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MICROPROPAGATION

CO_2-enriched microenvironment affects sucrose and macronutrients absorption and promotes autotrophy in the *in vitro* culture of kiwi (*Actinidia deliciosa* Chev. Liang and Ferguson)

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Abstract In traditional *in vitro* culture, the low CO_2 concentration inside the vessels restricts photosynthesis and necessitates the addition of sucrose to the culture medium as the main energy source, thus bringing about changes in the absorption of mineral elements from the culture medium. In this study, we investigated macronutrient absorption and sugar consumption in *Actinidia deliciosa* Chevalier Liang and Ferguson cv. Hayward (kiwi), cultured on medium supplemented with varying amounts of sucrose (0, 10, and 20 g l^{-1}) under both heterotrophy and autotrophy, flushed with different concentrations of CO_2 (non-ventilation, 300, 600, and 2,000 μl l^{-1}). In ventilated systems with 20 g l^{-1} of sucrose, sucrose absorption was less than under non-ventilation. The lowest rate of sucrose absorption was recorded when the explants were cultured on medium supplemented with 20 g l^{-1} of sucrose and flushed with 600 μl l^{-1} CO_2. Absorption of NO_3^-, PO_4^{3-}, and Mg^{2+} were high (maximum) at the end of the culture period (40 d) in explants flushed with 600 μl l^{-1} CO_2 that have been cultured 20 d in the presence of sucrose and then transferred to a sucrose-free medium. These autotrophic conditions promoted maximum plant growth in terms of both fresh and dry mass as well as the length and number of shoots and leaves. The study shows that to maintain an optimum regime of mineral nutrition for prolonged culture of kiwi *in vitro*, an increased amount of these three ions should be supplemented in Murashige and Skoog's medium.

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Introduction

Automation of *in vitro* culture is a must to produce high quality plants at competitive prices. In the vitroponic system, explants are cultured in liquid medium supported on cellulose plugs, which enables the introduction and extraction of the culture medium to be controlled. However, other parameters are also required to be controlled to improve the quality of microplants. These include the amount and quality of light, gases, and relative humidity inside the culture vessels (microenvironment; Arigita et al. 2002, 2003), as well as the composition of the culture medium to adapt it as much as possible to the specific requirements of the explants in each developmental stage.

Since traditional *in vitro* culture is characterized by low CO_2 concentration and photosynthetic photon flux density (PPFD) inside the vessels, sucrose is added to the culture medium as the main carbon (energy) source. Otherwise, explants grown under heterotrophic conditions develop various anatomical and physiological disorders that do not allow proper operation of the photosynthetic machinery and even result in desiccation during acclimatization *ex vitro* (Kubota and Kozai 1992). The organic and mineral compositions of the culture medium are particularly important to improve differentiation and optimize explant growth. It is well known that the amount of nutrients present in the culture medium must be sufficient to foster growth throughout the entire culture period.

The media used for *in vitro* culture are usually defined with a high content in some nutrients (nitrogen), while low

in others (phosphate). The correct qualitative and quantitative composition of the medium is very important for successful development of the explants. The nutrients present in the culture medium should not limit growth during the culture period. However, nutritional requirements differ with species, tissues, and physiological stages of the plant (George and Sherrington 1984). It is, therefore, very important to know these nutritional requirements when modifying the micro-environmental factors that can directly or indirectly affect nutrient uptake. These are (1) change from heterotrophic to autotrophic nutrition, which affects sucrose and ion absorption (Kozai et al. 1991); (2) changes in the medium pH, which can alter ion solubility (Kozai et al. 1991); and (3) increasing evapotranspiration of the explants through ventilation, thus lowering environmental relative humidity *versus* traditional culture in a water vapor saturated atmosphere. It has been shown that cellulose plugs used as explant support in liquid medium can influence mineral uptake because of ion adsorption by the cellulose fibers (Nordstrom and Eliasson 1986).

The nutritional requirements for the species can be studied by analyzing nutrient absorption during culture and reformulating the medium according to the results obtained. This approach has been used in some studies on *in vitro* nutrition (Schmitz and Lörz 1990; Dussert et al. 1995; Dantas et al. 2001). In traditional *in vitro* culture of kiwi, i.e., sucrose supplemented, gelled culture medium, capped vessels, and non-controlled microenvironment, there are few studies on nutrient absorption (Moncaleán et al. 2003). In this study, we investigated the dynamics of macronutrient absorption and sugar consumption from the medium which will allow us to ascertain the appropriate contribution of nutrients and sugar to promote organogenesis and the production of high quality plant from explants cultured in ventilated systems under controlled CO_2 atmospheres and in the presence of different sucrose concentrations in liquid medium.

Materials and Methods

Plant material and culture conditions. Apical shoots (10 mm long) of *Actinidia deliciosa* Chev. Liang and Ferguson cv. Hayward were excised from microplants grown on gelled (7.0 g l^{-1} agar) MS (Murashige and Skoog 1962) medium supplemented with 20.0 g l^{-1} sucrose, 4.44 μM N^6-benzyladenine (BA), and 2.89 μM gibberellic acid (GA_3). The growth regulators were filter-sterilized after adjusting the pH to 5.6 and sterilization at 120°C for 20 min. Four explants were cultured in 350 ml polycarbonate Magenta GA-7 (Magenta Corp., Sigma, St. Louis, MO) vessels, which contained a bed of cellulose plugs (Baumgartner Papier, Crissier, Switzerland) and 70 ml of the same liquid medium supplemented with sucrose at 0 (E_0), 10 (E_{10}), and 20 g l^{-1} (E_{20}) or either 20 g l^{-1} for the first 20 d and then changed to one half MS fresh medium without sucrose and hormones for the rest of the culture period (E_{20-0}; Arigita et al. 2002). The non-ventilated culture vessels were closed with polypropylene caps, and the ventilated ones with the same caps provided with one bacteriological filter of 10 mm of diameter and 0.22 μm of pore size (Magenta Corp.).

The plants grown in non-ventilated vessels with 20 g l^{-1} sugar in the medium (E_{20}^{0}) were used as reference because these are the most common culture conditions in traditional micropropagation of kiwi. The Magenta vessels were kept inside specially designed glass boxes for 40 d under a 16-h photoperiod (150 μmol m^{-2} s^{-1} PPFD at the culture level) at 25°C. The relative humidity was maintained at 75–80% (digital hygrometer Damion 306106) in the ventilated vessels, whereas it was near saturation inside the non-ventilated ones. The control of relative humidity was achieved by bubbling the incoming air stream through a water column at a fixed height to get 75–80% relative humidity inside the glass boxes.

