Changes in $^{13}$C/$^{12}$C of oil palm leaves to understand carbon use during their passage from heterotrophy to autotrophy

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The carbon isotope composition of leaf bulk organic matter was determined on the tropical tree *Elaeis guineensis* Jacq. (oil palm) in North Sumatra (Indonesia) to get a better understanding of the changes in carbon metabolism during the passage from heterotrophy to autotrophy of the leaves. Leaf soluble sugar (sucrose, glucose and fructose) contents, stomatal conductance and dark respiration, as well as leaf chlorophyll and nitrogen contents, were also investigated. Different growing stages were sampled from leaf rank −6 to rank 57. The mean values for the $\delta^{13}$C of bulk organic matter were $-29.01 \pm 0.9\%$ for the leaflets during the autotrophic stage, $-27.87 \pm 1.08\%$ for the petioles and $-28.17 \pm 1.09\%$ for the rachises, which are in the range of expected values for a C$_3$ plant. The differences in $\delta^{13}$C among leaf ranks clearly revealed the changes in the origin of the carbon source used for leaf growth. Leaves were $^{13}$C-enriched at ranks below zero (around $-27\%$). During this period, the ‘spear’ leaves were completely heterotrophic and reserves from storage organs were mobilised for the growth of these young emerging leaves. $^{13}$C-depletion was then observed when the leaf was expanding at rank 1, and there was a continuous decrease during the progressive passage from heterotrophy until reaching full autotrophy. Thereafter, the $\delta^{13}$C remained more or less constant at around $-29.5\%$. Changes in sugar content and in $\delta^{13}$C related to leaf ranks showed an interesting similarity of the passage from heterotrophy to autotrophy of oil palm leaves to the budburst of some temperate trees or seed germination reported in the literature.

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The oil palm (*Elaeis guineensis* Jacq.) is one of the most productive tree crops, presenting a very high yield (40 t FFB ha$^{-1}$; FFB: fresh fruit bunches) related to its high photosynthetic activity (from 23 to 30 $\mu$mol m$^{-2}$ s$^{-1}$) for a C$_3$ plant$^{1,2}$ and an important carbon allocation to fruits (fruits grouped in ‘bunches’). Seasonal variation in bunch production occurs in relation to climatic factors and internal trophic conditions (e.g. reserve carbon pool size and photosynthesis). As the annual bunch production is crucial for planters, studies into new methods of dealing with its potential links between leaf photo-assimilates, trunk carbon pool and bunches, they did not involve a precise investigation of existing carbon fluxes. Therefore, there was a clear need for a more thorough investigation of the carbon allocation pathways at the tree scale.

A collaborative project (ISOPALM*) was thus initiated on the oil palm in North Sumatra, focusing on carbon bunch filling at the tree level inside the ‘leaf-trunk-fruits’ system. ISOPALM aims at studying three main issues: changes in carbon use during leaf development, trunk reserve mobilisation, and metabolic changes linked to oleosynthesis during fruit maturation. In the present work, which has also been investigated in adult trees.$^{3,5}$ Starch (located mainly in the trunk top) and glucose (in the trunk bottom) were identified as the main components in the trunk.$^5$ The size of the reserve carbon pool might drive the bunch filling (in the case of a high reserve) or provoke abortion (in the case of a low reserve).$^{3,8}$ Although these studies revealed potential links between leaf photo-assimilates, trunk carbon pool and bunches, they did not involve a precise investigation of existing carbon fluxes. Therefore, there was a clear need for a more thorough investigation of the carbon allocation pathways at the tree scale.

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*ISOPALM: Ecophysiological Study in Oil Palm Yield using Carbon Isotope Discrimination*, collaborative project between IOPRI, CIRAD and UPS XI.
corresponds to the first part of ISOPALM, we focused on the changes in the carbon source during leaf growth. Oil palm leaves play a major role not only for photosynthesate production, but also in that they may affect bunch production through variations in leaf emission rates.\textsuperscript{10} Oil palm presents a specific growth pattern with a continuous succession of leaf buds from an apical meristem, twice a month on average. The oil palm leaf has a considerable growth duration (up to 35 months) consisting of four developmental stages: initiation (duration of about 24 to 25 months), fast elongation (4 to 5 months), full opening of the leaf (half a month), and the functional phase (from 16 to 24 months). Leaves are identified at each developmental stage by a rank number in the crown. During the full opening stage, which also corresponds to an important mass acquisition and to the development of the petiole and rachis, the leaf acquires its autotrophy. As competition between heterotrophic leaves (named ‘spears’) and other sink organs (roots, trunk, bunches) could lead to a decrease in bunch production, all metabolic indications bearing on the switch of leaves from heterotrophy to autotrophy would thus have to be investigated in light of oil palm phenology.

