Ann Sci For (1993) 50, 309-318

Technical note

An experimental system for the quantitative ^{14}C-labelling of whole trees *in situ*

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(Received 8 April 1992; accepted 10 March 1993)

Summary —The first part of this paper provides a brief review of the requirements that apply to ^{14}C-labelling chamber technology, particularly for tree labelling, and of the means that can be used to meet them. Two main points are considered: the quality of the plant chamber environment – the necessity of thermal and hygrometric regulations is discussed – and the possibility of determining the exact amount of $^{14}CO_2$ assimilated by the plant. The authors then describe a simple system allowing the quantitative labelling of entire trees, without temperature- or hygrometry-regulating devices which can be used in the morning. The CO_2 concentration is maintained at its natural level throughout the labelling procedure through an injection of cold CO_2 operated by an IRGA-driven computer. This system was successfully used for the labelling of grafted walnut trees.

assimilation chamber / control of CO_2 level / photosynthesis

Résumé — Un système expérimental permettant le marquage quantitatif au ^{14}C d'arbres entiers *in situ*. *Ce système, utilisé pour le marquage de noyers greffés de 3 ans (surface foliaire : 1,7 m^2), se compose d'une chambre d'assimilation et d'un dispositif d'injection de CO_2 à commande électronique permettant une régulation continue de la concentration en CO_2 (fig 1). Ne comportant pas de dispositif de régulation thermique, il n'est utilisé que pendant la matinée. Malgré une augmentation significative de la température au cours du marquage (fig 2), la photosynthèse est peu perturbée, comme le montre la figure 3 : le taux d'assimilation (pente des segments décroissants) reste régulier. La chambre d'assimilation, en PVC de 2 mm monté sur un cadre d'acier, forme un cylindre fermé (hauteur, 2 m; diamètre, 1,44 m), constitué de 2 moitiés s'accolant l'une à l'autre par un joint de caoutchouc. Lors de la fermeture, le joint est comprimé par une série d'écrous disposés tout au long de la suture. Le cylindre, soutenu par un portique métallique, contient l'ensemble de la frondaison. Une ouverture à la base du cylindre permet le passage du tronc, l'étanchéité étant assurée par un film de polyéthylène de 0,03 mm et un joint en mastic souple «Terostat». Des considérations*

Abbreviations: IR: infrared; PAR: photosynthetically active radiations; IRGA: infrared gas analyser; FMW: fresh matter weight. The mention of trade or firm names in this publication does not constitute endorsement or approval by the French Ministry of Agriculture.

théoriques permettent d'estimer à quelque 3% la radioactivité perdue par fuites lors du marquage. La régulation de la teneur en CO_2 répond à un double but. D'une part, en limitant l'écart par rapport aux conditions naturelles, on perturbe le moins possible la répartition biochimique et spatiale des assimilats. D'autre part, la totalité du ^{14}C étant injectée instantanément dès le début de l'opération, la régulation consiste à injecter du carbone «froid» pour compenser la photosynthèse, et l'équation (1) *(paragraphe «Injection de CO_2») donne à tout moment la quantité totale de ^{14}C restant dans la chambre. Ainsi, 99,3% de la radioactivité a disparu lorsqu'on a renouvelé 5 fois la totalité du CO_2 présent dans la chambre, ce qui était réalisé en 4 h environ. Le CO_2 est fourni par la réaction d'une solution de Na_2CO_3 gouttant dans un flacon d'acide sulfurique à 33% (fig 1). L'efficacité du dégagement gazeux est améliorée par une agitation magnétique et un barbotage de l'air de la chambre prélevé par une pompe. L'injection initiale du carbonate marqué, de forte radioactivité spécifique (1,85 GBq/mmole; 74 MBq par arbre, pesant chacun 2 kg de MS) ne modifie pas la teneur totale en CO_2 de la chambre. Puis le réservoir de carbonate est empli de solution «froide», 1 M, délivrée selon les besoins de la régulation par une électrovanne. Celle-ci est pilotée par un micro-ordinateur (fig 1) munie d'une carte d'acquisition de données (Micromac 4000, Analog Devices) qui enregistre par ailleurs la température, le PAR incident et la teneur en CO_2 de la chambre mesurée par un IRGA. Ce système libère quelques gouttes de carbonate dès que la teneur en CO_2 descend au-dessous de 350 vpm, ce qui permet une régulation efficace (fig 3). Les aspects quantitatifs des marquages ont été validés par 2 moyens indirects : d'une part, en vérifiant que la radioactivité résiduelle de l'air à la fin du marquage est conforme à l'équation (1); d'autre part, en retrouvant dans les arbres traités, quelques heures après marquage, 90% de la radioactivité injectée.*