The glass boxes were flushed with a hydrocarbon-free gas mixture of 210 ml l^{-1} O_2, 780 ml l^{-1} N_2 and CO_2 at 300 (E^{300}), 600 (E^{600}), and 2,000 ($E^{2,000}$) μl l^{-1} (Praxair SA, Madrid, Spain) at a flow rate of 30 ml min^{-1} (flowmeter GT 1350 Sho-Rate, Brooks Instrument B.V., Ede, Netherlands), resulting in the measured CO_2 concentration inside the Magenta vessels remaining constant after flushing for 15 min as shown by sampling at 10, 20, 30, and 40 d. For measuring CO_2 concentration inside the Magenta vessels, a silicone septum was fixed in a lateral hole facing another septum sited in the walls of the ventilated glass box. Sampling of the inner atmosphere of the Magenta vessels was by means of a special syringe for gases inserted through both septa. The gas samples were then injected into a Shimadzu model GC-3 gas chromatograph (Shimadzu Scientific Instruments, Columbia, MD) equipped with a thermoconductivity detector and a molecular sieve column. The carrier gas was nitrogen at a flow rate of 300 ml min^{-1}. The detector signal was quantified in a Shimadzu C-R5A Chromatopac integrator by using the external standard method. CO_2 identification was based on the retention time compared with calibration standard (Mandel Scientific Instruments, Columbia, MD).

The number of shoots per explant, main shoot length, number of leaves per explant, and leaf size (from the petiole to the apex) were measured after 40 d in culture.

pH measurement. The pH of the culture medium was measured after autoclaving (day 0) and at 10, 20, 30, and 40 d in the different treatments with a pH meter (Crison micropH 2001, Barcelona, Spain) connected to a Hamilton flushtrode 238060 (Teknokroma, Barcelona, Spain) electrode.

Estimation of sucrose and reducing sugars. Each sample of the medium at the different periods and culture conditions were taken from different culture vessels and analyzed for sucrose, D-glucose, and D-fructose according to Bergmeyer and Bernt (1974), using the Enzymatic BioAnalysis kit (Boheringer-Mannheim, Barcelona, Spain). The absorbance was measured at 340 nm with a Perkin-Elmer double-beam spectrophotometer model 550 SE (Perkin-Elmer, Wellesley, MA). The uptake of sugars was calculated from these data and was expressed as milligrams per plant. Sugars left in the spent medium were expressed as g l^{-1}.

Nutrient analysis. Aliquots of the medium at different periods (0, 10, 20, 30, and 40 d) and culture conditions were taken from different vessels, diluted 100-fold with distilled water (Milli-Q 185 plus system, 18 MΩ cm^{-1} resistivity, Millipore Iberica SA, Madrid, Spain), and used to quantify residual MS macronutrients (NO_3^-, SO_4^{2-}, Cl^-, PO_4^{3-}, NH_4^+, K^+, Ca^{2+}, and Mg^{2+}) by capillary electrophoresis (CE). Both cation and anion analyses were performed in a capillary ion analyser (CIA Quanta 4000E; Waters Chromatography SA, Milford, MA) with a 60 cm×75 μm uncoated fused silica capillary column (Waters Chromatography SA) connected to a Millennium 2010 processing data station.

Cation analyses were performed with 5 mM UV-Cat 1 (Waters Chromatography SA) and 6.5 mM α-hidroxyisobutyric acid (pH 4.0), adding 53 mg of 18-crown-6-ether to avoid NH_4^+ and K^+ comigration. The column was maintained at room temperature, and the CE system was operated at 20 kV voltage and 4 μA current. Cation detection was monitored at 185 nm. Anion analyses were performed with 4.6 mM chromate and 0.46 mM OFM-Anion BT (Waters Chromatography SA; pH 8.0). The column was maintained at 25°C, and the CE system was operated at −20 kV voltage and 19±1 μA current. On-column UV absorbance was monitored at 254 nm. Both cation and anion samples were filtered and injected hydrostatically at 9.8 cm for 10 s. The percentage of macronutrient absorption was calculated: [(initial amount)−(amount left at the end of the experiment)]×100/initial amount.

Statistical analysis. All organogenesis data are the mean of at least two independent experiments with 30 replicates each. One-way analysis of variance was used to test differences between treatments. When significant differences were found (α=0.05), means were separated by least significant difference. The mean of three analyses with three repeats each one was used for other cases. Quantitative data were analyzed by the non-parametric Kruskal–Wallis test for *n* independent group and the Mann–Whitney test for two independent group analysis. Differences were considered significant at the 5% level using the Statistical Package for the Social Sciences (SPSS) 7.5 for Windows statistical package (SPSS Inc., Chicago, IL).

Results

Organogenesis. Explants cultured under non-ventilation for 40 d in 20 g l^{-1} sucrose (E_{20}^{0}) developed two or more shoots, the main shoot being about 3 cm long (Table 1). Survival rate was high, and half of the leaves were longer than 1 cm. Sucrose elimination (E_0^{0}) or reduction (E_{10}^{0} and E_{20-0}^{0}) from the culture medium produced shorter plants with even smaller leaves and reduced survival rate to 40%, 65%, and 70%, respectively (Table 1).

At 300 μl l^{-1} CO_2, the best results for shoot length and number and size of leaves were for E_{20-0}^{300} when compared to non-ventilated ones (Table 1). Low (E_{10}^{300}) or no sucrose (E_0^{300}) in the culture medium produced smaller explants and E_0^{300} registered poor survival (Table 1).

Explants grown at 600 μl l^{-1} CO_2 and no sucrose in the culture medium (E_0^{600}) had very high mortality (77%; data not shown), whereas the best explants in length, number, and size of leaves were those grown at E_{20-0}^{600} with a survival rate of 95% (Table 1).

At 2,000 μl l^{-1} CO_2 survival rate was high except $E_{20-0}^{2,000}$ (25%, data not shown); however, the lowest values were measured for the other parameters considered (Table 1).

It is interesting to point out that all explants arrested growth upon transfer from 20 g l^{-1} to sucrose-free medium, but they soon recovered and, finally, in some cases, performed the best.

