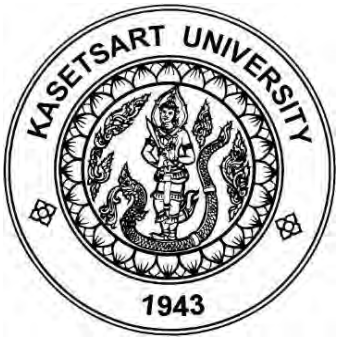

THESIS

USING $^{13}CO_2$ PULSE-LABELLING TO TRACK CARBON FROM PHOTOSYNTHATES TO LATEX IN RUBBER TREES (*HEVEA BRASILIENSIS* MUELL.ARG)

ORNUMA DUANGNGAM

GRADUATE SCHOOL, KASETSART UNIVERSITY
Academic Year 2022

THESIS APPROVAL
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DEGREE: Doctor of Philosophy (Horticulture)
MAJOR FIELD: Horticulture
DEPARTMENT: Horticulture

TITLE: Using $^{13}CO_2$ Pulse-Labelling to Track Carbon from Photosynthates to Latex in Rubber Trees (*Hevea brasiliensis* Muell.Arg)

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THESIS

USING $^{13}CO_2$ PULSE-LABELLING TO TRACK CARBON FROM PHOTOSYNTHATES TO LATEX IN RUBBER TREES (*HEVEA BRASILIENSIS* MUELL.ARG)

ORNUMA DUANGNGAM

A Thesis Submitted in Partial Fulfillment of
the Requirements for the Degree of
Doctor of Philosophy (Horticulture)
Graduate School, Kasetsart University
Academic Year 2022

Ornuma Duangngam: Using $^{13}CO_2$ Pulse-Labelling to Track Carbon from Photosynthates to Latex in Rubber Trees (*Hevea brasiliensis* Muell.Arg). Doctor of Philosophy (Horticulture), Major Field: Horticulture, Department of Horticulture. Thesis Advisor: Professor Poonpipope Kasemsap, Ph.D.
Academic Year 2022

Rubber trees are the main source of natural rubber, extracted from the latex that exudes from the trunk after tapping. Tapped trees require large amounts of carbon (C) to regenerate latex, which represent an additional C demand because of the amount of latex that flows out and the richness in C of rubber. Therefore, C supply is key for sustainable latex production, but the source of latex C remains poorly known. Does it come directly from the leaves where CO_2 is assimilated or from starch stored in the wood? Pulse labelling trees with $^{13}CO_2$ are valuable approaches to study carbon allocation; however, it is challenging for trees in the field. Thus, the objectives of this study were to determine the contribution of C sources in latex biosynthesis (recent photosynthates versus stored carbohydrates) and if there is a shift in their respective contribution and in C dynamics during the tapping period. Large temperature-controlled chambers (35–45 m^3) that enclosed the entire crown of a tree, provided a reliable estimate of tree crown photosynthesis, and ensured efficient $^{13}CO_2$ labelling were developed and tested. Whole tree crowns of 4-year-old rubber trees were pulse-labelled with $^{13}CO_2$ for 30–40 minutes. Labelling was performed in June when latex production was low *(*start of tapping) and in October, when it was high. The ^{13}C contents were quantified in the foliage, phloem sap, latex and trunk wood. The labelling experiment showed that, in both labelling periods, ^{13}C was recovered in the latex just after labelling, indicating that part of the photosynthates were directly allocated to latex. However, significant amounts of ^{13}C were still recovered in latex after 60 days and the peak was reached significantly later than in phloem sap, demonstrating the contribution of a 'reserve' pool as another source of latex C. In June, the latex C came from a pool where recent C (photosynthates) mixed with older C (stored starch) but a significant part of recent C was nevertheless invested in storage. In contrast, in October, the recovery of ^{13}C was faster and stronger in latex, indicating a high contribution of recent C coming directly from leaf photosynthesis. The contribution of new photosynthates to latex regeneration was therefore faster and higher when the latex metabolism was well established in October compared to June. To conclude, ^{13}C pulse-labelling proved efficient to study the origin of the latex C. Overall, latex C comes from a pool where newly assimilated C mixes with older one, but their respective contribution varies seasonally. An improved understanding of C dynamics and source-sink relationships in rubber trees is crucial to adapt tapping system practices and ensure sustainable latex production.

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Student's signature Thesis Advisor's signature

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Ornuma Duangngam

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LIST OF ABBREVIATIONS

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A_L	Total leaf area (m^2)
B_L	Total leaf biomass (kg)
C	Carbon content on a mass basis (%)
C1	The initial amount of label assigned to a rapid turnover ^{13}C pool (mg ^{13}C g^{-1} C)
C2	The amount of label assigned to a slow turnover ^{13}C pool (mg ^{13}C g^{-1} C)
C_{170}	The trunk girth at 1.7 m from the ground
C_a	Ambient CO_2 concentration
C_i	Intercellular CO_2 concentration
E	Transpiration rate (mm day^{-1})
ET	Evapotranspiration (mm)
GPP	Gross primary production (kgC m^{-2})
IWUE	Inherent water use efficiency (kgC kPa m^{-2} mm^{-1})
LMA	Leaf mass per area (kg/m^2)
LP	Labelling Period (June and October, 2016)
MRT	The mean residence time (days).
N	Nitrogen content on a mass basis (%)
P_{atm}	The atmospheric pressure (Pa)
P_{crown}	Net crown CO_2 exchange rates (μmol $tree^{-1}$ s^{-1})
P_{leaft}	Net leaf CO_2 exchange rates (μmol CO_2 $m^{-2}s^{-1}$)
PAR	Photosynthetically active radiation (μmol m^{-2} s^{-1})
PF	Polar fraction (soluble sugar and amino acid)
PPFD	Photosynthetic photon flux density (μmol m^{-2} s^{-1})
R	The ideal gas constant (8.314 J K^{-1} mol^{-1})
R_{sample}	The ratio of ^{13}C to ^{12}C in the sample (plant material)
$R_{standard}$	The ratio of ^{13}C to ^{12}C in the standard

LIST OF ABBREVIATIONS (Continued)

RH_{in}	Relative air humidity inside chamber (%)
RH_{out}	Relative air humidity outside chamber (%)
VPD	Daylight vapour pressure deficit (kPa)
VPDB	Vienna Pee Dee Belemnite reference
T_{air}	Air temperature (°C)
T_{in}	Air temperature inside chamber (°C)
T_{out}	Air temperature outside chamber (°C)
Trunk CR	Carbohydrate reserves in trunk wood (mostly starch)
Trunk PF	Polar fraction in trunk wood (soluble sugar and amino acid)
Trunk ST	Structural compounds in trunk wood
$x(^{13}C)$	The ^{13}C atom fraction
$x^{E}(^{13}C) =$	The excess ^{13}C(mg ^{13}C g^{-1} dry mass)
$\Delta^{13}C$	Carbon isotope discrimination (‰)
$\delta^{13}C$	Carbon isotope composition (‰)
δ_{air}	Carbon isotope composition of CO_2 in the atmosphere (‰)
δ_{plant}	Carbon isotope composition of the plant organic matter (‰)

INTRODUCTION

The rubber tree (*Hevea brasiliensis* Muell.Arg.) is the only commercial source of natural rubber. World production of natural rubber was 14.6 million tons in 2022 (IRSG 2023), mostly in Asia (> 90%). Thailand is the world's number one natural rubber producer with 4.85 million tons and exporter with about 3.86 million tons, which accounted for 36% of global natural rubber production. It represents the second world's rubber plantation area with 3.95 million ha reported by the Rubber Authority of Thailand (RAOT) in 2022. Natural rubber usually contains about 25–50 % dry matter. It is obtained after coagulation of the latex, the cytoplasm of laticifer vessels, which are arranged in concentric rings near the phloem in the inner bark. The latex exudes from any injured organs, particularly from the trunk after the bark is cut (Hébant and De Fay 1980). Large quantities of latex can be collected from the trunk by regular tapping. Latex contains mainly rubber particles (~30–50% w/w fresh latex; 87 % w/w dry latex) dispersed in the cytoplasmic serum (Hepper and Audley 1969).

In terms of composition, the latex mostly consists of cis-1,4-polyisoprene which represents about 35 % of its fresh weight or 87 % of its dry weight. The remaining 5–6 % w/w fresh latex or 13 % w/w dry latex are non-isoprene molecules such as lipids, proteins, carbohydrates and minerals (d'Auzac 1989; Bottier 2020; Sakdapipanich 2007). Latex and rubber particles are then made of a large proportion of carbon (C).

Latex is extracted using a multi-annual tapping system that can continue for 15–30 years by regular tapping of the tree bark, after an initial unproductive period of 5–9 years called the immature phase, depending on the clone used and on the environmental conditions (Paardekooper, 1989; Vrignon-Brenas et al. 2019). Rubber tapping is the method of bark incision to extract the latex from laticiferous vessels located in the phloem of the bark. In Thailand, different tapping systems are used in each region (Chantuma et al. 2011, 2017; Doungmusik and Sdoodee 2012; Obouayeba et al. 2009; Sainoi et al. 2017; Soumahin and Obouayeba 2009). Following tapping, the exuded latex is regenerated *in situ*.

In trees, the C assimilated by leaves is allocated to all sink organs for the purpose of respiration, growth, storage and defense (Kozlowski T.T. 1992). As the C requirement of different organs and processes vary temporally due to normal functioning or unexpected stress, trees must adjust C allocation to survive. In rubber tree, latex production and collection by tapping represent an additional C sink because the latex that flows out of the trunk must be regenerated by *de novo* synthesis (d' Auzac et al. 1997). Tapping is known to activate latex metabolism (Annamalainathan, Krishnakumar, and Jacob 2001) and the highest production of latex is frequently reached several months after the beginning of the tapping period (i.e., in October–November in Thailand). Latex regeneration consumes huge amounts of carbon in the form of soluble sugars inside the laticifer cells (d' Auzac et al. 1997). Intensive tapping systems are tailored for each rubber clone type, mainly by monitoring the changes in sucrose content in laticifers, the first limiting factor for regeneration of the exported latex, as sucrose is both the source of energy for the laticifers' metabolism and the precursor of the isoprene biosynthesis (d'Auzac 1989; Gohet et al. 1996).

However, to forecast the effect of tapping systems in the longer term, a better understanding is needed of the allocation of carbon resources between tree growth, reserves and latex regeneration. It has been known for decades that a negative relationship exists between annual latex production and annual wood biomass production (Gooding 1952; Karling 1934; Silpi et al. 2006). Preserving a good balance between growth and latex regeneration is a key for long-term rubber production (Gohet et al. 1996; Lacote et al. 2004; Obouayeba et al. 2012).

Moreover, tapping activates accumulation of starch in wood and bark, suggesting that rubber trees adjust the allocation of C to storage to satisfy metabolic demand for latex production, at the expense of growth (Chantuma et al. 2009; Silpi et al. 2006, 2007). The carbohydrates stored in the parenchyma xylem cells in the trunk are mostly made up of soluble sugars and starch (Chantuma et al. 2009; Silpi et al. 2007). Other compounds, including cyanogenic glucosides, have also been suggested to play the role of C buffer in the inner bark to regenerate latex (Kongsawadworakul et al. 2009). Finally, it is still not clear whether all the soluble sugars used for latex

regeneration come from stored carbohydrates (reserves) or if recent photosynthates transferred from the foliage to the trunk inner bark via the phloem sap are also used. Moreover, the concentrations of soluble sugars and starch vary with the season.

Rubber is a deciduous tree with annual defoliation in dry season, with leaves first changing color and then falling. During the defoliation period, local farmers stop tapping latex from their trees, usually by December–January depending on genetic material, planting density and environmental condition (Guerra-Hincapié et al. 2020; Priyadarshan 2017). In the dry season which includes tapping rest (i.e., from February to April), small changes take place in the total nonstructural carbohydrate concentrations in the trunk. Just after re-foliation and the beginning of tapping period (i.e., March–May), starch concentrations are low while soluble sugar concentrations are high, whereas after several months of tapping (i.e., October–November), starch concentrations are high and soluble sugar concentrations are low (Chantuma et al. 2009; Silpi et al. 2007).

A recent study showed that, in rubber tree, seasonal dynamics of natural carbon isotopic composition ($\delta^{13}C$) of latex were not related to those of leaf soluble sugars, suggesting that C in latex does not all originate directly from recently assimilated C in leaves but also from a reserve pool in which new C is mixed with older C (Kanpanon et al. 2015).

Pulse labelling the foliage with $^{13}CO_2$ makes it possible to trace the fate and dynamics of the labelled assimilates into the whole plant, its different organs, and metabolites. This method allow to study where and how rapidly the labelled C (i.e., recently assimilated C) is allocated among the different competing pools (Desalme et al. 2017; Epron et al. 2011; Epron, Laclau, et al. 2012; Kagawa, Sugimoto, and Maximov 2006; Keel et al. 2007, 2012; Studer, Siegwolf, and Abiven 2014; Tsuji et al. 2022). Because ^{13}C is not radioactive it poses no safety issues and can be easily used in field experiments. Allocation priorities and C dynamics can be estimated by comparing isotopic signals among organs, compartments or metabolic pools and by calculating kinetic parameters (e.g. mean residence times) from changes in isotopic signals after

labelling (Dannoura et al. 2011; Desalme et al. 2017; Epron et al. 2016). This makes it possible to trace recently assimilated C into both fast and slow cycling compounds and to calculate the transfer velocity and mean residence times of C in the metabolites (soluble compounds, starch, structural compounds) (Epron, Bahn, et al. 2012).

Based on past experience (Dannoura et al. 2011; Epron et al. 2016; Plain et al. 2009), the main challenges for measuring crown photosynthesis and pulse-labelling trees are the size of the chamber that can enclose the whole tree crown and the climate control inside the chamber. One of the first attempts to measure whole tree photosynthesis was on young apple trees several decades ago (Hei necke and Chiders, 1937); subsequently, other systems were developed, but limited to young, small trees (Corelli-Grappadelli and Magnanini 1993; Dreyer and Daudet 1984; Li et al. 2022) or to short-term measurements (Barton et al. 2010, 2012; Pérez-Priego et al. 2010) that excluded the possibility to perform $^{13}CO_2$ labelling. The size of the chamber is crucial for trees planted at low density, such as rubber tree or fruit trees, because they generally develop large crowns, requiring chambers of several tens of cubic meters. In addition, climate control is crucial in tropical conditions because the temperature inside a closed chamber rises rapidly without proper control. Thus, the current study aimed to develop and test a system including a large closed-chamber to measure photosynthesis of an entire tree crown and pulse label the tree with $^{13}CO_2$ in a rubber plantation in Eastern Thailand.

In the present study, the goal was to determine the contribution of C sources involved in the latex biosynthesis (recent photosynthates versus stored carbohydrates) and to assess if there is a shift in the respective contribution of recently assimilated C and reserves during the tapping period. This research developed and tested a system including a large closed-chamber to measure photosynthesis of an entire tree crown and pulse label the tree with $^{13}CO_2$ in a rubber plantation. Two different periods were compared: (i) 2 months after tapping started, when latex production is low and ii) 5 months after tapping started, when latex production is high, to identify any differences in C allocation and dynamics. The hypothesis was that the ^{13}C would be recovered rapidly after labelling in the latex, implying that some of the new photosynthates would

be rapidly allocated to latex but that the ^{13}C peak would be reached later because of the mixing of new (^{13}C) and older C (^{12}C) in a common pool of reserves before being allocated to latex. The expected result was that the ^{13}C allocation pattern and ^{13}C dynamics would change between the two labelling periods in response to changing C sinks due to an increase in latex production during the tapping period.

OBJECTIVES

This project aims to improve our understanding of the carbon allocation of latex in rubber trees (*Hevea brasiliensis* Muell.Arg.).

1. To develop and test the $^{13}CO_2$ pulse labelling system of a (4 year old) rubber tree.

2. To determine the contribution of C sources involved in the latex biosynthesis (recent photosynthates versus stored carbohydrates).

3. To determine if there is a shift in their respective contribution and in C dynamics during the tapping period.

LITERATURE REVIEW

Carbon allocation and plant production

Carbon (C) allocation is a major issue in plant ecology. During photosynthesis carbon dioxide (CO_2) is taken up from the air and converted to nonstructural carbohydrates (NSC) that serve as both energy carrier and as a building block for anabolic processes like growth, defense and storage or exchanges with other organisms (e.g., leaves, stems, roots) (Hartmann and Trumbore 2016).

Plant carbohydrates are categorized into 2 forms of structural and non-structural carbohydrates. The structural carbohydrates are polysaccharides or long-chained molecules (cellulose, hemicellulose, and pectin), that constitute the structure of the plant cells and of the supporting plant structure they form, such as stalks and stems. Non-structural carbohydrates, made of soluble sugars (glucose, fructose, and sucrose) and insoluble polymers (starch), are the major substrates used for both primary and secondary plant metabolism and storage (Hartmann et al. 2020; Hartmann and Trumbore 2016). However, some species have different strategies, storing lipids (Herrera-Ramírez et al. 2021). Plant growth and survival depend more on the partitioning of C resources between the different plant parts and function than on the total amount of assimilated C. In agriculture particularly, the productivity depends mainly on the harvest index, the amount of harvested biomass divided by the total amount of biomass. However, the processes of C partitioning among the different sinks remain poorly understood and this constitutes a weakness in process-based tree models (Génard et al. 2010).

C fluxes are generally assessed by the variations of the size or by the activity of the sink organs (like fruit growth). Though, such estimations may have a bias if re-allocation occurs, such as the mobilisation of reserves, especially in trees. Conversely, fluxes from the source organs to the sinks can be measured directly by the use of labelled compounds. The use of CO_2 enriched with the stable isotope ^{13}C allows tracking photo-assimilated ^{13}C atoms into metabolites and their transfer through the

phloem to the sinks. Such an approach is used to calculate transfer velocity and the proportion of recently assimilated C in the biomass synthesized after labelling. This method has long been restricted to the laboratory or to small plants, but it has recently been extended to large field grown trees, providing major information on C allocation processes (Dannoura et al. 2011; Epron et al. 2011). However, these studies did not tackle the question of the influence of variations in sink activity on C assimilation and allocation patterns. What happens when an additional sink competes with other sinks and functions? Answering such a question requires short term adjustment of sink activity without major perturbation of the whole plant functioning. The rubber tree is a very relevant model tree, thanks to its product, the latex. When the latex vessels located in the bark are severed by the tapping practice, the latex flows out and is collected before being regenerated *in situ*. This induces an artificial C sink because latex has a high C content and is not naturally exuded from the tree without tapping. Such specificity has been used to study the dynamics of carbohydrate reserves (for example (Silpi et al. 2007)).

