microC-flux: Short-term carbon fluxes within foliage tissues of drought-tolerant conifers

1. Summary

Trees and particularly northern hemisphere conifers play crucial roles for the terrestrial carbon (C) cycle. However, most of our mechanistic understanding of C-fluxes in the air-plant-soil continuum is based on studies using herbaceous plants and a general understanding of C-fluxes between cells and tissues within plant organs is largely lacking. Such understanding of pathways and dynamics of C assimilate translocation in *e.g.* foliar tissues is important for assessing the ability of different plants to maintain cellular homeostasis in an increasingly stressing environment. In particular, assimilate transport in conifer needles, which contain an inner flow-control barrier in the form of an endodermis, is not well understood. The microC-flux project will investigate the involvement of transitory starch pools in the translocation dynamics of carbon assimilates within needle tissues of pine foliage. The general objectives include a) significant methodological progress in ecophysiological research using stable isotopes, by developing a new methodology for the microlocalization of ^{13}C-enrichment in starch grains; b) elucidation of short-term C-dynamics and phloem loading within conifer tree foliage; and c) formulation of a physically consistent explanation of the predominantly opposing radial transports of water and sugars within conifer needles and the role of starch pools in mediating these transports. Therefore, on the basis of successful previous trials, both the conditions for $^{13}CO_2$ assimilation during pulse-chase experiments and microlocalization of ^{13}C-enrichments by means of nanoSIMS ion microprobe imaging will be optimized, as to ensure adequate labelling of transitory starch pools in conifer foliage. The optimized methodology will be used to analyse within-needle C-fluxes, in the framework of pulse-chase experiments performed on pine saplings of two species with contrasting drought stress tolerance and xeromorphy. Foliage and twig material will be sampled to analyse the diel C-assimilate fluxes from needles to the stem tissues, combining a) temporally and spatially highly resolved nanoSIMS imaging of ^{13}C freshly incorporated in and translocated to needle starch pools, b) compound-specific C isotope analysis and c) tissue-level quantification of starch pools. In parallel to the experimental part, a novel open-source model for carbon and water transport within pine needles will be developed to test hypotheses related to the underlying transport mechanisms and the degree of involvement of starch pools in the translocation of water and assimilates between the mesophyll and vascular bundle. With this project, the technological frontier of microlocalization of stable isotopes in plants will be pushed to unprecedented scales down to the sub-organelle level. The expected results will shed light on mechanisms controlling the transport of water and assimilates in foliage and its dependency on needle xeromorphy and drought-stress tolerance. Beside these mechanistic insights, the new technological advances, especially the use of gaseous (instead of so far solute) ^{13}C-sources during nanoSIMS imaging assessments, will enable many more groundbreaking applications in plant isotope research.

Keywords: xerotolerant pines, δ^{13}C, nanoSIMS, plant physiology modelling, Transitory starch pools, C-fluxes

2. Research plan *(excerpts)*

2.1 Current state of research in the field

Vast expanses of boreal forests at higher latitudes in the Northern hemisphere are dominated by conifers. Given the many xerotolerant taxa in this group, conifers can also be dominant in dry forests at lower latitudes, which are strongly affected by rising temperatures and recurring droughts [1, 2]. To assess the ability of

conifers to persist in increasingly harsh conditions, we not only need to understand their water relations, but also their carbon (C) transport and storage processes [3, 4]. However, mechanistic understanding of the regulation of the C assimilate fluxes and of storage and storage release in trees remains limited [5, 6]. Numerous studies have documented the magnitude of C-fluxes between autotrophic source tissues and sinks in storage stem/root tissues or further in the soil rhizosphere communities (reviews in [7-9]). They have been primarily based on pulse-chase experiments using C or oxygen (O) isotope labels, mostly using herbaceous plants. We thus know that new assimilates can be incorporated in plant tissues within minutes and circulated through the tree-soil continuum within 1 to more than 10 days, depending on environmental conditions, season, species, tree size and age or functional traits. We know that transport and distribution of new assimilates is slower in conifers than in broadleaved trees, but additional research is needed to understand the underlying processes in conifers [6, 7]. Along the whole C assimilate-flux pathway, up to 80 % of gross primary production is lost by respiration or emission of biogenic volatile organic compounds [7]. However, there are still large uncertainties regarding the details of C-fluxes, especially in the case of C-exchange between tissues in the same plant organ, which may contribute to *e.g.* interspecific discrepancies according to species-specific histological and cytological traits [10]. Moreover, during pulse-chase experiments, the influence of pulse timing on the labelling yield, given the diel physiological activity of assimilating foliage, is still mostly unknown. This latter knowledge gap is also due to the lack of highly time-resolved data, as most studies have a daily to weekly resolution, whereas studies focusing on the diel cycle at higher resolution are mostly lacking. More data on short-term assimilation and translocation dynamics of freshly acquired C, especially in the case of trees, is thus direly needed to better understand short-term C fluxes in plant organs.

