J. Amer. Soc. Hort. Sci. 127(6):963–970. 2002.

In Situ Estimation of Carbon Balance of In Vitro Sweetpotato and Tomato Plantlets Cultured with Varying Initial Sucrose Concentrations in the Medium

Chieri Kubota[1]
Department of Plant Sciences, The University of Arizona, Tucson, AZ 85721-0036

Makiko Ezawa and Toyoki Kozai
Department of Bioproduction Science, Chiba University, Matsudo, Chiba 271-8510, Japan

Sandra B. Wilson
Department of Environmental Horticulture, Indian River Research and Education Center, Institute of Food and Agricultural Sciences, University of Florida, 2199 South Rock Road, Fort Pierce, FL 34945-3138

Additional index words. CO_2 concentration, environmental control, *Ipomoea batatas*, *Lycopersicon esculentum*, photoautotrophic, photomixotrophic, photosynthesis, respiration, sugar

Abstract. The effects of initial sucrose (suc) concentrations in the medium (S_0) on the carbon balance and growth of sweetpotato [*Ipomoea batatas* (L.) Lam. 'Beniazuma'] and tomato (*Lycopersicon esculentum* Mill. 'HanaQueen') plantlets were studied under controlled environmental conditions. Plantlets were cultured with 0, 7.5, 15, or 30 g·L⁻¹ of S_0 under high photosynthetic photon flux (160 to 200 μmol·m⁻²·s⁻¹) and CO_2 enriched (1400 to 2050 μmol·mol⁻¹) conditions. Net photosynthetic rate per leaf area (P_l) decreased and dry weight per plantlet (W_d) increased with increasing S_0, but did not differ significantly between S_0 of 7.5 to 30 g·L⁻¹ for sweetpotato or 15 to 30 g·L⁻¹ for tomato. Carbon influxes and effluxes of the plantlets by metabolism of medium suc and/or photosynthesis, and respiration were estimated based on measurements of in situ and steady state CO_2 exchange rates and sugar uptake during culture. At S_0 from 7.5 to 30 g·L⁻¹, photosynthesis was responsible for 82% to 92% and 60% to 67% of carbohydrate assimilation for sweetpotato and tomato, respectively. Estimated carbon balances of plantlets based on the estimated and actual increases of moles of carbon in plant tissue demonstrated that in situ estimation of carbon balance was reasonably accurate for sweetpotato at S_0 of 0 to 15 g·L⁻¹ and for tomato at S_0 of 0 g·L⁻¹ and that the actual contribution of photosynthesis for tomato at high S_0 might be lower than the values estimated in the present experiment. Results showed that initial suc concentration affected the relative contribution of photosynthesis on their carbon balances and that the responses were species specific. The failure of validation at S_0 in a range specific to each species suggested the need for further study on carbon metabolism of in vitro plantlets cultured with sugar in the medium.

In vitro plant culture environments are recognizably different from greenhouse or field environments. The uniqueness includes the presence of sugar in the medium and use of the relatively airtight vessels, which results in the accumulation of ethylene and water vapor, and the accumulation or depletion of CO_2. Measurement of CO_2 concentration inside vessels containing chlorophyllous plantlets showed that CO_2 concentrations inside the vessel dropped below ambient levels and often fell as low as the CO_2 compensation point during the light period (Desjardins et al., 1988; Fournioux and Bessis, 1986; Fujiwara et al., 1987; Infante et al., 1989; Kozai and Sekimoto, 1988). Therefore, regardless of their photosynthetic ability, chlorophyllous plantlets grown in vitro are required to grow photomixotrophically; supported by relatively large amounts of available sugar in the medium and limited CO_2. Such a physiological phenomenon of having two carbon sources (photomixotrophy) is not the norm for ex vitro plants, except for parasitic plants, such as *Striga hermonthica* (Del.) Benth. (Pageau et al., 1998). Consequently, the in vitro environment serves as a unique model for studies focusing on photosynthesis and carbon metabolism. A quantitative understanding of the relative contribution of each carbon source gives important information on the carbon metabolism of in vitro plants.

For more practical reasons, a quantitative understanding of sugar uptake and photosynthesis is necessary to enhance plantlet growth with a minimal input of energy and other resources, thereby maximizing the biomass production efficiency. Reducing suc concentration in the medium and increasing photosynthetic photon flux (*PPF*) and CO_2 concentration in the headspace have reportedly enhanced net photosynthetic rates and growth of plantlets (Kozai, 1991). However, increasing the medium suc concentration may increase dry matter accumulation of the plantlets. When growth promotion is attempted while minimizing production costs, a decision must be made whether one should promote plantlet photosynthesis by controlling light and CO_2 levels, or enhancing carbon uptake from the medium by increasing the sugar supply, or both.

In vitro photosynthesis has been measured many ways including an in situ measurement method based on CO_2 balance in the vessel and the use of open flow or closed measurement systems, as reviewed by Desjardins et al. (1995). The in situ measurement method of net photosynthetic rates (Fujiwara et al., 1987; Fujiwara and Kozai, 1995) is preferred over open flow or closed measurement systems, to minimize the possible disturbance of the in vitro environment. For photomixotrophically cultured plantlets, net photosynthetic rates give a limited assessment of plantlet carbon balance because plantlets use sugar in the medium as carbon source in addition to CO_2. Moreover, net photosynthetic rates measured as CO_2 exchange rates include dark respiration rates which dissimilate sugar absorbed from the medium (Fujiwara et al., 1995). To optimize the environmental conditions for photomixotrophic plantlets, it is necessary to determine their carbon balance with each

Received for publication 3 Jan. 2002. Accepted for publication 21 Aug. 2002. We thank Nicca Chemical Co. Ltd., Japan, for providing plant materials and the Japan Society for the Promotion of Science for financial support.
[1]Corresponding author; e-mail ckubota@ag.arizona.edu.

carbon flux (influx and efflux) under various environments.

The carbon balance of photomixotrophic and photoautotrophic plantlets has been studied for rose, *Rosa multiflora* L. (De Riek et al., 1991); potato, *Solanum tuberosum* L. (Fujiwara et al., 1995; Wolf et al., 1998); and gardenia, *Gardenia jasminoides* Ellis. (Serret et al., 1996, 1997) using several different methods. De Riek et al. (1991) quantified components of plantlet carbon flow (net carbon fixed by photosynthesis, carbon released by respiration, and medium carbohydrate accumulated in dry weight and respired as CO_2) using ^{14}C labeled suc and $^{14}CO_2$. Use of carbon isotopes can be straightforward for determining carbon influx and efflux separately, and the proportion of tissue carbon originating from different sources. However, the analysis requires specially designed vessels or chambers and use of radioisotopes, limiting practical application of the method.

