

Sugar metabolism, photosynthesis, and growth of *in vitro* plantlets of *Doritaenopsis* under controlled microenvironmental conditions

Kong-Sik Shin · So-Young Park · Kee-Yoeup Paek

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Abstract Suboptimal environmental conditions inside closed culture vessels can be detrimental to *in vitro* growth and survival of plantlets during the acclimatization process. In this study, the environmental factors that affected *Doritaenopsis* plantlet growth and the relationship between growth and sugar metabolism were investigated. Cultures were maintained under heterotrophic, photoautotrophic, or photomixotrophic conditions under different light intensities and CO₂ concentrations. Photoautotrophic growth of *Doritaenopsis* hybrid plantlets could be promoted significantly by increasing the light intensity and CO₂ concentration in the culture vessel. The concentration of different sugars in the leaves of *in vitro*-grown plantlets varied with different cultural treatments through a 10-wk culture period. Starch, reducing sugars, and nonreducing sugar contents were higher in plantlets grown under photoautotrophic and photomixotrophic conditions than in heterotrophically grown plantlets. Net photosynthesis rates were also higher in photoautotrophically and photomixotrophically grown plantlets. These results support the hypothesis that pyruvate, produced by the decarboxylation of malate, is required for optimal photoautotrophy under high photosynthetic photon flux density. Growth was greatest in plantlets grown under CO₂-enriched photoautotrophic and photomixotrophic

conditions with high photosynthetic photon flux density. The physiological status of *in vitro*-grown Crassulacean acid metabolism (CAM)-type *Doritaenopsis* showed a transition from C3 to CAM prior to acclimatization.

Keywords *Doritaenopsis* · Photoautotrophic tissue culture · Sugar metabolism · Photosynthesis

Introduction

Micropropagation is routinely used for rapid multiplication of stock plant material to produce a large number of genetically and physiologically uniform plants. However, the low survival rate of regenerated plants can limit large-scale micropropagation of some plant species (Hazarika 2006). The culture vessel environment is characterized by high relative humidity, poor gas exchange between the culture vessel and the microclimate chamber, low CO₂ concentration, low photosynthetic photon flux density (PPFD), high sugar concentration in the culture medium, and high ethylene. These conditions may affect tissue vigor and shoot multiplication and can impede the successful transfer of micropropagated plants to *ex vitro* conditions (Kozai *et al.* 1997; Kozai *et al.* 2005; Park *et al.* 2011). Two of the most common problems with *in vitro* propagation are that the leaves of micropropagated plants may be chlorophyll deficient and contain more water than *ex vitro*-grown leaves, a condition termed “hyperhydricity.” Hyperhydricity shows altered leaf anatomy and can generate aeration stress that depletes oxygen levels in the cells. Several types of abnormal structures have been observed in *in vitro*-grown leaves, such as a reduced number of palisade cell layers, irregular stomata, chloroplast degeneration, and the cuticle is either very thin or absent (Chakrabarty *et al.* 2006; Dewir *et al.* 2006; Ivanova and Van Staden 2010).

K.-S. Shin
Biosafety Division, National Institute of Agricultural
Biotechnology, RDA, Suwon 441-707, Republic of Korea

S.-Y. Park (✉) · K.-Y. Paek
Department of Horticultural Science, Chungbuk National
University, Cheongju 361-763, Republic of Korea
e-mail: soypark7@chungbuk.ac.kr

S.-Y. Park · K.-Y. Paek
Research Center for the Development of Advanced Horticultural
Technology, Chungbuk National University, Cheongju 361-763,
Republic of Korea

Light intensity and quality are important factors for plant growth and development *in vitro* (Macedo *et al.* 2011). Low light conditions inside culture vessels are comparable to shade conditions. Shade-acclimated leaves undergo physiological changes that enable efficient use of limited light resources, but they are predisposed to photodamage by sudden exposure to high light conditions. This scenario can occur when culture-adapted tissues are transferred to soil under greenhouse conditions. The result of this damage is reduced net CO₂ assimilation after transfer to high light conditions, possibly for the entire life of the leaf (Van Huylbroeck 1994; Van Huylbroeck *et al.* 2000).

Conventional *in vitro*-grown heterotrophic plants have a low photosynthetic rate, and require the addition of sugar to the culture medium to serve as a carbon source. This affects their acclimatization during conversion of the plant tissue from heterotrophic to autotrophic growth. The low photosynthetic rate of plants *in vitro* is not due to low photosynthetic efficiency but results from the low CO₂ concentration in culture vessels (Park *et al.* 2011; Xiao *et al.* 2011).

