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Fagus sylvatica and *Picea abies* differ in their non-structural carbon build up and use

Master's Thesis

to gain the academic grade as a

Master of Science in the Study Program *Evolution, Ecology and Systematics* (EES)

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submitted by

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List of abbreviation

Abbreviation	Definition
°C	Celsius
C	Carbon
$\delta^{13}C$	Isotopic ratio of ^{13}C (‰)
$\Delta^{14}C$	Isotopic ratio of ^{14}C (‰)
CO_2	C dioxide
DBH	diameter at breast height
d.w.	dry weight
HPLC	high-performance liquid chromatography
NSC	non-structural C
O_2	gaseous oxygen
Resp	respiration
RH	relative humidity
rpm	revolutions per minute
RQ	respiratory quotient
RR	respiration rate
ws	water solubles

List of materials

Clear plexiglass Chambers (company)

Glass flasks 115ml (LouwersHanique, Hapert, Netherlands)

Fittings 12,7mm Ultra-Torr (Swaglock, Solon, USA)

CO_2 Sensor GEMP251/252 (Vaisala, Hamburg, Germany)

PC-Software Insight (Vaisala, Hamburg, Germany)

O_2 sensor Fibox 4 trace (Presens, Regensburg, Germany)

PreSens Measurement Studio 2 (Presens, Regensburg, Germany)

Synthetic air (20.5 % O_2, 0% CO_2; Westfalen AG, Münster, Germany)

Gasmixture (20.94 % O_2, 403 ppm CO_2; Westfalen AG, Münster, Germany)

Gasmixture (5 % N_2; Westfalen AG, Münster, Germany)

Gasmixture (1720 ppm CO_2; Westfalen AG, Münster, Germany)

Gasmixture (8950 ppm CO_2; Westfalen AG, Münster, Germany)

Gasmixture (10000 ppm CO_2; Westfalen AG, Münster, Germany)

Gasmixture (150000ppm CO_2; Westfalen AG, Münster, Germany)

Gasmixture (50000ppm CO_2; Westfalen AG, Münster, Germany)

Isotope Ratio Mass Spectrometer IRMS; Delta+ XL; (Thermo Fisher Scientific, Bremen, Germany)

Modified gasbench with Conflow III and GC (Thermo Fisher Scientific, Bremen, Germany)

12-ml Labco extainer (Labco Ltd, Lampeter, UK)

Auto-sampler CTC Combi-PAL autosampler (CTCAnalytics, Zwingen, Switzerland)

Laboratory air standard on the VPDB scale [Jena Reference Air Set-06 (JRAS-06)] 00 (Jena, Germany)

Graphitization-vacuum-line (In-house development, Jena, Germany)

Accelerator Mass Spectrometer *MICADAS* (Ionplus, Dietikon, Switzerland)

Sonorex Super 10P DK 255 P Ultrasonic Bath 160W (Bandelin, Berlin, Germany)

Ball mill MM400 (Retsch, Haan, Germany)

Amicon® Ultra 0.5ml Centrifugal Filter units Lot R9MA03310 (Merck, Darmstadt, Germany)

Amicon® Ultra 15ml Centrifugal Filters Lot R3MA06116 (Merck, Darmstadt, Germany)

Microcentrifuge tubes with socket screw caps Lot 210321540-1 (VWR, Radnor, USA)

Balance XPR205 (Mettler-Toledo, Greifensee, Switzerland)

Balance XPR2 (Mettler-Toledo, Greifensee, Switzerland)

Hermle Z 233 MK-2 Refrigerated Microlitre Cenrifuge (Thermo Fisher Scientific, Waltham, Massachusetts USA)

Thermomixer comfort (Eppendorf, Hamburg, Germany)

Eppendorf® Research® plus Pipette (Eppendorf, Hamburg, Germany)

100-1000µl M27146E

20-200µl N26105E

0.5-5ml N21459E

UltiMate 3000 (Dionex Softron GmbH, Germering, Germany)

Elementar vario TOC cube (Elementaranalyse systeme GmbH, Langenselbold, Germany)

IAEA-CH-6 (H. Polach, Australian National University, Canberra)

Ryeflour Lot #BC381 (European reference material, Geel, Belgium)

α-Amylase from Bacillus licheniformis Lot #SLBZ8583 (Sigma Aldrich, St. Louis, USA)

Amyloglucosidase from Aspergillus niger Lot #SLCH6247 (Sigma Aldrich, St. Louis, USA)

Short thread vials, ND9 (VWR, Radnor, USA)

Multoclear-13 PVDF 0.2 mm rosa (CS-Chromatographie Service GmbH, Langerwehe, Germany)

Silvercapsules (Elementar Analysensysteme GmbH, Langenselbold, Germany)

Smooth wall tin capsules (Elementar Analysensysteme GmbH, Langenselbold, Germany)

1. Introduction

Intensification of environmental stresses associated with climate change increase the risk of tree mortality. A key factor for the ability of trees to survive stress is the use of stored carbon (C). In Central Europe, a combination of drought years, heat waves, and bark beetle outbreaks in recent years has led to extensive dieback, particularly of *Picea abies* (Krejza *et al.*, 2021). *Fagus sylvatica* trees on the contrary show greater resilience to environmental stresses. The species difference has many reasons, among them the shallower rooting of the *Picea* that leads to poorer water supply (Nultsch, 2012).
Another factor influencing resilience is C storage (Wiley, 2020). Drought conditions can reduce stomatal conductance and therefore C assimilation (Kreuzwieser and Gessler, 2010; Boyd *et al.*, 2013). To maintain respiration and metabolism trees are thus forced to rely on their C reserves. However, in my thesis I explored whether different storage dynamics between Picea and Fagus can explain their contrasting survival.

C can be stored in various compounds like carbohydrates, starch, lipids, proteins, and amino acids that can be collectively defined as non-structural C (NSC). NSC stock size reflects a balance between C assimilation (source) and C sinks such growth and respiration (Hoch, Richter and Körner, 2003). Furthermore, variation exists between storages, with the hypothesis of a fast C pool vs. slow C pool. The former describes freshly assimilated C with a fast turnover that is used for metabolism and growth, with the second being a slow C pool mainly containing old, stored C (Richardson *et al.*, 2015). Therefore, the characteristics of the tree organs determine which of the storage types are mainly found in the respective organs.
Genus *Fagus* is considered a 'starchy tree', with predominantly starch reserves (Sinnott, 1918). *Fagus* has dense wood with a complex structure, not like evergreen trees. The leaves are quite soft and fragile, as they are also only on the tree for one season only and must be formed anew every year (Nultsch, 2012). Genus *Picea* is considered a 'fatty' tree, with a high abundance of lipids. It has a shallow root system, and its needles are heavily thickened with many anatomical attributes designed for colder and drier habitats (Nultsch, 2012).

I chose *Fagus sylvatica* and *Picea abies* as my target species because of their ecological and economic status in Germany. *Fagus sylvatica* is a broadleaved tree species, which has a coverage of 11% in Germany (NABU, 2021). It outcompetes other species by building up a dense crown resulting in a shaded forest ground, as well as the possibility to stay in the juvenile

stage in unfavourable environmental conditions. It can also cope with a variety of environmental stresses like C limitation (Fig. 1). *Picea abies* is much used in the forestry industry due to its fast growth and properties (soft and light wood) and has a coverage of 25% in Germany (NABU, 2021). It is native to only a few spots in Germany, one of them is the Thuringian Forest. It has a shallow root system, and its needles are heavily thickened, filled with photosynthetic parenchyma and many anatomical attributes designed for colder and drier habitats (Nultsch, 2012).

I investigate how, when and which reserves are used in *Fagus sylvatica* and *Picea abies*, with the aim to clarify not only differences between evergreen and broadleaved trees, but also to reveal differences at an organ level and to see variability between aboveground and belowground organs. To test if species and organs use NSC differently, I cut leaves, branches, and roots, and forced them to use C storages. I investigated the effects of C limitation in individual organs by measuring stable C isotopes and radiocarbon of the respired CO_2 of roots, leaves, and branches as well as the content of respired CO_2 and absorbed O_2. I extracted sugar and starch out of solid samples, which are then analysed for total organ C (TOC), component distribution of fructose, sucrose, and glucose, stable C isotopes, and radiocarbon.

I measured respirated CO_2 and used O_2 from the samples and calculated the respiration rate (RR) and respiratory quotient (RQ). The RR tells us how much C was respired and if this correlates with changes in the RQ, which is an indicator which types of reserves are used.
For example, when C assimilation is halted the use of storage is reflected by NSC concentration decrease. Assuming the NSC are used mostly for respiration, the respired CO_2 must mirror the NSC change. Moreover, gases exchanged in respiration contain information about metabolism, respiration sources, and use of stored NSC.
Another way to examine C usage is to test for stable C isotopes (^{13}C) and radiocarbon (^{14}C). While ^{13}C can tell us more about C usage in a metabolic context and about involved enzymes, ^{14}C indicates if fast or slow C-pools are used. Examining isotope distribution has a lot of advantages, since it can tell us a great deal about the stress response of the species. Plants discriminate ^{13}C and ^{14}C against ^{12}C due to kinetic fractionation (Tcherkez, Mahé and Hodges, 2011). A higher abundance of ^{13}C indicates changes in stomatal conductance, triggered by drought structural differences or shading, but it also can tell us more about C source for respiration, since different sources have different isotopic signatures (Francey and Farquhar, 1982; Tcherkez, Mahé and Hodges, 2011). For example, respirated air that originates origin comes from lipids is more ^{13}C-depleted, while respirated air from carbohydrate metabolism is

^{13}C-enriched (Gleixner *et al.*, 1993; Bowling, Pataki and Randerson, 2008). It is similar in radiocarbon, where a higher depletion indicates older C.

Finally, I extracted sugar and starch out of solid samples, which are then analysed for TOC component distribution of fructose, sucrose, and glucose, stable C isotopes and radiocarbon.

These methods should help to answer various questions, such as:

'Do trees prefer recent fixed C first or do they also use old reserves right away?',

'Do evergreen and broadleaved species differ in their preferred age of C reserves?', or

'Do roots prefer older or younger C reserves compared to branches or leaves?'.

I hypothesise that, I) As time goes on, the reserves are getting older, since the last C molecules that goes in also goes out first, II) C reserves are older in belowground tissue, than aboveground tissue, III) Whole tree C content is lower in *Picea abies* then *Fagus sylvatica*, IV) C storage is bigger in belowground tissue than aboveground tissue.

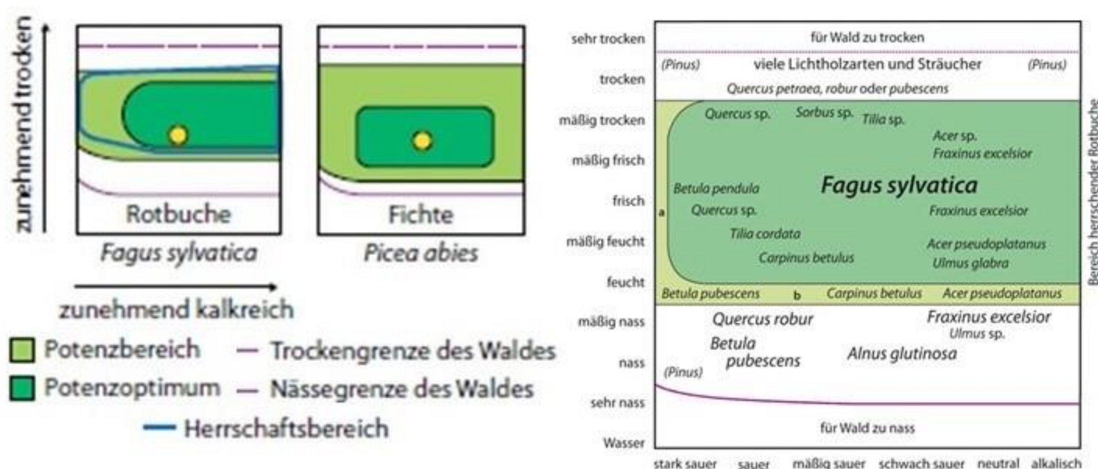

Figure 1: Ecogram by Ellenberg (modified) with free competition, the x-axis shows the gradient from acid soil to alkaline soil, the y-axis shows the gradient of water saturation of the soil, from wet to dry, the light green area shows the area where the trees can potentially live, the dark green area shows the potency optimum. The existence optimum, i.e., the area where the species can prevail and even can become dominant, is indicated by the blue circle, the yellow dot indicates average conditions, i.e., when all tree species thrive equally well (Bartsch and Röhrig, 2016, p.71 and 72).