We have thus investigated carbon use during leaf growth using stable carbon isotopes ($^{13}C/^{12}C$). The natural abundances of stable carbon isotopes have already been used to study photo-assimilate allocation and reserve mobilisation in trees\textsuperscript{11} as well as whole plant carbon allocation patterns.\textsuperscript{12,13} Recent studies\textsuperscript{13–17} on carbon isotope discrimination during post-photosynthetic processes have strengthened the idea that allocation pathways could be deduced from the observation of isotopic signature variations among organs. Concerning carbon allocation, behind each organ growth and development there are carbon fluxes between autotrophic sources and heterotrophic sink tissues. During their respective evolution, they depend on each other’s activity. Growth and development of autotrophic tissues are performed using the reserve carbon pool, which is filled in return by photosynthetic source activities. Because spatial and temporal variations in the $^{13}C/^{12}C$ ratio of organic matter integrate all metabolic and diffusion/transport processes in relation to environmental conditions, these variations could be used to investigate the sink-source relationship.

Accordingly, two main hypotheses were tested in the present study (i.e. first stage of the ISOPALM project). The first is that $\delta^{13}C$ would decrease with increasing leaf age with a respective pattern for leaflets, petioles and rachises; a study performed by Badeck et al.,\textsuperscript{14} based on data from more than 80 species, revealed that heterotrophic organs were significantly $^{13}C$-enriched compared with autotrophic ones. It was also shown in some trees and herbs\textsuperscript{17} that leaf laminae are $^{13}C$-depleted compared with main veins. Different hypotheses\textsuperscript{18–20} are currently being tested\textsuperscript{13,14,21–23} in order to determine the origin of such a difference in carbon isotope composition between heterotrophic and autotrophic tissues. Our aim was to test this difference between the autotrophic (leaflets) and heterotrophic (rachis and petiole) parts of oil palm leaves during their development.

The second hypothesis deals with changes in carbon use during autotrophic acquisition of photosynthetic organs. We aimed at comparing oil palm leaf functioning during passage from heterotrophy to autotrophy in tropical conditions, with two contrasting examples such as bean seedling ontogeny under controlled conditions and beech tree budburst in the temperate zone. For both examples, this fundamental process involves a switch between reserve mobilisation and photosynthesis onset. The onset of leaf photosynthesis probably plays an important role in the $^{13}C$-depletion of leaf organic matter.\textsuperscript{13} Damesin and Lelarge\textsuperscript{12} showed a $^{13}C$-enrichment in leaf organic matter of beech trees at budburst due to reserve mobilisation from the trunk and a subsequent $^{13}C$-depletion after the onset of leaf photosynthesis. Such a pattern has also been observed in tree rings with early-wood being $^{13}C$-enriched while the late-wood formed from photo-assimilates was $^{13}C$-depleted.\textsuperscript{24} More recently, a progressive $^{13}C$-depletion in leaf organic matter was elegantly demonstrated during leaf autotrophy acquisition in bean seedlings.\textsuperscript{13} We thus expected a variation in carbon isotope composition of leaf organic matter during leaf development in oil palm reflecting a progressive change in the relative contribution of the remobilised carbon reserves ($^{13}C$-enriched) and the new photo-assimilates ($^{13}C$-depleted).

Because of the considerable duration of leaf growth mentioned above, it is difficult to follow changes in the functioning of the leaf during its development. However, the position of leaves in the crown (i.e. leaf ranks) offers a complete sequence of leaf developmental stages on the same tree and at the same time. In order to test the above hypotheses, we thus investigated the carbon isotope composition of leaf organic matter sampled from different leaf ranks of oil palm trees. Leaf sugar, chlorophyll and nitrogen contents, leaf dark respiration, and stomatal conductance measurements were also carried out as an aid to the interpretation of the respective $\delta^{13}C$ evolutions in leaflets, petiole and rachis bulk organic matter, during the passage from heterotrophy to autotrophy, and the functioning of this process, in oil palm, compared with other $C_3$ plants. At the young stages, leaves being heterotrophic will probably reflect the isotopic signature of the reserve metabolites (e.g. starch in the trunk or sucrose, generally $^{13}C$-enriched),\textsuperscript{11,13} remobilised for the development of young leaves. During autotrophy acquisition, carbon coming from photosynthesis, which will be more $^{13}C$-depleted, will change the signature of the growing leaves with a difference within leaflets, petioles and rachises. In this work, we aimed at understanding such a change in the isotopic signal of oil palm during leaf growth and especially around rank one (Fig. 1) in relation to carbon use.

**EXPERIMENTAL**

**Plant material and ecological conditions**

Samples were taken during two periods, from February to June 2003 and from May to July 2004, on clone material *Dura x Pisifera* (Deli x La Mé type: MK 60: LM007T x DA 128 D) at Aek Pancur Research Station (3° 30′ N, 98° 48′ E; 25 m above sea level, North Sumatra, IOPRI, Indonesia).Ten oil palm trees planted in 1995 in a genetic trial were used for the
experiments. Each leaf was sampled on one occasion during either the first or the second period. The ‘trees’ could then be considered as repetitions where the factor rank was concerned. For some experiments, more than one measurement was carried out on the same leaf (see below and the figure legends for details).

