2006 International Oil Palm Conference
Commodity of the past, today, and the future
June 19-223, 2006

AGRONOMI

SESSION II

* Application of carbon isotope discrimination on sugars and organic matter for identifying leaf rank Physiological Differences in SINK-Source Metabolism in Oil Palm

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Emmanuelle Lamade, Setiyo I. Eko, Razak Purba, G. Simangunsong, Sébastien Girard, Ghashghaie Jaleh, Max Hill, and Gabriel Cornic

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IOPRI

CIRAD

Oral Communication : Full paper

Application of carbon isotope discrimination on sugars and organic matter for identifying leaf rank physiological differences in sink-source metabolism for oil palm.

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Summary

For the first time, **carbon isotope discrimination** $^{13}C/^{12}C$ is used for oil palm to investigate precisely carbon allocation from source organs to sink 's one and to identify metabolites belonged to a **reserve pool** , involved *in fine* in the **filling of the bunches**. **Isotope signature** is measured first on organic matter which is supposed to integrate total metabolism pathways during the elaboration of the organs. Then, same processes are done on soluble sugars (sucrose, fructose, glucose), starch and lipids. This project IOPRI-CIRAD-UPS XI is composed by three main experimentations (i) the study of the link from **heteretrophy** to **autotrophy** during the **leaves** development (ii) the **spatial** and

seasonal variations of metabolites and their isotope signatures on different parts of **the trunk** (iii) the study of the **oleosynthesis** during fruits maturation. Only the first part (i) is presented in this work which reveals that **carbon isotope** is a very pertinent tool to follow carbon assimilate inside each organs and may be of great help to found the main factor which is responsible for the **sex ratio** at individual scale including genetic and environment effects.

Key words : carbon isotope discrimination,oil palm, carbon allocation, reserves, sink-source organs,

Introduction

Carbon isotope composition was used, for the first time on oil palm, *Elaeis guineensis* Jacq. in North Sumatra (Indonesia) to get a better understanding of the major ecophysiological keys involved in the growth and development of this tropical tree and more particularly on **carbon allocation to the bunches and the use of reserves** (Lamade, 2003, Lamade et al. 2004). Some studies shows already that isotope discrimination or composition (Fig. 1) can be used as a tracer to study whole plant carbon allocation patterns (Arndt & Wanek 2002).

This study is a further step to an on-going research joint programme with CIRAD/UPS and IOPRI on the elaboration of a specific phenological model (***PHENOPALM***) based on the seasonal variations of the internal trophic level and the inter-organ competition for carbon supply (Lamade et al. 2002, Lamade et al. 2006). Oil palm is a monoïc crop which present alternatively male/female/abortion reproduction cycles, depending on endogenous factors, genetic components and environmental conditions. The yield is a function of the whole plant carbon allocation. Natural isotope marking at dry matter components level and metabolites (sugars, starch, lipids) could be useful to follow preferential pathways for carbon allocation from source supply to sink organs.

Already some recent studies dealed with the use of carbon isotope as a tracer for assimilate allocation and reserve mobilisation (Damesin & Lelarge, 2003) and we are attended to follow them.

Normally, the isotope composition is specific at tree level and organ level (Ghashghaie et al. 2002). Because preliminary references as the trends of variation in the isotope

composition of organs (leaves, stem, roots, and fruits) are needed, first investigations beard on **leaves and more specifically the passage from heterotrophy to autotrophy.** During the change from heterotrophy to autotrophy, expression of photosynthesis and growth are suspected of evolution. At young stage leaves will most probably present a rich $\delta^{13}C$ composition; The leaves are the initial source of any carbon entering the plant but the main question to answer is to **identify the origin and localisation** (trunk, petiole, leaf directly) of the **substrates** used during the filling of each fruit. Former study (Scheidecker,1954; Scheidecker et al.,1958) conducted on carbohydrates composition of all organs revealed, for the leaves, a strong evolution from the young to the senescent leaves: a high content of soluble and insoluble sugars (respectively 3.5 % of DM, 6.1 % of DM) for the young leaves (from rank 1 to 4), as well as for reductor sugars (3.1 % of DM) compared to the oldest (rank 23-25: soluble and insoluble, 3.2 %-4.3 % of DM and 1.7 % of DM for reductor 's one). By looking at the carbon isotope composition during the growth of each leaf, question as the identification of the substrates used (photoassimilates or reserve stock) for each step could be solved.

Other organs as oil palm trunk were well investigated first by Henson et al (1999) in Malaysia, especially on soluble sugars, carbohydrate, starch and acid-hydrolysable polysaccharides. They found changes with height (from the bottom at 1.06 m to the top, 4.6) with only sugars (137 mg g^{-1} DM to 285 mg g^{-1} DM at 4 m). Starch is maximal in the middle of the trunk (77 mg g^{-1} DM at 2.26 m) whereas no special trends could be noticed for other acid-hydrolysable polysaccharides. The oil palm trunk was suspected to be the main reserve organ. A second part, undertaken in this work is to follow carbon isotope composition of metabolites **along the trunk height and during climatic seasonal variation.** For the trunk , it is very important to evaluate the variation along years also and to identify possible influence of the meteorological seasonal variation on the isotope composition (or discrimination) according to other works as Schleser et al. (1999) or Dupouey et al. (1993) or Porte and Loustau (2001) and more recently Damesin & Lelarge (2003). The oil palm trunk is suspected, until now to be the main reserve organ (Dufrêne, 1989; Breure, 1987).

Isotope discrimination variation during fruit maturation of sugar/lipid ratio during its maturation could be useful to identify the substrate used to fill bunches (use of reserves) following the work of Gebbing et al (1998) and those of (DeNiro M.J. & Epstein S. 1977). Oleosynthesis as far as energy cost is concerned, is a very important sink for oil palm (the cost can be estimated at around 130 kg of CH_2O per year per tree).
Starch seems to play a role in the apparition of the lipid in the mesocarp but this question is still controversial and need further investigations. The use of the isotope composition of each element could permit to eliminate this uncertainty.

IOPRI-CIRAD-UPS project

Carbon isotope discrimination :
« a method to investigate : (1) the transpiration yield of the dry matter production and (2) the use of the reserves in oil palm

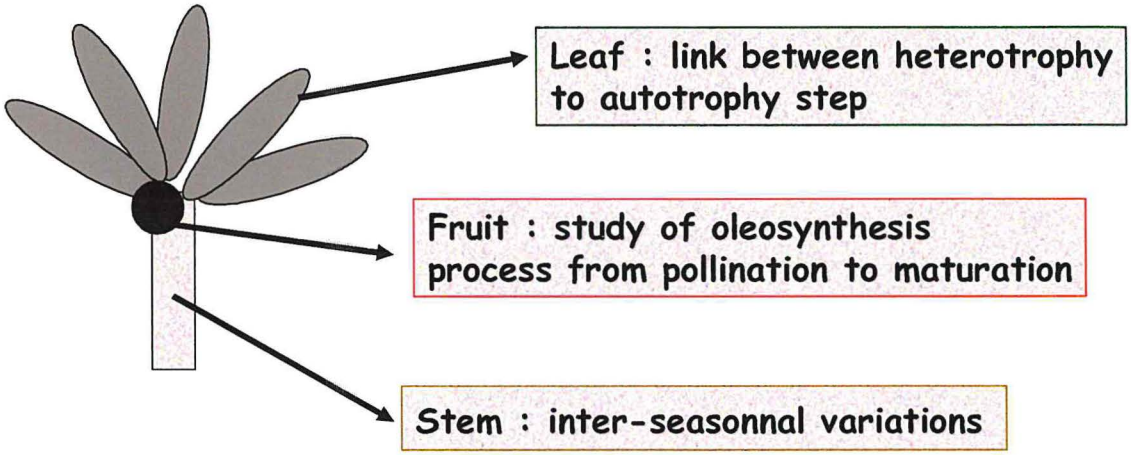


Fig. 1. General programme for the use of carbon isotope discrimination to investigate oil palm carbon metabolism (Lamade, 2003).

