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Nicha Kanpanon

LA COMPOSITION ISOTOPIQUE EN CARBONE EST-ELLE UN INDICATEUR ECOPHYSIOLOGIQUE PERTINENT DE L'EFFICIENCE D'UTILISATION DE L'EAU DE L'HEVEA?

IS CARBON ISOTOPE COMPOSITION A RELEVANT ECOPHYSIOLOGICAL INDICATOR OF GENETIC VARIATION IN WATER USE EFFICIENCY OF RUBBER TREE?

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LIST OF ABBREVIATIONS

A	Net CO_2 assimilation rates (μmol CO_2 $m^{-2}s^{-1}$)
C	Carbon content on a mass basis (%)
C_a	Ambient CO_2 concentration
C_i	Intercellular CO_2 concentration
C:N	Carbon and nitrogen ratio
E	Transpiration rate (mm day^{-1})
ET	Evapotranspiration (mm)
GPP	Gross primary production (kgC m^{-2})
g_m	Mesophyll conductance (mol m^{-2} s^{-1})
g_w	Stomatal conductance to water vapour (mol m^{-2} s^{-1})
IWUE	Inherent water use efficiency (kgC kPa m^{-2} mm^{-1})
J_{max}	Maximal light driven electron flow (μmol $m^{-2}s^{-1}$)
LA	Leaf area (cm^2)
LMA	Leaf mass per area (kg/m^2)
N	Nitrogen content on a mass basis (%)
N_A	Nitrogen content on an area basis
QTL	Quantitative trait locus
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
SWP	Soil water was still available (kPa)
V_{cmax}	Maximal carboxylation rate (μmol CO_2 m^{-2} s^{-1})
VPD	Daylight vapour pressure deficit (kPa)
VPDB	Vienna Pee Dee Belemnite reference
VPD_L	Leaf-to-air vapour pressure deficit (kPa)
WUE_i	Intrinsic water use efficiency (μmolC/molH_2O)
$\Delta^{13}C$	Carbon isotope discrimination (‰)
$\delta^{13}C$-B	Carbon isotope composition of bulk leaf material (‰)
$\delta^{13}C$-L	Carbon isotope composition of latex (‰)
$\delta^{13}C$-S	Carbon isotope composition of leaf soluble compounds (‰)
δ_A	Carbon isotope composition of CO_2 in the atmosphere (‰)
δ_P	Carbon isotope composition of the plant organic matter (‰)

IS CARBON ISOTOPE COMPOSITION A RELEVANT ECOPHYSIOLOGICAL INDICATOR OF GENETIC VARIATION IN WATER USE EFFICIENCY OF RUBBER TREE?

I. INTRODUCTION

The rubber tree (*Hevea brasiliensis*) is a major tree crop in Southeast Asia. Globally, it covers 9.82 million hectares mainly exploited by smallholders (UNCTAD, 2013). Thailand is the world leader of exportation of rubber. Rubber trees grow in tropical area. The optimal conditions are met in southern Thailand (between 1500 and 2000 mm of annual rainfall and about 3 month of dry season (Rao and Vijayakumar, 1992). However, plantations are now expanding throughout the country. The rubber plantations in non-traditional areas in the Northeast, North, East and West may be vulnerable to drought. Drought stress has been reported to impair the growth of rubber trees (Chandrashekar *et al*., 1998; Manmuen *et al*., 1993) and latex production (Wichichonchai and Manmeun, 1992). For example, in the Northeast of Thailand, the dry season lasts for approximately six months and the annual rainfall is less than optimal for rubber. Mitigating the problem by installing an irrigation system is only possible in limited areas, so the selection and breeding of rubber trees that use water more efficiently in these non-traditional areas is one option that is of paramount importance. There are genetic variations related to physiological characters that may be used in breeding program for a more efficient use of water by rubber plantations.

Among these physiological characters, water use efficiency at leaf level refers to the net amount of carbon assimilated by photosynthesis (A) per unit of water transpired (transpiration rate, E) during the same period (WUE) (Farquhar *et al*., 1989). The intrinsic water-use efficiency (WUE_i) is defined as the ratio between A and stomatal conductance (g_w) which is more closely associated with physiological responses because E depends both on stomatal conductance that is a leaf properties and on vapour pressure deficit, a climate variable. A high water use efficiency (WUE) enables trees to reduce their water consumption and thus to use soil water longer during the dry season. WUE is a complex

character, and carbon isotopic discrimination is a proxy for WUE which is easy to measure and therefore possible to be used in breeding programs.

Good relationships between carbon isotope discrimination and WUE_i were reported for C_3 plants species such as rice, wheat and poplar (Xu *et al*., 2009; Khazaei *et al*., 2009; Monclus *et al*., 2005). Today, carbon isotope discrimination is widely used as a proxy for intrinsic water-use efficiency and the knowledge can be applied in research on selection and breeding plants with higher water use efficiency. (Monclus *et al*., 2006; Roussel *et al*., 2009; Chen *et al*., 2011).

This project aims to study carbon isotope composition ($\delta^{13}C$) in several clones of rubber (*Hevea brasiliensis*) in order to bring knowledge for selection and breeding of water efficient rubber trees. Our main question is "Is carbon isotope composition a relevant ecophysiological indicator of genetic variation in water use efficiency of rubber tree?" and for that, we will specifically try to answer the following questions:

1. Does $\delta^{13}C$ predict well leaf level intrinsic water-use efficiency (WUE_i)?

We will establish the relationship between $\delta^{13}C$ and WUE_i at leaf level in a pot experiment including 10 clones setup in Thailand (Kasetsart University, Bangkok). This will allow us to determine how $\delta^{13}C$ can successfully predict leaf level intrinsic water-use efficiency (WUE_i)

2. Is there genetic variability for $\delta^{13}C$ among genotypes of *Hevea brasiliensis*?

We will study $\delta^{13}C$ in a larger number of clones in a trial where rubber trees from a germplasm collection of *Hevea brasiliensis* are cropped to enlarge the potential genetic variability, and where we are expecting to find a large range of leaf $\delta^{13}C$ among genotypes.

3. Is latex $\delta^{13}C$ related to leaf $\delta^{13}C$ in tapped and untapped trees? Can latex $\delta^{13}C$ be used as a proxy of WUE (spatially integrated and higher temporal resolution)? Is latex $\delta^{13}C$ related to canopy WUE?

We will relate temporal changes at both diurnal and seasonal scales in $\delta^{13}C$ of both leaf soluble carbon and latex in tapped and untapped trees in a site equipped with an eddy flux tower where canopy WUE can be computed, and evaluate the possibility to use latex $\delta^{13}C$ as a proxy of WUE.

II. LITERATURE REVIEW

1. General information on rubber tree

The rubber tree or Para rubber tree, *Hevea brasiliensis* Muell Arg., is the only species commercially planted for natural rubber production. In the wild, it may grow up to over 40 m and live for over 100 years, but in plantations rubber trees rarely exceed 25m height, and they are replanted after 25-35 years when yield falls (Webster and Paardekooper, 1989). It is the most important economic member of the genus *Hevea* and a member of the Euphorbiaceae family.

The rubber tree originates from the Amazon rain forest in South America, where climate is equatorial. Annual rainfall ranges from 1,900 to 2,700 mm per year, with a wet season from November to April and a dry season from June to August in Rondonia (Culf *et al*., 1998, Roberts *et al*., 2002). However, it is now planted mainly in South-East Asia where annual rainfalls range between 1500 and 2500 mm (Rao and Vijayakumar, 1992). The optimal climatic conditions for the rubber tree are 1500 mm or more rainfall and 25-28^{o}C of mean temperature with less than 3 month of dry season (Rao and Vijayakumar, 1992). This is the climate of the traditional planting area in southern Thailand. However, the plantations are nowadays expanding into non-traditional areas such as the north-eastern Thailand despite relatively low annual rainfall (below 1400 mm according to the Thai Meteorological Department) and a long dry season that may be adverse for growth and rubber yield (Rao *et al*., 1998).

Rubber tree plantations are made of clonal material propagated by bud grafting. Actually, only the shoot is clonal, as the rootstock is made of unselected seedlings. The main clone planted at smallholdings in Thailand, including the Northeast, is RRIM 600 (Rubber Research Institute Malaysia, clone number 600). Priyadarshan *et al*. (2005) studied the potential yield of several rubber clones in marginal environments suffering from severe winds, low temperatures and high evaporation in north-eastern India. Clone RRIM600 appeared to be able to adapt well to various conditions, and produced moderate yield in all marginal environments mentioned. This clone is then considered the reference to select new clones with higher yield potential and that can adapt to water limited conditions.

2. Latex

The commercial product, natural rubber, is a polymer, the cis-polyisoprene, made mainly of very long chains of isoprene. It is obtained from the latex of rubber trees. Latex is actually the cytoplasm of specialized cells organized in anastomosed vessels localized in the inner bark of the tree where the rubber formation takes place (Hébant and De Faÿ, 1980). Latex is collected by regularly tapping the bark of trees, severing the vessels and allowing latex to flow out for several hours until coagulation plugs the cut vessels. It is not directly linked to biomass accumulation contrary to most other crops (fruit, wood, leaf etc.). As dry rubber is made of 88% of carbon and as its synthesis requires energy, its production depends on the availability of carbohydrates in the tissues engaged in latex synthesis (Tupý, 1988). Sucrose content of the latex is one of the main parameters used to assess the physiological status of tapped trees and adapt tapping systems ('Latex diagnosis', Jacob *et al*., 1995, 1998). Latex biosynthesis will be described in a more detailed way in the third experiment (Chapter V).

3. What are the bases of rubber plantation productivity?

The productivity per surface area will depend first on the density of trees that can be tapped. Then it will vary with clone yield potential and tapping practices. The density of tappable trees will first depend on the survival rate of the young rubber trees. Secondly, although latex yield is not directly related to the size of the trees, rubber trees can be tapped only when their trunk is big enough. In Thailand the standard is an average trunk girth of 50 cm at 1.5 meters height above ground (Chardrashekar *et al*., 1998). This maturity is usually achieved around 5-6 years after planting in traditional conditions and around 8-10 years in sub-optimal conditions (Chardrashekar *et al*., 1998). Then, surviving capacity, mainly during the first years after planting, and fast growth are important parameters for farmers. If growth is slow, farmers have to wait a long time without income from the plantation. Adverse climate conditions encountered in sub-optimal areas such as northern and northeastern Thailand are likely to affect tree survival and growth (Wichichonchai and Manmeun, 1992).

Latex flow following tapping is affected by climate. Several meteorological parameters have then been related to yield: temperature, sunshine duration, evaporation and vapor pressure deficit are negatively correlated, whereas only rainfall is positively

correlated (Sanjeeva Rao *et al*., 1998). Accordingly, several researchers reported that rubber yield decreases in the dry season and in dry areas. (Chandrashekar *et al*., 1990; Gururaja Rao *et al*., 1990)

As rubber tree growing is extending to new areas with dryer climate area such as in northern and northeastern Thailand, latex yield may be impaired (Wichichonchai and Manmeun, 1992).

Finally, although rubber is a secondary metabolite which does not directly depend on biomass accumulation, the productivity of the plantations depends on the efficiency of carbohydrate supply. This is particularly true with low frequency tapping systems which require the tree to regenerate huge amounts of latex at each tapping time (Jacob *et al.*, 1989). Under limiting water conditions, the interactions between water use and photosynthesis may then be of first importance.

4. Photosynthesis in rubber trees

There were many studies about photosynthesis in rubber trees. The light response curves showed that there are genetic differences in maximum net photosynthetic rate (Ceulemans *et al*., 1984). Net photosynthetic rate also increased with increase in CO_2 concentration (Devakumar *et al*., 1998). The net photosynthetic rate among clones related to stomatal conductance (Nataraja and Jacob, 1999) and it was positively correlated to girth and yield after fifth year of tapping (Samsuddin *et al*., 1987). The maximum photosynthesis of tapped trees was higher than that of untapped trees. Tapping can increase leaf and canopy photosynthesis as the demand for assimilates from latex-producing tissue increases when latex is removed through tapping. Thus increased sink demand stimulates assimilate synthesis through photosynthesis (Gunasekara *et al*., 2007)

Samsuddin and Impens (1979) found that the photosynthetic rate depends not only on environmental variation but also on leaf age. The decrease in photosynthetic rate in aging leaves was associated with a decrease in stomatal conductance. Kositsup *et al*. (2010) found that not only temperature, but also age-related effect and position within the canopy need to be taken into account to assess photosynthesis in rubber trees. They also confirmed the strong impact of stomatal conductance on seasonal changes in net assimilation rate.

5. How can rubber trees adapt to water constraints?

Climate, and especially high temperature and drought, may affect photosynthesis of rubber tree. Under water stress conditions, very low stomatal conductance and severe inhibition of photosynthesis and transpiration occurred (Krishna *et al*., 1991). Sangsing *et al*. (2004a) found that net CO_2 assimilation rates, stomatal conductance and transpiration rate showed a declining curve as a function of increased water stress. Selection for drought-tolerant clones has become a major challenge in rubber breeding programs to ensure the sustainability of natural rubber production. A key is to better understand the physiological bases of water economy in rubber trees. Trees can adapt to water scarcity by resistance, regulation, avoidance and efficiency mechanisms.

There is relationship between stomatal function and plant hydraulic property (Sperry *et al*., 1993; Cochard *et al*., 2002). Water stress increases the tension gradient of water flow along shoot, inducing xylem cavitation, and resulting in embolism and loss of functional vessels. Then resistance to xylem cavitation is a way to resist to water stress. Jinagool *et al*. (2015) found no clonal variability and a small phenotypic plasticity for xylem vulnerability to cavitation in rubber tree branches. However, clonal differences in xylem vulnerability to cavitation were found in petioles, and clones also showed differences in stomatal response and in leaf shedding behavior in response to drought treatment.

Regarding water loss, Isarangkool Na Ayutthaya *et al*. (2011) demonstrated that hydraulic limitation hypothesis (Jones 1998, Sperry *et al*., 1998) was sufficient to describe water use regulation of a mature rubber tree stand in water-limited conditions. Their results confirmed the relevance of this approach to synthesize the overall behavior of rubber trees under drought. This approach was refined by Sopharat *et al*. (2015) who found that under high VPD a strong regulation of transpiration occurs even when soil water is available.

Several researchers studied the effects of soil and atmosphere drought on water relations of rubber trees. The lower values of predawn xylem water potentials ($\psi_{predawn}$) have been noticed in seasonal drought but no threshold for water stress or transpiration decline was clearly identified (Chandrashekar *et al*., 1990; Gururaja Roa *et al*., 1990).

Kumagai *et al*. (2015) compared rubber trees water relations in northeastern Thailand and Cambodia. In the seasons when actual water balance was negative, the deficit was compensated with soil water from the previous season at depths of 0–2 m (Thailand site) and 0-3 m (Cambodia site). There was a stricter stomatal regulation in Cambodia, where the risk of water induced xylem embolism was higher.

6. Water use efficiency (WUE)

Selection of genotypes which use water more efficiently is one key option in non-traditional planting areas where water resource may be scarce. At leaf level, scientists consider the ratio of photosynthesis rate to transpiration rate, A/E, and the ratio of photosynthesis rate to stomatal conductance of CO_2, A/g_w, often referred as intrinsic water use efficiency, WUE_i (Seibt *et al.*, 2008). At plant level the ratio of biomass increment to transpiration rate can be relevant and at farm level that can be the ratio of yield of a given product to water consumption.

Much of the exchange of water and CO_2 is determined at the leaf level, where stomata co-regulate the influx of CO_2 through photosynthesis and the loss of water to the atmosphere by transpiration. As transpiration is determined by both atmospheric dryness (vapour pressure deficit, VPD) and stomatal characteristics it does not relate directly to photosynthesis. For this reason, WUE_i is defined as the ratio of photosynthesis to stomatal conductance to water, thereby accounting for the effects of VPD on transpiration (Seibt *et al.*, 2008).

$$WUEi = A/g_w \qquad [1]$$

Intrinsic water use efficiency (WUE_i), the leaf-level ratio of photosynthesis to stomatal conductance (an indication of leaf transpiration), has been widely used to screen for heritable genotypic variation in water use characteristics of crops.

