The source of latex. Tracing carbon from leaf photosynthesis to latex metabolism in rubber trees using carbon stable isotopes

Philippe Thaler[1,2], Ornuma Duangngam[2], Poonpipope Kasemsap[2,3], Jate Sathornkich[2,3], Chompunut Chayawat[2], Duangrat Satakhun[2], Pierrick Priault[4], Dorine Desalme[4], Pisamai Chantuma[5] , Jaleh Ghashghaie[6] and Daniel Epron[4]

1 CIRAD, UMR Eco&Sols, F-34060 Montpellier, France.
2 Hevea Research Platform in Partnership, Centre of Thai-French Cooperation on Higher Education and Research, Kasetsart University, Bangkok10900, Thailand.
3 Department of Horticulture, Faculty of Agriculture, Kasetsart University, Bangkok 10900, Thailand.
4 Ecologie et Ecophysiologie Forestières, Faculté des Sciences et Technologies, Université de Lorraine, UMR INRA – UL 1137, F-54506 Vandoeuvre-les-Nancy, France.
5 Chachoengsao Rubber Research Center, Rubber Authority of Thailand, Sanam Chaiket, Chachoengsao 24160, Thailand.
6 Ecologie, Systématique et Evolution, Université Paris-Sud, CNRS UMR 8079, Orsay cedex 91405, France

Abstract

One of the main challenges for the future of Natural Rubber production is the scarcity of skilled manpower to tap the trees. The only way to cope with such issue is to reduce the tapping frequency. The key is the carbon supply to the latex producing tissues. With low tapping frequencies, the latex exported at each tapping day is higher than in traditional systems. Then the trees must mobilize huge amount of carbon at each tapping. Does the latex carbon come directly from the primary sources, the leaves where C is assimilated through photosynthesis, or from reserve pools as wood starch, or both? Knowing the actual C sources and knowing the pathways towards latex is then necessary to manage the tapping systems.

Stable isotopes and especially 13C are widely used in plant science as tracers but studying their natural abundance also provides insightful information on tree physiology.
We first compared the seasonal variations in natural abundance in tapped and untapped latex of RRIM600 trees to the variations in their leaves which vary according to climate and phenology. We showed that latex δ13C was higher and varied much less than that of leaves in tapped trees. The lack of correlation between variations in δ13C in leaves and in latex suggests that recent photosynthates are mixed in a large pool of stored carbohydrates that are involved in latex regeneration after tapping.

We then did a field labeling of full crowns of 3y-old tapped rubber trees with 13CO2 to trace the carbon from its assimilation in the leaves to the tree sinks and particularly to latex. Such experiment, using a specifically designed chamber, has never been done on rubber trees. Three trees (RRIT 408) were labelled in June and three other in October. We sampled leaves, phloem, wood and latex to analyze their 13C content and determine the dynamics of carbon allocation from leaves to reserves and latex. The latex samples will be followed during one year. The presentation describes the methodology and preliminary results showing that the labelling operation was successful.

1. Introduction

Carbon allocation and rubber productivity

One of the main challenges for the future of Natural Rubber production is the scarcity of skilled manpower to tap the rubber trees. In Thailand a consensus is emerging that the current labour intensive tapping systems have no future. There will simply not be enough tappers in a near future and their cost is going to increase. Whatever the possible genetic gain in the next coming generation of clones, the only way to cope with such increase in labour cost and scarcity is to reduce the tapping frequency (Gohet and Lacote2015).
Moving from the current 3d/4, 2d/3 or d/2 to less intensive systems like d/3 (tapping 1 day, 2 day rest), d/4, d/5 or even d/7 will be the only solution to significantly raise the production per tapping day and then the labour productivity (Gohet and Lacote. 2015). This will provide higher wages for the workers and higher return for the owners. It will also lower significantly the number of necessary tappers, as each worker will tap much larger areas.
It is well known that high latex yield per tree and per surface area is possible with low tapping frequency systems compensated by appropriate ethylene stimulation (d'Auzac *et al*. 1997). However, their design and management requires appropriate knowledge of the underlying physiological processes.