Importance of natural rubber in Thailand

Rubber (*Hevea brasiliensis* Muell.Arg.) is not only an interesting species for studies on C allocation in trees, it is a major tree crop and an important economic sector in many countries. Rubber plantations cover around 14 million ha and produced a total of 14.6 million metric tons in 2022 (IRSG 2023). Natural Rubber (NR) still accounts for 47% of the worldwide elastomer sale market (IRSG, 2021). The superior qualities of natural rubber to the synthetic polymers make it indispensable for automotive and aeronautic industries (e.g. trucks and planes tires), mechanics (e.g. joints and anti-seismic bracket), and in medical industry (e.g. latex gloves) (Martius et al. 2021, IRSG, 2021). Mainly, the demand is driven by the strong increase in the natural rubber consumption in Asia which currently accounts for 75% of the total worldwide consumption (13.8 million tons in 2021), whereas the supply is ensured by plantations of rubber trees in the inter-tropical zone, mostly in South-East Asia (84%). The prospect is good for these countries to invest in the development of rubber tree cultivation, not only to supply their national market, but also to export the surplus.

However, the recent evolution of rubber prices has been marked by high fluctuations which create an unstable environment for the full commodity chain (Pinizzotto et al. 2021).

Since 1997, Thailand has been the first producer of natural rubber with an estimated total output of 5 million tons and plantation area at 3.95 million ha in 2021, representing around 38% of the world production (13.9 million tons) (IRSG 2021). Of this amount, around 90% are exported as raw material, making rubber a major export commodity. Rubber wood also provides substantial additional incomes for the farmers at the time of tree logging. Rubber production in Thailand is mainly built on small-scale farmers (smallholders). Thus, it is estimated that rubber production involves, either directly or indirectly, about 10% of the total Thai population (6 million people or 1 million families). In all, it is indisputable that rubber production plays a very important role in Thailand economy (Chambon et al. 2013; Somboonsuke, Wettayaprasit et al. 2013).

The rubber tree

Hevea brasiliensis Muell.Arg. is a tropical tree and a perennial plant species from the Euphorbiaceae family. It is native to the Amazon Basin in Brazil and adjoining countries. Rubber grows best at temperatures of 20–28°C with a well-distributed annual rainfall of 1,800–2,000 mm. It grows satisfactorily up to 600 metres above sea level (but is capable of growing much higher to at least 1,000 metres near the equator) and will perform on most soils providing adequate. Its required temperature and rainfall define its prime growing area as between the 10° latitudes on either side of the equator, but it is cultivated much further north (Guatemala, Mexico and China) and south (Sao Paulo region of Brazil). In Thailand, the traditional area for rubber tree cultivation is located in the south with some areas in the east, which annual rainfall about or more than 1,600 mm, and a dry season period lasting only 1–2 months. The non-traditional rubber planting area is located in the northeast and the north of Thailand, with annual rainfall less than 1,600 mm, long dry periods (more than 4 months) (Chantuma et al. 2017). Mature *Hevea* trees in rubber plantations are commonly 20–30 metres high, and

stems smooth and straight; bark grayish; leaves alternate, trifoliate, petioles 7.5–10 cm long (Reed 1976). *Hevea* tends to be damaged by high winds. Such trees flower once a year and after insect cross-pollination produce large fruits containing several thimble-sized seeds with hard outer coats. In most plantations, rubber plants are semi-clonal. They are multiplied in nurseries by bud grafting on a seedling rootstock and then transplanted at normal density (500–600 tree ha^{-1}) in plantation plots.

Hevea is a deciduous tree with annual shedding (defoliation) of senescent leaves in dry season. Annual defoliation and refoliation cycles occur in rubber trees after 3–4 years of age, which render the trees leafless for a short period. Each year, the entire canopy is shed at the beginning of the dry season (Ridgman 1989). Leaf fall is normally followed within 2 weeks by the terminal buds bursting and by the expansion of new leaves within further week. The period of refoliation lasts approximately one month from the emergence of the leaf to the mature leaf stage, depending on genetic material, planting density and environmental condition (Guerra-Hincapié et al. 2020; Priyadarshan 2017; Ridgman 1989). Latex yields normally fall slightly at the onset of leaf fall and are markedly reduced during refoliation. There are relationships between long term latex yield and climatic parameters such as minimum temperature, maximum temperature, sunshine hours, and relative humidity (Gohet et al. 2015; Rao and Vijayakumar 1992; Ridgman 1989). This is due to the physiology of the rubber tree itself with changes in total non-structural carbohydrates, particularly starch, depleted following bud break and re-foliation in the stem wood (Chantuma et al. 2009; Silpi et al. 2007). The decrease in total carbohydrate concentration after refoliation indicates a net mobilization both for direct incorporation in new flushes (including leaf and flowers) and to sustain the increased growth respiration (Lacointe et al. 1993). Actually, the farmers stop tapping during the refoliation period to avoid competition for carbohydrates between leaf growth and latex yield.

The rubber trees respond to drought stimuli, which generate variation in leaf phenology at different times and locations. Priyadarshan (2017) indicated that the phenological phases respond to the latitudinal position where plantations are located. In the north of the equator, the defoliation occurs in February–March, mainly associated

with low humidity and high transpiration rate, while in the south of the equator in September–October, after winter with low temperature and water deficiency. The annual defoliation and re-foliation period is generally lasting from January to March, local farmers stop tapping latex from their trees, usually by late January or early February to April in Chachoengsao area, eastern of Thailand (Chantuma et al. 2017; Silpi et al. 2006). Additionally, leaf flushing phenology is delayed with higher temperatures during November–December (Zhai and Xu 2023). Depending on conditions, the initial growth takes 5–8 years to reach 'maturity', which is defined as the stage when tapping can be started (‘opening’). The opening corresponds to the moment when canopy closes and trunk girth increment slows down. It is also the time when the work of the tapper can be covered by the earnings of the rubber collected. Opening the trees too early would be prejudicial to the further growth and would condemn the trees to be tapped at a small size for years long with a limited production (Obouayeba et al. 2012). These results also highlight the fact that the competition between vegetative growth and rubber yield is important especially as the tapping is early. In practice, the technical standard is to open the trees when the trunk has reached about 50 cm circumference at the height of 1 metre above ground level (Chantuma et al. 2017).

Latex

Natural rubber latex is a cloudy white colloidal suspension, present in all the organs of the rubber tree. The latex is the cytoplasm of specialized cells known as laticifers that constitute the laticiferous vessels (LV) located within the phloem tissue mostly in the inner soft bark (ISB). In the bark of the trunk, the latex vessels make mantels that are emitted successively within the phloem tissue by the cambium (C). Laticiferous vessels of rubber trees are arranged in an articulate form in concentric rings in the phloem (Figure 1).

Natural rubber particles make 90% of the total solid content of the latex. Fresh natural latex is a white opaque fluid of density between 0.97 and 0.98 depending on the rubber content. It is almost neutral having a pH in the range of 6.5–7.0 (D’Auzac and Jacob, 1989). Latex contains mainly rubber particles (~30–50% w/w fresh latex; 87%

w/w dry latex) dispersed in the cytoplasmic serum (Hepper and Audley 1969). The rubber fraction is the most abundant, followed by the C-serum and the lutoids. In terms of composition, the fresh latex contains about 60% of water, 35% of cis-1,4-polyisoprene and 5–6% of non-isoprene molecules, i.e. mostly including proteins, lipids, carbohydrates and minerals (Gooding 1952; Nawamawat et al. 2011).

Interestingly, the rubber particles of *Hevea* latex exhibit a bimodal size distribution with the presence of large and small particles named cream and skim fractions, respectively, Figure 2 (Bottier 2020; Singh et al. 2003).

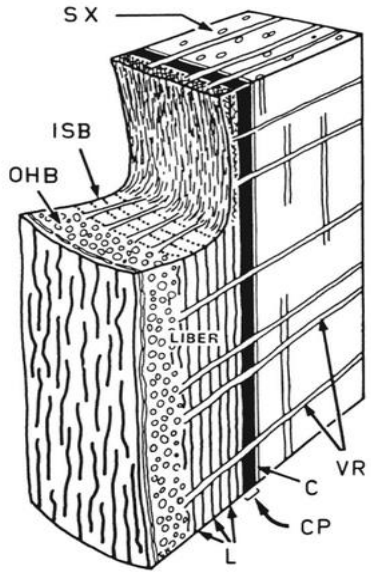

Figure 1 The general organization of the trunk bark and wood of *Hevea brasiliensis* Muell.Arg. at the tapping cut site; (C) cambium, (CP) conducting phloem, (ISB) inner soft bark, (L) laticiferous vessels, (OHB) outer hard bark, (SX) secondary xylem and (VR) vascular ray.

Source: (d'Auzac 1989)

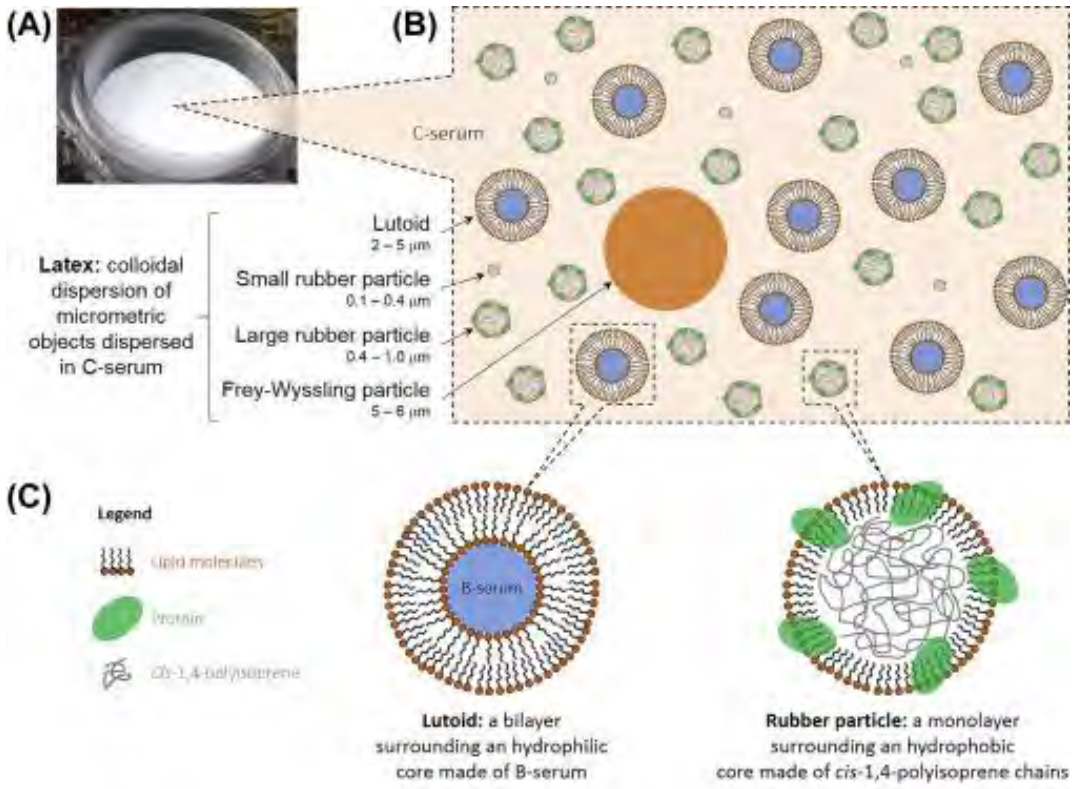

Figure 2 Latex of *Hevea brasiliensis* Muell.Arg. seen at various scales. (A) Picture of a cup of latex in a rubber tree field at the macroscale. (B) Schematic view of the latex at the mesoscale showing lutoids, rubber particles and Frey-Wyssling particles dispersed in the aqueous C-serum. (C) Schematic views of the major constituents of latex at the microscale: a lutoid and a rubber particle. The diagrams in (B) and (C) are not drawn to scale.

Source: (Bottier 2020)

Tapping and tapping system

Rubber trees are usually tapped by cutting a spiral groove in the bark halfway or more around the stem, as deep as possible but without injuring the tree's cambium. The tapping corresponds to a wound in the tree bark. Only a thin slice of bark, 2–3 mm thick is shaved off to cut open the laticiferous cells in the phloem. Latex gushes out of the tree when laticifer cells are severed during tapping. This flow is due to the very high

pressure inside the laticifer cells compared to the outside. The latex is collected by allowing it to run into a cup attached to the trunk. After some hours the coagulation of latex plugs this wound and the flow stops. To restart flow from a tapping cut in a subsequent tapping, all that is needed is to cut a thin shaving of the bark along with which the plugs of coagulated latex are also removed and release the latex upward into the new cut (Premakumari and Panikka, 1992; John, 1992; Kush, 1994).

The reference system is tapping trees on half-spiral alternate days (S/2 d2 by the international notation) Vijayakumar et al. (2009). However, in high-yielding budded clones prone to tapping-panel dryness (TPD), reduced tapping intensity of once in 3 days is recommended though, in general, small growers prefer higher frequencies.

Latex is extracted using a multi-annual tapping system that can continue for 15–30 years by regular tapping of the tree bark (Vrignon-Brenas et al. 2019). The incision of bark is made from left to right at an angle of 35°, downward at a height of 150 cm from the bud union (RRIT, 2018), by removing the thin bark at 0.5–1 mm from the cambium, will open a maximum number of vessels, and to avoid damage to the bark (Tupý 1985). In Thailand, trees are usually tapped at night. Tapping late during the day will reduce the latex yield due to increased transpiration leading to lower turgor pressure.

The tapping systems used to harvest vary in the different rubber production countries. Therefore, choosing a tapping system is an essential factor to determine the yield and physiology of rubber trees. Appropriate tapping systems are defined by clones and environment (Gohet et al. 1996; Lacote et al. 2004, 2010; Obouayeba et al. 2009). The tapping cut of rubber trees can have variable lengths. For example, in full spiral (S) the cut is made on the whole circumference of the tree, in half spiral (S/2) and third spiral (S/3), the cut is made on the half and the third circumference, respectively. The other main parameter is the tapping frequency, expressed as d1 (every day), d2 (every other day), 2d3 (tap two days, rest one day), etc. When low tapping frequencies, such as d4 or d5, are used, the decrease in cumulated production is compensated by the use of chemical stimulants (ethephon (2-chloroethyl phosphor-

nic acid), a precursor of ethylene) that increase the flow duration and then the yield at each tapping.

In Thailand, different tapping systems are used in each region (Chambon et al. 2013). The systems recommended by the Rubber Research Institute of Thailand (RRIT); such as S/2 d2, S/2 2d3, S/3 2d/3 and S/3 2d3 with ethephon are not often implemented by rubber smallholders (Somboonsuke et al. 2013). Currently, high frequency tapping systems (S/3 2d/3 or S/3 3d/4 mainly) are commonly used (Chantuma et al. 2011) because they can compensate for the reduction of actual tapping days due to weather variability, notably heavy rains during the rainy season, that leads to loss of revenue. However, such intensive systems may have adverse effects. First, frequent tapping induces more bark consumption and shortens the duration of tapping of the trees. Actually, the lifespan of a rubber plantation is about 20 years in Thailand, leading to shorter rotations than in areas using lower tapping frequencies (Obouayeba, Soumahin, and Coulibaly 2010; Sainoi et al. 2017; Soumahin and Obouayeba 2009). This has negative economical, but also environmental impacts, as each logging/replanting sequence degrades the soil (Panklang et al. 2022). Second, tapping has a huge effect on the tree growth. As both the vegetative growth producing wood biomass and latex production require large amounts of carbon, they are in competition for carbohydrates. Therefore, trunk growth is one index to determine a good balance between latex yield and good health of rubber trees. As the decrease of girth increment after resumption of tapping leads to a decrease in growth rate within only two weeks (Silpi et al. 2006), it is inferred that the tapping itself (repeated wounding of the trunk) affects growth. Hence, high tapping frequencies may have a stronger negative impact than lower ones.

Therefore, low frequency tapping systems (Gohet et al. 1996; Lacote et al. 2010; Obouayeba et al. 2010; Sainoi et al. 2017; Soumahin and Obouayeba 2009) have been developed. They are adapted to some major risks to rubber production, namely a short producing period and the lack of manpower. Several researchers have been working to improve these systems, particularly by better fitting the use of chemical stimulation by 2-chloroethyl phosphonic acid (ethephon), an ethylene generator applied to the tapping

panel, necessary with low frequency tapping systems. In particular, Gohet et al. (1996) proposed a framework to adapt the chemical stimulation to the physiological types of rubber tree clones. Ethephon releases ethylene gas that enhances latex yield because it increases the duration of latex flow after tapping, with a slower latex coagulation, and by activating latex cell metabolism (d'Auzac et al., 1997). This compensates for the loss of land productivity due to decreased tapping frequency and greatly enhanced labor productivity, as the yield per tree per tapping is much higher. Consequently, both small-scale planters and agro-industrial plantations use low tapping frequencies (d3 to d5) together with ethephon stimulation, in many countries worldwide (Sivakumaran and Chong, 1994, Jetro and Simon, 2007, Lacote et al., 2010, Traore et al., 2011). However, mostly for socio-economic reasons (small size of the farms, difficulties to share workers) these systems are not frequent in Thailand despite positive results, notably by Sainoi et al. (2017), showing that low frequency tapping systems (d3) with stimulation resulted in an equivalent yield in cumulative latex production compared with the other tapping systems and also had higher latex production per tapping. Bark consumption was less in the low frequency tapping systems leading to the possibility of lengthening the economic lifespan of the tapping panels of the tree.

The increasing cost and decreasing availability of workforce to tap rubber worldwide, including in Thailand, make low tapping frequency systems more and more necessary for the sustainability of natural rubber production. This is why large estates and several R&D institutions are conducting research on very low tapping frequencies, from D6 to D12 (twice a month) (Chantuma et al. 2017; Gohet, Chambon, and Lacote 2016; Phearun et al. 2019). Such systems would change the physiological functioning of the tapped trees, particularly regarding water and carbon dynamics.

Carbon allocation and rubber productivity

Tapping for latex production requires de novo latex synthesis that consumes a huge amount of carbon. A balance of C source (soluble sugars) inside the latex-producing vessels is therefore the key of rubber tree productivity, as sucrose is both the source of energy for latex metabolism and the precursor of the isoprene molecule (d'

Auzac et al. 1997). It has been shown that a direct competition for carbohydrate assimilates exists between rubber production and growth. The extent of this competition is well known and mainly depends on the latex sink size or metabolic activity, which itself depends on the clone and on the tapping systems (tapping frequency, hormonal stimulation etc.). Therefore, there are interactions between carbohydrate accumulation, partition and utilization for latex production and tree development.

Recent works in Thailand have shown that tapping affects growth, latex cell metabolism, activates reserve (starch) accumulation and the dynamics of carbohydrate contents between wood and bark too (Chantuma et al. 2009, 2011; Silpi et al. 2006, 2007). Tapping creates an additional sink that, contrary to expectations, increases the stored starch in wood and soluble sugars in bark. Starch acts as the long term storage in the wood and as local buffer in bark. Soluble sugars will be used as a carbon source for starch storage in wood and latex synthesis in bark (Chantuma et al. 2009; Silpi et al. 2007). Thus high starch accumulation ability could result in a long term latex yield, because the tree will be in a better balanced condition.