Starch and sugars, as the end products of photosynthesis and given their involvement in transitory storage and translocation of assimilated C, play key roles regarding C-fluxes within many plants and most trees [8, 11]. Starch is being stored inside of two convertible types of plastids, namely the amyloplast in stem and root organs and the chloroplast in light-exposed foliage and thin bark tissues. Chloroplasts show abundant membrane material, large chlorophyll amounts and transitory starch storage, with monotonic starch accumulation during the day and depletion during the night [12-15]. Steady changes in the starch grain size are indicated by the void space observed around starch grains [16]. Interestingly, in some highly time-resolved pulse-chase experiments using beech trees, the ^{13}C-enriched starch fraction in foliage remained stable during the remaining daytime-hours after the labelling and then exponentially decreased during the following night (Gessler et al. unpubl. data). Highly time-resolved data on the dynamics of transitory starch storage, especially in the case of trees, is largely missing and the within foliage dynamics of transitory starch stores remains mostly unknown. As primarily shown in herbaceous models but likely also valid for trees (subproject team WSL unpubl. data), transitory starch cycling fulfils two main functions: it acts (1) during the day as an overflow mechanism, removing sugars from mesophyll cells and allowing the plant to maximize assimilation, and (2) during the night as the main C-source in the absence of assimilation, thus resulting in a relatively steady day and night supply of carbon to exporting phloem tissues [17-19]. ^{13}C pulse-chase experiments using conifer trees suggest that the freshly acquired and non-respired ^{13}C does not only enter transitory storage pools but also contributes to daytime C-supply of phloem [20, 21].

Starch biosynthesis is unique to plants and produces starch grains with a complex semi-crystalline structure and special features compared to other sugar reserves [19, 22]. Chemically, starch consists of amylopectin and amylose polymers made of glucose α-1,4-linked and α-1,6-branched, arranged in stacks of alternate crystalline (with amylopectin) and amorphous (also including amylose) lamellae. The stacks form concentric shells or rings 100-400 nm thick around the *hilum* grain core, which are added and removed sequentially by disruptive phosphorylation, proceeding concurrently with glucan hydrolysis to maltose and glucose moieties, until the next crystalline ring is exposed [19]. Forming well segregated and solid structures within plastids, starch grains show an interesting microlocalization potential for studying the ^{13}C dynamics within transitory stores and C-fluxes between cells. Recently, highly time- and space-resolved data on freshly acquired C in algal symbionts of cnidarian coral organisms was obtained by means of high-resolution SIMS (Secondary Ion Mass Spectrometry; nanoSIMS) and MALDI_MS (Matrix Assisted Laser Desorption Ionization_Mass Spectrometry) imaging in the course of pulse-chase experiments [23, 24]. Using bicarbonate solutes highly spiked with ^{13}C, these studies have thus confirmed the high potential of these technologies to achieve breakthrough advances on isotope tracing into specific C-compounds at cellular and subcellular level [9]. However, comparable evidence in higher plants labelled with a gaseous $^{13}CO_2$ source – and then less efficiently spiked - is still wanting. If the isotopic enrichment limitation caused by using weaker ^{13}C labelling can be overcome, especially the nanoSIMS 50 ion microprobe, given its high sensitivity and spatial resolution, may enable the study of within-organ C-fluxes between different cell types and tissues in higher plants.

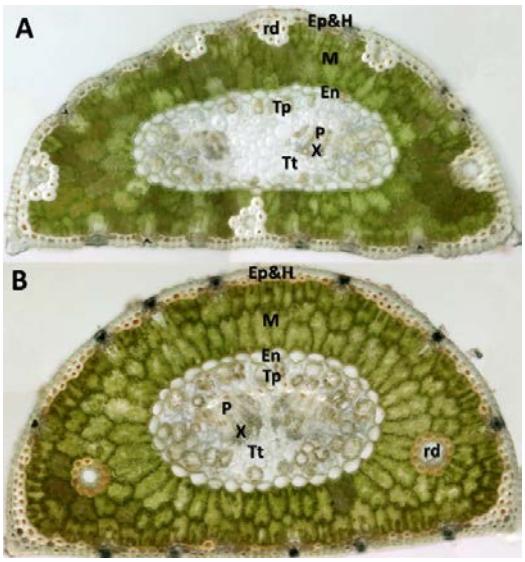

Fig. 1. Histological structure of *Pinus sylvestris* (**A**) and more xeromorphic *Pinus nigra* (**B**) needles. Outer needle tissues: Epidermis and hypodermis (Ep&H), mesophyll (M), resin ducts (rd). Vascular bundle and inner needle tissues: endodermis (En), transfer parenchyma (Tp) transfer tracheids (Tt), Phloem (P) and xylem (X). Vollenweider (unpubl.).

For the microlocalization of ^{13}C-enrichments by means of nanoSIMS technology or the analysis and modelling of within-organ C-fluxes, conifer needles provide an optimal plant model, given several structural, histological and functional features [5]. These properties include: 1) structurally and functionally distinct transitory starch stores between the C-assimilating mesophyll and exporting phloem (M, En, Tp in Fig.1) with mostly similar chloroplast structure; 2) mainly radial and 2-dimensional routes for inwards assimilate and outwards mineral sap (=water) flow across the endodermis (En) and within the mesophyll (M) [25];3) a linear arrangement of tissues parallel to the needle axis and a single central vascular bundle, with individual needle cross-sections thus representative of the structural arrangement of whole needle; 4) basically similar needle structure but enhancement of xeromorphic traits in genotypes with contrasted drought tolerance. In *Pinus nigra vs. sylvestris* (Fig. 1B *vs.* 1A),

these enhancements thus include thicker hypodermis layers, higher needle compactness - but also a larger vascular bundle and higher proportion of phloem and xylem [26-28]. In comparison to angiosperm leaves and their structurally and functionally more complex network of interlaced assimilating and transport tissues [16, 29, 30], conifer needles provide a structurally simpler and more uniform experimental model.