Sugars in the culture medium. When the explants were grown in non-ventilated cultures (E_{10}^{0} and E_{20}^{0}), sucrose depletion from the culture medium was fast for the first 20 d (70%); at the 40th d, 1.0 and 3.0 g l^{-1} sucrose remained in the culture medium initially supplemented with 10.0 or 20.0 g l^{-1} sucrose, respectively (Fig. 1). As sucrose disappeared from the culture medium, glucose and fructose increased in equal amounts up to the 20th d. Subsequently, there was slightly more fructose than glucose (Fig. 1).

In contrast, in ventilated cultures when the medium contained 20 g l^{-1} sucrose, nearly half of the initially added sucrose remained in the medium after 40 d of culture when the explants were grown under treatments E_{20}^{600} and $E_{20}^{2,000}$ (Table 2). However, with 10 g l^{-1} sucrose, the amount of sucrose remaining in the culture medium under ventilation was similar to the non-ventilated one (E_{10}^{0}). There were significant differences in glucose and fructose between ventilated, with 20 g l^{-1}, and non-ventilated, with 10 g l^{-1} (Table 2).

Table 1. Organogenesis and survival of kiwi microplants after 40 d in *in vitro* culture under varying environmental conditions (CO_2-flushing) with different concentrations of sucrose

Treatment[z]	Main shoot length	Number of shoots/explant	Number of leaves	Number of leaves longer than 1cm	Survival (%)
E_0^0	2.11±0.06 a	2.08±0.09 a	8.03±0.31 a	2.93±0.40 a	40
E_{10}^0	2.12±0.04 a	1.89±0.08 a	7.58±0.25 a	2.96±0.44 a	65
E_{20}^0	2.74±0.10 b	2.32±0.08 b	9.33±0.20 b	4.66±0.35 b	96
E_{20-0}^0	2.20±0.08 a	2.03±0.08 a	8.01±0.35 a	3.66±0.27 a	70
E_0^{300}	2.04±0.06 a	2.36±0.09 b	10.16±0.45 c	3.47±0.41 a	44
E_{10}^{300}	1.85±0.17 a	2.43±0.09 b	10.25±0.51 c	5.58±0.49 bc	50
E_{20}^{300}	2.75±0.19 b	2.44±0.09 b	11.86±0.53 d	6.09±0.36 c	93
E_{20-0}^{300}	3.32±0.17 c	2.41±0.18 b	11.14±0.55 cd	7.25±0.28 d	94
E_{10}^{600}	2.59±0.14 b	2.62±0.10 c	13.90±0.44 e	6.72±0.31 cd	90
E_{20}^{600}	3.34±0.17 c	3.12±0.09 d	13.62±0.55 e	7.10±0.17 d	90
E_{20-0}^{600}	3.64±0.19 d	3.09±0.11 d	15.03±0.49 f	7.85±0.21e	95
$E_{10}^{2,000}$	1.41±0.03 e	1.32±0.08 e	7.67±0.35 a	3.5±0.18 a	95
$E_{20}^{2,000}$	1.57±0.04 e	1.30±0.08 e	8.66±0.32 a	5.14±0.16 b	96
$E_{20-0}^{2,000}$	1.90±0.07 ae	1.35±0.08 e	9.09±0.3 ab	6.57±0.11 c	95

[z] E_0^0, E_{10}^0, and E_{20}^0 =non-ventilated cultures with 0, 10, and 20 g l^{-1} sucrose in the medium, respectively; E_0^{300}, E_{10}^{300}, and E_{20}^{300} =as before but flushed with 300 µl l^{-1} CO_2; E_{10}^{600} and E_{20}^{600} =as before but flushed with 600 µl l^{-1} CO_2; $E_{10}^{2,000}$ and $E_{20}^{2,000}$ =as before but flushed with 2000 µl l^{-1} CO_2; E_{20-0}^0, E_{20-0}^{300}, E_{20-0}^{600}, and $E_{20-0}^{2,000}$ =non-ventilated cultures and flushed with 300, 600, and 2000 µl l^{-1} CO and 20 g l^{-1} sucrose for the first 20 d followed by culturing on one half MS (Murashige and Skoog 1962) medium without sugar for the rest of the culture period

Each value represents the mean ± standard error of 60 data. Common *letters* within a *column* are not significantly different at $p \leq 0.05$ (least significant difference test)

About a threefold increase in dry mass occurred when the explants were grown under E_{10}^{600} as compared to E_{10}^0 (Table 3). The highest increase in dry mass was recorded in ventilated cultures containing 20 g l^{-1} sucrose and flushed with 600 µl l^{-1} CO_2 (E_{20}^{600}). At 2,000 µl l^{-1} CO_2 ($E_{10}^{2,000}$ and $E_{20}^{2,000}$), however, the increase in dry mass was less, and the results were similar to those of non-ventilated systems (Table 3). When sugars absorption was compared with dry mass increase, the lowest ratios were obtained for plants cultured in media containing 10 or 20 g l^{-1} sucrose under 600 µl l^{-1} CO_2 (Table 3).

pH changes during culture. After sterilization, there was a marginal drop in pH from 5.6 to 5.4 in the culture medium regardless of the amount of sucrose added. Generally, pH decreased for the first 20 d (data not shown) and then reached a plateau until the 40th day, when the pH values were higher in non-ventilated cultures and lower in cultures flushed with 2,000 µl l^{-1} CO_2 (Table 4). Intermediate and similar pH values were obtained after 40 d when the cultures were flushed with 300 and 600 µl l^{-1} CO_2. However, the lowest pH values were recorded in ventilated cultures flushed with 2000 µl l^{-1} CO_2, even in control medium without explants (Table 4).

Absorption of macronutrients. Explants cultured under non-ventilation absorbed NH_4^+ rapidly during the first 10 d, slowing down until the end of the culture (Table 5). As NH_4^+ absorption decreased, absorption of NO_3^- increased from the tenth day up to the end of culture. At the 40th day, in non-ventilated cultures, when the medium contained 20 g l^{-1} sucrose (E_{20}^0), absorption of the added NH_4^+ was 52%, while the amount of NO_3^- absorbed was 72% (Table 5). Explants changed at day 20 to one half MS medium (E_{20-0}^0) absorbed 7% NH_4^+ and 34% NO_3^- of the freshly added ions.