Many microclimate environmental factors can be controlled to enhance micropropagation efficiency. Since photoautotrophic micropropagation was developed more than two decades ago, numerous studies have been conducted to investigate the effects of the culture vessel microenvironment on plant growth and development and to devise strategies for improving these conditions (Zobayed *et al.* 2004; Yoon *et al.* 2009; Kozai 2010). Plant transpiration and nutrient uptake are critically important for a successful photoautotrophic micropropagation. Although physiological changes in some photoautotrophic plants grown *in vitro* have been studied (Norikane *et al.* 2010; Badr *et al.* 2011), there are few reports on plant growth and physiological changes during micropropagation of orchid species.

The purpose of this study was to investigate environmental factors during *in vitro* culture of *Doritaenopsis*, an

important ornamental orchid in the floriculture industry. The effects of light intensity and CO₂ enrichment on the growth of *Doritaenopsis* plantlets were explored for possible correlations with sugar metabolism. In addition, the physiological parameters of different culture systems that are currently used for *Doritaenopsis* were studied to understand their relationships to growth.

Materials and Methods

Plant material and culture medium. Seeds of *Doritaenopsis* ‘New Candy’ and *Doritaenopsis* ‘Mary Anes×Ever Spring’ hybrids were germinated on Hyponex medium (Kano 1965; 6.5 N–4.5 P–19 K at 1 g L⁻¹ and 20 N–20 P–20 K at 1 g L⁻¹) with 0.5 g L⁻¹ activated charcoal (Sigma, St. Louis, MO) and 7 g L⁻¹ phytoagar (Duchefa, Haarlem, Netherlands). After 4 mo, five seedlings (about 1.5 cm in shoot length and 0.2 g fresh weight) were cultured in each of several 250 ml Erlenmeyer flasks. For all treatments, at least five flasks were used for each treatment within a replicate, with three replicates. All cultures were maintained for 10 wk in a growth chamber (Dasol Scientific Co. Ltd., Gyeonggi, Republic of Korea) at 24±1°C with 16-h light/8-h dark photoperiod. After 10 wk of culture, leaf area, leaf length, root length, and number of roots were recorded from each plantlet grown under different environmental conditions.

Light intensity, sucrose, and CO₂ concentration. Three different growth conditions (heterotrophic, photomixotrophic, and photoautotrophic) were applied by varying sucrose, light, and CO₂ (Table 1). For heterotrophic cultures, 30 g L⁻¹ sucrose was added to the basal Hyponex medium, and the culture vessels were covered with sterile aluminum foil and sealed with a wrap to block air exchange. The

Table 1. Description of the treatments used in this study

	Treatment code	CO ₂ concentration outside the vessel (μmol mol ⁻¹)	PPFD (μmol m ⁻² s ⁻¹)	Sucrose (g L ⁻¹)	Gas exchange (times/h)
	Photoautotrophic				
	AL	350±20 ^z	40±2	–	2.83
	AH	350±20	120±5	–	2.83
	ALC	1,500±100	40±2	–	2.83
	AHC	1,500±100	120±5	–	2.83
	Photomixotrophic				
	ML	350±20	40±2	30	2.83
	MH	350±20	120±5	30	2.83
	MLC	1,500±100	40±2	30	2.83
	MHC	1,500±100	120±5	30	2.83
	Heterotrophic ^y				
	HL	350±20	40±2	30	0.25
	HH	350±20	120±5	30	0.25

PPFD photosynthetic photon flux density, A autotrophic, M mixotrophic, H heterotrophic, L low light, H high light, C CO₂ enrichment

^zValues reported as ±standard error

^yConventional culture (sealed vessel and a medium with sucrose) is used as control

conditions for heterotrophic growth included low heterotrophic conditions ($40 \pm 2 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPF and $350 \pm 20 \mu\text{mol mol}^{-1} \text{CO}_2$) and high heterotrophic conditions ($120 \pm 5 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPF and $350 \pm 20 \mu\text{mol mol}^{-1} \text{CO}_2$).

For photomixotrophic cultures, 30 g L^{-1} sucrose was added to the basal Hyponex medium which was used for seed germination and the culture vessel (250 ml Erlenmeyer flask) was sealed as before, except a small hole (1 cm diameter) was cut in the top and covered with a membrane filter (0.2 μm pore size; Micropore™, Seoul, Republic of Korea) to allow gas exchange to occur. The rate of gas exchange was calculated as the hourly ventilation rate divided by the air volume of the culture vessel according to Zobayed *et al.* (2000). The gas exchange for the photomixotrophic experiments was calculated to be 2.83 times/h. The conditions for photomixotrophic growth included low photomixotrophic conditions ($40 \pm 2 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPF and $350 \pm 20 \mu\text{mol mol}^{-1} \text{CO}_2$), high photomixotrophic conditions ($120 \pm 5 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPF and $350 \pm 20 \mu\text{mol mol}^{-1} \text{CO}_2$), low photomixotrophic conditions enriched with CO_2 ($40 \pm 2 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPF and $1,500 \pm 100 \mu\text{mol mol}^{-1} \text{CO}_2$), and high photomixotrophic conditions enriched with CO_2 ($120 \pm 5 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPF and $1,500 \pm 100 \mu\text{mol mol}^{-1} \text{CO}_2$).

