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The effect of different closure types, light, and sucrose concentrations on carbon isotope composition and growth of *Gardenia jasminoides* plantlets during micropropagation and subsequent acclimation *ex vitro*

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Abstract

The growth of *Gardenia jasminoides* Ellis plantlets and the development of photoautotrophy during two successive culture stages (shoot multiplication and root induction) *in vitro* was analyzed. We examined the effects of changes in growth conditions (type of tube closure, light, and sugar levels) on the development of photoautotrophy and growth during micropropagation and sought to establish whether they affected later acclimation to conditions *ex vitro*. During the two stages *in vitro*, plantlets were grown in tubes under two different PPFD (50 and 110 μmol m^{-2} s^{-1}), in media with three different sucrose concentrations (0, 1.5, and 3.0%, w/v) and with two different CO_2 levels inside the tubes (controlled by either tightly closed caps or loosely sealed caps, and with an external CO_2 concentration of 750 μmol mol^{-1}). The development of photoautotrophy was assessed by determining the difference between the stable carbon isotope composition ($\delta^{13}C$) of sugar cane sucrose used as a heterotrophic carbon source and that of leaflets grown *in vitro*. Plantlets from the root-induction stage showed a more highly developed photoautotrophy than those from the shoot- multiplication stage. At both stages, utilization of closed caps was the treatment which most stimulated development of photoautotrophy in plantlets. Also, lowering PPFD or sucrose concentration induced a greater degree of photoautotrophic development, the strongest effect being observed in plantlets cultured inside loosely sealed tubes. During acclimation *ex vitro*, plantlets taken from loosely sealed tubes *in vitro* performed better than those cultured inside tightly sealed tubes. The former, as well as recording a larger increase in fresh weight during this stage, also showed more negative $\delta^{13}C$ in the newly developed leaves, which would seem to indicate a better water status during acclimation. Present results validate the usefulness of $\delta^{13}C$ analysis of leaflets as a simple technique in assessing the development of photoautotrophy during culture *in vitro*. In addition, $\delta^{13}C$ analysis can be extended to evaluate growth conditions during acclimation to *ex vitro* conditions.

Abbreviations: LS-T – culture in loosely-sealed tubes under an external CO_2 concentration of 750 μmol mol^{-1}; TS-T – culture in tightly-sealed tubes at external CO_2 concentration of 750 μmol mol^{-1}; Δ - stable carbon isotope discrimination; $\delta^{13}C$ – ratio of $^{13}C/^{12}C$ relative to PeeDee belemnite standard; MS medium – Murashige and Skoog medium; p_i/p_a – ratio of intercellular to atmospheric partial pressure of CO_2; PPFD – photosynthetic photon flux density; L-PPFD – culture with 50±5 μmol m^{-2} s^{-1} PPFD; H-PPFD – culture with 110±10 μmol m^{-2} s^{-1} PPFD; N-Suc – medium with no sucrose; L-Suc – medium with 0.5% sucrose; H-Suc – medium with 3% sucrose; IAA – indole-3-acetic acid; BA - N^6–benzyladenine

Introduction

Explants, shoots and plantlets *in vitro* (hereafter plantlets) were previously considered to have inadequate photosynthetic ability for the provision of a positive carbon balance, and to require sugar as a carbon and energy source for hetero- or mixotrophic growth during multiplication and rooting stages (Grout and

Ashton, 1978). Recent research, however, has revealed that chlorophyllous plantlets *in vitro* have sufficient photosynthetic ability to develop photoautotrophy, but that this photosynthetic activity is restricted, in the main, by the low concentration of CO_2 in the vessel during the photoperiod and also, by sugar in the medium (Desjardins et al., 1988; Hdider and Desjardins, 1994; Kozai, 1991a, b; Kubota and Kozai, 1992; Solárová, 1989). This gives rise to the apparent paradox whereby sugar in the culture medium can be considered as being the main cause of the high production cost of plantlets in conventional mixotrophic micropropagation (for example, because of the loss of many plantlets due to contamination). On the other hand, the growth of plantlets *in vitro* can often be greater under photoautotrophic conditions than under heterotrophic conditions (see Kozai, 1991a, b). Furthermore, the photosynthetic ability of plants *in vitro* may favour further acclimation to conditions *ex vitro* (Kozai, 1991a, b; Kozai et al., 1990a). However, other experiments reported (Debergh et al., 1992) neither confirm that CO_2 is a limiting factor *in vitro* nor that a more photoautotrophic plantlet can overcome the transplantation shock (acclimation) more easily. Indeed, Capellades et al. (1990a, b) have shown that the least photoautotrophic plants of *Rosa multiflora* give the best results during the acclimation stage.

Although it is widely accepted that changing environmental factors may affect the development of photoautotrophy *in vitro* (see Kozai, 1991a, b), it is difficult to assess the extent to which the photoautotrophy attained by a given *in vitro* cultured plantlet is determined by these growth conditions. Single or rapid-measuring criteria cannot usually reflect the complex process of development of photoautotrophy, because they are either indirectly related or short-term indicators. More direct or accurate approaches to the quantification of the overall (time-integrated) photosynthetic contribution to *in vitro* plantlet growth (for example radioactive labelling) involve complicated, expensive and time-consuming techniques (Serret et al., 1996, and references mentioned therein).

Tracing with compounds weakly labelled with stable carbon isotopes means that precise, long-term monitoring of different carbon sources in plants can be undertaken (Deléens et al., 1994). Thus, for *in vitro* mixotrophic cultures of a C_3 plant, such as *Gardenia jasminoides*, the metabolic origin of carbon in leaflets (either from photosynthesis or from sucrose in the culture medium) may be traced thanks to the slight difference between carbon sources in natural isotope composition (see Serret et al., 1996). Therefore, the two carbon sources participating in *in vitro* plantlet growth can be distinguished by their natural isotope composition: on the one hand, heterotrophic products enriched in ^{13}C, originating from sugar cane (a C_4 plant) sucrose in culture media, and, on the other, photosynthetic products within the range typical of a C_3 plant (see Farquhar et al., 1989).

Tracing with stable isotopes was undertaken here to assess the extent to which changing growth conditions (*i.e.* CO_2 concentration, sucrose and light levels) during two different *in vitro* micropropagation stages (*i.e.* shoot multiplication and root induction) affect the degree of photoautotrophy of gardenia plantlets. In addition, the carbon isotope composition of leaves developed during acclimation to *ex vitro* conditions was used to assess the development of water stress during this same period of acclimation. This has been identified as the main stress that plants are exposed to during this period (Fujiwara et al., 1993). For C_3 plants, the carbon isotope composition is an integrated indicator of the ratio of intercellular to atmospheric partial pressure of CO_2 (p_i/p_a) (Farquhar, et al. 1982; 1989). The ratio p_i/p_a, in turn, is strongly affected by water status during growth. Both decreased water availability and increased evapotranspiration result in higher (less negative) $\delta^{13}C$ in plant material because of their effects on stomatal conductance and photosynthetic capacity (Condon et al., 1992). Therefore, from the analysis of $\delta^{13}C$ on leaves it should be possible to infer the occurrence of water stress during acclimation.