2. Material and Methods

2.1 Study site and sample collection

I sampled leaves, branches, and roots from five replicates of each Fagus and Picea trees growing at Hermannsberg (50°42’3” N, 10°36’52” E, 850 m a.s.l.), Germany. Trunk diameter at breast height (DBH) was estimated with measuring tape and was in average (± SD): *Fagus* 139 ± 32 cm, *Picea* 121 ± 25 cm. *Fagus* samples were collected on July 29th, 2021, and *Picea* samples on August 31st ,2021. Leaves and branches were collected from breast height to 2 m above the ground. The diameter of breast hight (DBH) is a common method to measure trunk diameter at roughly the same hight to standardize the results. I sampled roots from the same trees, making sure the collected roots were from the target individuals. For achieving that I followed the roots to its attachment at the main root. In addition, indicators like colour and smell helped me with identification. All excised samples were stored in a cool box with ice until the next day. Next day, roots were washed from soil and fine roots (≤ 2mm) were separated for further analysis.

2.2 Design of the experiment

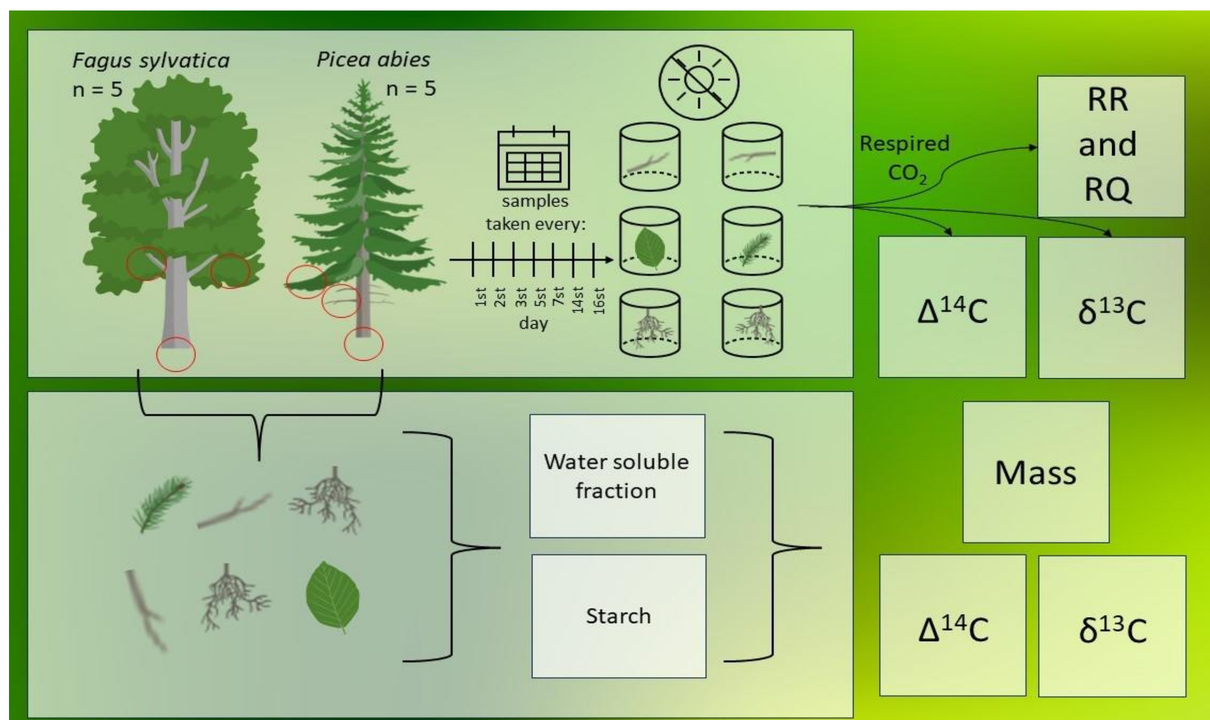

Figure 2: Graphic of experimental design, RR = respiration rate, RQ = respiratory quotient

The design of the experiment is conducted similar as Hilman et al. 2021. Over two weeks, I measured respiration and NSC pools in harvested organs that were stored in the laboratory in

wet paper and aluminium foil (Fig. 2). To measure respiration, I incubated samples of trees in an airtight assemble to collect respired CO_2 in glass flasks. This happens in a dark environment to avoid photosynthesis for C starvation. The on-line incubation period lasted two weeks and gas samples were extracted and measured on the 1st, 2nd, 5th, 7th, and 16th day for *Picea abies* and on the 1st, 2nd, 3rd (excluding roots) and 14th day. With the CO_2 and O_2 values I calculated the RR, RQ, and measured $\delta^{13}C$, and $\Delta^{14}C$. In parallel I extracted NSC from additional stock of replicated samples. Measurements of TOC and of glucose, fructose, sucrose, and starch content were carried out. Furthermore, mass content, $\delta^{13}C$, and $\Delta^{14}C$ were measured for the water-soluble fraction and starch This happened at the 1st, 2nd, 5th, 7th, and 16th day for *Picea abies* and on the 1st, 2nd, 3rd (excluding roots) and 14th day. Respiration and extraction applied at the same times. The reason for the different measurement dates between *Picea* and *Fagus* is the proceeding moulding of the samples in *Fagus* that took place way quicker than I thought. I removed these parts and secured the rest. This is important, as mould also practise extracellular digestion to respire, which could influence the results. Since there was now less material available for this reason, I dispensed with intermediate measurements and saved the rest for the last measurement. This way I have a comparison between the beginning and the end of the experiment.

2.3 Respiration

For ^{14}C measurements it is important that every surface and container that touch the samples is free of external C for the radiocarbon measurements. The reason is the low abundance of ^{14}C and therefore an easy contamination with foreign C. Furthermore, the particle accelerator is very sensitive, and could detect foreign C which in return would bias the results. To prevent this, I washed glass flasks in an Ultrasonic bath (Sonorex Super 10P DK 255 P Ultrasonic Bath 160W, Bandelin, Berlin, Germany) to remove dirt. Afterwards, flasks were baked for 5 h at 500 °C. Then, I flush the flasks with synthetic air (20.5% O_2, 0% CO_2; Westfalen AG, Münster, Germany) three times to ensure that any C that could be inside is removed.

I measured gas exchange and isotopic signatures of respired CO_2 using closed-system incubations. To measure CO_2 and O_2, samples were placed in plexiglass incubation chambers were filled with tree organs (the range was between 0.6 mg and 2.5 mg sample) and flushed with synthetic air 20.5% O_2 which has no C. It is to displace atmospheric air and makes sure that measured C is only from the samples. Then two 115 ml glass flasks were connected to the chamber and fixed with fittings (12.7 mm Swaglock Ultra-Torr) to ensure an airtight assemble.

Flasks were opened, and tree organs incubated in the dark at room temperature until the next day. One glass to measure the concentration of stable C isotopes and the second glass to measure radiocarbon. I measured *Fagus* samples less times then *Picea*, since senescence was advanced more quickly in *Fagus* organs. To make sure that I had at least comparative samples for the first and last day, I waived on intermediate measurements and saved the remaining samples for the last measurement.

For CO_2 and O_2 measurements, different air standards with different CO_2 and O_2 levels (Westfalen AG, Münster, Germany) were measured to calibrate the CO_2 Sensor (GEMP251/252, Vaisala, Hamburg, Germany) and O_2 sensor (Fibox 4 trace, Presens, Regensburg, Germany) and to have references for the measured values. I collected 20 ml air with a syringe from the air-tight assemble and injected into the customized system with the attached CO_2 and O_2 sensor. After the incubation was completed, both glass flasks were closed, and new flasks attached. Because of technical difficulties with the CO_2 sensor, the CO_2 dataset is incomplete and therefore unreliable. Consequently, I used the CO_2 measurements that were done simultaneously with the $\delta^{13}C$ measurements by the team of the BGC Isolab. Both, O_2 and CO_2 values needed to be corrected for different reasons, for instance water vapor increases the pressure in the flasks, which is detected by the sensitive spectrometer. I corrected the water vapour according to Helm *et al.*, 2021 by first using the Clausius-Clapeyron relation (equation (1)),:

$$es\ (hPa) = 6.11 * \exp\left(\frac{(17.502 * T)}{(T + 240.97)}\right) \tag{1}$$

where es describes the saturation water vapor pressure and T the temperature in °C.

Then I used es (hPa) in equation (2) to get the corrected end value of O_2:

$$O_{2_{corr}} = \frac{S * es(hPa)}{es(hPa) - p} \tag{2}$$

where S is the measured O_2 value and p is environmental pressure in [Pa].

In addition to the values for O_2, the CO_2 values must also be mathematically corrected. The team of the BGC Isolab took 100 µl air sample from the flask and measured the $\delta^{13}C$ and total CO_2 concentration in a mass spectrometer (Isotope Ratio Mass Spectrometer, IRMS; Delta+ XL; Thermo Fisher Scientific). To transfer the flask air that is in atmospheric pressure into IRMS vials, the flasks air was first expanded to a vacuumed headspace. After the pressure was

recorded, argon was injected to the headspace to create overpressured air mixture that can be expanded into the vials. This procedure dilutes the CO_2 concentration. I corrected this effect by calculating out the added argon. The $\delta^{13}C$ results are not affected by this, since the argon does not affect the number of present ^{13}C in the sample. To correct the samples for argon, I used equation (3):

$$CO_{2_{corr}} = \frac{CO_2 * Ar}{f} \tag{3}$$

where CO_{2corr} is the corrected CO_2 value in [ppm] CO_2 is the measured CO_2 in [ppm], Ar is the added argon in [bar] and f is the pressure in the glass flask in [bar].

I used the corrected CO_2 values then to calculate the RR by using equation (4) from Hilman *et. al.* 2022:

$$RR = \frac{\Delta CO_{2_{corr}}}{t} * \frac{V * BP * M_C}{T * W * R} \tag{4}$$

Where ΔCO_{2corr} is the corrected CO_2 concentration ($ppm/10^6$), t is the incubation time in days, V is the Volume of the incubation chamber [cm³], BP is the local barometric pressure [hPa], M_C is the molar mass of C (12 mg $mmol^{-1}$), T is incubation temperature (295°K), W is the weight of sample [g] and R is the ideal gas constant (83.14 ml hPa k^{-1} $mmol^{-1}$).

I also used the corrected values for the calculation of RQ with equation (5):

$$RQ = \frac{O_{2_{corr}}}{CO_{2_{corr}}} \tag{5}$$

C has two stable forms, ^{13}C and ^{12}C, which differ by an additional neutron in ^{13}C. This makes the atom heavier, leading it to be discriminated against in biological processes such as respiration. The reason is that ^{12}C, due to its lighter weight, reacts and diffuses faster. Lighter mass has a higher movement speed and bond strengths are smaller (Schuur, Druffel and Trumbore, 2016). To report ^{13}C abundance, the $^{13}C/^{12}C$ ratio in a sample is compared to the ratio in a standard (Pee Dee Belemnite (PDB)) material and expressed as $\delta^{13}C$ (Schuur, Druffel and Trumbore, 2016) in equation 6:

$$\delta = \left[\frac{\left[\frac{^{13}C}{^{12}C}\right]_{sample}}{\left[\frac{^{13}C}{^{12}C}\right]_{standard}} - 1 \right] * 1000 \quad (6)$$

In preparation for the $\Delta^{14}C$ measurements, I extracted the C from the second glass flask via the graphitization-vacuum-line (In-house development). The separation is based on cool traps, where the sample is first cooled down to a temperature where water vapor freezes to remove water vapor and then cooled down even further where CO_2 gets solid. Other compartments of the respired air are still in their gas form a can be therefore removed with a vacuum pump. Afterwards the frozen CO_2 is heated up again, so that it is gaseous again. It is further fed into an airtight glass vile with iron filings, where the CO_2 is collected by freezing it again using liquid nitrogen. Finally, 250 mbar of H_2 is added to the sample and iron powder to initiate a process called graphitization. The iron powder serves as a catalyst on whose surface the graphite is deposited during the reduction of C in the presence of H_2 and at temperatures of around 550 °C to graphite. This step is crucial because graphite generates constant C-ions from small mass samples and low contamination rate. This leads to more precise results (Chung and Kim, 2013). Samples are measured with an Accelerator Mass Spectrometer (*MICADAS* by Ionplus, Dietikon, Schweiz). If results showed outliers, meaning values that first which has a large distance to the rest of the values, and second that is outside of former reported values, I repeated the procedure a second time with the backup sample for these samples and measured them again to see if the outliers were artifacts or genuine.

2.4 NSC extractions

For the same reasons as for the respirated samples, it is just as important that the equipment for NSC extraction is clean for the $\Delta^{14}C$ extractions, as well. I used microcentrifuge tubes with socket screw caps. This is not favourable since plasticware release high amounts of C. It was not possible to use glassware, as our centrifuge is not designed for this, but we solved this problem by washing the plasticware thoroughly in the ultrasonic bath with distilled water and measured the total organic C (TOC) in the washing water to monitor their cleanness. If the TOC in the water was < 0.5 mg/mL, we stopped the washing process. If not, we continued until the threshold was met.