For photoautotrophic cultures, no sugar was added to the basal Hyponex medium. The culture vessels were closed with aluminum foil containing a small hole that was covered with a membrane filter (1 cm diameter), the same as for the photomixotrophic cultures. The conditions for photoautotrophic growth included low photoautotrophic conditions ($40 \pm 2 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPF and $350 \pm 20 \mu\text{mol mol}^{-1} \text{CO}_2$), high photoautotrophic conditions ($120 \pm 5 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPF and $350 \pm 20 \mu\text{mol mol}^{-1} \text{CO}_2$), low photoautotrophic conditions enriched with CO_2 ($40 \pm 2 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPF and $1,500 \pm 100 \mu\text{mol mol}^{-1} \text{CO}_2$), and high photoautotrophic conditions enriched with CO_2 ($120 \pm 5 \mu\text{mol m}^{-2} \text{s}^{-1}$ PPF and $350 \pm 20 \mu\text{mol mol}^{-1} \text{CO}_2$).

CO_2 concentrations in the culture chamber were monitored and controlled using an infrared-type CO_2 controller (Dasol Scientific Co. Ltd., Republic of Korea). Liquid CO_2 was supplied by Praxair Korea Co. Ltd. (Seoul, Republic of Korea). Metal halide and fluorescent lamps were used as light sources.

Acclimatization. Following the 10-wk culture period, plantlets were transplanted to round pots ($9 \times 10 \text{ cm}$) that contained pine tree bark (average particle size, $>5 \text{ mm}$) as culture soil. During the acclimatization period, the PPF in the day was adjusted to $100 \pm 20 \mu\text{mol m}^{-2} \text{s}^{-1}$, and the day/night temperature was $25 \pm 2/22 \pm 2^\circ\text{C}$.

Chlorophyll fluorescence. Plantlets were kept in the dark for 30 min before chlorophyll measurement. The chlorophyll fluorescence of the abaxial side of fresh leaves was measured

(Chakrabarty *et al.* 2006). Modulated fluorescence was measured using a PAM-200 chlorophyll fluorometer fitted with a DA-200 leaf clip holder (Heinz Walz, Effeltrich, Germany). Data were analyzed by a software provided by the manufacturer. Minimal fluorescence (F_0) was measured in dark-adapted leaves using incident light of $<0.1 \mu\text{mol m}^{-2} \text{s}^{-1}$; maximal fluorescence (F_m) was measured after a 1-s saturating pulse ($>3,500 \mu\text{mol m}^{-2} \text{s}^{-1}$) in the same leaves. Maximal variable fluorescence ($F_v = F_m - F_0$) and photochemical efficiency of photosystem II (PSII) (F_v/F_m) were calculated for dark-adapted leaves.

Net photosynthetic rate. The amount of CO_2 absorbed by *in vitro*-grown plantlets was calculated by measuring the CO_2 concentration in the culture container halfway through the light and dark photoperiods. The concentration of CO_2 was measured by using a gas chromatograph (HP 6890) equipped with a $25 \text{ m} \times 0.53 \text{ mm}$ PoraPlot Q capillary column and a thermal conductivity detector (Hewlett Packard, Wilmington, DE). Helium was used as carrier gas and set to a flow speed of 13 mL min^{-1} . Other parameters included an oven temperature of 90°C and an injector temperature of 200°C . The net photosynthetic rate (NPR) of plantlets was calculated according to Kozai and Iwanami (1988).

Malic acid and citric acid contents. Malic acid and citric acid were analyzed using high-performance liquid chromatography (HPLC). One gram of leaf tissue was finely ground, 2 ml of distilled water was added, soluble materials were extracted for 30 min at 60°C , and then the solution was filtered through a $0.2 \mu\text{m}$ membrane filter. The organic acids in the filtrate were measured using HPLC (Waters 600S controller, Waters 626 pump, Waters Co., Milford, MA) (Gomis *et al.* 1992) with a Nova-Pack C18 column ($3.9 \times 300 \text{ mm}$, Waters Co.), with a mobile phase of $0.2 \text{ M KH}_2\text{PO}_4/\text{H}_3\text{PO}_4$ (pH 2.4) at a flow rate of 0.5 mL min^{-1} , and measurements were performed using ultraviolet light at 215 nm. Totally the tests were repeated three times for each experimental group. Data was expressed with mean and standard errors in the figures to show statistical significance between the treatments.