Serret et al. (1996, 1997) used a stable carbon isotope (^{13}C) and estimated the percentage of carbon in leaflet tissue originating from photosynthesis. A similar method using ^{13}C to evaluate the "degree of photoautotrophy" in terms of percent tissue carbon derived from photosynthesis has also been tested (Wolf et al., 1998). Use of ^{13}C is suitable for in situ evaluation of carbon balance without altering culture conditions. However, percentages of carbon originating from photosynthesis and medium sugar over the total carbon in the plantlet (residuals after respiratory carbon loss) may not provide enough information for understanding the contribution of photosynthesis in photomixotrophic plantlet growth. Because the culture vessel represents a relatively closed system, carbon from the metabolism of medium suc in plantlets through respiration may be refixed by photosynthesis, resulting in a low estimate of degree of plantlet photoautotrophy (Serret et al., 1996). Furthermore, most sugar absorbed from the medium was reportedly released as CO_2, resulting in small amounts of carbon originating from medium sugar accumulating in the plantlets as indicated in the study using ^{14}C-suc (Borkowska and Kubik, 1990; De Riek et al., 1991).

To understand photomixotrophic growth of plantlets under different culture conditions, a quantitative approach for determining carbon influx by photosynthesis and sugar uptake is necessary. Fujiwara et al. (1995) developed a culture system where plantlet CO_2 exchange rates were continuously measured by employing forced ventilation in the vessel. This method required a number of assumptions to estimate the gross photosynthetic rate from CO_2 exchange rates during light and dark periods, but alternatively, allowed the estimation of carbon influx from photosynthesis. Unfortunately, forced ventilation disturbs the culture environment, resulting in misleading interpretation of results. To estimate carbon exchange in culture systems using natural ventilation, in situ measurement of CO_2 exchange rates is preferred over methods which use forced ventilation. The objectives of our research were to determine the effects of initial suc concentration (S_0) on in situ plantlet carbon balance estimated by in situ and steady state CO_2 exchange rates and medium sugar concentration.

Materials and Methods

Culture conditions. Sweetpotato (*Ipomoea batatas*, 'Beniazuma') and tomato (*Lycopersicon esculentum*, 'HanaQueen') were selected as model plants because both species have been micropropagated using photoautotrophic conditions (Afreen-Zobayed et al., 1999; Kubota et al., 2001). Culture conditions selected were specific for each species. Sweetpotato single node cuttings with a leaf (FW: 80 ± 15 mg) and tomato terminal shoots (FW: 80 ± 15 mg) were cultured for 20 and 15 d, respectively. Cylindrical polycarbonate vessels (volume: 480 mL) and lids (Nicca Chemical Co. Ltd., Fukui, Japan) were modified by drilling two holes (10 mm in diameter) in addition to the original hole (8 mm in diameter) for each lid and covering the holes each with a gas permeable membrane disk (pore size 0.5 mm; Milliseal, Millipore Japan). The number of air exchanges of the vessel (Kozai et al., 1986) was increased accordingly on days 6 and 11 by removing adhesive plastic tape that covered the gas permeable membrane disks, resulting in 1.3 (days 0 to 5), 1.9 (days 6 to 10), and 4.7 (days 11 to 20) air exchanges/h for sweetpotato; 1.3 (days 0 to 5) and 4.7 (days 6 to 15) air exchanges /h for tomato. Suc was added to the Murashige and Skoog (1962) medium at 0, 7.5, 15, or 30 $g \cdot L^{-1}$ before adjusting the pH (5.7 for sweetpotato; 5.9 for tomato). Vessels containing the liquid medium (100 mL per vessel) and cellulose plugs (20 plugs per vessel; Sorbarod, Baumgartner Papiers SA, Switzerland) were autoclaved for 20 min at 120 °C.

After transferring explants, vessels were placed under fluorescent tubes (FPL27EX-N, Matsushita Electric Co., Osaka, Japan) mounted horizontally above the shelf inside a growth chamber (AEL-3280, Advantec Co., Tokyo). The *PPF* was adjusted to 100 (days 0 to 10) and 200 (days 11 to 20) $\mu mol \cdot m^{-2} \cdot s^{-1}$ for sweetpotato; 100 (days 0 to 5) and 160 (days 6 to 15) $\mu mol \cdot m^{-2} \cdot s^{-1}$ for tomato under a 16 $h \cdot d^{-1}$ light period. Air temperatures inside the vessel during the light/dark period were 28.0/27.0 °C for sweetpotato and 24.0/23.0 °C for tomato, respectively, and were monitored by a 0.1-mm copper-constantan thermocouple inserted into a vessel placed in the center of the shelf. Relative humidity in the culture room ranged from 70% to 80%. The CO_2 concentration in the culture room was enriched to 1400, 1800, and 2000 $\mu mol \cdot mol^{-1}$ on days 0 to 5, 6 to 10, and 11 to 20, respectively, for sweetpotato; 2050 $\mu mol \cdot mol^{-1}$ on days 0 to 15 for tomato.

Growth measurements. Initial fresh weight was measured for each explant and initial dry weight of the explant (W_0) was estimated using the percent dry matter over the fresh weight obtained from 10 explants sampled separately. Dry weight was measured after drying fresh samples at 80 °C for more than 48 h. Plantlet fresh and dry weights and leaf areas were recorded 15 d (tomato) and 20 d (sweetpotato) after the start of the experiment. Leaf area was measured using image analysis software (LIA32, Nagoya University) on black and white images that were photocopied when harvested. The change in dry weight was calculated for each plantlet by subtracting W_0 from the final dry weight per plantlet (W_d), and converted into mole carbon per plantlet (D_d) with an assumption that 90% of dry matter was equivalent to carbohydrate [$(CH_2O)_n$] (Murayama et al., 1984).

Sugar concentration of the medium. After autoclaving the medium, and harvesting plantlets, 10 mL of medium was sampled from each vessel and filtered through a 0.22 µm (pore size) membrane. Concentrations of suc, glucose (gluc), and fructose (fruc) in the sampled media were measured using a HPLC with a refractive index detector (L6000, Hitachi Ltd., Tokyo) and a Gelpack GL-C611 column (Hitachi Chemical Co., Tokyo) maintained at 70 °C. Quantification was determined using a D-2000 Chromato-Integrator (Hitachi Ltd., Tokyo) and regression equations describing the suc, gluc, and fruc calibration lines. Prior to sampling the medium, each vessel was weighed. Medium volume was calculated from specific mass of the medium ($g \cdot L^{-1}$) and the mass of the medium obtained by subtracting mass of plantlets, dried plugs, and empty vessel from the total mass of the vessel.

The absorbed mass of sugar per plantlet was calculated from sugar concentrations and medium volume, and was converted to absorbed mole carbon through sugar uptake from the medium (U_n, mol C /plantlet):

$U_s = \{n_s \times S_0/M_s \times V(0) - [n_s \times S_s(t)/M_s + n_g \times S_g(t)/M_g + n_f \times S_f(t)/M_f] \times V(t)\} \times 1/N$ [1]

where, n_s, n_g, and n_f are mole carbon per mole of suc, gluc, and fruc (n_s = 12, and $n_g = n_f$ = 6); S_0 is the initial suc concentration of the medium before autoclaving (0, 7.5, 15, or 30 g·L^{-1}); $S_s(t)$, $S_g(t)$, and $S_f(t)$ are suc, gluc, and fruc concentrations (g·L^{-1}) on day t; M_s, M_g, and M_f are molecular mass of suc, gluc, and fruc (g·mol^{-1}); $V(0)$ and $V(t)$ are medium volume per vessel on days 0 (0.1 L) and t; N is the number of plantlets per vessel. Total moles of carbon originating from suc added per vessel was assumed to be unchanged during autoclaving.