I measured TOC, NSC content and isotopic signatures of NSC reserves. Tree samples were dried in an oven at 60 °C for a week and stored in a desiccator until further preparations. After grinding the samples with ball mill (Retsch MM400, Haan, Germany), samples were dried a second time afterwards at 60 °C over night to remove moisture that might have accumulate in the grinding process and stored at any time in the desiccator.
Of each tree sample I weighed 100 mg into 2 ml micro-centrifuge tubes, on an analytical balance, readability 0.1 mg (Balance XPR205, Mettler-Toledo, Greifensee, Switzerland).
To ensure that the results are viable, I used different standards with known $\delta^{13}C$, $\Delta^{14}C$ and mass-values. These are two internal in-house standards: Std2, a mixture of leaves from maple, hazelnut beech, oak, and ash trees, and Std3, a mixture of pine needles. I weigh 100 mg of our internal standard and three concentrations of our sucrose standard IAEA-CH-6 (H. Polach, Australian National University, Canberra), 1 mg, 3 mg, and 5 mg and I included as a standard for the starch extraction a rye flour standard (Sigma Aldrich, St. Louis, USA), with 2 mg.

I performed the sugar extractions according to the instructions of Landhäuser et. al. (2018) with a few modifications since his protocol is not intended for $\Delta^{14}C$ analysis. I used water instead of the original ethanol because ethanol introduces C to the sample that might change isotopic signatures. I added 1.5 ml distilled water to the sample and then put it in a Thermomixer (Eppendorf, Hamburg, Germany) for 10 min. at 35 °C, instead of the original 85°C to extract the sugars. The temperature decrease is important because I used the same sample for sugar- and starch extraction, and high temperature can lead to a gelatization and loss of starch into the sugars fraction. I transferred 1 ml of the supernatant in a second 2 ml screw caps tube. The pellet was washed two more times to remove any residual sugar extracts that still might be in the sample and could bias the results of the starch extraction. The supernatant of the washing step is discharged, and the pellets was kept dried for the starch extraction.
To measure glucose, fructose, and sucrose concentrations in the water-soluble fraction aliquot from the 1-ml sample was measured with a high-performance liquid chromatography (HPLC) (UltiMate 3000, Dionex Softron GmbH, Germering, Germany). Besides the identified sugars the water-soluble fraction contains many other unidentified sugars and other compounds like organic and amino acids that might be used as storage. Furthermore, the isotopic measurements are not differentiating between the soluble compounds and are valid to the entire sluble fraction. Hence, I Estimated the amount of total water-soluble C by measuring total organic C (TOC) in the 1-ml samples with an elementar vario TOC cube (Elementaranalyse systeme GmbH, Langenselbold, Germany).

To turn the sugars concentrations from mg/mL into percent dry mass, I used equation (7):

$$\%sugar_{glu+fru+suc}\left(\frac{w}{w}\right) = \frac{\left([glucose] + [fructose] + \frac{[sucrose] * 360.3}{342.3}\right) * V_{extract}}{(W * 10^3)} * 100\% \qquad (7)$$

where [glucose], [fructose] and [sucrose] are the concentrations of glucose, fructose, and sucrose in the sample in µg/mL, $V_{extract}$ is the extract volume in mL (1.5 ml).

To extract starch from the pellet I used the enzyme digestion method (Landhäuser et. al. 2018). This method uses α-amylase to convert starch to soluble glucans. We made few alterations to the protocol, since the enzyme has a $\Delta^{14}C$ value that could affect the measured value. Therefore, washing steps of the enzyme and starch extract had to be added. I diluted α-Amylase (Sigma Aldrich, St. Louis, USA) with distilled water and cleaned the enzyme three times with Amicon® Ultra 15 ml Centrifugal Filters Lot R3MA06116 (Merck, Darmstadt, Germany) to remove stabilizers and other low-molecular weight compounds from the enzyme (Wanek, Heintel and Richter, 2001). I mixed the cleaned enzyme with distilled water and added 1 ml of the enzyme solution to the sample. Samples were then incubated in the thermomixer for 30 minutes at 85°C and cooled down to room temperature afterwards. Meanwhile, I dried the Amicon® Ultra 0.5 ml centrifugal filter unit (Merck, Darmstadt, Germany). To achieve a high yield and remove the enzyme from the extraction, I filtered the complete supernatant with the Amicon® Ultra 0.5 ml centrifugal filter unit. I pipetted the samples into silver capsules (Elementar Analysensysteme GmbH, Langenselbold, Germany) for the $\Delta^{14}C$ measurement and smooth wall tin capsules (Elementar Analysensysteme GmbH, Langenselbold, Germany) for the $\delta^{13}C$ measurement.

The second enzyme step uses amyloglucosidase to convert glucans to glucose. I emulsified amyloglucosidase (Sigma Aldrich, St. Louis, USA) with sodium acetate buffer solution, and added 0.5 ml of the solution to 0.1 ml sample from the first enzyme step, which is then incubated in the thermomixer. I let it to cool down to room temperature and added 0.6 ml of chloroform to precipitate the protein enzymes, vortexed it and centrifuged it. I transferred the aqueous phase into a new tube. From there, I pipetted 0.5 ml sample into an HPLC-vial for glucose measurements with the HPLC.

To calculate the volume of starch (V_{starch}) I used equation (8):

$$V_{starch} = \frac{V3 + V4}{V3 * V2} \qquad (8)$$

where V2 is the volume of α-amylase solution (1 ml), V3 the volume of the aliquot of the α-amylase digestion (0.1 ml) and V4 the volume of amyloglucosidase solution (0.5 ml).
To calculate the concentration of the starch-digested glucose I used equation (9):

$$\% \, starch \left(\frac{w}{w}\right) = \frac{([glucose\ hydrolysate] * V_{starch})}{(W * 10^3) * 0.9} 100 \tag{9}$$

Where [glucose hydrolysate] being the concentration of glucose hydrolysate in the solution after starch digestion (µg/mL), the factor 0.9 is used to mathematically turn the glucose equivalent to starch.

To report the $\delta^{13}C$ of both, water solubles and starch, equation (6) was used again. Radiocarbon values for the extracts however needed some additional corrections, since I discovered impurities in the extraction samples. This was solved with the sugar standards.
The sugar standards have an $\Delta^{14}C$ of 500‰, if the value is different, that means that one or more steps in the method introduced external C to the samples. If such impurities were found in the $\Delta^{14}C$-sugar and starch samples, I corrected for that with a mass balance approach. So called dead C is C without any ^{14}C-Atoms and has an isotopic signature of -1000 ‰.
In equation (10) and I calculated the $\Delta^{14}C$ signature of the impurity:

$$\Delta^{14}C_{cont} \frac{m_{cont} * W_{\Delta 14C}}{meas_{\Delta 14C}} \tag{10}$$

Where m_{cont} is the mass of the contamination in [mg], $W_{\Delta 14C}$ is the weight of the sample in the silver capsule, and $meas_{\Delta 14C}$ is the measured $\Delta^{14}C$ signature of the sample.
I included this value then in equation (11), where I calculate the supposed $\Delta^{14}C$ values of the standards ($\Delta^{14}C_S$):

$$\Delta^{14}C_S = \frac{\Delta^{14}C_{standard} * W_{\Delta 14C} + (-925‰) * \Delta^{14}C_{cont}}{W_{\Delta 14C}} \tag{11}$$

where $\Delta^{14}C_{standard}$ is the original $\Delta^{14}C$ signature of the sugar standard (500‰) and -925 ‰ the radiocarbon signature of the contamination. I got the value of -925 ‰ by trial and error. When entering the correct value, the $\Delta^{14}C$ values of the standards should get the value 500 ‰, since this is their supposed $\Delta^{14}C$ signature. Since -1000 ‰ is the value of dead C, I started there and

worked my way down in steps of 15. At -925 ‰, the values looked good, and I therefore used this value in equation (12), where I finally calculated the corrected $\Delta^{14}C$ values of the samples:

$$\Delta^{14}C_{corr} = \frac{(W_{\Delta 14C} * meas_{\Delta 14C} - (-925‰) * m_{cont})}{m_{sample}} \quad (12)$$

2.5 Statistics

I applied linear mixed effects model with the *R* function '*lmer*' where organ, species and day are explanatory variables and tree individual a random effect. Significant differences among the species and organs were assessed based on estimated marginal means. To test normality, I used the Shapiro-wilk test, to test variance homogeneity I used the levene-test, and I checked with a scatter plot and QQ-plot for any skewness of the data. Data was transformed with log if necessary.

For statistical analysis and data representation I used Microsoft Excel, RStudio (ver. 2021.09.2+382;) and the R packages. I used *tidyverse* (Wickham *et al.*, 2019), *tidyr* (Wickham, Vaughan and Girlich, 2023) and *ggplot* (Wickham *et al.*, 2016) für data representation, *openxlsx* (Dragulescu and Arendt, 2020) for data import, *emmeans* (Russell *et al.*, 2023) for post-hoc tests, *car* (Fox *et al.*, 2023) to test for homogeneity of variance and *lme4* for the linear mixed effects model (Bates *et al.*, 2015).

3. Results

3.1 TOC and NSC

On the first day, leaves of *Fagus sylvatica* had TOC values two-times higher with 8.7% in leaves than roots with 4.5 % and branches with 4.7 % (Leaves-Branches $p = <.0001$; Leaves-Roots $p = <.0001$; Fig. 3). Similar applies to *Picea* with 10.5 % in needles, 5.8% in roots ($p = < .0001$), and 4.5 % in branches ($p = <.0001$). Differences in TOC between roots and branches are only marginal ($p = 1.0$) with 0.8% within *Picea* and 0.7 % within *Fagus.*

The decrease of roots and branches differs marginally between the species. While *Fagus sylvaticas* leaves have a high usage with 4.2 % between the first and last day, *Picea abies* leaves usage lay at 2.8 % between the first and last day (Fig.3). *Fagus sylvatica* roots have 2.1 % less TOC after two weeks, whereas *Picea abies* roots lose 2.8 % TOC. *Fagus* branches on the other hand have 1.2 % TOC left after two weeks, while it is 1.6 % TOC for *Picea abies*.

Notable are the variations of *Picea abies*' needles that don't occur in other organs or in *Fagus sylvatica*. The minimum and maximum of these variations between the first and 7th day have a difference of 1 %.

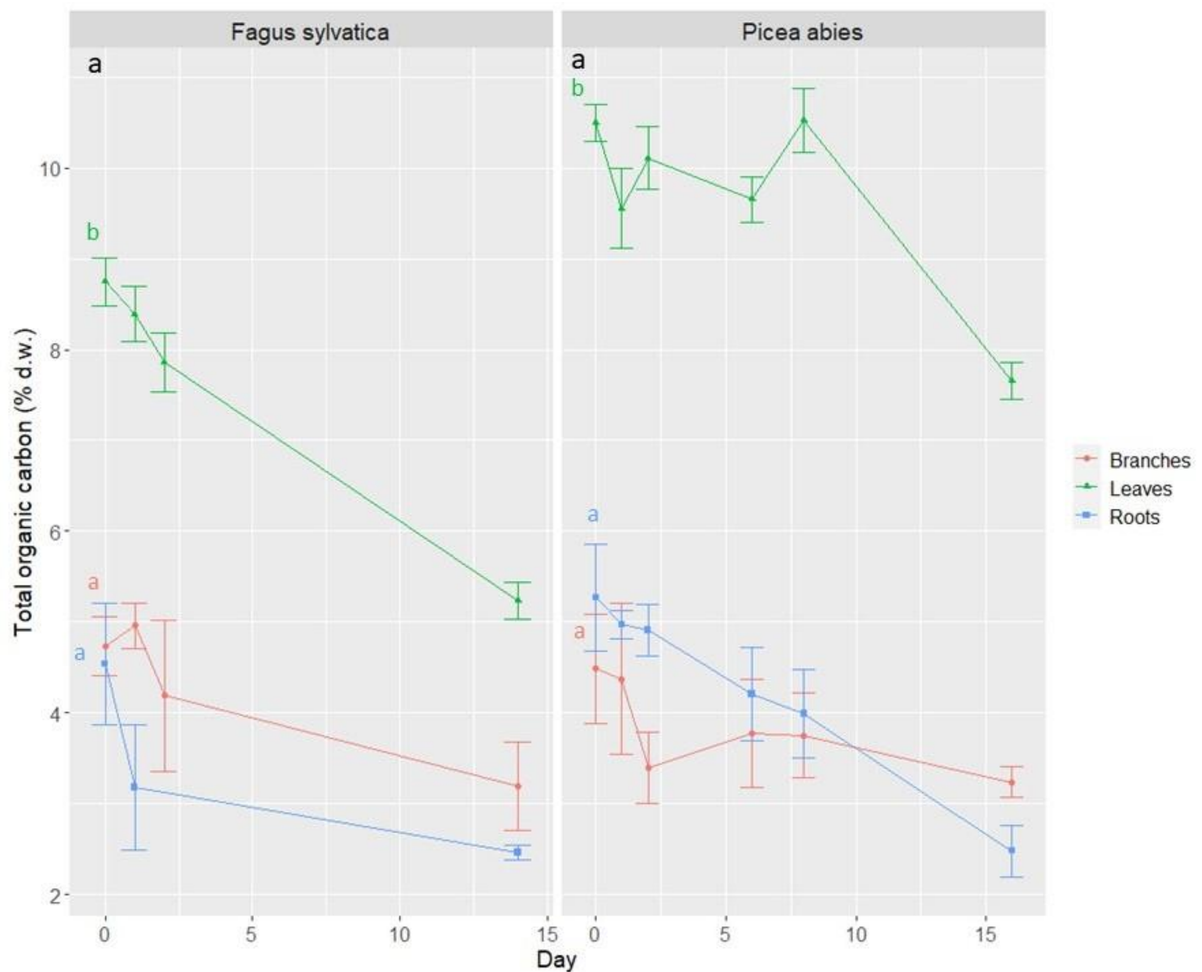

Figure 3: Total Organic C (%) of Fagus sylvatica and Picea abies organs during the two weeks experiment in % per dry weight (d.w.). To avoid fast desiccation tissues were kept moist. Fagus sylvatica leaves and branches started to decompose at day 3, roots at day 2, all Picea abies sample, black letters at the top left indicate the differences between the means of the respective species, while the letters of the colour of the respective graphs indicate differences between the means of the organs, differences among the species and organs were assessed based on estimated marginal means.