Carbohydrate extraction and analysis. Leaves were frozen in liquid nitrogen, transferred to mortars containing liquid nitrogen and ground with a pestle. The liquid nitrogen was allowed to evaporate and the ground tissues were extracted in 3 volumes of 80% ethanol to separate the ethanol-soluble fraction (containing sugars) and the ethanol-insoluble fraction (containing starch). The ethanol-soluble supernatant was removed after centrifugation for 15 min at $8,000 \times g$. The supernatant was passed sequentially through a C₁₈ Sep-Pak cartridge (Waters, Milford, MA) and a $0.2 \mu\text{m}$ membrane filter. Samples were injected into a Waters HPLC

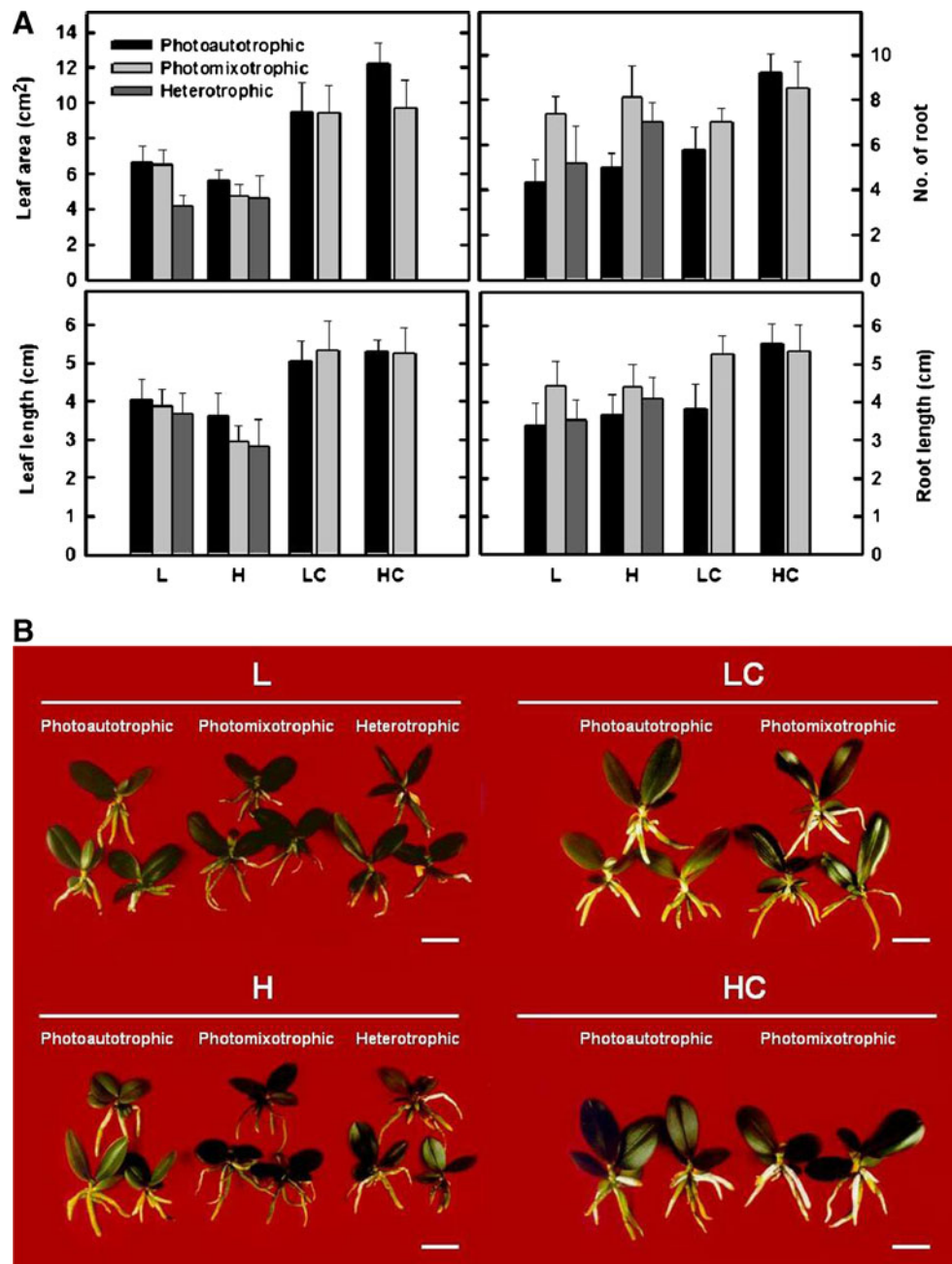
(Waters 626 pump and 600 s controller) using a Rheodyne 7725I injector equipped with a 20 μl sample loop. The isocratic system was operated at 1 mL min^{-1} using degassed 80% acetonitrile as the mobile phase. Soluble sugar separation was achieved using a carbohydrate WATO44355 ($250 \times 4:6 \text{ mm}^2$) stainless steel column (Waters). Soluble sugars were measured using a refractive index detector (Waters 410) (Ding *et al.* 1998). For starch determination, the precipitate was washed three times with 2–3 volumes of 40% ethanol to remove soluble sugar residues. Starch was solubilized using HCl–DMSO pretreatment (Lambrechts *et al.* 1994) and hydrolyzed by incubation with amyloglucosidase (50 units mL^{-1} in 0.1 M Na-acetate buffer pH 4.2) for 48 h

at 55°C, with occasional agitation. Starch was then determined as glucose using the dinitrosalicylic acid assay (Chaplin 1986).