chambre d'assimilation / régulation de la concentration en CO_2 / photosynthèse

INTRODUCTION

During the past 40 years ^{14}C has been widely used as a tracer in studies of carbon flows in biological or biochemical systems, in which its radiations can be used in imagery (autoradiography) or quantitatively counted in liquid scintillation or gas-flow counters. We will here discuss only global studies of carbon flows, in which the ^{14}C enters the plant system through the natural pathway, *ie* photosynthesis. The basic procedure in this case consists of feeding the plants with ^{14}C-enriched CO_2.

After a brief review of the constraints related to ^{14}C labelling, and of the main progress made in labelling chamber technology in order to meet them, particularly for trees, this paper presents a system allowing quantitative labelling which has been used successfully at our laboratory in Clermont-Ferrand.

This labelling system was designed to investigate carbon flows in 3- to 4-yr old walnut trees. Particularly, our aim was to trace the incorporation of photosynthate-derived carbon into carbohydrate reserves *vs* structural compounds at different times, as well as spring remobilization of the labelled reserves (Lacointe *et al*, 1993).

GENERAL CONSTRAINTS RELATED TO ^{14}C LABELLING

Airtight chambers are utilised in the quantitative feeding of plants with labelled CO_2 ($^{14}CO_2$ or $^{13}CO_2$). Enclosing plants in a closed illuminated chamber leads to rapid modification of the atmosphere due to depletion of CO_2 by photosynthesis and accumulation of a significant amount of heat and water vapour; the rate of photosynthesis can be significantly altered by these modifications in the environment.

Although the aim of feeding experiments is generally not to evaluate the photosynthetic rate (well known gas exchange methods are far more suitable for this purpose), it is necessary to maintain a sufficiently high rate of photosynthesis in order to achieve maximal exhaustion of the labelled CO_2 by the plants. Furthermore, a significantly reduced assimilation rate could disturb the natural pattern of chemical and spatial partitioning of assimilated C (Geiger and Fondy, 1991). Then at least partially regulating the most critical parameters of the environment may become necessary even for feeding periods of short duration. For long-term feeding experiments, due to significant alteration in most of the physiological functions when the environmental conditions are changed, the temperature and humidity of the air will have to be regulated.

A within-chamber environment allowing photosynthesis

Light conditions

The materials used to construct the chambers (transparent plastics) have photosynthetically-active radiation (PAR) transmission factors ranging between 70 and 90% (Dogniaux and Nisen, 1975), which involves some reduction in the photosynthetic rate with respect to open air conditions. In labelling experiments this reduction is assumed to have only little effect (if any) on the fate of the incorporated C in the plant (which is the question under study). For reasons of cost and ease of handling PVC was chosen.

Air temperature conditions

Due to very low transmittance of the plastic materials in the thermal IR range (between 2.5 and 25 μm; Dogniaux and Nisen, 1975), and low convection (closed circuit conditions), the temperature of the air inside the chambers can be increased by 5 to 15°C with respect to the outside in conditions of high solar irradiance. When excessive, this increase in temperature can lead to reduced or even negative net photosynthetic rates, the latter rendering impossible any labelling experiment in the absence of an additional cooling system.