In cultures flushed with 300 and 600 µl l^{-1} CO_2, absorption of NH_4^+ and NO_3^- was higher than in the non-ventilated cultures, even in those explants grown on sucrose-free medium after 20 d (E_{20-0}^{300} and E_{20-0}^{600}; Table 5). However, absorption of NH_4^+ and NO_3^-, by those grown at 2,000 µl l^{-1} CO_2, was similar to the non-ventilated, although more NH_4^+ and NO_3^- were absorbed by $E_{20-0}^{2,000}$ than by E_{20-0}^0 (Table 5). Explants E_0^{600} and $E_0^{2,000}$ were not recorded, as mortality was more than 75% in all cases.

Mg^{2+} absorption by microplants grown under non-ventilation was low for the first 10 d of culture, subsequently increasing up to 55% in the case of E_{20}^0 (Table 6). Microplants flushed with 300 and 600 µl l^{-1} CO_2 absorbed more Mg^{2+} than the non-ventilated ones. E_{20-0}^{300} and E_{20-0}^{600} performed better than those cultured under non-ventilation (Table 6). At 2,000 µl l^{-1} CO_2, Mg^{2+} absorption decreased, but the measured values were higher than those of explants cultured under non-ventilation.

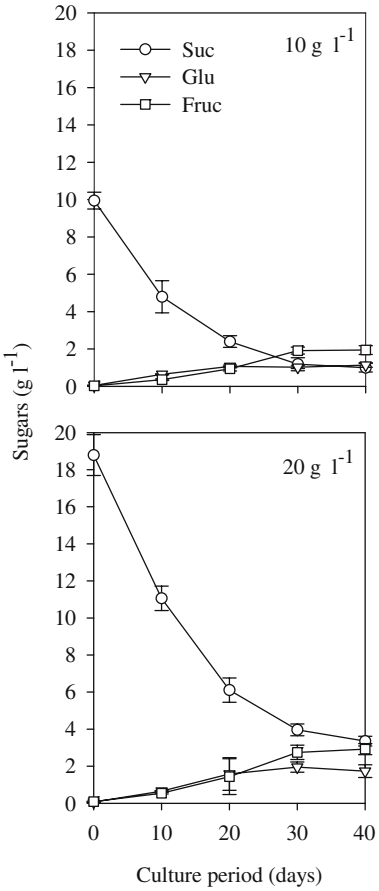

Figure 1. Changes in sucrose, glucose, and fructose concentrations in media used for kiwi culture under non-ventilation conditions. Initial sucrose concentration was 10 g l^{-1} (*top*) or 20 g l^{-1} (*bottom*).

Ca^{2+} absorption in non-ventilated cultures was slow for the first 20 d, subsequently increasing to 44% in microplants $E_{20}{}^{0}$ after the 40th day (Table 6). Flushing with CO_2 increased Ca^{2+} absorption and also improved the absorption capacity of explants cultured on sucrose-free medium after 20 d ($E_{20-0}{}^{300}$, $E_{20-0}{}^{600}$, and $E_{20-0}{}^{2,000}$; Table 6). Ventilation with 300, 600, and 2,000 µl l^{-1} CO_2 did not improve K^+ absorption when compared with those non-ventilated. Explants transferred, after 20 d, to one half MS medium free of sugars showed maximum absorption at $E_{20-0}{}^{300}$ (Table 6).

More than 70% of the added phosphate was taken up by kiwifruit microplants in all treatments except $E_{20-0}{}^{0}$ (Table 7). Cl^- absorption was always less than 20% under all the assayed conditions, with some periods of excretion to the medium (data not shown). Absorption of $SO_4^=$ was low, in all assayed conditions, while at the end of the culture, only 35% of the initial amount added to the medium had been absorbed (data not shown).

Table 2. Sugars in the medium after 40 d in *in vitro* culture of kiwi under varying environmental conditions (CO_2-flushing) with two concentrations of sucrose

Treatment[z]	Sucrose (gl^{-1})[y]	Fructose (gl^{-1})[y]	Glucose (gl^{-1})[y]
$E_{10}{}^{0}$	0.99±0.23 a	1.94±0.23 a	1.14±0.12 a
$E_{20}{}^{0}$	3.35±0.26 c	2.92±0.29 b	1.73±0.34 b
$E_{10}{}^{300}$	1.32±0.09 ab	1.66±0.21 a	1.05±0.21 a
$E_{20}{}^{300}$	7.93±0.33 d	2.24±0.27 ab	1.15±0.43 a
$E_{10}{}^{600}$	1.56±0.21 b	1.86±0.22 a	1.04±0.26 a
$E_{20}{}^{600}$	9.65±0.63 e	2.47±0.37 ab	1.19±0.35 ab
$E_{10}{}^{2,000}$	1.38±0.26 ab	1.65±0.27 a	1.07±0.35 a
$E_{20}{}^{2,000}$	8.75±0.74 de	1.93±0.37 a	1.02±0.25 a

[z] Same as in Table 1

[y] Each value represents the mean ± standard error of nine data. Common *letters* within a *column* are not significantly different at $p \leq 0.05$ (Mann–Whitney test)

Discussion

MS medium (Murashige and Skoog 1962) is used for culturing many woody and herbaceous plant species. However, sometimes it becomes deficient in macronutrients for some of these species (Sha et al. 1985; Singha et al. 1987) and so must be adapted to the requirements of the cultured explants (Anderson 1984; Driver and Kuniyuki 1984). Moreover, new experimental conditions (liquid medium and autotrophy) affect explant nutrition by modifying the absorption of nutrients.

It has been proposed that hexoses resulting from sucrose hydrolysis are an easily available source for absorption and cellular metabolism (Desjardins et al. 1995). Sucrose is hydrolyzed (1%) when the culture medium is autoclaved. During culture, hexoses are also gradually hydrolyzed by invertases located in the cell wall or in the plasmalemma (Goldner et al. 1991; Wendler et al. 1991). In general, significant levels of free invertases were not detected in the culture medium, although this could sometimes occur (Goldner et al. 1991; Wendler et al. 1991). Both glucose and fructose are readily assimilated by explants, but we showed that kiwi plants consumed more glucose, since there was more fructose than glucose in the spent medium after 40 d in culture. In some species, sucrose is completely hydrolyzed before absorption (Stafford and Fowler 1983; Schmitz and Lörz 1990; Moncaleán et al. 2003). However, other species such as *Fragaria* (Kozai et al. 1991) and *Malus* (Karhu 1997) do not show preferences in the absorption of either of the reducing sugars or in coconut, which preferably consumes fructose (Dussert et al. 1995).