Results and Discussion

Growth and morphological features of plantlets. For plantlets grown under CO_2 -enriched photoautotrophic and photomixotrophic conditions, the leaf area, leaf length, and root length were greater than for plantlets grown in heterotrophic conditions (Fig. 1A, B). In general, all growth parameters were highest in plantlets grown under CO_2 -

Figure 1. Leaf area, leaf length, root length, and number of roots per plantlet (A) and growth of *Doritaenopsis* hybrids cultured for 10 wk under different culture conditions. Plantlets were cultured under photoautotrophic (0% sucrose), photomixotrophic (3% sucrose), and heterotrophic (3% sucrose) conditions. Scale bar = 2 cm. *L* low PPF ($40 \pm 2 \mu\text{mol m}^{-2} \text{s}^{-1}$), *H* high PPF ($120 \pm 5 \mu\text{mol m}^{-2} \text{s}^{-1}$), *LC* low PPF + CO_2 enrichment ($1,500 \pm 100 \mu\text{mol mol}^{-1}$), *HC* high PPF + CO_2 enrichment ($1,500 \pm 100 \mu\text{mol mol}^{-1}$).



enriched photoautotrophic and photomixotrophic conditions with high PPFD. The leaves were smaller in L and H treatments (no CO₂ enrichment) compared with the leaves from LC and HC treatments (with CO₂ enrichment). Plantlets grown on medium containing sugar showed more and longer roots than those from the treatments that had no sugar. Statistically, the greatest root growth was observed in HC conditions for both photoautotrophic and photomixotrophic treatments.

The heterotrophic cultures, where no gas exchange occurred between the inside and outside of the culture vessel, showed the poorest growth and leaf area decreased to approximately 56% that of the plantlets grown in photoautotrophic and photomixotrophic conditions. Conventional micropropagation techniques are typically performed using small culture vessels under low PPFD conditions without ventilation (Xiao *et al.* 2011). Many studies indicate that photoautotrophic growth in cultured plantlets can be significantly promoted by increasing the CO₂ concentration and the light intensity, and by removing growth retardant gas such as

ethylene from the vessel (Lian *et al.* 2002; Yoon *et al.* 2009; Xiao *et al.* 2011).

Physiological and biochemical features of tissue samples. Measurement of the photosynthetic efficiency with chlorophyll fluorescence. Chlorophyll fluorescence was used to determine the photosynthetic efficiency. F_o , which is an indicator of the antenna size (number of pigment molecules) of PSII, was lower under heterotrophic treatment conditions after 10 wk of culture. The F_v/F_m ratio, which provides an estimate of the basal efficiency of the PSII photochemical process, was also lower under heterotrophic treatment conditions. The AHC and MHC cultures maintained values of 0.75–0.8 F_v/F_m , and showed the highest photosynthetic efficiency and the highest amount of photosynthesis. However, for the treatments exposed to high light intensity but without CO₂ enrichment, the value of F_v/F_m was lower (Fig. 2). The tissues subjected to high light intensity in the heterotrophic condition showed a significant decrease of the early value of F_v/F_m . Chakrabarty *et al.* (2006) studied hyperhydricity in apple and suggested that the