Carbon dioxide concentration inside the vessel and CO_2 exchange rate. Carbon dioxide concentrations inside and outside of the vessel (C_{in} and C_{out}, respectively) were measured for a 0.25 mL gas sample using a gas chromatograph with a flame ionized detector and a reaction furnace for converting CO_2 into CH_4 (GC-9A, Shimadzu Co., Osaka, Japan). Measurements were made during light and dark periods every 5 d when the C_{in} and C_{out} were considered stable (steady state conditions). Net photosynthetic rate (P_n) and dark respiration rate (R_d) per plantlet were calculated as described by Fujiwara et al. (1987) and Fujiwara and Kozai (1995).

Carbon moles taken up and released by plantlet CO_2 exchanges. Plantlet CO_2 exchange rates during culture were estimated based on the simulated changes in C_{in} as described below. The C_{in} under steady state conditions ($C_{in,s}$) during light and dark periods of each day were predicted for each vessel by fitting $C_{in,s}$ on days 0, 5, 10, 15, and 20 with time in days using polynomial equations. The $C_{in,s}$ on day 0 was given as median of the $C_{in,s}$ on day 5 and the C_{out} during light and dark periods. Diurnal changes of C_{in} were simulated for each day, assuming that C_{in} at the onset of the light period was equal to $C_{in,s}$ of the previous dark period, and that it decreased linearly and reached a steady state value 2 h after onset of the light period. These assumptions on changes of C_{in} during the culture period were based on the previous observations on diurnal and periodic changes in C_{in} of the vessels containing plantlets (Fujiwara et al., 1987; Fujiwara and Kozai, 1995; Kozai and Sekimoto, 1988; Kubota et al., 2001). The cumulative CO_2 exchange rate during the light period was estimated by integrating the difference of C_{out} and C_{in} over light period according to the dynamic and steady state methods for estimation of in vitro CO_2 exchange rate (Fujiwara at al., 1987; Fujiwara and Kozai, 1995). The R_d was calculated using $C_{in,s}$ during the dark period. Cumulative CO_2 exchange rate during the dark period was estimated as R_d multiplied by dark period (8 h) assuming that R_d remained constant during the dark period.

Moles of carbon fixed through photosynthesis and released through dark respiration and photorespiration during culture periods were estimated from the cumulative CO_2 exchange rate during light and dark periods, according to the following assumptions: 1) gross photosynthetic rate (P_g) is the sum of P_n, R_d, and photorespiration rate (R_p), 2) R_d is constant throughout the day, and 3) R_p is equivalent to 15% of P_g.

Estimates of moles of carbon fixed through photosynthesis and released through respiration involved a number of assumptions based on typical diurnal changes in CO_2 concentration inside vessels containing photosynthetically active plantlets. Therefore, validation of this method was made by comparing increases in moles of carbon per plantlet estimated from the carbon balance and those derived from the actual dry weight increase of the plantlets. Carbon balance of in vitro plantlets was expressed as follows:

$$U_s + U_p - L_d - L_p - D_b = 0 \quad [2]$$

where, U_p is carbon absorbed during photosynthesis (mol C/plantlet); L_d and L_p are carbon released during dark respiration and photorespiration (mol C/plantlet), respectively; and D_b is the carbon increase in plantlet tissue (mol C/plantlet). The percentage of U_p over the sum of U_s and U_p was calculated and expressed as percent

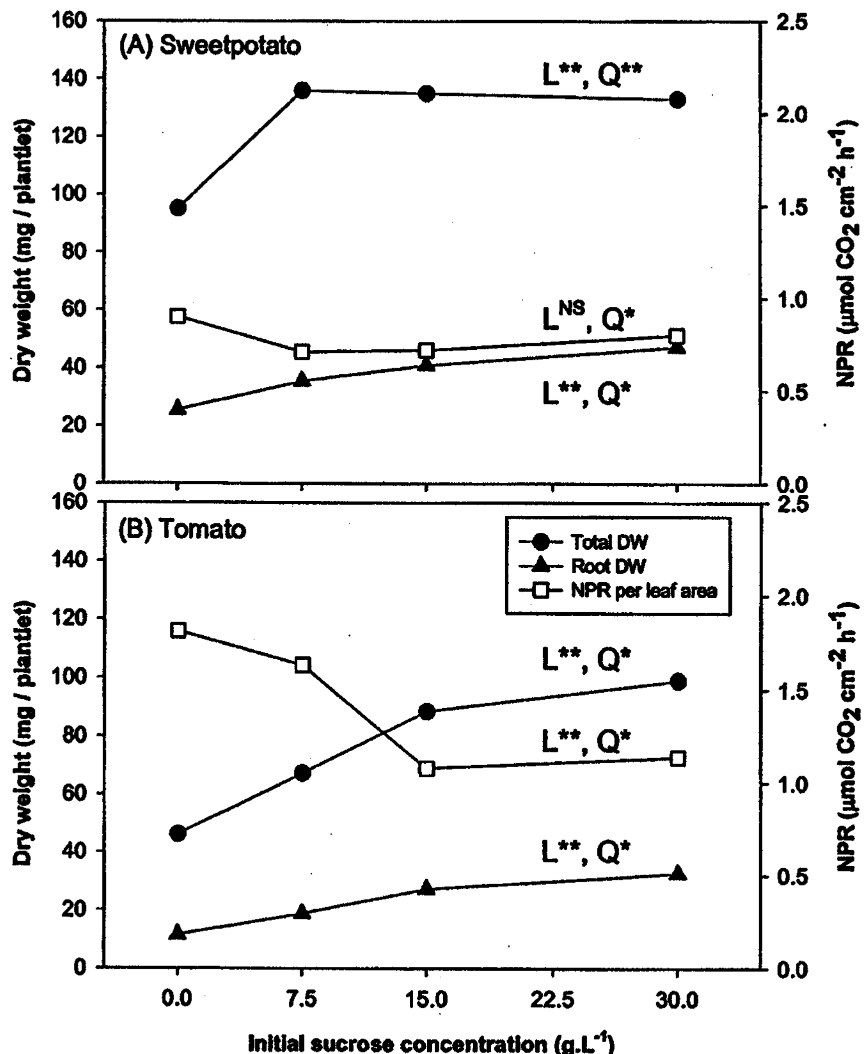

Fig. 1. Dry weight and net photosynthetic rate per leaf area during the light period of sweetpotato on day 20 (A) and of tomato on day 15 (B), as affected by initial sucrose concentration (0, 7.5, 15, or 30 g·L^{-1}). L^{ns}, L*, L**, Q^{ns}, Q*, Q** = nonsignificant or significant linear (L) or quadratic (Q) response at $P \le 0.05$ or 0.01, respectively.