In many tree species, physiological adaptations to drought include the accumulation of osmotically active substances and/or the presence of particular compatible solutes, among them cyclitols (Merchant et al. 2006). Actually, drought-induced tree mortality can be due to carbon starvation and hydraulic failure (Hartmann 2015). Carbon starvation is often referred to as the depletion of non-structural carbohydrates (NSC) in response to stomatal closure, reduced C assimilation and sustained C storage dependency during longer droughts (Hartmann 2015; McDowell et al. 2008) and is now considered as one of the main mechanism involved in climate-driven tree mortality. If such direct effect has not yet been observed in rubber trees, latex production can also decrease the available carbon for of osmotic adjustment and xylem refilling, increasing the vulnerability of trees. It is known that drought induces hydraulic failure in rubber trees (Kunjet et al. 2013; Sopharat et al. 2015) and that can increase the risk of C starvation, particularly in tapped trees.

Therefore, rubber production is regulated by the processes in photosynthate accumulation, metabolic partition and utilization in latex production. The management of the trade-off between latex production and biomass increment is a key of the productivity of rubber plantations (Gohet et al. 1996; Silpi et al. 2006). To better understand its physiological bases and forecast the long-term effects of tapping systems, one must be able to actually measure C fluxes within the rubber tree, from assimilation in leaves to biomass sinks and to latex, with a particular focus on reserves. Pioneering work has been done in Thailand thanks to the use of the stable carbon isotope ^{13}C (Kanpanon et al. 2015).

^{13}C carbon isotope and its composition ($\delta^{13}C$) in plants

Carbon from atmosphere consists of stable carbon isotopes ^{12}C and ^{13}C, counting for 98.9% and 1.1%, respectively. The amount of ^{13}C is expressed using isotope composition ($\delta^{13}C$, measured in ‰).

$$\delta^{13}C_{sample} = [R_{\text{sample}} / R_{\text{standard}}) - 1] \quad [1]$$

The standard is a reference limestone (Pee Dee limestone) at South Carolina State in the United States ($R_{standard;\ VPDB} = 0.0111802$), and the sample is either the plant (bulk tissues or organic molecules) or the CO_2 in air. The ratio of ^{13}C to ^{12}C found in plant is less than in air. $\delta^{13}C$ of C_3 plants averages -27‰ showing however large variations, and $\delta^{13}C$ of air is -8‰. $\delta^{13}C$ has negative values because both plants and air have ^{13}C in a less proportion than that found in the reference limestone.

The difference between CO_2 in air and carbon in plant tissue is related to fractionations that take place during photosynthesis and it is called carbon isotope discrimination ($\Delta^{13}C$, expressed in ‰). Carbon isotope discrimination during photosynthesis is the difference in isotope composition between the source of carbon (air) and the sink of carbon, which is the leaf carbohydrates and, more generally, the leaf biomass, with Equation 2:

$$\Delta = \frac{\delta_{air} - \delta_{plant}}{1 + \delta_{plant}} \approx \delta_{air} - \delta_{plant} \quad [2]$$

One of the reasons that plants fix $^{13}CO_2$ less than $^{12}CO_2$ is that $^{13}CO_2$ is heavier than $^{12}CO_2$ and thus $^{13}CO_2$ diffuses more slowly than $^{12}CO_2$ (4.4‰ fractionation). However, the most important reason is that the RuBisCO enzyme (ribulose-1, 5-bisphosphate carboxylase/oxygenase) tends to fix $^{12}CO_2$ more than $^{13}CO_2$ with a fractionation of about -30‰, while the enzyme PEP carboxylase (phosphoenol pyruvate carboxylase) tends to fix $^{13}CO_2$ more than $^{12}CO_2$ too, but in much less proportion. The average value for both carboxylations is assumed to be 27‰ in C_3 plants. Due to the contribution of these different fractionation steps, the overall fractionation during C_3 plant photosynthesis, also called ^{13}C discrimination ($\Delta^{13}C$), typically ranges from 18 to 25‰, and is mainly influenced by the ratio between internal and external CO_2 concentration (Farquhar and Richards 1984). Isotope ratio mass spectrometer (IRMS) allows fast, convenient and accurate measurements of $\delta^{13}C$. Therefore, although ^{13}C is a stable isotope, contrary to ^{14}C, it can be used in plant physiology, either in natural composition or through labelling.

Labelling with stable isotopes

Stable isotopes, especially ^{13}C, are widely used in ecology as tracers in trophic webs (Cerling et al. 2007; Dawson et al. 2002). Pulse-labelling with stable C isotopes has proven to be a valuable tool for understanding C dynamics, as it allows labelled carbon dioxide (CO_2) to be traced throughout plants (Epron et al. 2011; Epron, Laclau, et al. 2012). The fate of carbon in the soil plant system can be followed by pulse-labelling plants in the field with $^{13}CO_2$ for a short period of time (< 1 day). The ^{13}C assimilated by plants during the pulse labelling can then be tracked in the whole plant, down to the respiratory fluxes during the following days and weeks (chase period).

In particular, $^{13}CO_2$ pulse-labelling experiments have been widely used to assess the temporal dynamics of labelled photoassimilates in trees growing in both natural and perturbed conditions such as drought, increased temperature, and elevated CO_2

(Blessing et al. 2015; Epron, Laclau, et al. 2012; Furze et al. 2019; Hesse et al. 2019; Streit et al. 2013). However, previous studies have often used potted saplings or small trees (Barthel et al. 2011; Corelli-Grappadelli and Magnanini 1993; Endrulat et al. 2010; Li et al. 2022), and individual branches or the crown (Kagawa et al. 2006; Plain et al. 2009; Epron et al. 2011). Few ^{13}C labelling experiments have been conducted on large whole-trees in the field (Epron et al. 2016; Warren et al. 2012). Recently, Carbone et al. (2007) and Högberg et al. (2007) pulse labelled small boreal conifers growing in the field (with $^{14}CO_2$ and $^{13}CO_2$, respectively), to resolve the relative roles of new photosynthetic products as sources of below-ground and above-ground respiration. The accurate determination of residence and transfer times of carbon in the atmosphere–plant–soil system requires frequent measurements of the isotopic composition of evolved CO_2 during the chase period following the short-term labelling with $^{13}CO_2$. Up to now, both cost and time required for analyzing air samples by mass spectrometry in the laboratory limit frequency and duration of isotopic measurements in experiments studying either variations of natural isotopic abundance during seasons or changes in isotopic enrichment after a pulse labelling.

The recent development of tuneable diode laser absorption spectrometers (TDLAS) allows *in situ* simultaneous measurements of effluxes of $^{13}CO_2$ and $^{12}CO_2$ at a high frequency. TDLAS have recently been used to examine ecosystem functioning (Bowling et al. 2003, Griffis et al. 2004, Barbour et al. 2007, Bahn et al. 2009, Marron et al. 2009), and this is a promising tool for tracking ^{13}C in respiratory fluxes after pulse labelling (Bahn et al. 2009).

Short-term ^{13}C pulse labelling is a widespread approach to trace the fate of recently assimilated C. Here, whole plants (Lippu 1994, Keel et al. 2006, Sangster et al. 2010, Epron et al. 2011, Glaser et al., 2012) or parts thereof (Nogués et al., 2006, Streit et al., 2013) are exposed to a highly ^{13}C-enriched atmosphere, usually for periods ranging from minutes to several hours. This generates strong isotope labelling, which can be detected within the C pools in plants and soil (see review by Epron et al. 2012). These techniques shed new light into many not yet fully understood physiological mechanisms such as phloem loading/unloading including transfer

velocities and time lags between C assimilation and respiration (Plain et al. 2009, Kuzyakov and Gavrichkova 2010, Barthel et al. 2011), partitioning of C fluxes to heterotrophic and autotrophic respiration (Kuptz et al. 2011, Biasi et al. 2012) or translocation, storage and remobilization of assimilated C during the season (Kuzyakov and Domanski 2000, Kagawa et al. 2006, Keel et al. 2012). These and further isotope labelling studies traced the isotopic composition of certain plant tissues, non-structural carbohydrates, including soluble sugars and starch, and cellulose as representative of the structural C pool, yielding important insights into fundamental metabolic processes (Keel et al. 2012; Richardson et al. 2013).

Moreover, combining ^{13}C pulse labelling with compound-specific ^{13}C analyses opens up new horizons in the research on partitioning mechanisms of C between metabolites and metabolic groups, their formation and turnover times (Brüggemann et al. 2011). Isotope application continues to have high potential, especially in quantifying C allocation into individual compound classes (Streit et al. 2013). It can contribute significantly to expanding this understanding of mechanisms underlying the C allocation processes in plants.

Carbon isotope composition ($\delta^{13}C$) in rubber trees

Kanpanon (2017) found that the range of $\delta^{13}C$ at leaf level among 10 commercial clones of rubber trees was narrow (-29.9 ‰ to -31.5%) and there was a correlation between leaf $\delta^{13}C$ and intrinsic water use efficiency (WUE_i) under high vapour pressure deficit only. Therefore, on these 10 clones, the prediction of WUEi by leaf $\delta^{13}C$ would have low precision. However, when using a more genetically diverse collection of 49 wild genotypes, there were larger leaf $\delta^{13}C$ variations among the genotypes at all seasons (Kanpanon et al. 2017). The leaf $\delta^{13}C$ was rather stable with a good correlation between rainy and dry season. In rainy season, there was a positive significant correlation between leaf $\delta^{13}C$, leaf mass per area and leaf nitrogen. The genetic variability of leaf $\delta^{13}C$ is then promising for breeding if a good correlation between leaf $\delta^{13}C$ of leaf and WUE can be established (Kanpanon et al. 2017).

Kanpanon et al. (2015) investigated the diurnal and seasonal variation of $\delta^{13}C$ in 20-year-old tapped and untapped rubber trees and showed that latex $\delta^{13}C$ was unrelated to that of leaf soluble sugars, suggesting that the C in latex does not all originate directly from C recently assimilated in leaves but comes also from a reserve pool. However, the relative importance of the two sources is not yet known. Therefore, knowledge of seasonal differences and evolution from early tapping to more established situations is of great importance.

MATERIALS AND METHODS

1. Materials

1.1 Plant material; RRIT 408 clone of rubber trees (*Hevea brasiliensis* Muell.Arg.)

1.2 A portable gas exchange analyzer (Li6400XT; LI-COR Inc., Lincoln, NE, USA)

1.3 Air conditioner (TRANE; 36,000 BTU/h; WI, USA)

1.4 Air temperature and the relative humidity sensor (HMP 50, Vaisala, Helsinki, Finland)

1.5 Data logger (CR 1000; Campbell Scientific Inc.; Logan, UT, USA)

1.6 Quantum sensor (LI-190 R; LI-COR Inc., Lincoln, NE, USA)

1.7 The 35–45 m^3 of close chamber system, made of 150 µm-thick transparent polyethylene film

1.8 Diesel generator (25 kVA single phase diesel generator)

1.9 $^{13}CO_2$. (99.299 %, Cambridge Isotope Laboratory Inc., Andover, MA USA)

1.10 A infrared gas analyzer (LI-840; LI-COR Industries; Lincoln, NE, USA)

1.11 An absolute barometric pressure sensor (BMP 280; Bosch Sensortec; Reutlingen, Germany)

1.12 A air flow meter (DryCal DC-Lite; BIOS International Corporation; Butler, NJ, USA)

1.13 Ball-grinder (MM400, Retsch, Germany)

1.14 Vacuum centrifugation (vacuum concentrator CentriVap, LabConco, Kansas City, MO, USA)

1.15 Leaf area meter (LI-3100A, LI-COR Inc., Lincoln, NE, USA)

1.16 Freeze dryer (Maxi-Dry plus, HetoHolten, Allerod, Denmark)

1.17 Elemental analyser coupled to a continuous flow isotope ratio mass spectrometer (vario ISOTOPE cube coupled to the IsoPrime 100, IsoPrime Ltd, Cheadle, UK)

1.18 Tin capsule (6*4 mm)

1.19 Eddy-flux tower

1.20 Microwave oven

1.21 Methanol/chloroform/water mixture (12/5/3, v/v/v)

1.22 Methanol/chloroform (1/1, v/v)

1.23 Sulphuric acid (0.6 M H_2SO_4)

1.24 Hydrochloric acid (2% w/v)

1.25 Hydrochloric acid (6 M HCl)

2. Methodology

2.1 Study site and plant material

The experiment was performed at the Chachoengsao Rubber Research Center (CRRC), Rubber Authority of Thailand, Chachoengsao province (13°34′ N, 101°27′ E, 69 m elevation) in eastern Thailand. In this area, the reported mean annual air temperature and cumulative rainfall were 27.1°C and 1,247 mm, respectively, with a dry season from December to April (Thai Meteorological Department, 2019). Rainfall, temperature, and global radiation were measured throughout the experiment by the station 'Rubber Flux Chachoengsao', Asia flux network, and are shown in Figure 4. Average rainfall is less in traditional area (southern of Thailand). Wintering of the trees, namely the defoliation and re-foliation period is generally lasting from mid-January to April in Chachoengsao area.

A monoclonal plantation of rubber trees (*Hevea brasiliensis* Muell.Arg., clone RRIT 408) was planted in 2012 with a spacing of 7 m between tree lines and 2.5 m between trees (568 trees ha^{-1}), on a sandy-clay-loam soil (Kabin Buri series), Figure 3. Tapping for latex production began in May 2016 with the incision of the bark at a height of 1.20 m from the ground. In this study, the tapping process was started from 4 years after planting (earlier than regular trees) and the rubber tree trunk circumference was about 19–24 cm (Table 1). Every three days, the trees were tapped with a half spiral downward cut (i.e., 'S/2 d3', a common tapping system). The 9-month tapping season starts in early May and ends in January when defoliation and refoliation occur. A total of seven 4-year-old trees were selected for the labelling experiment in two

different periods (six trees) and one tree for testing the chamber (Table 1). These trees were tapped before the normal age and size for practical reasons. They were not too big, in order to easily handle the chamber and system for labelling but big enough for a significant latex production and to follow the ^{13}C in latex. Normally, the tapping process start from 7 years old after planting or the rubber tree circumference are about 50 cm at 1 m from the ground in this area (Chantuma et al. 2017).

2.2 Experimental design and treatments of pulse-labelling in rubber tree with $^{13}CO_2$

The experiment was arranged with two treatments (labelling periods) comprising three replications.

(i) Three trees were labelled on 25, 26, and 27 June 2016 (early in the rainy season, low latex yield). At that period, i.e. about 2 months after tapping began , the foliage was well developed and active and the tree girth increment was maximum (Silpi et al. 2006).

(ii) Three trees were labelled on 5, 6, and 7 October 2016 (late in the rainy season, high latex yield). At that period, i.e. 5 months after the beginning of tapping, latex production was higher than in June, as expected (Figure 5)

Figure 3 The rubber tree (4 year old) and the rubber plantation (RRIT 408 clone) used for the experiment at the Chachoengsao Rubber Research Center (CRRC), Chachoengsao province, Thailand.

Table 1 Growth characteristics of six rubber trees pulse-labelled with $^{13}CO_2$ in June, 2016 (#1–#3) and in October, 2016 (#4–#6) and non-labelled rubber tree (#7).

Labelling period	June 2016			October 2016			Non
Tree	#1	#2	#3	#4	#5	#6	#7
Date	25 Jun	26 Jun	27 Jun	5 Oct	6 Oct	7 Oct	14 Jun
Girth at 1.8 m (cm)	19.5	21.0	20.1	22.5	23.5	21.0	19.0
Tree height (m)	6.0	6.0	5.7	5.1	5.3	6.3	5.5
Crown wide (m)	4.2	4.0	3.8	3.8	4.2	4.0	3.7
Leaf area (m^2)	20.0	20.0	22.5	22.8	26.9	21.3	-
Leaf mass (kg)	2.5	2.3	2.5	3.0	2.8	2.6	-

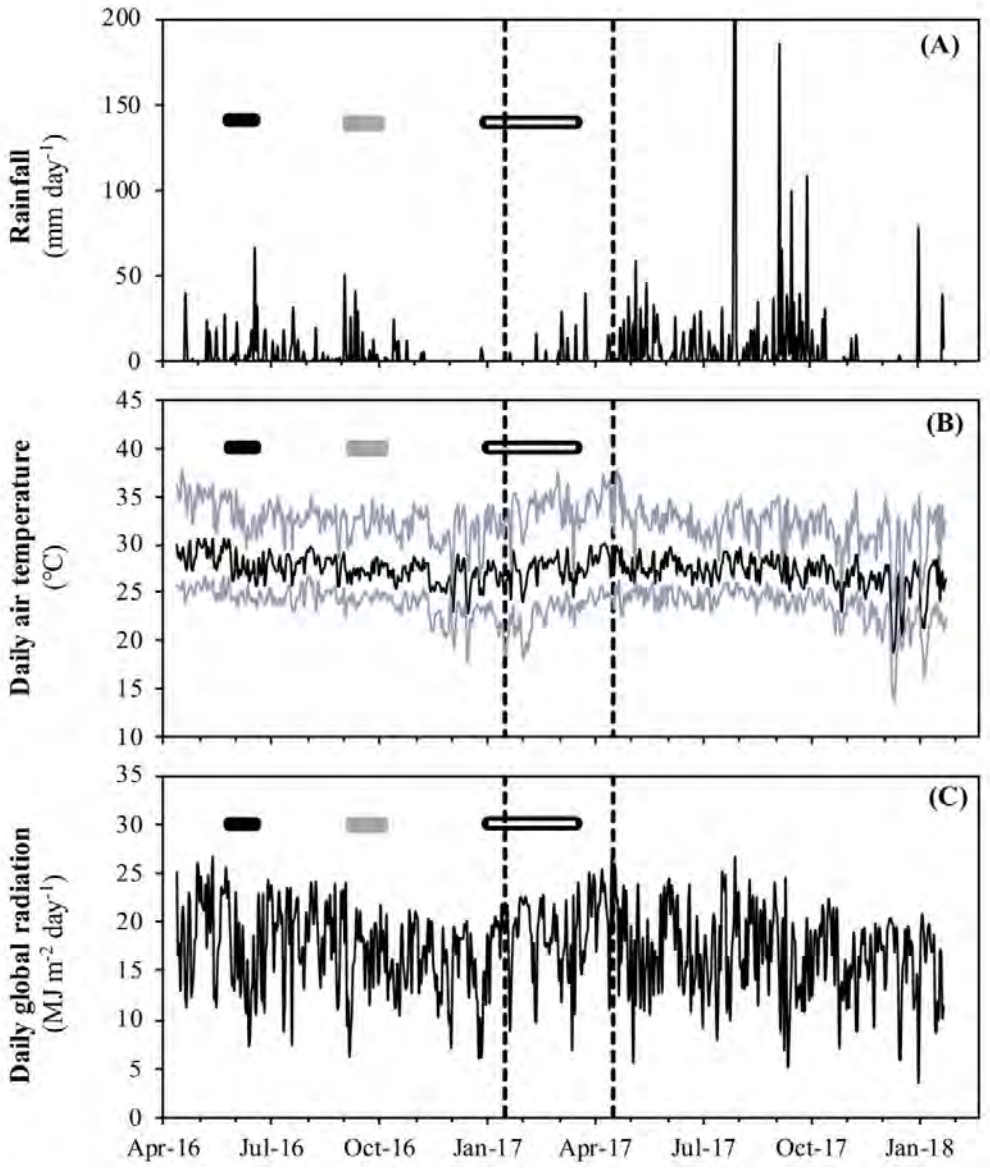

Figure 4 Meteorological conditions during the course of the experiment. (A) Rainfall, (B) mean daily air temperature (black line), minimum and maximum daily air temperature (grey lines) and (C) daily global radiation. Black and grey horizontal bars depict the two labelling periods in June and in October during which three trees were labelled sequentially. Labelling dates for each tree are listed in Table 1 and Table 2. The empty horizontal bars indicate the period from leaf fall until new leaves become mature (January–March 2017). Vertical dashed lines indicate the period when trees were not tapped (tapping rest; February–April 2017).