Studies about C-fluxes within foliar organs will contribute to elucidating the still elusive mechanisms of radial transfer and phloem loading of assimilates in conifers, as compared to angiosperm trees [5, 25, 31]. In both groups, there is a symplastic continuum between mesophyll and phloem cells, with numerous plasmodesmata connecting tissues and cells [29, 30]. However, in contrast to symplastic passive phloem loaders, the endodermis bundle sheath, with locally suberized/lignified cell walls, prevents apoplastic radial transport in conifer needles, acting as a flow-controlling barrier, similar to the root endodermis [25]. As a consequence, the inwards assimilate and outwards water fluxes are constrained through a bottleneck formed by the plasmodesmata sleeve in the two periclinal endodermis cell walls and the phloem loading mechanism in conifer needles may show differences to that observed in angiosperm trees (*i.e.* symplastic passive loading). As indicated by daytime transfer of freshly fixed C at the start of a chase period during pulse-chase experiments [20, 21], it appears that there is opposing advective water (acroptetal) and diffusive assimilate (basipetal) transport co-occurring during daytime through the numerous but tiny plasmodesmata of the endodermis cell walls. There might be different possibilities for the assimilates to be transported from the mesophyll to the phloem (against the net radial advective flow of water): (1) Diffusive assimilate transport may occur at the same time but in opposite direction as water transport through the same plasmodesmatal pathways, which would strongly restrict the transport rates[1]; (2) Assimilate transport through the endodermis may be stronger at night when water transport rates are low (Fig. 3). This latter alternative may involve particular roles for the transitory starch pools within the vascular bundles, for enabling constant phloem loading. During day time, a large portion of new assimilates would be thus converted to starch and stored in the mesophyll, while phloem loading could be largely supplied by starch pools in the vascular bundle (in endodermis and transfer parenchyma). At night, the mesophyll starch would be degraded, the soluble sugar products transferred to the vascular bundle and partitioned between phloem loading and replenishing of the starch pools within the endodermis barrier (as suggested by the large size of starch grains in the endodermis and transfer parenchyma; Vollenweider, unpubl. data). Recent evidence from oxygen isotope data [10] suggests that sucrose transported through the needle endodermis is broken down and converted before phloem loading, lending support to the above-mentioned hypothesis. Similar to studies on longitudinal phloem transport [5, 32], such within-organ C-fluxes and their interactions can be explored using a combination of pulse-chase experiments, nanoSIMS assessments and modelling of water and carbon transport between the mesophyll and the central vascular bundle.

According to a recent modelling study, water transport outside the vascular bundles of leaves is predominantly apoplastic and partly supported by gas diffusion [33], so the endodermis itself, in conifer needles, is the main bottleneck where the opposing flows of water and sucrose may pose a difficulty. Modelling studies on roots confirm the function of endodermis tissues as bottleneck for water transport, where all water has to pass

[1] Given channel size constraints in plasmodesmatal sleeves, this should still be true in the case of active assimilate transport and/or route segregation for water and assimilates

through the symplast before reaching the vascular tissues [34, 35]. This also emphasizes the importance of osmotic gradients, in addition to pressure gradients, for the control of radial water transport in roots, whereby pressure differences drive water flow in the apoplast, while pressure and osmotic differences in combination drive water flow across membranes in the symplast [36]. Note that the plasmodesmata numbers and dimensions in cell walls of the needle endodermis have a strong influence on the flow velocity of water against which diffusive transport of sucrose takes place during the day [37]. Whilst significant understanding on longitudinal phloem loading and transport has been emerging recently [5, 32], the mechanisms of radial assimilate transport from mesophyll to phloem still need to be deciphered. A spatially-explicit framework of solute and water transport considering the tissue hydraulic anatomy has been developed for roots [34, 38], and compartmentalized mathematical frameworks exist for simulating coupled time series of labelled and unlabelled pool sizes in the different compartments (incl. starch), depending on assumptions about the transfer kinetics [39, 40]. However, combining spatially explicit modelling of radial assimilate and water transport with kinetic modelling of metabolic conversions between sugar and starch has not been attempted yet, to our knowledge.

2.2 Microlocalization of C-isotopes by means of high-resolution SIMS imaging – the proof of concept

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In a pulse-chase experiment in the summer of 2016, young trees growing in semi-controlled conditions were exposed (pulse) to 500 ppm $^{13}CO_2$ (20 % enrichment) for two hours in the morning, after one-hour CO_2 depletion (down to ~300 ppm), for increasing the labelling yield (peak labelling enrichment in the water-soluble fraction - mainly sugars - by the end of pulse: $\delta^{13}C = 300\ ^0/_{00}$). For the microlocalization of ^{13}C-enrichment in starch grains of chloroplasts, pine needle samples harvested during the first 24 hour-chase period, chemically fixed and processed similar to samples for electron microscopy [16], were analysed using a nanoSIMS 50 ion microprobe *(…)* at LIST (Fig. 2). *(…)* However, only a few samples exhibited ^{13}C enrichments in starch grains superior to background (range: 16.5-406.1 $^0/_{00}$) during the nanoSIMS assessments (Fig. 2). It appeared that the photosynthesis recovery during the pulse period of the 2016 experiment had remained too low for allowing significant labelling of starch inlays, considering the aforementioned significant enrichment in the sugar fraction and starch grain-filling as an overflow mechanism. The ^{13}C enrichments observed within starch grains were found in mesophyll only, and typically showed up as thin outer rings about 400 nm thick at the grain periphery (Fig. 2C). This evidence suggested that only the last layer of amylopectin/amylose stacks had been labelled [22]. Preliminary control assessments by means of atomic force microscopy (AFM) demonstrated that the low $\delta^{13}C$ yield during SIMS imaging could be primarily attributed to low $^{13}CO_2$ uptake during the pulse period of the labelling experiment (Valle, unpubl. results). These trials, beside initiating a constructive collaboration with LIST, indicated the feasibility of within-organ assessments of ^{13}C enrichments by means of high-resolution SIMS imaging using a gaseous $^{13}CO_2$ source, provided improvements regarding the 1) control of experimental conditions, 2) exposure to the ^{13}C-enriched CO_2 source and 3) sample processing prior to nanoSIMS investigations.