During the experiment, the oil palm trees were around 8–9 years old and thus considered to be adult. The climatic requirements for maximum yield are a daily temperature between 22–24°C (minimum) and 29–33°C (maximum), a solar radiation of about 15 MJ m⁻² day⁻¹ and an annual rainfall greater than 2000 mm. In North Sumatra, the climatic characteristics present on average a solar radiation of 16 MJ m⁻² day⁻¹, a mean annual temperature of 25°C and an annual total rainfall around 2800 mm well distributed over the year. The clone trees sampled were typical of a Deli x La Mé cloned material chosen as ‘control’ in many genetic trials planted in Sumatra by IOPRI (Indonesian Oil Palm Research Institute). Phenological recordings were started at the same time with the observations of dates of each developmental stage of all incremented units (one unit was composed of a leaf and its adjacent inflorescence).

During phenological recordings, the oil palm leaves were numbered as ‘ranks’ according to their emergence from the crown centre (Fig. 1). The leaves are organised in eight spirals. Leaf ‘rank 1’, starting point of the ‘spiral 1’, is the youngest visible leaf (the leaf reaches ‘rank 1’ when 2/3 of the leaflets are individualised) and it is considered to be the first ‘functional’ (i.e. autotrophic) leaf by the planters. The leaf emission rate (from 15 to 21 leaves per year) depends mainly on water availability (a dry period will provoke an accumulation of spear leaves), temperature (low temperature will decrease the growth rate) and also probably on the carbon reserve at tree level.

The leaves were sampled from rank –6 (small spear leaf) to rank 57 (old leaf) representing different developmental stages and thus different ages. The leaves being large (around 6 m² per leaf), each leaf from was divided into ten segments from the base to the tip of the rachis (delimited by two specific points, C and A, Fig. 2) following the same methodology as for leaf area determination. Only one leaflet was collected per segment (total for 1 leaf: 10 leaflets collected), then mixed and cut into small pieces. The rachis was also divided in ten segments and completely collected. Each petiole was cut into small pieces and collected. The spear leaves (from rank –6 to 0) were entirely cut into small pieces to facilitate powdering. All the samples were then carefully disinfected by ozone, put directly into liquid nitrogen and stored at –80°C before being dried in an oven for a minimum of 2 days at a temperature of just below 80°C. The samples were then ground (Type MM200, Retsch, Haan, Germany) to obtain a fine powder for carbohydrate content measurements and isotopic analyses. Sampling was always carried out before 11:00 am.

**Carbohydrate extraction**

The finely ground powder was used for carbohydrate extraction following the methodology already described. Briefly, 100 mg of each sample were suspended in distilled water in Eppendorf vials and maintained for 20 min in ice.
After centrifugation, the supernatant containing the water-soluble fraction was used for soluble sugar measurements and the pellets for starch extractions. Quantification of individual soluble sugars (sucrose, fructose and glucose) was performed by HPLC (high-pressure liquid chromatography). The water-soluble fraction was filtered (filter HV 0.45 μm type, Millipore, Molsheim, France) before injection into the HPLC system. For each sample, 25 μL of the filtered extract were injected into a Sugar Pak-1 column (6.5 mm diameter and 300 mm length, Waters, Guyancourt, France). The calibration curves established with standard sugars allowed quantification of the individual sugar content for each sample. For starch purification, the pellet was suspended in HCl (6 N) to solubilise the starch which was then precipitated using methanol. Carbon isotope analyses were then carried out on purified and freeze-dried starch.

**Carbon isotope analyses**

The carbon isotope composition of bulk organic matter of the leaflets, petioles and rachises as well as of the starch extracted from the leaflets was determined using an NA-1500 elemental analyser (Carlo-Erba, Milan, Italy) coupled to an isotope ratio mass spectrometer (VG Optima, Micromass, Villeurbanne, France). The carbon isotope compositions were calculated as the deviation of the carbon isotope ratio (13C/12C, called R) from the international standard V-PDB: 

$$\delta^{13}C = 10^3 \frac{[R_{sample} - R_{standard}]}{R_{standard}}.$$ 

A laboratory reference compound (glutamic acid) was measured every 12 samples in order to correct for any offset of the mass spectrometer.

**Dark respiration and stomatal conductance**

Dark respiration was measured using a semi-closed system connected through a magnesium perchlorate desiccant to a LCA2 type CO2 infrared gas analyser (IRGA) (Analytical Development Co., Hoddesdon, UK). An entire leaflet still inserted into a respiration chamber (PVC tube – drainpipe) opaque to sunlight, with a volume of 0.01163 m3. This respiration chamber was well ventilated by two fans placed outside the chamber, around point B (Fig. 2), was totally connected to the IRGA. Air was pumped three times successively at 3 min intervals. The respiration chamber was connected to the IRGA. Air was pumped three times successively at 3 min intervals. The respiration rate was obtained from the increase in CO2 concentration between each time interval, divided by the area of the leaflet included in the chamber. Leaf ranks 9-10, 17, 25 and 33, belonging to one tree, were investigated during the daytime after shade acclimation. Around 15 measurements were performed per leaflet. After respiration measurement, the leaflet was sampled for leaf area determination. Measurements of leaf diffusive resistance for water vapour were made with a model LI-1600 steady-state porometer (Li-Cor Inc., Lincoln, NE, USA) along spiral 1, from 7.00 to 10.00 am on attached leaflets. For these measurements (around 400, with at least 50 measurements per rank except for leaf rank 49), leaflets around point B were chosen with regard to their potential for maximal gas exchanges. The rectangle in Fig. 2 indicates the upper third part of the leaflets, which were inserted into the porometer chamber during the measurements.