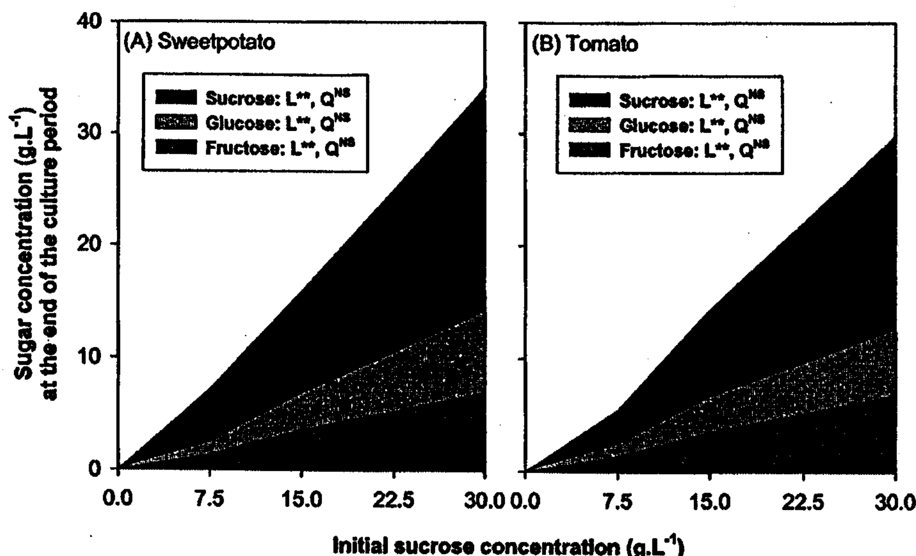

Fig. 2. Final sucrose, glucose, and fructose concentrations ($g\cdot L^{-1}$) for sweetpotato (A) and tomato (B) plantlets as affected by initial sucrose concentration (S_0). L^ns, L*, L**, Q^ns, Q*, Q** = nonsignificant or significant linear (L) or quadratic (Q) response at $P \leq 0.05$ or 0.01, respectively.

contribution of photosynthesis [$\%P$; $\%P = U_p/(U_s + U_p)$]. The D_b, estimated by solving Eq. [2], was compared with the increase of moles of carbon in dry weight (D_d).

Statistical analysis. There were five and six vessels for each treatment in experiments using tomato and sweetpotato plantlets, respectively. Vessels containing four plantlets were considered replicates. Experiments for sweetpotato and tomato were conducted separately each with a completely randomized experimental design. All data within each experiment were subjected to analysis of variance (ANOVA), and effects of initial suc concentration were analyzed by partitioning into orthogonal multiple contrasts using the Statistical Analysis System (Version. 6.12, SAS Institute).

Results and Discussion

Plantlet growth and net photosynthetic rate. Total (W_d) and root dry weight of sweetpotato and tomato plantlets increased whereas net photosynthetic rate per leaf area (P_l) decreased when suc was added to the medium (Fig. 1). The dry weight and net photosynthetic rate of the plantlets cultured without sugar in the medium ($S_0 = 0$) may be further enhanced at a higher CO_2 concentration and PPF more favorable to photosynthesis.

For sweetpotato plantlets, W_d increased and P_l decreased with addition of suc to the medium, but both stabilized at S_0 greater than 7.5 $g\cdot L^{-1}$ (Fig. 1A). For tomato plantlets, W_d and P_l stabilized around 15 to 30 $g\cdot L^{-1}$ of S_0. The increase of W_d was associated with the decrease of P_l, probably because the plantlets had greater relative carbon influx from sugar in the medium at higher S_0. Decreased photosynthetic activities due to the sugar added to the medium have been reported with many species (Cristea et al., 1999; Kozai, 1991; Pospisilova et al., 1992). Langford and Wainwright (1987) demonstrated that decreasing the medium suc concentration (10, 20 or 40 $g\cdot L^{-1}$) increased the net photosynthetic rate of in vitro grown shoots for two rose cultivars (*Rosa* hybrid). Net photosynthetic rate per leaf area of tomato plantlets was about 60% lower with 30 $g\cdot L^{-1}$ than with no suc when examined under similar culture conditions as in the present experiment (Kubota et al., 2001).

The biochemical aspect of depression of photosynthesis by sugar in the medium has been extensively studied. Cappellades et al.

Table 1. CO_2 concentrations (C_{in}, $\mu mol\cdot mol^{-1}$) inside the vessel during light and dark periods measured under steady state conditions on days 5, 10, 15, and 20 (day-20 measurement was for sweetpotato plantlets only) as affected by initial sucrose concentration of the medium (S_0). Simulation of the diurnal change of CO_2 concentration inside the vessel and the subsequent estimation of cumulative CO_2 exchange rates were made using the values obtained for each replication.

	Light period				Dark period			
S_0	Day 5	Day 10	Day 15	Day 20	Day 5	Day 10	Day 15	Day 20
Sweetpotato ($n = 6$)^z								
0	1010	1680	600	300	1640	1990	2220	2990
7.5	1090	1680	570	245	1680	1990	2350	4080
15	1120	1700	780	310	1710	1940	2250	3410
30	1230	1880	845	390	1650	1940	2300	3380
Significance								
L	**	**	**	**	NS	NS	**	NS
Q	NS	NS	NS	NS	NS	NS	NS	NS
Tomato (n = 5)								
0	1970	1510	890	---	2490	2410	2670	---
7.5	2040	1310	540	---	2550	2600	3140	---
15	2065	1260	660	---	2610	2710	3220	---
30	2200	1360	450	---	2680	2860	3940	---
Significance								
L	**	NS	NS	---	**	**	**	---
Q	NS	NS	NS	---	NS	NS	NS	---

^z Except for dark period on day 5 (n = 4).
*,** Significance at $P \leq 0.05$ or 0.01, respectively; L or Q indicates linear or quadratic response, respectively.

(1991) showed that rose plantlets grown on a high suc concentration (50 g·L^{-1}) medium had high accumulation of starch in the leaves resulting in reduced photosynthesis. Accumulation of carbohydrate in leaves has a strong correlation with inhibition of CO_2 assimilation, although the exact mechanism is not known (Foyer and Galtier, 1996). Wilson et al. (2000) demonstrated potential differences in carbohydrate status between tomato and sweetpotato plantlets cultured under similar culture conditions. They reported that leaves of tomato plantlets cultured with 30 g·L^{-1} suc had significantly higher soluble sugar (suc, gluc, and fru) and starch concentrations than those without sugar in the medium, but that leaves of sweetpotato with and without sugar in the medium did not show significant differences in soluble sugar and starch concentrations. Desjardins et al. (1995) suggested that the most probable cause of reduced photosynthesis with sugar in the medium was on a series of biochemical reactions leading to the down-regulation or feedback inhibition of ribulose bisphosphate carboxylase (rubisco). In the present experiment, reduction of P_i observed for sweetpotato plantlets may have been caused by enzymatic activities mediated by sugar absorbed from the medium rather than carbohydrate accumulation in the leaves, assuming that carbohydrate did not accumulate in the sweetpotato leaves with sugar in the medium as found by Wilson et al. (2000). For tomato, both increased carbohydrate levels and limited rubisco activities remain as possible reasons for a reduction of net photosynthetic rate. Further biochemical analyses will be needed to determine the actual limiting factor of photosynthesis at high S_0 for these species.