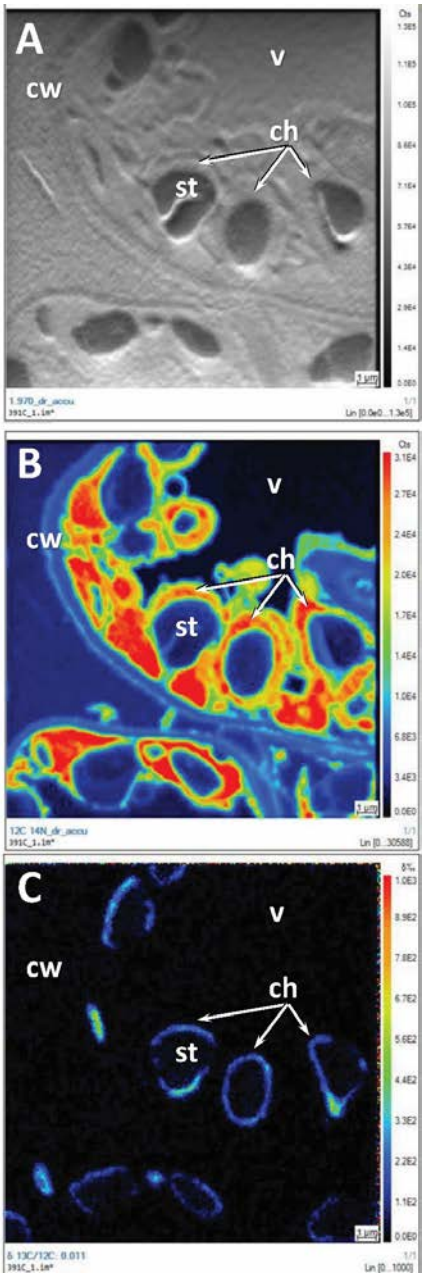

Fig. 2. Isotope and element mapping by means of nanoSIMS during the assessment trials of ^{13}C-enrichment within starch (st) grains of mesophyll cell chloroplasts (ch) from Scots pine needle, 2.5 hours after the end of pulse period. **A.** Secondary electron (SE) image. **B.** ^{12}C-^{14}N isotope map. High values because of high N-content outline the embedding of transitory starch grain stores within the chloroplast matrix. **C.** $\delta^{13}C$ map. Average starch grain enrichment: 99.7 $^{0}/_{00}$. About 0.4 µm thick enrichment shells around starch grains suggested ^{13}C-incorporation restricted to the latest formed ring. Other structures: cell wall (cw), vacuole (v). Vollenweider et al. (unpubl).

The suitedness of high-resolution SIMS imaging technology for analysing subcellular ^{13}C enrichments was further confirmed during comparative trials performed at LIST and LTA, University of Geneva, using the same samples and recent Time Of Flight-SIMS instruments. Imaging the same portions of mesophyll, the ^{13}C enrichments in starch grains, as documented by nanoSIMS, could not be detected, as a likely consequence of insufficient mass resolution (differentiation of ^{13}C and ^{12}CH ions) at the needed spatial resolution (less than 100 nm; Vollenweider et al. unpubl.).

Evidence obtained during all these SIMS trials at LIST and LTA thus provided invaluable know-how and contributed to designing some work package (WP) actions (WP1, see below).

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2.3 Detailed research plan

2.3.1 Project scope and general objectives

The microC-flux project scope is the short-term dynamics of assimilated C within the foliage tissues of conifer trees, as a function of needle xeromorphy and drought stress tolerance. It includes several general objectives:

a) Significant methodological progress in ecophysiological research using stable isotopes, by developing a new microlocalization methodology based on high-resolution SIMS imaging, as to microlocalize stable isotope ratios at subcellular level and quantify C-fluxes within plant organs.

b) Elucidation of short-term (and diel) dynamics of radial C transfer and phloem loading in pine needles.

c) Physically consistent explanation of the opposing day-time radial transport of water and sugars within conifer needles, associated limitations for gas exchange and the role of transitory starch pools in this transport

The project combines experimental and modelling approaches, namely:
i) Pulse-chase labelling experiments using a $^{13}CO_2$-enriched C-source. Highly resolved data (both temporarily and spatially) will be produced during assessments of short-term C-fluxes, from the subcellular to foliage level and by means of high-resolution SIMS imaging and $\delta^{13}C$ measurements. This work will be coordinated with compound-specific $\delta^{13}C$ assessments, realized using bulk needle and twig bark samples. In parallel, the foliage non-structural carbohydrates (NSC), needle structure and foliar gas exchange in relation with drought tolerance traits will be measured.
ii) Modelling of short-term C-fluxes within needles, considering different starch and sugar pools and a physics-based representation of water and soluble NSC transport between needle tissues. The model will be parametrized and its assumptions tested using data from the experiments.