Materials and methods

Plant material and growth conditions

Micropropagated gardenia (*Gardenia jasminoides* Ellis) plantlets were derived from 2 cm-long shoot tips, grown in the greenhouse. Plantlets were aseptically grown inside Pyrex tubes (52 ml). During shoot multiplication, explants of approximately 74±6 mg (mean ± SE fresh weight of 11 samples), consisting of 2-3 leaflets and having a minimum shoot length of 1.5 cm, were cultured in 13 ml of MS medium (Murashige and Skoog, 1962) with 8.0 g l^{-1} of agar (Difco) and different sucrose concentrations (3%, 0.5% and 0%, w/v). The vitamins used in the culture were thiamine HCl (50 mg l^{-1}, m-Inositol (5 g l^{-1}), Ca pantothenate (0.5 g l^{-1}) and glycine (0.2 g l^{-1}). The growth regulator used during this phase was BA (1.0 mg l^{-1},

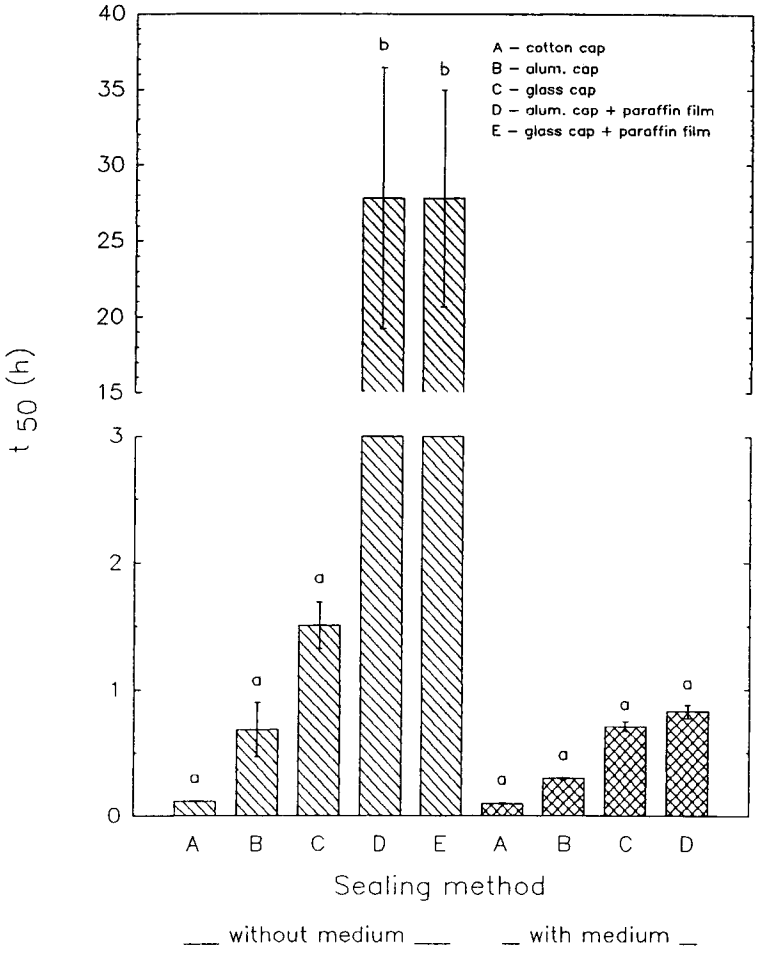

Figure 1. Influence of different methods of tube sealing and the presence or not of agarized media on the time in hours required for half of injected CO_2 to escape (t_{50}). The volumes of the empty tubes and the medium used were 52 ml and 13 ml, respectively. The measurements were run at 23°C and the initial concentration of CO_2 was approximately 2% (v/v). Values of t_{50} are means ± SE of at least 18 (empty tubes) or 5 (tubes with medium) measurements. Means not shearing a common letter are significantly different ($p \leq 0.05$) by Duncan comparison Test. Sealing methods assayed were A: cotton cap, B: aluminium cap, C: glass cap, D: aluminium cap sealed with paraffin film, and E: glass cap sealed with paraffin film.

Fluka) and pH was adjusted to 5.5. After four weeks of culture, root formation *in vitro* was induced by transfer to the same medium modified to contain half strength macroelements and 2.0 mg l^{-1} IAA (Fluka) and pH 5.0. Thus, explants of about 171±15 mg (mean ± SE), derived from plantlets grown during the shoot multiplication stage, were subcultured for a further four weeks. During root induction, sucrose levels were maintained at the same level as during shoot multiplication. In both culture stages, cane sugar was used as a sucrose source in the preparation of the different media. In addition, a fourth medium with 3.0% sugar beet (a C_3 plant) sucrose was also prepared and assayed for shoot multiplication. Plantlets were maintained in a growth chamber (E-15, Conviron), with a 12-h photoperiod. Growth chamber temperature was kept constant (22±2°C) through the light/darkness period. Illumination was provided by fluorescent cool white tubes (F72T12/CW/VHO 160W, Sylvania) supplemented with incandescent bulbs (Sylvania). Light intensity was adjusted by the use of a shadow mesh. For each sucrose concentration in the culture media and during both culture stages, two photosynthetic photon flux densities (PPFD) were assayed: 50±5 μmol m^{-2} s^{-1} (low PPFD) and 110±10 μmol m^{-2} s^{-1} (high PPFD) inside the culture tubes, at the level of cul-

ture medium. The CO_2 concentration inside the growth chamber was maintained around 750 μmol mol^{-1} during both stages. Culture tubes were loosely closed with aluminium caps (Sero-Tap 24-26 mm, Selecta), provided with an inner spring which prevented tight closure, thus allowing gas exchange with the surrounding atmosphere. Then, half of the tubes were kept loosely closed (LS-Tubes) while the other half were tightly sealed with paraffin film (Parafilm, American Nat. Can, Greenwhich, CT.USA) to prevent gas exchange (TS-Tubes). Differences in permeability to gas exchange using these two closure systems were tested (and compared with other conventional systems of tube closure such as glass caps), under two experimental conditions: tubes with and without the agarized medium. The half time for gas retention (t_{50}) for the CO_2 was measured (Figure 1) following Jackson et al. (1994) by tracking the decline in concentration over time of CO_2 previously injected into the tube with a portable infrared gas analyzer (IRGA, LICOR 6200, LI-COR, Lincon, Nebraska, USA).

Acclimation was performed in the growth chamber described above for a period of 4 weeks. Plantlets coming from the root induction stage were transplanted in 0.055 l pots, covered with a transparent plastic to prevent dehydration, and then placed within the growth chamber. The substrate was a mixture of peat:perlite (1:1, w/w). Pots were watered daily to field capacity. The plastic cover was gradually opened to provide a final relative humidity of 60%. The PPFD was 60±5 μmol m^{-2} s^{-1} during the first week and thereafter it was progressively increased to 325±15 μmol m^{-2} s^{-1}. Temperature at the level of plants was kept constant (23±2°C) through light/darkness period.

Leaflets from both micropropagation stages together with leaves from acclimation were evaluated for relative composition of stable carbon isotopes. In addition, total fresh and dry weights of the aerial part of plantlets were measured at the end of each stage. Measurements of dry weight were taken after drying samples at 60°C for 48h. During root induction, the degree of rooting was evaluated as the total fresh weight of roots per plantlet. For acclimated plants the total fresh and dry weights of the aerial part, as well as the fresh weight of roots, were measured.

^{13}C composition

Newly developed leaflets were sampled at the end of both micropropagation stages. Two distinct samples were collected per plant at the acclimation stage: first, the last leaflet to appear during the root induction stage and which expanded further during acclimation and second, the first leaf to have reached full developement during acclimation. Samples were dried at 60 °C and ground to a fine powder. The $^{13}C/^{12}C$ ratios were determined by mass spectrometry at Isotope Services, Inc., (Los Alamos, NM 87544, USA). Results were expressed as $\delta^{13}C$ values (‰), where:

$$\delta^{13}C(‰) = [(\text{R sample/R standard}) - 1] \times 1000 \quad (1)$$

R being the $^{13}C/^{12}C$ ratio. A secondary standard, calibrated against Peedee belemnite (PDB) carbonate, was used for comparison. Samples of at least 2 mg were used. Whenever possible 2-3 samples, each consisting of 1-3 plantlets, were analyzed for both kinds of tube closure and PPFD levels, culture medium and micropropagation stage. In addition, sucrose (either cane or beet sugar) from the culture medium was analyzed ($\delta^{13}C$ sugar) and the difference between the carbon isotope composition of the plantlets and the sucrose was further calculated as $\delta^{13}C$ sugar - $\delta^{13}C$ leaves. Values of $\delta^{13}C$ for the cane sugar and beet sugar sucrose were −11.29 and −23.54 ‰, respectively. During acclimation, analysis was carried out on plants coming from each of the treatments assayed during root induction. Within a given treatment, and for the two distinct leaves assayed at this stage, 2-3 samples, each consisting of one leaf, were analyzed.