Generally, water-soluble sugars make up around 40% of the TOC, with sucrose and starch being the biggest reserves. This varies in *Fagus sylvatica* between 30 % and 42 %, in *Picea abies* between 26 % and 65 %. Sugar and starch content and depletion varies by species and organ (Fig. 4 A, B, C, Fig 5).

Fagus sylvatica organs contain between 1.5 % - 2% sugar per mg dry matter, while *Picea abies* organs contain approximately 2 % - 3 % sugar per mg dry matter at the beginning of the experiment (Fig 4 A, B, C). Sugar content in *Fagus* is inconsistent in its development, with roots having a decreasing trend, leaves increasing until day two, with a sudden drop afterwards. Sugar content in *Fagus* branches increase until the third day, reaching a plateau afterwards and containing 1 % more sugar after 14 days (Fig. 4 B). It should be noted that the sugar reserves

within the *Fagus* leaves increase rapidly after the second measurement by 2.4 % before they drop again by 3.9 % (Fig. 4 A). On the contrary, all organs of *Picea abies* are showing decreasing trends in their sugar content, with roots having the steepest decline.
Starch content is decreasing in both species and in all organs, although the decrease in *Picea* is only marginal but significant ($p = < .0001$). Same goes for *Fagus* ($p = < .0001$). *Fagus* has, with an exception in roots, more starch reserves than *Picea*. Especially in *Fagus* branches, starch content is the highest with 1.6 % at the beginning. *Fagus sylvatica* biggest reserves in branches and leaves are sucrose and starch with a mean C (glucose equivalents) of 1.6 % and 1.7 % sucrose in branches, and 0.8 % starch and 2.2 % sucrose in leaves (Fig. 5). *Picea abies* has a balanced distribution of fructose, sucrose, glucose, and starch in the aboveground organs. *Picea* leaves show a high glucose content on the first day (mean C of 1.5%). Whereas *Fagus* tapped mainly sucrose, *Picea* uses all kinds of sugar without any preference.

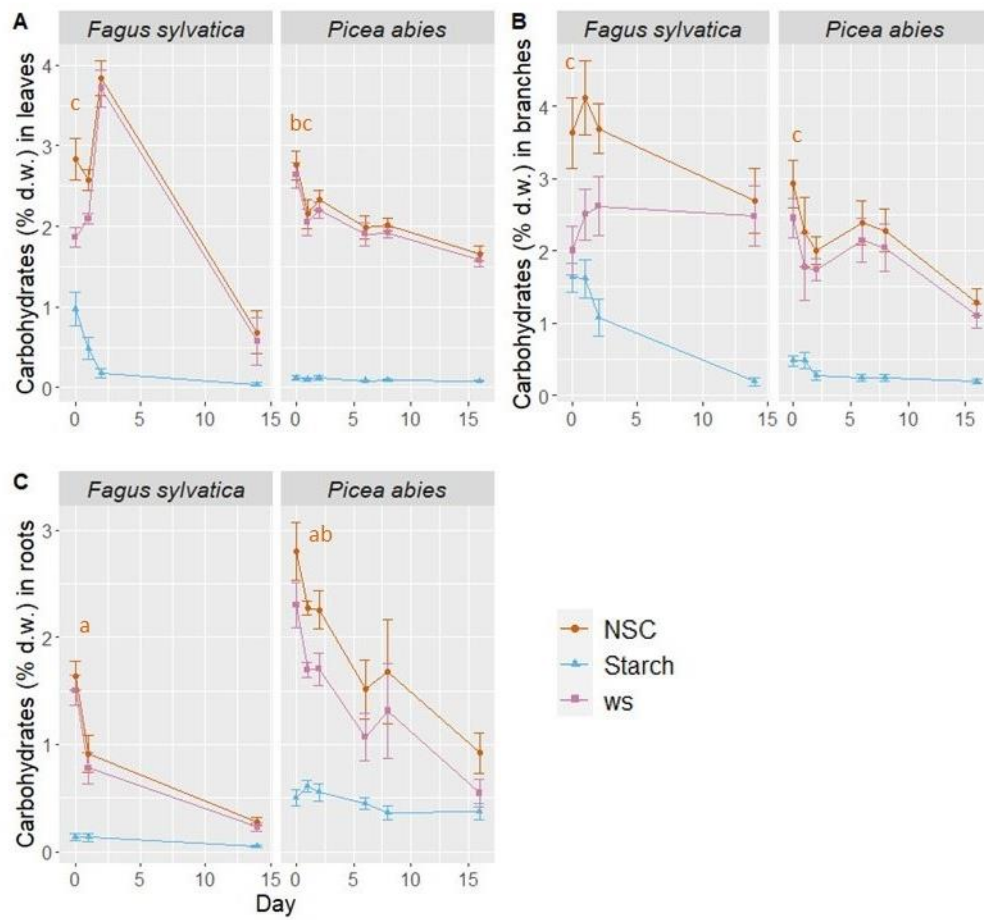

Figure 3: Total Non-structural carbon (NSC), starch and water solubles (ws) course over two weeks of Fagus sylvatica and Picea abies in A)leaves, B) branches and C) roots in % per dry weight (d.w.), the letters of the colour of the NSC graph indicate differences between the means of the NSC, differences among the species were assessed based on estimated marginal means.

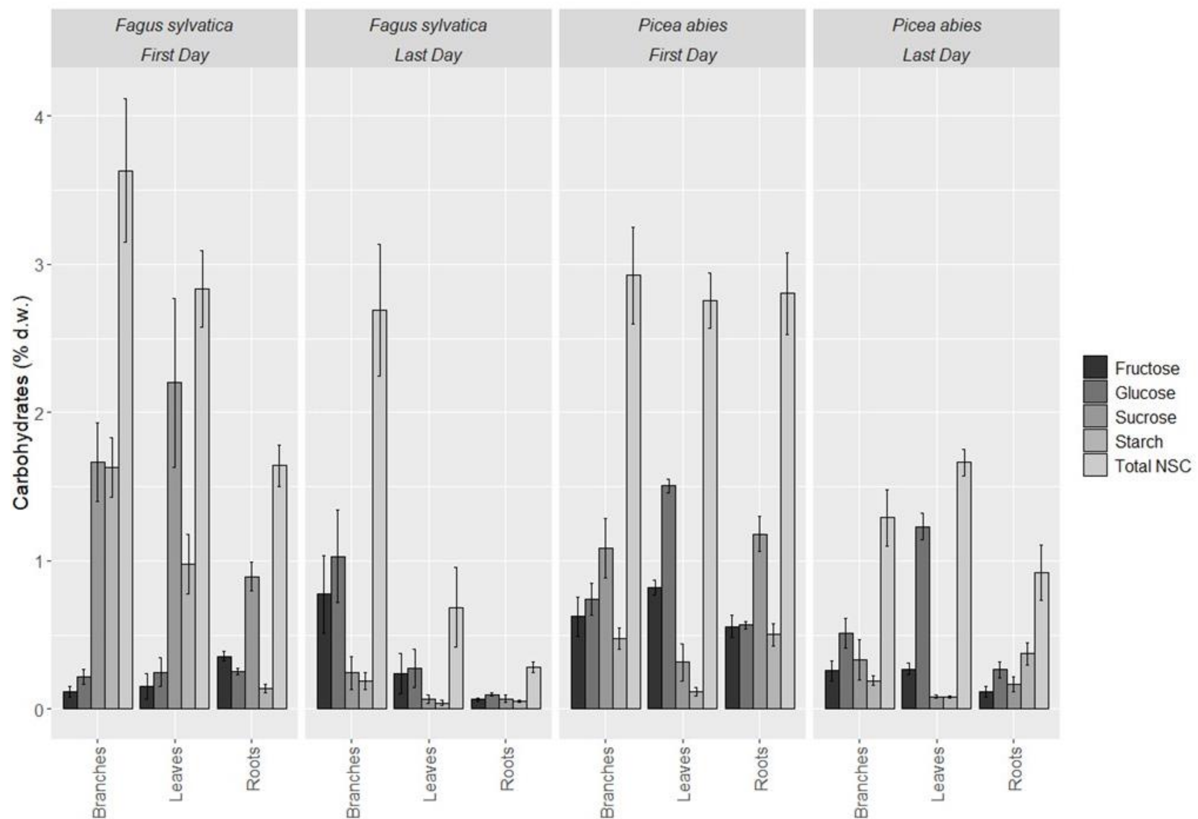

Figure 4: Carbohydrates of Fagus sylvatica and Picea abies separated in Fructose, Glucose, Sucrose, Starch and all combined as Total Non-structural carbon (NSC) in % per dry weight (d.w.) during two weeks experiment

At first, *Fagus sylvatica* initial RR was faster than the RR of *Picea abies* in leaves and branches, but differences disappeared until day 14-16 (Fig. 6). *Fagus* has a different strategy over the two-week experiment than *Picea abies* for the most part, but the significance level was at ($p = 0.07$).

At the beginning, all organs of *Fagus* have different respiration rates, with a CO_2 output varying between 7.5 mg C g^{-1} day^{-1} in leaves and 2.6 mg C g^{-1} day^{-1} in roots on the first day (Fig. 5). After two weeks incubation, the RR of all organs are approaching each other, with 1.8 mg C g^{-1} day^{-1} for leaves and branches, and 1 mg C g^{-1} day^{-1} in roots after two weeks.

Differences of RR in *Picea* are not as big as in *Fagus*, with only 3.6 mg C g^{-1} day^{-1} respired in roots, 2.6 mg C g^{-1} day^{-1} respired in leaves and 1.4 mg C g^{-1} day^{-1} respired in branches.

Picea abies shows a similar RR in roots as in *Fagus* roots over the two-week period, with *Fagus* roots dropping its RR by 1.9 mg C g^{-1} day^{-1} and *Picea* roots dropping by 2.0 mg C g^{-1} day^{-1} (Fig. 5). *Picea* needles don't change their RR at all in two weeks, branches only by 0.6 mg C g^{-1} day^{-1}. *Fagus* has a rapid decrease in respirated C in aboveground organs by 2.8 mg C g^{-1} day^{-1} (branches) and 6.2 mg C g^{-1} day^{-1} (leaves), while *Picea*, display decreases in the woody organs

of 0.6 mg C g^{-1} day^{-1} in branches and 1.4 mg C g^{-1} day^{-1} in leaves. On the other hand, roots of both species have a similar decline rate of ~ 1.9 mg C g^{-1} day^{-1}.