The microC-flux project is needed to address several knowledge gaps in our current mechanistic understanding and in the ecologically essential case of conifer foliage, regarding the 1) within needle spatial and temporal dynamics of assimilate transport and storage, 2) C-transfer and phloem-loading within conifer foliage and 3) importance of pulse timing during pulse-chase experiments, considering the diel changes in the foliar physiology.

The research part is divided into 3 work packages (WPs), with one focusing on methodological issues and two others on short-term C-fluxes within conifer foliage. The project hypotheses are described in Chapter 2.3.2, with the main hypothesis and underlying conceptual model illustrated in Fig. 3, whereas the interactions between work packages and hypotheses are shown in Fig. 4 (2.3.4). The selected tree species are two important Eurasian species, namely Scots pine (*Pinus sylvestris*), and Austrian pine (*Pinus nigra*), the latter with similar needle structure (Fig. 1) but amplified needle xeromorphy and drought-stress tolerance.

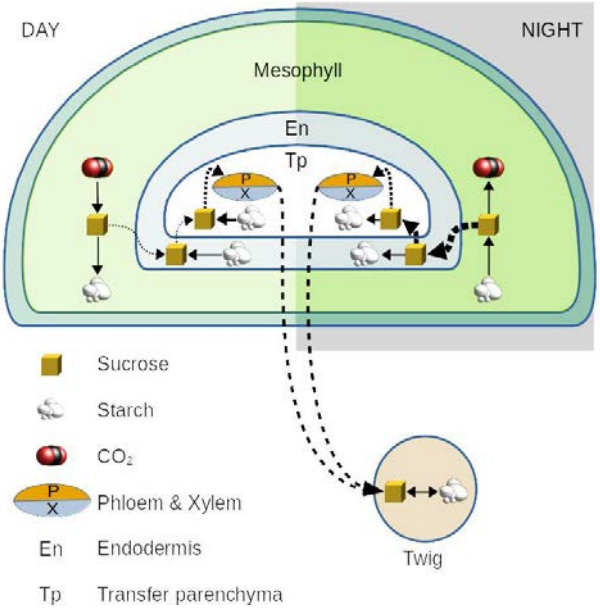

Fig. 3. Schematic functional compartmentalization of hypothesized (H1-4) sucrose-starch conversions (solid arrows) and sucrose transport (dashed arrows) between mesophyll, phloem and twig in pine foliage. Potential day-night separation of dominant processes is highlighted by shading, the conversion and transport rates by arrow thickness. Note that the sucrose transport from both phloem elements to the twig is expected during the day and night. Water transport and the differentiation between symplastic and apoplastic transport is left out for clarity.

2.3.2 Project hypotheses

Each hypothesis in the microC-flux project includes two mutually exclusive A/B process alternatives:

H1) Within needles, the C-export from mesophyll to phloem (A) involves transitory storage within starch pools of endodermis and transfer parenchyma / (B) proceeds without entering transitory storage within tissues of vascular bundle.

H2) Day and night, the C-supply of phloem within conifer needles (A) is rather stable and does not show any significant diel variation / (B) shows a clear diel pattern, with more C-exportation at night

H3) During the day, (A) the mesophyll-to-bundle sheath diffusive transfer of assimilates through the plasmodesmata sleeve in endodermis cell walls is refrained by the incoming bundle sheath-to-mesophyll advective flow of water leading to reduced assimilate transfer across the endodermis compared to night-time / (B) day-time net water fluxes across the endodermis do not inhibit assimilate transfer in the opposite direction.

H4) During the night, (A) the transitory starch pools in the endodermis and transfer parenchyma are being replenished by carbon originating from transitory starch pools in the mesophyll / (B) the carbon from hydrolysed transitory starch pools in the mesophyll is entirely directed to phloem.

H5) Under conditions of high water demand and supply, Austrian pine, being more xeromorphic than Scots pine and thus showing higher proportions of vascular tissues [26-28], exhibits (A) higher rates of both transpiration and C-fixation in the mesophyll, stronger C-transfer rates to bundle sheath tissues, and a stronger day-night separation of sucrose transport across the epidermis (at night) and mobilisation of starch pools inside the vascular bundle (during the day) / (B) reduced water flux and potentially higher water use efficiency, but similar patterns of sucrose-starch interactions.

2.3.3 WP1: Maximizing ^{13}C enrichment and optimizing its detection in conifer needle tissues

Specific objectives: On the basis of preliminary ^{13}C-microlocalization evidence and trials (see 2.2.2), the main objectives in WP1 include several method developments and testing to maximize mesophyll starch labelling during the ^{13}C-pulse exposure and improve the ^{13}C detection and yield during nanoSIMS assessments. A novel ^{13}C-exposure and imaging methodology will be developed, allowing us breakthrough progress in plant isotopic research (project objective 2.3.1a).

