Rubber trees must mobilize a huge amount of carbon to regenerate the latex after tapping. Knowing the C sources and pathways towards latex will help managing tapping systems. We labelled 4y-old tapped rubber trees with $^{13}$CO$_2$ and analyzed $^{13}$C content in leaves, phloem, wood and latex during one year to determine the dynamics of C allocation. The peak of $^{13}$C in latex 10-15 days after labelling in June indicated that newly assimilated C was mixed in a pool of reserves before being used to regenerate latex. The earlier (6-8 days) and much higher peak in October showed that when the regeneration metabolism was well established the transfer of recent assimilates was faster. In both cases $^{13}$C was recovered more than 40 days after labelling, demonstrating the contribution of reserves. $^{13}$C recovery in soluble sugars and quebrachitol, an important osmoticum, are followed-up to specify their dynamics.

Keywords: carbon allocation, carbohydrates, latex regeneration, reserves, stable isotopes.

INTRODUCTION

Carbon Allocation and Rubber Productivity

One of the main challenges for the future of Natural Rubber production is the scarcity of skilled labor to tap the rubber trees. Whatever the possible genetic gain in the next coming generation of clones, the only way to cope with such increase in labor cost and scarcity is to reduce the tapping frequency (Gohet and Lacote 2015).
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). With low tapping frequencies, the latex exported at each tapping day is higher than in traditional systems. Then the trees must mobilize a 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.

The question that arises 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 amounts 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.

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 $^{13}\text{C}$ 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 $^{13}\text{C}$ allows tracking photo-assimilated $^{13}\text{C}$ 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 has 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). Appropriate methodologies have been developed for in situ pulse labelling of 10-m-tall trees with a large crown labelling chamber and adapted to tropical conditions (Epron et al., 2016). 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). In rubber, our team showed that the seasonal dynamics of carbon isotopic composition of latex were not related to that of leaf soluble sugars. This showed that C latex does not come from direct photosynthates but rather from a pool where recent C is mixed with older one (Kanpanon et al., 2016). Such pool is likely made of starch reserves.

**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 an ongoing field labelling experiment.
MATERIAL AND METHOD

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 d2, 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 (Thaler et al., 2016) a labelling system according to Plain et al., (2009). The large crown closed chamber (about 35 m$^3$) 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. It was followed by a 20-30 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 was made of PE sheet over a frame and was about 35 m$^3$. The inside temperature was regulated by an air conditioner.

**Sampling**

For each tree, we determined 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, C-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 $^{13}$C 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 2cm$^2$) 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 Desalme et al., (2017).

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 H$_2$SO$_4$ to coagulate the latex and separate dry rubber from C-serum. H$_2$SO$_4$ was 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.

The $^{13}\text{CO}_2 / ^{12}\text{CO}_2$ ratio of all samples ($R_S$) 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 ($\delta^{13}\text{C}$) was expressed relative to this reference:

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

RESULTS AND DISCUSSION

As shown in Thaler et al., (2016), we successfully kept chamber temperature and humidity close to external conditions. Leaf CO$_2$ assimilation was steady and within expected range before labelling, indicating limited leaks from the chamber and appropriate climate control. We therefore considered that the rubber trees within our chambers were not stressed and behave as in normal atmosphere.

$^{13}\text{C}$ in Leaves and Phloem

The experiment is still going on, as sampling will end in October 2018. Not all the harvested samples have been analyzed yet. However, the first results indicated that the labelling procedure was successful with a significant increase in $^{13}\text{C}$ in the leaves just after opening the chamber. In June, $\delta^{13}\text{C}$ was up to 440 and 1730 ‰ in bulk leaves and leaf soluble sugar, respectively. In October, it was up 305 and 1365 ‰ in the same samples, as compared to -26‰ before labelling (Fig 2). This was lower than the results of Plain et al., (2009) with beech, or Epron et al., (2016) with tropical eucalypt, but enough to track $^{13}\text{C}$ in the trees. The $\delta^{13}\text{C}$ decreased rapidly in leaves, showing a fast transfer of recently assimilated photosynthates from these matures leaves. This was confirmed by the dynamics of $\delta^{13}\text{C}$ in trunk phloem (Fig 2), showing a peak one day after labelling and a rapid decrease thereafter.
Figure 2. δ^{13}C in bulk leaves (leaves C), leaf soluble compounds (leaves PF), phloem sap (phloem C) and bulk latex (latex C). Trees 1 to 3 were labelled in June (beginning of tapping season), trees 4 to 6 were labelled in October (full tapping season). The vertical dotted bar indicates the labelling day (D0).

The kinetics of $^{13}$C label recovered in total organic matter in leaves (Fig 3) showed a mean residence time of 20.0 h in June and 28.8h in October. The export rate in June was then faster. Kinetics of $^{13}$C in bulk phloem and soluble sugars were similar in June and October (not showed), with a mean residence time of 39-45 hours, similar to that reported in temperate tree species (Dannoura et al., 2011).
Tracking carbon from photosynthesis to latex with $^{13}$C field labelling experiment

Figure 3. Kinetics of $^{13}$C recovered in leaf total organic matter. Mean residence time (MRT) of $^{13}$C was calculated with a 2-compartment kinetic model in June (trees 1-3) and in October (trees 4-6).