Our investigations about the use of natural carbon isotope ($^{13}C/^{12}C$) have been started on oil palm trees in Aek Pancur Research Station (IOPRI) where first biomass sampling have be realised on clone MK60. No previous study on oil palm may serve as a reference, preliminary works was to identify first sources of variation of the isotope composition or discrimination at the organ scale. The main point is to understand the substrate (carbohydrates) which is principally involved in the organogenesis process for oil palm. For the leaf, emphasis was beard on the passage from **heterotrophy to autotrophy**.

For this first step, the study is not involving the root system. Differences within organs (leaflets, rachis, petiole, fruits, stem) and for one organ within development stage (leaf rank, degree of maturation of the fruit, stem height) are expected: O'Leary (1981) noted differences between $*^{13}C$ of photosynthetic tissue and woody one (around 2-4 ‰), suggesting a fractionation step between leaves and wood. To determine carbohydrates reserve locations, we need to get information about specific fractionation process for oil palm at each organ level. This first part is involved in a complete programme aims to map carbon isotope discrimination of specific metabolites

and dry matter at tree level for identify the main substrat and its origin (apical meristem zone, "heart" ....) and secondary to complete the metabolite pathways conducting to,first, the filling of the bunches then involve in the variation of inflorescence cycles and aborption phenomenom.

Materials and methods

Generality about stables isotopes

The main biological elements (C, N, O, H...) do exist under different isotope status (Fig. 2). Natural abundance is variable within different organic compartments (for example carbone) and inorganic ones of the biosphere (atmosphere, flora, fauna, C3 and C4 plants). Also this variation can be observed within individuals, organs and then among molecules belonging to the same organism.

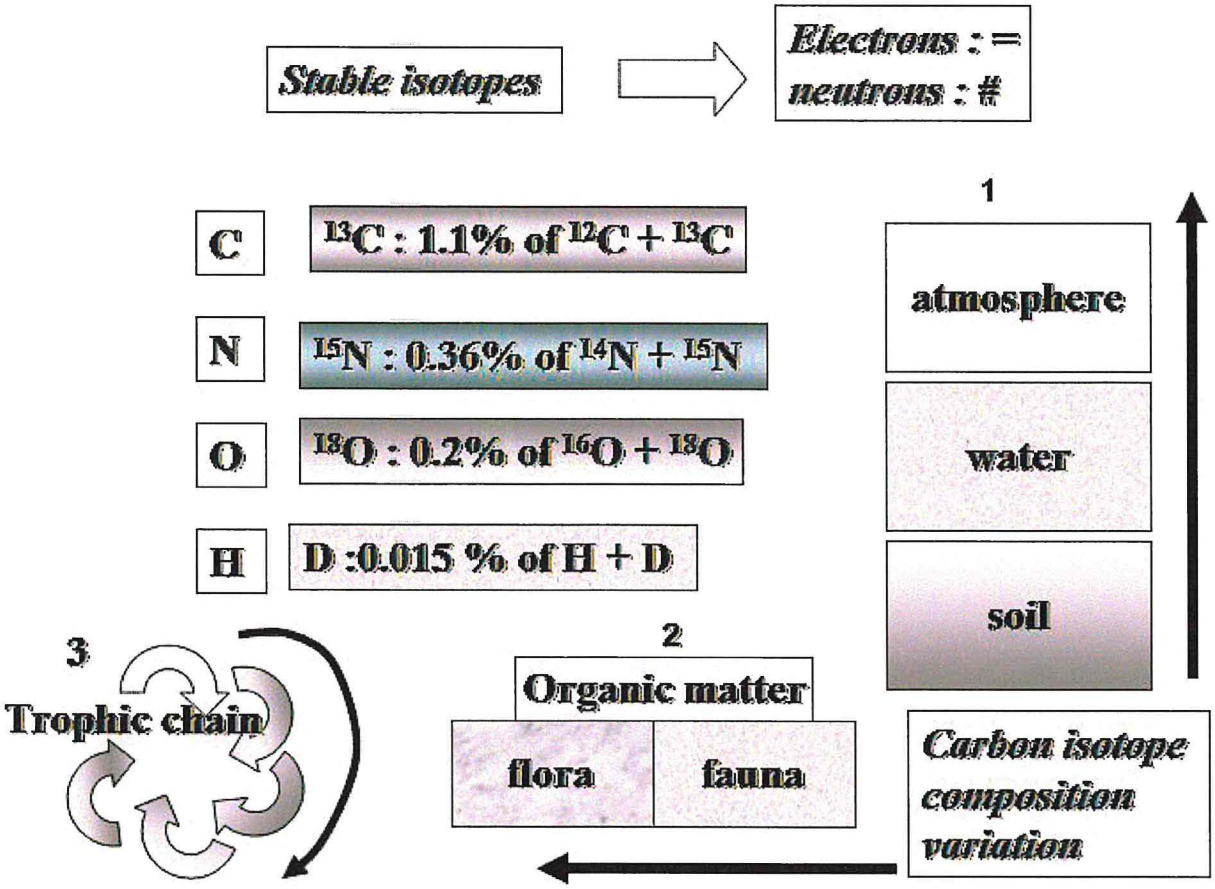


Fig. 2. Variation and proportion of natural stable isotopes (C,N,O,H) in the Biosphere, in Organisms and along Trophic Chain (from Ghashghaie et al., 2001).

This variation are due to isotopic "fractionation" or "discrimination". The global fractionation during photosynthesis (Fig. 2) will shows the isotopic signature of the limiting stage. Stable isotopes could be considered as very powerfull to analyse the biological functioning of plants at different scale from cells to ecosystems. It is a permanent dating on long term of the photosynthetic products and the reserves pools used further for the development of any plants.

C3 and C4 plants could be easely distinguished by their isotope signature. For C4 plants (Sugarcane, Maize, Sorghum, Millet....) , PEP carboxylase is the enzyme of the first carboxylation , Δ will be around 3-4‰ less than for C3 plant (Wheat, Barley, Rice, Potatoes, **Oil Palm**, Sugarbeet..) ,Rubsico is concerned, wih Δ around 19-20 ‰.