A few authors have tried to solve this problem which can become critical for long feeding periods especially when intense radiative conditions are encountered.

Lister *et al* (1961) interposed water filters to absorb part of the IR radiations from the light source. This system is viable for indoor labelling but unsuitable in the field. Palit (1985) used occasional spraying of cold water, whereas Lister *et al* (1961), Warembourg and Paul (1973), Geiger and Shieh (1988) made use of different types of heat exchangers to regulate the temperature. All these systems, well adapted to small-sized chambers (a few litres), would become problematical if used with chambers several cubic meters in size, as necessary to label whole trees.

However, even for small chambers, since the only requirement is that of no significant reduction in photosynthesis, most authors did not include any cooling device in their feeding system and tried simply to limit overheating, *ie* to operate preferentially in the morning. This is approach that was adopted for our system.

Air humidity conditions

When exposed to high solar irradiance, well watered plants inside a closed chamber convert a large proportion of the incident radiative energy into latent heat by transpiration, leading to complete saturation of the volume of the chamber by water

vapour in a few min and to heavy condensation on the walls which constitute the cold elements of the system. Since the leaves absorb most radiation, they become warmer so that no condensation occurs on them. These physical conditions at leaf level (high temperature and low water saturation deficit) are known to be generally favourable to photosynthesis (provided the temperatures do not become excessive). Then one can assume that regulating the humidity of the air *per se* would generally be unnecessary for feeding experiments of short duration. On the contrary, for long-duration feeding experiments, a system of complete air conditioning (temperature and hygrometry) is necessary. A few authors (Webb, 1975; Kuhn and Beck, 1987; Geiger and Shieh, 1988) regulated the relative humidity in the labelling chamber, using a cooled vapour trap. For our feeding experiments which were designed to last ≈ 4 h it was decided to leave the hygrometry unregulated.

Regulating the CO_2 concentration

Since exhaustion of the ambient CO_2 by photosynthesis in feeding experiments leads to decreased photosynthetic rates, maintaining the CO_2 concentration at normal values is necessary. Achieving accurate regulation of CO_2 requires continuous measurement of its concentration (using an IRGA) and an injection system. Rough control of the ambient CO_2 can be achieved by temperate injection of chemical reactants (Warembourg and Paul, 1973; Smith and Paul, 1988; Schneider and Schmitz, 1989) or by the use of cylinders of diluted CO_2 and mass-flow regulators (Webb, 1975; Geiger and Shieh, 1988; Hansen and Beck, 1990). Though less accurate, the former solution was chosen for our system because of its simplicity of operation.

Making the labelling quantitative

Depending on the objectives of the experiment, it may or may not be important to regulate the isotopic ratio of the assimilated CO_2 (specific activity in case of $^{14}CO_2$).

In long-term labelling experiments steady state has to be reached, hence the isotopic ratio of the photosynthetic CO_2 must be held constant, but the total amount of incorporated C is generally of no importance. On the other hand, in short-term labelling experiments achieving quantitative labelling, *ie* knowing how much ^{14}C the plant has actually taken up may be of importance, particularly for experiments with destructive sampling; but keeping the isotopic ratio constant is generally unnecessary.

In order to make a short-term labelling quantitative, the first step is to accurately determine the total quantity of $^{14}CO_2$ injected into the labelling system. The CO_2 can be directly injected as gas from a syringe (Balatinecz *et al,* 1966) or a pressurized cylinder (Webb, 1975; Kuhn and Beck, 1987). Alternatively, it can be released from the reaction of ^{14}C-carbonate with excess acid (Lister *et al,* 1961; Hansen, 1967; Warembourg and Paul, 1973; Glerum and Balatinecz, 1980; Langenfeld-Heyser, 1987; Smith and Paul, 1988; Lacointe, 1989; Schneider and Schmitz, 1989; and many others). In the latter case, due to the higher density of CO_2 as compared to air, the atmosphere in the reaction vessel must be chased efficiently. This problem was solved by forcing the chamber atmosphere into the reacting solution (fig 1).