**Leaf chlorophyll and nitrogen contents**

The chlorophyll content of leaflets was measured with a portable SPAD-502 (Konica Minolta Sensing, Inc., Paris, France) on four trees, on spiral 1 (leaf ranks 1, 9, 17, 25, 33, 41 and 49) with six repetitions per leaf. The results were expressed in SPAD units because no direct leaf chlorophyll analysis calibration was available for oil palm. The SPAD unit is calculated based on the amount of light transmitted by the leaf in two wavelength regions (blue: 400-500 nm, and red: 600-700 nm) in which the absorbance of chlorophyll is different. The leaf nitrogen content was measured on the same samples according to the usual routines for oil palm ‘leaf diagnosis’ (nutrient status of oil palm tree determination from leaf nitrogen content) was carried out.

**Biomass of leaf parts**

In order to follow the general growth pattern of oil palm leaves, the leaf biomass measurements were completed by dissection of an oil palm crown, carried out on one tree of about 12 years old (Quencez, unpublished results), but similar to the material used for the above measurements. Total leaf sampling was done from leaf rank –2 to 9, then 17, 25 and 33. The leaflets (all of them), rachises and petioles were then dried and weighed separately as explained above.

**Statistical analyses**

Mixed analysis of variance tests were performed on δ13C (on leaflets, petioles and rachises) with the ranks (all leaf ranks studied), dates (2003, 2004) and their respective interactions as ‘fixed effects’ and with the ‘trees’ (10 trees) nested into the ranks and their interaction with ‘dates’ nested into the ‘ranks’ as ‘random effects’ (mixed procedure, SAS Inc., Cary, NC, USA). Mean comparisons were performed with the ‘LSD’ test with $P < 0.05$.

**RESULTS**

**Leaf growth**

Since the leaves in a crown were at different developmental stages, we were able to establish, by dissection of the whole crown of an oil palm tree, the general growth pattern of the leaflets, petioles and rachises as a function of the leaf rank (Fig. 3). The evolution of the whole leaf dry weight followed a classical logistic curve ($Y = 3.8/(1 + (1.01 \exp(-0.43 \text{ Leaf rank})))$) from rank –2 to rank 9 (from –12 to –2: simulated dotted line). The whole leaf dry weight of ranks 17 and 25 appeared to be lower than that of rank 9 due to the general trend of oil palm growth showing an increase in the leaf weight along years followed by a stabilisation when the tree became adult. The spear leaf (including rachises and leaflets) started to grow quickly at around rank –12 and reached the maximum weight at rank 0 (1.8 kg dry weight (DW)). Then the leaflets and the rachises were individualised corresponding to leaf replenishment at rank 1 (rachis: 1.1 kg DW; leaflets: 1.03 kg DW) with the spear leaf becoming a leaf. The petiole
started to grow quickly at rank −2 (23 g DW) until rank 2 (24% of DW increase per rank) then continued to grow slowly until rank 9. At rank 9, the petiole had a higher DW (1.5 kg DW) than the rachis (1.1 kg DW) and the leaflets (0.95 kg DW). The total leaf weight (including petiole, rachis and leaflets) reached a maximum value at rank 8 (3.6 kg DW). The main difference in growth rate between the leaf parts was observed after the heterotrophic stage with a 53% increase in DW for the petiole biomass but only a 9% increase for the rachis, while the leaflets stopped growing at rank 1. While the maximum growth for all other leaf parts occurred from rank −2 to rank 1 during the passage from autotrophy to heterotrophy, by its growth after this transition, the petiole could enhance light interception by the leaf.

Dark respiration and stomatal conductance

Clear general trends for dark respiration ($R_d$) and stomatal conductance ($g_s$) were observed across leaf ranks (Figs. 4(A) and 4(B), respectively). For stomatal conductance, a maximum value was observed for leaf rank 1 ($149 ± 15.5$ mmol m$^{-2}$s$^{-1}$), with a plateau until rank 10 and then a progressive decrease until reaching almost complete stomatal closure ($g_s = 8 ± 1.5$ mmol m$^{-2}$s$^{-1}$) around rank 50 (Fig. 4(B)). A linear relation between $g_s$ and the leaf rank was obtained ($g_s = -2.40$ (Leaf rank) + $136.1$, $r^2 = 0.91$). For the dark respiration, a maximum value was observed for rank 10 ($2.25$ μmol m$^{-2}$s$^{-1}$), and there was then a net decrease until rank 33 with a minimum value of $0.3$ μmol m$^{-2}$s$^{-1}$ (respiration data for early and late stages are missing). The initial decrease in respiration was quite fast, i.e. more than 50% from rank 10 to rank 17.