Inherent water use efficiency (IWUE) at the ecosystem level can be calculated according to Beer *et al*. (2009) from daytime integrated values of gross primary production (GPP*)* and evapotranspiration (ET), and mean daylight vapour pressure deficit (VPD):

$$IWUE = \frac{GPP}{ET} \times VPD \qquad [2]$$

A high water use efficiency will enable trees to reduce their water consumption and thus to use soil water longer during the dry season. During the long season with high water vapor pressure deficit and low soil water content that characterized the dry tropical climate of north-eastern Thailand, leaf stomatal conductance and net CO_2 assimilation are expected to decrease (Sangsing *et al*., 2004a; Chen *et al*., 2010). As a consequence, intrinsic water use efficiency is thought to increase during the dry season. In rubber trees, this was confirmed by Sangsing *et al*. (2004a) who found that water use efficiency (WUE) was high under severe stress, due to a more rapid decrease of *E* than *A*. Drought also reduced leaf water potential, which was positively related to stomatal conductance.

Finally, the integration of physiological knowledge into breeding programs seems interesting to select rubber trees adapted to dry climatic conditions. However, breeding trees is a long process as parameters such as growth or yield should be assessed on the long term. Breeders are then looking for early selection methodologies based on genetic markers, for example quantitative trait loci (QTL). QTLs were used for growth, initial latex production and related traits in rubber breeding (Rattanawong *et al*., 2009). Researchers are now trying to use QTLs for physiological parameters such as water use efficiency (WUE). However, measurements of large numbers of genotypes are required to establish QTLs (about 200 in Rattanawong *et al*., 2009). For such purpose, measurements of actual WUE can be inconvenient. Researchers must wait a long time if WUE is based on the measurement of biomass, and estimating the water use by a large number of trees is not really feasible. On the other hand, gas exchange measurements are difficult and hardly compatible with large number of samples, particularly in tropical field conditions. Then the use of indicators or proxy of the interesting physiological functions is required. Several researches showed good relationships between carbon isotope discrimination and WUE in C_3 plants species such as rice, wheat, poplar tree (Monclus *et al*., 2005; Khazaei *et al*., 2009; Xu *et al*., 2009) but information is lacking in rubber trees.

7. Carbon isotope composition ($\delta^{13}C$)

Carbon from atmosphere consists of carbon isotopes ^{12}C and ^{13}C, approximately 98.9% and 1.1% respectively. The ratio of ^{13}C to ^{12}C found in plant is less than in air. The amount of ^{13}C is expressed using isotope composition ($\delta^{13}C$, measured in ‰).

$$\delta^{13}C_{sample} = [(R_{sample} / R_{standard}) - 1] \quad [3]$$

The standard is a reference limestone (Pee Dee limestone) at South Carolina State in the United States, and the sample is either the plant (bulk tissues or organic molecules) or the CO_2 in air. $\delta^{13}C$ of C_3 plants averages -27‰ showing however large variations, and $\delta^{13}C$ of air is -8‰. $\delta^{13}C$ are 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

$$\Delta = \frac{\delta_{air} - \delta_{plant}}{1 + \delta_{plant}} \approx \delta_{air} - \delta_{plant} \qquad [4]$$

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), but 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), as:

$$\Delta = 4.4 + 22.6 \frac{C_i}{C_a} \qquad [5]$$

Isotope ratio mass spectrometer (IRMS) allows fast, convenient and accurate measurements of $\delta^{13}C$.

According to the Fick's law for diffusion, leaf CO_2 assimilation rate depends on leaf stomatal conduction for CO_2 and the gradient of CO_2 concentration between air and inside the leaf:

$$A = g_C(C_a - C_i) \qquad [6]$$

and thus WUE_i can be written:

$$WUEi = (C_a/1.6)(1 - C_i/C_a) \qquad [7]$$

Combining equations 5 and 7 shows that the isotope composition ($\delta^{13}C$) of the plant material is linearly and positively related to the intrinsic water use efficiency (WUE_i) of the plant. Fig. 1 shows relationship between $\delta^{13}C$ of leaf and WUE_i in six poplar species.

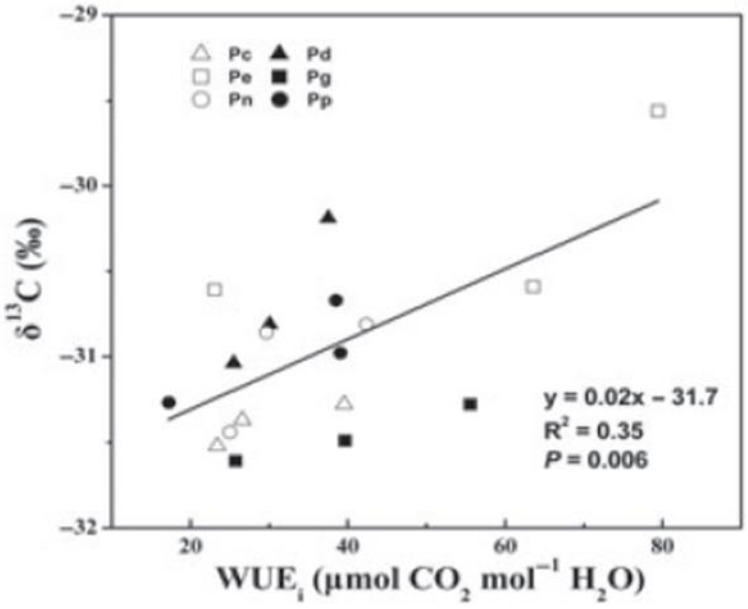


Figure 1 Carbon isotope composition (‰, $\delta^{13}C$) and intrinsic water use efficiency (WUE_i) for *P. cathayana* (Pc), *P. deltoides* (Pd), *P. euramericana* (Pe), *P. alba* x *P. glandulosa* (Pg), *P. nigra* (Pn) and *P. popularis* (Pp) (Cao *et al*., 2011).

The carbon isotope discrimination of plant organic matter is therefore widely used as a proxy for water-use efficiency. It can be used for seeking genotypes exhibiting high water use efficiency for selection and breeding programs (Monclus *et al*., 2005; Chen *et al*., 2011). To our knowledge such approach has never been published in rubber tree.

III. DOES $\delta^{13}C$ PREDICT WELL LEAF LEVEL WATER-USE EFFICIENCY?

1. Abstract

Carbon isotope composition ($\delta^{13}C$) is often considered as an indicator of leaf intrinsic water-use efficiency (WUE_i) which can be assessed on a large number of individuals and thus used in selection program for breeding genotypes with higher water use efficiency. The relationship between $\delta^{13}C$ and WUE_i has been studied in several species, including trees, but it has not yet been studied in rubber trees. Confirming that $\delta^{13}C$ predicts well clonal differences in WUE_i is therefore a prerequisite for implementing this parameter in future breeding programs. Bulk leaf $\delta^{13}C$ and leaf gas exchange were measured on young saplings of 10 rubber clones grown outdoor in pots (Kasetsart University, Bangkok, Thailand). WUE_i was estimated at low and high VPD. The range of $\delta^{13}C$ among 10 clones was narrow, the highest value being -29.9 ‰ in clone RRIT-CH-39-2085 and the lowest -31.5‰, in clone RRIT-CH-36-980. Our data did not show any relationship between bulk leaf $\delta^{13}C$ and WUE_i measured at low VPD but at high VPD, positive but weak correlation between $\delta^{13}C$ and WUE_i was observed. We concluded that on these 10 clones, the prediction of WUE_i by $\delta^{13}C$ would have low precision.

2. Introduction

The rubber plantations now expanding to non-traditional areas in the Northeast, North, East and Westparts of Thailand are vulnerable to dryer conditions prevailing in these areas as compared to traditional ones. Drought stress affects the growth of rubber trees (Chandrashekar *et al*., 1998; Manmuen *et al*., 1993) and the latex production (Wichichonchai and Manmeun, 1992). The water balance of rubber plantations in these new areas can be negative during long dry seasons, threatening the survival of the plantations (Sopharat *et al*., 2015; Kumagai *et al*., 2015). Mitigating the problem by installing an irrigation system is only possible in limited areas, so the selection and breeding of rubber trees that use water more efficiently in these non-traditional areas seems a relevant option. A high water use efficiency (WUE) will enable trees to reduce their water consumption and thus to use soil water longer during the dry season.

WUE represents the balance between photosynthetic carbon gain and water loss by transpiration. At the leaf level, the intrinsic water use efficiency (WUE_i) is defined as the ratio of net photosynthesis to stomatal conductance (Seibt *et al.*, 2008):

$$WUEi = A/g_w \qquad [1]$$

WUE_i is thus a complex and dynamics characters and its estimation from gas exchange measurement is time consuming. As stated in the general introduction, measuring WUE is difficult and hardly compatible with the size of samples necessary for breeding programs or to identify genetic markers. For such reasons, the use of an indicator, or proxy of WUE_i that can be easily measured is required. It as being theoretically demonstrated that the carbon isotope composition ($\delta^{13}C$) of C_3 plants is strongly related to its WUE_i (Farquhar *et al.,* 1982) which is easy to measure on a large number of samples using isotope ratio mass spectrometer (IRMS). $\delta^{13}C$ of the photosynthetic products is lower than $\delta^{13}C$ of CO_2 in the atmosphere (δ_A) because of two major fractionations steps that take place during photosynthesis and imprint $\delta^{13}C$ of the plant organic matter (δ_P). These main fractionations occur during the diffusion of CO_2 into the leaf (4.4‰) and during carboxylation (27.0‰). 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 influence by the ratio between internal CO_2 concentration (C_i) and atmospheric CO_2 concentration (C_a).

$$\Delta^{13}C = \delta_{air} - \delta_{plant} = 4.4 + 22.6\ C_i/C_a \quad [4\text{-}5]$$

According to the Fick's law for diffusion, leaf CO_2 assimilation rate depends on leaf stomatal conduction for CO_2 and the gradient of CO_2 concentration between air and inside the leaf:

$$A = g_C(C_a - C_i) \qquad [6]$$

and thus WUE_i can be written:

$$WUEi = (C_a/1.6)(1 - C_i/C_a) \qquad [7]$$

Combining equations 4-5 and 7 shows that the isotope composition of the plant material is linearly and positively related to the intrinsic water use efficiency of the plants. It was shown that $\delta^{13}C$ predicts well differences in WUE_i in oak (Ponton *et al*.,

2002; Roussel *et al*., 2009), but also clonal difference in terms of biomass production per unit water loss in wheat (Farquhar and Richards 1984; Khazaei *et al*., 2009) and in poplar (Rasheed *et al*., 2013). However, clonal variations in bulk leaf $\delta^{13}C$ may not only reflect differences in WUE_i. The relationship between bulk leaf $\delta^{13}C$ and WUE_i may be affected by difference in mesophyll conductance (Evans *et al.,* 1986) and post-photosynthetic fractionations (Badeck *et al*., 2005).

Our objective was therefore to confirm that bulk leaf $\delta^{13}C$ predicts well leaf-to leaf differences in WUE_i. This is a prerequisite for using this parameter in future breeding programs. Bulk leaf $\delta^{13}C$ and leaf gas exchange were measured on young saplings of 10 rubber clones growing in pot outdoor at Kasetsart University, Bangkok, Thailand.

3. Material and methods

a. Study site and plant material

The experiment was conducted in April 2015 at Kasetsart University, Bangkok, central Thailand.

Ten clones of rubber (*Hevea brasiliensis*) were selected, including the two most common commercial clones in Thailand, RRIM 600 and RRIT 251 that were used as standards and compared to 8 new clones bred at the Chachoengsao Rubber Research Center (CRRC) of the Thai Department of Agriculture, namely RRI-CH-36-1463, RRI-CH-36-980, RRI-CH-39-2085, RRI-CH-39-334, RRI-CH-36-1035, RRI-CH-36-1286, RRI-CH-36-1541 and RRI-CH-36-1515. Table 1 shows the parental origin of these clones. The plants were grown at CRRC nursery according to usual practices in Thailand, i.e. budded on seedling rootstocks in polybags. One-year-old plants (with two developed leaf flushes) were transplanted into small pots (diameter 25.4 cm and height 22.8 cm) with Pakchong soil series from National Corn and Sorghum Research Station, Nakornrachaseema province, and kept for 6 weeks outdoor under a neutral shading net at Kasetsart University, Bangkok (Fig. 2). The trees were watered every day by a drip irrigation system. The measurement was done on the second fully developed flush of leaves. This second flush was grown during March and April 2015, i.e. during the dry season, when the average air temperature was 29.9 °C and rainfall was 313 mm (Monthly Current Report March and April 2015, Thai Meteorological Department).

Table 1 The parental origin of 10 clones of rubber (*Hevea brasiliensis*) used in the study.

Clones	Mother plants	Father plants
RRIM600	Tjir 1	PB 86
RRIT251	-	-
RRI-CH-36-1463	-	-
RRI-CH-36-980	PB 235	RRIM 600
RRI-CH-39-2085	RRIC121	RRIC7
RRI-CH-39-334	RRII203	PB235
RRI-CH-36-1035	PB 235	RRIM 600
RRI-CH-36-1286	RRIT 21	PB 235
RRI-CH-36-1541	--	
RRI-CH-36-1515	$PB_{unknown}$	RRIT4

Unknown (-)



Figure 2 The rubber tree plants used to assess the relationships between $\delta^{13}C$ and WUE_i. The grafted clones were cultivated in pots under neutral shading net at Kasetsart University, Bangkok (April 2015).

b. Leaf gas exchange

Gas exchanges were measured on attached leaves with two portable photosynthesis systems (LI-6400, LI-COR, Lincoln, Nebraska, USA). Intrinsic water use efficiency (WUE_i) was calculated from the ratio of net CO_2 assimilation rate (A, μmol m^{-2} s^{-1}) to stomatal conductance (g_w, mol m^{-2} s^{-1}) in a 2 × 3 cm leaf chamber. We used 5 replications (one leaf on five different trees) for each clone. We recorded A and g_w under two contrasted conditions on each leaf, namely low leaf-to-air vapour pressure deficit (VPD_L= 0.7 kPa) and high VPD_L (1.8 kPa). The CO_2 concentration was 415 μmol mol^{-1} and photosynthetic photon flux density 1200 μmol m^{-2} s^{-1} supplied with red blue light emitting diodes (*6400-02B* LED light source). Leaf temperature and air humidity were varied from 26 to 30°C and from 60 to 80% to generate the two contrasting VPD_L conditions.

c. Sampling and sample preparation for stable isotope analysis

Leaves were collected and stored in a chilled box after measurement of leaf gas exchanges. Leaf area was measured with a leaf area meter, LI-3100A (LI-COR Inc., Lincoln, NE, USA) before oven drying for 72 h at 50 °C. The dry leaves were then weighed and leaf mass per area (LMA) was calculated as the ratio between leaf dry mass and leaf area. After drying, the leaf samples were ground to fine powder and about 1 mg was weighed in 6*4 mm tin capsule and stored in desiccators for $\delta^{13}C$ and nitrogen determination.

The dry leaf powder embedded in the tin capsule was combusted in a furnace and leaf nitrogen and $\delta^{13}C$ 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) at the PTEF laboratory, Nancy, France.

4. Results

a. Leaf gas exchange

The ranges of CO_2 assimilation rates (*A*) were similar at high and low VPD_L. At low VPD_L, RRI-CH-36-1286 had the highest *A*, (9.5 μmol m^{-2} s^{-1}) and RRI-CH-36-980 the lowest (5.9 μmol m^{-2} s^{-1}). At high VPD_L, RRIT-CH-36-1541 had the highest *A*, (9.7 μmol m^{-2} s^{-1}), and RRI-CH-36-980 the lowest (6.3 μmol$m^{-2}s^{-1}$). The average stomatal

conductance (g_w) among the 10 clones was lower at high VPD_L (0.13 mol $m^{-2}s^{-1}$) than at low (0.16 mol m^{-2} s^{-1}). g_w among the 10 clones ranged from 0.11 mol $m^{-2}s^{-1}$ to 0.20 mol $m^{-2}s^{-1}$ at low VPD_L and from 0.10 mol $m^{-2}s^{-1}$ to 0.16 mol $m^{-2}s^{-1}$ at high VPD_L. *A* and g_w were positively correlated (Fig. 3) at both low and high VPD_L but the relation was curvilinear.