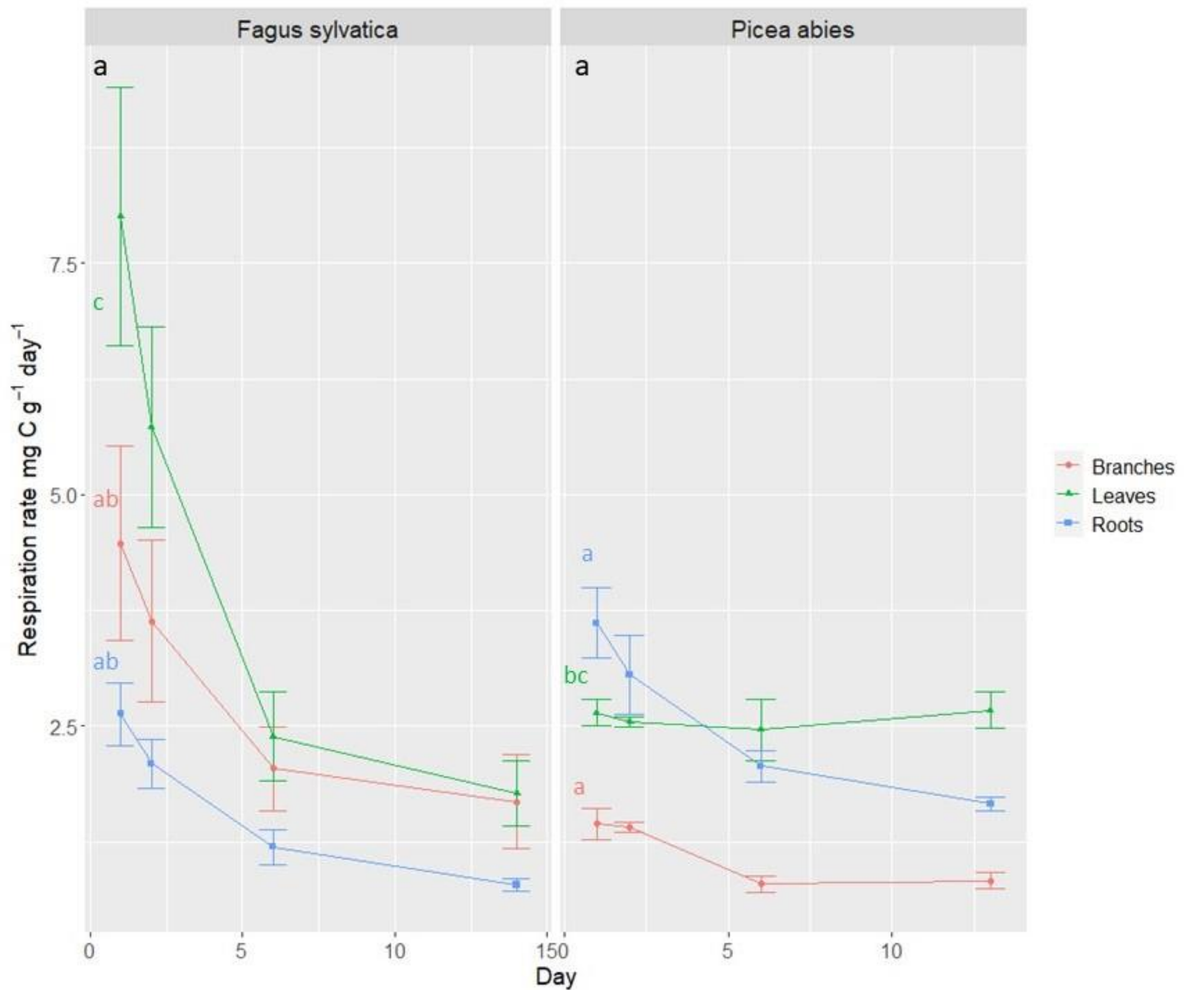

Figure 5 : Respiration rate of Fagus sylvatica and Picea abies organs in mg Carbon (C) g^{-1} day^{-1}, black letters at the top left indicate the differences between the means of the respective species, while the letters of the colour of the respective graphs indicate differences between the means of the organs, differences among the species and organs were assessed based on estimated marginal means.

At the beginning, the RQ of all organs in both species start at around 0.9, except for roots of *Fagus sylvatica*, which have a start value of 1.1 (Fig. 7). Until day two, all organs are dropping in their RQ, in *Picea abies* all with roughly the same dropping rate (0.6). *Fagus* on the other hand is also dropping in its RQ but not as uniform as in *Picea*. Afterwards, the RQ in *Fagus* is increasing again, until values of 1.3 (roots), 1 (leaves) and 0.8 (branches) are reached. However, *Piceas* RQ is instead dropping until 0.6 in leaves, 0.7 in roots and 0.7 in branches is reached.

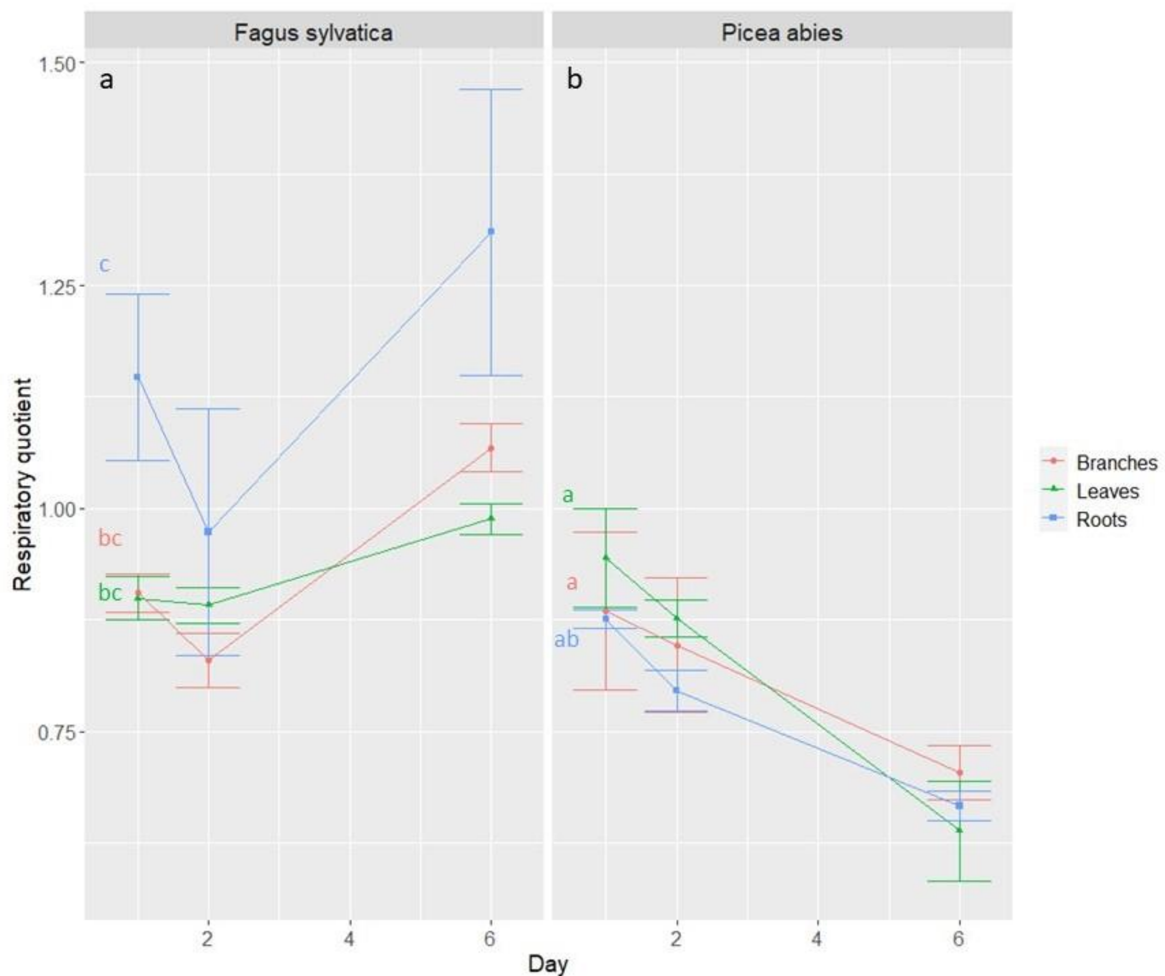

Figure 6: Respiratory quotient (RQ) of Fagus sylvatica an Picea abies, the y-Axis the unitless. The graph is ending after six days, due to due to very large variable data afterwards, black letters at the top left indicate the differences between the means of the respective species, while the letters of the colour of the respective graphs indicate differences between the means of the organs, differences among the species and organs were assessed based on estimated marginal means.

3.2 Stable C isotopes $\delta^{13}C$

On the first day, branches and leaves of both species follow similar curves in their $\delta^{13}C$ signature of respirated air, reaching a plateau after the 6th day (Fig. 8). Roots of *Fagus sylvatica* have a starting $\delta^{13}C$ value of 27.0 ‰, while *Picea abies* has 28 ‰, a difference of 0.9 ‰. All woody organs show an overall increase over time (*Picea* roots = 0.6 ‰, *Picea* branches 0.8 ‰, *Fagus* roots = 0.5 ‰, *Fagus* branches = 1.0 ‰).

Branches in both species and *Picea* roots develop a plateau, whereas leaves of both species get more depleted (*Fagus* by 2.1 ‰, *Picea* by 1.3 ‰, Fig. 8). It is to note, that *Fagus* leaves show an increase by 1.5 % on day two, with a rapid depletion afterwards, which has no significance ($p = 1.0$) whereas *Picea* leaves show a drop by 1.5 ‰. Roots of both species have the highest $\delta^{13}C$ values (between 28.0 ‰ – 26.0 ‰) compared to the above-ground organs. The development of $\delta^{13}C$ follows a similar course in all organs of both species.

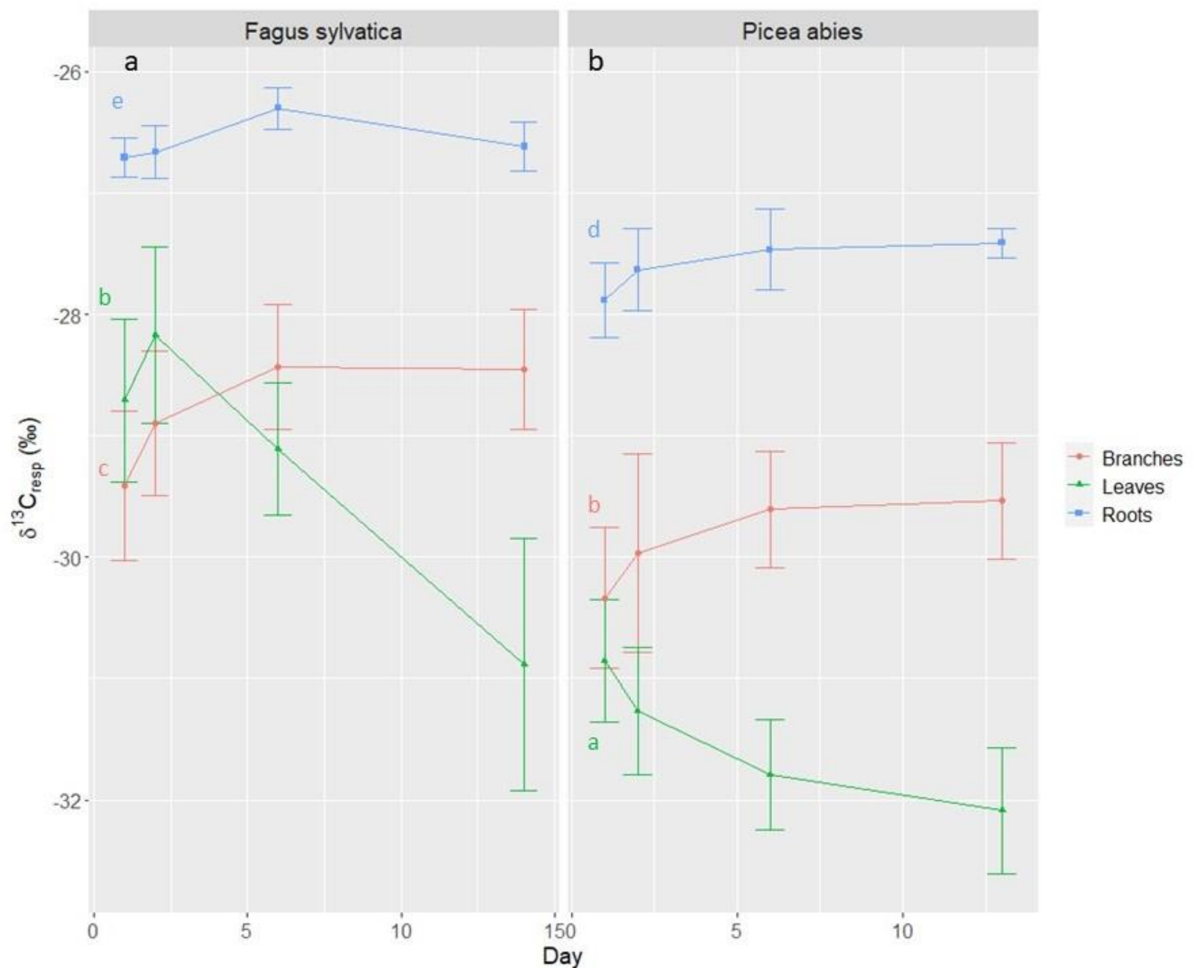

Figure 7: Respiratory δ13C (‰) of Fagus sylvatica and Picea abies, black letters at the top left indicate the differences between the means of the respective species, while the letters of the colour of the respective graphs indicate differences between the means of the organs, differences among the species and organs were assessed based on estimated marginal means.