The key is the carbon supply to the latex producing tissues. 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 (d'Auzac *et al*. 1997). There is a direct competition for carbohydrate assimilates 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…).

With low tapping frequencies, the latex exported at each tapping day is higher thant in traditional systems. Then the trees must mobilize huge amount of carbon at each tapping, even if the total over one year will be the same. The capacity to attract rapidly such amount (sink strength), the uploading capacity and the availability of the resource in the vicinity of the tapping cut are then the determining factors of yield. 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 1996, Silpi *et al*. 2006). But its physiological bases remain poorly understood.

The question that arise immediately is "Where does the latex carbon come from?". Does it come directly from the primary sources, the leaves where C is assimilated through photosynthesis? Or from reserve pools as wood starch? Or both? If higher amount are necessary in a short time with low tapping frequency, will this amount be available?
Knowing the actual C sources and knowing the pathways towards latex is then required. We need to measure carbon fluxes within the tree.

Recent works in Thailand have shown that tapping affects both growth, latex cell metabolism and activates reserve (starch) synthesis too (Silpi *et al.* 2006, 2007, Chantuma *et al.* 2009). Thus high starch accumulation ability could result in a long term latex yield, because the tree will be in a better balanced condition.

Stable isotopes and plant physiology.

Stable isotopes and especially ^{13}C are widely used in plant science as tracers (Dawson *et al*. 2002; Cerling *et al*. 2007). 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 approach is used to calculate transfer velocity and the proportion of recently assimilated C in the biomass synthesized after labelling.

This method have long been restricted to the lab 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). The CATS project represented a significant step forward, as it developed a methodology that proved appropriate for in situ pulse labelling of 10-m-tall trees with a large crown labelling chamber. Labelling was done at different dates during the growing season on three tree species exhibiting contrasted phenology (Epron *et al*. 2011). Analyses by isotopic ratio mass spectrometry and isotopic ratio infrared spectrometers were performed to trace ^{13}C in respiratory efflux, in the different compartments of the tree (leaf, root, branch and stem) and their different metabolic components (soluble compounds, starch, proteins, structural compounds, respired CO_2).

Moreover, even without labelling, the measure of natural abundance of ^{13}C in the different organs, tissues or metabolites can also provide information on C assimilation and use. This is because every biophysical process creates fractionation, i.e. a change in the proportion of ^{13}C to ^{12}C (namely isotopic composition, $\delta^{13}C$) from the sources to the products. For example the ^{13}C content of leaf soluble sugars (product of the photosynthesis) is different from that of the atmosphere (source). The carbon isotopic composition of latex could be compared to that of photosynthates and of reserves (starch). The seasonal dynamics of such indicators could give indication on the main source of latex carbon.

Objectives

The aim of the study is to improve our understanding of the metabolism of latex in rubber tree (*Hevea brasiliensis*) as related to tapping system. There is a need to identify which carbon source (stored carbohydrates versus recent photosynthate) is involved in latex biosynthesis, what therefore determines the carbon availability for latex synthesis and how a shift between these two sources occurs depending on climate conditions, tree phenology and tapping intensity. We present herein results from a first study on the dynamics of natural isotopic composition and preliminary results from an ongoing field labeling experiment.

2. Material and Methods

1- ^{13}C natural abundance in rubber trees.