In the present study, there was a higher rate of sugar absorption in microplants grown under non-ventilated conditions for the first 20 d of culture. Under these nearly heterotrophic conditions, the development of explants was mostly dependent on the energy supplied by the exogenous

Table 3. Absorption of sugars, dry mass increase, and their relationship in kiwi cultured *in vitro* under varying environmental conditions (CO_2-flushing) with two concentrations of sucrose

Treatment[z]	Sugars absorption (mg plant^{-1})[y]	Dry mass increase (mg plant^{-1})[y]	Sugars/dry mass increase
E_{10}^{0}	104±8 a	67±6 a	1.55±0.26 ab
E_{20}^{0}	209±16 c	97±9 b	2.15±0.36 b
E_{10}^{300}	102±8 a	95±8 b	1.07±0.17 a
E_{20}^{300}	135±10 b	136±11 c	0.99±0.15 a
E_{10}^{600}	93±7 a	183±10 d	0.51±0.06 c
E_{20}^{600}	100±8 a	215±15 d	0.46±0.068 c
$E_{10}^{2,000}$	98±8 a	56±4 a	1.75±0.26 ab
$E_{20}^{2,000}$	136±10 b	78±7 ab	1.74±0.28 ab

[z] Same as in Table 1

[y] Each value represents the mean ± standard error of nine data. Common *letters* within a *column* are not significantly different at $p \leq 0.05$ (Mann–Whitney test)

sucrose (Maretzki et al. 1974), which was also necessary for the formation of new structures (George and Sherrington 1984). Sucrose at 20 g l^{-1} was more than sufficient to maintain the metabolic machinery and plant growth of *A. deliciosa* cultured *in vitro*. At the end of the culture, 20% of the added sucrose remained in the medium when it was initially supplemented with 20 g l^{-1} sucrose. However, plants cultured on medium containing 10 g l^{-1} sucrose absorbed 90% of the initially added sucrose, indicating that sucrose at this concentration was not sufficient to ensure the growth and development of plants, as shown by the lower increase in dry weight, fewer and shorter shoots, and smaller leaves

Table 4. Effect of CO_2-flushing on pH[z] of the medium after 40 d of *in vitro* culture of kiwi at different sucrose concentrations

Sucrose (g l^{-1})	CO_2 (µl l^{-1})			
	0	300	600	2,000
0	4.78±0.05 b	5.25±0.04 a	ND	ND
10	4.56±0.07 c	3.96±0.06 d	4.00±0.07 d	3.62±0.07 e
20	4.42±0.07 c	3.96±0.06 d	4.00±0.07 d	3.69±0.07 e
20–0[y]	4.40±0.06 c	4.14±0.07 d	4.12±0.07 d	3.89±0.06 d
20[x]	5.25±0.04 a	5.21±0.03 a	5.17±0.04 a	3.89±0.06 d

ND not determined

[z] Each value represents the mean ± standard error of nine data. Common *letters* are not significantly different at $p \leq 0.05$ (Mann–Whitney test)

[y] 20 g l^{-1} sucrose for the first 20 d followed by culturing on one half MS (Murashige and Skoog 1962) medium without sugar for the rest of the culture period

[x] pH of the culture medium without explants

after 40 d of culture. A similar consumption rate of sugars was also described in other species (Kozai et al. 1991; Dussert et al. 1995).

It is clear that plant growth under non-ventilated conditions was at the expense of the sugars added to the culture medium, even when the vessels were not hermetically closed, thus allowing photosynthesis to some extent (Arigita et al. 2002). In contrast to the results obtained in *Fragaria* (Kozai et al. 1991), when no sucrose was present in the medium at the very beginning of the culture to induce autotrophic nutrition, a reduction in shoot length and dry mass in kiwi was observed, and E_0^{300} registered high mortality (56%; Arigita et al. 2002). Our findings are supported by those obtained in rose, in which sucrose at 10 g l^{-1} in the medium diminished plant dry mass by about 50% (Capellades et al. 1991) and those obtained in coconut in which elimination or minimization of sucrose in the culture medium resulted in plants with higher photosynthesis, although this improvement was not sufficient to confer higher *ex vitro* survival or growth (Fuentes et al. 2005b). It is possible that these plants did not have sufficient reserves in the *in vitro* formed leaves to initially maintain *ex vitro* growth. Under ventilated conditions, sucrose was necessary for plant growth, at least for the first few days, presenting a sort of mixotrophic carbon nutrition.

Culture ventilation (300 and 600 µl l^{-1} CO_2) decreased sugar absorption and, more importantly, the ratio between sugars and dry mass. This indicates a shift from a heterotrophic to an autotrophic regime in which the plants are able to fix CO_2 and use it to produce the necessary energy for their growth and development (Keutgen et al. 1997; Pospisilova et al. 1997; Rival et al. 1997). As CO_2 increased from 300 to 600 µl l^{-1} CO_2, there was an increase in dry weight as well as improvement in plant quality, i.e., more and longer shoots and more and larger leaves. A reduction in growth rate occurred when the explants were grown at 2,000 µl l^{-1} CO_2, and, therefore, the accumulation of dry mass was not as much as compared to those grown at 600 µl l^{-1} CO_2; under such conditions, it has been shown that there is less RUBISCO concomitant with a reduced photosynthesis (Arigita et al. 2002).