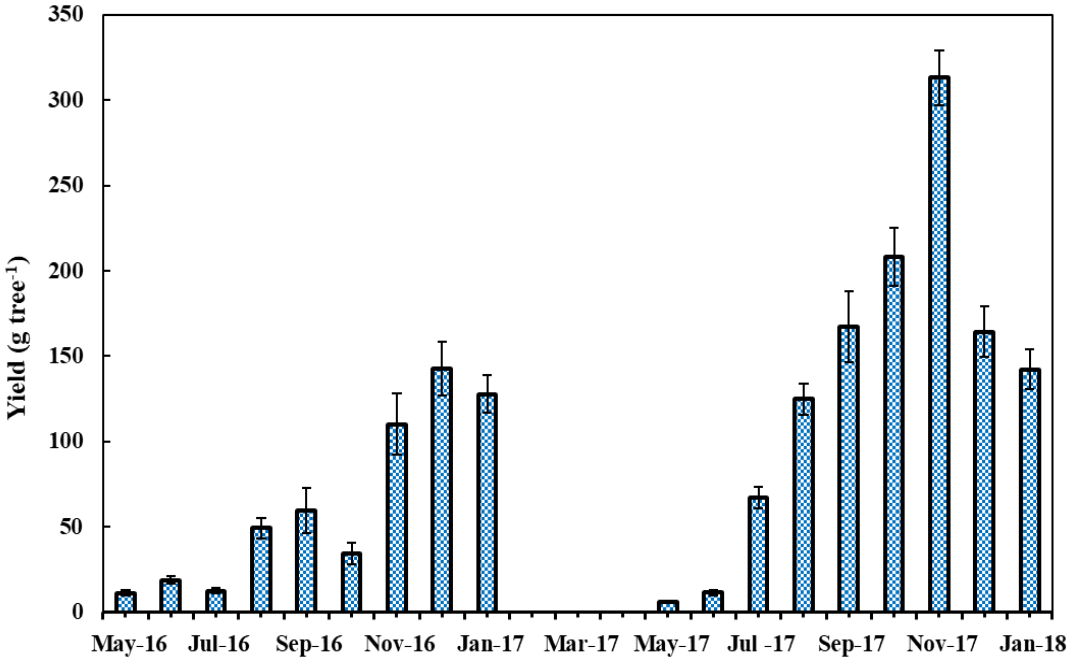

Figure 5 Mean monthly latex production throughout the experiment. Vertical bars represent means ± standard errors (n=12 trees).

2.3 Design of large chamber to measure tree crown CO_2 exchange rates and pulse-label trees with $^{13}CO_2$

The whole crown of the tree was enclosed in a chamber attached to scaffolds surrounding the tree (Figure 6, Figure 7 and Figure 8), using a design adapted from previous studies on eucalypt and beech trees (Epron et al. 2016; Plain et al. 2009). The chamber, made of 150 µm-thick transparent polyethylene film, consisted of four lateral walls (3.0–3.8 m in height, depending on the size of the crown) and a top that was heat-sealed to the lateral walls. The chamber's total volume was in the range 35–45 m^3 in a design similar to that of Plain et al. (2009) and Epron et al. (2016).

Immediately before the measurement, the crown was covered by the chamber, which was pulled tightly over the chamber support frame made from 1.3 cm diameter steel pipe. The pipes were cut to the desired lengths and connected to each other forming an approximately cubic shape. The size of the chamber support frame was adjusted to fit the size of each crown. The floor of the chamber (12 m^2) was made

of four plywood sheets (8 mm thick) that were cut out in one corner to accommodate the trunk. The floor was supported by 1.3 cm diameter steel pipes arranged in a square shape and covered by a sheet of transparent polyethylene film which was sealed with the chamber walls using clips and duct tape (Figure 8 and Figure 10).

A split-type air conditioner (TRANE; 36,000 BTU/h; WI, USA), powered by a 25 kVA single phase diesel generator (Figure 10), was used to limit the increase in air temperature inside the chamber compared to that of the outside air and to avoid water condensation. The outdoor unit was fixed to a frame located next to the chamber at the height of its floor and the indoor unit was placed inside the chamber. In addition, two axial fans and two air blowers were placed inside the chamber to ensure the air was well mixed inside the chamber.

Air temperature (T_{air}) and the relative humidity (RH) were measured with one probe outside the chamber and two probes inside the chamber (HMP 50, Vaisala, Helsinki, Finland). The photosynthetic photon flux density (PPDF) was measured outside and inside the chamber (LI-190 R; LI-COR Industries; Lincoln, NE, USA). Microclimatic data were stored every minute using a data logger (CR 1000; Campbell Scientific Inc.; Logan, UT, USA), Figure 12.

Moving the entire chamber, including the scaffolding frame and air conditioning unit, from tree to tree took a few hours, allowing measurement of one tree per day at least, with replications for statistical purposes.

2.3.1 Measurements of tree crown CO_2 exchange rates

Whole-canopy gas exchange measurements were performed on six rubber trees on June 25, 26 and 27 and on October 5, 6, and 7 between 8 and 11 a.m. just after the tree was tapped, early and late, respectively, in the rainy season. After closing the chamber, the decrease in CO_2 concentration inside the chamber due to the photosynthesis of the crown leaves was monitored for 20 minutes in June, 2016 and for 10–15 minutes in October, 2016 before starting $^{13}CO_2$ injection (Figure 6 and

Figure 7). The CO_2 concentration was measured using an infrared gas analyzer (LI-840; LI-COR Industries; Lincoln, NE, USA) and the values were stored on the datalogger every minute. One additional rubber tree was measured on June 14 for 82 minutes until the concentration in the chamber decreased from ambient to below 100 μmol mol^{-1}. This tree was not labelled with $^{13}CO_2$.

The slope of the decrease in CO_2 concentration was used to calculate net crown CO_2 exchange rates (P_{crown}, in μmol $tree^{-1}$ s^{-1}) based on Equation 3:

$$P_{\text{crown}} = \frac{\Delta[\text{CO}_2]}{\Delta \text{t}} \frac{V \times P_{\text{atm}}}{R \times (T_{\text{air}} + 273.15)} \quad [3]$$

where $\frac{\Delta[CO_2]}{\Delta t}$ is the slope of the linear variations in CO_2 concentrations over time, V is the system volume (chamber, tubes, and analyzer, with the latter two being negligible, in m^3), T_{air} is the air temperature (°C), R is the ideal gas constant (8.314 J K^{-1} mol^{-1}), and P_{atm} is the atmospheric pressure in Pa measured using an absolute barometric pressure sensor (BMP 280; Bosch Sensortec; Reutlingen, Germany).

Figure 6 Overview of the large closed-chamber designed to field measure photosynthesis of whole tree crowns and pulse label trees with $^{13}CO_2$, installed on a rubber tree.

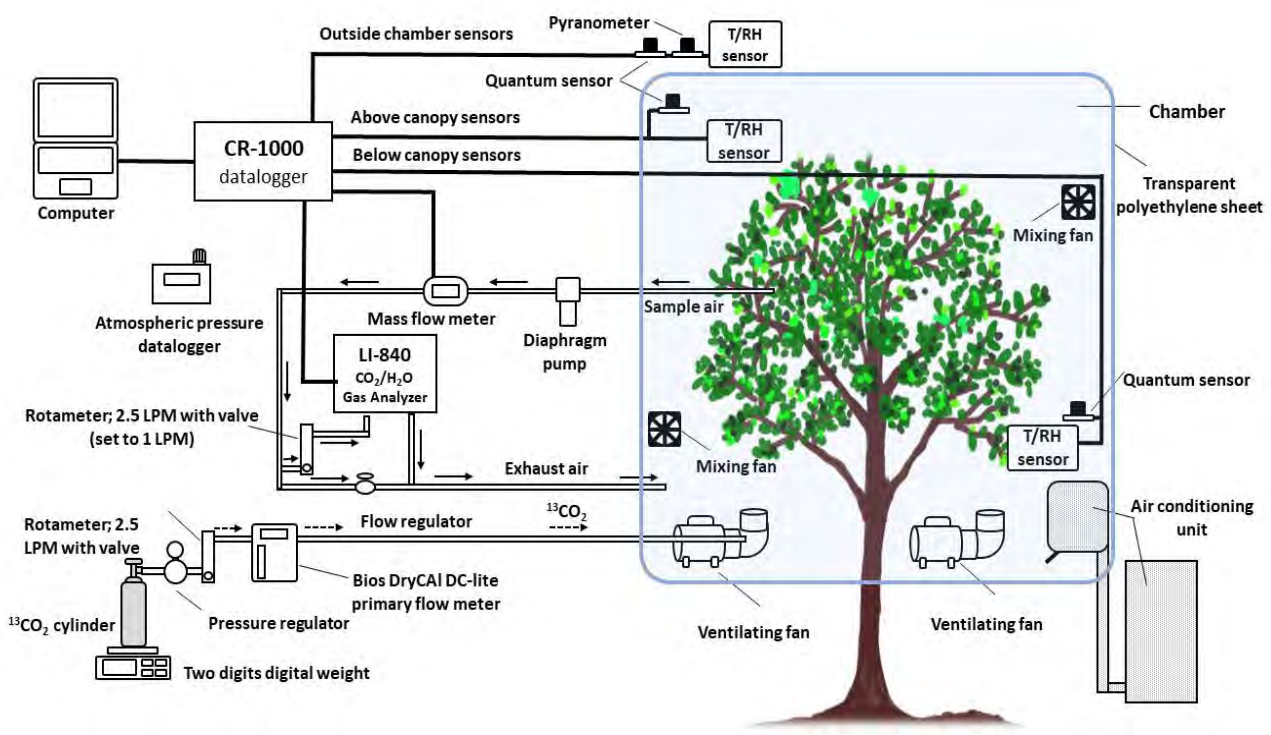

Figure 7 Schematic diagram of overview of large closed-chamber designed for field measurement of photosynthesis of whole tree crown and to pulse label tree with $^{13}CO_2$. Chamber made of 150 μm-thick transparent polyethylene film and attached to scaffolds surrounding the tree excluded for clarity. Air cooling ensured by air conditioning unit and air mixing using two axial fans and two air blowers inside chamber. Sensors measuring air temperature, humidity, and photosynthetic photon flux density installed inside and outside the chamber and connected to datalogger. CO_2 concentration inside the chamber is measured using infrared gas analyzer. $^{13}CO_2$ from gas cylinder injected into labelling chamber just above air blower, using rotameter and primary air flow meter. Drawing by Jate Sathornkich and Thippawan Angsiri.

Figure 8 The large closed-chamber designed for field measurement. (A) The scaffolds were placed surrounding the rubber tree. Air conditioning unit, two axial fans, two air blowers and sensors (air temperature, humidity, and PPFD) were installed inside chamber frame. (B) The cubic shape of chamber made of 150 µm-thick transparent polyethylene film, which was pulled tightly over the top of canopy and chamber support frame. The size of the chamber support frame was adjusted to fit the size of each crown. (C) The floor of the chamber was sealed with the chamber walls using clips and duct tape.

2.3.2 Pulse-labelling rubber tree with $^{13}CO_2$

In situ pulse labelling was performed between 8 and 11 a.m. on each tree by injecting $^{13}CO_2$ just after the tree was tapped (Figure 11). Pulse labelling was performed on three trees in June, 2016 and three trees in October, 2016 by injecting $^{13}CO_2$ in the chamber just after the initial crown CO_2 exchange measurements had been completed. Almost-pure $^{13}CO_2$ (99.299 %; Cambridge Isotope Laboratory Inc.; Andover, MA, USA) was constantly injected into the labelling chamber using a primary air flow meter (DryCal DC-Lite; BIOS International Corporation; Butler, NJ, USA) at a rate adjusted to balance the estimated net rate of CO_2 consumption by crown photosynthesis and for 45–70 min to deliver approximately the same amount of $^{13}CO_2$ to each labelled tree (18 L of $^{13}CO_2$ per tree, equivalent to approximately 9.3 g of ^{13}C or 32 g of $^{13}CO_2$). The masses of $^{13}CO_2$ injected into the chamber were confirmed each time by weighing the gas cylinder just before and after labelling. The $^{13}CO_2$ was delivered close to an air blower to facilitate mixing. After the injection period, the chamber remained closed for an additional 15 minutes for the tree to assimilate part of the remaining $^{13}CO_2$ in the labelling chamber. Then, the chamber was opened and removed. Notably, the CO_2 concentration in the chamber could not be measured during the labelling because the gas analyzer was designed for measuring $^{12}CO_2$ and had low sensitivity to $^{13}CO_2$ (Figure 10 and Figure 11).

Figure 9 The control system and data logger to measure tree crown CO_2 exchange rates and pulse-label trees with $^{13}CO_2$.

Figure 10 (A–C) Air cooling ensured by air conditioning unit, (B) the compressor part was placed outside chamber but (C) the cooling part and (D) air mixing using two air blowers were placed inside chamber.

Figure 11 $^{13}CO_2$ from gas cylinder was constantly injected into the labelling chamber using a primary air flow meter at a rate adjusted to balance the estimated net rate of CO_2 consumption by crown photosynthesis.

Figure 12 Sensors measuring air temperature and humidity below canopy (left), and photosynthetic photon flux density above canopy (right).

2.4 Leaf photosynthesis

Net CO_2 assimilation was measured using a portable gas exchange analyzer (Li6400XT; LI-COR Inc., Lincoln, NE, USA) with the photon flux density set at 1,400 μmol m^{-2} s^{-1}; the CO_2 concentration inside the chamber was 390 μmol mol^{-1} (SD = 5), the leaf-to-air vapor pressure difference was 2.0 kPa (SD = 0.4), and the leaf temperature was 32°C (SD = 2). Net CO_2 assimilation was measured on 16 leaflets on each tree, selected at four intercardinal positions (north-east, north-west, south-west, and south-east), two heights (the upper and lower halves of the crown) and two leaf whorls (first and second whorls), see Figure 13.

Figure 13 Leaf photosynthesis of rubber tree by using a portable gas exchange analyzer (Li6400XT; LI-COR Lincoln, NE, USA).

2.5 Crown leaf area and mass

The periodic pattern of rubber shoot development (Combe and du Plessix 1974; Hallé and Martin 1968) gives the branches a sub-verticillate arrangement (whorl). In a given clone, the number of leaves per whorl is rather stable, resulting in a good correlation between the number of whorls counted by sight and the leaf area of a tree. Therefore, the total leaf area (A_L) was estimated using the equation proposed by Srisondee (2019) which makes it possible to predict the leaf area with excellent accuracy. They reported a root mean squared error of 3.8 (R^2 = 0.95) when the leaf areas measured on 38 trees were compared to the predicted values using Equation 4:

$$A_L = 0.01948 \times C_{170}^{1.298} \times F_{>3}^{0.5042} \quad [4]$$

where $F_{>3}$ is the number of leaf whorls with more than three leaves and C_{170} is the trunk girth at 1.7 m from the ground. Total leaf biomass (B_L) was estimated from A_L by considering the relationship between leaf biomass and the leaf area of foliage samples collected for ^{13}C analysis.

2.6 Sample collection and preparation for ^{13}C analyses

Leaf samples (bulk leaf and leaf polar fraction)

Leaves were collected from each tree before labelling (D–1) and immediately after labelling (D0) and 24, 48 and 96 h after labelling. Two samples were collected from each tree at each sampling date, except at D0 when four samples were collected. Each sample comprised eight leaves, each leaf comprising three large leaflets, sampled from eight different positions in the crown (in the lower and upper crown sections in the same four intercardinal positions as mentioned earlier).

The leaf samples were immediately stored in a chilled box and transported to the laboratory where leaf area was measured using a leaf area meter (LI-3100C; LI-COR; USA). The samples were dried in a microwave oven at 800 W for 2 min, weighed

and then ground into fine powder using a ball-grinder (MM 400; Retsch, Haan, Germany). The leaf mass per area (LMA) was calculated as the ratio of leaf dry mass to leaf area of the sampled leaves to estimate total leaf biomass of tree crowns.

The ^{13}C analyses were performed by placing tin capsules containing 1 mg of dry leaf powder in an elemental analyzer coupled to a continuous flow isotope ratio mass spectrometer (vario ISOTOPE cube coupled to the IsoPrime 100; IsoPrime Ltd; Cheadle, UK).

The leaf polar fraction (PF, including soluble sugars, amino acids and organic acids) was specifically purified from dry leaf by the sequential biochemical procedure based on the polar properties of compounds of interest described in Desalme et a. (2017). Briefly, an initial extraction of 100 mg of dry powder in 800 µL methanol/chloroform/water (MCW, 12/5/3, v/v/v) was performed to separate soluble and insoluble compounds. The compounds soluble in MCW were further separated according to their polarity by adding 1 mL of methanol/chloroform (1/1, v/v) and 400 µL of ultra-pure water. After decantation and centrifugation (2,000 g, 5 min, 15°C), the upper phase containing polar compounds (PF) was recovered and dried under vacuum centrifugation (vacuum concentrator CentriVap, LabConco, Kansas City, MO, USA). The dried PF was diluted with ultra-pure water and an aliquot corresponding to 0.6 mg of dry extract was transferred into a tin capsule (Elemental Microanalysis, Cambridge, UK; 6 × 4 mm; ref. D1006 BN/139877), freeze-dried followed by ^{13}C analysis of the leaf PF.

Bark extract samples

A piece of bark (about 2.7–4 cm^2) was collected from each tree at a height of 180 cm above the ground (60 cm above the tapping cut) the day before labelling (D−1) and 24, 48, 72 and 120−168 h after labelling (Figure 14). The change in the date of the last sampling was due to operational constraints. Soluble compounds were extracted from the bark using the exudation method (Dannoura et al. 2011, 2019). Briefly, the samples were infused in 2 mL of distilled water at ambient temperature in the dark for

5 h. The piece of bark was then removed, oven-dried at 65 °C and weighed. The extracts were filtered through nylon cartridges (Whatman, 0.2 µm, diameter 25 mm), vacuum-evaporated for 4 h (Maxi-Dry plus, HetoHolten, Allerod, Denmark) and weighed. The dried extracts were diluted with ultra-pure water and an aliquot corresponding to 0.6 mg of dry extract was transferred into a tin capsule, freeze-dried and followed by ^{13}C analysis of the bark extract.