Leaf nitrogen and chlorophyll contents

The nitrogen and chlorophyll contents measured on the leaves of spiral 1, on four trees, showed different evolutions as a function of the leaf rank (Fig. 4(C)). The leaf nitrogen content had a maximum at rank 9 ($0.07%$, n = 24), then showed a constant decrease until rank 41 ($0.23%$, n = 24). The SPAD measurements, indicating the chlorophyll content of the leaves, showed an opposite pattern with a clear increase until a maximum value at rank 33 ($74.8 ± 3.1$ SPAD unit, n = 24). The minimum value was found for the youngest leaf ‘rank 1’ ($52.01 ± 7.17$, n = 24). For the older leaves, from rank 25 to 49, there was a decrease in the nitrogen content of the leaves but no decrease in the chlorophyll content.
from rank -6 to rank -2. During the heterotrophic phase, both sucrose and glucose amounts decreased rapidly reaching minimum values (almost zero) at rank 0, while the decrease for fructose (Fig. 5(C)) was smooth and its minimum value at rank 0 was higher (3.9 ± 0.5 mg g⁻¹ DW). During autotrophy acquisition, from rank 0 to 1, a sharp increase was observed for all the sugars (at rank 1: glucose = 6.23 ± 0.53 mg g⁻¹ DW; fructose = 13 ± 0.96 mg g⁻¹ DW; sucrose <1 mg g⁻¹ DW) followed by another smoother increase from rank 1 to 2. At the autotrophic period, a transient plateau was observed between rank 2 and 5 (total sugars: around 30 mg g⁻¹ DW) before the sugar content increased again (Fig. 5(D)). Maximal values (except for a surprising peak for fructose at rank 6 of 26 ± 0.72 mg g⁻¹ DW) were observed for glucose and sucrose at rank 25 (around 23 mg g⁻¹ DW), while, for sucrose, a plateau was reached at leaf rank 30 (3.0 mg g⁻¹ DW). A slight decrease was then observed at rank 42 for fructose and glucose.

**Carbon isotope signature of leaf starch**

The isotopic composition of the starch remained almost unchanged across leaf ranks with a mean δ¹³C value of −25.6 ± 0.4‰ (Fig. 6(A)). A relatively higher δ¹³C content in starch was observed at rank −3 (−24.8 ± 0.4‰) during the rapid growth stage of the leaf. A slight δ¹³C-depletion was then noticed until rank 1 reaching −26.1 ± 0.8‰, significantly different (P < 0.03) from rank −3. After the passage to autotrophy, despite a high value at rank 9 (−24.7‰), there was a slight increase and then a stabilisation around −25.5‰. The slightly higher value at rank 9 should be viewed with caution because there was no replication for this rank.

**Carbon isotope signature of bulk organic matter**

The δ¹³C values (means ± SD) measured on the bulk organic matter of the leaflets, rachises and petioles were plotted against leaf ranks (Figs. 6(A), 6(B), and 6(C)). A net decrease in the isotopic signature of the leaflets was observed across the ranks. This variation with ranks was significant (Table 1) with P < 0.0001, but not the variation between the trees sampled (random effect: Z = 1.38 n.s. (not significant)). No date effect (P = 0.6 n.s.) was observed (Table 1), indicating no statistical difference between the samples analysed in 2003 and 2004.

Interestingly, three distinct functional phases emerged from δ¹³C evolution (Fig. 6(A)) among leaf ranks: (i) the heterotrophic phase between rank −6 and 0, with the highest δ¹³C values around −27‰; (ii) the switch from heterotrophy to autotrophy, corresponding to a sharp decrease between rank 0 (δ¹³C of −27.8 ± 0.3‰) and rank 2 (δ¹³C of −28.7 ± 0.4‰); and (iii) the full autotrophic phase of the leaves, starting at rank 2 during which δ¹³C remained low although a surprising δ¹³C-enrichment was noticed from rank 12 to 18 with a maximum value at rank 17 (−28.4 ± 0.4‰) significantly different (P < 0.07) from rank 21 to 57. After rank 21, the leaflet δ¹³C slightly decreased before stabilising around −29.8‰ until the oldest leaf analysed (rank 57).

The rachises and petioles (Figs. 6(B) and 6(C)) presented the same pattern as the leaflets, with a δ¹³C-depletion across leaf ranks. A net but not significant (Tables 2 and 3) decrease

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**Figure 5.** Variation of soluble sugar contents across the leaf ranks. The total soluble sugar content (D) is the sum of the individual sugars analysed using HPLC: sucrose (A), glucose (B) and fructose (C) in mg per g of leaflet DW. Data points correspond to the mean values per leaf rank and the error bars correspond to ± SD (n = 97 sampled from 10 trees). White symbols correspond to the heterotrophic phase, grey symbols to the rank 1 and black symbols to the leaf autotrophy.
was observed for rachis and petiole from rank 1 until the last leaf rank analysed (49 for rachises and 41 for petioles). The δ^{13}C values for petiole and rachis were not determined during the heterotrophic phase because of the difficulty in separating the leaf parts. At rank 1, the δ^{13}C values of the rachis (−27.4 ± 0.4‰) and the petiole (−27.3 ± 0.45‰) were slightly higher than the value of the leaflets at the same rank and thus close to those of the leaflets at the heterotrophic stage. For the petioles, the δ^{13}C first rapidly declined from rank 3 to 9 (−29.01‰) and then remained unchanged until rank 42, while rachises presented first a stable feature until rank 9 (−27.8 ± 0.4‰) followed by a δ^{13}C-depletion until rank 15 (−29.3‰), then remained constant until rank 49. The rachises and petioles remained δ^{13}C-enriched compared with leaflets for all leaf ranks except between ranks 15 and 25, corresponding to the δ^{13}C-enrichment of the leaflets around rank 17.