Removal of sucrose after 20 d of culture improved the plant growth, suggesting that kiwi explants need sugars during the initial stages of development to accumulate starch reserves and develop the efficient photosynthetic machinery necessary for CO_2 fixation to become adapted to the new conditions (Van Huylenbroeck and Debergh 1996). However, an initial high sugar content in the medium can impair the carboxylation rate of explants (Langford and Wainwright 1987; Cournac et al. 1991) and have negative effects on the plants that may be related to increased plant susceptibility to feedback inhibition because of an excess of carbohydrates stored in the leaves and low sugar exporta-

Table 5. NH_4^+ and NO_3^- concentration left in the medium along the *in vitro* culture of kiwi and percentage of absorption at 40 d under varying levels of CO_2-flushing and sucrose

CO_2 (μl l^{-1})	Culture period (days)	NH_4^+ (mM)[z]				NO_3^- (mM)[z]			
		Sucrose (g l^{-1})				Sucrose (g l^{-1})			
		0	10	20	20–0[y]	0	10	20	20–0[y]
0	0	20.34±0.43	20.22±0.40	20.31±0.43		39.20±1.58	39.30±1.43	39.19±1.67	
	10	16.41±0.38	15.70±0.47	14.77±0.36		36.70±1.71	36.64±1.32	36.46±1.53	
	20	14.19±0.45	13.89±0.57	12.69±0.42	11.40±0.45[x]	31.20±1.96	30.46±1.21	30.07±1.49	21.04±1.12[x]
	30	12.81±0.50	12.35±0.62	11.15±0.45	11.63±0.43	23.15±1.53	22.88±1.62	21.25±1.30	22.65±1.40
	40	12.10±0.60 a	11.50±0.53 a	9.84±0.64 a	10.63±0.47 a	15.03±1.46 a	14.59±1.21 a	10.83±1.11 a	13.90±1.05 a
		41%	43%	52%	7%[w]	62%	63%	72%	34%[w]
300	0	20.37±0.44	20.19±0.56	20.25±0.63		38.94±1.11	37.90±1.29	37.49±1.19	
	10	17.41±0.42	16.45±0.51	15.95±0.41		34.64±1.61	34.27±1.49	34.07±1.32	
	20	14.74±0.31	14.15±0.36	11.53±0.57	10.72±0.48[x]	29.28±1.83	28.91±1.56	26.96±1.59	20.84±1.32[x]
	30	13.50±0.43	12.38±0.47	9.54±0.46	8.98±0.42	22.67±1.19	21.24±1.22	16.98±1.33	14.75±1.30
	40	12.51±0.41 a	10.62±0.53 ab	7.95±0.48 b	7.15±0.40 b	15.41±1.30 a	13.67±1.14 a	8.56±0.99 a	5.04±0.98 b
		39%	47%	61%	33%[w]	60%	64%	77%	76%[w]
600	0	ND	19.96±0.49	20.26±0.42		ND	37.90±1.25	37.49±1.33	
	10	ND	15.28±0.31	13.50±0.34		ND	34.27±1.67	34.07±1.43	
	20	ND	12.64±0.41	10.69±0.47	10.70±0.46[x]	ND	28.91±1.82	26.96±1.56	21.14±1.42[x]
	30	ND	10.96±0.53	8.47±0.57	7.23±0.48	ND	21.24±1.49	15.36±1.37	11.70±1.33
	40	ND	9.83±0.69 b	6.09±0.54 c	4.62±0.50 c	ND	12.86±1.59 a	4.58±1.09 b	6.77±1.14 bc
			51%	70%	57%[w]		66%	88%	68%[w]
2,000	0	ND	20.24±0.47	20.35±0.47		ND	38.94±1.43	38.44±1.51	
	10	ND	15.68±0.37	14.44±0.36		ND	35.22±1.62	34.65±2.01	
	20	ND	13.07±0.43	12.06±0.48	10.68±0.39[x]	ND	31.98±1.48	28.94±1.38	21.14±1.43[x]
	30	ND	11.93±0.55	10.90±0.60	9.97±0.48	ND	24.86±1.30	19.74±1.40	17.86±1.46
	40	ND	11.09±0.51 ab	9.62±0.43 a	8.52±0.35 b	ND	16.46±1.62 a	10.17±1.67 a	8.40±1.54 c
			45%	53%	20%[w]		58%	74%	60%[w]

ND not determined

[z] Each value represents the mean ± standard error of nine data. Common *letters* within a *column* are not significantly different at $p \leq 0.05$ (Mann–Whitney test)

[y] Same as in Table 4

[x] Ion concentration of the new fresh medium (one half MS)

[w] Percentage of absorption from day 20 after the medium was changed

tion out of the leaf (Capellades et al. 1991; Fuentes et al. 2005a). This in turn induces the loss of photosynthetic activity (Xu et al. 1994; Arigita et al. 2002; Fuentes et al. 2005b), the decrease in the amount of rubisco and its carboxilation efficiency (Fuentes et al. 2005b) or even structural disorders (Dimassi-Theriou and Bosabalidis 1997). As the accumulation of sugars in the leaves is higher under low light intensity (Dimassi-Theriou and Bosabalidis 1997), the new autotrophic conditions and higher PPFD will diminish these disorders, thus increasing photosynthesis (Fuentes et al. 2005a).

An inherent problem associated with liquid culture medium is its low buffering capacity compared with agar-gelled medium (Skirvin et al. 1986; Singha et al. 1987). In the present study, the observed decline in pH after autoclaving to values lower than those observed by Singha et al. (1987) was not enough to affect ion solubility. For the first few days of culture, a decrease in pH occurred because of extrusion of H^+ from the explants. It has been shown that this proton extrusion is observed each time the medium is renewed (Williams 1993). After 20 d, stabilization occurred at a higher pH in non-ventilated than in ventilated systems flushed with 300 and 600 μl l^{-1} CO_2. The lowest pH was recorded at 2,000 μl l^{-1} CO_2 because of CO_2 dissolving in the culture medium (Leva et al. 1984). Under different conditions, similar effects were also reported in kiwi (Moncalean et al. 2003), *Ptilotus* (Williams et al. 1990), and *Cucumis* (Skirvin et al. 1986).