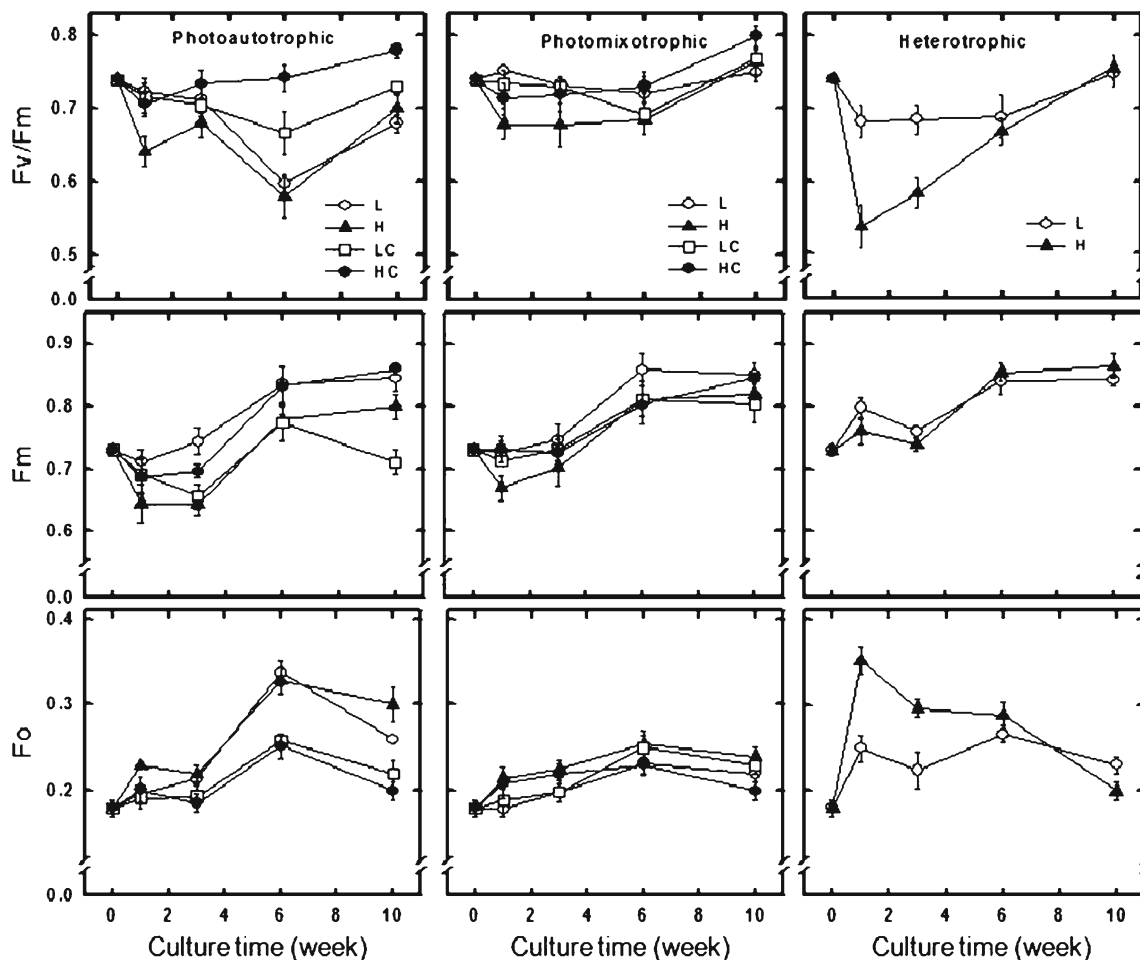


Figure 2. Fluorescence parameters (F_v/F_m , F_o , and F_m) during *in vitro* culture of *Doritaenopsis* hybrid plantlets. Vertical bars represent mean \pm standard error of plantlets from three replicate vessels.

substantial collapse of photosynthesis that occurred in heterotrophic treatments largely resulted from a severe disruption of the photosystem (or at least of PSII), and was probably not the result of any major effects on other aspects of the photosynthesis.

Net photosynthetic rate. The photosynthetic rate was affected by different culture conditions. For plantlets grown in the photoautotrophic and photomixotrophic treatments, depending on the time within the photoperiod, CO₂ absorption decreased during the light cycle and CO₂ absorption increased during the dark cycle (Fig. 3). The plant materials that were cultured in CO₂-enriched conditions (such as AHC and MHC) showed a high CO₂ absorption throughout the culture compared to the other treatments, and CO₂ absorption was increased in plantlets grown in photoautotrophic treatments, especially at Week 8 (Fig. 3). Plantlets grown under strong light with CO₂-enriched condition (HC) seemed to exhibit the Crassulacean acid metabolism (CAM)-type photosynthetic pattern. *Doritaenopsis* is CAM type plant in nature. In general, CAM plants photosynthesize by absorbing CO₂ during the night (Osmond 1978). However, activation of CAM or C3 metabolism depends on the microenvironment, so *in vitro*-grown plantlets can show C3-type photosynthesis patterns under certain conditions (Malda *et al.* 1999) and transition to a CAM-type photosynthetic pattern upon exposure to bright light and CO₂ supplementation.

Photoautotrophic micropropagation is defined as micropropagation without sugar in the culture medium, where carbohydrate accumulation of tissue cultures and subsequent growth is dependent fully on photosynthesis and inorganic nutrient uptake (Zobayed *et al.* 2004; Arigita *et al.* 2010; Kozai 2010). Thus, it also can be called

photosynthetic micropropagation in sugar-free medium (Xiao *et al.* 2011).

In AHC and MHC treatments, malic acid and citric acid content remained high after 6 wk culture (Fig. 4). An increased CO₂ concentration increased the rate of malic acid synthesis and its concentration in the cytoplasm (Zobayed *et al.* 2000). Our result supports the hypothesis that pyruvate, produced by the decarboxylation of malate, is required for optimal photoautotrophy under high PPFD. Apparently, the pyruvate produced by other metabolic pathways is insufficient to support optimal growth under high PPFD conditions. During heterotrophic (and presumably photomixotrophic) growth, the principal mode of glucose catabolism is *via* the pentose phosphate pathway. Malate was the dominant fixation product in photoautotrophic cultures of soybean grown under elevated CO₂ concentrations (Horn and Widholm 1994).