**Figure 6.** Variation of the carbon isotope composition (δ^{13}C) of starch (A: triangles) and of organic matter (OM) across leaf ranks (A: circles, B and C). Circles correspond to δ^{13}C of leaflets (A), rachises (B), and petioles (C). In C, leaflets (solid line) and rachises (dotted line) are presented again for comparison. Data points correspond to the mean values per leaf rank and the error bars correspond to ± SD; A, starch: n = 65 sampled from seven trees. For leaflet OM (A), n = 376, and 24 ranks were sampled from ten trees. For rachises (B), n = 84, and 16 ranks were sampled from nine trees, and for petioles (C), n = 57, and 16 ranks were sampled from nine trees. White symbols correspond to the heterotrophic phase (A), grey symbols (A, B and C) to the rank 1 and black symbols to the autotrophic phase.

**Table 1.** Analysis of variance: mixed procedure with SAS 9.1 performed on δ^{13}C of OM of the leaflets

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Num DF: degrees of freedom of the numerator, Den DF: degrees of freedom of the denominator.

n.s.: non significant; ***: significant at P < 0.001.

**Table 2.** Analysis of variance: mixed procedure with SAS 9.1 performed on δ^{13}C of OM of the petioles

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Covariance parameter estimates

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Num DF: degrees of freedom of the numerator, Den DF: degrees of freedom of the denominator.

n.s.: non significant; +: significant at P < 0.05; **: significant at P < 0.01.

**Table 3.** Analysis of variance: mixed procedure with SAS 9.1 performed on δ^{13}C of OM of the rachises

<table>
<thead>
<tr>
<th>Effect</th>
<th>Num</th>
<th>Den</th>
<th>F</th>
<th>P &gt; F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ranks</td>
<td>15</td>
<td>18</td>
<td>1.16</td>
<td>n.s.</td>
</tr>
<tr>
<td>Dates</td>
<td>1</td>
<td>10</td>
<td>0.97</td>
<td>n.s.</td>
</tr>
<tr>
<td>Ranks’Dates</td>
<td>4</td>
<td>18</td>
<td>0.31</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

Covariance parameter estimates

<table>
<thead>
<tr>
<th>Parm cov</th>
<th>Z</th>
<th>P (Z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trees(Dates)</td>
<td>1.53</td>
<td>n.s.</td>
</tr>
<tr>
<td>Trees’Dates(Ranks)</td>
<td>0.07</td>
<td>n.s.</td>
</tr>
<tr>
<td>Residual</td>
<td>3.80</td>
<td>***</td>
</tr>
</tbody>
</table>

Num DF: degrees of freedom of the numerator, Den DF: degrees of freedom of the denominator.

n.s.: non significant; ***: significant at P < 0.001.
DISCUSSION

The main objective of the present work was to study carbon use during the leaf growth of oil palm trees. The leaf position in the crown (i.e. leaf rank) being a direct identifier of leaf age in oil palm trees, we were able to investigate a complete sequence of leaf ages. We measured the carbon isotope signature of bulk organic matter, which integrates the leaf growth conditions, across the leaf ranks in order to examine the changes in leaf functioning during ontogeny and mainly during the switch from heterotrophy to autotrophy. In addition, we measured other leaf physiological characteristics (i.e. soluble sugar contents, nitrogen and chlorophyll contents, stomatal conductance and respiration), which bring important indications on carbon use during leaf growth. Three main stages (i.e. leaf heterotrophy, transition phase and leaf autotrophy) with significant changes in carbon isotope signature of leaf organic matter related to changes in carbon metabolism and leaf functioning were revealed, which are discussed to relate these to already known processes on other plants. Differences in $^{13}$C between leaflets, rachises and petioles are also discussed in relation to post-photosynthetic discriminations and autotrophy versus heterotrophy functioning.

$^{13}$C-enrichment during leaf initiation and elongation: the heterotrophic phase before rank 1

Different studies have already shown that leaf development in oil palm trees is initially very slow, then becomes faster during elongation at rank $-12$ when the leaf grows very quickly from 10 cm to 6 m long in 4-5 months. The present work showed that, during the end of the elongation phase, the leaf sugar contents were very low presumably because of their use for leaf growth. The rapid increase in spear leaf biomass required a carbon allocation presumably from both heterotrophic organs (trunk apex) and autotrophic ones (older functional leaves), which needed a strong gradient for sugar transfer. The decrease in the leaf sucrose concentration (from 3.5 mg g$^{-1}$ at rank $-6$ to 0 mg g$^{-1}$ DW at rank 0) suggested that sucrose participates strongly in leaf growth and respiration during this fast elongation stage. Sucrose could either come from the degradation of starch, located at the top of the trunk, or directly from the trunk apex which showed a high concentration of sucrose compared with glucose and fructose.