Table 6. Mg^{2+}, Ca^{2+}, and K^+ concentration left in the medium along *in vitro* culture of kiwi and percentage of absorption at 40 d under varying levels of CO_2-flushing and sucrose

CO_2 ($\mu l\ l^{-1}$)	Culture period (days)	Mg^{2+} (mM)[z]				Ca^{2+} (mM)[z]				K^+ (mM)[z]			
		Sucrose ($g\ l^{-1}$)				Sucrose ($g\ l^{-1}$)				Sucrose ($g\ l^{-1}$)			
		0	10	20	20–0[y]	0	10	20	20–0[y]	0	10	20	20–0[y]
0	0	1.44±0.10	1.41±0.11	1.47±0.09		3.11±0.13	3.15±0.16	3.02±0.12		19.21±0.54	19.85±0.44	19.78±0.46	
	10	1.36±0.11	1.32±0.13	1.33±0.08		2.98±0.07	2.99±0.19	2.96±0.10		17.66±0.40	18.06±0.44	17.77±0.44	
	20	1.07±0.07	1.02±0.09	0.94±0.09	0.74±0.06[x]	2.76±0.11	2.73±0.20	2.68±0.15	1.90±0.20[x]	15.71±0.49	15.15±0.54	14.53±0.52	11.90±0.45[x]
	30	0.89±0.09	0.86±0.11	0.76±0.07	0.69±0.07	2.38±0.20	2.29±0.13	2.15±0.12	1.98±0.18	12.53±0.61	11.96±0.40	10.79±0.47	11.69±0.44
	40	0.77±0.06 a	0.75±0.09 a	0.66±0.07 a	0.59±0.06 a	2.02±0.14 a	1.91±0.12 a	1.68±0.13 a	1.45±0.11 a	9.58±0.45 a	8.99±0.55 a	7.32±0.38 a	8.50±0.43 a
		47%	47%	55%	20%[w]	35%	39%	44%	24%[w]	50%	55%	63%	29%[w]
300	0	1.46±0.09	1.51±0.04	1.48±0.06		3.14±0.10	3.14±0.05	3.17±0.04		19.90±0.46	19.84±0.55	19.95±0.39	
	10	1.30±0.11	1.35±0.09	1.34±0.05		3.00±0.15	2.98±0.07	2.89±0.11		18.18±0.40	18.37±0.42	18.68±0.46	
	20	1.18±0.11	1.08±0.07	0.99±0.09	0.73±0.07[x]	2.71±0.11	2.68±0.17	2.62±0.16	1.92±0.21[x]	16.46±0.33	16.84±0.43	15.56±0.44	11.92±0.44[x]
	30	0.93±0.09	0.78±0.07	0.57±0.12	0.44±0.11	2.32±0.15	2.26±0.14	2.12±0.10	1.99±0.10	13.57±0.42	13.40±0.55	11.16±0.44	10.87±0.39
	40	0.64±0.07 a	0.46±0.07 b	0.32±0.08 b	0.15±0.05 b	1.75±0.15 a	1.62±0.13 ab	1.08±0.12 b	0.80±0.12 b	11.81±0.52 b	11.00±0.48 b	7.37±0.54 a	6.60±0.43 b
		56%	70%	78%	79%[w]	44%	48%	66%	58%[w]	41%	45%	63%	45%[w]
600	0	ND	1.52±0.10	1.47±0.11		ND	3.12±0.08	3.19±0.10		ND	19.97±0.52	19.89±0.39	
	10	ND	1.40±0.11	1.30±0.09		ND	2.92±0.17	3.07±0.12		ND	17.59±0.42	17.11±0.44	
	20	ND	1.05±0.07	0.93±0.11	0.75±0.06[x]	ND	2.64±0.11	2.66±0.15	1.92±0.21[x]	ND	14.66±0.50	14.05±0.55	11.95±0.46[x]
	30	ND	0.73±0.10	0.48±0.11	0.34±0.10	ND	2.10±0.15	2.03±0.11	1.91±0.12	ND	12.61±0.67	11.73±0.46	10.81±0.55
	40	ND	0.48±0.09 b	0.25±0.09 b	0.18±0.08 b	ND	1.48±0.14 b	1.03±0.13 b	0.80±0.12 b	ND	10.93±0.44 b	9.58±0.57 b	8.59±0.48 a
			68%	83%	76%[w]		52%	68%	58%		45%	52%	28%[w]
2,000	0	ND	1.50±0.07	1.47±0.10		ND	3.16±0.11	3.15±0.19		ND	19.85±0.39	19.96±0.47	
	10	ND	1.41±0.09	1.40±0.08		ND	2.97±0.20	3.00±0.16		ND	17.65±0.42	17.19±0.43	
	20	ND	1.09±0.10	1.04±0.19	0.73±0.08[x]	ND	2.69±0.13	2.73±0.12	1.91±0.18[x]	ND	14.87±0.49	14.46±0.53	11.89±0.41[x]
	30	ND	0.79±0.07	0.73±0.08	0.64±0.07	ND	2.38±0.18	2.23±0.17	2.02±0.14	ND	13.19±0.31	12.46±0.41	12.57±0.29
	40	ND	0.63±0.12 ab	0.51±0.05 c	0.39±0.04 c	ND	1.80±0.15 ab	1.30±0.18 a	1.16±0.14 c	ND	11.31±0.39 b	10.26±0.53 b	11.02±0.40 c
			58%	65%	47%[w]		43%	49%	39%[w]		43%	49%	7%[w]

ND not determined

[z] Each value represents the mean ± standard error of nine data. Common *letters* within a *column* are not significantly different at $p \leq 0.05$ (Mann–Whitney test)

[y] Same as in Table 4

[x] Ion concentration of the new fresh medium (one half MS)

[w] Percentage of absorption from day 20 after the medium was changed

Table 7. PO_4^{3-} concentration left in the medium along *in vitro* culture of kiwi and percentage of absorption at 40 d under varying levels of CO_2-flushing and sucrose

CO_2 (µl l^{-1})	Culture period (days)	PO_4^{3-} (mM)[z]			
		Sucrose (g l^{-1})			
		0	10	20	20–0[y]
0	0	1.22±0.05	1.21±0.06	1.24±0.03	
	10	0.97±0.06	1.09±0.09	1.06±0.05	
	20	0.72±0.07	0.85±0.10	0.76±0.10	0.64±0.05[x]
	30	0.43±0.03	0.50±0.07	0.38±0.06	0.42±0.06
	40	0.27±0.04 a	0.38±0.05 a	0.29±0.04 a	0.23±0.03 a
		78%	69%	77%	64%[w]
300	0	1.23±0.03	1.24±0.04	1.23±0.04	
	10	1.05±0.05	1.02±0.07	0.97±0.07	
	20	0.71±0.09	0.67±0.05	0.55±0.05	0.65±0.06[x]
	30	0.49±0.07	0.46±0.06	0.28±0.07	0.23±0.06
	40	0.29±0.04 a	0.31±0.06 a	0.14±0.06 b	0.11±0.05 b
		76%	75%	89%	83%[w]
600	0	ND	1.23±0.04	1.25±0.04	
	10	ND	1.04±0.09	0.95±0.06	
	20	ND	0.63±0.05	0.44±0.12	0.66±0.05[x]
	30	ND	0.42±0.07	0.24±0.07	0.18±0.07
	40	ND	0.29±0.05 a	0.13±0.03 b	0.11±0.07 b
			76%	90%	83%[w]
2,000	0	ND	1.24±0.04	1.24±0.04	
	10	ND	1.06±0.09	0.97±0.05	
	20	ND	0.63±0.07	0.48±0.06	0.63±0.07[x]
	30	ND	0.47±0.08	0.29±0.05	0.24±0.05
	40	ND	0.36±0.05 a	0.20±0.02 ab	0.16±0.04 ab
			71%	84%	74%[w]