Evaluation of the degree of photoautotrophy

The proportion of C atoms incorporated by photosynthesis to total C atoms in a recently developed leaflet cultured *in vitro* was determined on the basis of the isotope dilution equation (see Serret et al., 1996). Thus, plantlets were cultivated under two differently labelled sucroses (sugar beet and sugar cane), within the range of natural difference in $\delta^{13}C$. Then, the proportion ($a \leq 1$) of leaflet carbon coming from photosynthates was calculated as follows:

$$a = \frac{\delta^{13}\text{Csb-leaflet} - \delta^{13}\text{Csb-sucrose}}{\delta^{13}\text{Cphotosynthates} - \delta^{13}\text{Csb-sucrose}} = \frac{\delta^{13}\text{Csc-leaflet} - \delta^{13}\text{Csc-sucrose}}{\delta^{13}\text{Cphotosynthates} - \delta^{13}\text{Csc-sucrose}} \quad (2)$$

and then,

$$a = \frac{\delta^{13}\text{Csc-leaflet} - \delta^{13}\text{Csb-leaflet}}{\delta^{13}\text{Csb-sucrose} - \delta^{13}\text{C sc-sucrose}} + 1 \quad (3)$$

with $\delta^{13}C$ sb- and $\delta^{13}C$ sc-leaflet being the carbon isotope composition of leaflets grown in MS media with either sugar beet or sugar cane sucrose; $\delta^{13}C$ sb- and $\delta^{13}C$ sc-sucrose the isotope composition of sugar beet and sugar cane sucrose used; and $\delta^{13}C$ photosynthates, the carbon isotope composition of carbon proceeding from photosynthesis. Values of *a* and $\delta^{13}C$ photosynthates were calculated, for leaflets cultured during the shoot multiplication stage with 3.0% sucrose at L-PPFD and the two different tube closures assayed, using equations [2] and [3].

For the remaining culture conditions studied, in order to halve the number of $\delta^{13}C$ analyses performed, a ranking of photoautotrophy among the set of culture conditions studied was established from the difference between $\delta^{13}C$ of sucrose and $\delta^{13}C$ of leaflets ($\delta^{13}C$ sucrose -$\delta^{13}C$ leaflets) only from plantlets cultured with sugar cane sucrose (see Serret et al., 1996).

CO_2 concentration inside the tubes

For the set of treatments assayed, the CO_2 concentration inside the culture tubes was measured during root induction by means of a portable IRGA (LICOR 6200). The tubes used for CO_2 measurements were closed using the aluminium caps described earlier (see growth conditions), in which a serum cap was inserted to permit headspace sampling during the experiments. A small volume of air from inside the tube was sampled with a gas-tight syringe and then injected into the IRGA. The CO_2 concentration of the sample was calculated from the variation in CO_2 caused by injection and measured by the IRGA following corrections to allow for changes in air pressure and volume due to dilution in the gas exchange system (170 ml volume). From each culture tube, three successive extractions were performed at intervals of about 2h: the first one being during the hour before the onset of light period. The first and second extraction consisted of 1 ml air each and this represented about 2.5% of the total air inside the culture tube (39 ml air volume = 52 ml tube volume - 13 ml gel volume), and the third which consisted of 2 ml air in order to improve the accuracy of measurements affected by low CO_2 concentration.

Analysis of data

Data on t_{50}, CO_2 concentration inside the tubes, $\delta^{13}C$ and weight of plantlets were analyzed by ANOVA using Number Cruncher Statistical System (ver. 5.03).

Results

CO_2 exchange in culture tubes and the effect darkness and light variation on CO_2 concentration inside the tubes

Figure 1 shows the effect of different methods of sealing tubes and the presence or absence of gel on the t_{50} obtained after injecting sufficient gas to raise the initial internal concentration to approximately 2% CO_2. Sealing the caps with paraffin film significantly ($p \leq 0.05$) increased t_{50}. Thus, the tubes without gel showed values ranging from over 25 h, for caps sealed with paraffin film, down to less than 1.5 h for caps without paraffin film sealing. For the tubes with aluminium caps, sealing with paraffin film increased the t_{50} more than 40 times. However, when t_{50} was evaluated in tubes with medium, no significant effect of film sealing was observed and t_{50} values were less than 1 h for all the closures assayed. In fact, there was a significant ($p \leq 0.05$) interaction between the presence of gel and the utilization of paraffin film.

The CO_2 accumulated at the end of the dark period (see filled bars in Figure 2) inside the tightly closed (TS-Tubes) tubes was around 6500 μmol mol^{-1} (mean value across treatments), 5-10 times higher than that in the loosely closed (LS-Tubes) tubes, whereas the CO_2 concentration inside the growth chamber (i.e. outside the tubes) was kept constant at around 750 μmol mol^{-1}. The very high concentration inside the tightly closed tubes may be due to the low tranfer (mainly by diffusion) of CO_2 through them. In contrast, after several (3-4) hours of light-period, CO_2 concentration inside the tightly closed tubes dropped to a level around 200 μmol mol^{-1}, whereas in the loosely closed tubes levels were almost twice these. Difference in CO_2 concentration inside the tubes due to the type of sealing was highly significant ($p \leq 0.001$), during both the dark and the light period. In addition, for the light period the concentration of sugar also had a significant ($p \leq 0.001$) effect on the CO_2 concentration. Significant ($p \leq 0.05$) interactions existed between sugar concentration and PPFD level and the kind of tube closure.

Effect of carbon source on carbon isotope composition of leaflets: development of photoautotrophy by plantlets in vitro

Carbon isotope composition of leaflets varied widely according to the growth conditions and culture stage (Table 1). Leaflets with higher (less negative) $\delta^{13}C$

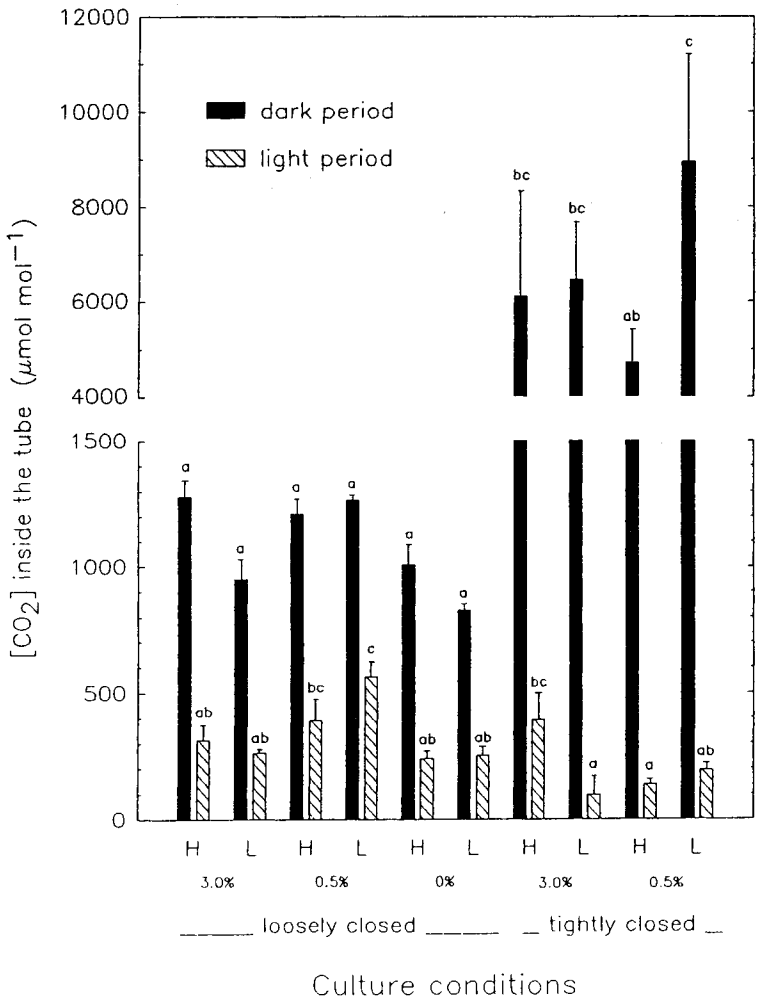

Figure 2. CO_2 concentration inside the culture tubes measured during root induction in the set of treatments assayed by means of a portable infrared gas analyzer (IRGA). Values represent the CO_2 concentration the hour before the onset of light period (dark bars) and the lowest concentration recorded after 4 h of photoperiod (dashed bars). Values are means ± SE of 4-5 tubes. Treatments assayed were the two different PPFD (H, L) levels, three sucrose (0%, 0.5%, 3.0%) concentrations and the two kinds of tube closure detailed in Materials and Methods. Within either, dark- or light-period, means not shearing a common letter are significant different ($p \leq 0.05$) by Duncan comparison Test.