Secondly, the injected CO_2 must not leave the system during the labelling. Hence the chamber – and circuit when present – must be airtight, which is also important to avoid pollution problems, particularly indoors. Air-tightness is generally

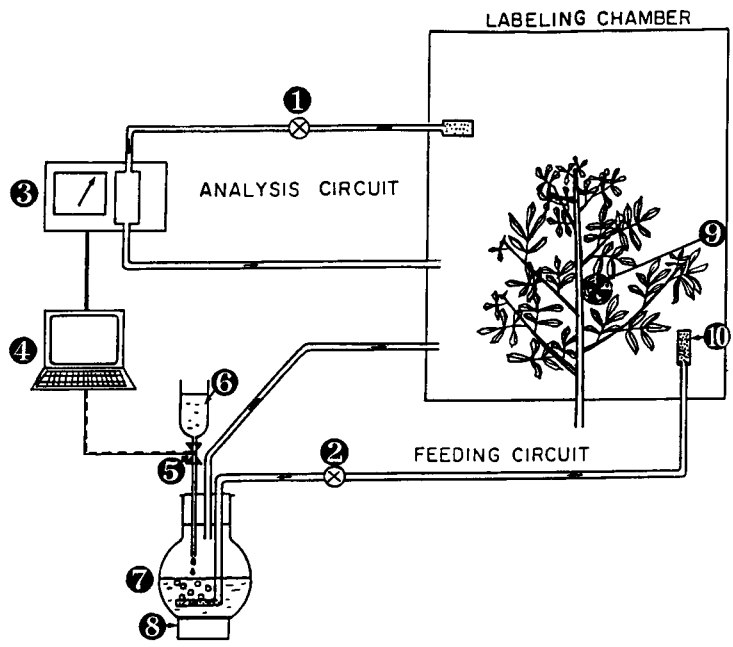

Fig 1. The equipment used for the ^{14}C-labelling of walnut trees *in situ*. 1 and 2, pumps; 3, infrared gas analyser (IRGA); 4, computer controlling; 5, magnetic valve; 6, Na, Na_2CO_3 solution; 7, H_2SO_4 solution; 8, magnetic stirrer; 9, fan; 10, porous cap.

not a real problem with solid chambers, but can be with chambers made of plastic film, due to the possibility of small tears or holes and rather large changes in volume allowed. The above-mentioned materials including plastic films, generally exhibit a sufficient impermeability to CO_2, *eg* $1.04 \cdot 10^{-4}$ $cm^3 \cdot m^{-2} \cdot min^{-1} \cdot Pa^{-1}$ for a 0.03-mm polyethylene film (Daudet, 1987).

Many authors have not carried out more controls, either because they were not interested in the exact quantity incorporated (Balatinecz *et al,* 1966; Langenfeld-Heyser, 1987), or because they allowed ^{14}C-assimilation for a time which they either assumed or knew to be long enough for a complete exhaustion of the $^{14}CO_2$ in the chamber (*eg* 6 h for Hansen, 1967; 30 min for Palit, 1985). However, some authors further investigated the actual amount of ^{14}C taken up by measuring the level of $^{14}CO_2$ still in the system at the end of the labelling period. Before opening the chamber, they forced its atmosphere into a CO_2-trapping circuit generally containing KOH or $Ba(OH)_2$ (a common procedure to avoid pollution, particularly indoors) and then measured the radioactivity trapped by the alkali (Glerum and Balatinecz, 1980).