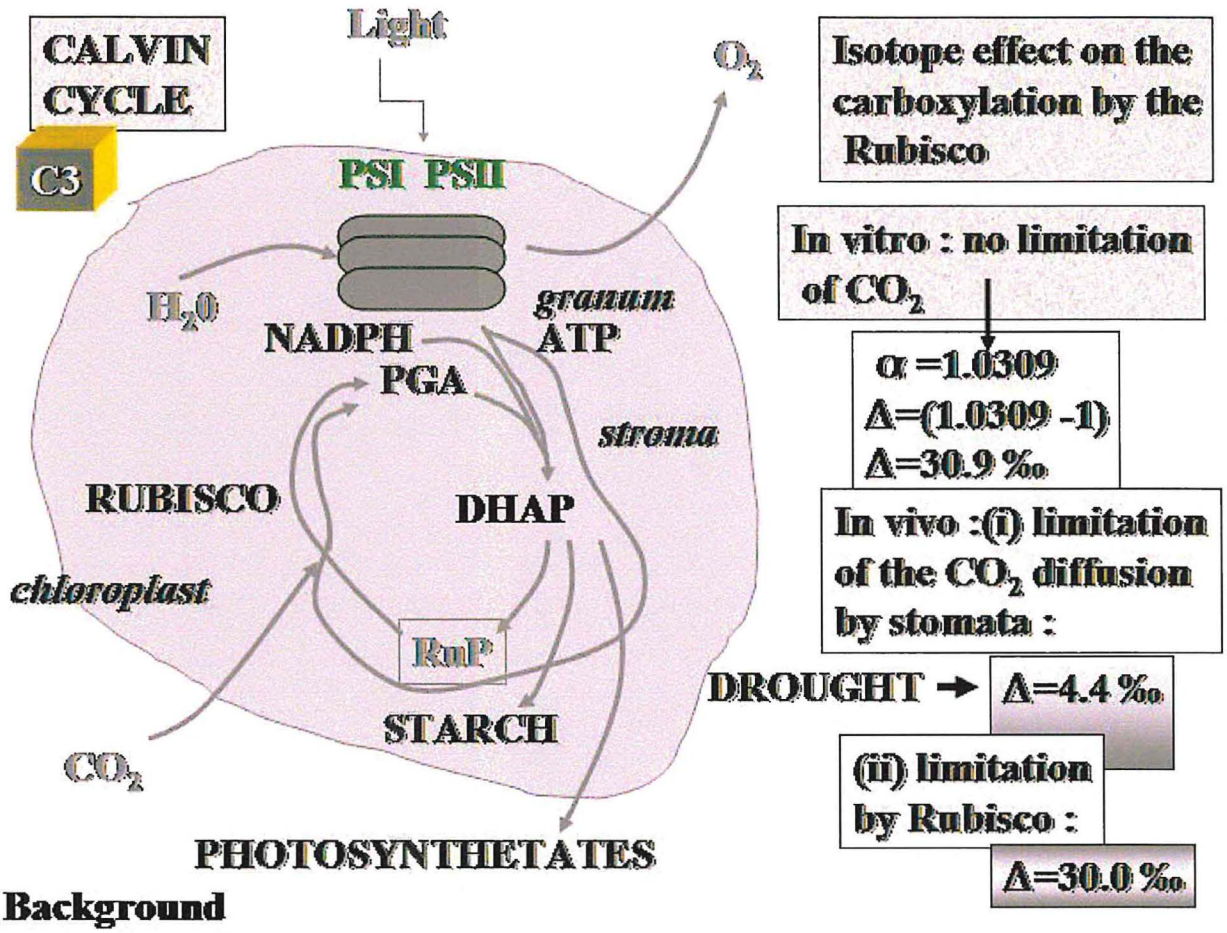


Fig. 3. Cell metabolism and isotope fractionation of carbon pathway for C3 plants (from Ghashghaie et al. , 2001)

Plant materials and ecological conditions

Sampling were done from February to June 2003, and following years 2004 and 2005 on clonal material *Dura x Pisifera* (MK 60: LM007T x DA 128 D) at Aek Pancur Research Station (3°30' N, 98°48' E; 25 m a.s.l., Nord Sumatra, IOPRI, Indonesia), on 8 oil palm trees, year planting of 1995 (Photo 1) and on 5 trees of " *Dura*"," *Tenera*" and "*Pisifera*" types on leaf, trunk, roots and fruits. Only leaves results are reported in this first paper. Climatology characteristics may be resumed as usual conditions in North Sumatra at this latitude with a radiation about 16 Mj m^{-2} day^{-1} and an annual total rainfall around 2800 mm well distributed over the year (Lamade & Setiyo, 1996). The clone trees chosen were not presenting any abnormality and were typical of a *Deli x La Mé* material.

Photo 1. Clone studied (MK60) with carbon isotope and phenology (Aek Pancur Research Station, IOPRI, 2005)

Leaves (rachis, petiole, leaflets)

For the leaves, different growing stages were sampled from rank -6 to rank 45. Each leaf from the point C to A was divided in ten segments following the same methodology as for leaf area determination (Tailliez and Koffi, 1992). Collected segments were then carefully washed in ozone water and directly put in liquid nitrogen and stored at -80°C until dried in oven during 10 days minimum at temperature below 80°C, then grinded 3 times. For leaves from rank 1 to 5, leaflets, petioles and rachis were individualised. Segments were cut in small pieces. Each leaf in the crown, from rank -6 to 45 is suspected to receive the same amount of sun. All samplings were done always before 11 h 00 am. More or less the spiral n° 1 was investigated for all trees studied.

Photo 2 and 3 : sampling methods (Lamade et al. 2004) for carbon Isotope (Aek Pancur Research Station, IOPRI)

Carbon isotope analyses

Explanations about the Farquhar 'model (1982) and the dependent theory for carbon isotope analysis are well described in Ghashghaie et al. (2001). The carbon isotope response composition is done by the following formula :

$\delta^{13}C$ (‰) = [(Rs/RVPDB)-1] x 1000

where Rs and RVPDB are the molar abundance ratios of carbon isotopes $^{13}C/^{12}C$ of one sample and the standard VPDB.

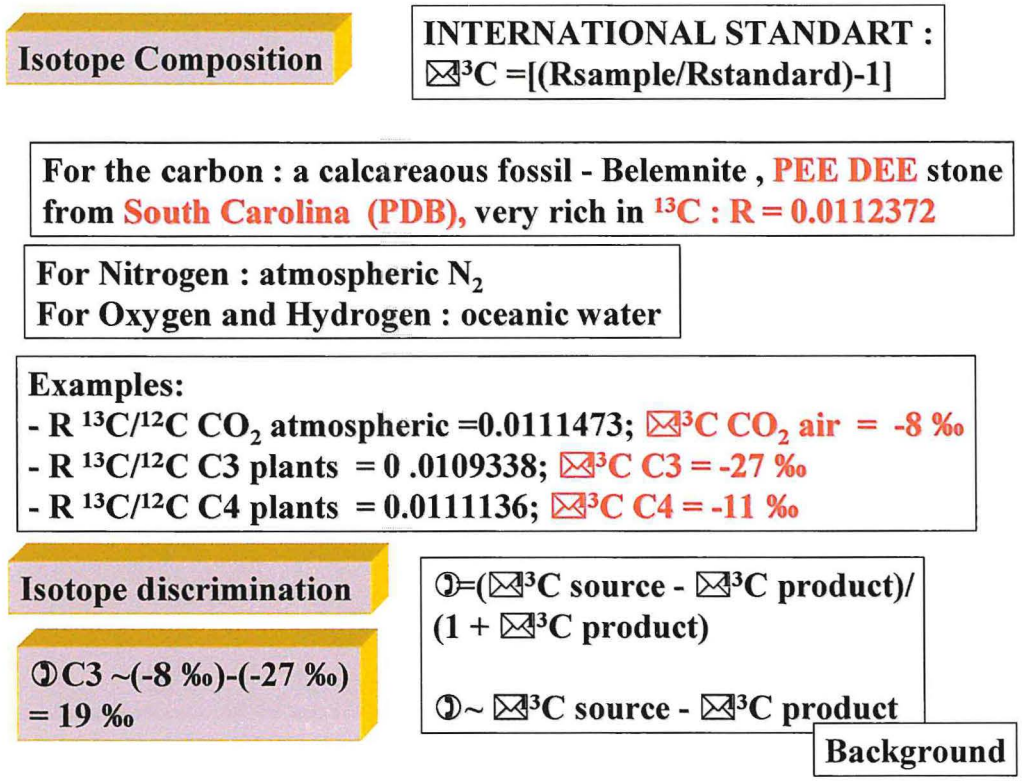


Fig. 4. Some definitions about carbon isotope : composition, discrimination, ratio ect...(Ghashghaie, 2001)

Carbon Isotopes analyses were done at the technical platform of IBP (Institut de Biotechnologie des Plantes, Orsay, France). The $\delta^{13}C$ of organic matter as well as carbohydrates (starch, glucose, sucrose, fructose) was determined using a continuous flow ANCA-MS (Roboprep on-line Dumas combustion and Tracermass MS; Europa Scientific Ltd, Crewe, UK) and a mass spectrometer (VG Isotech, Middlewich,UK).