values were the consequence of a greater utilization of sugar cane sucrose as a carbon source ($\delta^{13}C$ sugar cane sucrose = -11.29 ‰), whereas those with lower (more negative) $\delta^{13}C$ values fixed more CO_2 from the surrounding atmosphere. From the mean $\delta^{13}C$ values of leaflets for each growth condition and micropropagation stage (Table 1), the difference ($\delta^{13}C$ sugar - $\delta^{13}C$ leaflets) was calculated, using the $\delta^{13}C$ sugar cane as a constant (Figure 3).

For plantlets grown inside loosely sealed tubes (LS-Tubes) in presence of 0.5% or 3.0% sugar, values of ($\delta^{13}C$ sugar - $\delta^{13}C$ leaflets) during the root induction stage were higher than those during the shoot multiplication stage (Figure 3A, B). This would suggest a more highly developed photoautotrophic metabolism during root induction than in the previous (shoot multiplication) stage. Based on ($\delta^{13}C$ sugar - $\delta^{13}C$ leaflets) values, the culture of plantlets inside loosely closed tubes (LS-Tubes) which facilitated gas exchange through the tube was the factor which stimulated the development of photoautotrophy most during *in vitro* culture, particularly during the later (i.e. root induction) stage (Figure 3A, B). Thus, the ($\delta^{13}C$ sugar - $\delta^{13}C$ leaflets) value increased from TS- to LS-Tubes around 2.5 and 4.0 times during shoot multiplication and root induction, respectively (Figure 3C). However, during

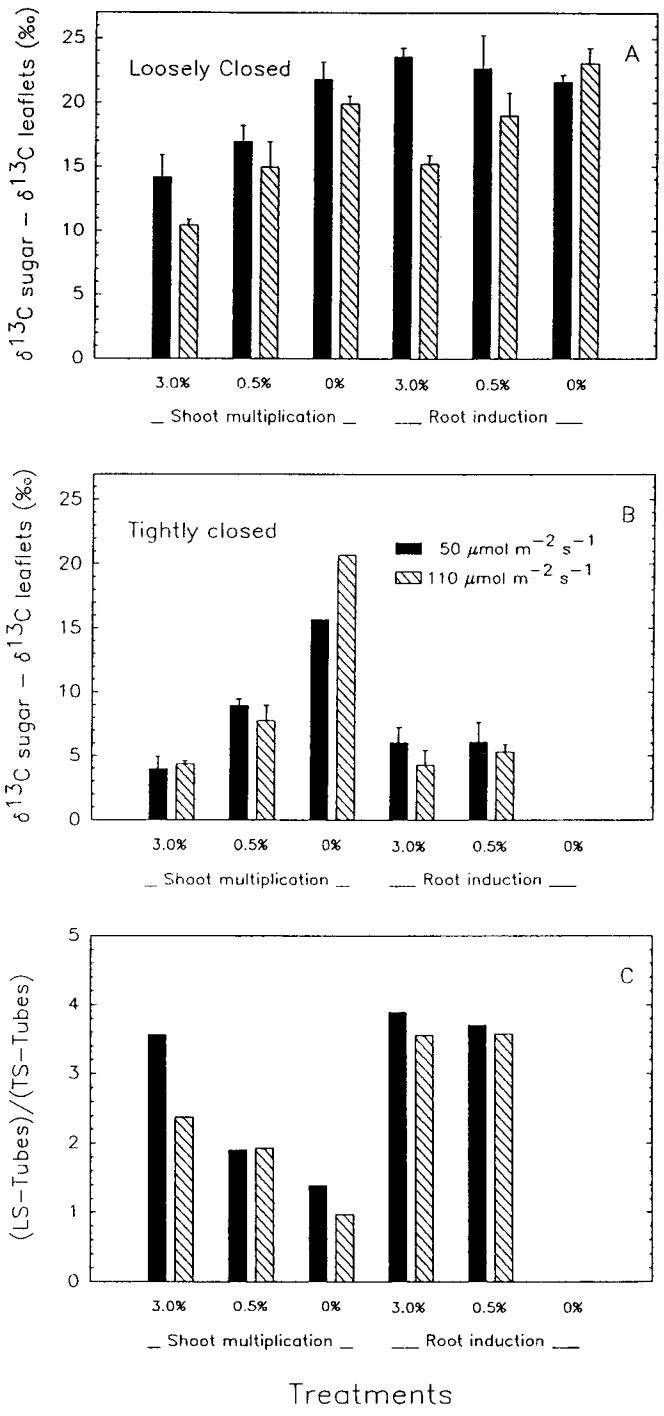

Figure 3. Effect PPFD and sucrose levels on the difference between $\delta^{13}C$ of sugar cane sucrose in culture medium and $\delta^{13}C$ from dry matter of gardenia leaflets ($\delta^{13}C$ sucrose - $\delta^{13}C$ leaflets) developed during shoot multiplication and root induction stages within loosely sealed (LS-Tubes: *Figure 3A*) and tightly sealed (TS-Tubes: *Figure 3B*) tubes. Except for shoot multiplication with no sucrose (only one sample available), values are means ± SE of 2-3 samples, each consisting of 1-3 plantlets. For each treatment and culture stage the effect of increasing the inside CO_2 concentration during light period (by using tubes with loose caps) on induction of photoautotrophy is represented by the ratio between the ($\delta^{13}C$ sucrose - $\delta^{13}C$ leaflets) difference in LS-Tubes and TS-Tubes leaflets (*Figure 3C*)

Table 1. Effect of tube closure, PPFD levels, and sucrose concentration on medium on stable carbon isotope composition ($\delta^{13}C$, ‰) of gardenia leaflets developed during shoot multiplication and root induction culture periods.