Further progress was achieved through measuring the $^{14}CO_2$ level not only at the end of the labelling, but continuously during the labelling period. Lister *et al* (1961) used both an IR gas analyser for estimat-

ing the total CO_2 level and a Geiger-Müller tube for volumic radioactivity, whereas Kuhn and Beck (1987) used only an IRGA to measure the decrease in the CO_2 level (and calculate that of the $^{14}CO_2$) within the chamber. As mentioned above (see *Regulating the CO_2 concentration*), some authors used an IRGA to regulate the CO_2 level inside the chamber throughout the labelling period.

When the injected CO_2 was of constant specific radioactivity, this allowed long-duration labelling under steady-state conditions (Warembourg and Paul, 1973; Webb, 1975; Geiger and Shieh, 1987; Smith and Paul, 1988). On the other hand, when all the $^{14}CO_2$ was injected at the beginning of the experiment and the continously injected CO_2 was only $^{12}CO_2$ (Hansen and Beck, 1990), this allowed a precise calculation of the total ^{14}C taken up by the plant under conditions of minimum perturbation. This was the basis of the system we designed for the labelling of whole trees.

DESCRIPTION AND PERFORMANCES OF THE LABELLING SYSTEM

The labelling system is composed of an assimilation chamber and an electronically-controled CO_2 injection device allowing continuous regulation of the inside CO_2 concentration (fig 1). It has been used on 3-yr-old grafted walnut trees with 1 trunk and 4/5 branches and a total leaf area of ≈ 1.7 m^2. The trees were grown outdoors in 200-l containers.

The assimilation chambers

Two chambers were used alternatively, allowing either local labelling of a branch section or global labelling of the whole above-ground part.

The chamber used for the local labelling was an open cylinder made of 2-mm PVC (PAR transmission factor = 85%). Its height was 0.50 m and its diameter 0.34 m (vol = 45 l). This cylinder was extended at each end by a 0.03-mm polyethylene film junction, allowing gas-tight sealing on the branch with Terostat 9010 sealing profile (Teroson, France).

The chamber used for global labelling was a closed cylinder (height = 2 m; diameter = 1.44 m; vol = 3.25 m^3), made of 2-mm PVC set on a steel frame. It consisted of 2 halves hanging from a portable support, which could be joined together *via* rubber joints. Airtightness was achieved by compressing the joints with screws. There was an opening in the cylinder bottom for the stem, and airtightness was achieved through plastic film junction and sealing as for the small chamber.

Despite ample precautions, we could not assume that airtightness was absolute, either for the large or for the small chamber, due to preexisting small holes in the plastic film parts and/or leaks induced by differential thermal dilatation of the rigid parts of the chambers. No precise measurement of leakage was made for the chambers but an estimate of the upper limit of total radioactivity lost due to these leaks can be given, assuming equipressure between the inside of the chamber and atmosphere, when thermal dilatation of the air in the chamber occurs. In such conditions, an increase in temperature of 15–20°C during the course of feeding (*cf* fig 2), could lead to a leakage of 6% of the air in the chamber; we can expect a lesser relative loss of total radioactivity (≈ 3%) since the specific radioactivity of the CO_2 decreases continuously during the feeding period.

In both chambers the atmosphere was homogeneized by a fan, and there were 4 openings for the in- and outlet tubes of 2 closed circuits: one for CO_2 level monitoring and one for CO_2 injection (fig 1). The

tubing was made of polyamide (Rilsan), which was chosen for its impermeability to CO_2.

CO_2 injection

Total amount of RA required per tree

The total amount of radioactivity required was determined according to the sensitivity of the least sensitive method used for ^{14}C measurement. Two methods were used in the experiment: liquid scintillation for soluble compounds, and argon–methane flow counting for insoluble compounds. The less sensitive method is the latter, which was used in a previous experiment on walnut seedlings (Lacointe, 1989). This study showed that an accurate measurement of the RA incorporated in all organs (including new spring organs) required ≈ 1 μCi (37 kBq) $^{14}CO_2$ fed per g plant DM as an order of magnitude. Since the DM weight was ≈ 2 kg, the amount injected was determined as 74 MBq for each tree.