In a first study we examined both daily and seasonal variations of carbon isotope composition of the trunk latex (δ13C-L), leaf soluble compounds (δ13C-S) and bulk leaf material (δ13C-B) in twenty-year-old tapped and untapped RRIM 600 trees tapped in S/2 d/2. The specific methods were described in Kampanon et al. 2015. Briefly, trunk latex was sampled in May 2013, just after the beginning of tapping season, in August 2013, in November 2013 and in April 2014, before the beginning of the new tapping season. At each season, samples were collected 21 times over five-day periods. Each sampling period encompassed one tapping day,

except in April 2014 where the tapping season had not started yet. Four sun leaves (eight leaflets) were sampled in the upper part of the crown by climbing each tree twice a day at 7:00 and 15:00 over of five-day periods. Twenty µ l of the latex solution was directly transferred in a 6 × 4 mm tin capsule and oven-dried over night at 50°C. Leaf samples were grinded to fine powder and one mg was weighed in a tin capsule for bulk leaf 13C analysis. The leaf soluble compounds (including amino acids, organic acids, soluble sugars) was extracted in methanol/chloroform/water mixture.

The $^{13}CO_2/^{12}CO_2$ ratio of all samples (RS) were determined using an elemental analyser coupled to a continuous flow isotope ratio mass spectrometer (vario ISOTOPE cube coupled to the IsoPrime100, IsoPrime Ltd, Cheadle, UK), using an internal working standard that was related to the international Vienna Pee Dee Belemnite reference (VPDB). The carbon isotope composition (δ13C) was expressed relative to this reference:

$$\delta^{13}C = \frac{R_S - R_{VPDB}}{R_{VPDB}}$$

Following this first study, we are currently conducting a similar one but with younger trees (8 y-old) at opening. The latex sampling schedule is tighter, every two weeks over one year (May 2016-April 2017), to better catch seasonal patterns linked to climate, phenology and tapping. Moreover, we will compare $\delta^{13}C$ in latex to $\delta^{13}C$ of leaf soluble sugars (marker of the current photosynthetic activity) and to that of carbohydrate stored in the trunk wood and bark (starch and soluble sugars).

2- Labelling rubber trees with $^{13}CO_2$

The study takes currently place in the Chachoengsao Rubber Research Station (RAOT, western Thailand). Rubber trees (*Hevea brasiliensis*), clone RRIT 408 are tapped in S/2 D/2, creating a diversion of carbohydrate resource towards the regeneration of the exported latex. As the size of fully mature trees would make the labelling process tremendously difficult, we chose 4 y-old trees, with an average trunk girth of 20 cm at 1 m from ground and a mean height of 6 m. Although such trees were much below the standard girth for tapping (50 cm at 1 m) and younger than the average opening age in CRRC (7 y-old), we expected that they would produce significant amount of latex.

In-situ labelling with $^{13}CO_2$ was done on 3 trees at two dates: i) one month after opening (June 2016, rainy season). At that period, the tree foliage was well developed and active and the girth increment was maximal (Silpi *et al*. 2006). This was about 3 months after the beginning of the rainy season; ii) during high latex production (October 2016, rainy season). Labelling was done few hours after tapping between 8 and 11 a.m. Short term pulse of almost pure $^{13}CO_2$ (99% CIL, Andover, USA) was carried out. We adapted a labelling system according to Plain *et al*. (2009). The large crown closed chamber (about 30 m3) was made of transparent polyethylene film. The film was installed around each tree immediately before labelling by fitting the chamber on a frame and a platform attached to scaffolds (fig 1). The chamber was tight to the frame by clips and around the trunk by tape. An air conditioning system was used to maintain inside-chamber air temperature near the outside air temperature, and to avoid water condensation inside chamber. Air temperature and air relative humidity along with PAR were recorded inside and outside the chamber during the labelling period. The CO_2 concentration in the chamber was monitored using IRGA (Licor 820). The amount of injected $^{13}CO_2$ was controlled and monitored by a volumetric flow meter. To calculate the flow rate and injection duration, we first measured the CO_2 assimilation rate in natural atmosphere within the closed chamber during 5 minutes. We then regulated the $^{13}CO_2$ flow

rate just above the assimilation rate and adjusted the injection duration to reach 33g of $^{13}CO_2$ per tree. Fast mixing was ensured by two air blowers and 2 fans. The injection duration ranged 40-60 minutes in June. I was followed by a 30 min assimilation time. We then open the chamber and immediately started post-labelling sampling.