Task 1.1: Maximizing the needle gas-exchange

Rationale: Given the lower ^{13}C yield during pulse-chase experiments 1) in conifer *versus* broadleaved trees as a consequence of slower foliar physiology [7] and 2) in the case of a gaseous instead of solute ^{13}C source [23], we will precisely determine the combination of environmental factors and key functional properties in selected pine tree foliage which, in interaction with diel needle physiology, primarily contributes to higher rates of leaf gas exchange and thus $^{13}CO_2$ uptake and transpiration rates. ^{13}C labelling experiments in general will be carried out following published protocols established by the team members (*e.g.* [45, 53])

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Task 1.2: Maximizing starch labelling during labelling experiments

Rationale: Beside high rates of gas exchange, the high levels of starch labelling needed for the microlocalization of ^{13}C-enrichments within conifer needles largely rely on adequate exposure conditions during the pulse-chase experiments, as indicated by past experience (see 2.2). Adding to existing exposure methods [9], we will specifically adapt the experimental design developed during past projects - also studying C- and O-fluxes in conifers [21] - to the specific environment, species-specific responses and research objectives in WP2-3. The tested parameters will include the 1) labelling chamber design, 2) photosynthesis recovery after initial CO_2 depletion, 3) pulse duration and determination of the most appropriate timing in relation to day length (*e.g.* 12 or 16 h) and diel leaf physiology and 4) response variation in the two pine species. The suitability of tested approaches will be evaluated in terms of starch labelling yield by the end of the pulse period and confirmed by effective microlocalization trials using nanoSIMS, completed in the framework of the next task (1.3).

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Task 1.3: Improvement of sample preparation and ^{13}C microlocalization during nanoSIMS assessments

Rationale: The measurement trials performed at LIST have demonstrated the capacity of the nanoSIMS 50 ion microprobe of ACP for microlocalizing and quantifying ^{13}C enrichments in starch grains of pine needles, after exposure to a $^{13}CO_2$ enriched C-source (cf. 2.2.2). However, the AFM characterisation trials have detected some topography and sectioning artefacts in measurements carried out on thin sections (200-250 µm) deposited on silicon wafer. Here, we propose to study the behaviour of starch grains, presenting a semi-crystalline structure, under a focused ion beam. Their pulverization rate has to be put in relation to the measured SIMS intensities, as well as the topography of the samples. To exclude potential losses of material during sample preparation, we plan to test different methods, including (i) cryo-fixation [shock freezing] and low temperature embedding, and (ii) chemical fixation and room temperature embedding. Furthermore, we will improve subsequent processing of the sample resin block by ultra-microtomy for optimizing sample thickness, geared towards measurements of the $^{13}C/^{12}C$ ratio in the semi-crystalline structure of starch grains. We will benchmark this approach against test measurements carried out on surfaced sample blocks. This latter preparation method offers the advantage of requiring no contact of sample with water during processing (in contrast to ultra-microtomy), and thus avoiding critical sample contamination. We will carry out the topography measurements before and after SIMS bombardments via AFM. In addition, we will visualise in 3D the isotopic distribution in the starch grain by combining AFM and SIMS, which will allow us to add a spatial dimension to the measured SIMS intensity.

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2.3.4 WP2: Experimental assessments of short-term C-fluxes and circadian starch dynamics in conifer foliage

Specific Objectives: The WP2 forms the experimental core of the microC-flux project (project objective 2.3.1b). Based on methodological optimization achieved in WP1, the specific objectives of WP2 include the 1) completion

of three pulse chase experiments using a ^{13}C-spiked (99% ^{13}C) CO_2 source; 2) [2]analysis of diel dynamics of freshly assimilated carbon at subcellular level within pine needle tissues and at needle and twig organ level; 3) characterisation of diel variation in the subcellular to needle-level starch dynamics in relation to leaf gas exchange; and 4) characterisation of response variation as a function of needle xeromorphy and drought stress tolerance traits. This WP will also provide parametrization and validation data for modelling in WP3 (cf. Fig. 4).

Task 2.1: Pulse-chase experiments

Rationale: Based on the know-how acquired in WP1, two short-term diel pulse-chase experiments will be performed. They will be followed by a third pulse-chase experiment to test differences in the fluxes and timing of C-translocation between the two species (hypothesis 5) under different atmospheric water demand (i.e. water pressure deficit; VPD), and carbon supply (light intensity). The focus will be set on a high time resolution of observations (experiment 1), responses at species level (experiment 2) and effects of environmental conditions on intensity of water fluxes and sucrose transport for contrasted needle xeromorphy traits ([26, 27]; experiment 3). These three experiments are needed to address all experimental questions while tallying the number of samples and timing of analysis to analytical capacities available at LIST ACP. For the sake of data comparability, the first two exposure experiments in WP2 will be performed within a short time period and the harvested material will be analysed during subsequent years. The gas exchange data from the three exposures will be used as input for model simulations in WP3 (cf. Fig. 4).

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Task 2.2: Analysis of diel variation in ^{13}C-enrichment in tissue starch pools of conifer needles by high-resolution SIMS imaging

Rationale: The ^{13}C-enrichment within starch pools at four needle tissue locations (outer mesophyll/inner mesophyll/endodermis/transfer parenchyma) from samples collected in Task 2.1 (Experiments 1, 2) will be assessed by means of high-resolution SIMS imaging, according to methodological improvements achieved in Task 1.3 of WP1. This data will be used to characterize the dynamics of within-needle C-fluxes and test several project hypotheses (1, 4, and 5 in Chapter 2.3.2). It will also contribute to the model-based testing of Hypothesis 3 in WP3 (cf. Fig. 4). Moreover, the nanoSIMS assessments will provide subcellular evidence on C-incorporation into starch grains and its spatio-temporal variation (Fig. 3), as a function of starch pool location, needle tissue specificities and degree of needle xeromorphy. All assessments (as well as the other microscopical analysis described in task 2.4 and 2.5) will be conducted on samples harvested (Task 2.1) at needle mid-position, to avoid potential disturbance by inhibited longitudinal assimilate transport towards needle tips [5].