On the first day, water solubles and starch are more $\delta^{13}C$ depleted in branches and leaves than roots (Fig. 9). The difference between roots and leaves of *Fagus* lies at 2.1 ‰ and 2.5 ‰ between *Fagus* root and branch. In *Picea* the difference between roots and needles lies at 3.5 ‰ and 1.1 ‰. For *Picea abies*, the course of the graphs of $\delta^{13}C$ of starch follows the $\delta^{13}C$ of water solubles. The values on the first day are also similar. *Fagus sylvatica* on the other hand has a different trend in each organ than in $\delta^{13}C$ of water solubles. Results of starches $\delta^{13}C$ values show variation between species and organs in *Fagus sylvatica*, as well as in *Picea abies* (Fig. 9). *Picea* has similar decreasing developments in all organs but showing opposite trends in comparison to the respirational $\delta^{13}C$ results. The organs of *Fagus sylvatica* however show all different developments and similar trends as the respiratory $\delta^{13}C$ results.

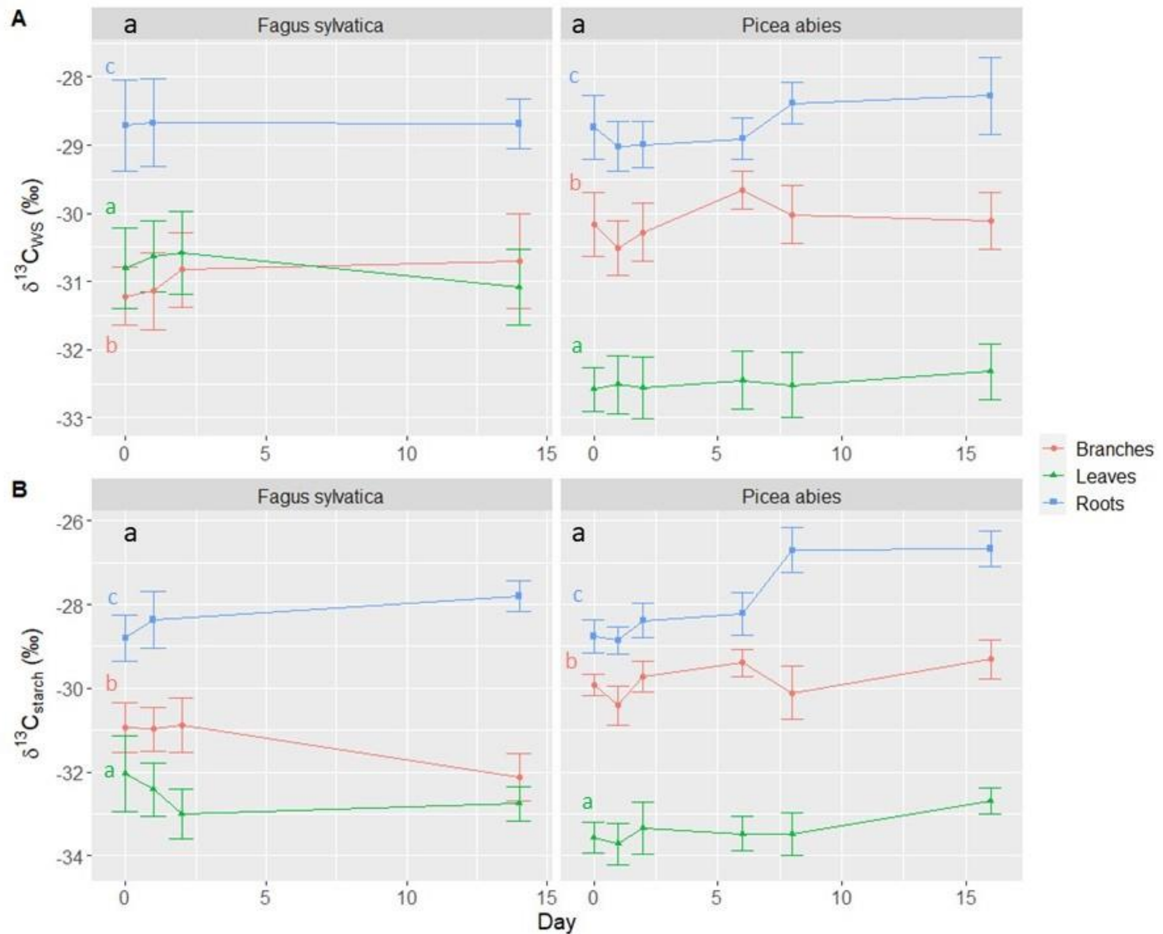

Figure 8: $\delta^{13}C$ of A) water-solubles and B) starch, black letters at the top left indicate the differences between the means of the respective species, while the letters of the colour of the respective graphs indicate differences between the means of the organs, differences among the species and organs were assessed based on estimated marginal means.

3.3 Radiocarbon

In the dataset of the $\Delta^{14}C$ of respired CO_2 were a few outliers, i.e., data points that had quite unusually high values compared to the rest (Fig. 10). These samples were measured a second time and results were more in line with the rest of the data. Unfortunately, we were not able to find out the reason for the high outliers in the first measurement and the post-hoc test revealed significant differences between species, but not between organs (all p-values = > 0.05). However, since this the dataset is not completely unrealistic, and differences between species are significant ($p = 0.02$), I decided to still include it. This should be kept in mind, while viewing this graph. At the beginning, the respired air of all organs of both species has $\Delta^{14}C$ values between -10.0 ‰ and 0.0 ‰ (Fig. 10). Afterwards, there is no clear trend among the organs in their respective species. While $\Delta^{14}C$ values of *Fagus sylvaticas* woody organs are either increasing or having nearly the same value after two weeks as at the beginning, leaves are decreasing by 1.5 ‰. *Picea abies* on the other hand shows decreasing trends in all its organs,

with roots forming a plateau after the second day, branches having an increase of 9.1 ‰ from day one to two, decreasing afterwards again by -14.5 ‰, and leaves having the most severe drop by 15.0 ‰.

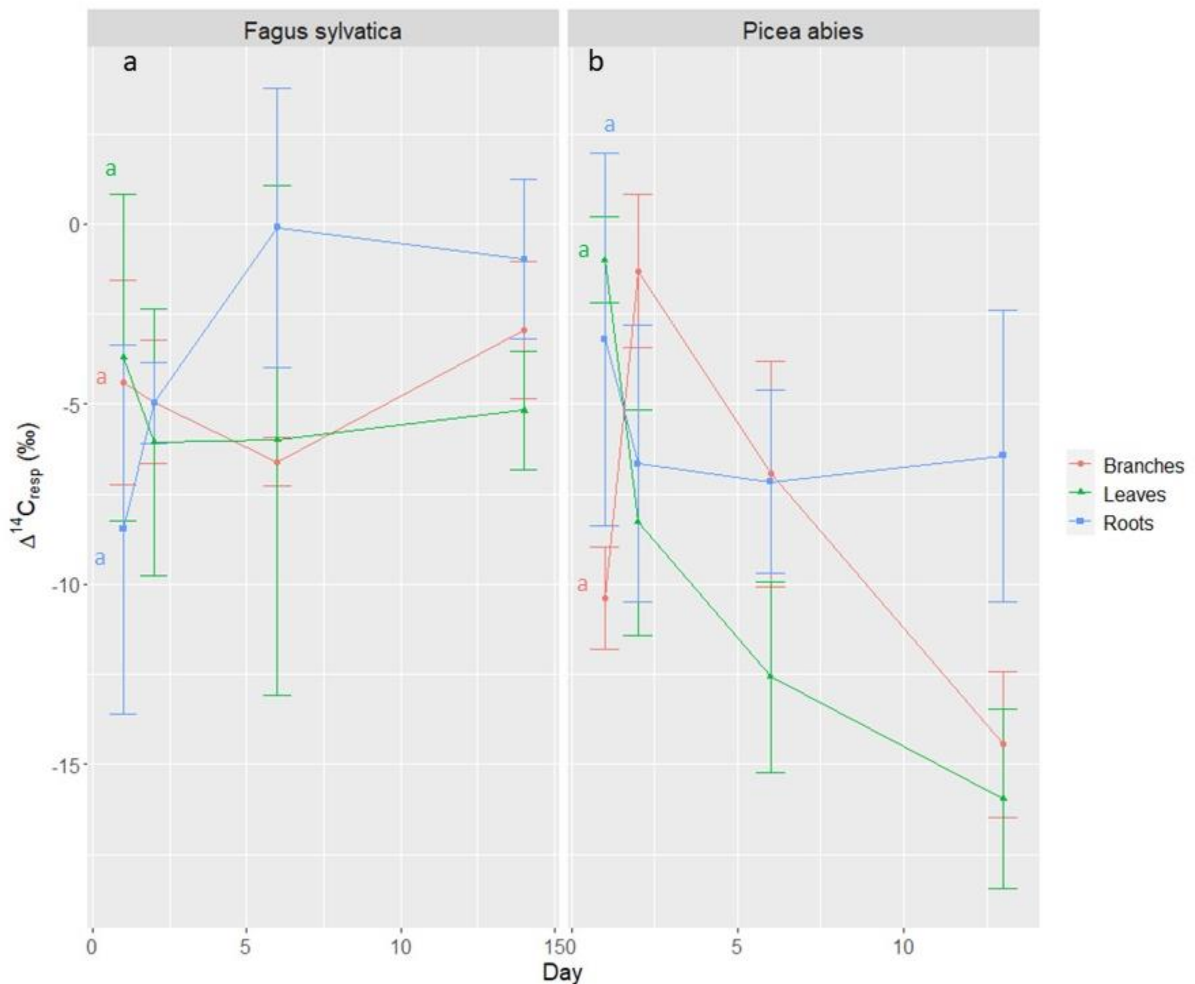

Figure 10: $\Delta^{14}C$ of respired CO_2, black letters at the top left indicate the differences between the means of the respective species, while the letters of the colour of the respective graphs indicate differences between the means of the organs, differences among the species and organs were assessed based on estimated marginal means.

On the flip side, we can see different developments of the $\Delta^{14}C$ in water solubles of both species over two weeks (Fig. 11). At the beginning, all *Picea abies* organs have a similar $\Delta^{14}C$ signature, ranging between 3 ‰ - 8 ‰. *Fagus sylvatica* organs on the other hand differ a lot in their beginning $\Delta^{14}C$ values, with roots having 22 ‰, leaves 4.2 ‰ and branches 9.2 ‰.

While organs of *Fagus* have constant or decreasing $\Delta^{14}C$ values over two weeks, *Picea* organs show increase, with roots having the biggest rise by 28.8 ‰.

On one hand, *Fagus* has minor changes of $\Delta^{14}C$ content between the first day and the end of the experiment in branches (0.9 ‰) and roots (3.8 ‰). On the other hand, leaves get more depleted by 9.8 ‰. *Picea* has a clear upward trend, with all water solubles getting older from the first day forward. After the second day, a plateau begins to form. *Fagus* show either a downward trend, like the leaves, and therefore reserves are getting younger, or a plateau from the start like branches and roots. It must be considered that roots and branches of *Fagus* drop

in their Δ^{14}C signatures after the first day and only rise again after the second day, so that the initial value is almost reached again. After two weeks, the oldest reserves can be found in *Picea* roots with 35‰ and the youngest in the leaves of *Fagus* with -5‰.