	H-Suc LS-Tubes	H-Suc TS-Tubes	L-Suc LS-Tubes	L-Suc TS-Tubes	N-Suc LS-Tubes	N-Suc TS-Tubes[x]
			Shoot Multiplication			
H-PPFD	-21.73±0.50^{c}	-15.68±0.21^{ab}	-26.24±1.99^{d}	-19.05±1.21^{bc}	-31.22±0.57^{ef}	32.00^{ef}
L-PPFD	-25.44±1.78^{d}	-15.26±1.02^{a}	-28.27±1.24^{de}	-20.26±0.52^{c}	-33.11±1.35^{f}	-26.98^{d}
			Root Induction			
H-PPFD	-26.53±0.69^{b}	-15.57±1.18^{a}	-30.26±1.79^{bc}	-16.61±0.62^{a}	-34.35±1.19^{cd}	—
L-PPFD	-34.85±0.72^{d}	-17.35±0.98^{a}	-33.97±2.60^{cd}	-17.42±1.49^{a}	-32.90±0.53^{cd}	—
			Acclimation[y]			
			1st leaf developed			
H-PPFD	-28.36±0.65^{b}	-24.17±0.98^{a}	-29.18±0.23^{b}	-29.37—^{b}	-29.87±0.57^{b}	—
L-PPFD	-28.28±0.69^{b}	—	-30.71±1.25^{b}	-24.60±1.12^{a}	-30.75±0.31^{b}	—
			2nd leaf developed			
H-PPFD	-29.20±0.44^{a}	-28.44±0.18^{a}	-28.68±0.35^{a}	-28.77 —^{a}	-29.17±0.48^{a}	—
L-PPFD	-28.78±0.54^{a}	—	-29.44±0.35^{a}	-29.10±0.65^{a}	-29.06±0.17^{a}	—

Values of $\delta^{13}C$ from leaves of plants acclimated to conditions *ex vitro* are also included. Values represent the mean ± SE of 2-3 samples, each sample consisting of at least two leaflets or leaves, respectively. For each growth period means within a column not sharing a common letter are significantly different ($p \leq 0.05$) by Duncan comparison Test, when ANOVA was also significantly ($p \leq 0.05$) different. Abbreviations of growth conditions assayed: LS-Tubes and TS-Tubes, culture in tubes with unsealed and sealed caps, respectively; L-PPFD and H-PPFD, culture with 50±5 μmol m^{-2} s^{-1} PPFD and 110±10 μmol m^{-2} s^{-1} PPFD, respectively; N-Suc, L-Suc and H-Suc, media with 0%, 0.5% and 3% of sucrose, respectively.

[x] Due to the small growth of these plants only one analysis was performed with all leaflets pooled. This growth condition (N-Suc TS-Tubes) was discarded in the further stages. [y] Treatments refer to growth conditions during culture *in vitro*.

shoot multiplication within TS-Tubes, a dramatic loss of photoautotrophy was also evident as the sucrose concentration increased (Figure 3B). This loss of photoautotrophy was partially compensated by growing with LS-Tubes (Figure 3A vs 3B). During shoot multiplication there is therefore an important interaction between the tube closure and sugar (and to a lesser extent PPFD). In contrast, large stimulation of photoautotrophy was observed during root induction, independently of sugar concentrations and PPFD levels. At this stage, the type of tube closure appears to be the main factor controlling the development of photoautotrophy (Figure 3C). Although the PPFD was the factor in which the effect was least evident, L-PPFD (compared with H-PPFD) somewhat stimulated the development of photoautotrophy in plantlets from both stages when cultured with LS-Tubes and with the highest sucrose (H-Suc) concentration (Figure 3).

Because the type of tube closure during *in vitro* culture was the strongest factor inducing photoautotrophy in the plantlets (Figure 3), the effect of this treatment on the performance of photoautotrophy was examined more closely by culturing LS- and TS-Tube plantlets with sugar beet sucrose (Table 2). Thus, during shoot multiplication at 3% sucrose and 50 μmol m^{-2} s^{-1} PPFD, the relative contribution of photosynthates to total carbon content of leaflets increased from 36.3% to 92.7% when plantlets were cultured inside tightly sealed or loosely sealed tubes respectively (Table 2).

Changes in carbon isotope composition during acclimation

In order to evaluate the response of plantlets during acclimation, the carbon isotope composition of the first two leaves which developed through the acclimation period was analyzed. The range of $\delta^{13}C$ values measured was lower than that of leaflets *in vitro*, as CO_2 was the only source of carbon available to plants. Variations in $\delta^{13}C$ were probably due to the development of stress periods during acclimation. Values of $\delta^{13}C$ from the first leaf developed were significantly higher in plants coming from some of the treatments with air-tight tubes compared with values recorded for plants cultured in loosely-closed tubes under the same PPFD and with the same sucrose levels (Table 1). The sec-

Table 2. Carbon isotope composition of photosynthates ($\delta^{13}C$ photosynthates) and the relative contribution of photosynthates (% photosynthates) to carbon content of gardenia leaflets growing during shoot multiplication with 3.0% sucrose, L-PPFD and two different types of tube closure (loosely and tightly sealed tubes: LS-Tubes, TS-Tubes).

	$\delta^{13}C$ sucrose	Leaflets	
		LS-Tubes	TS-Tubes
$\delta^{13}C$ sugar cane (‰)	−11.29	−25.44	−15.26
$\delta^{13}C$ sugar beet (‰)	−23.54	−26.33	−23.06
% photosynthates (%)		92.7	36.3
$\delta^{13}C$ photosynthates (‰)		−26.55	−22.22

Plantlets cultured *in vitro* were subcultured in an MS medium containing 3.0% of either sugar beet or sugar cane sucrose. For calculations of $\delta^{13}C$ photosynthates and% photosynthates, $\delta^{13}C$ values of sugar beet and sugar cane sucrose as well as those from leaflets grown under either MS medium in the presence of different types of sugar were used as detailed in equations [2] and [3] of Material and Methods.

ond leaf which developed during acclimation showed no differences in $\delta^{13}C$ between plantlets from different culture conditions *in vitro*. $\delta^{13}C$ values were as low as those of the first leaf previously developed under LS-Tubes *in vitro*.

Effect of culture conditions in vitro on weight of plantlets during micropropagation and further acclimation

During shoot multiplication for a given PPFD and sucrose level over 0%, plantlets cultured within TS-Tubes showed higher final fresh weights than those in LS-Tubes, whereas at 0% sucrose, LS-Tubes gave rise to higher weights. In contrast, during root induction the response to tube closure during culture was reversed. Thus, for a given PPFD and sucrose level, plantlets cultured with LS-Tubes showed higher fresh weight (Table 3) than those cultured in TS-Tubes. H-PPFD plantlets also showed higher fresh weights than L-PPFD plantlets. The sucrose level in the media had no effect and even N-Suc plantlets showed a tendency to higher fresh weights. During root induction, the main factor affecting the percentage of dry weight compared to fresh weight was the sucrose concentration in the media. This percentage decreased from a value somewhat higher than 20% to about 16% as sucrose in the media was reduced from 3% to 0%. Total fresh weight after acclimation tended to be higher in plants coming from LS-Tubes plantlets *in vitro* than that in plants from TS-Tubes. Dry weight of acclimated plants was around 20% of fresh weight irrespective of their growth conditions during micropropagation. Total fresh weight of plantlets after root induction could be of relevance due to its effect on the fresh weight of plants acclimated *ex vitro*. Thus, for the set of treatments assayed, when considered together, there was a positive correlation (r^2=0.51, n=8, $p \leq 0.05$) between total fresh weight of plantlets at root induction and that of acclimated plants.

Discussion

The relative contribution of photosynthesis to total carbon accumulation by leaflets cultured inside tightly closed tubes during root induction under H-Suc and L-PPFD conditions was 36% (Table 2). This value fits within the range of variation (due to the cultivar chosen) in *Rosa multiflora* growth under tight-closure usage conditions, caused by CO_2-fixation (25-60%), and measured using ^{14}C labelling (De Riek et al., 1991; Debergh et al., 1992). Using an indirect approach involving the daily measurements of net CO_2 uptake, Fujiwara et al. (1987) also concluded that for a set of 12 different ornamental plants growing in closed vessels the main source of carbon for plantlet growth was sucrose. When cultured inside LS-Tubes, the contribution of external CO_2 to the carbon assimilated by leaflets increased to almost 95%, which shows the strong effect of this treatment. The effect of varying other growth conditions in gardenia micropropagation, such as increasing PPFD during shoot multiplication, did not yield such a marked improvement in the contribution of external CO_2(Serret et al., 1996).