Root growth increased with increasing S_0 (P linear ≤ 0.01; P quadratic ≤ 0.05) (Fig. 1). Shoot to root dry weight ratio was 2.8, 2.9, 2.3, and 1.8 for sweetpotato; and 3.1, 2.6, 2.3, and 2.0 for tomato at S_0 of 0, 7.5, 15, and 30 g·L^{-1}, respectively, indicating that more carbohydrate was allocated to roots with increasing S_0 in both species. Accumulation of soluble sugar and starch in roots was reported for sweetpotato and tomato plantlets cultured with 30 g·L^{-1} suc in the medium as compared with no suc (Wilson et al., 2000). High total soluble sugars were observed in leaf and root tissue of tomato and in root tissue of sweetpotato when cultured with 30 g·L^{-1} suc (Wilson et al., 2000). Piqueras et al. (1998) reported that *Calathea luisae* Gagnep. plantlets had higher starch concentration in roots and stems than in leaves, while suc concentration was highest in the stems, followed by leaves and roots. Although root dry weight of sweetpotato and tomato similarly responded to S_0, carbon partitioning and thereby soluble sugar and starch concentrations may have been different between species.

Sugar concentration and medium volume. During autoclaving, a portion of the original suc present was hydrolyzed to gluc and fruc in all media containing sugar. Specific sugar concentrations after autoclaving were 6.5, 14.4, and 29.5 g·L^{-1} for suc; 0.7, 1.0, and 1.4 g·L^{-1} for gluc; and 0.7, 1.1, and 1.4 g·L^{-1} for fruc at S_0 of 7.5, 15 and 30 g·L^{-1}, respectively. The increase in total sugar (suc, gluc, and fruc) concentrations after autoclaving was 5% to 10%. This indicates that, in addition to hydrolysis of suc, a small portion of water may have evaporated from the vessel during autoclaving or condensed on the inner wall of the vessel, resulting in an increase in the total sugar concentration.

After day 20 (sweetpotato) and 15 (tomato), suc still comprised the majority of the sugar in the medium and it was 4.8, 9.2, and 20.0 g·L^{-1} for sweetpotato; and 3.1, 7.8, and 17.3 g·L^{-1} for tomato at S_0 of 7.5, 15 and 30 g·L^{-1}, respectively (Fig. 2). No sugar was detected in the medium without suc. All sugar concentrations at the end of culture increased with increasing S_0 (P linear ≤ 0.01). Medium volume at the end of culture was not affected by S_0, and was 15% to 20% less in sweetpotato and 13% to 16% less in tomato than the initial volume (data not shown). Based on the suc, gluc, and fruc concentrations and residual medium volume, the total mass of sugar left in the medium was 77%, 87%, and 99% (0.58, 1.31, and 2.96 g/vessel) of the initial values (0.75, 1.5, and 3.0 g/vessel) for sweetpotato; and 64%, 82%, and 85% (0.48, 1.23, and 2.54 g/vessel) for tomato at S_0 of 7.5, 15, and 30 g·L^{-1}, respectively. Although the culture period for tomato was shorter than that for sweetpotato, more sugar remained in the medium for sweetpotato than for tomato. The low consumption of sugar (most sugar remained unconsumed in the medium) was probably due to CO_2 enrichment and high PPF that promoted photosynthesis by the cultured plantlets. Under conditions with lower PPF and more limited CO_2 (without CO_2 enrichment and/or lower ventilation of the vessel), the plantlets may rely more on sugar in the medium as the major carbon source, and the sugar consumption would be higher than those in the present experiment. This hypothesis had been confirmed for tomato in a separately conducted experiment (Ezawa, 1997), where tomato plantlets were cultured under low PPF (100 µmol·m^{-2}·s^{-1}) in vessels with a low number of air exchanges (1.6 h^{-1}). Under these conditions, the plantlets absorbed 32% of initial sugar when cultured at 30 g·L^{-1} of S_0, while they absorbed 15% of initial sugar at the same S_0

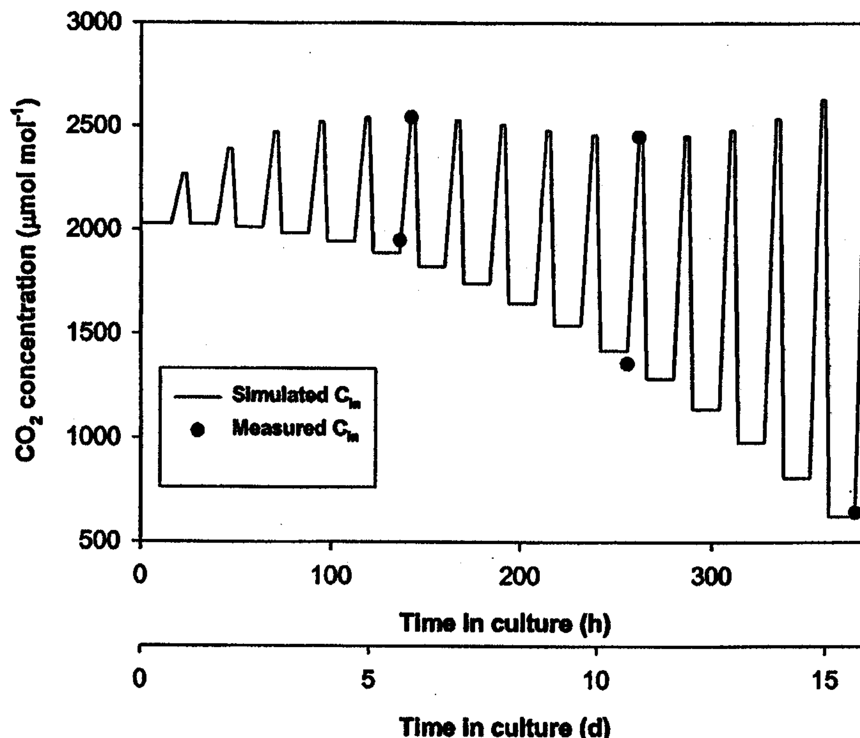

Fig. 3. Simulated changes in CO_2 concentration (C_{in}) inside a vessel containing four tomato plantlets without sugar in the medium (S_0 = 0). The C_{in} during light and dark periods was assumed to decrease/increase linearly and reach steady state conditions 1 and 6 h, respectively, after onset of the period. CO_2 concentration outside the vessel was 2050 mmol·mol^{-1} throughout the culture period.

Table 2. Carbon balance of sweetpotato and tomato plantlets as affected by initial sucrose concentration of the medium (S_0). Absorbed mole carbon by sugar uptake from medium, U_s, and by photosynthesis, U_p; Moles of carbon released by dark respiration, L_d, and by photorespiration, L_p; Percent contribution of photosynthesis, $\%P$ [$\%P = 100 \times U_p/(U_s + U_p)$].

	Absorbed carbon (mmol C/plantlet)		Contribution[z]	Released carbon (mmol C/plantlet)	
S_0	U_s	U_p	$\%P$	L_d	L_p
Sweetpotato for 20 days (n = 6)					
0	0.0	6.2	100	2.7	0.93
7.5	1.6	6.9	82	3.7	1.04
15	1.9	6.2	77	3.2	0.92
30	0.6	5.7	92	3.2	0.85
Significance					
L	**	NS	**	NS	NS
Q	NS	*	**	NS	*
Tomato for 15 days (n = 5)					
0	0.0	3.6	100	2.0	0.54
7.5	2.4	5.0	67	3.0	0.75
15	2.6	5.3	67	3.5	0.79
30	4.5	6.0	60	4.7	0.91
Significance					
L	*	NS	**	*	NS
Q	**	**	**	**	**

[z]Data (percentage) were transformed by arcsinebefore analysis.