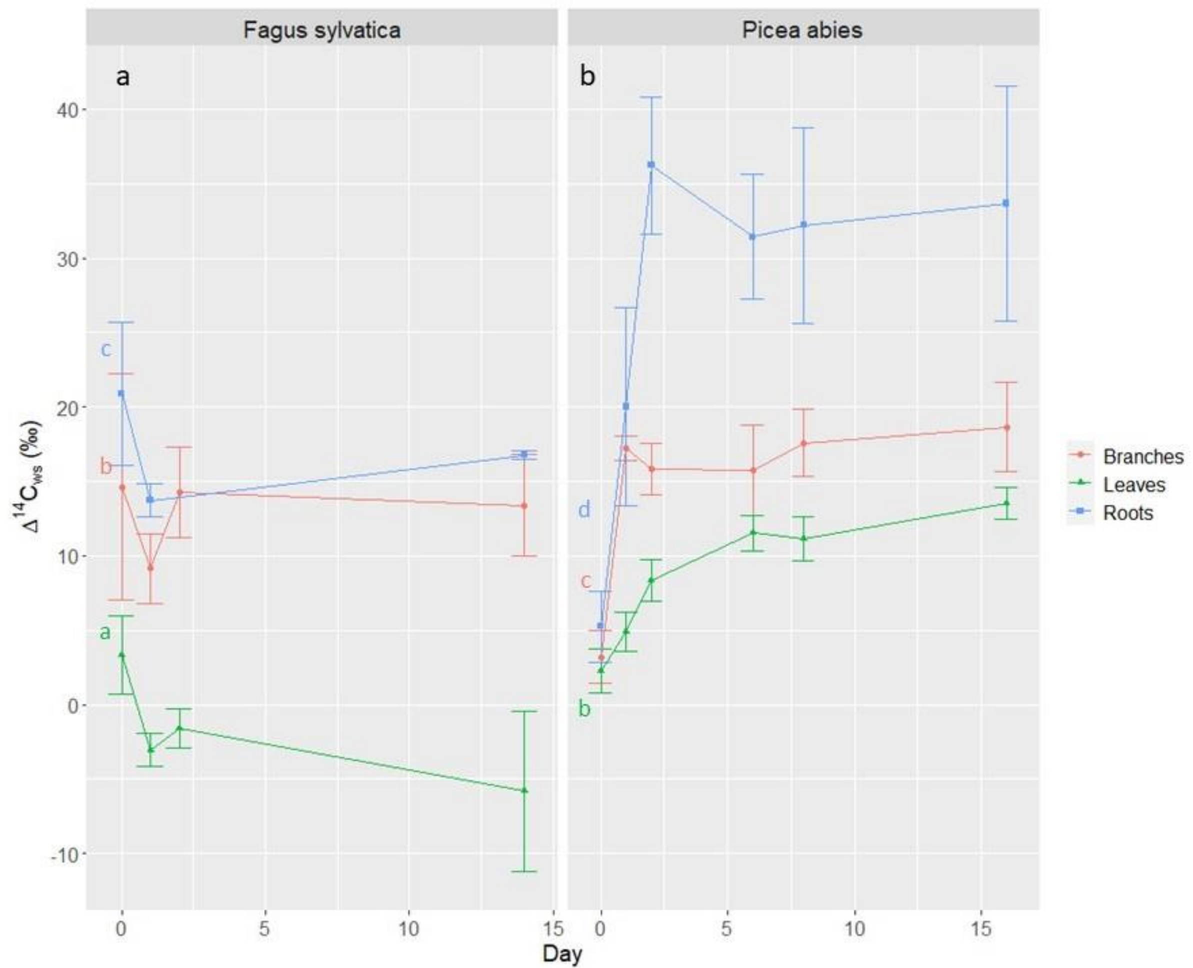

Figure 11: Δ^{14}C signatures of water solubles (ws) in ‰, black letters at the top left indicate the differences between the means of the respective species, while the letters of the colour of the respective graphs indicate differences between the means of the organs, differences among the species and organs were assessed based on estimated marginal means.

On the first day of the measurements, the branches have the same Δ^{14}C signature in starch of 15.1 ‰ (Fig 12). In *Fagus sylvatica*, after a drop on the second day, this increases again to a value of around 22.1 ‰. After that, there are no more values. The leaves of *Fagus* show a similar course, starting from 8.5 ‰ with a final value of 20 ‰ and thus only a difference of 2.0 ‰ to the branches. Root values are missing.

Starch in *Picea abies* on the other hand shows a Δ^{14}C signature of 24.8 ‰ on the first day, which shows a decreasing trend over the two weeks with upward and downward variability ending at 15.1 ‰. Starch in the branches, like *Fagus*, shows an initial value of 14.9 ‰ which also ends with variation over the two weeks at 21 ‰. Thus, the starch of both the branches and the leaves shows similar Δ^{14}C signatures between the two species. The starch within the roots

shows an initial $\Delta^{14}C$ signature of 30.0 ‰ which ends with a strong variability over the two weeks measurement period at 36.1 ‰.

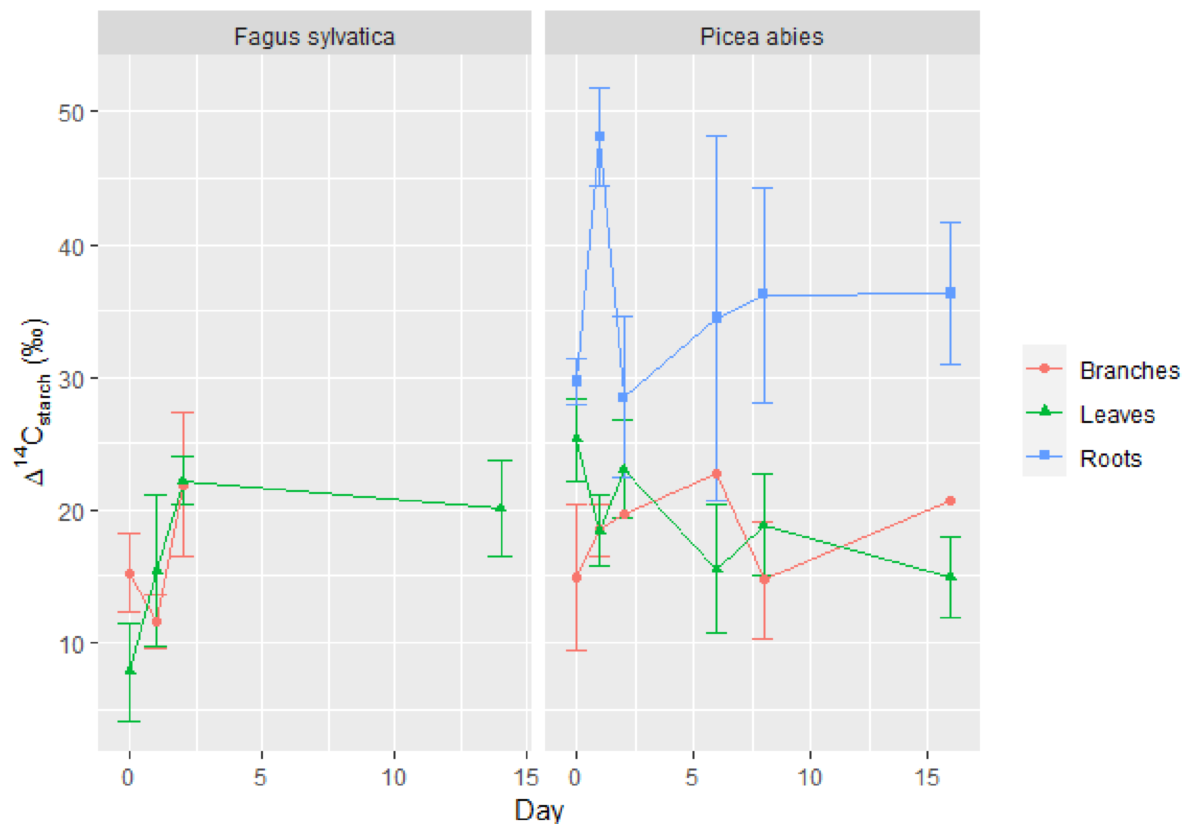

Figure 12: $\Delta^{14}C$ signature of starch in ‰ over two weeks, due to missing data, this graph is incomplete. Roots of Fagus sylvatica *are not shown and branches of* Fagus sylvatica *are only shown up to the third measurement.*

4. Discussion

4.1 *Picea abies* discriminates carbon by age, while *Fagus sylvatica* draws on a mixed C pool

Results of $\Delta^{14}C$ measurements in water solubles and respired CO_2 suggest that under C limitation, *Fagus sylvatica* accesses mixed C pools to maintain metabolism, whereas *Picea abies* discriminates by age and prefers newly assimilated C at the beginning. My data shows that $\Delta^{14}C$ values of water solubles and respired air stays relatively constant, except for *Fagus* roots which shows an age trend, while the respired CO_2 of *Picea* gets younger with time. This leads me to believe that *Fagus* mostly does not discriminate its C reserves by age, but *Picea* does. This confirms the last in-first-out hypothesis, described by Lacointe *et al.*, 1993, for *Picea abies* but is contrary to *Fagus sylvatica*. *Fagus sylvatica* not following the hypothesis is partly in line with different previous studies where mixed pools in broadleaved trees were found in

stem respired CO_2 and sap (Muhr *et al.*, 2013, 2015), while others found separated pools in broadleaved trees (Carbone *et al.*, 2013). Roots of *Fagus* not following the same principle as the aboveground organs could be explained by roots being considered the organ containing reserves that are used in catastrophic events, rather than for everyday metabolism which explained the older C found in roots in other studies (Furze *et al.*, 2019). However, the sugar and starch content in roots dropped in the two weeks experiment but the $\Delta^{14}C$ content of the water solubles stayed rather constant. Additionally, previous studies found a separation of young and old C (Richardson *et al.*, 2015). This leads me to believe that other types of reserves than sugar were used in roots for respiration that were not measured, and this leads to stable $\Delta^{14}C$ values for water solubles, but age differentiated values in $\Delta^{14}C$ of respired air in *Fagus* roots. Richardson describes the slow C pool as C which is turned over quickly and is used for growth and metabolism, and the slow pool as C which consists of old reserves. This could mean that the hypothesis of a \`slow\` and \`fast\` C pool in *Fagus* roots and in *Picea abies* is also supporting the hypothesis by Richardson *et. al*. 2015.

Reasons for these differences are versatile, from structural differences in the anatomy of both species and resulting life strategies, different allocation strategies to contrasting shifts of C storage usage during stress. Differences in anatomy are often adaptations to different habitats which has an impact on C allocation (Hartmann and Trumbore, 2016; Jiao *et al.*, 2020). *Fagus* natural habitat is very broad from the south of Sweden to the north of Greece, to the west it reaches Northern Spain and to the east its growing as far as Ukraine. Therefore it grows in different climatic conditions which lead to different adaptations like structural differences of the parenchyma. On the other hand, the natural habitat of *Picea* is dry and cold areas where growing season are too short for annual leaves and the climate effects the properties of the wood (Sandak *et al.*, 2015). Even though trees normally can outcompete other species more easily in their natural habitat, this is mostly not the case for *Picea* as seen in Fig. 1. It is known that the growing conditions influence the properties of the parenchyma in trees, which in turn influences transport and storage of NSC (Plavcová *et al.*, 2016; Zhang *et al.*, 2022). Wood of gymnosperms have an anatomically simpler design, with missing wood fibre and vessels, and tracheids taking care of stability and conductance at the same time, while having resin canals as a pronounced network which grow through the wood (Nultsch, 2012). Angiosperms on the other side have more pronounced wood parenchyma, wood fibres and vessels. Structure and proportion of parenchyma can be described as an adaptation to different climatic conditions and are a factor of NSC distribution (Godfrey *et al.*, 2020). Therefore, the more nuanced parenchyma in *Fagus* could explain the the higher content of NSC in aboveground organs and the mixing of the C pools since transportation and usage of freshly assimilated C is more effective. With less

pronounced parenchyma like in *Picea*, it is more difficult and takes longer to tap reserves, so new assimilated C is used first. The simpler structure of *Picea* wood can also lead to less efficient water transportation compared to *Fagus*. As a result, *Picea* must use more NSC to maintain water conductivity and must therefore mainly use older reserves for respiration in the long run. In drought, sugars are accumulated in the xylem (Tomasella *et al.*, 2020).
Regarding the photosynthetic active tissue, *Fagus* sheds its leaves in autumn and thus relies on its C reserves for several months. This means no large reserves of old C can be formed in leaves, otherwise they would get lost in the shedding. Thus, the transition between young and old C could be less pronounced. I also found leaves to have the youngest C in water solubles of all organs (Fig. 11). *Picea abies* keeps its needles for several years, therefore these can be used as a storage organ for older C. Moreover, keeping its photosynthetic tissue leaves the ability to assimilate new C, if the climate conditions allow it, so it could always have fresh C in reserve. This could be practical if sudden changes in the environment occur, with the emphasis that the quantity varies by climate conditions. If stressful environments occur, this means that *Picea* always has fresh C on reserves but is maybe lacks flexibility when it comes to kinds of C storage are tapped (fresh vs old), which could lead to a lower resilience compared to *Fagus*. Roots have contrasting morphology, with *Fagus sylvatica* forming taproots that can dig deep into the ground, reaching groundwater, whereas *Picea* has a shallow root system which takes up water from topsoil layers (Nultsch, 2012). This led to less efficient water uptake rates which lead to more C must be invested to maintain water flow in the tree.

4.2 *Fagus sylvatica* loses lots of NSC at the beginning of carbon limitation, but has a better outlook then *Picea abies* in the long run

At the beginning, NSC content is more variable across organs in *Fagus sylvatica* than in *Picea abies* which is accompanied by a higher consumption rate of said NSC reserves. When trees face stressful environments, they reduce respiration since this is a costly process. This happens in all organs of *Fagus sylvatica* and marginally in branches and roots of *Picea abies*. The needles of *Picea* however kept their RR steady. At the same time, *Fagus* used more sugars and starch than *Picea*. Since the changes in RR in *Picea* is only marginal or not present, *Fagus* will save more sugars in the long run, even though it lost a high proportion at the beginning, due to high RR at the start. To compensate for this, *Fagus sylvatica*, known as a tree with lots of starch reserves (Sinnott, 1918), can reduce starch to glucose by starch hydrolysis which can then be used for metabolic pathways and respiration (Thalmann and Santelia, 2017). Furthermore, fructose is also produced from glucose with the help of the enzyme's hexokinase or fructokinase

(Stein and Granot, 2018; Huang *et al.*, 2023). This is partially reflected in *Fagus* leaves and branches (Fig. 5), where starch content is decreasing and glucose- and fructose level are rising. Since fructose levels are only rising by 0.1 % in leaves, it can be assumed that in this compartment only a small part of glucose was transformed into fructose. The change of metabolites is also mirrored in the isotopic signature.