Results are expressed in δ units versus PDB (Belemnite from Pee Dee formation in South Carolina)

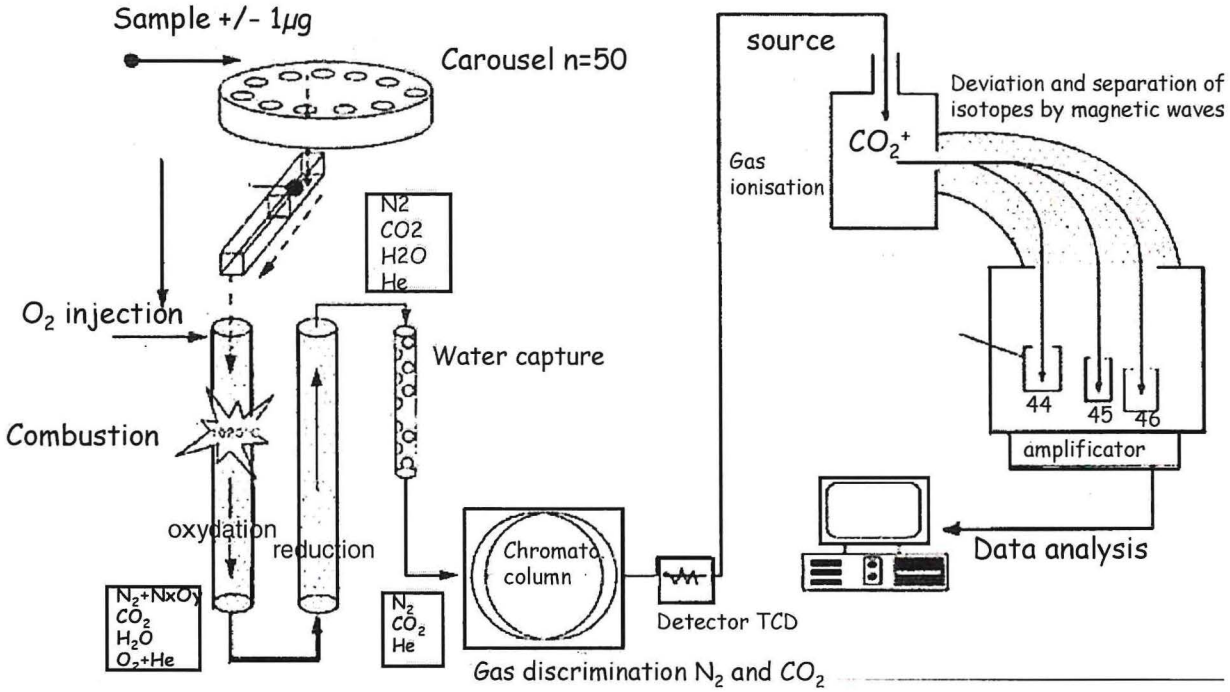
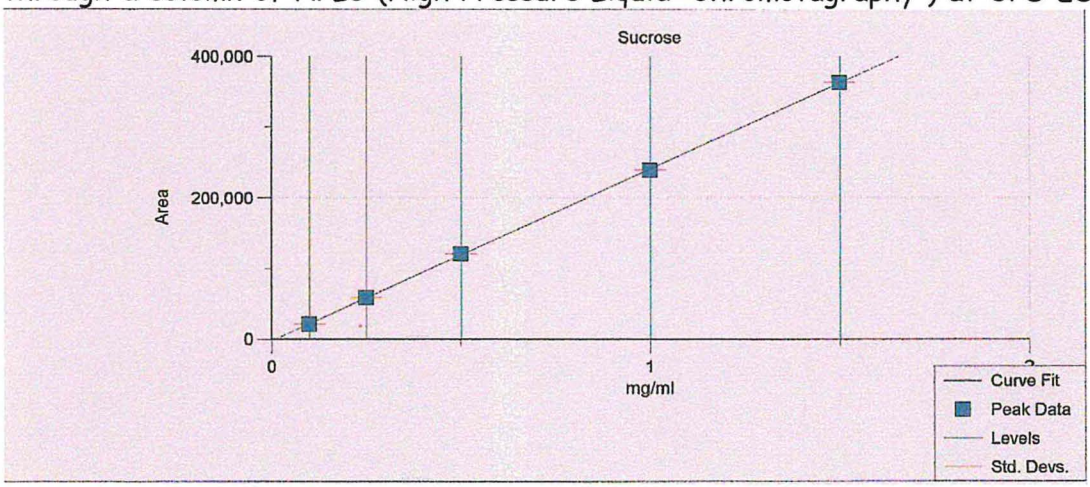


Fig. 5. Isotope analysis experimental design for IMRS (Ghashghaie et al., 2001)

Soluble sugars analyses

On collected samples, fine grinding was performed again and solubles sugars extractions were done following exactly Max Hill's methodology described in Duranceau *et al.* (1999). Separation and quantification of sucrose, fructose and glucose were performed through a colomn of HPLC (High Pressure Liquid Chromotagraphy) at UPS ESE CNRS-



UMR 8079 (Orsay, France). Calibrations for all sugars (Fig. 6) where done before trought the colomm and show perfect results.

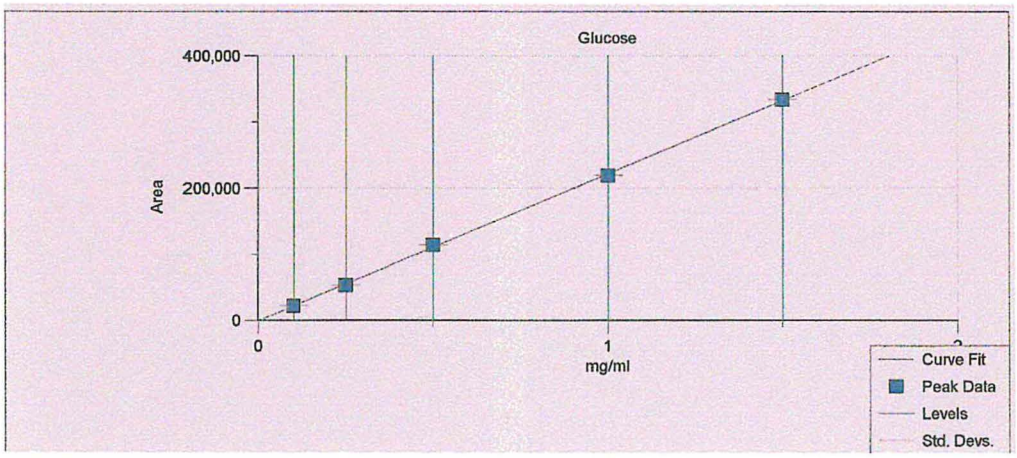


Fig 6. Example of the good calibration of the HPLC column for sucrose and glucose determination at UPS XI (Orsay,France)

Leaf gas exchange

Dark respiration has been measured with a closed system (fig. 7) already well described in Lamade (1999). The spiral one has been investigated during the day time.