Changes in carbohydrate content during culture period. The sugar concentration in leaves of *in vitro*-grown plantlets through 10-wk culture varied with treatments (Figs. 5 and 6). Starch, reducing sugar, and non-reducing sugar contents were higher in plantlets grown under AHC and MHC conditions, with increases after 6 wk culture. Starch, reducing soluble sugar, nonreducing sugar, and total sugar content were higher in photoautotrophic and photomixotrophic conditions than in the leaves and roots of plantlets grown under heterotrophic conditions (Fig. 6). These variations were obvious because NPR rates were very high in photoautotrophically and photomixotrophically grown plants, as previously reported (Lian *et al.* 2002).

A very good correlation was observed between starch content and high light and CO₂ (AHC and MHC) culture conditions. The photomixotrophic and photoautotrophic groups under high light intensity showed that the starch

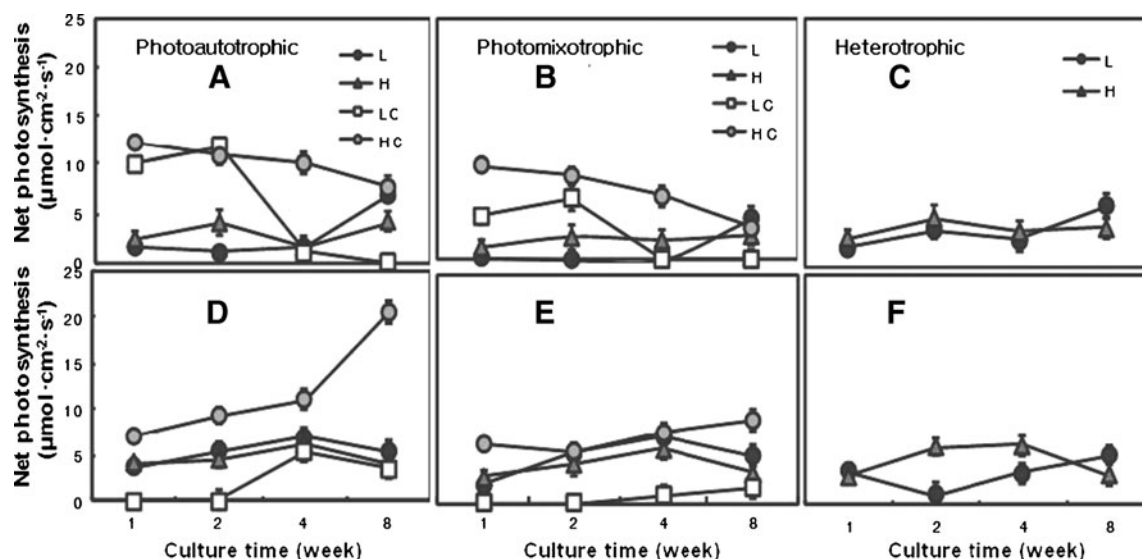


Figure 3. Changes in CO₂ uptake during light (A–C) and dark (D–F) periods in *Doritaenopsis* hybrid plantlets during *in vitro* culture. Vertical bars represent mean ± standard error of plantlets from three replicate vessels.