Figure 1. Field $^{13}CO_2$Labelling chamber installed on a 6m high, 4 y-old rubber tree clone RRIT 408 at CRRC, Thailand. The chamber is made o PE sheet over a frame and is about 35 m^3. The inside temperature is regulated by an air conditioner.

• Sampling

For each tree, we will determine i) the quantity and ^{13}C composition of starch and soluble sugars in the trunk wood (xylem) and in the phloem; ii) the quantity and ^{13}C composition of bulk latex, dry rubber, serum and soluble sugars in serum.

- Leaf, wood and bark were collected just before labelling to get the base isotopic content of each tissue (D-1). 12 leaves were collected immediately after removing the chamber for D0 (maximum 13C concentration) and from D1 to D4. Bark was collected at 1.7 m with a chisel every day during 3 days then at D6 after labelling. Inner bark samples (about 2cm²) were put in distilled water to collect phloem extract according to Dannoura *et al*. (2011). Wood was collected at 1.7 m with a core borer (3 cm long) at D-1 and then twice a year to analyse non structural carbohydrates (starch and soluble sugars) according to Silpi et al (2007) and the carbon isotopic composition of these carbohydrates according to Plain *et al*.(2009).
- Latex was sampled by micro-punctures with a decreasing frequency from every day during the first 3 days after labelling to every month during one year. Drops of latex were collected after puncturing the bark with a needle (about 2 mm thick) just below the tapping cut. The first 2 drops were discarded. 2 drops were collected in 1 ml of distilled water for bulk latex ^{13}C analyses according to Kampanon et al. (2015). 10 other drops were collected in 0.6 N of H2SO4 to coagulate the latex and separate dry rubber from serum. H2SO4 wes used instead of the usual TCA to avoid adding organic acid that would change the carbon isotopic content. The coagulate was washed under distilled water and dried in oven before an aliquote was put in tin capsule for isotopic analyses. The solution was either vacuum-evaporated or kept liquid.

3. Results and discussion

13C natural abundance in latex of tapped and untapped trees

The results of the first study of seasonal variations have been published in Kanpanon et al (2015) and are summarized herein. The second study is undergoing.

Fig 2 shows that latex $\delta^{13}C$ of tapped trees was clearly different from that of untapped ones and that the later was stable during the year. This re-enforced the hypotheses that latex of untapped trees is not renewed. Its $\delta^{13}C$ reflects that of its source at the moment of its synthesis. However latex $\delta^{13}C$ of tapped trees was higher and less variable than that of leaf soluble compounds that reflect the current photosynthetic activity.