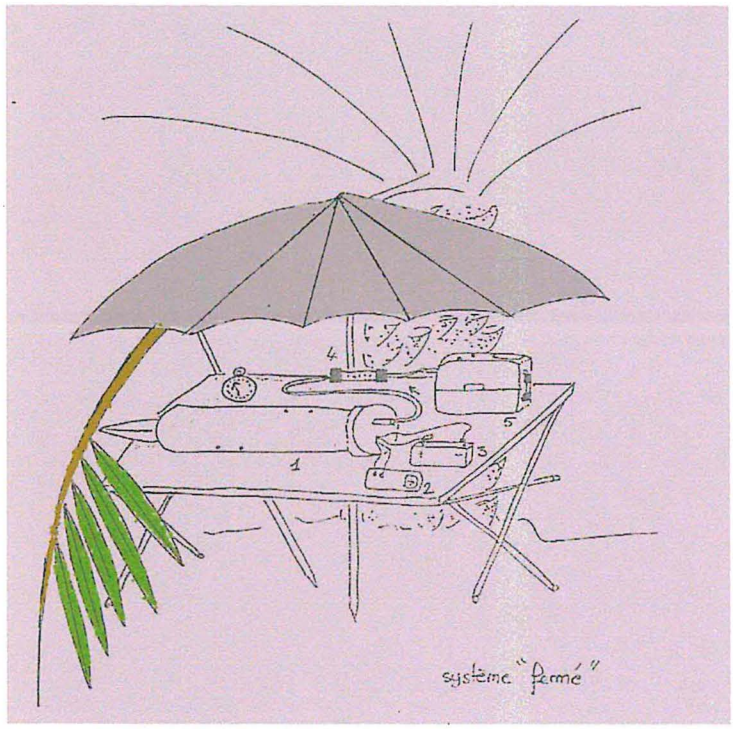


Fig. 7. Experimental design of dark respiration measurements with a closed system for one leaflet , *in situ*.

Results and Conclusion

Organic matter

Analyses were first performed first on total organic matter. The mean value for $\delta^{13}C$ for total organic matter for all samples was -27.46 ‰ (s.d. 0.93), which is normally expected for a C3 plant like oil palm. Differences within trees and organs (inside a same tree) and development stage (for a same organ) were revealed: especially a nice gradient could be pointed along the leaf ranks. An expected gradient can be seen from leaflets (mean value: $\delta^{13}C$=-28.22‰, s.d. =1.01) to rachis ($\delta^{13}C$=-27.04‰, s.d. =0.38) and petioles ($\delta^{13}C$=-26.91‰, s.d. = 0.56) with a difference of 1.6 ‰ between photosynthetic tissues and others.

The 13C-deletion in leaves compared to other organs is in agreement with the data in the literature.

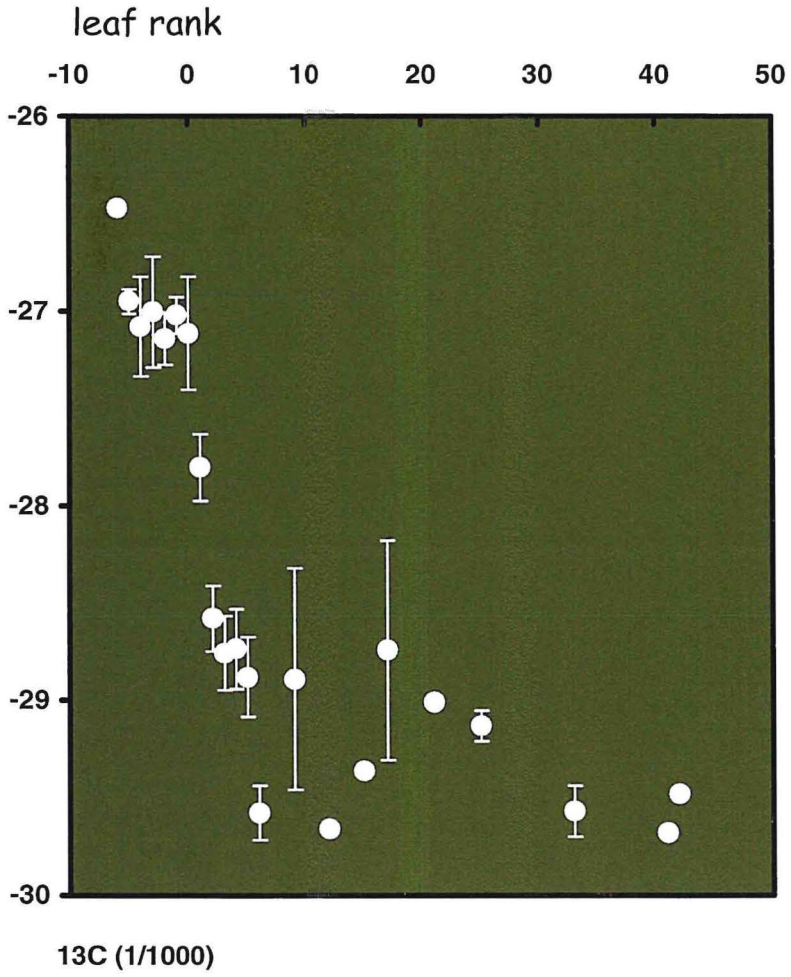


Fig. 7. Variation of the carbon isotope discrimination of the organic matter along the leaf rank

A slight depletion could be observed when the leaf is reaching the full opening at rank 1 ,then during the passage from heterotrophy to autotrophy. The isotope signature of the leaf organic matter is depending on the CO_2 partial pressure in the intercellular cell space. More this pressure is important more the discrimination against the heavy isotope ^{13}C will be high and Δ 13C more negative. On very young leaves (-6 to 0),the air is not diffusing enough because leaflets, rachis are totally closed and the petiole is not individualized yet. The other reason of the high signature of the young leaves is the presence of other carbohydrates (soluble sugars) which present always higher isotope signature (fig. 7).

The $\delta^{13}C$ gradient along the leaf ranks could reflect (i) an increase in stomatal conductance and thus an increase in photosynthetic carbon isotope disimination during leaf maturation and/or (ii) changes in carbon metabolism. The labeling experiments will then allow us to determine (i) the changes in carbon metabolism during the different stage of leaf growth and fruit formation and (ii) the carbon allocation in oil palm. This result points out the importance of the stomatal conductance for oil palm metabolism.. ^{13}C fractionation of organic matter and stomatal conductance, temporal and seasonal variations must be investigated also as well as genetic comparisons behaviour.

Soluble sugars

Very few amont of **sucrose** (fig. 8) was found in the leaves (from 0.259 mg/ml, in average, at rank -6, 0 mg/ml at rank 0 and 0.2866 mg/ml at rank 33) but the pattern reveals that sucrose can be the sugar used for the **growth** and the **development** of heterotrophic organs (roots, trunk , fruits) due to very high concentrations at the tops of the trunk in the meristem part (around 6 mg/ml) . But after the rank 10, sucrose remain in an acceptable concentration not far from the youngest leaves.