*,**Significance at $P \leq 0.05$ or 0.01, respectively; L or Q indicates linear or quadratic response, respectively.

under a high number of air exchanges (4.7 h^{-1}) and high PPF (160 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

Steady state CO_2 concentration inside the vessel ($C_{in,s}$) and estimation of changes in C_{in} during culture. In both plant species, $C_{in,s}$ measured during the light period decreased with time, except for sweetpotato during days 5 to 10 (Table 1). Increased $C_{in,s}$ during the light period in sweetpotato for days 5 to 10 was due to an increase in C_{out} (from 1400 $\mu mol \cdot mol^{-1}$ on day 5 to 1800 $\mu mol \cdot mol^{-1}$ on day 6). Although differences among S_0 were relatively small, $C_{in,s}$ during the light period increased with increasing S_0 (P linear ≤ 0.01), except for tomato on days 10 and 15 in which no significant influence of S_0 was observed. The $C_{in,s}$ attained a range of 250 to 390 $\mu mol \cdot mol^{-1}$ in sweetpotato and 450 to 890 $\mu mol \cdot mol^{-1}$ in tomato by the end of culture. The $C_{in,s}$ measured during the dark period increased with time in sweetpotato. The S_0 did not affect $C_{in,s}$ during the dark period except for day 15. In tomato, $C_{in,s}$ during the dark period increased with time regardless of S_0 except for 0 $g \cdot L^{-1}$, and with increasing S_0 (P linear ≤ 0.01).

The time course of changes in C_{in} was simulated for each vessel based on the measured $C_{in,s}$ by our assumptions (Fig. 3). The C_{in} during the dark period was simulated as reaching steady state conditions 6 h after initiation of the dark period, although changes in C_{in} during the dark period did not affect the estimation of cumulative CO_2 exchange rates since the R_d was assumed to be constant. Simulated results were considered representative of the typical changes in C_{in} of the vessels containing plantlets under natural ventilation conditions.

Plantlet carbon balances. The S_0 affected U_s, U_p, $\%P$, and L_p, but did not affect L_d for sweetpotato (Table 2). The $\%P$ decreased and attained 77% to 92% when suc was added to the medium. For tomato plantlets, S_0 affected U_s, U_p, $\%P$, L_d, and L_p (P quadratic $\leq$ 0.01). The $\%P$ decreased with S_0, and attained 60% to 67% for S_0 of 7.5 to 30 $g \cdot L^{-1}$. When the plantlets were cultured with sugar in the medium, the range of U_s was relatively higher for tomato (2.4 to 4.5 mmol C per plantlet) than for sweetpotato (0.6 to 1.6 mmol C per plantlet), while W_d was relatively higher for sweetpotato (133 to 136 mg per plantlet) than for tomato (67 to 99 mg per plantlet). This observation corresponded to the higher $\%P$ for sweetpotato (77% to 92%) than for tomato (60% to 67%).

Mole fractions of U_s to the carbon added as sugar in the medium (6.5, 13, and 26 mmol C per plantlet were added at 7.5, 15, and 30 $g \cdot L^{-1}$ of S_0, respectively) can be considered as indicating the sugar utilization efficiency, and were 0.25, 0.14, and 0.02 $mol \cdot mol^{-1}$ for sweetpotato; and 0.37, 0.20, and 0.17 $mol \cdot mol^{-1}$ for tomato at 7.5, 15, and 30 $g \cdot L^{-1}$ of S_0, respectively, decreasing with increasing S_0. In *R. multiflora*, 50% to 75% of tissue carbon was derived from sugar uptake from the medium; however, 68% to 84% of sugar taken up from the medium was used for respiration (De Riek et al., 1991). Borkowska and Kubik (1990), using ^{14}C-suc, showed that sour cherry (*Prunus cerasus* L.) plantlets absorbed 23% of sugar added to the medium but only 5% of the original activity of the medium remained in dry weight, indicating that most of the carbon absorbed as sugar from the medium had been respired. In the present experiment, the ratios of L_d to U_p increased with increasing S_0 (0.44, 0.54, 0.52, and 0.56 for sweetpotato; 0.56, 0.60, 0.66, and 0.78 for tomato at S_0 of 0, 7.5, 15, and 30 $g \cdot L^{-1}$, respectively), indicating increased R_d with increasing S_0. High dark respiration rates have been reported for many species when cultured with sugar in the medium (Cristea et al., 1999; Galzy and Compan, 1992; Kubota et al., 2001). If the sugar taken up from the medium merely enhances the dark respiration rate and is mostly released as CO_2 to the atmosphere, addition of sugar in the medium reduces the efficiencies of energy and sugar incorporated to dry matter in plantlets, especially chlorophyllous plantlets that have already gained photosynthetic ability.

The higher $\%P$ in sweetpotato than in tomato suggests that controlled environments (e.g., CO_2 enrichment and increased PPF) can more effectively enhance the dry weight increase of sweetpotato plantlets, but that increasing sugar concentration in addition to CO_2 enrichment and increased PPF may be necessary to effectively increase dry weight of tomato plantlets. De Riek et al. (1991) used ^{14}C-labeled suc and $^{14}CO_2$ to show that 75% of the dry weight carbon originated from suc (25% was from photosynthesis) for rose plant-

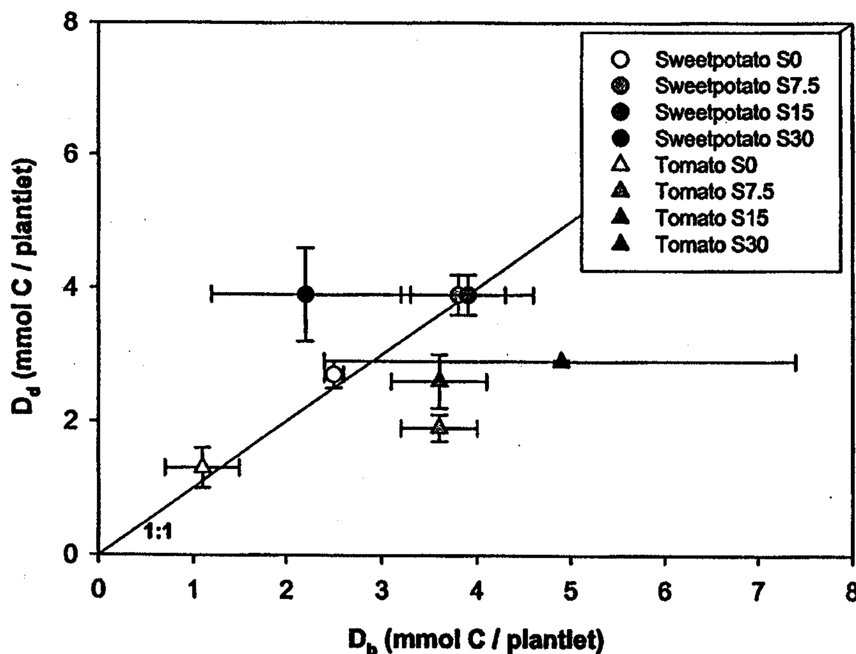

Fig. 4. Increase of moles of carbon per plantlet calculated from the balance equation, D_b ($D_b = U_s + U_p - L_d - L_p$), and from dry weight increase, D_d. U_s and U_p are absorbed mole carbon by sugar uptake from medium and by photosynthesis, respectively; L_d and L_p are released mole carbon by dark respiration and by photorespiration, respectively. Means ± SD are shown (n = 6 and 5 for sweetpotato and tomato, respectively).