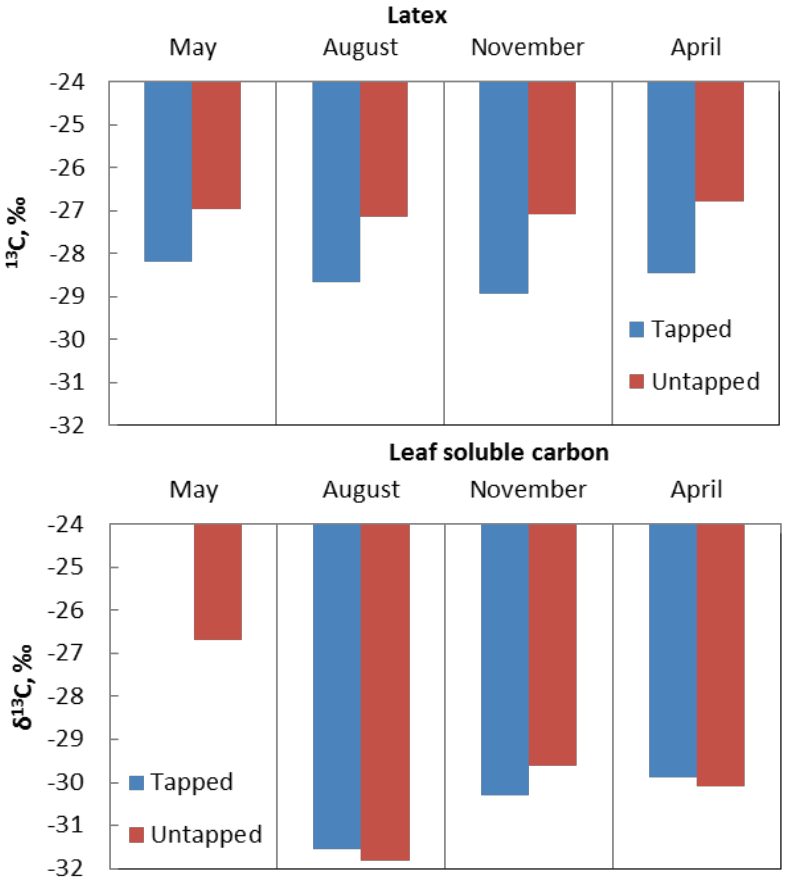

Figure 2. Mean carbon isotopic composition $\delta^{13}C$ (‰) of latex (top) and leaf soluble carbon (bottom) of tapped and untapped Hevea trees clone RRIM 600 sampled in May, August, November 2013 and April 2014. From Kampanon *et al*. 2015.

There was clearly no correlation between isotopic composition of latex and of leaf soluble compounds (Fig 3). This showed that latex carbon does not come directly from recent photosynthesis but from a mixed pool of carbohydrate within the tree. This strongly suggested that reserves (starch) are involved in the supply of sucrose to laticifer vessels to regenerate the latex exported by tapping. Results of the ongoing experiment will tell if this hypothesis is true.

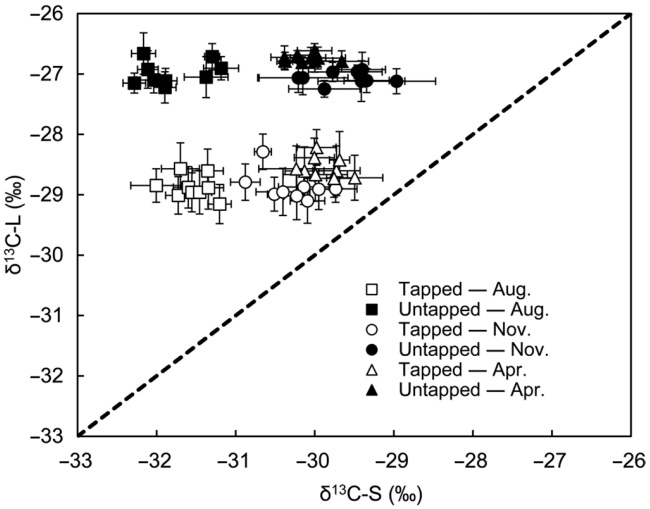

Figure 3 Relationship between carbon isotope composition of latex (δ^{13}C-L) and of leaf soluble compounds (δ^{13}C-S) in August 2013 (squares), November 2013 (circles) and April 2014 (triangles) in tapped (open symbols) and untapped (closed symbols) 20-year-old rubber trees. Each value is the average (with standard error) of samples collected at 7:00 and 15:00 h on five tapped and five untapped trees. The dotted line represents the 1 : 1 relationship. Kampanon *et al. 2015*

Moreover, the δ^{13}C of leaf (bulk and soluble compounds) differed between tapped and untapped trees (data not shown), indicating that tapping affects the leaf metabolism as well.

Preliminary results of the labelling experiment.

We successfully kept chamber temperature and humidity close to external conditions and leaf CO_2 assimilation was steady and within expected range before labelling, indicating limited leaks from the chamber and appropriate climate control (data not shown). We therefore considered that the rubber trees within our chambers were not stressed and behave as in normal atmosphere. Note that we could not properly measure chamber CO_2 concentration during labeling as our IRGA did not detect $^{13}CO_2$, but only $^{12}CO_2$.