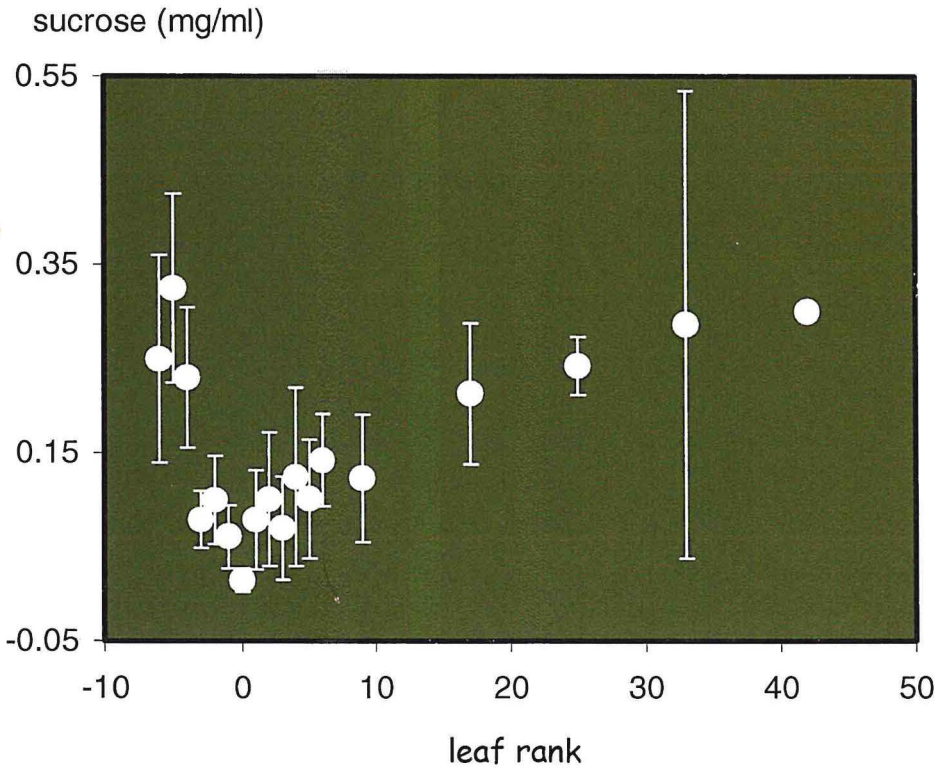
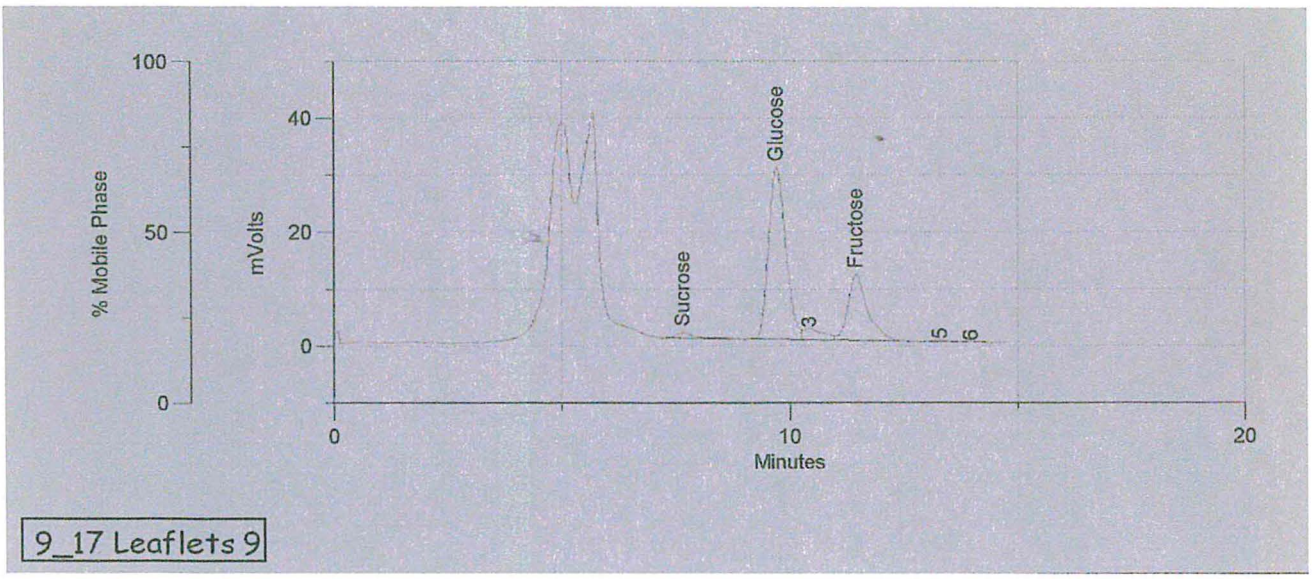


Fig. 8. Variation of sucrose concentration along the leaf rank.

Then another hypothesis may not be neglected too : sucrose could be translocated very quickly from very active leaves (rank 1 to 10) which will be, then, the major "source' of carbon to all heterotrophic organs (roots,trunk, fruits, young leaves). The "sink" leaves are of course the youngest before the rank 0.

Glucose is also fluctuating lineary with leaf rank with a clear increase from rank 0 (0.01 mg/ml in average) to rank 17 -33 (maximum between 2 and 2.5 mg/ml) . It seems that for oil palm,it may be the first sugar coming from photosynthetic activity. Still glucose is present at high level in older leaves over the rank 40, this point have implication for recommendations for pruning. The 42 leaves recommended generally in Indonesia remain adequate with te consideration of the glucose pattern. (see below the result of the separation with the HPLC column in UPS X,Orsay, France)



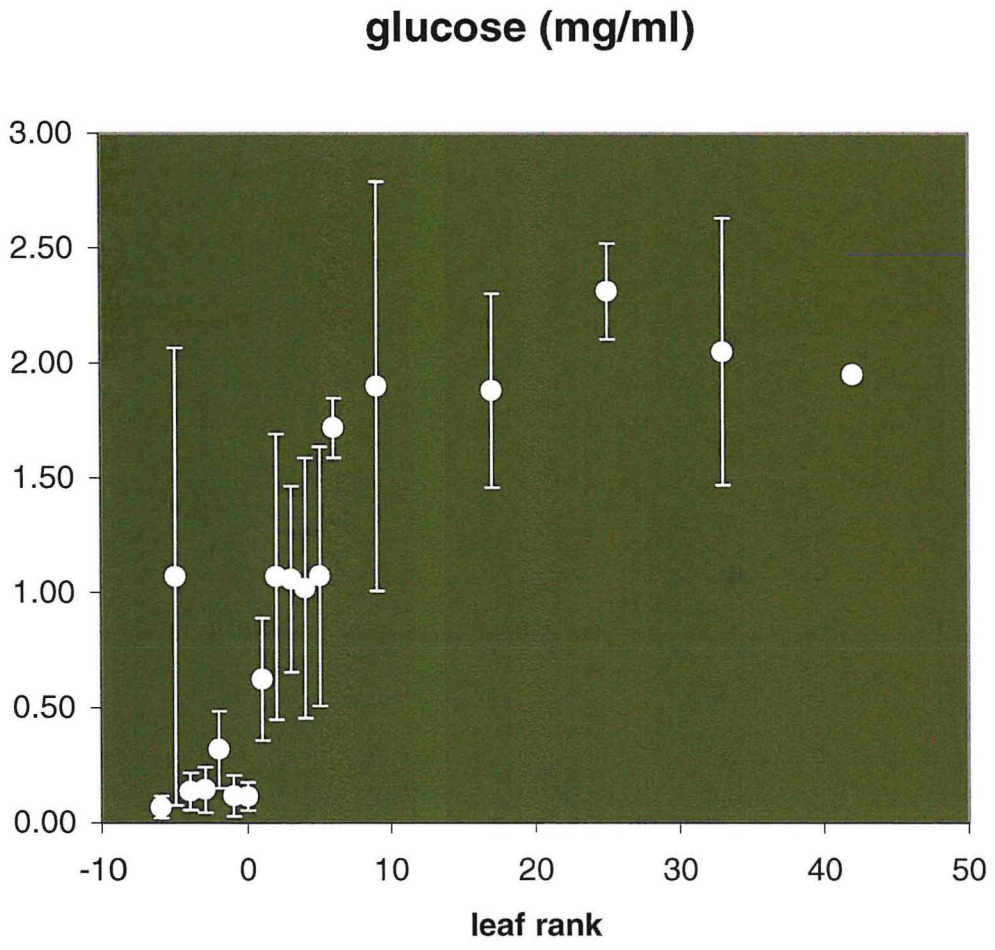


Fig. 9 Variation of glucose concentration along leaf rank.