The higher $^{13}$C of leaf organic matter (OM) (around $-27\%$, close to the $^{13}$C value of starch) observed during this stage reinforced the hypothesis that the trunk might supply $^{13}$C-enriched sugars (sucrose and starch are known to be $^{13}$C-enriched compared with OM) to the heterotrophic spears. Other studies on the oil palm trunk have revealed an inverse gradient for glucose (increasing gradient towards the bottom trunk) compared with sucrose, suggesting a putative role for glucose in reserve mobilisation (for the root system development). The high concentration of glucose usually found at the periphery of the trunk central cylinder could indicate that glucose participates in the construction of the leaf sheath that encircles the stem apex. In fact, oil palm behaved as other trees, when comparing our results with those of other authors, with regard to reserve remobilisation which is considered to be a key process in the metabolism of perennial trees during the growth of heterotrophic organs.

Another hypothesis is that adult autotrophic leaves (source) supplied carbon to young heterotrophic leaves with photosynthetic carbohydrates originally $^{13}$C-depleted, which were then enriched in $^{13}$C during remobilisation and transportation, as suggested by many authors. Fructose was also believed to participate in leaf growth: its concentration in heterotrophic leaves was higher (from 3 to 7 mg g$^{-1}$ DW) than that of other sugars. It might also participate through important translocation, perhaps from the first 12 autotrophic leaves to the leaves under elongation. Similar features were observed in temperate trees before bud formation and during budburst. A slight $^{13}$C-depletion of about 1% in leaf starch from rank $-3$ ($-24.8\%$) to rank 0 ($-26\%$) up to rank 1 ($-26.1 \pm 0.8\%$) was observed, which was unusual compared with what happens during budburst of temperate trees, where $^{13}$C-depletion occurs after the growing phase. This indicated that the starch measured during the heterotrophic phase could have been a product of a progressive mixing of increasing amounts of $^{13}$C-depleted photo-assimilates stemming from adult leaves (1 to 4 containing high starch concentrations: 60 mg g$^{-1}$ DW) to carbohydrates originating from the trunk reserve ($^{13}$C-enriched).

In addition, it should be highlighted that for heterotrophic organs, some (re)fixation of CO$_2$ by PEPC could change the isotopic signal of these organs. PEPC activity has been shown to be high in heterotrophic organs, which could explain at least partly the higher $^{13}$C of OM in such organs (spear leaves in the present case) than in autotrophic ones.

Switch from heterotrophy to autotrophy at rank 1 strongly changes leaf isotopic signature

This transition stage clearly showed rapid changes in leaf functioning, i.e. metabolism, reserve remobilisation, growth and photosynthetic activity. These changes occurred between rank 0 and rank 1 when the leaf reached its maximum length and all the leaflets progressively opened, starting at the tip. The chlorophyll content was not at its maximum rate at rank 1 but started to increase rapidly. During this phase, leaf growth might be very sensitive to environmental conditions (drought) and internal trophic state. Interestingly, the leaflets and rachises reached their maximum biomass at rank 1, whereas the petioles increased both in length and biomass. All the parts were then completely individualised, i.e. separated. However, the leaves remained fragile because of a lack of lignification, achieved after rank 2. Leaflets opened their stomata, located at the abaxial side of the lamina, and started to assimilate CO$_2$ at rank 1, i.e. thus becoming autotrophic.

Photosynthesis increased rapidly, depending on leaf N content, which was high, when chlorophyll, rather low at that time, started to increase. A rapid transition was observed.
during this phase with a significant decrease in the $\delta^{13}C$ of leaf OM compared with the $^{13}C$-enriched level of the heterotrophic phase, indicating the carbon newly produced by photosynthesis for leaf growth. The amounts of individual sugars, which were at their minimum level, strongly increased from rank 0 to rank 1, as a result of photosynthetic activity. The stomatal conductance, maximal at rank 1, indicated a very low resistance to CO2 diffusion in the leaf during this short period. The high photosynthetic discrimination against $^{13}C$, expected to be high, related to maximal stomatal conductance was in accordance with the model developed by Farquhar et al. The $\delta^{13}C$ of OM at rank 1 was $-27.7 \pm 0.3\%$. This resulted from a combination of sugars mobilised during the growth and the beginning of the photosynthetic activity with a limitation by the Rubisco sugars mobilised during the growth and the beginning of the leaf OM compared with the $^{13}C$-enriched level of the sources during this short phase.

The higher $\delta^{13}C$ values observed for rachises and petioles remained surprisingly constant; it became slightly $^{13}C$-enriched from rank 0 and remained $^{13}C$-enriched even after rank 9. The stability of the starch isotopic signature meant that the origin of the starch pool might have been the same for all leaf ranks. $^{13}C$-enrichment in starch has been known to result from isotope fractionation during aldolase reaction, i.e. the hexoses formed in the chloroplasts and consequently the starch synthesised have been $^{13}C$-enriched, while the trioses left behind which are then transported to the cytosol and form the sucré were $^{13}C$-depleted.38 According to the model developed by Tcherkez et al.39 the isotope effect of aldolase, and thus the $^{13}C$ enrichment in transitory starch, increases with the increase in starch synthesis rate (i.e. hexose flux to starch synthesis compared with sucrose). We did not measure the starch content in leaves, but the observed $^{13}C$-enrichment in leaf starch and its stability despite the $^{13}C$-depletion in leaf OM could indicate a high starch synthesis in these fully autotrophic leaves. Earlier studies5,6 have shown a high starch concentration in all leaves with a gradient from young leaves (around 60 mg g$^{-1}$ DW for rank 1 to 4) to older leaves (around 40 mg g$^{-1}$ DW for rank 23–25). The high and constant leaf sugar contents during this stage could indicate that the leaves, still photosynthetically active, synthesised the starch and sugars which could be partly transported to the sink organs but maintained a high sugar content, thus ensuring the sugar gradient between the source (leaves) and the sink (bunches, roots, trunk and/or young growing leaves) organs.