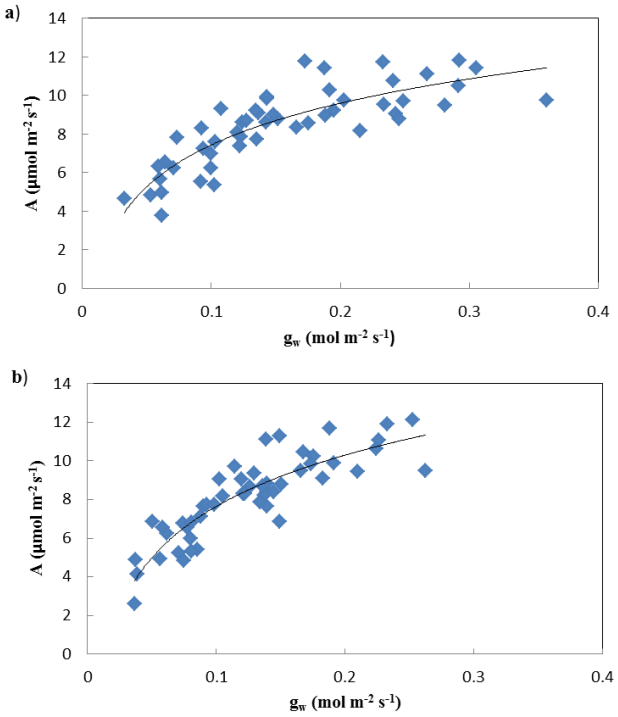


Figure 3 Relationships between net CO_2 assimilation (*A*) and stomatal conductance (g_w) at a) low VPD_L, b) high VPD_L. Each point represents one leaf sampled on 5 trees from each of the 10 clones.

The variations of WUE_i were negatively and non-linearly related to the variation in g_w, both at low VPD_L ($R^2 = 0.82$) and at high VPD_L ($R^2 = 0.67$), as expected because WUE_i is the ratio between *A* and g_w. In contrast, while we would expect positive relations between *A* and WUE_i, we found also negative but rather weak relations ($R^2 = 0.33$ and 0.24, respectively, Fig. 4), which suggest that leaf to leaf variation in WUE_i are driven more by leaf-to leaf variation in g_w than by variations in *A*.

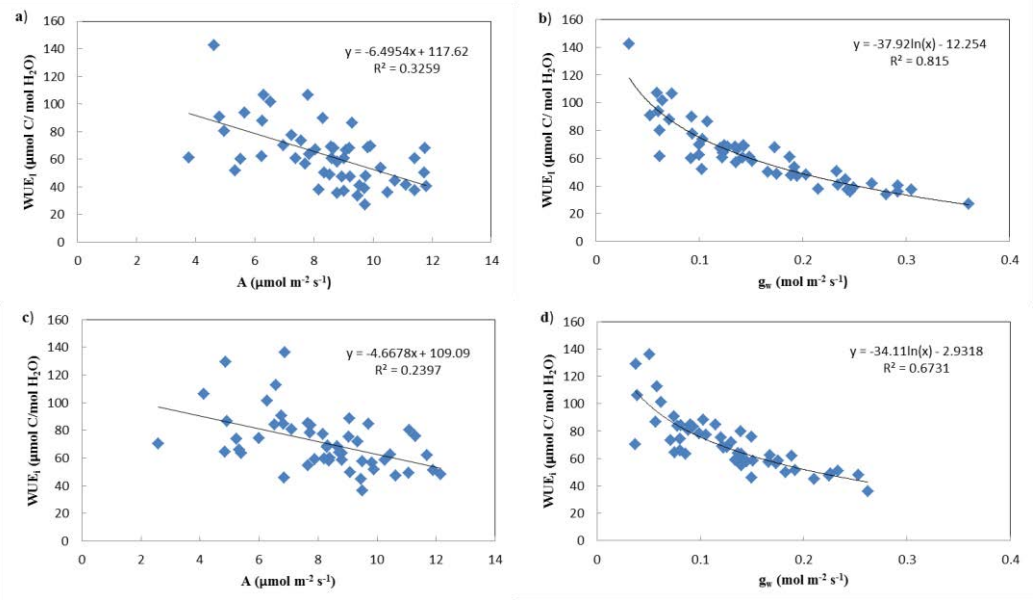


Figure 4 Relationships between intrinsic water use efficiency (WUE_i) and net CO_2 assimilation (A) at a) low VPD_L, c) high VPD_L, and relationships between intrinsic water use efficiency (WUE_i) and stomatal conductance (g_w) at b) low VPD_L, d) high VPD_L. Each point represents one leaf sampled on 5 trees from each of the 10 clones.

b. Water use efficiency (WUE_i) and carbon isotope composition ($\delta^{13}C$)

The range of $\delta^{13}C$ among 10 clones was small, the lowest $\delta^{13}C$ was -31.5‰ in RRIT-CH-36-980 and the highest was -29.9 ‰ in RRIT-CH-39-2085. There were large variation of WUE_i between replications in the same clone, but a low range among clones, with the lowest and highest average of WUE_i among 10 clones being 59.5 µmol C/molH_2O (RRIT-CH-36-980) and 81.9 µmol C/molH_2O (RRIT-CH-36-1463) at low VPD_L and 54.1 µmol C/molH_2O (RRIT-CH-36-1035) and 68.5 µmol C/molH_2O (RRIT-CH-36-2085) at high VPD_L respectively. There was a weak but significant positive linear relation between WUE_i and $\delta^{13}C$ ($R^2 = 0.19$, $p<0.001$) at high VPD_L but the relation was not significant at low VPD_L (Fig. 5).

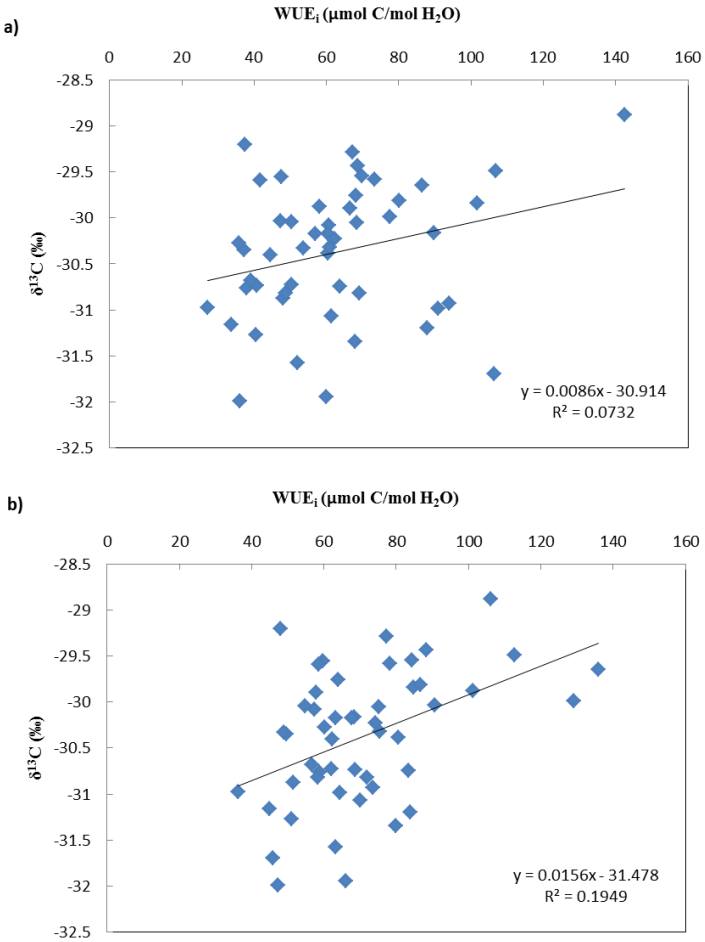


Figure 5 The relation between intrinsic water use efficiency (WUE_i), leaf carbon isotope composition ($\delta^{13}C$), a) low VPD_L and b) high VPD_L. Each point represents one leaf sampled on 5 trees from each of the10 clones.

c. Leaf carbon isotope composition ($\delta^{13}C$) and leaf traits

Table 2 shows correlation coefficients between leaf carbon isotope composition ($\delta^{13}C$) and other leaf traits. The nitrogen content on an area mass basis (N_A) was positively and significantly correlated with leaf mass per area (LMA; r = 0.65). Bulk leaf carbon isotope composition ($\delta^{13}C$) was not significantly correlated coefficients to both the leaf mass per unit area (LMA) and the nitrogen content on an area mass basis (N_A) in this study.

Table 2 Correlation coefficients between leaf carbon isotope composition ($\delta^{13}C$) and other leaf traits (leaf mass per area, LMA; leaf area, LA and nitrogen content on an area basis (N_A).

	$\delta^{13}C$	LMA	N_A
$\delta^{13}C$	1	0.2	0.26
LMA		1	0.65*
N_A			1

Stars (*) indicate significant coefficients.

5. Discussion

The relationship between net assimilation rate (A) and stomatal conductance (g_w) was curvilinear, with a linear trend at low g_w and a saturation trend at high g_w. Such kind of curvilinear relation has often been reported (Epron and Dreyer 1993; Roussel *et al*., 2009) and suggests that leaves with high rate of photosynthesis displayed lower WUE_i. This was confirmed by the weak negative correlation between A and WUE_i. The variations of WUE_i were more driven by the variation in g_w rather than by variations in A, both at low and at high VPD_L. This stronger effect of g_w than that of A on WUE_i was also observed in poplar clones (Monclus *et al*., 2006). Both results highlight that the different leaves differed in the intensity of the stomatal control of A and WUE_i. The lack of correlation between $\delta^{13}C$ and N_A or LMA in our rubber clones may indicate that the difference in $\delta^{13}C$ among clones would be more related to differences in stomatal properties than in differences in photosynthetic capacity. Similarly, in pedunculate oak, the tight correlation between $\delta^{13}C$ and stomatal dimension suggested that genetic differences in water use efficiency within this family was due to differences in stomatal properties (Roussel *et al*., 2009).

A positive linear relation is expected between leaf intrinsic water use efficiency (WUE_i) and the isotope composition ($\delta^{13}C$) of photosynthetic products because both are strongly related to the ratio between internal and atmospheric CO_2 concentration (Farquhar *et al*., 1982). The isotope signature of photosynthetic product is thought to imprint $\delta^{13}C$ bulk plant material. Strong relationships between bulk leaf $\delta^{13}C$ and water

use efficiency has been reported among genotypes in oaks (Ponton *et al*., 2002; Roussel *et al*., 2009). However, we only found a weak correlation between $\delta^{13}C$ and WUE_i across our 10 genotypes of rubber tree when WUE_i was measured at high vapour pressure deficit (VPD_L) and no correlation when it was measured at low VPD_L. A similar discrepancy between the expected and the observed relations has been reported for poplars where a weak linear relationship was found only under moderate drought conditions (Monclus *et al*., 2006). In our study, the fact that bulk leaf $\delta^{13}C$ was correlated to WUE_i only at high VPD_L may be due to the fact that the leaves on which bulk $\delta^{13}C$ was measured have grown, and thus have accumulated organic matter, during the dry season when VPD_L was high.

Several reasons may account for the lack of strong relations between $\delta^{13}C$ and WUE_i. First of all, the range of $\delta^{13}C$ among our 10 clones was quite narrow (1.6 ‰), with the highest $\delta^{13}C$ values been -29.9 ‰ in RRIT-CH-39-2085 and lowest been -31.5% in RRIT-CH-36-980. By comparison, Roussel *et al*. (2009) has compared oak progenies that exhibited a 2 ‰ difference in $\delta^{13}C$. The low range of $\delta^{13}C$ we measured would translate in a range of about 20 µmol C/molH_2O or less for WUE_i according to equations 2 and 4. This is actually what we recorded both at low VPD_L (22.4 µmol C/molH_2O) and high VPD_L (14.4). The observed range in bulk leaf $\delta^{13}C$ in these 10 clones was narrower than the one we observed when comparing 49 clones from Amazonia germplasm growing in a common garden (Chapter IV). Using clones exhibiting a larger range of $\delta^{13}C$ and WUE_i would be necessary to better test the existence of a close coupling between $\delta^{13}C$ and WUE_i among genotypes of rubber trees. However, Monclus *et al*., (2006) failed to find a strong relation between $\delta^{13}C$ and WUE_i despite much higher ranges of $\delta^{13}C$ (2.7‰) among their poplar genotypes.

Moreover, the occurrence of a finite mesophyll conductance to CO_2 may shift the expected slope of this relationship (Evans *et al*., 1986). Mesophyll conductance (g_m) of rubber trees has not yet been reported in the literature. We did not try to assess it in this study but it is well documented that g_m is often low in trees (Epron *et al*., 1995). However, because g_m is strongly correlated to both stomatal conductance and net photosynthesis, differences in g_m among clones and cultivar are not expecting to change the ranking of the different genotypes (Lauteri *et al*., 1997; Barbour *et al*., 2010). Thus, despite the potential occurrence of differences in g_m among genotypes or rubber trees, this

is not expected to limit the possibility of using $\delta^{13}C$ as a qualitative tool for ranking genotypes according to their potential water use efficiency.

As mentioned above, the isotope signature of photosynthetic product is thought to imprint the isotope composition of bulk plant material. However, some additional drifts may occur because of post-photosynthetic fractionations during leaf metabolism. Under such circumstances, the isotope composition of C compounds remaining in leaves differed from the isotope respiration of C compounds that are either respired or exported to the other plant organs (Badeck *et al*., 2005). In addition, the different leaf compounds exhibited their own isotope signature according to these post-photosynthetic fractionations that occur along their biosynthetic pathways. Thus changes in leaf composition will affect the isotope composition of the bulk leaf. In our third experiment, we found on the RRIM600 clone that the leaf soluble compounds of tapped trees were slightly enriched in ^{13}C compared with bulk leaf (0.4‰) while the opposite trend was observed for leaves of untapped trees (Kanpanon *et al*., 2015, chapter V). The question arises whether differences in leaf metabolism among clones may change the biochemical content of the leaves, thus accounting for clonal variation in bulk leaf $\delta^{13}C$ that is not related to differences in WUE_i. This should be addressed in future research projects.

6. Conclusion

In conclusion, we failed to find a robust relation between bulk leaf $\delta^{13}C$ and WUE_i, which remained a prerequisite before using bulk leaf $\delta^{13}C$ as criteria for breeding for more water efficient rubber trees. Several additional studies should be conducted in the future. First, it would be necessary to increase the number of clones and to enlarge the range of bulk leaf $\delta^{13}C$ values among them. Second, it would be interesting to measure on line carbon isotope fractionation to check whether potentially low and variable mesophyll conductance is partly offsetting the relation between water use efficiency and photosynthetic carbon discrimination. Finally, it would be wise to compare bulk leaf $\delta^{13}C$ with the $\delta^{13}C$ of leaf soluble sugar to check whether or not post-photosynthetic fractionations are altering the relation between bulk leaf $\delta^{13}C$ and WUE_i.

IV. IS THERE GENETIC VARIABILITY FOR $\delta^{13}C$ AMONG RUBBER TREE GENOTYPES?

Large variation in carbon isotope composition of bulk leaf material of rubber trees (*Hevea brasiliensis*) from a germplasm collection: an opportunity for improving water use efficiency in future breeding programs.

1. Abstract

Rubber tree (*Hevea brasiliensis*) is an important economic crop in Thailand. As the plantations extend to new and dryer areas, physiological parameters to help breeding for adapted genotypes are required, such as water use efficiency (WUE). Carbon isotope composition of bulk leaf ($\delta^{13}C$) can be used as an indicator of intrinsic water use efficiency (WUE_i) of plants. A prerequisite step is to analyze the genetic variability of $\delta^{13}C$. We compared the carbon isotope composition of bulk rubber tree leaf ($\delta^{13}C$) in rainy and dry seasons in a collection of 49 wild genotypes of rubber in Northern Thailand. There were large $\delta^{13}C$ variations among the genotypes at all seasons. $\delta^{13}C$ ranged between -33.3‰ to -30.0‰ in rainy season and -33.1‰ to -29.9‰ in dry season. $\delta^{13}C$ was rather stable with a good correlation between rainy and dry season. In rainy season, there was a positive significant correlation between $\delta^{13}C$, LMA and leaf nitrogen (N_A). The average $\delta^{13}C$ was significantly different between two genetic clusters previously identified in this population. This study shows that the genetic variability of $\delta^{13}C$ is promising for breeding if a good correlation between $\delta^{13}C$ of leaf and WUE is established.