Fig 4 shows that the canopy assimilated a high amount of $^{13}CO_2$, as shown by the huge increase in δ^{13}C, from about -26‰ before labelling to about + 350 ‰ at chamber opening. These amounts were lower than that recorded by Plain *et al*. (2009) for beech trees (over 1000 ‰), however they are sufficient to recover ^{13}C in the sink organs and tissue.

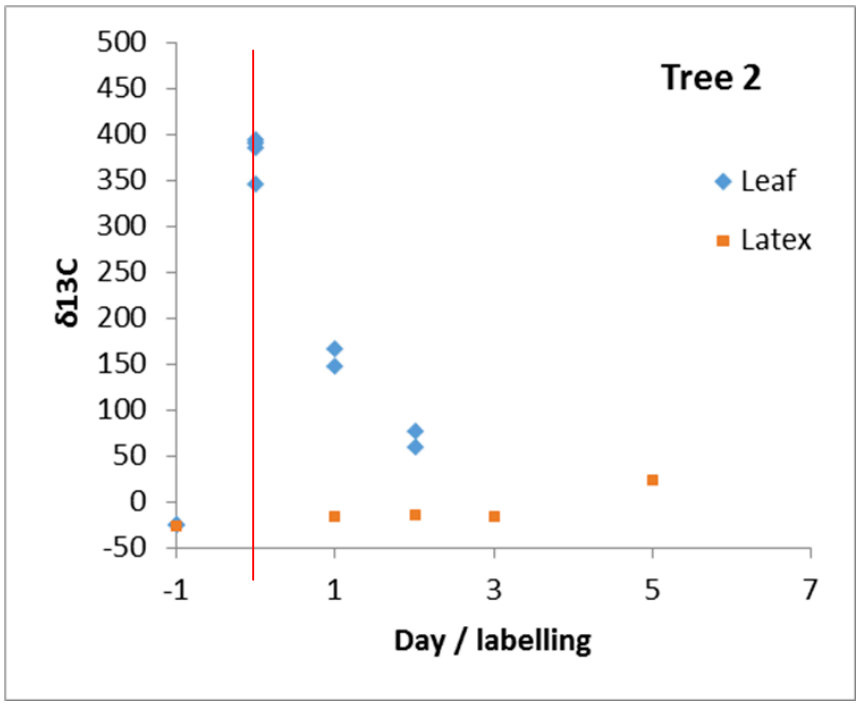

Figure 4. Example of the evolution of the carbon isotopic composition ($\delta^{13}C$ in ‰) in rubber tree bulk leaves and bulk latex after pulse-labelling with $^{13}CO_2$ in June 2016. The vertical bar indicate labelling day (D0).

Moreover we could detect an increase in bulk latex $\delta^{13}C$, from about -26‰ before labelling to about +25‰ 5 days after labelling. Such increase indicated a rapid transfer of the assimilated carbon from the leaves to the latex, then showing a contribution of recent photosynthates to latex metabolism. However, these preliminary results do not tell if such carbon was already incorporated into rubber or just uploaded into latex cytosol. Moreover, we cannot exclude the contribution of phloem sap as the latter is inevitably sampled together with latex itself when bark is severed.

4. Conclusion

The first study comparing ^{13}C natural abundance in leaves and latex of tapped and untapped trees clearly indicated that the latex carbon comes from a pool a mixed carbon, likely involving starch reserves. The latter will be analyzed for $\delta^{13}C$ as well, to determine its actual contribution as a source of carbon for latex regeneration.

The field labelling experiment with $^{13}CO_2$, a world first in rubber tree, was successful as shown by the high $\delta^{13}C$ in leaves just after labelling and the early recovery of ^{13}C in latex.
We are then very confident that these experiments are opening tremendous perspectives for natural rubber research, not only for ecophysiology and tapping systems but for rubber to better understand NR biosynthesis and NR structure.