We found a quite similar gradient with the chlorophyll content (Fig. 10) of the leaves directly related to photosynthetic activity which strengths the role of the glucose as a

direct assimilate products from the '**source leaves**'. We will follow some pertinent theories as developed by Tcherkez et al. (2004) when all analyses will be performed.

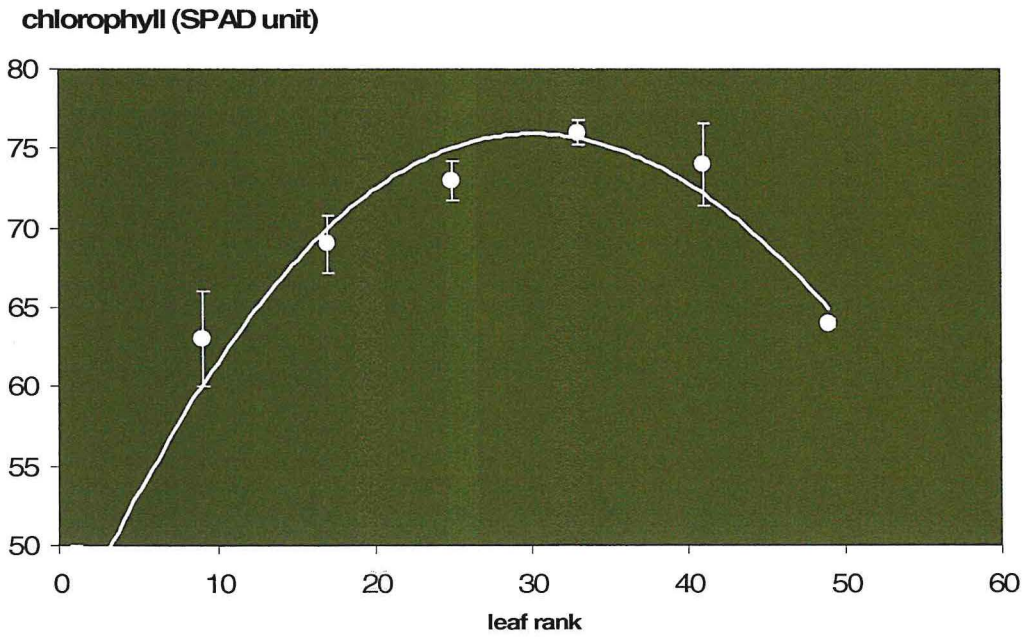


Fig. 10. Variation of leaf chlorophyll content along leaf rank.

Frutose concentration in leaves is variable with leaf rank as glucose but appears already during heterotrophic stage, which means that this sugar plays a role also in the

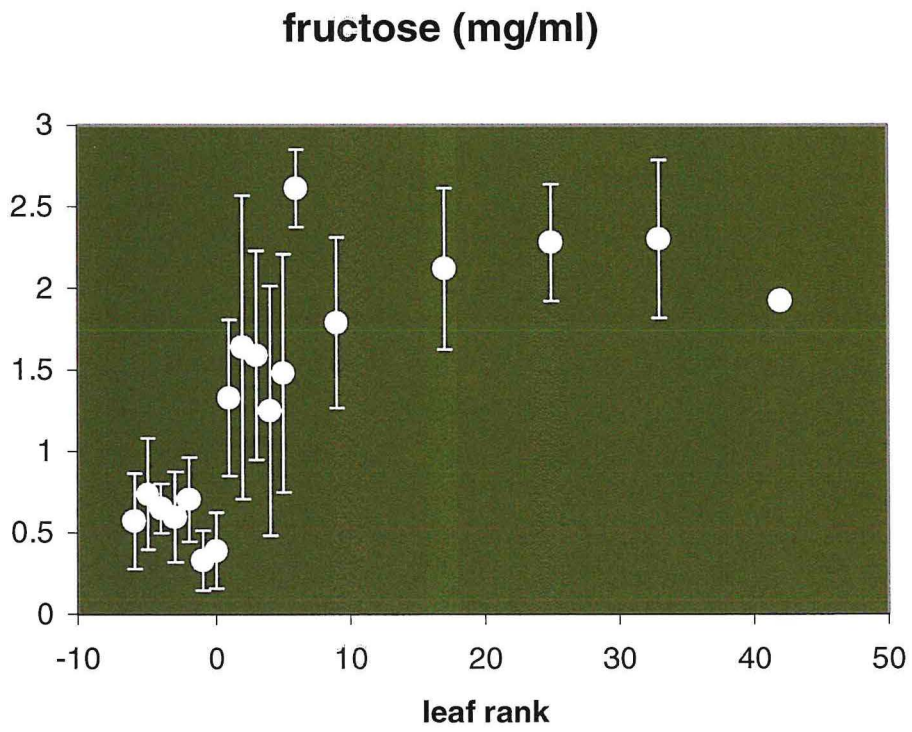


Fig. 11. Variation of fructose concentration along leaf rank.

elaboration of heterotrophic organs. Fructose is already quite high before the rank 10 and does not "diseappears" after until older leaves.

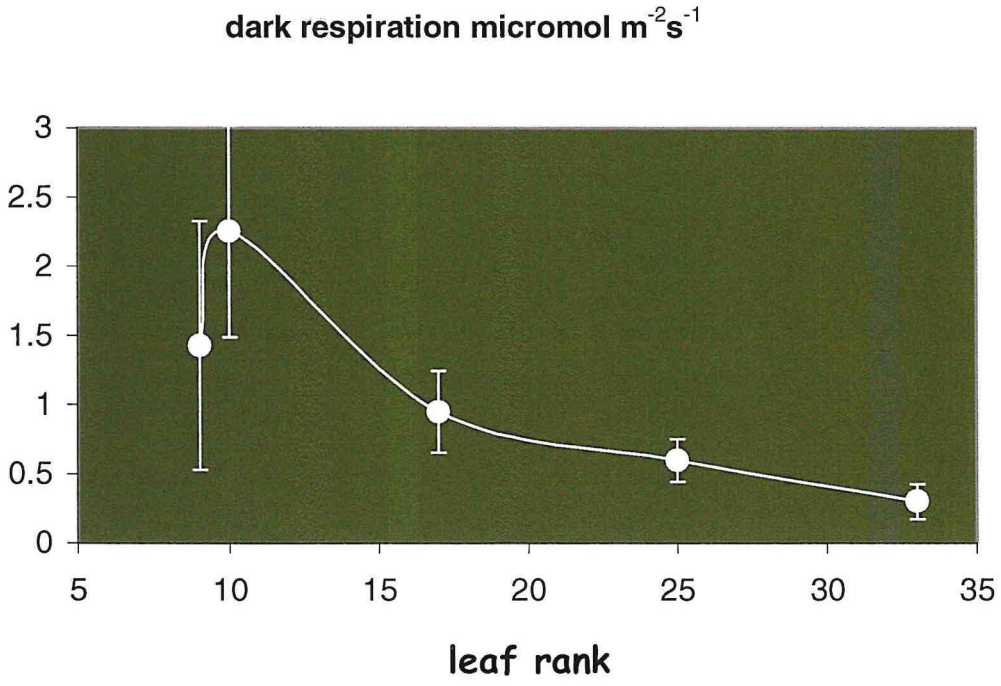


Fig. 12. Variation of the dark respiration of the leaflets along leaf rank gradient.