2. Introduction

Thailand is the world leader of natural rubber with about a third of the global production. Since the eighties, rubber tree (*Hevea brasiliensis*) plantations have expanded throughout Thailand from the traditional southern area. The planting of rubber plantations in non-traditional areas in the Northeast, North, East and West induces risks linked to climate and particularly to drought. Drought stress has been reported to impair the growth of rubber trees (Chandrashekar *et al*., 1998; Manmuen *et al*., 1993) and latex production (Wichichonchai and Manmeun, 1992). Thus, this may decrease the expected income for

farmers and have an important impact on the country's economy. Selection for drought-tolerant clones has become a major challenge in rubber breeding programs to ensure the sustainability of natural rubber production. A key is to better understand the physiological bases of water economy in rubber trees. Trees can adapt to water scarcity by resistance, regulation, avoidance and efficiency mechanisms.

Regarding resistance, several researchers investigated the relations between rubber tree and water in dry conditions. Xylem vulnerability to cavitation is a trait related to drought-induced mortality. Jinagool *et al*. (2015) found no clonal variability and a small phenotypic plasticity for xylem vulnerability to cavitation in branches. However, clonal differences in xylem vulnerability to cavitation were found in petioles, and clones also showed differences in stomatal response and in leaf shedding behavior in response to a simulated drought. Isarangkool Na Ayutthaya *et al.* (2011) demonstrated that hydraulic limitation hypothesis was sufficient to describe water use regulation of a mature rubber tree stand in water-limited conditions. They showed that RRIM600 clone in northeast Thailand displayed an isohydric behavior. Sopharat *et al*. (2015) refined the study and showed that whatever soil water availability transpiration was regulated under high evaporative demand. Kumagai *et al*. (2015) compared how rubber trees regulate water in northeastern Thailand and Cambodia and showed that there was a stricter stomatal regulation in Cambodia, where the risk of water induced xylem embolism was higher.

Selection of genotypes which use water more efficiently is one key option in these non-traditional areas where water resource may be scarce. Water use efficiency (WUE) can be expressed in different ways. At plant level the ratio of biomass increment to transpiration rate can be relevant and at farm level that can be the ratio of yield to water consumption. At leaf level, scientists consider the ratio net carbon assimilation rate (A) per unit water transpired (transpiration rate, E) during the same period (Farquhar *et al*., 1989) or the ratio of photosynthesis rate to stomatal conductance of CO_2, A/g_w, often referred as intrinsic water use efficiency, WUE_i.

We consider here the intrinsic water use efficiency of plants (WUE_i), the ratio of CO_2 assimilation rate (A) to the stomatal conductance to water vapor (g_w):

$$WUEi = A/g_w \qquad [1]$$

Carbon isotope composition ($\delta^{13}C$) of C_3 plants is strongly related to its WUE_i (Farquhar *et al.,* 1982) which is easy to measure on a large number of samples using isotope ratio mass spectrometer (IRMS) and it is linearly and positively related to WUE_i. The carbon isotope discrimination of plant organic matter is therefore widely used as a proxy for water-use efficiency that can be used for seeking genotypes exhibiting high water use efficiency for selection and breeding programs (Roussel *et al*., 2009; Chen *et al*., 2011).

To our knowledge, such approach has not been used so far for rubber tree. Two steps are necessary for its implementation, (i) check the relationship between $\delta^{13}C$ in rubber leaves and WUE_i and (ii) assess the variability of $\delta^{13}C$ in rubber genetic resource. However, the current cultivated clones of rubber are all descending from a narrow initial population named 'Wickham clones'. To enlarge the genetic resource, ex-situ collections of the wild rubber tree *H. brasiliensis* were gathered more than 25 years ago mainly from populations in Southwest Amazonia (Priyadarshan and Gonçalves, 2003). The collections were distributed among several countries, including Thailand. Le Guen *et al.* (2009) studied the genetic diversity within this natural population to improve genetic resource management and sampling strategy in natural populations of *H. brasiliensis*. Lekawipat *et al.* (2003) showed that the wild accessions of the collection located in Thailand were more polymorphic than the cultivated ones and could be divided in 5 clusters on the basis of microsatellite markers.

This project aims to study carbon isotope composition ($\delta^{13}C$) variation in a large number of clones from wild populations of rubber trees originating from southwest Amazonia. The objective is to bring knowledge about the genetic variability of $\delta^{13}C$ among these populations and whether or not a large variability is potentially available in natural populations or in germplasm collections that could be used in future breeding programs.

3. Materials and methods

a. Study site and plant material

This research was done in a plantation at the Nong Khai Rubber Research Center (NRRC), Department of Agriculture, in the upper Northeast Thailand**.** The average

annual rainfall at Nong Khai (Thai Meteorological Department, 2014) is 1600mm, considered optimal for rubber tree, but the dry season lasts five to six months, much longer than the optimum of 3 months.

b. Plant material

The studied plot is a germplasm collection containing clones (genotypes) of wild rubber trees (*Hevea brasiliensis*) collected in 1981 in the Amazonian forest in the states of Acre, Rondonia and Mato Grosso in Brazil (Priyadarshan and Gonçalves, 2003; Lekawipat *et al*., 2003). The trees were propagated by bud-grafting and were 18 years old at the beginning of experiment (Fig. 6). Five trees from 230 wild accessions were planted at a spacing 2.5 x 7 meters. The present studies were limited to 49 clones (Table 3) that were selected according to their survival (at least 4 trees) and their distribution within the plantation to avoid trees growing at the edge of the plantations. After this selection, the subset contained 28 clones from the state of Rondonia, 20 clones from the state of Mato Grosso but only one from the state of Acre. Regarding the genetic classification established by Lekawipat *et al*. (2003), the unique representatives of cluster IV and V were present in our study. We also had 19 representatives of cluster II, 14 representatives of cluster III but only 2 representatives of cluster I, while 12 of our genotypes were not present in Lekawipat's study.



Figure 6 The germplasm collection containing clones (genotypes) of wild rubber trees (*Hevea brasiliensis*) at the Nong Khai Rubber Research Center (NRRC), Department of Agriculture, in the upper Northeast Thailand (2012).

Table 3 List of the *Hevea brasiliensis* clones (genotypes) sampled in this study. The genotypes were classified in the 5 genetic clusters identified by Lekawipat *et al*. (2003), when possible.

No.	Name	Type	Source	Cluster
1	AC/F/7 38/62	Wild	Acre / Feijo	III
2	RO/A/7 25/6	Wild	Rondonia/	II
3	RO/A/7 25/181	Wild	Rondonia/	II
4	RO/A/7 25/205	Wild	Rondonia/	V
5	RO/A/9 23/187	Wild	Rondonia /	-
6	RO/CM/10 44/265	Wild	Rondonia / Costa	II
7	RO/CM/10 44/669	Wild	Rondonia / Costa	II
8	RO/CM/11 63/7	Wild	Rondonia / Costa	II
9	RO/CM/11 63/182	Wild	Rondonia / Costa	II
10	RO/CM/11 63/189	Wild	Rondonia / Costa	II
11	RO/CM/11 63/366	Wild	Rondonia / Costa	II
12	RO/CM/12 62/20	Wild	Rondonia / Costa	II
13	RO/CM/12 62/180	Wild	Rondonia / Costa	II
14	RO/J/6 32/19	Wild	Rondonia / Jaru	-
15	RO/OP/4 20/16	Wild	Rondonia / Ouro	IV
16	RO/I/118 121	Wild	Rondonia / Ouro	-
17	RO/CM/11 63/149	Wild	Rondonia / Costa	II
18	RO/PB/1 2/68	Wild	Rondonia / Pimenta	II
19	RO/PB/1 2/83	Wild	Rondonia / Pimenta	II
20	RO/PB/1 2/219	Wild	Rondonia / Pimenta	II
21	RO/PB/2 3/223	Wild	Rondonia / Pimenta	II
22	RO/PB/2 3/250	Wild	Rondonia / Pimenta	II
23	RO/PB/2 3/260	Wild	Rondonia / Pimenta	II
24	RO/PB/2 3/301	Wild	Rondonia / Pimenta	II
25	RO/PB/2 3/351	Wild	Rondonia / Pimenta	II
26	RO/I/10 54	Wild	Rondonia /	-
27	RO/I/22 60	Wild	Rondonia /	-
28	RO/I/56 81	Wild	Rondonia /	-
29	RO/I/108 111	Wild	Rondonia /	-

30	MT/C/1 18/59	Wild	Mato Grosso /	III
31	MT/C/2 10/12	Wild	Mato Grosso /	III
32	MT/C/1 18/116	Wild	Mato Grosso /	III
33	MT/C/2 10/39	Wild	Mato Grosso /	III
34	MT/C/2 10/94	Wild	Mato Grosso /	III
35	MT/C/2 10/59	Wild	Mato Grosso /	III
36	MT/C/2 10/61	Wild	Mato Grosso /	III
37	MT/C/2 10/138	Wild	Mato Grosso /	III
38	MT/I/4 7/55	Wild	Mato Grosso /	-
39	MT/C/10 17/1	Wild	Mato Grosso /	III
40	MT/IT/12 26/70	Wild	Mato Grosso /	III
41	MT/IT/12 26/136	Wild	Mato Grosso /	III
42	MT/IT/16 34/72	Wild	Mato Grosso /	III
43	MT/IT/17 27/45	Wild	Mato Grosso /	-
44	MT/IT/15 28/175	Wild	Mato Grosso /	III
45	MT/VB/25A 57/67	Wild	Mato Grosso / Vila	I
46	MT/VB/25A 57/2	Wild	Mato Grosso / Vila	I
47	MT/I/16 22	Wild	Mato Grosso /	-
48	MT/I/44 43	Wild	Mato Grosso /	-
49	MT/I/45A 44	Wild	Mato Grosso /	-

c. Climatic data

Climate data was collected manually every day from December 2012 to December 2013. Temperature data was measured by thermometer, wet bulb dry bulb thermometer was used for measuring humidity and rainfall value was measured by the bucket method.

d. Tree measurement and latex yield

The trunk girth of all trees was measured at 170 cm above ground in March 2012, September 2012 and September 2013. All trees were tapped for latex yield in half-spiral every two days during wet seasons (1/2S d/2, standard method in Thailand). The latex was collected as coagulum which would be dried in 21 days by airing and weighted. A sample of each clone was used to estimate dry weight.

e. Sample preparation for stable isotope analysis

Four trees per clone were sampled from 6 to 9 December 2012 (beginning of dry season) and from 23 to 26 September 2013 (rainy season). One sunlit leaf was harvested at 4 directions in each tree and 2 leaflets were kept per leaf (8 leaflets per tree). The leaflets samples were immediately stored in a chilled box and brought back to a laboratory located at the station. Leaf area was measured with a leaf area meter, LI-3100A (LI-COR Inc., Lincoln, NE, USA) and oven dried for 72 h. at 50 °C. The dry leaves were then weighed and leaf mass per area (LMA) was calculated as the ratio between leaf dry mass and leaf area. After drying, one composite sample (8 leaflets/tree) was ground to fine powder and 1 mg was weighed in tin capsule (6*4 mm) and stored in desiccators for $\delta^{13}C$ analysis. Samples were sent to the PTEF laboratory in INRA (French National Institute for Agricultural Research) Nancy, France for $\delta\ ^{13}C$ analysis. The nitrogen content (N), the carbon content (C) and the carbon isotope composition ($\delta^{13}C$) of all samples 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).

The isotope composition ($\delta^{13}C$) is expressed relative to an international standard (V-PDB) derived from a reference limestone (Pee Dee Belemnite) at South Carolina State in the United States:

$$\delta^{13}C_{sample} = [(R_{sample} / R_{standard}) - 1] \quad [3]$$

with *R*, the ratio of ^{13}C to ^{12}C in the plant material (sample) and in the international standard ($R_{VPDB} = 0.0111802$).

f. Data analysis

Statistical analyses were performed using the statistical software SAS 9.1.3 (Statistical Analysis System). ANOVA was performed to test the differences in $\delta^{13}C$ between the two sampling times and the 49 clones with 4 replications. ANOVA between two genetic clusters was done on the mean data per clone with 19 clones in cluster II and 14 in cluster III. The Spearman rank correlation coefficient between the $\delta^{13}C$ values measured on the 49 clones at two sampling dates was computed using the statistical software R 2.15.1 (R Core Team 2012) and use to check the stability of the genotype ranking.

4. Results

a. Environmental conditions

From December 2012 to December 2013, the annual temperature averaged 25.3°C with the highest monthly average temperature in April (28.6°C) and lowest in December (21.9°C). The annual rainfall was 1962 mm with 4 months of high rainfall (1622 mm). The highest rainfall was recorded in July (530 mm) while there was no rainfall in February and in November (Fig. 7).

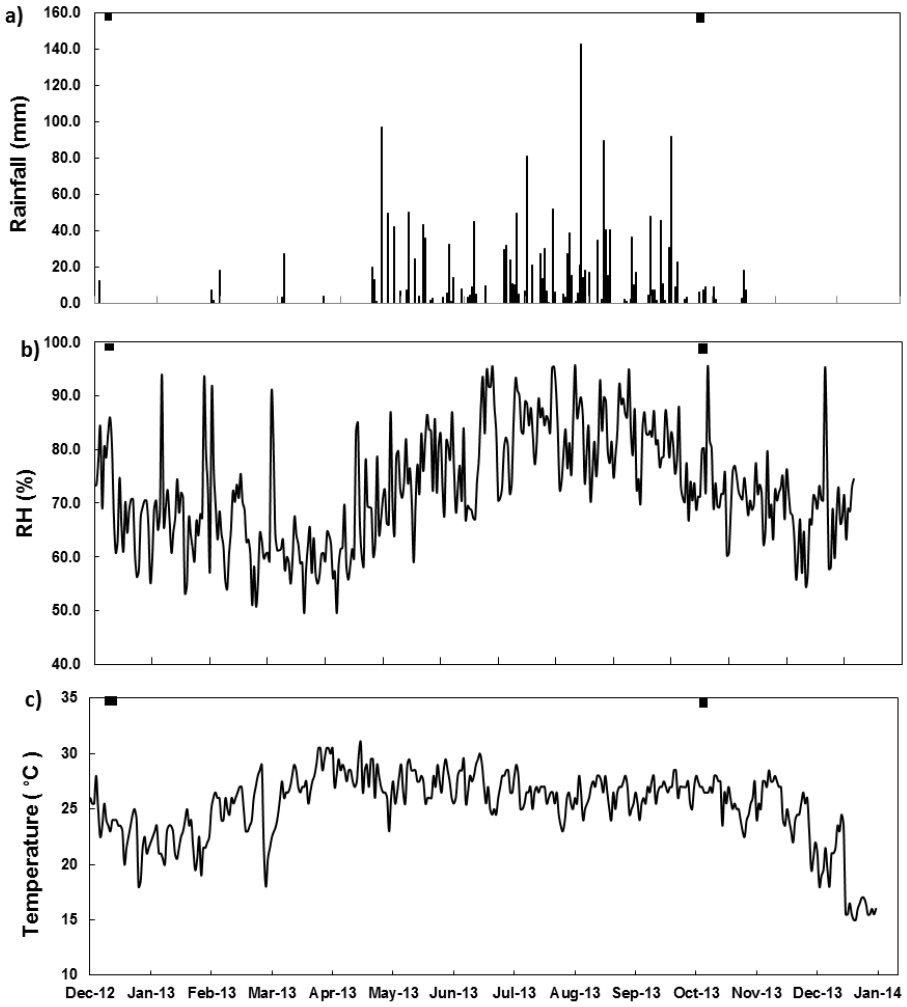


Figure 7 Annual courses of a) daily rainfall, b) mean daily air humidity and c) mean daily air temperature.

b. Variations in leaf carbon isotope composition among clones

The difference in $\delta^{13}C$ among the 49 clones was highly significant ($p<0.001$). The range of $\delta^{13}C$ (more than 3‰) was large among the 49 clones at both seasons, with a minimum value of -33.1‰ for clone MT/VB/25A-57/2 and a maximum of -29.9‰ for clone MT/C1-18/59 in December, and with a smaller value of -33.3‰ for the same clone in December and a maximum of -30.0‰ for clone AC/F/7-25/115 in September. Five clones in December and eight clones in September had $\delta^{13}C$ above -30.5‰, but only one clone belonged to these two groups (MT/C/2-10/94) (Fig. 8). At the opposite side of the range, in four clones in December and in two clones in September, $\delta^{13}C$ was less than -32.5‰, but again, only one clone belonged to these two groups (MT/VB/25A-57/2). While the effect of sampling time on $\delta^{13}C$ was not significant, the interaction between clones and sampling time was indeed significant ($p= 0.03$).