[2]The diel variation in amounts of labels invested in volatile organic compounds in the two tested species will be analysed in the framework of other ongoing research projects and collaborations (*e.g.* Univ. of Freiburg i.B., Basel and Bern). We will insert parts of the labelled twig into an inert chamber for VOC analysis, let pressurized air flow through the chamber and guide the air from the chamber outlet through adsorbent tubes filled with polydimethylsiloxane (PDMS) foam. Adsorbed VOC and the ^{13}C enrichment will then be measured with a GC-MS equipped with a thermo-desorption/cold injection unit by our partners. This work is not budgeted within the project but covered by own resources of the applicants and partners.

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Task 2.3: Analysis of circadian trajectories of freshly acquired C by means of compound-specific isotope analysis
Rationale: We will determine the circadian cycle of sugar and starch concentration and carbon isotopic composition at the organ level in the two tested species (needles and twig bark material from experiment 1-3 of task 2.1). This data is needed to better understand diel phloem loading processes, and the contribution of xeromorphy traits to drought stress tolerance (hypotheses 1, 2, 4, 5); moreover, it will contribute to resolving the ^{13}C diel pattern in sugars, which cannot be derived from nanoSIMS or leaf anatomical measurements. The amounts of ^{13}C-label and water lost by respiration and transpiration, respectively, will be quantified with the XiBox equipment (see Task 1.1) and this data will be used to characterize and model the interactions between C and water transport in needle tissues, as well as the carbon costs of assimilate transport (WP3; cf. Fig. 4).

(…)

Task 2.4: Characterisation of diel variation of starch pools at tissue level
Rationale: Estimates of starch grain frequency and size at tissue level in the two species will be determined by means of bright field microscopy, histochemical staining and colour image analysis. In combination with starch concentration measurements at needle level from task 2.3 and histological assessments from task 2.5, this data will be used to characterize starch pool diel dynamics at tissue level – especially the contribution of endodermis and transfer parenchyma starch pools to day-time C-phloem supply, and as a function of needle xeromorphy. Hence, this dataset will be used to further test the project hypotheses 1, 4, 5 (cf. 2.3.2). It is also needed for model-based assessment of the role of starch pools in sugar transport in WP3 (Fig. 4).

(…)

Task 2.5: Characterization of needle structure
Rationale: The histological structure of needle material sampled in the two tested pine species during the two pulse-chase experiments will be characterized by means of light microscopy and image analysis. This spatial information is needed for model parametrization in WP3 (cf. Fig. 4). It will also contribute to calculating tissue-level estimates of starch grain size and frequency (task 2.4) and to quantify differences in needle xeromorphy traits between the two tested species (hypothesis 5).

(…)

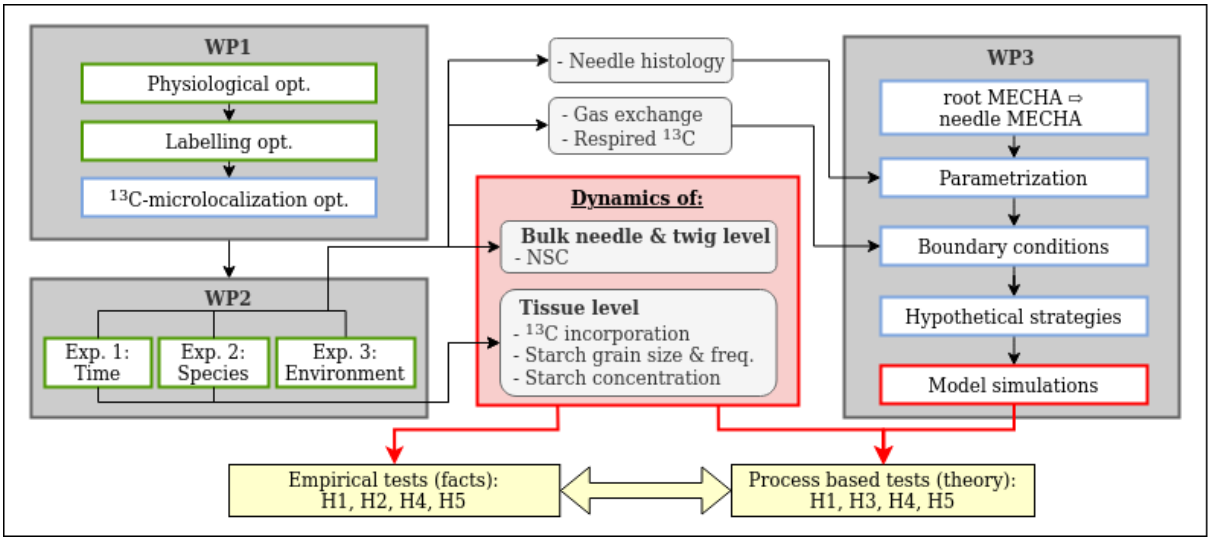


Fig. 4. Interaction between the empirical (WP1, 2) and theoretical (WP3) work packages, including the use of data (middle column) and model simulations for hypothesis and knowledge testing. Frame colours: Tasks carried out at WSL (green) or LIST (blue); data used for hypothesis testing (red).