Our results here, therefore, provide evidence to show that the development of photoautotrophy in micropropagated gardenia seems to be restricted by the tightness of the closure of tubes (which results in lower levels of CO_2 inside the tubes during the photoperiod compared with those of loosely sealed tubes) and in part by the presence of sugar in the medium, particularly during shoot multiplication (Figure 2, Table 2). Furthermore, plantlets from the root induction stage showed more highly developed photoautotrophic characteristics than those from the shoot multiplication stage. Similar conclusions have been reached previous-

Table 3. Effect of tube closure, PPFD levels, and sucrose concentration on medium on the total fresh weight (mg per plantlet) and the dry weight relative to fresh weight (%) of the aerial part of gardenia plantlets developed during shoot multiplication and root induction stages *in vitro* and the further acclimation to conditions *ex vitro*.

	H-Suc LS-Tubes	H-Suc TS-Tubes	L-Suc LS-Tubes	L-Suc TS-Tubes	N-Suc LS-Tubes	N-Suc TS-Tubes[x]
			Shoot Multiplication			
			(aerial part: total)			
H-PPFD	289 ± 38^{cd}	448 ± 68^{e}	244 ± 37^{bcd}	308 ± 18^{d}	188 ± 18^{abc}	113 ± 17^{a}
L-PPFD	248 ± 37^{bcd}	257 ± 31^{bcd}	177 ± 21^{ab}	236 ± 19^{bcd}	234 ± 33^{bcd}	98 ± 09^{a}
			Root Induction			
			(aerial part)			
H-PPFD	252±33	253±32	356±67	295±18	292±37	—
L-PPFD	283±11	154±31	223±37	218±51	299±28	—
			(relative dry weight)			
H-PPFD	21.2 ± 0.3^{c}	20.8 ± 0.7^{c}	17.5 ± 0.3^{ab}	17.4 ± 0.4^{ab}	16.3 ± 1.0^{ab}	—
L-PPFD	18.1 ± 1.8^{abc}	27.8 ± 1.5^{d}	21.0 ± 1.1^{c}	19.2 ± 0.9^{bc}	15.9 ± 0.7^{a}	—
			(roots)			
H-PPFD	129 ± 12^{c}	115 ± 18^{c}	64 ± 13^{ab}	49 ± 3^{a}	94 ± 11^{bc}	—
L-PPFD	92 ± 08^{bc}	63 ± 15^{ab}	106 ± 13^{c}	63 ± 9^{ab}	125 ± 15^{c}	—
			(total)			
H-PPFD	382±45	369±40	420±80	345±20	386±41	—
L-PPFD	374±19	218±36	348±43	281±59	424±25	—
			Acclimation[y]			
			(aerial part)			
H-PPFD	983 ± 174^{cde}	950 ± 67^{bcde}	793 ± 71^{bc}	—	861 ± 67^{bcd}	—
L-PPFD	1206 ± 127^{e}	—	699 ± 100^{b}	405 ± 40^{a}	1116 ± 81^{de}	—
			(relative dry weight)			
H-PPFD	19.8 ± 0.6^{ab}	18.4 ± 0.4^{a}	20.1 ± 0.7^{ab}	—	19.8 ± 0.5^{ab}	—
L-PPFD	20.9 ± 0.5^{b}	—	19.4 ± 0.6^{ab}	19.7 ± 0.6^{ab}	20.3 ± 0.3^{b}	—
			(roots)			
H-PPFD	508 ± 49^{bcd}	429 ± 40^{bc}	379 ± 40^{b}	—	438 ± 40^{bc}	—
L-PPFD	642 ± 70^{d}	—	390 ± 70^{b}	177 ± 30^{a}	555 ± 40^{cd}	—
			(total)			
H-PPFD	1491 ± 128^{cd}	1379 ± 98^{cd}	1171 ± 99^{bc}	—	1299 ± 148^{cd}	—
L-PPFD	1848 ± 196^{d}	—	937 ± 150^{ab}	582 ± 67^{a}	1669 ± 129^{d}	—

For plantlets during root induction and acclimation fresh weight of roots and total (aerial part plus roots) fresh weight was also recorded. Values represent the mean ± SE of at least 21 plantlets from shoot multiplication, 5 plantlets from root induction and between 5-24 (depending on treatments) from acclimation. For each growth period and parameter studied means not sharing a common letter are significantly different ($P\leq0.05$) by Duncan comparison Test, when ANOVA was also significantly ($P\leq0.05$) different. Columns without letters are not significantly different ANOVA.

[x] Due to the small growth of these plants only one analysis was performed with all leaflets polled. This growth condition (N-Suc L-CO_2) was discarded in the further stages.

[y] Treatments refer to growth conditions during culture *in vitro*.

ly for other micropropagated plants using more sophisticate and/or expensive methods such as monitoring of photosynthetic gas exchange throughout development or labelling with radioactive isotopes (De Riek et al., 1991; Kozai, 1991a, b; Kozai et al., 1992).

In contrast, the fact that lower PPFD can stimulate photoautotrophy might be somewhat surprising. Indeed, it is generally accepted that photoautotrophic growth can be stimulated by increasing quantum flux density (Lee et al., 1985), particularly when high PPFD is associated with CO_2 enriched conditions *in vitro* (Dubé and Vidaver, 1992; Kozai, 1991a, b). However, our results show that L-PPFD (50 μmol m^{-2} s^{-1} inside the tube) induced more photoautotrophic plantlets than H-PPFD (110 μmol m^{-2} s^{-1} inside the tube), when cultured in a medium with sucrose. In a previous study of gardenia, the effect of higher PPFD on photoautotrophy also remained unclear (Serret et al., 1996). Thus, the intermediate PPFD (110 μmol m^{-2} s^{-1}, outside the tube) enhanced photoautotrophy only during the shoot multiplication stage, while higher PPFD reversed this tendency. Otherwise, higher PPFD is associated with an increase of temperature inside tubes (Serret et al., 1996), which in most exposed leaves and conditions of low transpiration, may be even higher. The PPFD conditions during *in vitro* culture described elsewhere usually refer to measurements taken on the empty culture shelf and not to those taken inside a closed tube among other test tubes in a stainless wire frame. Therefore, the actual PPFD which reaches plantlets may be substantially lower. Thus, Fujiwara et al. (1989) reported that PPFD inside glass tubes of 47 ml volume closed with aluminium caps and placed on the culture shelf was 76% lower than the mean incident PPFD on the shelf surface.

The difference in degree of air-tightness provided by the caps is clearly stated from the difference in CO_2 concentration inside the tubes, during either the dark or the light period. Thus, whereas CO_2 concentration inside the growth chamber was more than twice that found in normal atmospheric conditions (750 μmol mol^{-1}), the CO_2 concentration after about 4 hours of light period was around 200 μmol mol^{-1} in the tightly-closed tubes and nearly twice that inside the loosely closed tubes (Figure 2). Steady-state CO_2 concentrations in relatively air-tight culture tubes between 100-200 μmol mol^{-1} or lower during the light period have been shown by various authors (Fujiwara et al., 1992; Kozai, 1991a; Kozai et al., 1990a; Kubota and Kozai, 1992; Righetti et al., 1993; Solárová, 1989). However, our results about t_{50} (Figure 1) suggest that the agarized medium is actually acting as a strong sink for CO_2, which may have important implications buffering (i.e. smoothing) the light-darkness pattern of CO_2 inside the culture tubes. Indeed, other authors (Debergh et al., 1992) reported that the CO_2 level inside tightly-closed jars followed a typical circadian shift depending upon the light-darkness period, though it always remained higher than the normal external atmospheric concentration (350 μmol mol^{-1}). Nevertheless, Debergh et al. (1992) used much lower PPFD (30 μmol m^{-2} s^{-1}, no specification as to whether this was inside or outside jars) than the former authors. Similarly, Kozai et al. (1990a) working with *Cymbidium* plantlets concluded that the steady-state CO_2 concentration during the photoperiod was lower inside the vessel than outside at any PPFD greater than 35 μmol m^{-2} s^{-1}.