Despite some discrepancies between the two seasons for clones exhibiting the highest and the lowest values of $\delta^{13}C$, the genotype ranking based on $\delta^{13}C$ values measured on the 49 clones between the two sampling dates was positive and significant at 0.05 ($\rho = 0.72$, Fig. 9). The $\delta^{13}C$ was rather stable with a good correlation between rainy and dry season.

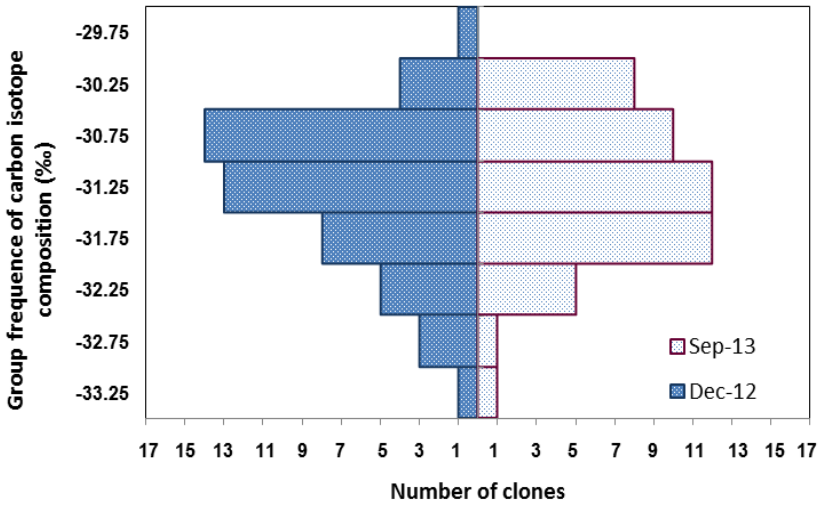


Figure 8 Frequency distribution of carbon isotope composition in bulk leaf ($\delta^{13}C$) in the dry season (December, left) and in the rainy season (September, right). N=49.

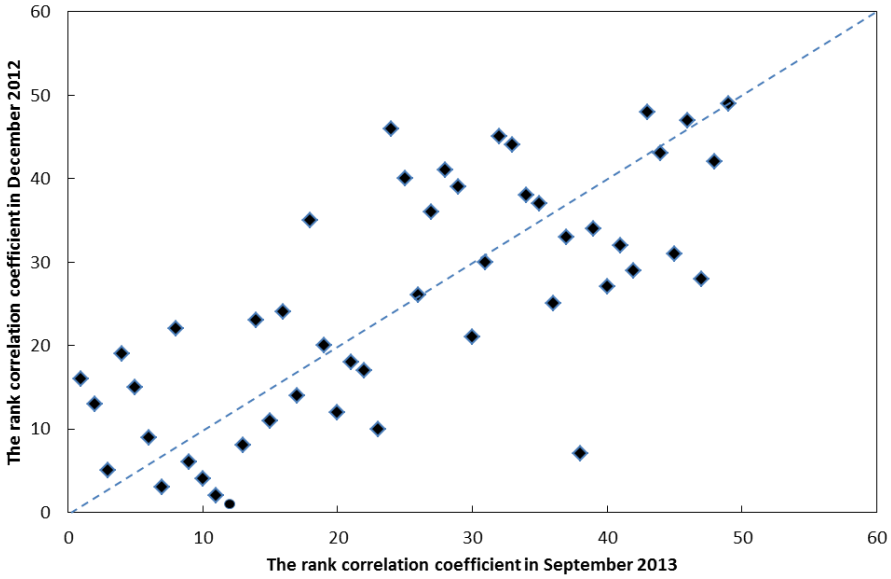


Figure 9 Relation between the genotype ranking based on $\delta^{13}C$ values measured on the 49 genotypes in the dry season (December 2012) and wet season (September 2013). Each point represents a clone. The dotted line represents the 1/1 line. The Spearman rank correlation coefficient was 0.72.

c. Relationships between leaf carbon isotope composition and other leaf traits, tree growth and rubber yield

There was no correlation between $\delta^{13}C$ and any other leaf traits in December 2012 but $\delta^{13}C$ was positively correlated with the leaf mass per unit area (LMA) and the nitrogen content on a leaf area basis (N_A) in September 2013 (Table 4).

d. Tree growth and rubber yield

The average girth relative growth rate (RGR) was 1.8% from March 2012 to September 2012 and 1.5% from September 2012 to September 2013 in the 49 clones. Mean latex yield was 8.47 gram/tree/tapping and 6.71 gram/tree/tapping in December 2012 and September 2013, respectively. RGR, latex yield and $\delta^{13}C$ were not correlated at both dates (data not shown).

e. Relationship between carbon isotope composition and genetic diversity

The $\delta^{13}C$ was not related to the state of origin (Acre, Rondonia and Mato Grosso). Table 5 shows the average $\delta^{13}C$ of the 49 studied clones ranked according to Lekawipat's dendrogram (Lekawipat *et al*., 2003). There was only one clone in cluster IV and V. The average of $\delta^{13}C$ value was -31.4‰ in December 2012 and -31.5‰ in September 2013 in

Cluster II (19 clones) and -30.9‰ in December 2012 and September 2013 in Cluster III (14 clones), respectively. The differences between the two clusters were significant at both seasons (p=0.02 in December 2012 and p=0.001 in September 2013).

Table 4 Correlation coefficients between leaf carbon isotope composition ($\delta^{13}C$), and other leaf traits (leaf area, LA; leaf mass per area, LMA; nitrogen content on a mass basis, N; carbon content on a mass basis, C; carbon and nitrogen ratio, (C:N) and nitrogen content on an area basis (N_A) in December 2012 and September 2013.

	Dec 12	Sep 13
LA	0.05	0.12
LMA	0.14	0.36*
N	0.13	0.09
C	0.01	0.12
C:N	-0.11	0.10
N_A	0.21	0.47*

Stars (*) indicate significant coefficients.

Table 5 The relation of carbon isotope composition 49 clones and similarity dendrogram of 68 *H. brasiliensis* wild accessions, calculated from data of 12 microsatellite markers, using UPGMA as the clustering method. (Lekawipat *et al.*, 2003).

Cluster	Number of clones	$\delta^{13}C$ (‰) Dec.-12	$\delta^{13}C$ (‰) Sep.-13
I	2	-32.9	-32.8
II	19	-31.4*	-31.5*
III	14	-30.9*	-30.9*
IV	1	-31.3	-31.4
V	1	-31.2	-30.6
-	12	-31.4	-31.2

Stars (*) indicate significant differences. Differences were tested only between the clusters with enough clones (cluster II and III).

5. Discussion

This study presents the first evaluation of the diversity of carbon isotope composition ($\delta^{13}C$) in leaves of rubber trees (*Hevea brasiliensis*). The carbon isotope composition ($\delta^{13}C$) of bulk leaf material ranged between -30‰ and -33‰ among 49 clones of a population of wild rubber genotypes (1981 Amazonian accessions, Priyadarshan and Gonçalves 2003, Lekawipat *et al*., 2003). This is in the lowest part of the range of -35‰ to -22‰ for C_3 plants (O'Leary, 1988), but is typical for tropical tree species (Medina & Minchin, 1980; Medina *et al*., 1991; Buchmann *et al*., 1997). Variability of $\delta^{13}C$ among genotypes in rubber tree was in the same range as that in poplar (Monclus *et al*., 2005) which was considered large (Marron *et al*., 2005; Monclus *et al*., 2005, 2006; Zhang *et al*., 2004) and honey mesquite (Pennington *et al*., 1999). As compared with recent clones bred from the Wickham population (chapter III), the range in this wild population was higher: Among those Wickham clones, the lowest $\delta^{13}C$ was -31.5% in RRIT-CH-36-980 and the highest was -29.9 ‰ in RRIT-CH-39-2085.

These low $\delta^{13}C$ found in leaves of tropical species is mainly due to wet conditions, which lead to higher ratios of stomatal conductance to photosynthesis, thus increasing the C_i/C_a ratio, which in turn leads to lower carbon isotope discrimination values in these areas. The rank correlation coefficient for $\delta^{13}C$ values between the two sampling dates was positive and significant. Although the growth rate and weather conditions changed, there were few changes in $\delta^{13}C$ values when the seasons changed. This low seasonal variability would make the use of carbon isotope method more robust to compare clones.

During the wet season, variation in $\delta^{13}C$ between clones seemed related to differences in photosynthetic capacity that was reflected by difference in leaf N content, and in the dry season, the variation in $\delta^{13}C$ among clones may be more controlled by differences in the g_w sensitivity to dry conditions. The measurements of photosynthetic capacities (maximal carboxylation rate, V_{cmax}, and maximal light driven electron flow, J_{max}) and WUE_i would be requested to drive to a firm conclusion (Monclus *et al*., 2005, 2006).

The lack of relationship between latex yield and trunk girth increment was not surprising when comparing clones. Latex is produced by a secondary metabolism and is not directly linked to biomass accumulation (George and Kuruvilla, 2000). The occurrence of genotypes without any latex yield from tapping despite a significant growth

has already been reported in wild genotypes (Gonçalves *et al*., 2006). Moreover, latex yield is influenced by antecedent weather conditions experienced by the trees, not necessarily by the instant conditions (Sanjeeva Rao *et al*., 1997), which could also account for the absence of relationship between leaf $\delta^{13}C$ and latex yield in our study.

The lack of relationship between $\delta^{13}C$ and growth that we have observed may have many causes and has been reported already for poplars (Monclus *et al*., 2005, 2006; Bonhomme *et al*., 2008). Soule and Knapp (2011) suggested that increased WUE_i associated with rising CO_2 can positively impact tree growth rates in the western United States and are thus an evolving component of forest ecosystem processes. And they concluded that if potential climate changes lead to increasing aridity in the western United States, additional increases in WUE_i associated with future increases in CO_2 might mitigate growth declines associated with drought conditions. Van der Sleen *et al*. (2015) present measurements of stable carbon isotopes and growth rings in the wood of 1,100 trees from Bolivia, Cameroon and Thailand. Measurements of carbon isotope fractions in the wood indicate that intrinsic water-use efficiency in both understorey and canopy trees increased by 30–35% over the past 150 years as atmospheric CO_2 concentrations increased. However, they found no evidence for the suggested concurrent acceleration of individual tree growth when analyzing the width of growth rings, showing that tree growth and water use efficiency are not necessarily correlated.

There was a significant difference between two identified genetic clusters, then a likely genetic basis for $\delta^{13}C$ (table 3). The genetic clusters partially but not completely match the geographic origin (state). The fact that the cluster effect was significant contrary to the state effect reinforces the validity of the genetic classification and also the probability of a genetic basis of $\delta^{13}C$.

6. Conclusion

There was a large variability in $\delta^{13}C$ of bulk leaf material among 49 wild rubber trees (*Hevea brasiliensis*) clones. The significant differences in mean $\delta^{13}C$ between genetic clusters indicated a probable genetic base of $\delta^{13}C$ in rubber trees. This was reinforced by the fact that the state of origin (Rondonia, Mato Grosso and Acre) had no significant influence. As $\delta^{13}C$ was rather stable among seasons, its use as a proxy of the intrinsic water uses efficiency (WUE_i) is promising. The lack of a negative correlation

between $\delta^{13}C$ and latex yield or tree growth rate indicates that there is a potential to improve water-use efficiency without reducing latex yield or wood production in rubber trees, if a good correlation is established between $\delta^{13}C$ and WUE_i for this species. Studies of this relationship on a large number of commercial rubber clones are then recommended.

V. IS LATEX $\delta^{13}C$ RELATED TO LEAF $\delta^{13}C$ IN TAPPED AND UNTAPPED TREES? CAN LATEX $\delta^{13}C$ BE USED AS A PROXY OF WUE? IS LATEX $\delta^{13}C$ RELATED TO CANOPY WUE_i?

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1. Abstract

Latex, the cytoplasm of laticiferous cells localized in the inner bark of rubber trees (*Hevea brasiliensis*), is collected by tapping the bark. Following tapping, latex flows out of the trunk and is regenerated, whereas in untapped trees, there is no natural exudation. It is still unknown whether the carbohydrates used for latex regeneration in tapped trees is coming for recent photosynthates or from stored carbohydrates, and in the former case, it is expected that latex carbon isotope composition of tapped tree will vary seasonally, whereas latex isotope composition of untapped trees will be more stable. Temporal variations of carbon isotope composition of trunk latex ($\delta^{13}C$-L), leaf soluble compounds ($\delta^{13}C$-S) and bulk leaf material ($\delta^{13}C$-B) collected from tapped and untapped twenty-year-old trees were compared. A marked difference in $\delta^{13}C$-L was observed between tapped and untapped trees whatever the season. Trunk latex from tapped trees was more depleted (1.6‰ on average) with more variable $\delta^{13}C$ values than that of untapped trees. $\delta^{13}C$-L was higher and more stable across seasons than $\delta^{13}C$-S and $\delta^{13}C$-B, with a maximum seasonal difference of 0.7‰ for tapped trees and 0.3‰ for untapped trees. $\delta^{13}C$-B was lower in tapped than in untapped trees, increasing from August (middle of the rainy season) to April (end of the dry season). Differences in $\delta^{13}C$-L and $\delta^{13}C$-B between tapped and untapped trees indicated that tapping affects the metabolism of both laticiferous cells and leaves. The lack of correlation between $\delta^{13}C$-L and $\delta^{13}C$-S suggests that recent photosynthates are mixed in the large pool of stored carbohydrates that are involved in latex regeneration after tapping.

2. Introduction

Rubber tree (*Hevea brasiliensis* Muell. Arg, Euphorbiaceae family) is a widely planted species in South-East Asia where annual rainfalls range between 1800 and 2500 mm (Rao and Vijayakumar, 1992). They are nowadays expanding on non-traditional areas such as the north-eastern Thailand despite lower annual rainfall and longer dry seasons (below 1400 mm on average from 1996 to 2014, Thai Meteorological Department 2015) that may limit rubber yield (Priyadarshan, 2003; Rao *et al*., 1998). Due to the high economic values of natural rubber production that generates incomes to smallholders in Thailand, farmers are adopting intensive latex harvesting schemes (Chantuma *et al*., 2011). Both the expansion of the planted area and the intensification of the harvest raise questions regarding sustained latex yield in rubber plantations.

Natural rubber is obtained from the latex that contains about 25-45% of rubber particles (De Faÿ *et al*., 1989). Latex is the cytoplasm of anastomosed laticiferous cells localized in the inner bark of the tree where the rubber formation takes place (Hébant and De Faÿ, 1980). Latex is collected by regularly tapping the bark of tree, allowing latex to flow out for several hours until coagulation plugs the laticifer vessels. Latex is present in all the organs of the tree and it does not exude without cutting. The residence time of latex in the bark of untapped trees is unknown. Tapping induces latex regeneration in the bark of trunk. Natural rubber is made of poly (cis-1,4-isoprene) at 95% but also contains small amounts of lipids (phospholipids, glycolipids, tocotrienols, etc), proteins, carbohydrates and inorganic compounds (Backhaus, 1985; Liengprayoon *et al*., 2013). Poly (cis-1,4-isoprene) is synthetized from isopentenyl pyrophosphate (IPP) which mainly derives from pyruvate through the mevalonate pathways in the cytoplast (Chappell 1995) but the occurrence of a non-mevalonate pathway in specialized plastids, the Frey-Wyssling particles, cannot be ruled out (Chow *et al*., 2012).