The dark respiration (Fig. 12) shows a pic around 9-10 leaf rank which correspond to the more active leaf as far as the photosynthesis is concerned (Lamade and Setiyo, 1997) and decrease strongly after the rank 15. Once again the older leaves show a low carbon cost and stay "**source organ**'" quite long.

Starch

A light decrease of $\delta^{13}C$ can be observed as well as for the organic matter depending of the leaf rank, but the $\delta^{13}C$ of the starch in the leaf is higher (2 ‰). This maybe explain (i) the starch exported to the heretotrophic leaves is enriched during the translocation (ii) the adult leaves are discriminating more following stomatal aperture.

Perspectives

Others results will be soon available to finish the isotope mapping of oil palm trees at individual level, particularly for the trunk where 11 points of sampling have identified and followed on trees studied. Some preliminary results show the importance of sucrose in the apex (fig. 12) and much more less in the bulbous base of the palm (fig. 13)

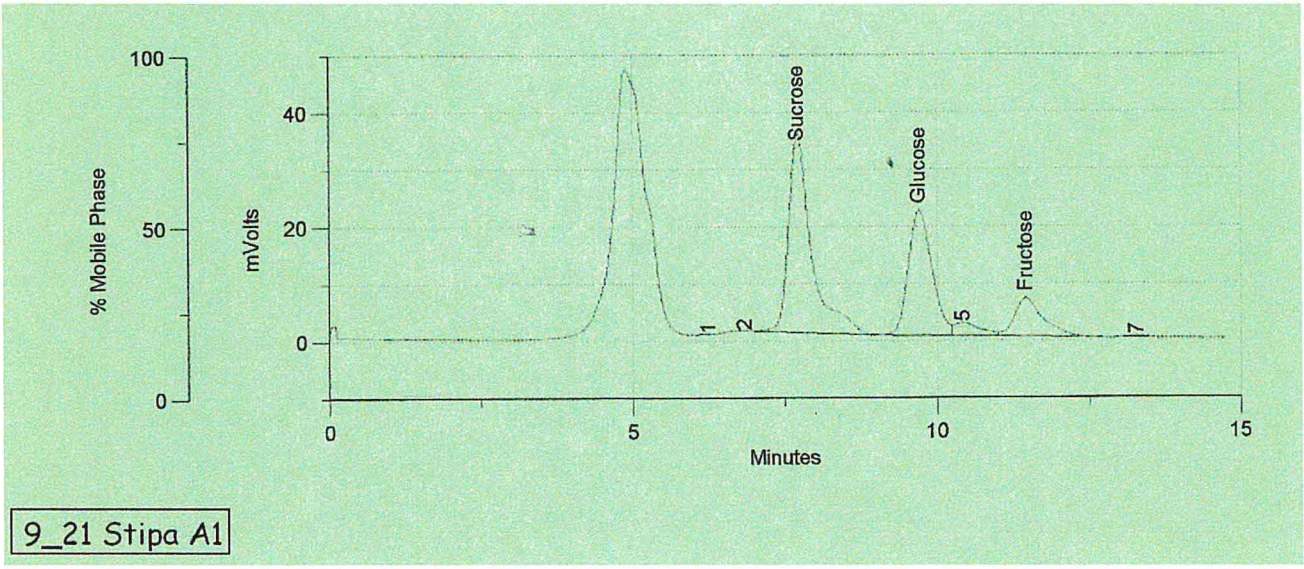


Fig. 12. Importance of the sucrose in the apex (meristem zone) of the trunk

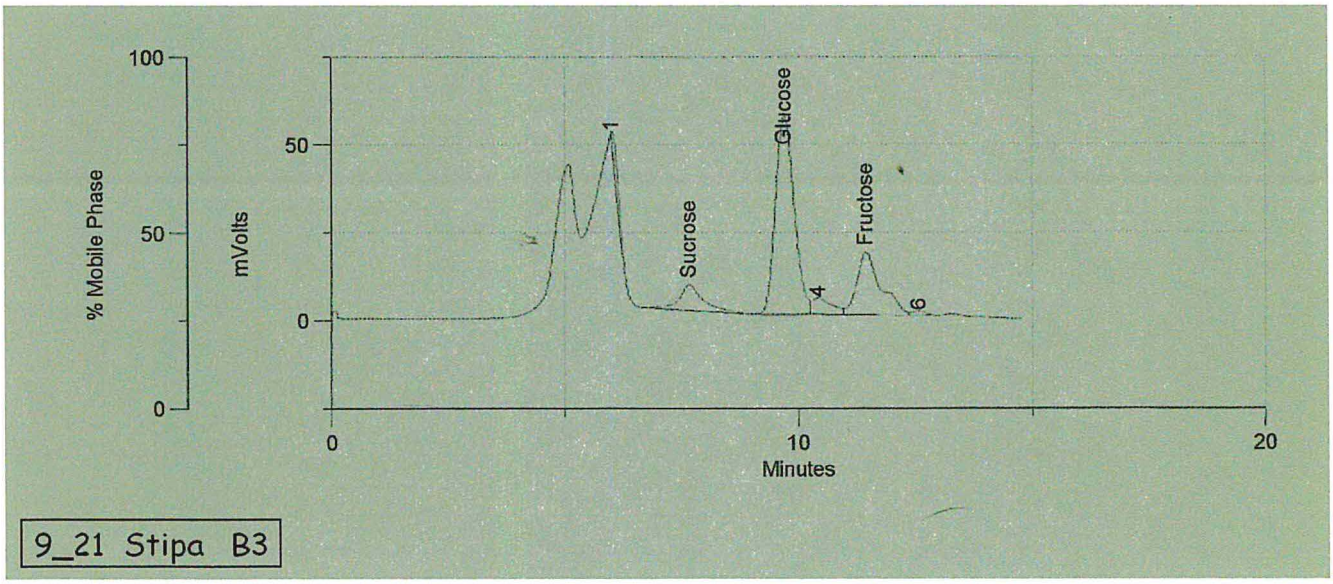


Fig. 13. Importance of the glucose on the peripheric zone of the bulbous base of the trunk.

Carbon isotope discrimination is already a pertinent tool to follow photosynthetic metabolites for oil palm in leaves (heterotrophic and autotrophic) and for the understanding of the filling of the bunches from sugars reserves. Other perspectives will be open with the use of this tool for *Dura/Tenera* discrimination and inheritance and for the identification of **drought tolerancy.**

The labeling experiments will then allow us to determine (i) the changes in carbon **metabolism** during the different stage of leaf growth and fruit formation and (ii) the carbon allocation in oil palm. Because the isotope signature is definitively integrated in the standing biomass, it will allow us to investigate with a great precision the metabolic pattern responsible of **the sexualisation** of the inflorescences.

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