During the dark period, the CO_2 respired by plantlets accumulated to concentrations around 1000 μmol mol^{-1} in all the loosely-closed tubes, whereas CO_2 accumulated to concentrations between 4000 to 9000 μmol mol^{-1} in the tightly-closed tubes. A similar range of CO_2 concentrations to those found in tightly-closed tubes has been reported for closed vessels (Fujiwara et al., 1987). Thus, the high accumulation of CO_2 during the dark period inside the tightly-closed culture tubes, would suggest that they may represent a relatively closed system. The sugar cane- sucrose broken down in the plantlets by respiration may accumulate during darkness and it might be further refixed by photosynthesis during light period. Such refixation results is an underestimation of the degree of photosynthetic development (i.e. photoautotrophy) attained by plants. In this regard, refixation of the CO_2 respired in a closed system such as the TS-Tubes should not lead to further discrimination of that reassimilated by the plantlet (Farquhar et al., 1989) and, therefore, $\delta^{13}C$ of photoassimilates would not be affected. Current methods, such as quantification of the photosynthetic contribution in micropropagated plantlets using the two labelled substrates ^{14}C- sucrose and ^{14}C-CO_2, may also underestimate the real photosynthetic capability of plantlets cultured in relatively airtight tubes or vessels. The underestimation may be due to the refixation of accumulated respired CO_2, providing this CO_2 is not flushed away periodically (De Riek et al., 1991). However, in spite of this, a reasonable ranking order of treatments and/or culture stages of the relative contribution of external CO_2 as a source of carbon in culture plantlets may still be assessed by means of this simplified approach. For a given kind of tube closure (i.e. with the same gas exchange character-

istics throughout the tube), this technique can provide a reliable ranking order of treatments (e.g. sucrose or PPFD levels) and/or culture stages of the relative degree of development of photoautotrophy.

In this study, the $\delta^{13}C$ of photosynthates for the root multiplication plantlets cultured within tightly-sealed tubes (TS-Tubes, treatment, Table 2) was 4.33‰ higher than that of plantlets cultured inside loosely-sealed tubes (LS Tubes). This may be due to the low diffusive conductance of CO_2 through the former tubes. It is interesting to note that this value is similar to the value proposed as the fractionation of carbon isotopes due to the differential diffusivities of CO_2 containing ^{12}C and ^{13}C across the stomatal pathway (see references in Farquhar et al., 1989).

It has previously been reported that the fresh and dry weights attained by plantlets *in vitro* is greater in CO_2 enriched treatments than in CO_2 non-enriched environments (Kozai et al., 1990b; Kozai et al., 1991a), regardless of the sucrose concentration in the culture medium (Kozai et al., 1991b). We observed this pattern only during the root induction stage (Table 3). During the earlier shoot multiplication stage, and with the exception of the sugar free medium, the opposite tended to be true. This may be related to the markedly lower development of photoautotrophy during shoot multiplication compared to that during root induction (see Table 1, Figure 3). Fujiwara et al. (1992) concluded that for potato plantlets *in vitro*, in which CO_2 and PPFD conditions were appropriate to the development of photoautotrophy, cultures grew and developed largely depending on sucrose in the culture medium and before the CO_2 balance became positive. Thus, during the shoot multiplication stage in gardenia, the concentration of sucrose in the medium has a positive effect on the final weight of plantlets as it is the main source of carbon. A delay in plantlet growth when cultured in sugar-free medium has also been reported for various species during shoot multiplication (Reuther, 1991). During root induction under CO_2 enriched conditions, and in media without sugar, plantlet yields show weights comparable to those cultured with sucrose. Similarly, Reuther (1991) found no retardation of rooting in *Pelargonium peltatum* and *Vitis vinifera* due to the lack of sucrose in the medium. Galzy and Compan (1992), however, reported an increase in growth during root induction for *Vitis vinifera* when cultured under mixotrophic (culture medium with sucrose) conditions compared with cultivation under photoautotrophic (sucrose-free culture medium) conditions.

During acclimation, plantlets with a more highly developed photoautotrophy (grown inside loosely-sealed tubes, under external CO_2 enriched conditions) are the most efficient whether this is measured by the final weight of plants (Table 3) or the lower $\delta^{13}C$ values in the first leaf to develop (Table 1). Our results support earlier findings reported in other micropropagated species following transplantation *ex vitro* (see Kozai, 1991a, b). However, contradictory results have been obtained for other species (Capellades et al., 1990 a, b; Debergh et al., 1992). The latter authors concluded that it was the least photoautotrophic plantlets that gave the best results during the acclimation stage. Capellades et al. (1991) proposed the explanation that the least autotrophic plantlets, grown under the specific conditions of their experiment, accumulated more starch in their chloroplast and were therefore better suited for further acclimation. However their results might be explained (at least in part) by the low PPFD (25-30 μmol m^{-2} s^{-1}) and the fact that there was no indication as to whether this condition was to be found inside or outside the jars. Higher PPFD during micropropagation is associated with substantially higher air temperatures inside the tubes with respect to those outside (Serret et al., 1996) and therefore presumably with higher rates of transpiration (Tanaka et al., 1992; Fujiwara and Kozai 1995). This in turn may result in plantlets *in vitro* expressing certain xerophytic or light-acclimatized traits (Desjardins, 1995; Serret et al., 1996; Trillas et al., 1995), similar to those reported for leaves *ex vitro* (Araus et al., 1986; Araus and Hogan, 1994). Such morphophysiological characteristics would further improve acclimation to *ex vitro* conditions.

By measuring $\delta^{13}C$ of the leaves which developed during acclimation to *ex vitro* conditions, the occurrence of water stress periods can be inferred. Indeed, higher (less negative) values of $\delta^{13}C$ would be expected as a consequence of water stress (Farquhar et al., 1989; Condon et al., 1992; Araus et al., 1993). Thus, for the first leaf to develop the significantly higher values of $\delta^{13}C$ in some plants coming from TS-Tubes compared with those of plants from LS-Tubes would seem to indicate that the former suffered water stress after transplantation. Growth *in vitro* under the more photoautotrophic conditions favoured by loosely-closed tubes (together with a high external CO_2 atmosphere), could stimulate the development of plant adaptations against an excessive loss of water by transpiration. Thus, a more strongly developed root system (Table 2), cuticle and epicuticular waxes, along with a better stomatal

functioning (Sutter, 1983; Capellades et al., 1990a, b; Desjardins, 1995) in more photoautothrophic plantlets (i.e. from LS-Tubes) seems to prevent further development of water stress during acclimation. The absence of differences in $\delta^{13}C$ in the second leaf suggests that all plants were already acclimated to the *ex vitro* conditions when this leaf developed. Acclimated plants coming from the different *in vitro* growth conditions showed, in general, lower ratios of aerial section to root fresh weights than were shown by plantlets after root induction. Values in all cases were around 2, although plants derived from TS-Tubes showed values 10-15% higher.

In summary, present results validate the utility of $\delta^{13}C$ analysis of leaflets, reported in an earlier paper (Serret et al., 1996), as a single technique in the assessment of photoautotrophy development during *in vitro* culture. In addition, the utilization of $\delta^{13}C$ analysis could be extended to evaluate growth conditions during acclimation *ex vitro*.