Control of CO_2 injection

CO_2 was generated through dropping a sodium carbonate solution from a burette into excess 33% sulfuric acid. The efficiency of CO_2 evolution was improved by a magnetic stirrer and by forcing the chamber atmosphere through the reacting solution with a pump.

The first step was the injection of all the ^{14}C-carbonate which induced only a slight increase in the total CO_2 concentration within the chamber (< 0.1% for the large, 6% for the small chamber) due to the high specific radioactivity of the carbonate (1.85 GBq/mmol ref CMM 54, CEA, France). The procedure then consisted of maintaining the total CO_2 concentration between 330 and 360 vpm until 99% of the injected $^{14}CO_2$ had been assimilated. Provided the total CO_2 level in the chamber remained constant, the radioactivity still present at any time could be easily calculated:

$$R = R_i \cdot \exp(-n/N) \quad [1]$$

R being the radioactivity still present, R_i the initial radioactivity injected, n the total amount of CO_2 injected from cold carbonate since the beginning, and N the amount of CO_2 constantly present in the chamber.

From this equation it can be derived that the radioactivity was exhausted by 99.3% for $n = 5N$, which was achieved within 4–5 h in the large chamber, or < 1 h in the small chamber.

The CO_2 level was continuously measured with an IRGA (Mark III, ADC, UK). A data processor system (Micromac 4000, Analog Devices, USA) connected to a microcomputer allowed the recording of physical parameters such as air temperature, incident PAR (Daudet, 1987) and monitoring of a magnetic valve. Whenever the CO_2 level dropped below 350 vpm, the valve opened and an unlabelled sodium carbonate solution was dropped into the acid, injecting cold CO_2 into the chamber. The molarity of the carbonate solution was 1 M for the large and 0.125 M for the small chamber.

An example of the time course of CO_2 concentration during feeding is given in figure 3. One can see that the stability of CO_2 was correct during most of the feeding period. Some dysfunction could occur due to poor stability of the flow of the sodium carbonate solution through the precision cock (see fig 1).

Variation of air temperature

In order to limit temperature increase, labellings were performed in the morning, and lasted < 5 h. Figure 2 shows the

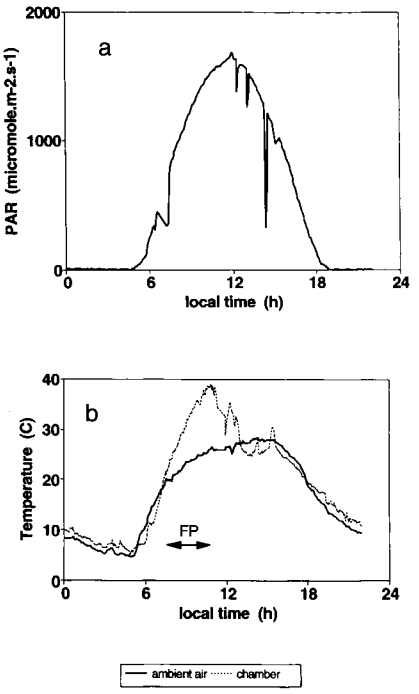

Fig 2. Time course of some physical parameters during the same day of feeding. a) PAR irradiance; b) air temperature inside (near the fan) and outside the chamber. The inside hygrometry was close to saturation. **FP:** feeding period during which the system was closed.