Picea is considered a 'fatty tree', with high lipid storages. Previous studies revealed that trees with high lipid content start to respire lipids (Hanf *et al.*, 2015; Huang *et al.*, 2021). This happens during the glyoxylate cycle, which allows respiration based on fatty acids (Kunze *et al.*, 2006). Considering that *Picea* used only ~ 1.5 % of the NSC within two weeks (Fig. 5), but the respiration rate remained relatively constant or decreased only minimally (Fig. 5), respirating around 35 mg C g^{-1} in two weeks in leaves, it is possible that *Picea* also used lipids for respiration. This is also supported by the RQ which indicates lipid use at values around 0.7, and that the needles were depleted of more $\delta^{13}C$ after two weeks. However, the data from the RQ require further explanation. Due to application difficulties at the beginning of the experiment, mainly affecting *Fagus* measurements, artefacts are clearly visible in the *Fagus sylvatica* results. Unfortunately, these could not be mathematically corrected. Nevertheless, it is evident that the first two measurements of *Fagus* and the complete measurements of *Picea* follow trends that are not completely unrealistic and similar results have been reported (Patterson *et al.*, 2018; Hilman *et al.*, 2021), so I still included the RQ.
The change of metabolites can also be examined by $\delta^{13}C$ measurements. Since the isotopic signature of metabolites varies between species (González *et al.*, 1999; Ghashghaie *et al.*, 2001; Werner *et al.*, 2009), we need to measure the $\delta^{13}C$ of fructose, glucose and sucrose to determine the dimension of influence on the $\delta^{13}C$ of respired air. Nevertheless, the metabolites have $\delta^{13}C$ signatures that can be distinguished from each other. For example, the stable isotope signatures of lipids are much lower than those of carbohydrates, no matter the species (Jacobson *et al.*, 1970; Gleixner *et al.*, 1998; Ghashghaie *et al.*, 2001). Although I can't exactly say for how much volume percent the metabolites affect the $\delta^{13}C$ values of the respired air, more general statements are possible. Isotope fractionation due to differnt physical properties of C isotopes, lead to different masses of the C, leading to kinetic fractionation, which in consequence leads to different $\delta^{13}C$ values. One example is the enzyme phosphoenolpyruvate carboxylase (PEPC). This enzyme is important for the biological C fixation where it refills intermediates of the Krebs-Cycle anaplerotically (Shi *et al.*, 2015). PEPC fixates bicarbonate, which is ^{13}C enriched, and leads to ^{13}C enriched products of the enzyme e.g., oxaloacetate or malate which are part of the Krebs cycle. From this ^{13}C-enriched fructose-6-phosphate and glucose-6-phosphate is

produced which is used to form starch. In the dark, the ^{13}C-enriched starch is then remobilized and transformed to sucrose which means there is a day-night cycle of $\delta^{13}C$. Roots as a sink organ are therefore more ^{13}C enriched than other organs which is also reflected in the data (Fig. 9)(Tcherkez, Mahé and Hodges, 2011). Branches on the other hand are much more variable considering their $\delta^{13}C$ values, changing with season or phenology. But it is to note, that roots are always more ^{13}C-enriched than the other organs and leaves are always the most ^{13}C-depleted. The only exception is the water-soluble fraction in *Fagus*, where branches and leaves have similar content. In *Picea abies* $\delta^{13}C$ values of both water soluble and starch are getting either ^{13}C-enriched or stay the same after some variability. Dark leaf respiration uses glucose-6-phosphate, leading to CO_2 which is ^{13}C-enriched, which is contrary to the respiratory data, where leaf emitted CO_2 gets more depleted after two weeks (Fig. 8). This could be explained by other processes like respiration of lipids, as already mentioned, or ^{13}C-depleted amino acids. The ^{13}C signature of lipids is more depleted than carbohydrates. In contrast however, the $\delta^{13}C$ values of roots and branches increased after two weeks. According to the literature, needles should have less lipids than roots or wood (Hoch, Richter and Körner, 2003). Respired CO_2 of leaves of *Fagus* and *Picea* are the only organs that were more depleted after two weeks, suggesting that they used lipids for respiration, even though leaves are the organs with the least amount of lipid storage. Leaves from broadleaved trees can accumulate small amounts of triacylglycerol which is metabolically active and thus could explain this depletion in leaves (Lin and Oliver, 2008).

4.3 Not all methods were successful

Although I have taken $\Delta^{14}C$ measurements on respired CO_2 and starch, I have not included the $\Delta^{14}C$ starch dataset in the discussion, and the $\Delta^{14}C$ respiration dataset with the emphasis that this data might contain artefacts. To use the radiocarbon method, a minimum amount of sample weight must be present. Unfortunately, this was not the case for starch in many cases, therefore many samples could not be measured. In addition, there were some samples that were just within the measurable range but were so small that even the smallest impurities led to large artefacts. This may be because we took the same sample for both sugar and starch extraction. Although we lowered the extraction temperature to 35 °C to minimise the gelatinization of the starch, it cannot be ruled out that a high quantity of starch was still lost through this step. We did this because often, especially with fine root samples, there is not enough material to take fresh samples for all measurements. In addition, senescence of *Fagus* leaves proceeded much

faster than expected which means that fewer sample could be used than was collected. However, one could have considered using fresh samples for at least the branch samples.
Another factor is the amount of enzyme. The enzyme amount in the Landhäuser protocol refers to a sample weight of 30 mg. We used 100 mg instead, to account for the minimum required sample content for the radiocarbon method. Since we had to wash the enzyme with a filter for the $\Delta^{14}C$ measurements, a larger amount of enzyme would lead to clogging of the filter and the enzyme would no longer have been sufficiently purified.
Another problem were the measured $\Delta^{14}C$ values of the respired samples. After the first results were inspected, outliers were measured a second time with backup samples. Here I found that the second measurement returned different values. But the same sample was used for the first measurement, as well as for the $\delta^{13}C$ measurement, where no outliers or relics could be detected. Unfortunately, I couldn't find a satisfactory explanation for this variability, so I included them in the discussion with a disclaimer. It might be possible, that the specific bottles were not cleaned sufficiently and some foreign C was left, however all glass flasks underwent the same cleaning procedure, so this is only a supposition.

5. Summary

C storages are essential for trees survival while facing biotic and abiotic stresses but data about age discrimination of these reserves are limited.
With accelerator mass spectrometry, stable C measurements, respiration measurements and NSC analyses, I examined how *Picea abies* and *Fagus sylvatica* deal with C limitation on an organ level if old C reserves are used right away or if freshly assimilated C is preferred and if evergreen and broadleaved trees differ in these aspects.
My data suggest that *Fagus sylvatica* has C reserves, which are mixed, meaning old and new C is blended, whereas *Picea abies* follows the last in-first out hypothesis. This could be due to different anatomical structures which leads to different C allocations and different demands on its reserves. *Picea*, which is growing naturally in cold and dry habitats has anatomical adjustments like needles, which could lead to C assimilation all year long. This is why it's relying more on new C than storages at the beginning. *Fagus* on the other hand has a broad habitat with different climate conditions, where flexibility in which C is used (new or old) is more important. Furthermore, facing C limitation, *Fagus sylvatica* follows a more conservative approach in NSC use by decreasing respiration to reduce C loss. *Picea abies* on the other hand advances towards a more radical tactic, keeping its respiration rate constant without major changes and therefore using fresh C first in form of sugars and switching to other reserves. I

assume that this lack of flexibility and the constant RR could lead to higher reserve usage in the long run and thus lead to a disadvantage.

While further large-scale studies are needed to find out whether these differences are applying on all deciduous and evergreen trees or if the differences are even more nuanced by e.g., family or genus, my data provides evidence that the showcased results are due to opposing life strategies. My estimates show possible explanations on why evergreen species follow the last in-first out hypothesis and suggest possible explanations why evergreens discriminate C by age, whereas broadleaved species do not under C limitation, which leads to a higher resilience in *Fagus*. This is particularly interesting in the eye of climate change, which affects *Picea abies* more severe than *Fagus sylvatica*. My data gives an explanation, how this could be linked to C usage. Moreover, the resilience of trees is linked to many factors, which are not included in this thesis like genetic diversity, forest management or soil quality. It would be interesting for future experiments to include these factors.

6. Zusammenfassung

Kohlenstoffspeicher sind für das Überleben von Bäumen unter biotischen und abiotischen Stressbedingungen von entscheidender Bedeutung, aber es gibt nur wenige Daten über die Altersunterscheidung dieser Reserven.

Mit Beschleuniger-Massenspektrometrie, Messungen der stabilen Kohlenstoffisotopen, Respirationsmessungen und NSC-Analysen habe ich untersucht, wie *Picea abies* und *Fagus sylvatica* mit der Kohlenstoffbegrenzung auf Organebene umgehen, ob alte Kohlenstoffreserven sofort genutzt werden oder ob frische assimilierter Kohlenstoff bevorzugt wird und ob sich immergrüne und laubabwerfende Bäume in diesen Fragen unterscheiden.

Meine Daten deuten darauf hin, dass *Fagus sylvatica* auf gemischte Kohlenstoffreserven zugreift, d. h. alter und neuer Kohlenstoff gemischt sind, während *Picea abies* der Last-in-first-out-Hypothese folgt. Dies könnte auf unterschiedliche anatomische Strukturen zurückzuführen sein, die zu einer unterschiedlichen Verteilung des Kohlenstoffs und einer unterschiedlichen Beanspruchung der Reserven führen. *Picea*, die von Natur aus in kalten und trockenen Lebensräumen wächst, hat anatomische Anpassungen wie Nadeln, die zu einer ganzjährigen Kohlenstoffaufnahme führen, so dass sie mehr auf neuen Kohlenstoff als auf Speicher angewiesen ist. *Fagus* hingegen hat ein größeres Habitat mit unterschiedlichen Klimabedingungen, in dem die Flexibilität bei der Nutzung des Kohlenstoffs (neu oder alt)

wichtiger ist. Darüber hinaus verfolgt *Fagus sylvatica* angesichts der Kohlenstoffbegrenzung einen konservativeren Ansatz bei der NSC-Nutzung, indem sie die Atmung verringert, um den Kohlenstoffverlust zu reduzieren. *Picea abies* hingegen geht zu einer radikaleren Taktik über, indem die Atmungsrate ohne größere Veränderungen konstant gehalten wird und daher zunächst frische Kohlenstoff in Form von Zuckern verwendet und dann auf andere Reserven umsteigt. Ich vermute, dass dieser Mangel an Flexibilität und die konstante RR langfristig zu einer höheren Reservenutzung und damit zu einem Nachteil führen könnten.

Obwohl weitere groß angelegte Studien erforderlich sind, um herauszufinden, ob diese Unterschiede für alle laubabwerfenden und immergrünen Bäume gelten oder ob die Unterschiede z. B. nach Familie oder Gattung noch definierter sind, liefern meine Daten Hinweise darauf, dass die gezeigten Ergebnisse auf entgegengesetzte Lebensstrategien zurückzuführen sind. Meine Daten zeigen mögliche Erklärungen dafür, warum immergrüne Arten der Last-In-First-Out-Hypothese folgen, und geben mögliche Erklärungen dafür, warum immergrüne Arten Kohlenstoff nach Alter diskriminieren, während Laubbäume dies unter Kohlenstofflimitierung nicht tun, was zu einer höheren Widerstandsfähigkeit bei *Fagus* führt. Dies ist besonders im Hinblick auf den Klimawandel interessant, der *Picea abies* stärker beeinträchtigt als *Fagus sylvatica*. Meine Daten geben eine mögliche Erklärung, wie dies mit der Nutzung von Kohlenhydraten zusammenhängen könnte. Außerdem hängt die Widerstandsfähigkeit von Bäumen von vielen Faktoren ab, die in dieser Arbeit nicht berücksichtigt wurden, wie z. B. genetische Vielfalt, Waldmanagement oder Bodenqualität. Es wäre interessant, diese Faktoren in künftigen Experimenten mit zu berücksichtigen.

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Herewith I declare that I prepared this thesis on my own, that I did not use any other sources and resources than those that are specified, that all arguments and ideas that were literally or analogously taken from other sources are sufficiently identified, and that the thesis in identical or similar form has not been use as part of an earlier course achievement or examination procedure.

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