Whatever the involved pathway, IPP derives from sucrose degradation to pyruvate (Jacob *et al*., 1989). Latex regeneration between two tapping events depends on the availability of sucrose (Obouayeba *et al*., 2009 ; Tupý, 1989). Sucrose content of latex is well-correlated to rubber yield and constitutes one of the main indicators used in latex diagnosis methods widely used to characterize tapping systems in rubber plantations or to assess yield potential of rubber clones (Chantuma *et al*., 2011; Jacob *et al*., 1989; Jacob *et al*., 1995; Lacote *et al*., 2010). However, it is still unknown whether the carbohydrates

used for latex regeneration in tapped trees are coming from recent photosynthates transferred from the foliage to the trunk inner bark via the phloem sap or if they are derived from carbohydrates stored in parenchyma xylem cells in the trunk, such as starch (Chantuma *et al*., 2009; Silpi *et al*., 2007) or cyanogenic mono- and di-glucosides (Kongsawadworakul *et al*., 2009).

Because carbon isotope discrimination during photosynthesis is positively and mechanistically related to the intercellular carbon dioxide concentration in leaves (Farquhar *et al*., 1982), the stable carbon isotope composition ($\delta^{13}C$) of plant organic materials has been widely used to compare time-integrated genetic and phenotypic variations in water use efficiency. (Farquhar and Richards, 1984; Le Roux *et al*., 1996; Rasheed *et al*., 2013). Variations in phloem sap $\delta^{13}C$ are also potentially reflecting temporal climate-driven variation in photosynthetic carbon isotope discrimination (Rascher *et al*., 2010). During the long season with high water vapour pressure deficit and low soil water content that characterize the dry tropical climate of north-eastern Thailand, leaf stomatal conductance and net CO_2 assimilation are expected to decrease (Chen *et al*., 2010; Sangsing *et al*., 2004a). As a consequence, intrinsic water use efficiency is thought to increase during the dry season, changing photosynthetic carbon isotope discrimination that it will imprint on $\delta^{13}C$ of photosynthetic products (Farquhar *et al*., 1982). The isotope signal fluctuation has proven to be useful to estimate lags between photosynthetic carbon assimilation and the use of the assimilated carbon for metabolic processes in trees (Ekblad and Högberg, 2001). It was shown that isotopic mixing of recent photosynthates into large C pools such as stored carbohydrates can delay the recovery of the isotope signals into respiration (Mencuccini and Hölttä, 2010; Wingate *et al*., 2010). While stable C isotope composition and its temporal variations in latex have not yet being addressed, short lags of a few days duration may be expected if the carbohydrate used for latex production is derived from sucrose directly carried by the phloem sap. In contrast, the temporal variation in photosynthetic carbon isotope discrimination should be almost fully dampened if carbohydrates used for rubber synthesis are derived from storage pools acting as a buffer between photosynthesis and rubber synthesis.

This study examined both daily and seasonal variations of carbon isotope composition of the trunk latex ($\delta^{13}C$-L), leaf soluble compounds ($\delta^{13}C$-S) and bulk leaf material ($\delta^{13}C$-B) in twenty-year-old tapped and untapped rubber trees in relation with

vapour pressure deficit, soil water content and inherent water use efficiency at ecosystem level. Because tapping for latex production induces *de novo* latex synthesis that consumes a large amount of carbon derived from photosynthesis, we postulated that δ^{13}C-L of tapped trees will exhibit more pronounced temporal variations than the composition of the trunk latex collected from untapped trees. We further postulate that comparing temporal patterns in δ^{13}C-L and δ^{13}C-S, the latter being driven by daily and seasonal changes in photosynthetic carbon isotope discrimination, will be promising for identifying which carbon source (stored carbohydrates versus recent photosynthate) is involved in latex regeneration.

3. Materials and methods

a. Experimental site and plant materials

The experiment was done at the Chachoengsao Rubber Research Centre (CRRC), Chachoengsao province (13°41′ N, 101°04′ E, 69 m elevation) in Thailand. Mean annual air temperature and cumulative rainfall were 28.1°C and 1328 mm (2001-2009, Thai Meteorological Department) with a dry season between December and April. The six ha plot is embedded in a 200 ha stand where a 25 m high eddy-flux tower was built in 2006 (Rubber Flux Chachoengsao, Asia flux network). A three-dimensional sonic anemometer-thermometer (CSAT3, Campbell scientific Inc.) and an open-path infrared gas analyzer (LI7500, Li-Cor Biosciences, Lincoln, NE, USA) were installed at 27.5m high (Fig. 10).

A monoclonal rubber tree (*Hevea brasiliensis* Müll. Arg.) plantation was installed in 1994 with tree spacing of 7 x 2.5 m with the clone RRIM 600, the most widely planted clone in Thailand, on a sandy-clay-loam soil (Kabin Buri series). Field capacities and wilting points (-1.5 MPa soil water potential) were respectively 28.0% and 12.0 % (v/v) at 0-30 cm, 30.2% and 13.7% at 30-60 cm, and 29.9% and 13.7% at 60-90 cm. Mean tree height and mean tree diameter (1.7 m high) were respectively 20 m and 22.6 cm in January 2013.

Tapping for latex production was initiated in 2003 in this plantation. Since then, the nine-month tapping season starts in early May and ends in January when defoliation and refoliation occur. Tapping consists in severing latex vessels by cutting the bark of the trunk downward over half-circumference spiral, allowing latex flowing out for three to five hours before the vessels are naturally plugged. Five trees that were tapped every

three days in half-spiral (1/2S d/3) and five never tapped trees were selected for this study. No ethylene-generating chemical was used to stimulate latex production.



Figure 10 The rubber plantation (RRIM 600 clone) used for the experiment, with the Rubber Flux Chachoengsao flux tower at the Chachoengsao Rubber Research Centre (CRRC), Chachoengsao, Thailand. (http://asiaflux.net/index.php?page_id=96)

b. Sampling

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 at regular four-hour intervals except in the middle of the night (no sampling at 3:00). Each sampling period encompassed one tapping day, except in April 2014 where the tapping season had not started yet. Trunk latex was collected by inserting a small plastic cannula (two mm inner diameter) into the bark of the trunk at 0.5 m above the soil for all trees (Fig. 11), which was 5 cm below the downward tapping cut for tapped trees. Latex was sampled below the tapping cut because this is the main latex drainage and latex regeneration area (Auzac, 1989). The two first drops were discarded and the two following drops were collected in an Eppendorf tube containing one ml of distilled water.

The tube was shaken and stored at 4°C until being processed. Four sun leaves (eight leaflets) from the four cardinal directions were sampled twice a day at 7:00 and 15:00 over a five-day period in the upper part of the crown by climbing each tree, except in May 2013 where no sample was collected. The leaf samples were immediately stored in a chilled box, brought back to a nearby laboratory, dried for two minutes in a microwave oven at 800 W and pooled as one sample.



Figure 11 Latex sampling, trunk latex was collected by inserting a small plastic cannula into the bark of the trunk 5 cm below the tapping cut.

Sample preparation for stable isotope analysis

Twenty µl of the latex solution was directly transferred in a 6 × 4 mm tin capsule and oven-dried over night at 50°C. Each leaf sample was ground to fine powder and one mg was weighed in a tin capsule for bulk leaf ^{13}C analysis. The leaf soluble compounds (including amino acids, organic acids, soluble sugars) was extracted by shaking 100 mg of leaf powder in a methanol/chloroform/water mixture (12/5/3, v/v/v). After centrifugation (10 min at 2000 g), the supernatant was mixed with methanol/chloroform (1/1, v/v). Water was added until phase separation occurred. The upper methanol/water phase was collected and evaporated. The dry extract was weighed, solubilized in water to a concentration of 30 g L^{-1} and 40 µl of this solution was pipetted into a tin capsule and

dried. All tin capsules were stored in a desiccator until analysis. The $^{13}CO_2/^{12}CO_2$ 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 ($\delta^{13}C$) was expressed relative to this reference:

$$\delta^{13}C_{sample} = [(R_{sample} / R_{standard}) - 1] \quad [3]$$

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 ‰.

Because this is the first study analyzing $\delta^{13}C$ in rubber tree leaves and latex, our methods were scrutinized for potential fractionation occurring during sample drying. An additional set of sample was collected on five trees. For each sample, a subsample was dried using our drying methods and a subsample was freeze-dried. The mean differences were 0.08‰ for latex and 0.03‰ for bulk leaves and they were not significant ($p>0.90$).

c. Inherent water use efficiency, rainfall and soil water stock.

Inherent water use efficiency (IWUE) at the ecosystem level was calculated according to Beer *et al*., (2009) from daytime integrated values of gross primary production (GPP) and evapotranspiration (ET), and mean daylight vapour pressure deficit (VPD):

$$IWUE = \frac{GPP}{ET} \times VPD \quad [2]$$

GPP and ET were calculated from half-hourly measurements of the net exchanges of carbon dioxide (NEE) and water vapour between the rubber stand and the atmosphere. CO_2 and H_2O fluxes were obtained from the eddy covariance between the fluctuations of vertical wind speed and air CO_2 concentration and water vapour measured at 20Hz, 6m above the canopy. Flux were calculated with the Edire software (University of Edinburgh, UK) and quality-checked according to the Euroflux procedure (Aubinet *et al*., 1999). Gap-filling and partitioning of NEE into GPP and Reco were performed using the on-line

platform hosted by the Max Planck Institute for Biogeochemistry in Jena. GPP was calculated as the difference between NEE and total ecosystem respiration, the latter being extrapolated from a fitted exponential relationship between night time CO_2 flux and air temperature (Reichstein *et al*., 2005).

Mean daylight VPD was calculated from air temperature and relative air humidity (CS215-L, Campbell Scientific, Inc., Logan, UT, USA) measured at the top of the eddy-flux tower along with rainfall (tipping bucket rain gauge ARG100, Campbell Scientific Inc). Soil water content was measured with 15 TDR probes (CS616,Campbell Scientific, Inc.) installed at 0.1, 0.3, 0.5, 0.7 and 0.9 m depths (three probes per depth) allowing us to calculate the soil water stock down to the depth of 1m. These data were collected every 30s and 30-min averages were stored on CR1000 dataloggers (Campbell Scientific, Inc).

d. Data analysis

Statistical analyses were performed using the statistical software R 2.15.1 (R Core Team 2012). Mixed-effect models were used to estimate the effect of the time of sampling, season and treatment (tapped and untapped) as fixed effects on $\delta^{13}C$ with trees as a random effect, using the 'lmer' function in the 'lme4' package (Bates *et al*., 2012). Contrasts were used to test relevant differences when the overall model was significant ($P < 0.05$). Linear models ('lm' function, 'stats' package) were used to examine the relationship between $\delta^{13}C$-S and either VPD or IWUE. Models including or not the treatment as a co-variable were further compared using the 'anova' function ('stats' package).

4. Results

a. Environmental conditions and inherent water use efficiency

The climate seasonality was markedly pronounced with six months of high rainfall (1425 mm), high mean daily air temperature (27.5°C), low mean daylight vapour pressure deficit (0.95 kPa) and high soil water stock (205 mm) from May to October while these values were respectively 248 mm, 26.1°C, 1.4 kPa and 165 mm from November to April (Fig. 12). The first sampling (6-10 May) was thus just after the transition between the dry and the wet season, the second sampling (19-23 August) was in the middle of the rainy season, the third sampling (25-29 November) at the beginning of the dry season and the last sampling (21-25 April, 2014) at the end of the dry season. The mean daylight vapour

pressure deficit was higher in May 2013 and April 2014 (1.49-1.45 kPa) than in August and November (1.03-1.0 kPa, Table 1). The inherent water use efficiency at ecosystem level (IWUE) was also higher in May 2013 and April 2014 (4.81-4.39 gC kPa $kg^{-1}H_2O$) than in August and November (2.27-2.42 gC kPa $kg^{-1}H_2O$, Table 6).

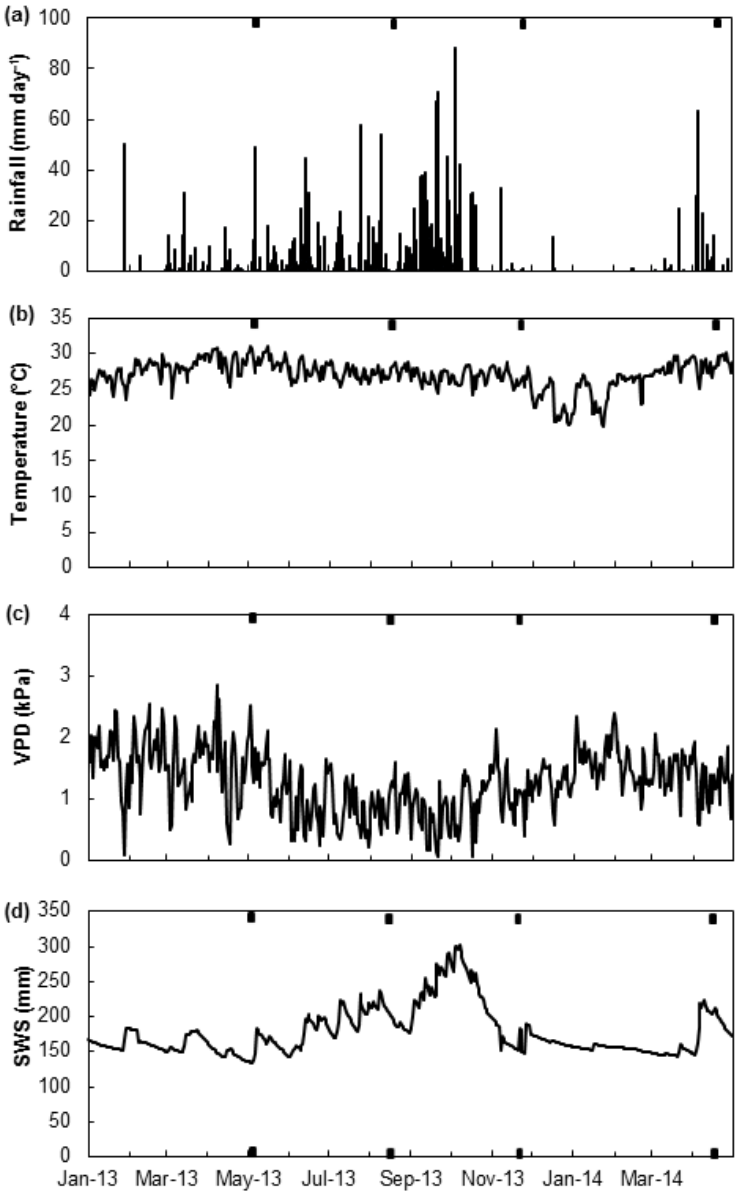


Figure 12 Seasonal courses of a) daily rainfall, b) mean daily air temperature, c) mean daylight vapour pressure deficit (VPD) and d) soil water stock (SWS)**.** The horizontal bars on show the four sampling periods. The area between the two dashed lines represents the available soil water. The major tick marks refer to the first of the month.

Table 6 Mean daylight vapour pressure deficit (VPD), soil water stock (SWS), inherent water use efficiency at ecosystem level (IWUE), carbon isotope composition of bulk leaf (δ^{13}C-B), of leaf soluble compounds (δ^{13}C-S) and of trunk latex (δ^{13}C-L) at four sampling seasons (leaves were not collected in May) in tapped and untapped twenty-year old rubber trees. Each value is an average (with standard error) of 45 (9 sampling times × 5 trees) replicates for leaves (bulk and soluble compounds) and 105 (31 sampling times × 5 trees) replicates for trunk latex.