The higher $\delta^{13}C$ values observed for rachises and petioles than for leaflets (except between ranks 15 and 25 where leaflets showed a surprising $^{13}C$-enrichment) were in agreement with published data concerning the generally observed $^{13}C$-enrichment in heterotrophic compared with autotrophic organs14,33 as well as in veins compared with laminae.17 This could be due either to the heterotrophy of the veins (the rachis in the case of the oil palm leaf) and petioles compared with laminae (leaflets) or probably to a higher anaplerotic activity in petioles and rachises similar to the reported data, for instance the C4-like functioning of the vascular photosynthetic cells in celery and tobacco petioles,40 and the high level of PEPc in the young twigs of beech and some other tree species.41 This could be one of the main reasons for $^{13}C$-enrichment in the veins, petioles and rachises of C3 plants.
Changes in $^{13}$C/$^{12}$C of oil palm leaves

Surprising changes in isotopic signature around leaf rank 17

One point which still remains to be elucidated is the surprising $^{13}$C-enrichment of the leaflets between rank 15 and 25 (with a peak at rank 17), while the $^{13}$C-content of petioles and rachises remained almost unchanged from rank 12 around their minimum values. It could be because of the change in the orientation of the leaf, from the vertical to around their minimum values. It could be because of the change in the orientation of the leaf, from the vertical to horizontal position. The position of the leaf in the crown and thus the rate of light interception could also affect its isotopic signature. Simulated incoming radiation at each leaf rank revealed an interesting gradient between leaves 0 and 34 in relation with sun elevation. Maximum radiation interception was observed between ranks 15 and 20 (around 3% intercepted light per rank), with leaf rank 34 still intercepting around 1% of incoming radiation. The high leaf N content between ranks 15 and 20 could indicate a high photosynthetic capacity, as already observed for the oil palm, of these leaves despite a relatively lower stomatal conductance. This should decrease the leaf internal CO$_2$ concentration and thus the photosynthetic discrimination, explaining the observed $^{13}$C-enrichment in leaflet OM. Thereafter, a slowdown in leaf $^{13}$C was noticed with the value remaining quite low even in older leaves similar to the value of rank 57. This observation concerned the general pattern of $^{13}$C variation with leaf age. Older leaves contained more chlorophyll than younger ones indicating their acclimation to low photon flux densities. Further investigations on photosynthetic activity and C$_i$/C$_a$ (ratio of intercellular to ambient CO$_2$ concentrations) together with light incidence at each leaf rank are needed to confirm this explanation.

CONCLUSIONS

The $^{13}$C of leaf OM is considered to reflect mainly the isotope fractionation associated with photosynthetic carbon fixation. However, recent studies clearly demonstrated the existence of post-photosynthetic discriminations, which could potentially affect the carbon isotope signature of organic matter explaining the isotopic differences between autotrophic and heterotrophic organs/tissues. Accordingly, the description of the passage from heterotrophy to autotrophy of the oil palm leaves revealed significant changes in their carbon isotope composition: (i) heterotrophic spear leaves (< rank 0) were $^{13}$C-enriched because of the remobilisation of reserve carbon from source organs (e.g. $^{13}$C-enriched carbohydrates from the trunk); (ii) when spears became leaves at rank 1 (i.e. leaflet replenishment), a rapid transition occurred with a $^{13}$C-depletion of OM and an increase in chlorophyll content; and (iii) autotrophic leaves were $^{13}$C-depleted suggesting they were mainly using photosynthates. Our results also confirmed the $^{13}$C-depletion in autotrophic tissues (leaflets) compared to heterotrophic ones (rachis and petiole) already reported for other species. But we showed huge changes during leaf development. The present results on the use of the carbon isotope composition for investigating the leaf growth and development at plant scale for oil palm open new insights into the carbon metabolism and allocation studies of other sink organs such as trunk, roots and bunches. The study of leaf growth was an important step, as it concerns the functioning of the trunk apex responsible for oil palm growth. The present data reveal interesting similarities in the way carbon is managed in oil palm leaves during the set up of functional photosynthetic capacities as compared to other C$_3$ plants, and suggests that developing heterotrophic leaves are mainly relied on reserve pools for their carbon supply.

In addition, our results suggest that when using bulk organic matter of leaves for ecological (carbon balance at ecosystem level) or agronomical (isotopic signature as indicator of leaf water use efficiency) studies, the difference between tissues and the developmental stages should be taken into consideration (see also Badeck et al. in this issue).

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