Figure 14 Bark sampling (left), a piece of bark was collected from each tree at a height of 180 cm above the ground and was infused in 2 mL of distilled water (right).

Latex samples

Latex was collected from the trunk of each tree the day before labelling (D−1) and several times over a period of 1 year after labelling at a decreasing frequency (every day during the first 3 days, every 3 days for 2 weeks, every 6 days for 1 month and every 15 days until the end of January 2018 (580 days after labelling in June and 480 day after labelling in October). Except for the first 3 days after labelling, latex samples were always collected on a tapping day at 6.30 h., i.e., before tapping. No

samples were taken during the period when the trees were not tapped (i.e. during the dry season, February–April, 2017). Latex was collected by inserting a small plastic cannula (internal diameter 2 mm) into the bark of the trunk at a height of 115 cm above the ground (Figure 15), i.e. 5 cm below the downward tapping cut, because this is the main latex drainage and latex regeneration area (d'Auzac 1989).

The two first drops were discarded and the two following drops were collected for ^{13}C analysis of the total organic matter in latex (hereafter called 'bulk latex') in a tube containing 1 mL of distilled water as described in Kanpanon et al (2015) and Duangngam et al. (2020). Twenty microliters of the latex solution were transferred into a tin capsule and oven dried at 50 °C overnight.

Figure 15 Latex sampling, trunk latex was collected by inserting a small plastic cannula into the bark of the trunk 5 cm below the tapping cut.

The next 10 drops were collected for ^{13}C analysis of rubber and serum in a tube containing 2 mL of sulphuric acid (0.6 M H_2SO_4) that was shaken and stored at 4 °C until processed. Mineral acid was used to coagulate the rubber and separate it from serum instead of the usual organic acid (trichloroacetic acid) to avoid adding C that would have changed the carbon isotopic content. The coagulated rubber was neutralised

by soaking it in water, oven dried at 70 °C for 24 h, and 0.7 mg was placed in a tin capsule. The solution of H_2SO_4 containing the serum was filtered through nylon cartridges (Whatman, 0.2 µm, diameter 25 mm) and used for ^{13}C analysis of serum.

Trunk samples

Trunk cores were taken at a height of 170–180 cm above the ground (0.5 cm diameter, 3.5 cm long including 0.5 cm of bark and 3 cm of wood) with a wood auger the day before labelling (D–1) and 1 week, 1 month, 4 months and 7 months after labelling (Figure 16). No trunk sampling was performed after 7 months in trees labelled in October. The trunk samples were immediately placed in a chilled box, stored at –20 °C until they were freeze-dried (Benchtop, Cryotec, Saint-Gély-du-Fesc, France), and ground. The trunk PF (including soluble sugars, amino acids and organic acids), structural compounds (ST) and carbohydrate reserves (including starch) were purified from 100 mg of dry trunk powder following the protocol described in Desalme et al (2017).

An initial extraction was performed in 800 µL MCW (12/5/3, v/v/v) to separate soluble and insoluble compounds. As described above for leaf samples, the compounds soluble in MCW were further separated according to their polarity by adding 1 mL of methanol/chloroform (l/l, v/v) and 400 µL of ultra-pure water. After decantation and centrifugation (2,000 g, 5 min, 15°C), the upper phase containing polar compounds was recovered. The compounds insoluble in MCW were sequentially separated. Proteins and pigments were discarded by dissolution in 300 µL of phosphate buffer. The remaining pellet after protein extraction was washed several times with hot ethanol to remove pigments, hydrolysed by acid treatment (1 mL 6 M HCl for 1 h) and centrifuged at 12,000 g for 30 min at 4°C. The HCl-hydrolysable fraction contained in the supernatant was precipitated with absolute methanol for one night at 4°C and then collected by centrifugation (12,000 g, 10 min, 4°C). The HCl-hydrolysable fraction contained starch, but also probably products derived from hydrolysis of hemicellulose, pectin, and gums (Richter et al. 2009), and constitutes the fraction called hereafter ‘carbohydrate reserves’ (CR). The remaining pellet after acid hydrolysis contained non HCl-hydrolysable structural compounds (ST). PF, CR and ST were vacuum-evaporated

(vacuum concentrator CentriVap, LabConco, Kansas City, MO, USA). Aliquots of dry CR and dry ST were placed in tin capsules for ^{13}C analysis. The dried PF was diluted with ultra-pure water and an aliquot corresponding to 0.6 mg of dry extract was transferred into tin capsules, freeze-dried followed by ^{13}C analysis of the trunk PF. The soluble sugars and carbohydrate reserves in the trunks were extracted from 20 mg of trunk dry powder using methanol 70 % following a protocol adapted from Shvaleva et al. (2006). After centrifugation of the extract (18,000 rpm, 10 min, 4°C), soluble sugars were recovered in the supernatant (supernatant 1) and carbohydrate reserves in the pellet. The carbohydrate reserves contained in the pellet were hydrolysed with hydrochloric acid 2% (100°C; 1 h) into soluble sugars and recovered in the supernatant (supernatant 2) after centrifugation. The concentrations of soluble sugars in both supernatants were assayed using the anthrone method (Yemm and Willis 1954).

Figure 16 Trunk wood sampling, trunk cores were taken at a height of 170–180 cm above the ground with a wood auger (left), a piece of sample was 0.5 cm diameter, 3.5 cm long including 0.5 cm of bark and 3 cm of wood (right).

2.7 ^{13}C analyses and calculations

All the ^{13}C analyses will be performed by placing tin capsules containing 0.6–1 mg of dry material in an elemental analyser coupled to a continuous flow isotope ratio mass spectrometer (vario ISOTOPE cube coupled to the IsoPrime100, IsoPrime Ltd, Cheadle, UK), see Figure 17 using an internal working standard that was related to the international Vienna Pee Dee Belemnite reference (VPDB). The carbon isotope composition ($\delta^{13}C$) was expressed relative to this reference using Equation 1:

$$\delta^{13}C_{sample} = [R_{\text{sample}} / R_{\text{standard}}) - 1] \quad [1]$$

The precision of the analyses (standard deviation), based on the repeated measurements (n = 12) of our internal standard during each sample run, was below 0.1 ‰.

The ^{13}C atom fraction, $x(^{13}C)$, was calculated from the carbon isotope composition ($\delta^{13}C$), expressed relative to the isotope ratio of the Vienna Pee Dee Belemnite standard (RVPDB = 0.0111802) (Coplen et al. 2002) using Equation 5:

$$x(^{13}C) = \frac{^{13}C}{^{12}C + ^{13}C} = \frac{(\delta^{13}C + 1) \times R_{\text{VPDB}}}{[(\delta^{13}C + 1) \times R_{\text{VPDB}}] + 1} \quad [5]$$

The excess ^{13}C atom fraction, ($x^E(^{13}C)$ was calculated as the difference between $x(^{13}C)$ of each sample collected from labelled trees and $x(^{13}C)$ measured in the same compartment before labelling (at D–1), multiplied by the carbon content of each sample (C) and the each sample mass (M), $x^E(^{13}C)$ was expressed in mg^{13}C g^{-1}C. In the foliage and in bark extract, $x^E(^{13}C)$ was also expressed in mg ^{13}C g^{-1} dry mass by considering the C content of the compartment. The total amount of ^{13}C in leaves at D0 (Crown ^{13}C; Table 3), was calculated from $x^E(^{13}C)$ (expressed in mg ^{13}C g^{-1} dry leaf) multiplied by total leaf biomass using Equation 6:

$$x^E(^{13}C) = \sum \left(x(^{13}C)_{lab} - x(^{13}C)_{unl}\right) \times C \times M \quad [6]$$

Figure 17 Isotope Ratio Mass Spectrometer (IsoPrime 100, IsoPrime Ltd, UK)

2.8 Analysis of tracer kinetics

The temporal dynamics of $x^E(^{13}C)$ in the foliage (bulk and PF), in the bark extract, in the serum and in trunk PF was fitted with an exponential decay function (Epron et al. 2016) as follows Equation 7:

$$x^E(^{13}C) = C_1\, exp\left(\frac{-t}{MRT}\right) + C_2 \qquad [7]$$

where C_1 is the initial amount of label assigned to a rapid turnover ^{13}C pool; C_2 is the amount of label assigned to a slow turnover ^{13}C pool (expressed in mg ^{13}C g^{-1} dry mass in leaves and in bark; or in mg ^{13}C g^{-1} C in serum and in trunk PF); t is the time elapsed after labelling; MRT is the mean residence time (expressed in hours in foliage and bark or in days in serum and trunk PF). The sum of C_1 and C_2 is the total amount of ^{13}C in each compartment.

The temporal dynamics of $x^E(^{13}C)$ in rubber and latex was fitted with an equation that included a logistic rise and an exponential decrease (Dannoura et al. 2019; Studer et al. 2014) using Equation 8:

$$x^E(^{13}C) = \mathrm{A} \times \frac{\exp\left(\frac{-t-B}{MRT}\right)}{1+\exp\left(-C \times (t-B)\right)} \quad [8]$$

where A is the theoretical maximum amount of ^{13}C at the peak (mg ^{13}C g^{-1} C); B is the peak time (days); C is the constant rate of the ^{13}C accumulation (mg ^{13}C day^{-1}); t is the time elapsed after labelling (days); MRT is the mean residence time (days). The short-term dynamics can be described by three phases: (i) lag phase (time needed for C transfer), (ii) phase dominated by ^{13}C import or net accumulation and (iii) a phase dominated by ^{13}C export or stationary phase (equilibrium between ^{13}C import and export).

2.9 Statistical analyses

All statistical analyses was performed using R 4.1.0 software (R Development Core Team, 2021). Linear models (based on the "lm" function in R) were used to estimate the initial slopes of the decrease in CO_2 concentration with time in the chamber. Significant differences in crown photosynthesis and excess ^{13}C in leaves between June and October were assessed using an analysis of variance (based on the "aov" function in R). A linear mixed-effects model with trees as the random effect was used to test for differences in the isotope composition of leaves between June and October (based on the "lmer" function in lme4 package in R).

The effects of position in the crown and the orientation or whorl on leaf photosynthesis were assessed using linear mixed-effects models with trees as the random effect. Linear regression between crown photosynthesis and either photosynthetic photon flux density, total leaf area, or the average net CO_2 assimilation of leaves were calculated based on the "lm".

Mixed-effect models, with trees as random effect, were used to estimate the effect of the labelling period (June or October) and time after labelling as the fixed effect on $x^E\left(^{13}C\right)$ in tree compartments (leaf, bark extract, latex and trunk) using the lmer function in the 'lmer4' package (Bates et al. 2014). Contrasts were used to test relevant differences when the overall model was significant ($P < 0.05$). Exponential decay functions and the logistic function was fitted to $x^E\left(^{13}C\right)$ using the nonlinear least-square method (nls function in the 'stats' package). Model parameters in the exponential decay model (C_1, MRT, C_2) and in the logistic model (A, B, C, MRT) will be tested for significance ($P < 0.05$), as well as the model itself (r^2 and root mean square error). At first, models were fitted on the whole dataset either without considering possible variations of model parameters between the two labelling periods or allowing the model parameters to adjust specifically for June and October labelling, using dummy variables. In all cases except for trunk PF, allowing the model parameters to adjust specifically provided a better adjustment (lower Akaike information criterion, AIC). We therefore further adjusted parameters of all models independently for June and October, except for trunk PF. Model parameters are reported with their 95% confidence intervals.

RESULTS AND DISCUSSION

1. Measuring photosynthesis of entire tree crowns and pulse label trees in large closed-chamber with $^{13}CO_2$ in the field: Design and testing

1.1 Environmental condition in chamber

The light transmission in the photosynthetically active range of radiation (PAR) of the polyethylene film used for the chamber walls was, as expected for this type of material, over 90 % of incoming photosynthetic photon flux density (PPFD, Figure 8). In addition, polyethylene is known to have only little impact on the spectral composition of the PAR passing through the film (Corelli-Grappadelli and Magnanini 1993). The air temperature (T_{air}) in the chambers was on average 1.2 °C above the temperature measured outside the chamber, with a maximum observed positive deviation of 3 °C during the approximately 90 minutes the chambers were closed (Figure 18 and Table 2). This indicated that the power of the air-conditioning system was strong enough to maintain the ambient temperature in large, unshaded crown chambers in tropical conditions. However, the counterpart of this efficiency was a fairly substantial drop in RH, which also showed rapid variations of large amplitudes due to the intermittent operation of the air conditioner (Figure 18). If, on average, the decrease in RH was limited to 8 %, the maximum drop during a cooling cycle could temporarily exceed 30 %. The main limitation of this system is that although the temperature was properly controlled, the air RH showed large fluctuations. The performance can be improved in the future by adding an ultrasonic mist generator controlled by a humidity probe.

1.2 Response of crown photosynthesis to decreased CO_2 concentration

The decrease in CO_2 concentration for the tree that was not labelled with $^{13}CO_2$ was monitored for 82 minutes until the concentration in the chamber decreased from near ambient (385 μmol mol^{-1}) to below 100 μmol mol^{-1} (Figure 9A). During the measurement, T_{air}, RH and PPFD averaged 30 °C (SD = 1.3), 51 % (SD = 11) and 1,330

μmol m^{-2} s^{-1} (SD = 630), respectively. The decrease in CO_2 concentration in the chamber was not linear and was best predicted by a third-degree polynomial function, with its first derivative being the slope ($\frac{\Delta[CO_2]}{\Delta t}$) which can be used in Equation 3 to calculate crown photosynthesis. Crown photosynthesis decreased when the CO_2 concentration decreased in the chamber, as expected for a C3 plant (Figure 19B). The CO_2 compensation concentration when the net crown photosynthesis reached 0 (when gross photosynthesis and respiration were balanced) was 70 μmol mol^{-1}. This value was in the upper range of typical values for leaves of C3 plants, which was expected because the crown also included branches that add additional CO_2 loss through their respiration (Bravdo 1971).

Table 2 The meteorological conditions recorded on the day of labelling. Mean air temperature (T_{air}), mean relative air humidity (RH) and mean photosynthetically active radiation (PAR). Subscripts In and Out mean inside and outside the chamber, respectively.

Date	Tree	T_{air-In} (°C)	RH_{In} (%)	PAR_{In} (μmol m^{-2} s^{-1})	$T_{air-Out}$ (°C)	RH_{Out} (%)	PAR_{Out} (μmol m^{-2} s^{-1})
25 Jun	#1	29.9	74	706	29.7	72	867
26 Jun	#2	30.2	64	775	29.5	76	882
27 Jun	#3	28.7	76	549	28.1	82	691
5 Oct	#4	32.0	55	1,000	30.7	72	1,049
6 Oct	#5	30.5	68	732	28.7	76	849
7 Oct	#6	30.6	61	803	28.5	74	919

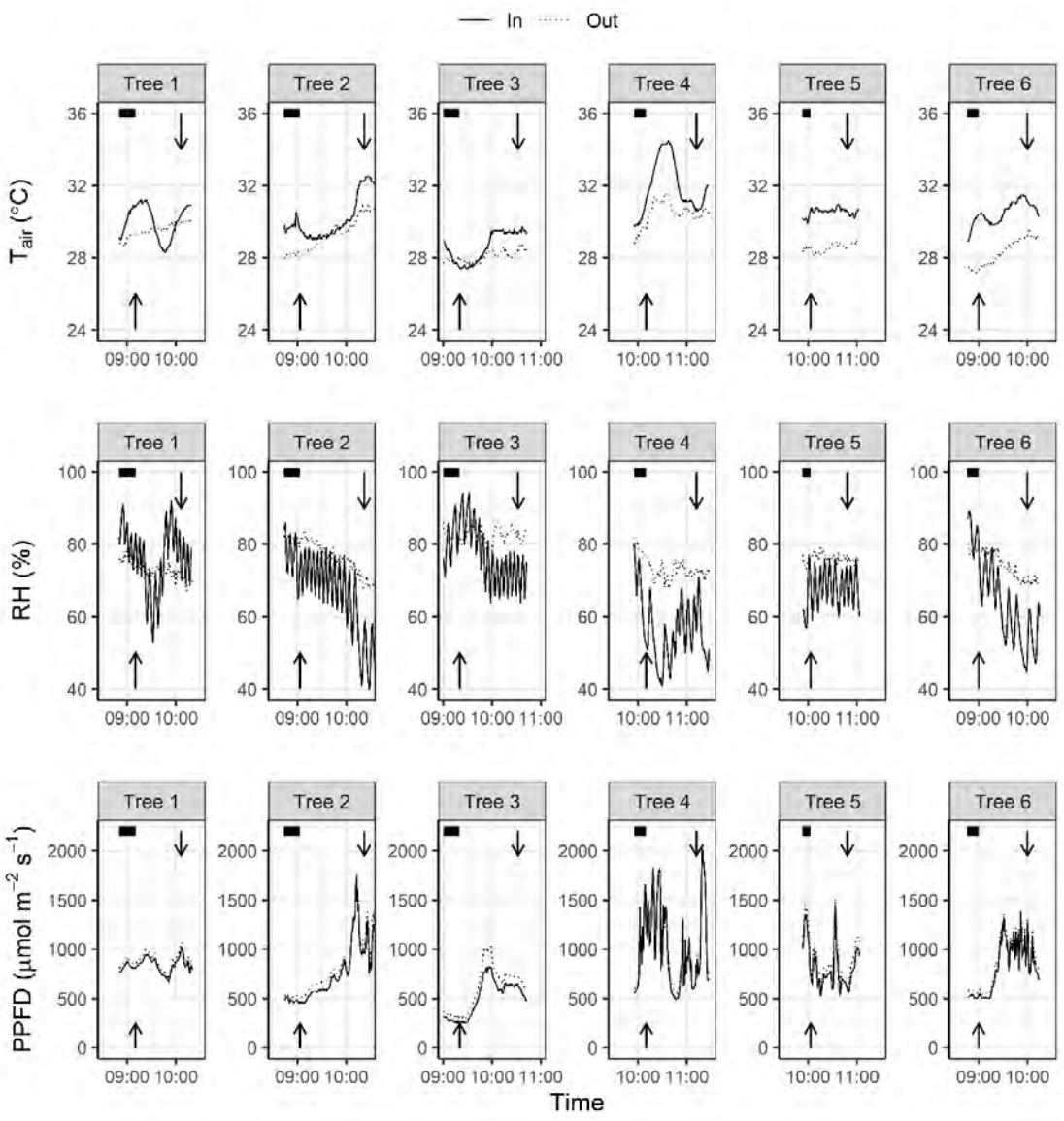

Figure 18 Air temperature (T_{air}), relative humidity (RH), and photosynthetic photon flux density (PPFD) recorded inside (solid lines) and outside (dotted lines) closed chamber. Trees #1 – #3 measured in June and trees #4 – #6 were measured in October. Black horizontal bar at top of each panel indicates when crown CO_2 exchange rate was calculated from decrease in CO_2 concentration in chamber. Two vertical arrows indicate beginning and end of $^{13}CO_2$ injection.