ND not determined

[z] Each value represents the mean ± standard error of nine data. Common *letters* within a *column* are not significantly different at $p \leq 0.05$ (Mann–Whitney test)

[y] Same as in Table 4

[x] Ion concentration of the new fresh medium (one half MS)

[w] Percentage of absorption from day 20 after the medium was changed

A minor decrease in pH of the medium without sucrose, concomitant with a reduction in plant growth, was possibly related to a lesser uptake of nutrients. However, no differences could be observed between the plants grown under ventilation at 300 or 600 µl l^{-1} CO_2 and 10 or 20 g l^{-1} of sucrose. The lowest pH was recorded in media flushed with 2,000 µl l^{-1} CO_2. Once stabilized, the pH of the medium was below the values that allow free availability of nutrients. It is widely accepted that the availability of Fe^{2+}, Mn^{2+}, Ca^{2+}, Mg^{2+}, and PO_4^{3-} is reduced at pH<7.0, and the availability of most of the nutrients is reduced at pH<5.0 (Williams 1993). Although the initial pH of the culture medium becomes lower over the culture period than the values considered optimal for ion absorption, the presence of EDTA increases ion solubility and renders their availability for the plants. Accordingly, those that show greater growth, E_{20}^{600} and E_{20-0}^{600}, have higher absorption (Arigita et al. 2002).

Studies carried out with plants grown in soil, sand, or hydroponics show that the pH of the medium is closely related to the amount of NH_4^+ and NO_3^- absorbed by the plants (Leifert et al. 1991), and there are differences in this aspect between different plant species (Leifert et al. 1992). The absorption of nitrogenous compounds occurs throughout the culture; initially NH_4^+ is preferred to NO_3^- as has been observed in other species (Mezzetti et al. 1991; Hdider et al. 1994; Moncaleán et al. 1999). This preferential absorption of NH_4^+ is justified by the lower energy cost required in ammonium assimilation (5 ATP per mole ammonium) than in nitrate reduction (15 ATP per mole nitrate; Moncaleán et al. 2003).

NH_4^+ is passively absorbed and incorporated into the cell metabolism through glutamine synthetase. Subsequently, an ATPase pumps H^+ out of the cells, thus decreasing the pH of the culture medium (Salsac et al. 1987). Acidification of the culture medium promoted NO_3^- uptake (George and Sherrington 1984), increasing after 20 d, while at the same time reducing NH_4^+ uptake. Early NO_3^- absorption will be useless, because it will not be readily reduced by poor photosynthetic machinery. We observed that ventilation favored the absorption of both forms of nitrogen concomitant with the highest plant growth. Similar results were also reported in strawberry (Kozai et al. 1991) and tobacco (Geiger et al. 1999), where there was an increment in NO_3^-

absorption with increasing autotrophy of the plants. Although MS medium is rich in nitrogen sources, the amount of NO_3^- can be a limiting factor for plant growth under the new culture conditions (ventilation at 300 or 600 μl l^{-1} CO_2 and 20 g l^{-1} sucrose or elimination of sucrose after 20 d of culture) if the plants are cultured for longer periods.

Another ion that is influenced by autotrophy is Mg^{2+}, which is required during the formation of chloroplast, as a component of chlorophylls (Mezzetti et al. 1991). In the present study, high Mg^{2+} absorption was found to be associated with foliar expansion and development of photosynthetic structures, in autotrophic ventilated plants (Arigita et al. 2002). Ca^{2+} absorption is related to transpiration (Williams 1993; Sarkar et al. 2005). It is known that a lack of root system and low matric potential of the culture medium induce apical necrosis, as the transpiration stream cannot support adequate Ca^{2+} uptake (McCown and Sellmer 1987). Under ventilated culture conditions, mass flow supplies enough Ca^{2+} for plant development and, therefore, no necrotic symptoms were observed.

There were no very big differences in K^+ absorption at the assayed conditions except when explants were transferred to sugar-free medium after 20 d (E_{20-0}). In these microplants, some kind of antagonism can be observed between K^+ absorption and NH_4^+ resulting from NH_4^+ charge accumulation inside the cells (Morard et al. 1999).

About 80–90% of the initial PO_4^{3-} was absorbed under all assayed conditions, and this is in agreement with the results obtained in other studies (Leifert et al. 1991; Barbas et al. 1993; Morard et al. 1998; Moncaleán et al. 2003). The highest PO_4^{3-} absorption was observed in ventilated systems (autotrophic), in which it is required for the formation of ATP and NADP in photosynthesis (Barbas et al. 1993). Furthermore, at the end of the culture, the PO_4^{3-} becomes insoluble at a low pH (<5.0) of the medium, and thus remains unavailable to the plants. Although involved in maintaining the ionic balance (Dussert et al. 1995), Cl^- did not appear to play this role in the present study, and most of the initially added Cl^- remained unabsorbed at the end of the culture. Similar results were also observed in other studies (Cozza et al. 1997; Sallanon et al. 1997). We could not observe any differences either in the rate of SO_4^{2-} absorption between different culture conditions assayed. Since more than 60% of the initially added SO_4^{2-} remained in the culture medium after 40 d of culture, it can be assumed that its availability was enough to foster optimum plant growth.

In conclusion, the current study showed that an improvement in the growth of kiwi plants occurred when they were cultured under ventilation with 600 μl l^{-1} CO_2 and becoming wholly autotrophic after elimination of sucrose from the culture medium at day 20. The plant growth was most vigorous under these conditions, which also promoted maximum absorption of Mg^{2+}, NO_3^-, and PO_4^{3-}. To extend the culture period to use microhedging in an automated system, more of these most absorbed ions are required to be added while replenishing the medium to foster optimal plant growth and development over protracted culture periods.

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