Outcomes / Perspectives for development

- Knowledge on the origin of the carbon of latex, current photosynthetis versus reserves. This will allow to better manage tapping calendar and to **forecast the yield** along the year according to weather.
- Determination of the fraction of the tree carbon that is available for rubber biosynthesis. This will give the **yield potential** of the trees, and particularly show which kind of clones can mobilize high resources at each tapping time and then be **suitable for low tapping frequency**. This is a key for the future of Rubber in thailand (see introduction).
- Link between tapping systems and carbon mobilization for latex regenaration. This will allow **long term diagnosis** of tapping systems and clone yield potential, in addition to latex diagnosis that give a short term information.

5. References

Chantuma P, Lacointe A., Kasemsap P., Thanisawanyangkura S., Gohet E., Clément A., Guilliot A., Améglio T. and Thaler P. (2009). Carbohydrate storage in wood and bark of rubber trees submitted to different level of C demand induced by latex tapping. Tree Physiology 29, 1021–1031

Dannoura M, Maillard P, Fresneau C, Plain C, Berveiller D, Gerant D, Chipeaux C, Bosc A, Ngao J, Damesin C, Loustau D and Epron D (2011). In situ assessment of the velocity of carbon transfer by tracing 13C in trunk CO2 efflux after pulse labelling: variations among tree species and seasons. New Phytologist 190, 181-192.

d'Auzac J, Jacob J-L, Prévôt J-C, Clément A, Gallois R, Crestin H, Lacote R, Pujade-Renaud V and Gohet E (1997). The regulation of cis-polyisoprene production (natural rubber) from Hevea brasiliensis. Recent research developments in plant physiology 1, 273-332.

Dawson T.E, Mambelli S, Plamboeck A H, Templer P.H and Tu K.P (2002). Stable isotopes in plant ecology. Annual Review of Ecology and Systematics 33, 507-559.

Cerling T.E, Ayliffe L;K, Dearing M.D, Ehleringer J.R, Passey B.H, Podlesak D.W, Torregrossa A.M and West A.G (2007). Determining biological tissue turnover using stable isotopes: the reaction progress variable. Oecologia 151, 175-189.

Epron D, Ngao J, Dannoura M, Bakker M R, Zeller B, Bazot S, Bosc A, Plain C, Lata J C, Priault P, Barthes L and Loustau D (2011). Seasonal variations of belowground carbon transfer assessed by in situ 13CO2 pulse labelling of trees. Biogeosciences 8, 1153-1168.

Gohet E. and Lacote R. (2015). Basics of rubber tree physiology and potential yield of clones. How to optimize land and labor productivity? Kasetsart University, May 8th 2015

Kanpanon N., Kasemsap P., Thaler P., Kositsup B., Gay F., Lacote R., Epron D. (2015). Carbon isotope composition of latex does not reflect temporal variations of photosynthetic carbon isotope discrimination in rubber trees (Hevea brasiliensis). Tree Physiology 35: 1166-1175. DOI: 10.1093/treephys/tpv070

Plain C, Gerant D, Maillard P, Dannoura M, Dong Y W, Zeller B, Priault P, Parent F and Epron D (2009). Tracing of recently assimilated carbon in respiration at high temporal resolution in the field with a tuneable diode laser absorption spectrometer after in situ 13CO2 pulse labelling of 20-year-old beech trees. Tree Physiology 29, 1433-1445.

Silpi U., Thaler P., Kasemsap P. Lacointe A., Chantuma A., Adam B., Gohet E., Thanisawanyangkura S., and Améglio T. (2006). Effect of tapping activity on the dynamics of radial growth of Hevea brasiliensis trees. Tree Physiology 26, 1579-1587.

Silpi U., Lacointe A., Kasemsap P., Thanisawanyangkura S., Chantuma P., Gohet E., Musigamart N., Clément A., Améglio T. and Thaler P. (2007). Carbohydrate reserves as a competing sink: evidence from tapping rubber trees. Tree Physiology 27, 881-889.