		May, 2013	Aug., 2013	Nov., 2013	Apr., 2014
Ancillary data	VPD (kPa)	1.49	1.03	1.00	1.45
	SWS (mm)	168	190	175	190
	IWUE (kgC $kg^{-1}H_2O$ kPa)	4.81	2.27	2.42	4.39
Bulk leaf δ^{13}C (‰)	tapped trees	-	- 31.8 ± 0.1 a	- 31.2 ± 0.1 b	- 30.0 ± 0.1 c
	untapped trees	-	- 30.3 ± 0.1 *a	- 30.2 ± 0.1 *a	- 29.5 ± 0.1 *b
Leaf soluble compounds δ^{13}C (‰)	tapped trees	-	- 31.6 ± 0.1 a	- 30.3 ± 0.1 b	- 29.9 ± 0.1 c
	untapped trees	-	- 31.8 ± 0.1 a	- 29.6 ± 0.1 *c	- 30.1 ± 0.1 b
Trunk latex δ^{13}C (‰)	tapped trees	-28.2 ± 0.1 c	- 28.8 ± 0.1 a	- 28.9 ± 0.1 a	- 28.5 ± 0.1 b
	untapped trees	- 27.0 ± 0.1 *ab	- 27.0 ± 0.1 *a	- 27.1 ± 0.0 *a	- 26.8 ± 0.0 *b

Stars (*) indicate significant differences between untapped and tapped trees for a given season. For a given treatment, different letters indicate significant differences between sampling seasons. There was no significant effect of sampling time within a season and no significant interaction between sampling time and seasons or treatments.

b. Carbon isotope composition of bulk leaf (δ^{13}C-B) and leaf soluble compounds (δ^{13}C-S)

δ^{13}C-B differed significantly between tapped and untapped trees for the three sampling seasons (leaves were not collected in May 2013). δ^{13}C-B were lower in tapped trees (-30‰ to -31.8‰) than in untapped trees (-29.5‰ to -30.3‰), with the lowest values in the middle of the rainy season in August (Table 6). On average, the difference between the two treatments was 0.8‰.

The differences in δ^{13}C-S between tapped and untapped trees were less pronounced that the differences in δ^{13}C-B and were only significant in November (0.7‰). On average, the difference between the two treatments was 0.1‰. The leaf soluble compounds of tapped trees were slightly enriched in ^{13}C compared with bulk leaf (+0.4‰) while the opposite trend is observed for leaves of untapped trees (-0.3‰). Similarly to δ^{13}C-B, the lowest values of δ^{13}C-S were recorded in August in the middle of the rainy season in both treatments (Table 6).

Within each sampling period, there was no significant effect of the time of sampling (morning versus afternoon) and no clear trend over the five days for either δ^{13}C-B or δ^{13}C-S (Fig. 13) despite day-to-day variations in mean daylight vapour pressure deficit (VPD) and inherent water use efficiency (IWUE). The linear regressions between δ^{13}C-S and either VPD or IWUE were not significant ($p > 0.05$) except when November data were excluded (Fig. 14). The regression parameters were not different between the two treatments and the relation was stronger between δ^{13}C-S and IWUE ($R^2 = 0.73$) than between δ^{13}C-S and VPD ($R^2 = 0.41$).

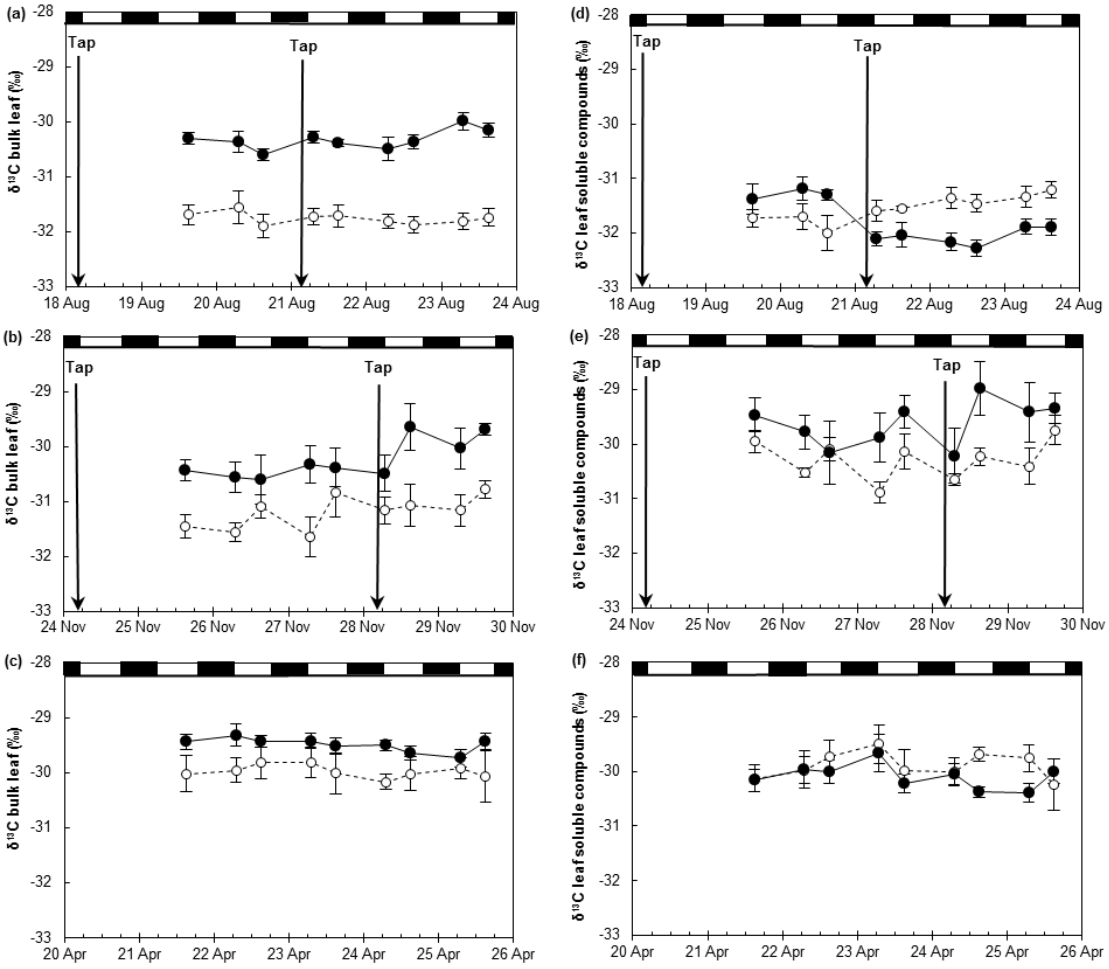


Figure 13 Mean carbon isotope composition of (a,b,c) bulk leaf (δ^{13}C-B) and (d,e,f) leaf soluble compounds (δ^{13}C-S) over the courses of five-day sampling periods in (a,d) August 2013, (b,e) November 2013 and (c,f) April 2014 for tapped (open symbols, dashed line) and untapped (closed symbols, solid line) twenty-year old rubber trees. Each value is an average (with standard error) of samples collected on 5 trees. The vertical arrows show the tapping times. Leaves were not collected in May 2013.

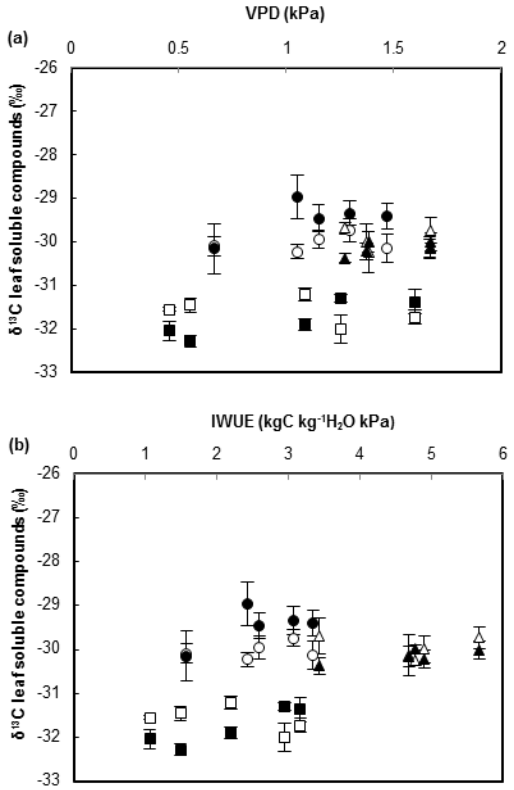


Figure 14 Relationships between carbon isotope composition of leaf soluble compounds (δ^{13}C-S) and a) mean daylight vapour pressure deficit (VPD), b) inherent water-use efficiency at the ecosystem level (IWUE) in August 2013 (squares), November 2013 (circles) and April 2014 (triangles) for tapped (open symbols) and untapped (closed symbols) twenty-year old rubber trees. For δ^{13}C-S, each value is a daily average (with standard error) of samples collected at 15:00 on 5 trees.

c. Carbon isotope composition of trunk latex (δ^{13}C-L)

A marked difference in δ^{13}C-L was observed between tapped and untapped trees whatever the season, even during the tapping rest. Trunk latex from tapped trees was more depleted than latex from untapped trees (1.6‰ on average, Table 6). Variations in δ^{13}C-L within each sampling period were not significant and did not exhibit any clear diurnal pattern (Fig. 15). Tapping events (arrows on Fig. 11) that occurred during all sampling periods except April did not have any clear imprint on δ^{13}C-L in tapped trees. δ^{13}C-L was more stable than δ^{13}C-S and δ^{13}C-B with a maximum seasonal difference of 0.7‰ for tapped trees (lowest values in August and November) and of only 0.3‰ for untapped trees (Table 6). The trunk latex was enriched compared with leaf soluble

compound by about 2‰ for tapped trees and 3.5‰ for untapped trees but the day-to-day variation in δ^{13}C-S was not recovered in δ^{13}C-L (Fig. 16).

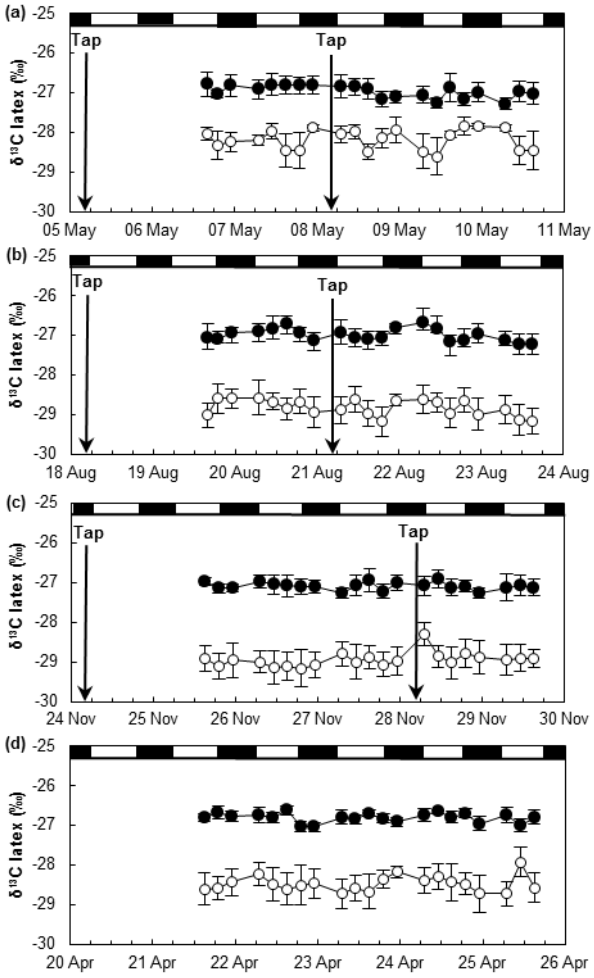


Figure 15 Mean carbon isotope composition of trunk latex (δ^{13}C-L) over the courses of five-day sampling periods in a) May 2013, b) August 2013, c) November 2013 and d) April 2014 in tapped (open symbols, dashed lines) and untapped (closed symbols, solid line) twenty-year old rubber trees. Each value is an average (with standard error) of samples collected on 5 trees. The vertical arrows show the tapping times.

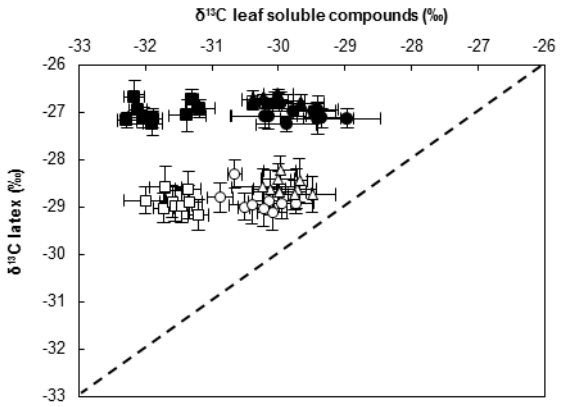


Figure 16 Relationship between carbon isotope composition of latex (δ^{13}C-L) and of leaf soluble compounds (δ^{13}C-S) in August 2013 (squares), November 2013 (circles) and April 2014 (triangles) in tapped (open symbols) and untapped (closed symbols) twenty-year old rubber trees. Each value is daily average (with standard error) of 10 (2 sampling times × 5 trees) replicates for leaf soluble compounds and 25 (5 sampling times × 5 trees) replicates for trunk latex collected on 5 trees.

5. Discussion

The isotope composition of leaf soluble compounds exhibited seasonal variations with more negative values in August during the rainy season in both tapped and untapped rubber trees. Soil water availability and vapour pressure deficit (VPD) are important environmental factors that affect stomatal functioning in higher plants and thus carbon isotope discrimination during CO_2 assimilation in C_3 plants (Cernusak *et al*., 2013), thus imprinting on the isotope composition of photosynthates (Brugnoli *et al*., 1988; Gaudillère *et al*., 2002). Thus, lower vapour pressure deficit and higher soil water availability during the rainy season, leading to low IWUE (2.27 gC kPa $kg^{-1}H_2O$), may have accounted for the 1.7‰ lower δ^{13}C-S in August as compared with values recorded in April at the end of the dry season (Table 6). A close relation was expected between δ^{13}C-S and IWUE across seasons because photosynthetic carbon isotope discrimination is related the intercellular CO_2 concentration (Farquhar *et al*., 1982). A discrepancy between IWUE and δ^{13}C-S was however observed in November (Table 6), where VPD was as low as in August but the soil water stock was much lower than in August and April. One reason for that might be that IWUE at ecosystem level accounts for whole canopy transpiration and evaporation at the soil surface while δ^{13}C-S was measured on sunlit

leaves from the top of the tree crown. Low soil water stock may have constrained stomatal conductance of sunlit leaves to avoid xylem pressure reaching the point of xylem dysfunction (Chen *et al*., 2010; Sangsing *et al*., 2004b).

δ^{13}C-S also exhibited day-to-day variations of 0.6 -1.2‰ at each sampling period (Fig. 12), but despite large day-to-day variations in weather conditions, the variations in δ^{13}C-S were not correlated to variations in mean daylight vapour pressure deficit or in IWUE (Fig. 14). One reason for that is probably that leaf soluble extracts contain other compounds such as organic and amino acids that are thought to have a higher residence time in leaves than photosynthetic products that are rapidly metabolized, respired or exported. In larch needles, the mean residence time of C was 2.2 - 2.3 days in sucrose and starch compared with 3.6 days in all sugars and sugar alcohols, and 6.1 in glucose (Streit *et al*., 2013). While the isotope signature of photosynthetic products would rapidly reflect environmental fluctuations, mixing of recent photosynthates in a larger pool of leaf soluble compounds would dampen and delay the isotopic signal in that pool (Wingate *et al*., 2010).

The fact that δ^{13}C-B was higher in April than at the two other sampling periods for both tapped and untapped trees may reflect the particular phenology of the rubber tree species that renews leaves every year over a period of one month: old leaves shed and new leaves unfold in January and early February (middle of the dry season) at our site (Chairungsee *et al*., 2013). Thus, leaves collected in April 2014 were grown during the dry season in contrast to leaves sampled in August and November. Low photosynthetic carbon isotope discrimination when leaf renewal occurs during the dry season may explain the high δ^{13}C-B of leaves sampled in April.