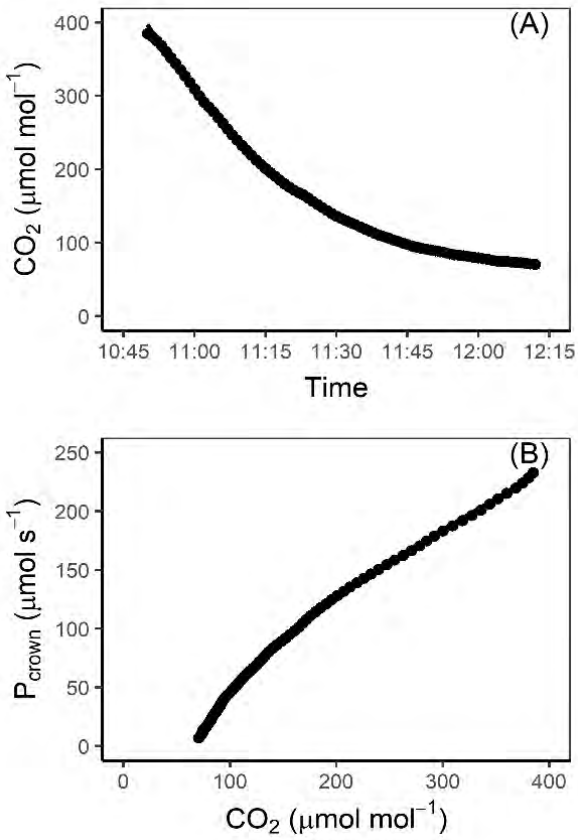

Figure 19 (A) Decrease in CO_2 concentration in chamber for 82 minutes after closure until concentration decreased from ambient to below 100 μmol mol^{-1}. (B) First derivative of third-degree polynomial function fitted to decrease in CO_2 concentration and used to calculate crown photosynthesis (P_{crown}) based on Equation 3. Measurement made on June 14 on tree (#7 in Table 1) not labelled with $^{13}CO_2$.

1.3 Variations of crown photosynthesis among trees at near ambient CO_2 concentration

The decrease in CO_2 concentration was monitored for 10–20 minutes on six trees that were labelled with $^{13}CO_2$ immediately after (Figure 20). The initial linear slope of the decrease of CO_2 concentration was used to calculate crown photosynthesis.

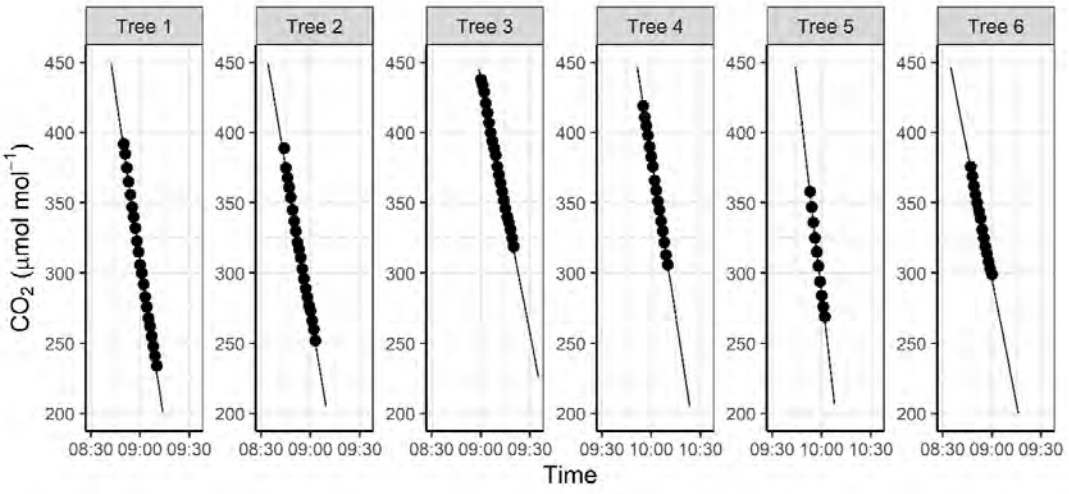

Figure 20 Decrease in CO_2 concentration in chamber for 10–20 minutes after closure and before injecting $^{13}CO_2$. Closed circles are measurements and solid lines are adjusted linear relationships between time and CO_2 concentrations. Trees #1 – #3 measured in June and trees #4 – #6 measured in October.

On average, crown photosynthesis was 183 µmol s^{-1} with a mean photon flux density during all measurements of 700 µmol m^{-2} s^{-1}. With a tree spacing of 7 m in rows 2.5 m apart, this was equivalent to 10.5 µmol m^{-2} s^{-1} on a ground area basis. Eddy flux tower estimates of gross primary production (GPP) at the same light intensity in a nearby 27-year-old rubber plantation was about 20 µmol m^{-2} s^{-1} from May to October (Wang et al. 2022). Two reasons could explain this twice as high value. First, net crown photosynthesis is the balance between gross leaf photosynthesis and crown respiration (leaves and branches), while respiration is not included in the GPP estimate. Second, the trees in the 27-year-old plantation were much taller (more than 20 m) and had a completely closed canopy with a maximum leaf area index of 6.2 during the rainy season (Wang et al. 2022), whereas canopy cover was only about 70 % of the ground area in the current experiment.

Crown photosynthesis varied between the six trees, but no significant differences were found between trees measured in June and those measured in October ($P = 0.18$). The variation of crown photosynthesis was well explained by the differences

in the average photon flux density between the different dates of measurement, as reflected by significant positive relationships (R^2 = 0.84 and P = 0.01 with PPFD measured outside the chamber, Figure 21A; R^2 = 0.78 and P = 0.02 with PPFD measured inside the chamber). Crown photosynthesis was not related to the RH inside the chamber (P = 0.14), but unexpectedly was related to RH measured outside the chamber (R^2 = 0.76 and P = 0.03), which could be explained by the strong negative correlation between photon flux density and relative humidity (R = -0.88, P = 0.02). In other words, a lower RH was recorded when conditions during the measurement were very sunny than when there was intermittent cloud cover. Although the effect of photon flux density was dominant, there was also a positive trend between crown photosynthesis and whole tree leaf area (R^2 = 0.53 and P = 0.09, Figure 21B).

1.4 Variations of leaf photosynthesis between and within tree crowns, and relationship with crown photosynthesis

Crown photosynthesis was also positively related to mean leaf photosynthesis (R^2 = 0.58 and P = 0.07, Figure 21C). Mean leaf photosynthesis exhibited large differences between the six tree crowns as shown above (Figure 21C), in the range 4.9–11.4 μmol m^{-2} s^{-1}. These values were in agreement with measurements conducted on seedlings and mature rubber trees (Alam, Nair, and Jacob 2005; Chen et al. 2010; Kositsup et al. 2009, 2010; Senevirathna, Stirling, and Rodrigo 2003). Within-crown variations were less pronounced than between-crown variations. Neither the position in the crown (upper or lower half of the crown), nor the orientation (north-east, north-west, south-west, or south-east), nor the whorl (first or second whorls) significantly influenced leaf photosynthesis (P > 0.1). This last result was in agreement with the lack of variation in the leaf nitrogen content per unit area with leaf age (Kositsup et al. 2009).

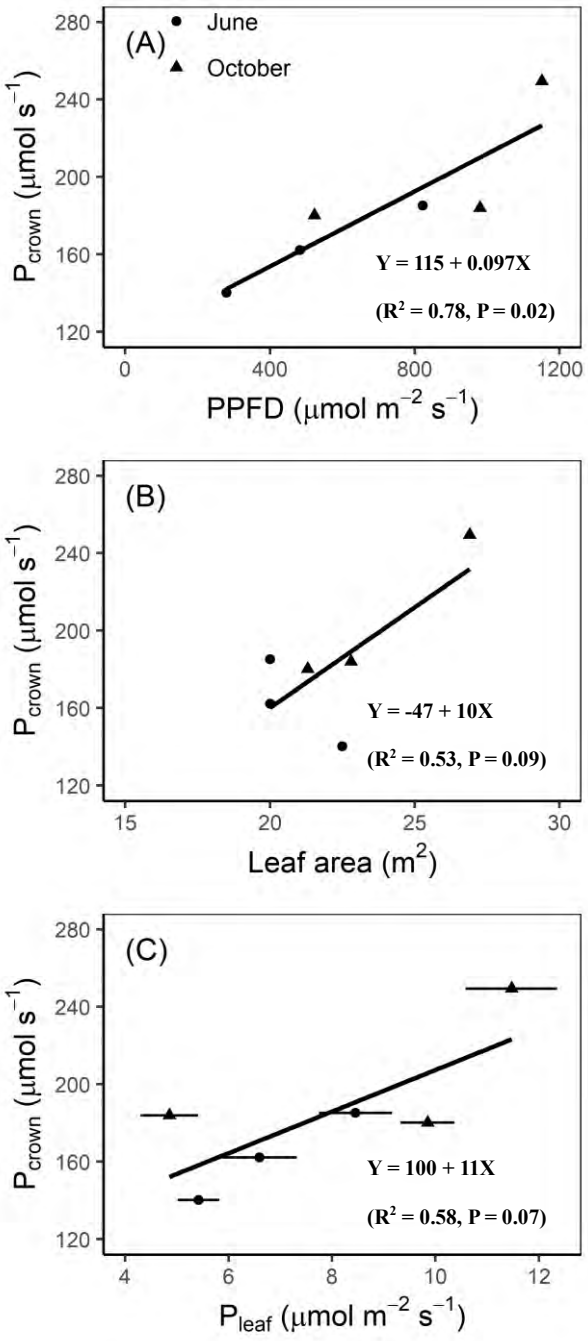

Figure 21 Relationship between crown photosynthesis (P_{crown}) and (A), photosynthetic photon flux density (PPFD), (B) total leaf area, and (C) average net CO_2 assimilation of 16 leaves for each tree (P_{leaf}; horizontal bars represent standard errors of mean). Circles show trees measured in June and triangles show trees measured in October. Linear regression lines are shown in each panel.

1.5 ^{13}C recovered in leaves

The total amounts of ^{13}C recovered in tree leaves, $x^E(^{13}C)$, were in the range 4.0–6.3 g tree^{-1} (Table 3), representing 43–68 % of the $^{13}CO_2$ injected in the chamber. Not all the injected $^{13}CO_2$ was supposed to be recovered in the foliage, because it was diluted in the pre-existing $^{12}CO_2$ in the chamber (330 ppm on average) and not all the $^{13}CO_2$ was assimilated by leaves before the chamber was opened. As mentioned above, it was not possible to measure the $^{13}CO_2$ concentration in the chamber because of the low sensitivity of the gas analyzer to $^{13}CO_2$. However, based on the rates of injection of $^{13}CO_2$ and crown photosynthesis measured before injecting $^{13}CO_2$, the remaining mass of ^{13}C in the chamber at the opening time was estimated at 3.2 ± 0.3 g. Thus, the total amounts of ^{13}C recovered in tree leaves accounted for 89 % of the assimilated $^{13}CO_2$ on average after accounting for the mass of ^{13}C remaining in the chamber. The fact that 43–68 % of the injected $^{13}CO_2$ was recovered in the leaves after the end of labelling highlighted the effectiveness of the design of the chamber and the labelling protocol.

The total amounts of ^{13}C recovered from the tree leaves, $x^E(^{13}C)$, were significantly higher in June than in October (5.8 ± 0.4 and 4.3 ± 0.2 g tree^{-1}, respectively, n = 6, P = 0.03), despite no significant differences in crown photosynthesis. The amounts of ^{13}C recovered in the tree leaves represented 63 % of the ^{13}C injected in the chamber in June on average, and 46 % in October. The reason for this difference remains unknown; however, seasonal differences in ^{13}C losses by either respiration or emission of volatile organic compounds cannot be excluded. Rubber trees are indeed strong emitters of isoprene and monoterpenes, especially in the wet season (Baker et al. 2005; Wang et al. 2007).

Table 3 Isotope composition ($\delta^{13}C$) of leaves collected immediately after labelling (mean ± standard error for 4 composite leaf samples for each tree) and excess ^{13}C in whole foliage of trees, $x^E(^{13}C)$, for 3 trees labelled in June, 2016 and 3 trees labelled in October, 2016. Numbers in bracket represent efficiency of labelling, calculated as ratio between $x^E(^{13}C)$ and the amount of ^{13}C injected in chamber. Differences between June and October tested using mixed-effect model with tree as random effect for $\delta^{13}C$ and linear model for, $x^E(^{13}C)$.

Month	Tree	$\delta^{13}C$ (‰)	$x^E(^{13}C)$ (g tree^{-1})
June	#1	432 ± 23	6.2 (68 %)
	#2	377 ± 11	5.0 (54 %)
	#3	443 ±1 0	6.3 (69 %)
October	#4	237 ± 19	4.1 (44 %)
	#5	307 ± 11	4.7 (51 %)
	#6	272 ± 7	4.0 (43 %)
Month	F-values	25.7	10.3
Effect	P-values	0.007	0.03

Note: F-values are the ratio of the between-group to the within-group variances of the linear model and the P-values are the probability of obtaining these F-values if the difference between the two months are not significant.

2. In situ $^{13}CO_2$ pulse labelling of rubber trees reveals a shift in the contribution of carbon sources in latex regeneration

2.1 The excess ^{13}C in the compartment of rubber trees

Excess ^{13}C in foliage

The total amount of ^{13}C assimilated by trees (Crown ^{13}C or $x^E(^{13}C)$) varied between 4.0 and 6.3 g tree^{-1} (Table 4). In leaves, the ^{13}C recovered in total organic matter (bulk) and polar compounds (PF) both in June and October decreased rapidly in the first 48 h after pulse labelling and remained relatively stable in the following hours (Figure. 22A and Figure 22B). The labelling period (LP; June vs October) had a significant effect on the excess ^{13}C recovered in bulk leaves and leaf PF and the decrease in ^{13}C excess over time differed in June and October (significant interactions between time and LP) (Figure 22A and Figure 22B; P<0.05). These decreases were adequately described by a two-pool exponential decay model (Equation 7) both in June and October (Table 4). In bulk leaves, there was more ^{13}C per unit dry matter partitioned in the rapid turnover ^{13}C pool (C1) in June than in October (the 95% confidence intervals did not overlap) but the mean residence time of ^{13}C (MRT) in this pool did not differ between June and October (the 95% confidence intervals overlapped, Table 4).

In bulk leaves and leaf PF, ^{13}C reached asymptotic C2 values that differed significantly from 0, indicating that a fraction of the assimilated ^{13}C was recovered in the rather stable pool. There was no difference in the C2 values in June and October (Table 4).

Excess ^{13}C in bark extract

The excess ^{13}C recovered in bark extract was maximal one day after labelling and decreased thereafter both in June and October (Figure 22C). This decrease was

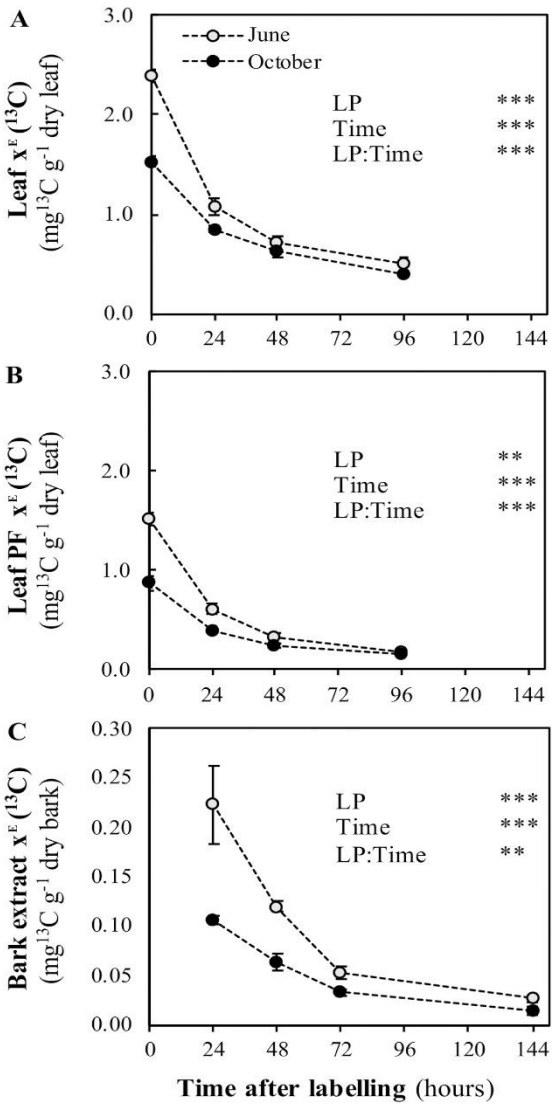

Figure 22 Short-term dynamics of excess ^{13}C ($x^E(^{13}C)$), expressed in mg ^{13}C g^{-1} dry mass, after $^{13}CO_2$ pulse labelling of rubber trees) in June (open circles) and in October (filled circles) in (A) total organic matter of leaves (bulk leaves), (B) leaf polar fraction (PF), and (C) bark extract fraction. PF includes soluble sugars, amino and organic acids. Data represent means ±SE (n=3 trees). For the last sampling of bark extract, the three values from the three replicates trees (which were sampled respectively 120, 144, or 168 h after labelling) were pooled and placed at 144 h on the graph. Asterisks indicate significant effect of the labelling period (LP), time and the LP–time interaction on excess ^{13}C in bulk leaves, leaf PF and bark extract (**P<0.01, ***P<0.001).

adequately described by an exponential decay model in which the C2 parameter characterizing the slow turnover pool was not significantly different from 0 (Table 4) indicating that assimilated ^{13}C was not recovered in a rather stable pool. MRT was not different in June (38.8 h) or in October (45.3 h) but higher than in leaves (Table 4).

Excess ^{13}C in latex

^{13}C enrichment of bulk latex, rubber and serum was observed one day after labelling (Figure 23). Excess ^{13}C and ^{13}C dynamics in bulk latex and rubber were close and were adequately described by combining a logistic rise and an exponential decay model (Equation 8). In both compartments, peak $x^E(^{13}C)$ was reached faster and was higher in trees labelled in October than in trees labelled in June (Figure 23A and Figure 23B; Table 5). Moreover, MRT of ^{13}C in latex and rubber was 2-fold longer in trees labelled in June (38.9 and 37.3 days; Table 3) than in trees labelled in October (17.3 and 15.9 days; Table 5).