2.3.5 WP3: Model simulations of water and carbon transport between the mesophyll and vascular bundle

Specific Objectives: In WP3, we will assess if the observed dynamics of water and carbon transfer between the mesophyll and the central vascular bundle can be explained by the physical processes of diffusion, osmosis and pressure-driven advection in pine needles, and in how far the involvement of dynamic starch pools along the pathway supports the transport processes (Project Objectives b and c, Section 2.3.1).

Task 3.1: Physics-based 3D model of radial water and solute transport including starch pools in a needle

Rationale: Considering the specific anatomy of the pine needles investigated here, we will formulate a 2-dimentional model of water and solute transport between the mesophyll and vascular tissues, simulating the processes of pressure-driven advection, concentration-driven diffusion and osmotic processes across cell membranes and symplastic compartments. We will focus especially on transport across the endodermis, where the lignification of cell walls inhibits apoplastic transport, forcing all intra- and intercellular transport through symplast and plasmodesmata. Advective day-time flow velocities in these plasmodesmata will be estimated by dividing observed needle transpiration rates by typical sizes and frequencies of plasmodesmata in plant cell walls. Modelling of carbon conversion between soluble sugars and insoluble starch along with advection-diffusion driven transport processes will enable us to simulate the dynamics of ^{13}C-labeled carbon abundance in starch grains and solute pools of different needle compartments. Comparison of simulations with observed data will be used to test Hypotheses 1, 3, 4 and 5. Note that our focus on radial transport processes is complementary to already existing models of longitudinal phloem loading and transport [5, 32].

(…)

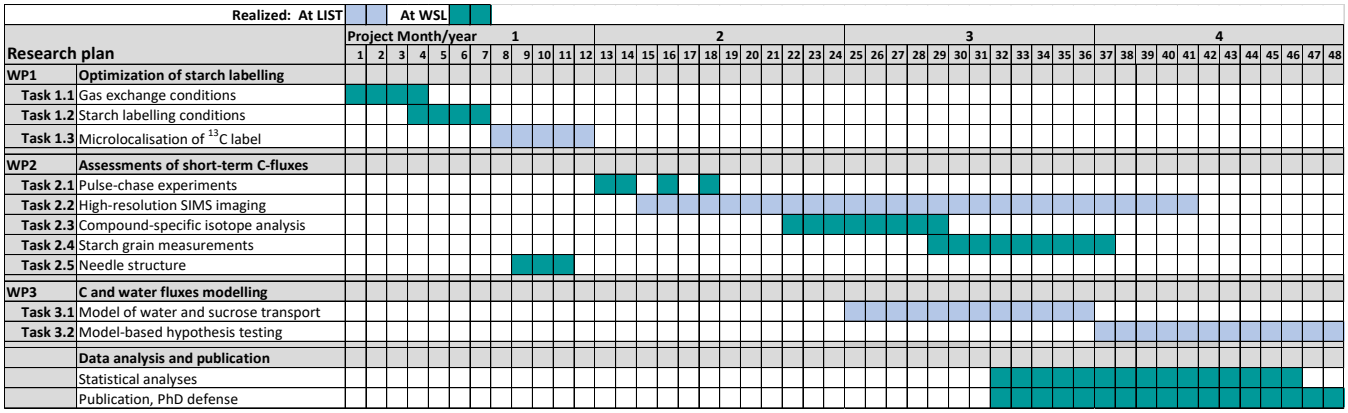
Task 3.2: Model evaluation and hypothesis tests about the involvement of starch pools in needle-scale water and solute transport

Rationale: Simulations of advection-diffusion through plasmodesmata will reveal if simultaneous transport of water and assimilates in opposite directions across the endodermis is physically possible along the same pathways (Hypothesis 3B), and what would be the minimum requirements for the abundance of plasmodesmata to support such transport. Modelling of hypothetical carbon conversion strategies between soluble sugars and insoluble starch along with advection-diffusion driven transport processes will enable us to explain the observed dynamics of starch pools and translocation dynamics of ^{13}C-labeled carbon between different needle compartments and test what is the most plausible involvement of starch pools in the process (Hypotheses 2-4), given the observations in WP2.

(…)

2.4 Schedule and milestones

Project schedule (Gantt chart):



Not shown: bi-yearly research update and planning meetings between the two subproject teams.

Project milestones (M):

WP1: M1.1: Optimization of needle gas exchange (month 4); M1.2: Conditions for highest starch labelling yield determined (month 7); M1.3: Sample preparation and ^{13}C label microlocalization optimized (month 12)

WP2: M2.1: Completion of needle structure measurements (month 11); M2.2: Completion of the three pulse-chase experiments (month 18); M2.3: Completion of the high-resolution SIMS imaging assessments (month 41); M2.4: Completion of the compound-specific isotope analysis (month 29); M2.5: Completion of starch grain measurements (month 37)

WP3: M3.1: Extension of MECHA model to pine needles and inclusion of starch pool dynamics; submission of technical paper (Month 36); M3.2: Simulation of starch and labelled CO_2 dynamics and model-based hypothesis tests based on experimental data; submission of scientific paper (Month 48)

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