The difference in δ^{13}C between leaf soluble extracts and bulk leaf samples in tapped trees (from +0.1 to +0.9‰, Table 6) is consistent with the general pattern showing that leaf soluble compounds are often more enriched than the bulk leaf materials (Bowling *et al*., 2008). However, this pattern was not observed in untapped trees. At two sampling periods (August and April), leaf soluble compounds were more depleted than the bulk leaf materials. While δ^{13}C-S were within the same range for both type of trees, bulk leaves sampled on untapped trees were less depleted than those sampled on tapped trees at all seasons. This is more likely revealing an effect of tapping in the biochemical composition of the leaves, even if their nitrogen concentrations were similar (3.3%, data

not shown), rather than difference in intrinsic water use efficiency that would have also imprinted on the leaf soluble extract.

Several compounds are known to be depleted in ^{13}C compared with photosynthetic products. This is the case for soluble compounds such as organic acids, amino acids or polyols, and insoluble compounds such as lignin and lipids while cellulose and starch are typically enriched (Badeck *et al*., 2005; Bowling *et al*., 2008; Devaux *et al*., 2009). Poly (cis-1,4-isoprene) is produced from acetyl CoA through the cytosolic mevalonate pathways (Chappell, 1995). Compounds that derived from acetyl CoA are all expected to be isotopically depleted compared with photosynthetic products as a result of fragmentation fractionation that occurs during pyruvate decarboxylation (DeNiro and Epstein, 1977; Tcherkez *et al*., 2004). We would have expected leaves of untapped trees to be more depleted than leaves of tapped trees if more photosynthates are available for leaf secondary metabolism when the sucrose demand for latex synthesis in the trunk is low. However, little is known about all fractionation steps occurring throughout the cytosolic mevalonate pathways, and this also holds true for the alternative plastidic non-mevalonate pathway.

The latex collected from the trunk is less depleted in ^{13}C than either the leaf soluble compounds (Fig. 16) or the bulk leaf materials despite the expected fractionation occurring during the oxidation of pyruvate (see above). The synthesis of trunk latex occurs in cells localized in the inner bark of the tree (Hébant and De Faÿ, 1980), either from sucrose transferred from the foliage to the trunk inner bark via the phloem sap or from stored carbohydrates in the trunk (Eschbach *et al*., 1986; Tupý, 1989). Whatever the source of carbon that is used for rubber synthesis, one would expect this source to be enriched in ^{13}C compared with leaf soluble carbon to account for the observed enrichment of latex collected in the trunk. ^{13}C enrichment of phloem sap compared with leaf soluble carbon has commonly been observed in trees (Brandes *et al*., 2006; Damesin and Lelarge, 2003; Gessler *et al*., 2007; Hu *et al*., 2010; Kodama *et al*., 2008; Wingate *et al*., 2010). Similarly, stored carbohydrates such as starch are commonly enriched compared with leaf soluble carbon (Damesin and Lelarge, 2003; Maunoury-Danger *et al*., 2010). The ^{13}C enrichment of latex compared with leaf soluble carbon suggests that starch might be the primary carbon source for latex regeneration in tapped trees, which is consistent with an

increase in starch concentration in the wood of tapped trees that was shown to occur at the expense of radial growth (Chantuma *et al*., 2009; Silpi *et al*., 2007; Silpi *et al*., 2006).

The isotope composition of latex collected in trunk of untapped trees remained fairly constant across the seasons (0.3‰ variation) while the variation was more pronounced in tapped trees (0.7‰), exhibiting lower values during or just after the rainy season than at the end of the dry season (May 2013 and April 2014, Fig. 15). This suggests that latex is not naturally metabolized in the tree. It remains overtime in untapped trees while tapping for latex production implies a continuous de novo latex synthesis. The isotope composition of the new latex is thought to be influenced by the isotope signature of the carbohydrates used for its synthesis. The fact that the isotope composition of the leaf soluble compounds of tapped trees increased by 1.3‰ between the middle of the rainy season (August) and the beginning of the dry season (November) while latex $\delta^{13}C$ remained constant suggested a long lag for the photosynthetic isotope signal to be transferred into the trunk latex. This lag is much longer than the few days expected for photosynthate to reach the site of latex collection owing to the fast transfer of carbon in broadleaved tree species (Dannoura *et al*., 2011; Epron *et al*., 2012). This is reinforced by the lack of correlation between $\delta^{13}C$-S and $\delta^{13}C$-L within each sampling period. This also suggests that latex synthesis relies more on stored carbohydrates than on recent photosynthates, but ^{13}C pulse labelling on tapped and untapped trees in the field will be useful to strengthen this conclusion.

Like bulk leaf materials, trunk latex sampled on tapped trees were 1-2 ‰ more depleted than latex sampled on untapped trees at all seasons (Table 6). While the concentration in nitrogen and carbon of the latex could not be determined precisely in our experiment because the weight of latex used for the analysis was not known with enough precision, the C:N ratio, which is not biased by the weighing inaccuracy, revealed that trunk latex from tapped trees contained more nitrogen relative to carbon than the latex of untapped trees (C:N of 81 and 131 respectively, data not shown), which is consistent with high activity, of several enzymes in laticiferous cell cytoplasm of tapped trees (Tupý 1989). Tapping is known to activate metabolism in laticiferous cells (Annamalainathan *et al*., 2001; Jacob *et al*., 1989), showing large increase in potassium and inorganic phosphorus content while magnesium and sucrose contents decrease (d'Auzac 1989; Tupý, 1989). Our results are thus consistent with an effect of tapping on the biochemical

composition of the latex, but understanding the cause of latex ^{13}C depletion in tapped trees requires further compound specific isotope analyses.

6. Conclusion

Our study compared both daily and seasonal variations of carbon isotope composition of trunk latex, bulk leaf material leaf soluble compounds in tapped and untapped rubber trees in the field. As far as we know, there was no earlier study investigating carbon isotope composition of latex and its temporal variations. $\delta^{13}C$-L was more stable than $\delta^{13}C$-S and $\delta^{13}C$-B and $\delta^{13}C$-L of tapped trees exhibited more pronounced seasonal variations than latex collected from untapped trees, indicating a long residence time of rubber particles when trunk latex is not collected. However, variations in $\delta^{13}C$-L and $\delta^{13}C$-S were not correlated at a daily time scale and were delayed and damped at a seasonal scale. This suggests that recent photosynthates are mixed in a large pool of stored carbohydrates that are involved in latex regeneration after tapping. This has important consequences for the management of rubber plantations, as reserve dynamics could give insight on long-term effects of harvesting systems, in addition to latex diagnosis which gives insight on short-term changes in latex metabolism. Both bulk leaf materials and trunk latex of tapped trees were more depleted in ^{13}C than those sampled on untapped trees, suggesting that tapping affects not only the metabolism of the anastomosed laticiferous cells but also leaf metabolism. The use of compound-specific isotope analysis on leaf extracts, latex and phloem sap, combined with ^{13}C labelling of photosynthetic products, will provide new insights into source-sink relations involved in latex biosynthesis.

VI. CONCLUSION

The general question was "Is carbon isotope composition a relevant ecophysiological indicator of genetic variation in water use efficiency of rubber tree?" It was developed in 3 sub-questions addressed in 3 experiments.

In the first experiment, we studied the relationship between $\delta^{13}C$ and leaf level intrinsic water-use efficiency (WUE_i) to test if $\delta^{13}C$ predicts well clonal differences in leaf level water-use efficiency. Bulk leaf $\delta^{13}C$ and leaf gas exchange were measured on young saplings of 10 rubber clones growing in pot. WUE_i was estimated at low and high VPD_L. The correlation between $\delta^{13}C$ and WUE_i was positive and significant at high VPD_L only, but correlation coefficient was low which means the prediction of WUEi by $\delta^{13}C$ would have low precision. This may be because the range of $\delta^{13}C$ among 10 clones was narrow, from -29.9 ‰ in RRIT-CH-39-2085 to -31.5% in RRIT-CH-36-980.

In the second experiment, we wanted to know if there was genetic variability for $\delta^{13}C$ among genotypes of *Hevea brasiliensis*. The number of clones was higher than in the first experiment and their genetic origin wider. We analyzed the genetic variability of $\delta^{13}C$ in bulk leaf sampled both in rainy and dry seasons in a collection of 49 wild genotypes of rubber. There were large $\delta^{13}C$ variations among the genotypes at all seasons. $\delta^{13}C$ ranged between -33.3‰ to -30.0‰ in rainy season and -33.1‰ to -29.9 ‰ in wet season. $\delta^{13}C$ ranking among clones was rather stable with a good correlation between data obtained on leaves collected during both the rainy and the dry seasons. Moreover, the average $\delta^{13}C$ was significantly different between two genetic clusters previously identified in this population. This study showed that the genetic variability of $\delta^{13}C$ was promising for breeding if a good correlation between $\delta^{13}C$ of leaf and WUE was established. However, $\delta^{13}C$ value in the Amazonian population that we tested are more negative than those observed on the already available commercial clone. Thus improving WUE by breeding new clones will first require identifying genotypes that exhibit less depleted composition in ^{13}C.

In the first and second experiment, we have studied carbon isotope in bulk leaf samples. In the third experiment, we investigated the relation between $\delta^{13}C$ in leaf and $\delta^{13}C$ in latex. We first checked whether latex $\delta^{13}C$ was related to leaf $\delta^{13}C$ in tapped and

untapped trees. Then we asked whether latex $\delta^{13}C$ can be used as a proxy of WUE, with the idea that latex $\delta^{13}C$ would be spatially integrated (at tree rather than at leaf level), that trunk latex is easier to collect on a large number of tall trees (collecting a representative sample of leaves requires climbing trees) and that latex $\delta^{13}C$ will provide higher temporal resolution if the latex production relies directly on carbohydrates produced by foliage photosynthesis. There was no earlier study investigating carbon isotope composition of latex and its temporal variations. We found that latex $\delta^{13}C$ ($\delta^{13}C$-L) was more stable than the latex $\delta^{13}C$ in leaf soluble compounds ($\delta^{13}C$-S) and in bulk leaves ($\delta^{13}C$-B). $\delta^{13}C$-L of tapped trees exhibited more pronounced seasonal variations than latex collected from untapped trees. However, variations in $\delta^{13}C$-L and $\delta^{13}C$-S were not correlated at a daily time scale and was delayed and damped at a seasonal scale. This has important consequences on the management of rubber plantations, as reserves dynamics could give insight on long-term effects of harvesting systems, in addition to latex diagnosis which gives insight on short-term changes in latex metabolism. We also found that both bulk leaf materials and latex of tapped trees were more depleted in ^{13}C than those sampled on untapped trees, suggesting that tapping affects not only the metabolism of the anastomosed laticiferous cells but also leaf metabolism. The use of compound-specific isotope analysis on leaf extracts, latex and phloem sap, combined with ^{13}C labelling of photosynthetic products, will provide new insights into source-sink relations involved in latex biosynthesis.

All together, the results showed that to make $\delta^{13}C$ a good predictor of WUE_i in rubber tree we need to test the relationship between $\delta^{13}C$ and WUE_i on a larger number of clones. The isotopic composition of bulk leaf material may not reflect the current gas exchange properties because it is influenced by the climate conditions that were prevailing when they were growing and getting mature, and then unlinked to current WUE_i. Working on leaf soluble carbohydrate may be more relevant (Farquhar *et al.*, 1982). However, we could not exclude from our third experiment that variation of bulk leaf $\delta^{13}C$ among genotypes was not only driven by differences in WUE_i but also by differences in biochemical composition of leaves. In such case, the leaf would not only reflect fractionation during photosynthesis that was linked to WUE_i but also post photosynthetic fractionation that occurs in leaves.

The fact that rubber trees are propagated by grafting buds of selected clones onto rootstocks grown from seeds is also a source of uncontrolled variability. The genetic origin of the rootstocks is either completely unknown or just partially controlled if the seeds are collected in monoclonal plots, where at least the mother parent is known. Moreover, as roots and root-shoot interactions can greatly influence water use efficiency at plant scale (Struve *et al*., 2006), and rubber trees are propagated by grafting buds of selected clones onto rootstocks grown from seeds is also a source of uncontrolled variability. The genetic origin of the rootstocks is either completely unknown or just partially controlled if the seeds are collected in monoclonal plots, where at least the mother parent is known. This will limit the range of the knowledge obtained from studies at leaf level and highlights the need to consider also the genetic variation of properties of the root system of rootstock materials by identifying traits that are contributing to the whole tree water use efficiency.

This thesis was the first study about carbon isotopic composition of latex. It was limited to bulk natural latex without separation of latex compounds. The next step will be 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 of climate conditions, tree phenology and tapping intensity.

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Is carbon isotope composition a relevant ecophysiological indicator of genetic variation in water use efficiency of rubber trees?

Abstract The rubber (*Hevea brasiliensis*) plantations extend to non-traditional area in Thailand where dryer conditions has been reported to impair the growth of rubber trees and latex production. Physiological parameters helpful for breeding adapted genotypes are required, such as water use efficiency (WUE). Carbon isotope discrimination is widely used as a proxy for WUE that can easily be used for selection and breeding programs for drought tolerance**.** Leaf $\delta^{13}C$ and leaf gas exchange were measured on young saplings of 10 rubber clones growing in pot in a common garden. The range of leaf $\delta^{13}C$ among 10 clones was narrow and the correlation between $\delta^{13}C$ and WUE_i was significant under high vapour pressure deficit only, which means the prediction of WUE by $\delta^{13}C$ would have low precision. There were large $\delta^{13}C$ variations among the genotypes at all seasons in a collection of 49 wild genotypes of rubber in Northeastern Thailand. $\delta^{13}C$ was rather stable with a good correlation between rainy and dry season. The genetic variability of $\delta^{13}C$ is promising for breeding if a good correlation between $\delta^{13}C$ of leaf and WUE can be established. The lack of correlation between $\delta^{13}C$ of latex ($\delta^{13}C$-L) and of leaf soluble compounds ($\delta^{13}C$-S) collected from tapped and untapped 20 year-old rubber trees suggests that recent photosynthates are mixed in the large pool of stored carbohydrates that are involved in latex regeneration after tapping. Thus $\delta^{13}C$ of latex is not a relevant indicator of WUE of rubber trees.

Keywords Carbon isotopes, *Hevea brasiliensis*, rubber tree, water use efficiency, selection and breeding

La composition isotopique en carbone est-elle un indicateur écophysiologique pertinent de l'efficience d'utilisation de l'eau de l'hévéa ?

Résumé : Les plantations d'hévéa (*Hevea brasiliensis*) s'étendent vers des zones non traditionnelles de production en Thaïlande où des conditions plus sèches ont pu conduire à une diminution de la croissance des arbres et de la production de latex. Des paramètres physiologiques utiles pour sélectionner des génotypes adaptés sont nécessaires, comme l'efficience d'utilisation de l'eau (WUE). La discrimination isotopique du carbone est largement utilisée comme proxy pour WUE et peut être aisément utilisée dans des programmes de sélection pour la tolérance à la sécheresse. $\delta^{13}C$ des feuilles et les échanges gazeux foliaires ont été mesurés sur de jeunes plants de dix clones d'hévéa cultivés en pot dans une pépinière. La gamme de $\delta^{13}C$ des feuilles entre ces dix clones était restreinte et la corrélation ente $\delta^{13}C$ et WUE_i était significative que sous fort déficit de pression de vapeur saturante, ce qui signifie que la prédiction de WUE par $\delta^{13}C$ serait peu précise. Il y avait une large gamme de $\delta^{13}C$ entre les génotypes dans une collection de 49 génotypes sauvages d'hévéa cultivés dans le nord-est de la Thaïlande en saison sèche et en saison des pluies. $\delta^{13}C$ était relativement stable avec une bonne corrélation entre les saisons. Cette étude montre que la variabilité génétique de $\delta^{13}C$ est prometteuse pour des futurs programmes de sélections si une bonne corrélation entre of $\delta^{13}C$ et WUE peut être établie. L'absence de corrélation entre de $\delta^{13}C$ du latex ($\delta^{13}C$-L) et des composés solubles extraits des feuilles ($\delta^{13}C$-S) prélevées sur des arbres saignés et non saignés âgés de 20 ans suggère que photosynthétats récemment produits se mélangent à un stock important d'hydrate de carbone impliqués dans la régénération du latex après la saignée. Donc, $\delta^{13}C$ du latex n'est pas un indicateur pertinent de WUE.

Mots-clefs : Isotopes du carbone, *Hevea brasiliensis*, efficience d'utilisation de l'eau, sélection