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References

Araus JL, Alegre L, Tapia L, Calafell R & Serret MD (1986) Relationships between photosynthetic capacity and leaf structure in several shade plants. Am. J. Bot. 73: 1760–1770

Araus JL, Brown HR, Febrero A, Bort J & Serret MD (1993) Ear photosynthesis, carbon isotope discrimination and the contribution of respiratory CO_2 to differences in grain mass in durum wheat. Plant Cell Environ. 16: 383–392

Araus JL & Hogan KP (1994) Comparative leaf structure and patterns of photoinhibition of the neotropical palms *Scheelea zonensis* and *Socratea durissima* growing in clearings and forest understory during the dry season in Panama. Am. J. Bot. 81: 726–738

Capellades M, Fontarnau R, Carulla C & Debergh PC (1990a) Environment influences anatomy of stomata and epidermal cells in tissue-cultured *Rosa multiflora*. J. Amer. Soc. Hort. Sci. 115(1): 141–145

Capellades M, Lemeur R & Debergh PC (1990b) Kinetics of chlorophyll fluorescence in micropropagated rose shootlets. Photosynthetica 24: 190–193

Capellades M, Lemeur R & Debergh PC (1991) Effects of sucrose on starch accumulation and rate of photosynthesis in *Rosa* cultured *in vitro*. Plant Cell Tiss. Organ Cult. 25: 21–26

Condon AG, Richards RA & Farquhar GD (1992) The effect of variation in soil water availability, vapor pressure deficit and nitrogen nutrition on carbon isotope discrimination in wheat. Aust. J. Agric. Res. 43: 935–947

Debergh PC, De Meester J, De Riek J, Gillis S & van Huylenbroeck J (1992) Ecological and physiological aspects of tissue-cultured plants. Acta Bot. Neerl. 41: 417–423

Deléens E, Cliquet JB & Prioul JL (1994) Use of ^{13}C and ^{15}N plant label near natural abundance for monitoring carbon and nitrogen partitioning. Aust. J. Plant Physiol. 2: 133–146

De Riek J, Van Cleemput O & Debergh PC (1991) Carbon metabolism of micropropagated *Rosa multiflora* L. In Vitro Cell. Dev. Biol. 27P: 57–63

Desjardins YA, Laforge F, Lussier C & Gosselin A (1988) Effect of CO_2 enrichment and high photosynthetic photon flux on the development of autotrophy and growth of tissue-cultured strawberry, raspberry and asparagus plants. Acta Hortic. 230: 47–57

Desjardins Y (1995) Overview of factors influencing photosynthesis of micropropagated plantlets and their effect on acclimatization. Carre F & Chagvardieff P (ed) *In* Ecophysiology and Photosynthetic *in vitro* Cultures. pp 145–160

Dubé SL & Vidaver W (1992) Photosynthetic competence of plantlets grown *in vitro*. An automated system for measurement of photosynthesis *in vitro*. Physiol. Plant. 84: 409–416

Farquhar GD, O'Leary MH & Berry JA (1982) On the relationship between carbon isotope discrimination and the intercellular carbon dioxide concentration in leaves. Aust. J. Plant Physiol. 9: 121–137

Farquhar GD, Ehleringer JR & Hubick KT (1989) Carbon isotope discrimination and photosynthesis. Annu. Rev. Plant Physiol. Plant Mol. Biol. 40: 503–537

Fujiwara K, Kozai T & Watanabe I (1987) Fundamental studies on environments in plant tissue culture vessels. (3) Measurements of carbon dioxide gas concentration in closed vessels containing tissue cultured plantlets and estimates of net photosynthetic rates of the plantlets. J. Agr. Met. 43: 21–30

Fujiwara K, Kozai T, Nakajo Y & Watanabe I (1989) Effects of closures and vessels on light intensities in plant tissue culture vessels. J. Agric. Met. 45: 143–149

Fujiwara K, Kira S & Kozai T (1992) Time course of CO_2 exchange of potato cultures *in vitro* with different sucrose concentrations in the culture medium. J. Agr. Met. 48: 49–56

Fujiwara K, Aitken-Christie J & Kozai T (1993) Water potential of Radiata pine shoots cultured *in vitro* under different relative humidities. Plant Tissue Culture Letters, 10(2): 144–150

Fujiwara K & Kozai T (1995) Control of environmental factors for plantlet production -with some mathematical simulation *In* F. Carre & P. Chagvardieff (ed) Ecophysiology and Photosynthetic *in vitro* Cultures (pp 109–120)

Galzy R & Compan D (1992) Remarks on mixotrophic and autotrophic carbon nutrition of *Vitis* plantlets cultured *in vitro*. Plant Cell Tiss. Org. Cult. 31: 239–244

Grout BWW & Ashton MJ (1978) Transplanting of cauliflower plants regenerated from meristem culture. II. Carbon dioxide fixation and the development of photosynthetic ability. Hortic. Res. 17: 65–71

Hdider C & Desjardins Y (1994) Effects of sucrose on photosynthesis and phosphoenolpyruvate carboxylase activity of *in vitro* culture strawberry plantlets. Plant Cell Tiss. Org. Cult. 36: 27–33

Jackson MB, Belcher AR & Brain P (1994) Measuring shortcomings in tissue culture aeration and their consequences for explant

development. In: Lumsden PJ, Nicholas JR & Davies WJ (eds) Physiology, Growth and Development of Plants in Culture (pp 191–203). Kluwer Academic Publ., Dordrecht.

Kozai T (1991a) Micropropagation under photoautotrophic conditions. In: Debergh PC & Zimmerman RH (eds) Micropropagation: Technology and Application (pp 447–469). Kluwer Academic Publ., Dordrecht

Kozai T (1991b) Photoautotrophic micropropagation. In Vitro Cell. Dev. Biol. 27P: 47–51

Kozai T, Oki H & Fujiwara K (1990a) Photosynthetic characteristics of *Cymbidium* plantlet *in vitro*. Plant Cell Tiss. Org. Cult. 22: 205–211

Kozai T, Takazawa A, Watanabe I & Sugi J (1990b) Growth of tobacco seedlings and plantlets *in vitro* as affected by *in vitro* environment. Environ. Control Biol. 28: 31–40

Kozai T, Iwabuchi K, Watanabe K & Watanabe I (1991a) Photoautotrophic and photomixotrophic growth of strawberry plantlets *in vitro* and changes in nutrient composition of the medium. Plant Cell Tiss. Org. Cult. 25: 107–116

Kozai T, Ohde N & Kubota C (1991b) Similarity of growth patterns between plantlets and seedlings of *Brassica campestris* L. under different *in vitro* environmental conditions. Plant Cell Tiss. Org. Cult. 24: 181–186

Kozai T, Fujiwara K, Hayashi M & Aitken-Christie (1992) The *in vitro* environment and its control in micropropagation. In: Kurata K & Kozai T RH (eds) Transplant Production Systems, (pp 247–282). Kluwer Academic Publ., Dordrecht

Kubota C & Kozai T (1992) Growth and net photosynthetic rate of *Solanum tuberosum in vitro* under forced and natural ventilation. HortSci. 27: 1312–1314

Lee N, Wetzstein HY & Sommer H (1985) Effects of quantum flux density on photosynthesis and chloroplast ultrastructure in tissue-cultured plantlets and seedlings of *Liquidambar styraciflua* L. towards improved acclimatization and field survival. Plant Physiol. 78: 637–641

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473–497

Reuther G (1991) Stimulation of the photoautotrophy of *in vitro* plants. Acta Hort. 300: 59–75

Righetti B, Magnanini E & Rossi F (1993) Photosynthetic carbon dioxide uptake and oxygen accumulation during *in vitro* culture of *Actinidia deliciosa* cv Tomuri. Environ. Exp. Bot. 33: 523–528

Serret MD, Trillas MI, Matas J & Araus JL (1996) Development of photoautotrophy and photoinhibition of *Gardenia jasminoides* plantlets during micropropagation. Plant Cell Tiss. Org. Cult. 45: 1–16

Solárová J (1989) Photosynthesis of plant regenerants. Diurnal variation in CO_2 concentration in cultivation vessels resulting from plantlets photosynthetic activity. Photosynthetica 23: 100–107

Sutter E (1983) Chemical composition of epicuticular wax in cabbage plant grown *in vitro*. Can J. Bot. 62: 74–77

Tanaka K, Fujiwara K & Kozai T (1992) Effects of relative humidity in the culture vessel on the transpiration and net photosynthetic rates of potato plantlets *in vitro*. Acta Hort. 319: 59–64

Trillas MI, Serret MD, Jorba J & Araus JL (1995) Leaf chlorophyll fluorescence changes during acclimatization of the rootstock GF677 (Peach x Almond) and propagation of *Gardenia jasminoides* In: E. F. Carre & Chagvardieff (ed) Ecophysiology and Photosynthetic *in vitro* Cultures. (pp 161–166)