The dynamics of ^{13}C in serum differed from those in bulk latex and rubber (Figure 23). The initial value of excess ^{13}C recovered in serum was higher in trees labelled in June than in trees labelled in October (Figure 23C and Table 5). $x^E(^{13}C)$ decreased rapidly in the serum in the first three days after labelling and remained rather stable over the following 60 days both in June and October (Figure 23C). The decrease was adequately described by a two-pool exponential decay model in which C2 accounted for only 3% of the total ^{13}C in June and for 4% in October (Table 5). The MRT of the mobile pool of ^{13}C in serum was lower in trees labelled in June (9.7 days) than in trees labelled in October (16.6 days). The MRT of ^{13}C was shorter in serum than in latex/rubber in the trees labelled in June, whereas there was no difference in the MRT of ^{13}C between serum and latex/rubber in the trees labelled in October (Table 4 and Table 5).

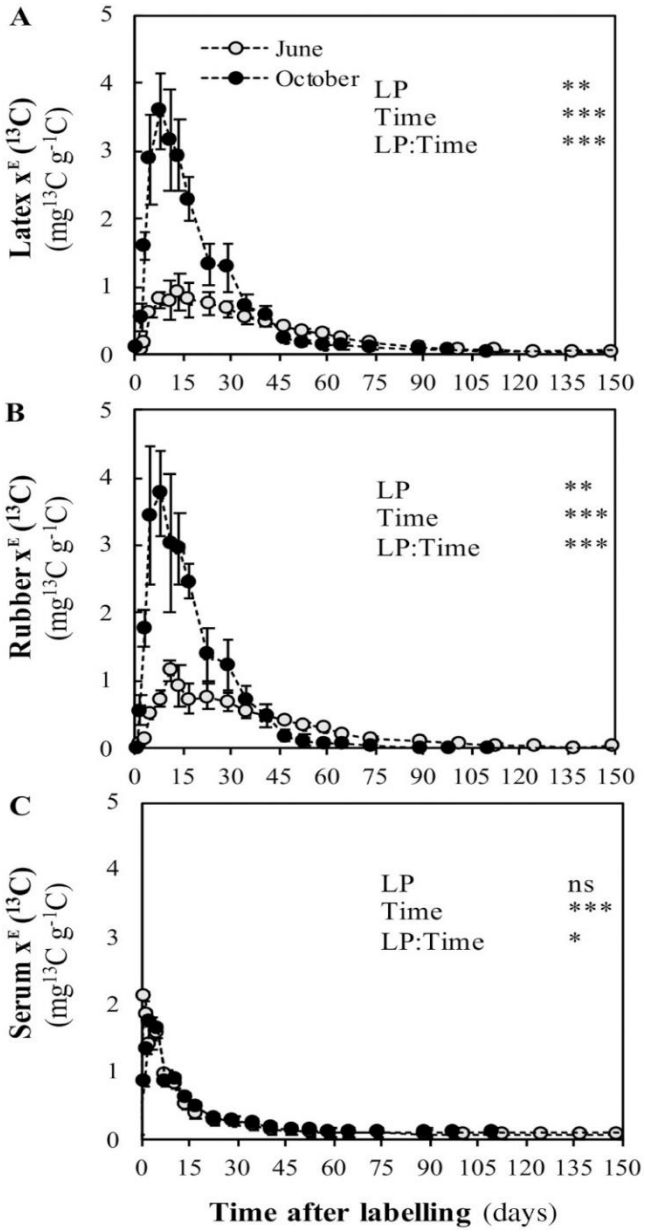

Figure 23 Time courses of excess ^{13}C ($x^E(^{13}C)$), after $^{13}CO_2$ pulse labelling of rubber trees (*Hevea brasiliensis* Muell.Arg.) in June (open circles) and in October (filled circles) in (A) latex, (B) rubber, and (C) serum. Data represent means ±SE (n=3 tree). The selected period was justified by the stability of $x^E(^{13}C)$ in latex, serum, and rubber after 150 days considering the actual scale of the graphs. Asterisks indicate a significant effect of the labelling period (LP), time and the LP–time interaction on excess ^{13}C in latex, rubber, and serum (ns, P>0.05; *P<0.05, **P<0.01, ***P<0.001).

Excess ^{13}C in the trunk and carbohydrate concentrations

The concentrations of soluble sugars and carbohydrate reserves in the trunks did not differ between trees labelled in June and trees labelled in October (Figure 24). The concentrations of soluble sugars did not change over the tapping season (around 20 mg g^{-1} dry mass) whereas the concentrations of carbohydrate reserves increased throughout the tapping season (Figure 24).

Enrichment in ^{13}C was observed in the total organic matter of the trunk cores in the samples collected one week after labelling (Figure 25A) and in the purified fractions: polar fraction (PF; Figure 25B), structural compounds (ST; Figure 25C) and carbohydrate reserves (CR; Figure 25D). The excess ^{13}C in trunk PF reached higher values (1.3–1.6 mg ^{13}C g^{-1} C) than in the other trunk fractions (maximum of 0.3 mg ^{13}C g^{-1} C). Excess ^{13}C in PF decreased significantly (by 80%) 40 days after labelling (Figure 25B) and the ^{13}C dynamics at both labelling periods was adequately described by a single exponential one-pool decay model (Table 4) with a MRT of 21.9 days (Table 4). In carbohydrate reserves, excess ^{13}C was significantly higher in trees labelled in June than in trees labelled in October (Figure 25D). There was no clear temporal dynamics of the excess ^{13}C in the total organic matter or in structural compounds, and in addition, no difference between trees labelled in June or in October (Figure 25A and Figure 25C).

2.2 Tracing the fate and dynamics of ^{13}C in tapped rubber trees from the leaves to latex

All the studied compartments in the young rubber trees were rapidly supplied with recently photo-assimilated ^{13}C (Figure 23, Figure 24 and Figure 25). The amount of excess ^{13}C recovered in the total organic matter of leaves (bulk leaves) and in the leaf polar fraction (PF) decreased rapidly just after labelling following a two-pool exponential decay model (Figure 22A and 22B, Table 4). This highlights the contribution of recently assimilated C in two types of C-molecules which differ in their MRTs or turnover rates in the rubber tree leaves. Most of the recent C (75–80% of the bulk fraction, 85–90% of polar fraction, Table 4) was invested in molecules that are

rapidly exported from the foliage via respiration, phloem transport or emission of volatile organic compounds (VOCs), especially in rubber trees which are strong emitters of isoprene and monoterpenes (Baker et al. 2005; Wang et al. 2007). The MRT of these compounds in the leaves was short (20–28 h, Table 4), as is commonly observed in boreal, temperate and tropical species (Dannoura et al. 2019; Desalme et al. 2017; Epron, Bahn, et al. 2012; Epron et al. 2016; Warren et al. 2012).

Recently assimilated C also provides 'building blocks' for constructing molecules with slower turnover, such as structural compounds, lipids and some proteins. In rubber tree leaves, 20–25% of the recently assimilated C was incorporated in these slow-turnover molecules (Table 4). In rubber, leaf flushes occur every two to three months and grow regularly (Hallé & Martin, 1968). Incorporation of ^{13}C in structural compounds was therefore expected after both June and October labelling. Rubber tree leaves contain rubber producing laticifers that remain in the leaf for a long time if the leaf is not injured (Webster and Baulkwill 1989). Rubber tree leaves also contain several other secondary metabolites including tannins, saponins, sterols, resins and flavonoids (Abulude, 2007; Wigati et al, 2014), which also contribute to the slow turnover pool. Part of the sucrose may not be exported from the leaves but stored in leaf vacuoles (Farrar and Farrar 1986), and further used to produce latex or cyanogenic monoglucosides in leaves (Kongsawadworakul et al. 2009). Some of these slow turning compounds are soluble in polar solvent and contribute 10–15% of the labelled C in the polar fraction (Table 4). Excess ^{13}C in bark extract peaked rapidly after labelling and disappeared completely (one-pool decay model) with a MRT of about two days (Figure 22C**,** Table 4), showing that the labelled molecules arrived promptly in this compartment and were rapidly exported from it. This confirmed that the bark extract was mostly made up of phloem sap (Gessler, Rennenberg, and Keitel 2004). The short MRT of ^{13}C in the bark extract of rubber trees is similar to that reported in several other tree species (Dannoura et al. 2011, 2019; Epron et al. 2016; Gessler et al. 2004; Högberg et al. 2008). Sucrose is the main carbohydrate transported in rubber trees (Tupý 1985; Zhu et al. 2018) but some of the cyanogenic monoglucosides, which are produced in the foliage, are also exported from the leaves and transported in the form of diglucosides in the phloem sap (Selmar, Lieberei, and Biehl 1988). They are

delivered to the laticifers in the bark where they are involved in latex metabolism in the regeneration area (Kongsawadworakul et al. 2009).

The dynamics of excess ^{13}C in the rubber particles (Figure 23B) clearly drove the dynamics of excess ^{13}C in the bulk latex (Figure 23A), which was expected because rubber particles are rich in C (80%) and account for 90% of the dry latex (Hepper and Audley 1969). As latex flows out of the tree only when the bark is cut (under tapping), the dynamics of ^{13}C in the latex results from inputs of photosynthates and reserves over time and losses in the form of the latex exported at each tapping day. Such dynamics of C in latex only concern the area of the trunk where there is regeneration of latex, i.e., the area of the trunk located some centimeters below the tapping cut (d'Auzac 1989). Therefore, the MRT of ^{13}C in latex is hypothesized to be infinite in untapped trees and in the region of the trunk far away from regeneration area in tapped trees but is likely to change in the regeneration area of tapped trees depending on the tapping system, particularly tapping frequency. Serum accounts for a minor part of the C content of bulk latex and the dynamics of excess ^{13}C in the serum was very different from those of latex (Figure 23C). Serum is the cytoplasm of the laticifers, and thus contains many of the substances normally found in plant cells, including carbohydrates, amino acids, proteins, organic acids, inorganic salts, lipids, nucleotides and nucleic acids (Archer et al. 1969). The amount of excess ^{13}C recovered in serum started to decrease just a few days after labelling (one day in June and three days in October), following a two-pool exponential decay model (Figure 23C, Table 4). The excess ^{13}C started to be recovered in rubber within one to two days after labelling (Figure 23B), indicating that part of the ^{13}C recovered in serum was directly incorporated in rubber. Sucrose provides the carbon skeleton and energy supply for biosynthesis (d' Auzac et al. 1997).

However, in the rubber, the excess ^{13}C peak was reached later (6.6 days after labelling in June and 4.2 days in October, Table 5) and ^{13}C was still found in the rubber more than 100 days after labelling (Figure 23B). These results confirm the hypothesis that rubber C does not only come from recent photosynthates, but also

from a pool of reserves where recent carbon is mixed with older carbon as suggested by Kanpanon et al (2015).

In the trunk of the rubber tree, ^{13}C-labelled compounds were found as early as six days after labelling in the polar fraction (PF), structural compounds (ST) and carbohydrate reserves (CR), and ^{13}C was still detected 210 days after labelling (Figure 25A–D). The excess ^{13}C in the trunk PF (one-pool decay model) decreased rapidly (MRT of ^{13}C was 19–25 days) (Figure 25B**,** Table 4) whereas excess ^{13}C evolved more slowly in structural compounds and carbohydrate reserves (Figure 25C and Figure 25D). Our results confirmed that rubber trees store large amounts of carbohydrate reserves in trunk parenchyma, especially during the tapping period (Figure 24). This seasonal trends are similar to those previously noted for starch (Chantuma et al. 2009; Silpi et al. 2007). Starch stored in the trunk parenchyma is therefore a good candidate for the supply of C for latex biosynthesis.

2.3 Temporal variation in allocation and dynamics of ^{13}C

There was no difference in the mean residence time (MRT) of ^{13}C in the foliage and in the phloem sap between June and October (Table 4). The MRT of ^{13}C in serum was longer in October than in June (Table 4), whereas the MRT of ^{13}C in rubber particles and bulk latex were shorter in October than in June (Table 5). Seasonal changes in climatic conditions are known to influence the dynamics of C transport and allocation in trees (Dannoura et al. 2011; Epron et al. 2016; Epron, Laclau, et al. 2012; Ruehr et al. 2009).

Table 4 Kinetic parameters of short-term ^{13}C dynamics in different compartments of the rubber tree : total organic matter of leaves (bulk leaf), leaf polar fraction including soluble sugars, amino, and organic acids (leaf PF), bark extracts, serum, and trunk PF after ^{13}C pulse labelling in June, 2016 and October, 2016.

Compartment	Labelling period	Mobile ^{13}C pool	Stable ^{13}C pool	MRT (95% CI)	Accuracy of the model	
		C_1 (95% CI)	C_2 (95% CI)		RMSE	R^2
		(mg ^{13}C g^{-1} DM or C)	(mg ^{13}C g^{-1} DM)			
Bulk leaf	June	0.198 (0.179–0.218)	0.051 (0.032–0.066)	19.8 h (13.9–27.0 h)	0.018	0.95
	October	0.119 (0.103–0.139)	0.038 (0.016–0.052)	27.9 h (17.9–43.9 h)	0.014	0.92
Leaf PF	June	0.141 (0.123–0.160)	0.017 (0.001–0.032)	20.6 h (13.1–30.9 h)	0.016	0.93
	October	0.075 (0.058–0.096)	0.015 (−0.005 to 0.028)	22.2 h (9.3–46.1 h)	0.015	0.80
Bark extract	June	0.409 (0.268–0.642)	NA	38.8 h (24.7–65.2 h)	0.031	0.85
	October	0.182 (0.147–0.226)	NA	45.3 h (35.3–59.5 h)	0.009	0.94
Serum	June	2.198 (1.987–2.402)	0.065 (0.03–0.10)	9.7 d (8.0–11.9 d)	0.21	0.85
	October	1.478 (1.329–1.634)	0.063 (0.02–0.11)	16.6 d (13.4–20.8 d)	0.20	0.82
Trunk PF	June/October	0.193 (0.161–0.234	NA	21.9 d (14.0-31.8 d)	0.021	0.90

Note C_1, excess ^{13}C recovered in the mobile pool at D0; MRT of ^{13}C; C_2, asymptotic remaining amount of ^{13}C recovered in the compartment after the pulse-chase period (stable pool). C_1, MRT, and C_2 (with 95% CI) were estimated separately for each labelling period (except trunk PF) by fitting experimental data (excess ^{13}C 'x^E (^{13}C)' over time, Figure 22) to a two-pool (mobile and stable ^{13}C pools) exponential decay model (Equation 7). When C_2 was not significantly different from 0, data were adjusted to a one-pool decay model and C_2 is equal to 0. The accuracy of the adjustments was tested with root mean square error (RMSE) and R^2. NA, not applicable in this context.

Table 5 Kinetic parameters of short-term ^{13}C dynamics in latex and rubber in the rubber tree after ^{13}C pulse labelling in June, 2016 (Jun) and October, 2016 (Oct).

Compartment	Labelling period	Kinetic parameters			MRT (95% CI)	Accuracy of the model	
		A (95% CI) (mg ^{13}C g^{-1} C)	*B* (95% CI) (day)	*C* (95% CI) (mg ^{13}C g^{-1} C day^{-1})		RMSE	R^2
Bulk latex	June	1.17 (1.06–1.30)	5.66 (4.72–6.81)	0.49 (0.36–0.69)	38.9 (32.9–45.9)	0.11	0.86
	October	4.73 (4.28–5.23)	4.18 (3.65–4.80)	0.86 (0.66–1.15)	17.3 (14.6–20.4)	0.35	0.89
Rubber	June	1.17 (1.05–1.32)	6.58 (5.51–7.81)	0.49 (0.36–0.68)	37.3 (30.9–45.0)	0.13	0.83
	October	5.37 (4.76–6.06)	4.09 (3.54–4.75)	0.96 (0.71–1.34)	15.9 (13.0–19.3)	0.45	0.88

Note *A*, theoretical excess ^{13}C at the peak; *B*, the date when the peak was reached; *C*, constant rate of ^{13}C accumulation in the compartment; MRT, mean residence time of ^{13}C. *A*, *B*, *C*, and MRT (with their 95% CI) were estimated for each labelling period by fitting experimental data (excess ^{13}C '$x^E(^{13}C)$' over time, see Figure 23A and Figure 23B with an equation that included a logistic rise and exponential decay model (Equation 8).The accuracy of the adjustments was tested with root mean square error (RMSE) and R^2.

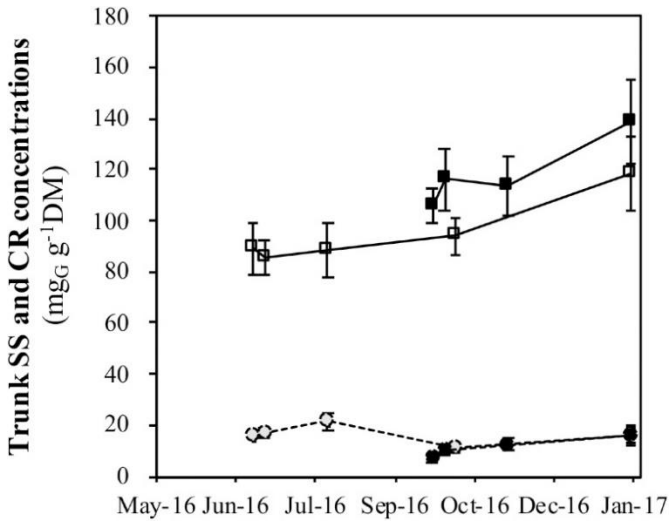

Figure 24 Mean concentrations of soluble sugars (circles) and carbohydrate reserves (squares) in the trunk wood of rubber trees (*Hevea brasiliensis* Muell.Arg.), expressed in mg glucose equivalent g^{-1} dry mass ($mg_G\ g^{-1}$ DM), after pulse labelling in June (open symbols) and in October (filled symbols). Data represent means ±SE (n=3 trees).

However, labelling was performed in June and October, early and late in the rainy season, during which climatic conditions did not change much (Figure 4), but during which the strengths of the different sinks are expected to change, thus modifying the allocation pattern. A lower rate of latex production was found in June (three grams per tree and per tapping event) compared to October (12 g) (Figure 5). This was expected because when a tree is tapped for the first time, the initial yield is low. Successive tapping at regular intervals increases the yield until a plateau is reached after some months (Webster and Baulkwill 1989). For practical reasons (the size of the chamber), this experiment was conducted on trees that were younger than the usual age when tapping starts (4 years old instead of 6 years old). For commercial tapping, rubber trees are generally opened when their girth reached around 45 cm measured 1.7 m above the ground. The girth of our trees was only 19.5–23.5 cm at this height. However, the yield obtained was 0.1 and 0.5 g tree^{-1} tapping^{-1} cm^{-1} of the cut in June and October, respectively, which was significant compared to standard yields of about 1 g tree^{-1} tapping^{-1} cm^{-1} of cut for trees with a girth of 50 cm (Lacote et al. 2010).