lets cultured with 30 g·L^{-1} suc under low PPF (35 μmol·m^{-2}·s^{-1}) without CO_2 enrichment (conventional culture conditions). This percentage varied with different cultivars within the same species. Lower sugar concentrations (Serret et al., 1997; Wolf et al., 1998) and osmotic potentials (Wolf et al., 1998) increased percent tissue carbon derived from photosynthesis of photomixotrophic plantlets. The percentage of tissue carbon derived from photosynthesis of potato plantlets was about 40% when cultured at 30 g·L^{-1} suc, while it was only 10% at 80 g·L^{-1} suc (Wolf et al., 1998).

Effects of physical environmental conditions on percent carbon derived from photosynthesis over the total carbon in dry weight have been well studied in gardenia. Gardenia plantlets at shoot multiplication stage were shown to have 36% photosynthates in leaflet dry matter when cultured in tightly sealed vessels, but 93% when cultured in loosely sealed vessels (Serret et al., 1997). Increasing *PPF* from 50 to 300 μmol·m^{-2}·s^{-1} increased this percentage from 16% to 56% for the gardenia leaflets (Serret et al., 1996). Our preliminary data for tomato plantlets (Ezawa, 1997) cultured under 100 μmol·m^{-2}·s^{-1} and 1.6 air exchanges /h in the vessel showed that contribution of photosynthesis (*%P*) was 24% when cultured at 30 g·L^{-1} suc, while it was 60% at the same suc level under 160 μmol·m^{-2}·s^{-1} and 4.7 air exchanges /h. Under conventional culture conditions (lower *PPF* and less ventilation), plantlets are generally dependent on sugar in the medium mainly due to limited availability of CO_2 in the head space and thereby the *%P* is expectedly smaller. Fujiwara et al. (1995) showed that the contribution from photosynthesis to dry weight increase, expressed as mass of carbohydrate on a basis of plantlet dry weight increase, was three to four times greater than the contribution from sugar taken up from the medium for potato plantlets cultured at 15 g·L^{-1} initial suc concentration under 100 μmol·m^{-2}·s^{-1} *PPF* and 1000 μmol·mol^{-1} CO_2 concentration. This result agrees with those obtained by us for sweetpotato at 15 g·L^{-1} of S_0 (the ratio of U_s to U_p was 3.3). In the present experiment, the *%P* can be further increased for both species by increasing CO_2 concentration and *PPF* inside the vessel.

Validation of the increase of moles of carbon per plantlet showed that the estimation using the carbon balance equation was reasonably accurate for plantlets cultured without sugar in the medium ($S_0 = 0$), regardless of species. This conclusion indicates that the assumptions made for simulating C_{in} and estimating cumulative photosynthesis and respiration were acceptable for no sugar in the medium. For sweetpotato plantlets, increased moles of carbon per plantlet, calculated according to the carbon balance equation (D_b), generally agreed with the increased moles of carbon calculated from the dry weight increase (D_d) except at the highest S_0 of 30 g·L^{-1} (Fig. 4) where the D_b were underestimated. For tomato plantlets, D_b agreed with D_d only at no sugar ($S_0 = 0$ g·L^{-1}), and was overestimated when cultured with sugar in the medium. The disagreement of D_b and D_d for tomato may be due to overestimation of photosynthetic carbon uptake (U_p) as indicated by Fujiwara et al. (1995). Dark respiration rate for plantlets cultured with sugar in the medium includes CO_2 through dissimilation of sugar in the medium. Therefore the present estimate of U_p based on the CO_2 exchange rate measured during light and dark periods may be an overestimate. For tomato, the differences between D_b and D_d were 1.7, 1.0, and 2.0 mmol C per plantlet at 7.5, 15, and 30 g·L^{-1} of S_0, which accounted for 40% to 70% of U_s. This finding may indicate that a significant portion of sugar absorbed from the medium was released as CO_2, as reported for other species under conventional culture conditions (Borkowska and Kubik, 1990; De Riek et al., 1991). If the 40% to 70% of U_s were respired as CO_2, 37% to 86% of dry weight carbon originated from suc (14% to 63% from photosynthesis) [e.g., (2.4 – 1.7)/1.9 = 0.37 at 7.5 g·L^{-1} of S_0]. For sweetpotato, there was little difference between D_b and D_d except at 30 g·L^{-1} of S_0. This finding may indicate that the majority of suc taken up from the medium remained in plant tissue without its release as CO_2. In this case, 15% to 49% of dry weight carbon originated from suc for sweetpotato. The relatively lower L_d/U_p ratio in sweetpotato than tomato shows that sweetpotato respired less CO_2 than did tomato per fixed mole of carbon, which may also indicate that less carbon from medium suc was released in sweetpotato than in tomato. If U_p was overestimated at high S_0 in tomato in the present experiment, actual U_p and *%P* may be lower than the presented values for tomato.

Another reason that could explain underestimation/overestimation is the possible diurnal changes in net photosynthetic and dark respiration rates, which were assumed to be at steady state for most of the time during the light and dark periods in the present study. Overestimation of photosynthesis and/or underestimation of dark respiration can lead to the overestimation of D_b, and underestimation of photosynthesis and/or overestimation of dark respiration can cause an underestimation of D_b. Diurnal changes in respiratory CO_2 release have been shown in many plant species including in vitro callus cultured in darkness (Wilkins and Holowinsky, 1965). There is also ample evidence that the level of carbon substrate in tissue is positively correlated with plant respiration rate (Amthor, 1989) and

photosynthesis (Foyer and Galtier, 1996). The high S_0 could enhance the carbohydrate concentration of the plantlets and thereby alter the diurnal changes in CO_2 exchange rates.

Nonphotosynthetic carbon fixation by phosphoenolpyruvate carboxylase (PEPC) has been reported for in vitro plantlets (Desjerdins et al., 1995). However, PEPC activity was not determined in the present experiment. Desjardins et al. (1995) found high PEPC activity when sugar was added to the medium for early growth stages of strawberry plantlets. A nonnegligible amount of CO_2 fixation by PEPC may cause overestimation of $\%P$, but further biochemical work is needed to separate photosynthetic and nonphotosynthetic carbon fixation.

The carbon balance of in vitro plantlets was estimated in situ with reasonable accuracy when grown with an initial medium suc concentration of 0 to 15 $g \cdot L^{-1}$ for sweetpotato and with no suc for tomato. Tomato plantlets were more dependent on sugar in the medium than sweetpotato. For both sweetpotato and tomato, the dry weight increased, but the net photosynthetic rate during the light period and the efficiency of sugar use from the medium decreased with increasing initial suc concentrations. Such analysis of carbon balance and use of resources for tissue culture is crucial for a better understanding of carbon metabolism in vitro and for selection and improvement of methods and systems for micropropagation.