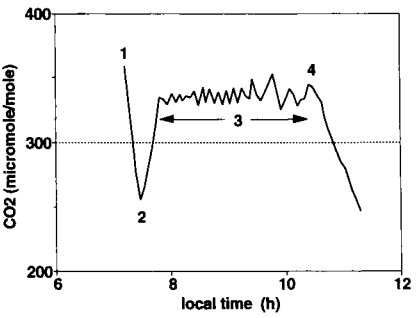

Fig 3. Time course of the CO_2 concentration in the chamber during the feeding of a whole tree. Different phases can be identified: 1, closing of the chamber; 2, injecting the $^{14}CO_2$ and starting the injection of cold CO_2; 3, regulation of the CO_2 concentration until exhaustion of the cold carbonate solution; 4, final phase after exhaustion of the carbonate solution.

increase of temperature inside the large chamber during a labelling day with very high solar irradiance. Although the air temperature reached 38°C inside the chamber at the end of the feeding period (> 12°C increase with respect to the ambient temperature), there was no significant alteration in photosynthesis as can be seen from figure 3: the assimilation rate, as derived from the parts with negative slopes, remained relatively regular throughout the labelling procedure. So did the kinetics of cold CO_2 injection operated by the system to keep the CO_2 concentration around 350 vpm (parts with positive slopes). This indicates that no major disturbance of photosynthesis and presumably of the general plant physiology occurred. In fact, the photosynthesis of walnut trees appears quite resistant to high temperature; nevertheless, negative values of net assimilation were observed one day when the inside temperature reached 45°C.

Validating the quantitative aspects of the feedings

Two indirect means could be used to estimate the amount of total radioactivity actually absorbed by the trees and compare it to the theoretical value as given in equation [1]:

– measuring the radioactivity that remained in the atmosphere of the chamber and in the different vessels at the end of the feeding period. At the end of a few local labellings, which according to equation

[1] were > 99.5% complete, the chamber atmosphere was forced into a KOH solution, then an aliquot was evaporated and assessed for radioactivity in an argon–methane flow counter (NU 20, Numelec, France). This method, although rapid, is not accurate for relatively concentrated solutions; however, it provides an order of magnitude. About 0.25% of the initially injected $^{14}CO_2$ was still in the chamber, which was in accordance with the theoretical value. The reaction vessel also retained a slight but measurable radioactivity: ≈ 0.3%, which stresses the importance of efficient stirring;

– sampling the tree soon after feeding in order to estimate the total radioactivity incorporated. Seven h after local labelling, in August 1989, 2 trees were harvested, fixed in liquid nitrogen and freeze-dried. After grinding, their total radioactivity was measured with the gas-flow counter: respectively, 88% and 91% of the injected radioactivity were recovered. The missing 10% was attributed to respiratory losses, although an experimental error of a few percent in assessing the total radioactivity of an entire tree cannot be discarded.

CONCLUSION

Use and performances of the system

The labelling system described exhibits 3 characteristics which have already been separately described by other workers, as mentioned above, but not together:

– a large assimilation chamber (> 3 m^3) allowing the labelling of large trees, namely grafted walnuts bearing some fruit. It remains handy enough to allow the labelling of a different tree every day;

– quantitative labelling. This can guarantee the complete assimilation of the injected CO_2, but it can also be stopped at any time (*eg* in case of excessive temperature increase) allowing the accurate amount of ^{14}C taken up to be determined;

– a CO_2 level constantly maintained at its natural value, thus limiting changes in the within-leaf partitioning between sucrose and starch which could affect export dynamics.

This system allowed us to investigate the spatial and chemical partitioning of assimilated carbon in walnut trees in August and October, when the trees exhibited contrasting daily net assimilation rates (Kajji, 1992). We also obtained interesting results on the long-term fate of the labelled carbon reserves, *eg* a differential mobilization rate of the starch reserves according to their formation time (Lacointe *et al,* 1993).

For the sake of simplicity no temperature regulation was included in our system and we assumed that in most cases this lack of thermal regulation had no effect on the process of redistribution of assimilates within the trees. Nevertheless, it is clear that incorporating such an improvement in the system would be of interest, as it would permit long-term labelling experiments or/and feeding during the warmest days.

ACKNOWLEDGMENT

The authors are most grateful to M Crocombette for providing technical assistance.

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