$^{13}$C in Latex

Although $^{13}$C started to be recovered in latex (both bulk latex and dry rubber fraction) 1 to 2 days after labelling, the peak occurred later, 10 to 15 days after labelling in June and 5 to 10 days in October (Fig 4). The $\delta^{13}$C was higher in October (200-400‰) than in June (50-100‰). Following this peak, the $^{13}$C content decreased slowly and significant labelling was still found more than 40 days after labelling.

Figure 4. $\delta^{13}$C in bulk latex, dry rubber and C-serum from D-1 to D40. Trees 1 to 3 were labelled in June (beginning of tapping season), trees 4 to 6 were labelled in October (full tapping season). The vertical dotted bar indicates the labelling day (D0).
These results are consistent with the hypothesis that most latex carbon does not come directly from recent photosynthesis, but from a pool where recent carbon is mixed with older one (Kampanon et al., 2016). This was particularly true when labelling was done shortly after tree opening, when latex yield was low (June, 2 g/tree/tapping). In October, 6 months after opening, the latex yield per tree was much higher (12 g/tree/tapping) and the recovery of $^{13}$C was faster and stronger, indicating a higher contribution of recent photosynthates or a faster mixing in the ‘common pool’ once the latex regeneration metabolism was well established. However, as the trees in the experiment started to be tapped at only 4 y-old, much before reaching the standard size (20 cm girth at 1 m high, instead of 50 cm), their reserve may have been low and rapidly depleted, explaining such a change between June and October. The fact that $^{13}$C was still found more than 40 days after labelling confirmed that a part of the assimilated carbon was stored before being used for latex regeneration. Rubber stores large amounts of starch in trunk wood parenchyma (Silpi et al., 2007, Chantuma et al., 2009). Therefore, the ‘common pool’ is expected to be constituted mainly of such starch reserves. This was partially confirmed by the $^{13}$C dynamics in wood soluble sugars and starch (Table 1). $\delta^{13}$C in wood soluble sugars reached 74-129‰ 6 days after labelling and was close to zero (then still above initial value) 45 days after labelling. Labelling of starch was lower and with a more irregular pattern. However, it was not possible to sample wood often enough to reflect the real dynamics of $^{13}$C in reserves, as repeated coring of the trunk of such small trees would damage them too much.

Table 1. $\delta^{13}$C (‰) in wood polar fraction (soluble sugars, amino and organic acids) and starch following June labelling.

<table>
<thead>
<tr>
<th>Days/labelling</th>
<th>Polar fraction</th>
<th>Starch</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D-1</td>
<td>D6</td>
</tr>
<tr>
<td>Tree 1</td>
<td>-25.0</td>
<td>91.9</td>
</tr>
<tr>
<td>Tree 2</td>
<td>-25.0</td>
<td>128.7</td>
</tr>
<tr>
<td>Tree 3</td>
<td>-25.0</td>
<td>73.8</td>
</tr>
</tbody>
</table>

To go further in understanding carbohydrates dynamics, we analyzed the $^{13}$C in bulk latex, the C-serum and the dry rubber fraction made mainly of cis-polyisoprene (Fig 4). Preliminary results showed that
the dynamics of bulk latex and dry rubber were very close, confirming
that most the latex carbon was rapidly incorporated into cis-polyisoprene.
The dynamics of $^{13}$C in C-serum in June was very similar to that in
phloem. This could indicate a fast transfer of recent carbon into latex
vessels. However, it was actually impossible at this scale to sample C-
serum without phloem sap and conversely, due to the imbrication of latex
and phloem vessels within the phloem tissue. Other methods are required
to address the specific issue of sucrose transport from phloem into latex
vessels.

Quebrachitol is a cyclitol found in higher concentration than every
sugar in latex serum (Bealing 1969). The role of quebrachitol is not really
known, but it is believed to be important in osmotic balance and water
influx following tapping and ethylene stimulation (Dusotoit-Coucaud et
al., 2010). Interestingly, we recovered very little $^{13}$C in quebrachitol in C-
serum, as compared to soluble sugars, with different kinetics in June and
in October (Fig 5). Analysis of the dynamics of quebrachitol
concentration in our sample are undergoing to better understand such
results.

Figure 5. $\delta^{13}$C in bulk C-serum, glucose, fructose and quebrachitol from C-serum.
Sucrose was hydrolyzed into glucose and fructose by H$_2$SO$_4$ used to
cogulate the latex. Trees 1 to 3 were labelled in June (beginning of tapping
season), trees 4 to 6 were labelled in October (full taping season).

**CONCLUSION**

This work was the first successful field labelling of the full canopy
of tapped rubber trees with $^{13}$CO$_2$. The methodology was proved efficient
to study the allocation of carbon from its uptake by photosynthesis in the
leaves to its incorporation into latex and rubber. Although the study is still going on, preliminary results showed interesting trends.

The kinetics of $^{13}$C recovery in latex were consistent with the hypothesis that most latex carbon comes from a pool of reserves where recently assimilated carbon is mixed with older ones. This reinforces the importance of following reserves (starch) dynamics.

Once sucrose was uploaded into latex vessels, its carbon seemed to be rapidly integrated into rubber. The fact that quebrachitol was not much labelled raises questions regarding the origin of this soluble compounds believed to play an important role in latex osmotic balance.

In situ labelling with stable isotopes appears a very promising methodology to unravel the effect of tapping on carbohydrate allocation and to understand and forecast the factors determining latex yield on the long term. This is particularly relevant in the context of labor scarcity necessitating low-frequency tapping systems.

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**REFERENCE**