Literature Cited

Afreen-Zobayed, F., S.M.A. Zobayed, C. Kubota, T. Kozai, and O. Hasegawa. 1999. Supporting material affects the growth and development of in vitro sweetpotato plantlets cultured photoautotrophically. In Vitro Cell. Dev. Biol. Plant 35:470–474.

Amthor, J.S. 1989. Respiration and crop productivity. Springer-Verlag, New York.

Borkowska, B. and M. Kubik. 1990. Utilization and accumulation of ^{14}C-sucrose in sour cherry shoots rooted in vitro. Scientia Hort. 44:261–267.

Cappellades, M., R. Lemeur, and P. Debergh. 1991. Effects of sucrose on starch accumulation and rate of photosynthesis in *Rosa* cultured in vitro. Plant Cell Tissue Org. Cult. 25:21–26.

Cristea, V., F. Dalla Vecchia, and N. La Rocca. 1999. Development and photosynthetic characteristics of a photoautotrophic *Chrysanthemum* culture. Photosynthetica 37:53–59.

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

Desjardins, Y., C. Hdider, and J. De Riek. 1995. Carbon nutrition in vitro. Regulation and manipulation of carbon assimilation in micropropagated systems, p. 441–447. In: J. Aitken-Christie, T. Kozai, and M.A.L. Smith (eds.). Automation and environmental control in plant tissue culture. Kluwer Academic, The Netherlands.

Desjardins, Y., F. Laforge, C. Lussier, and A. Gosselin. 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 Hort. 230:45–53.

Ezawa, M. 1997. Effects of initial sucrose concentration in the medium on the growth of plantlets cultured in vitro (in Japanese with English figures and tables). BS thesis, Chiba University, Japan.

Fournioux, J.-C. and R. Bessis. 1986. Influence de la teneur en gaz carbonique sur la morphogénèse en culture in vitro. Can. J. Bot. 64:2608–2616.

Foyer, C.H. and N. Galtier. 1996. Sucrose–sink interaction and communication in leaves, p. 311–340. In: E. Zamski and A.A. Schaffer (eds.). Photoassimilate distribution in plants and crops: Source–sink relationships. Marcel Dekker, New York.

Fujiwara, K. and T. Kozai. 1995. Physical microenvironment and its effects, p. 319–369. In: J. Aitken-Christie, T. Kozai, and M.A.L. Smith (eds.). Automation and environmental control in plant tissue culture. Kluwer Academic, The Netherlands.

Fujiwara, K., S. Kira, and T. Kozai. 1995. Contribution of photosynthesis to dry weight increase of in vitro potato cultures under different CO_2 concentrations. Acta Hort. 393:119–126.

Fujiwara, K., T. Kozai, and I. Watanabe. 1987. Fundamental studies on environments in plant tissue culture vessels. III. Measurements of carbon dioxide gas concentration in closed vessels containing tissue cultured plantlets and estimates of net photosynthetic rates of the plantlets (in Japanese with English summary, figures and tables). J. Agr. Meteorol. 43:21–30.

Galzy, R. and D. Compan. 1992. Remarks on mixotrophic and autotrophic carbon nutrition of *Vitis* plantlets cultured in vitro. Plant Cell Tissue Organ Cult. 31:239–244.

Infante, R., E. Magnanini, and B. Righetti. 1989. The role of light and CO_2 in optimizing conditions for shoot proliferation of *Actinidia deliciosa* in vitro. Physiol. Plant. 77:191–195.

Kozai, T. 1991. Autotrophic micropropagation, p. 313–343. In: Y.P.S. Bajaj (ed.). Biotechnology in agriculture and forestry. vol. 17. Springer-Verlag, New York.

Kozai, T. and K. Sekimoto. 1988. Effects of the number of air exchanges per hour of the closed vessel and the photosynthetic photon flux on the carbon dioxide concentration inside the vessel and the growth of strawberry plantlets in vitro (in Japanese with English abstract, figures and tables). Environ. Control Biol. 26:21–29.

Kozai, T., K. Fujiwara, and I. Watanabe. 1986. Fundamental studies on environments in plant tissue culture vessels. II. Effects of stoppers and vessels on gas exchange rates between inside and outside of vessels closed with stoppers (In Japanese with English summary, figures and tables). J. Agric. Meteorol. 42:119–127.

Kubota, C., K. Kakizaki, T. Kozai, K. Kasahara, and J. Nemoto. 2001. Growth and net photosynthetic rate of tomato plantlets during photoautotrophic and photomixotrophic micropropagation. HortScience 36:49–52.

Langford, P.J. and H. Wainwright. 1987. Effect of sucrose concentration on the photosynthetic ability of rose shoots in vitro. Ann. Bot. 60:633–640.

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

Murayama, N. T. Hirata, H. Yazaki, T. Tano, T. Horiguchi, N. Shimada, and K. Maeda. 1984. Plant nutrition (in Japanese). Buneido, Tokyo.

Pageau, K., P. Simier, N. Naulet, R. Robins, and A. Fer. 1998. Carbon dependency of the hemiparasite *Striga hermonthica* on *Sorghum biocolor* determined by carbon isotopic and gas exchange analyses. Austral. J. Plant Physiol. 25:695–700.

Piqueras, A., J.M. Van Huylenbroeck, B.H. Han, and P.C. Debergh. 1998. Carbohydrate partitioning and metabolism during acclimatization of micropropagated *Calathea*. Plant Growth Regul. 26:25–31.

Pospisilova, J., J. Solarova, and J. Catsky. 1992. Photosynthetic responses to stress during in vitro cultivation. Photosynthetica 26:3–18.

Serret, M.D., M.I. Trillas, J. Matas, and J.L. Araus. 1996. Development of photoautotrophy and photoinhibition of *Gardenia jasminoides* plantlets during micropropagation. Plant Cell Tissue Organ Cult. 45:1–16.

Serret, M.D., M.I. Trillas, J. Matas, and J.L. Araus. 1997. The effect of different closure types, light, and sucrose concentrations on carbon isotope composition and growth of *Gardenia jasminoides* plantlets during micropropagation and subsequent acclimatization ex vitro. Plant Cell Tissue Organ Cult. 47:217–230.

Wilkins, M.B. and A.W. Holowinsky. 1965. The occurrence of an endogenous circadian rhythm in a plant tissue culture. Plant Physiol. 40:907–909.

Wilson, S.B., C. Kubota, and T. Kozai. 2000. Effects of medium sugar on growth and carbohydrate status of sweetpotato and tomato plantlets in vitro, p. 258–265. In: C. Kubota and C. Chun (eds.). Transplant production in the 21st century. Kluwer Academic Publishers, The Netherlands.

Wolf, S., N. Kalman-Rotem, D. Yakir, and M. Ziv. 1998. Autotrophic and heterotrophic carbon assimilation of in vitro grown potato (*Solanum tuberosum* L.) plants. J. Plant Physiol. 153:574–580.