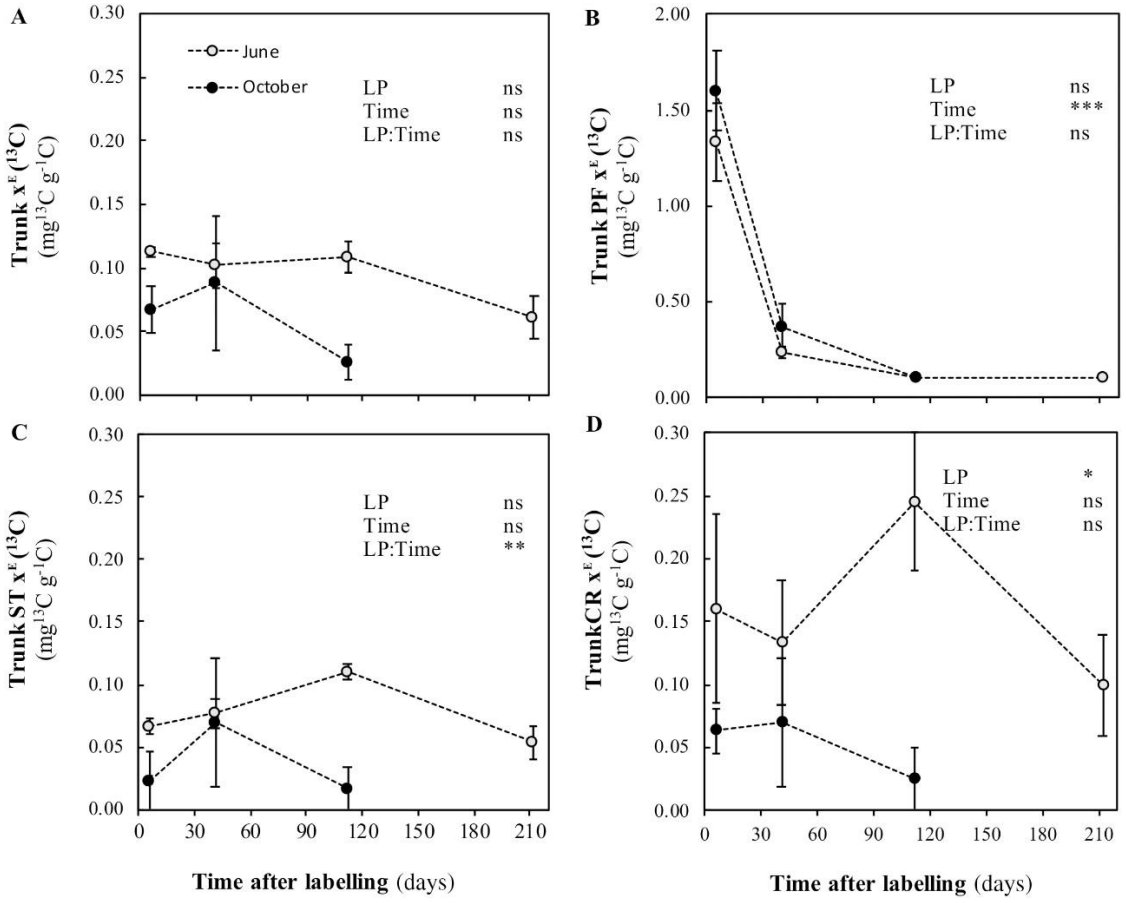

Figure 25 Time courses of excess ^{13}C ($x^E(^{13}C)$), expressed in mg ^{13}C g^{-1} C, after $^{13}CO_2$ pulse labelling of rubber trees (*Hevea brasiliensis* Muell.Arg.) in June (open symbols) and in October (filled symbols) in (A) total organic matter of the trunk (bulk trunk), (B) trunk polar fraction (PF), (C) trunk structural (ST), and (D) trunk carbohydrate reserves (CR). Data represent means ±SE (n=3 trees). Asterisks indicated a significant effect of the labelling period (LP), time and the LP–time interaction on excess ^{13}C in bulk trunk, trunk PF, ST, and CR (ns, $P>0.05$; *$P<0.05$, **$P<0.01$, ***$P<0.001$).

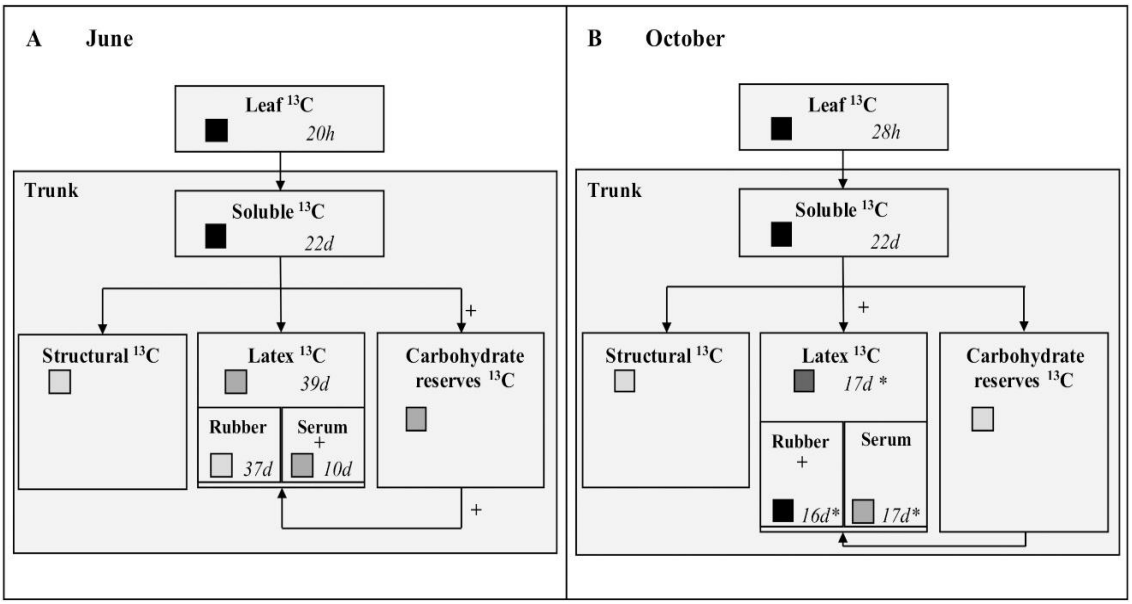

Figure 26 Schematic representation of the allocation of the recently assimilated C (traced under the form of ^{13}C) in the trunk among soluble compounds (soluble ^{13}C), growth (structural ^{13}C), storage (carbohydrate reserve ^{13}C), and latex production (latex ^{13}C) in rubber trees (*Hevea brasiliensis* Muell.Arg.), in June (A) and in October (B).

Note: See the detail of Figure 26, Arrows represent C fluxes between compartments. The size of boxes represents the amount of ^{13}C invested in growth, storage, or latex production. The shading of the square in each box represents the intensity of the labelling reached at the peak (from black: highly labelled to white: unlabeled). The mean residence time of ^{13}C (MRT), if calculated, is shown in italics and expressed either in hours in leaves or in days in trunk compartments. An asterisk following MRT of ^{13}C in October indicates a significant difference from MRT in June. Leaf ^{13}C is allocated to the trunk soluble compounds and then partitioned among structural growth, latex production, and carbohydrate reserve formation. (A) In June, the recently assimilated C is preferentially invested in carbohydrate reserve formation (+), resulting in higher excess ^{13}C in trunk carbohydrate reserves in June than in October. A part of the ^{13}C is also rapidly recovered in serum but not in rubber, suggesting that rubber C comes mainly from the carbohydrate reserve pool (+) in which new C has been mixed with old

C. (B) In October, latex production and concentrations of carbohydrate reserves are higher than in June (larger boxes). The recently assimilated C is preferentially invested in the latex in which it participates in rubber formation. The higher yield increases the sink strength of the laticifers in which rubber production occurs.

Leaf ^{13}C was allocated to the trunk soluble compounds and then partitioned among structural growth, latex production and carbohydrate reserve formation. The main findings of this study was summarized in a schematic representation of the allocation of the recently assimilated C (traced under the form of ^{13}C) in the trunk among soluble compounds (soluble ^{13}C), growth (structural ^{13}C), storage (carbohydrate reserves ^{13}C) and latex production in June and in October (Figure 26). Because excess ^{13}C in leaves, phloem (Figure 22) and serum (Figure 23C) was higher in trees labelled in June than in October, we expected that excess ^{13}C in rubber (Figure 23B) would be higher in June than in October. However, the initial excess ^{13}C and the maximum rate recovered in rubber were higher in trees labelled in October than in June (Figure 23B), suggesting a higher direct contribution of recent photosynthates to rubber (+) in October (Figure 16B).

This hypothesis is supported by the shorter time needed to reach the peak in excess ^{13}C in rubber and the faster disappearance of excess ^{13}C in rubber when labelling was performed in October than in June (Figure 23B, Table 5). In October, latex production (Figure 5) and carbohydrate reserve concentrations (Figure 24) are higher than in June (shown as larger boxes than in June in Figure 26). We thus suggest that recent photosynthates are allocated to rubber production in larger quantities after several months of tapping, i.e., once the latex regeneration metabolism is well established and carbohydrate reserves are full. In June, the recently assimilated C was preferentially invested in carbohydrate reserve formation (+; Figure 16A), resulting in higher excess in trunk carbohydrate reserves than in October (Figure 15D). Storage is considered as a high-priority and active process in rubber trees that accumulate C at the expense of competing sinks, i.e., growth and latex production (Chantuma et al. 2009; Silpi et al. 2007). A part of ^{13}C is also rapidly recovered in serum but less in rubber (Figure 23B, C), suggesting that rubber C comes mainly from the reserve pool (+;

Figure 26A) in which new C has been mixed with old C. Therefore, the priorities of C allocation in rubber trees are first storage, then latex production. Nevertheless, we cannot exclude that priorities in C allocation could change with time, when trees become taller and store larger amounts of C in their trunk, and the shift observed in C sources for latex regeneration over the tapping season for these young trees might be less significant in older trees.

Higher concentrations of ^{13}C in serum and its faster disappearance (shorter MRT) in June than in October (Figure 23C; Table 4) contrasted with the lower concentrations of ^{13}C and longer MRT in rubber in June compared with October (Figure 23B; Table 5), suggesting that the ^{13}C allocated to serum in June is not invested in molecules that are precursors for rubber synthesis, but in other C-compounds involved in laticifer metabolism or allocated to a pool of C storage. By contrast, the ^{13}C recovered in serum in October is hypothesized to be preferentially invested in molecules involved in the rubber synthesis (probably sucrose and, to a lesser extent, cyanogenic monoglucosides), explaining the earlier appearance of the peak in ^{13}C and higher excess ^{13}C observed in rubber in October than in June (Figure 23B; Table 5). Further investigations using compound-specific stable isotope analysis should clarify ^{13}C partitioning and dynamics in the different C-containing molecules in the serum.

$^{13}CO_2$ labelling allowed us to trace the fate and determine the dynamics of the recently assimilated C in the different organs and compartments of rubber trees, including leaves, phloem sap, trunk wood and latex. The results showed that even if the recently assimilated C was rapidly incorporated in latex and rubber, the C used for the regeneration of the latex come also from another source (stored carbohydrates) where old and new C are mixed. A difference in ^{13}C allocation and dynamics between June and October (1 month or 5 months after the beginning of tapping) was observed in latex and in wood starch. It was concluded that in October, when latex metabolism is well established and starch reserves in the trunk are complete, a greater proportion of recent photosynthates is used directly for latex regeneration. In June, photosynthates are preferentially invested in the formation of carbohydrate reserves in the trunk, and part

of reserves is remobilized in the inner bark to regenerate latex. The contribution of reserves to latex regeneration underlines the importance of further analysis of the patterns of allocation between growth, latex and reserves to be able to forecast the effects of different tapping systems on the carbon budget of the whole rubber tree.

CONCLUSION AND RECOMMENDATIONS

This study is the first experimental work investigating the carbon allocation in latex and its dynamics in rubber trees (*Hevea brasiliensis* Muell.Arg.) using $^{13}CO_2$ pulse-labelling experiment. The methodology was successful and the results were conclusive.

The large crown chamber designed in this study was tested on 4-year-old, 5–6 m tall, rubber trees in the field, both for measuring crown photosynthesis and pulse-labelling trees with $^{13}CO_2$. The rates of crown photosynthesis were consistent with measurements at leaves and ecosystems levels. Moving the entire chamber, including the scaffolding frame and air conditioning unit, from tree to tree took a few hours, allowing measurement of one tree per day at least, with replications for statistical purposes. The main limitation of this system is that although the temperature was properly controlled, the air relative humidity showed large fluctuations. The performance can be improved in the future by adding an ultrasonic mist generator controlled by a humidity probe. Nevertheless, more than one-half of the $^{13}CO_2$ injected in the chamber was recovered in the leaves after the end of the labelling, proving that the design of the chamber and the labelling protocols were effective.

Whole crown $^{13}CO_2$ pulse labelling was performed in June, when latex production was low, and in October, when it was high. ^{13}C content was quantified in the foliage, phloem sap, wood, and latex. In both labelling periods, ^{13}C was recovered in latex just after labelling, indicating that part of the carbohydrates was directly allocated to latex. However, significant amounts of ^{13}C were still recovered in latex after 100 day and the peak was reached significantly later than in phloem sap, demonstrating the contribution of a reserve pool as a source of latex C. The contribution of new photosynthates to latex regeneration was faster and higher when latex metabolism was well established, in October, than in June. However, the experiments were conducted on young rubber trees at the beginning of latex collection. An improved understanding of C dynamics and source–sink relationships in rubber

tree over several years after the onset of tapping is crucial to understand the importance of carbohydrate reserves, and to forecast and manage tapping systems in a changing environment, thus ensuring a sustainable latex production. For such purpose, survey of the seasonal and interannual dynamics natural abundance of ^{13}C in the different tree compartments can be a complementary approach, easier to implement on larger trees and over a longer period.

Perspectives and recommendations

What to infer from these results regarding rubber production?

A major evolution in natural rubber production is the necessary shift towards low frequency tapping systems. Due to competition with other activities (on farm and out farm), the workforce necessary for tapping the trees is less and less available (Tongkaemkaew et al. 2018) and its cost is increasing. It is then forecasted that Thai rubber farms will switch to lower frequency tapping systems that provide a higher yield per tapping day and therefore a higher daily income for the tappers (Sainoi et al 2017). Implementing such systems in the Thai context necessitate a considerable adaptative work that is not in our scope. However, many of the other main rubber producing countries already use lower tapping frequencies (i.e. d3 to d5) in estates as well as in family farms (Vijayakumar et al. 2009). That is the case for example in Cambodia (Phearun et al. 2019) or in Côte d'Ivoire. Researches are already implemented to develop very-low frequency systems, from d6 (weekly with a day off) to d12 (twice a month with a weekly day off). All these systems use Ethephon, an ethylene precursor, to compensate for the loss of tapping days by increasing the yield per tree per tapping day. Ethephon is well-known to prolong the latex flow (delay the coagulation on the tapping cut) and enhance the regeneration metabolism (Lacote et al. 2010; Obouayeba et al. 2010; Sainoi et al. 2017; Sainoi and Sdoodee 2012). However, there are so far some limits. With stimulation systems tailored (dose, frequency) to clones, it is possible to obtain the same yield per tree, and then per land area, in d3, d4 and even d5 than in intensive systems (i.e. d2, 2d3). However, from d6 the yield starts to decrease and the prototypes are not yet profitable under very low

tapping frequency (Gohet et al. unpublished results). If the duration of the latex flow appears to be one main limiting factor, there are also issues regarding the latex regeneration. The reference tapping frequency is d2 (every other day), because the regeneration time of the exported latex is evaluated to be around 40 h (d'Auzac 1989), then regeneration would be complete if trees are tapped every two days. Higher frequencies (2d3, 3d4) are usually combined with shorter tapping cut (1/3 of spiral instead of 1/2) to compensate. With such tapping frequencies (d2 and below), the regeneration process is rather regular as the trees are almost always regenerating latex, in small quantities. Reducing tapping frequencies disturb such regularity, as the trees export higher amount of latex each time, but less frequently. The pattern becomes an alternance of peaks of export/regeneration and long periods of rest. The lower the tapping frequency, the higher the peaks and the longer the lasting periods. Of course, such patterns lead to uneven metabolic demand. Could the current photosynthesis and the available reserves sustain such peaks of C demand? Conversely, would the latex metabolism not "sleep" during too long inactive periods?

Do these results provide clues about these possible responses?

The first answer is that although these results show that recent photosynthates are involved in latex regeneration, the contribution of reserves was confirmed. This is consistent with the results of Chantuma et al. (2009) and Silpi et al. (2007), who showed that tapped trees accumulated more reserves than untapped ones, while their growth was reduced. Therefore, the dynamics of reserves should be studied in detail to understand the responses of rubber trees to low tapping frequencies. One possibility would be to use the parameters of the function used to describe the kinetics of ^{13}C in latex, particularly the mean residence time (MRT).

What does C MRT means in latex and rubber?

In transport compartments such as phloem, MRT indicates the speed of transfer from the sources (canopy) to the sinks (growing organs, reserves, metabolism, respiration). In structural compartments, MRT indicates the turn-over of carbon in the

structures of the plant. How fast, the C that composes the structures is renewed or discarded. In storage compartments, the MRT indicates the balance between the supply of C to the reserve compounds (for rubber trees, mostly starch in wood parenchyma) and its use to sustain metabolic demand. But what about latex and rubber? When, and only when, the laticifer vessels are severed by tapping, the latex, i.e. the cytoplasm of the latex cells, flows out. The exported latex is partly replaced by latex coming from the rest of the anastomosed laticifer vessels (often mentioned as the displacement area, (d'Auzac, 1989). Therefore, a part of the ^{13}C kinetics relate to the direct flow of matter within the laticifer vessels. This component directly depends on the amount and frequency of the export, then on the tapping system. However, as the nucleus and the mitochondria of the latex cells remain in place, the latex content, made mainly of rubber particles and then of C, is regenerated in situ in what is called, without precise location, the regeneration area. This component of the kinetics should be similar to that of the sink organs, particularly the reserve compartments (starch), as the used compounds are to be regenerated in situ from imported sucrose. One difference is that in the reserves, starch must be hydrolyzed into sucrose to be mobilized, whereas latex is directly exported. It would not be easy to disentangle this complex pattern. However, at the tree scale the overall MRT will depend on the amount of exported latex and the frequency of exportation, then on the tapping system. Further research is necessary to assess if the ups and downs induced by low frequency tapping would result in higher or lower MRT as compared to smoother traditional systems. Possible variations in the other parameters of the kinetics (A, the excess ^{13}C at the peak; B, the date when the peak is reached) could also provide relevant information on how C resources are mobilized. It would be of particular interest to compare the kinetics under different tapping systems, particularly different tapping frequencies. Comparing clones known for their more or less active latex metabolism would also be relevant.

Finally, the experimental design, pulse-labelling approach and sampling strategy are also suitable for further studies on other tree species of interest in horticulture or silviculture, especially for addressing whole tree carbon balance and carbon allocation to specific sink organs or functions, such as, in addition to latex

regeneration, fruit maturation and storage of sugars. In addition, crown photosynthesis datasets provide valuable information for testing and validating functional 3-D models of trees.

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