5. Pearson correlation coefficients were calculated between T_{av}—average (**a**); T_{min}—minimum (**b**); T_{max}—maximum (**c**) temperatures and vapor pressure deficit (VPD) (**d**) versus δ^{13}C for pinitol (P), sucrose (S), glucose (G), fructose (F), total carbohydrates (PSGF) and their concentrations Pc, Sc, Gc, Fc, and organic acid compounds (δ^{13}C in malate (M), citrate (C) and their concentrations Mc, Cc) in declining mountain pines. Horizontal black lines on the top and bottom present significance levels of $p < 0.05$.

While δ^{13}C values were strongly affected by site-specific temperature conditions, concentrations of individual compounds were not climate sensitive. Interpretations of physiological adaptions to temperature in carbon isotopes might ultimately transfer from assimilates to tree rings [30]. Declining trees might be more sensitive to water shortage compared to healthy trees on the same site. This might cause earlier stomatal closure, according to the classical model for carbon isotope fractionation [33], and can result in an increase in δ^{13}C of sucrose as a response to drought, leading to a higher water-use efficiency in declining compared with healthy mountain pine trees. Higher concentration levels of hexose (glucose and fructose) can play a reserve function for survival mechanisms of declining compared to healthy mountain pine trees. Seasonal patterns of organic acid (malate and citrate) suggest increasing investment in maintenance and repair mechanisms. The seasonal cycle of carbohydrates and organic acids may be considered as an indicator for a modified carbon metabolism within the leaves and possibly within the other tree tissues, partially explaining the decline of mountain pine trees.

Our findings show differences in the seasonal variability of individual carbohydrates and organic acids in needles of declining compared to healthy mountain pine trees at the compound-specific level. We found that temperature plays a significant role in determining the seasonal carbon dynamics at the needle level. Lower seasonal δ^{13}C variability and lower concentration levels of sucrose in needles suggest less photosynthetic activity and sink carbon demand in declining compared to healthy mountain pine trees. Higher concentration levels of hexose (glucose and fructose) can play a reserve function for surviving mechanisms of mountain pine trees. Seasonal patterns of organic acid (malate and citrate) suggest increasing investment in maintenance and repair mechanisms. Therefore, the seasonal cycle of carbohydrates and organic acids can be considered to be an indicator of a modified carbon metabolism within the leaves and possibly within the other tree tissues, partially explaining the decline of mountain pine trees.

Further field studies with different tree species and direct measurements of leaf gas-exchange as well as measurements of starch concentrations are urgently needed to complete the eco-physiological explanation of tree-decline processes. Detailed information can be derived from the analysis of each individual compound by CSIA only, while using traditional bulk $\delta^{13}C$ analysis does not provide significant differences in carbon dynamic at the leaf level.

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Conflicts of Interest: The authors declare no conflict of interest.

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