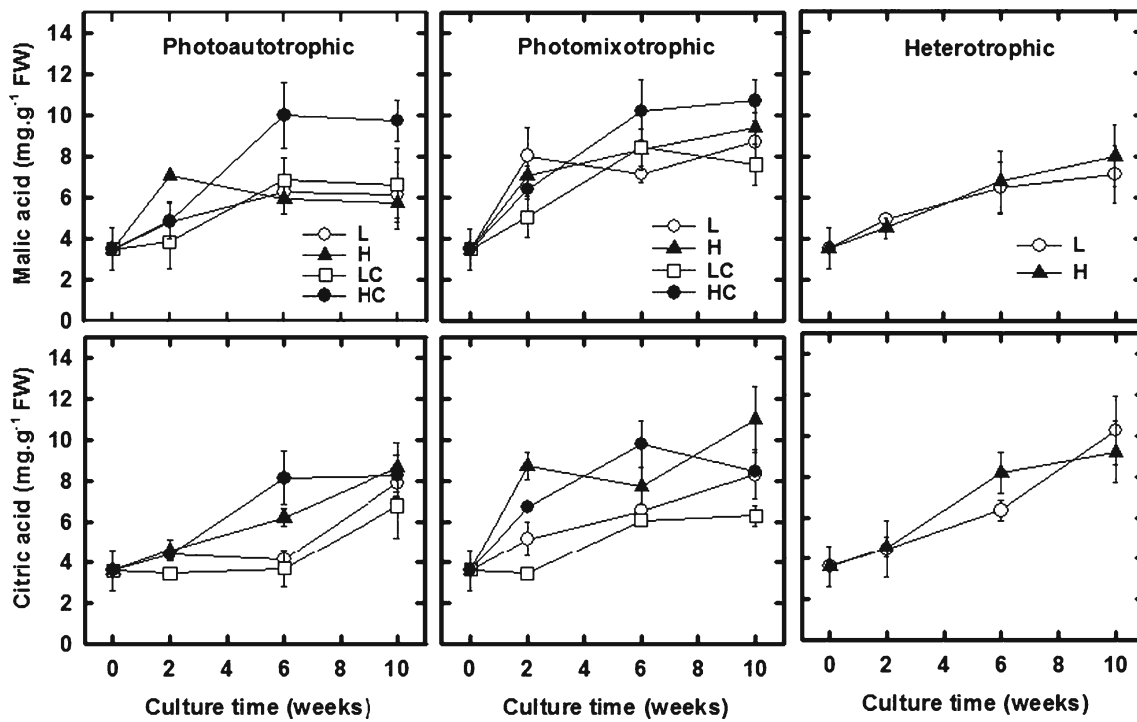


Figure 4. Changes in malic acid and citric acid contents in leaves of *Doritaenopsis* hybrid plantlets during the *in vitro* culture period. Vertical bars represent mean ± standard error of plantlets from three replicate vessels.

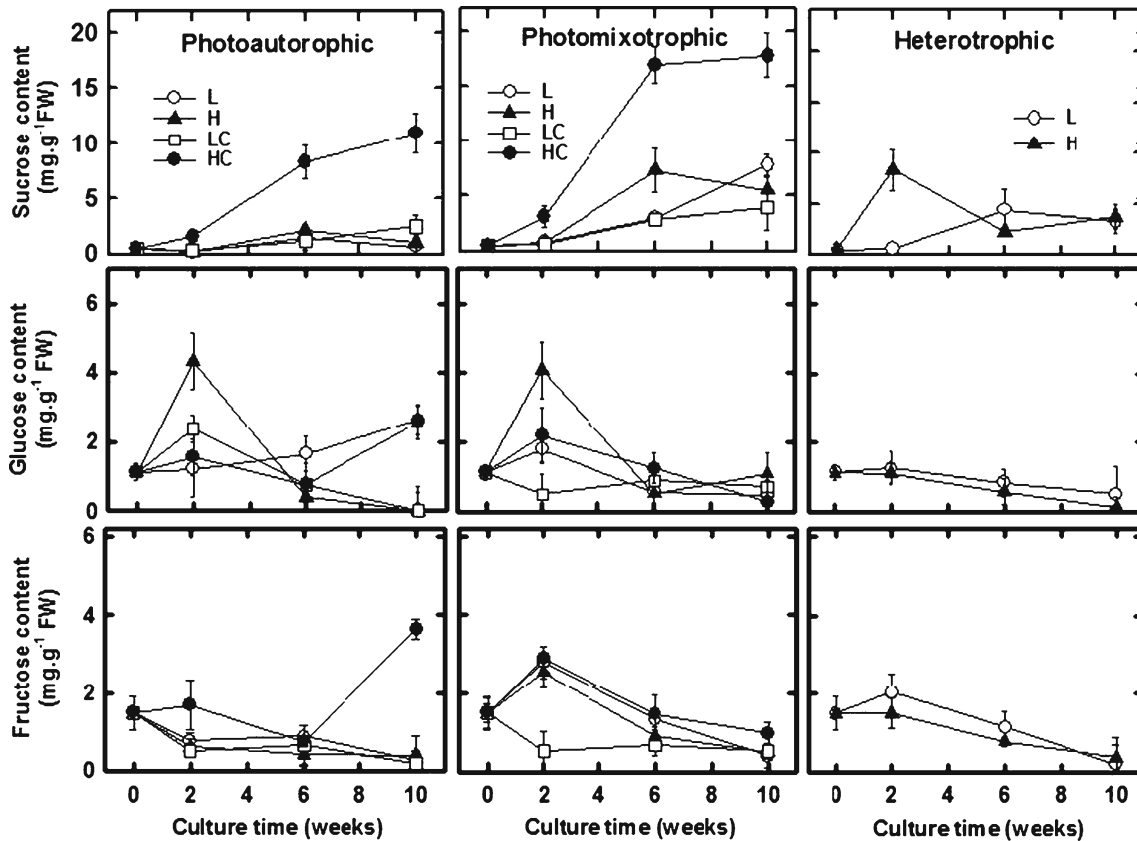


Figure 5. Changes in sucrose, glucose, and fructose contents in leaves of *Doritaenopsis* hybrid plantlets under photoautotrophic, photomixotrophic, and heterotrophic conditions over 10 wk culture. Vertical bars represent mean ± standard error of plantlets from three replicate vessels.

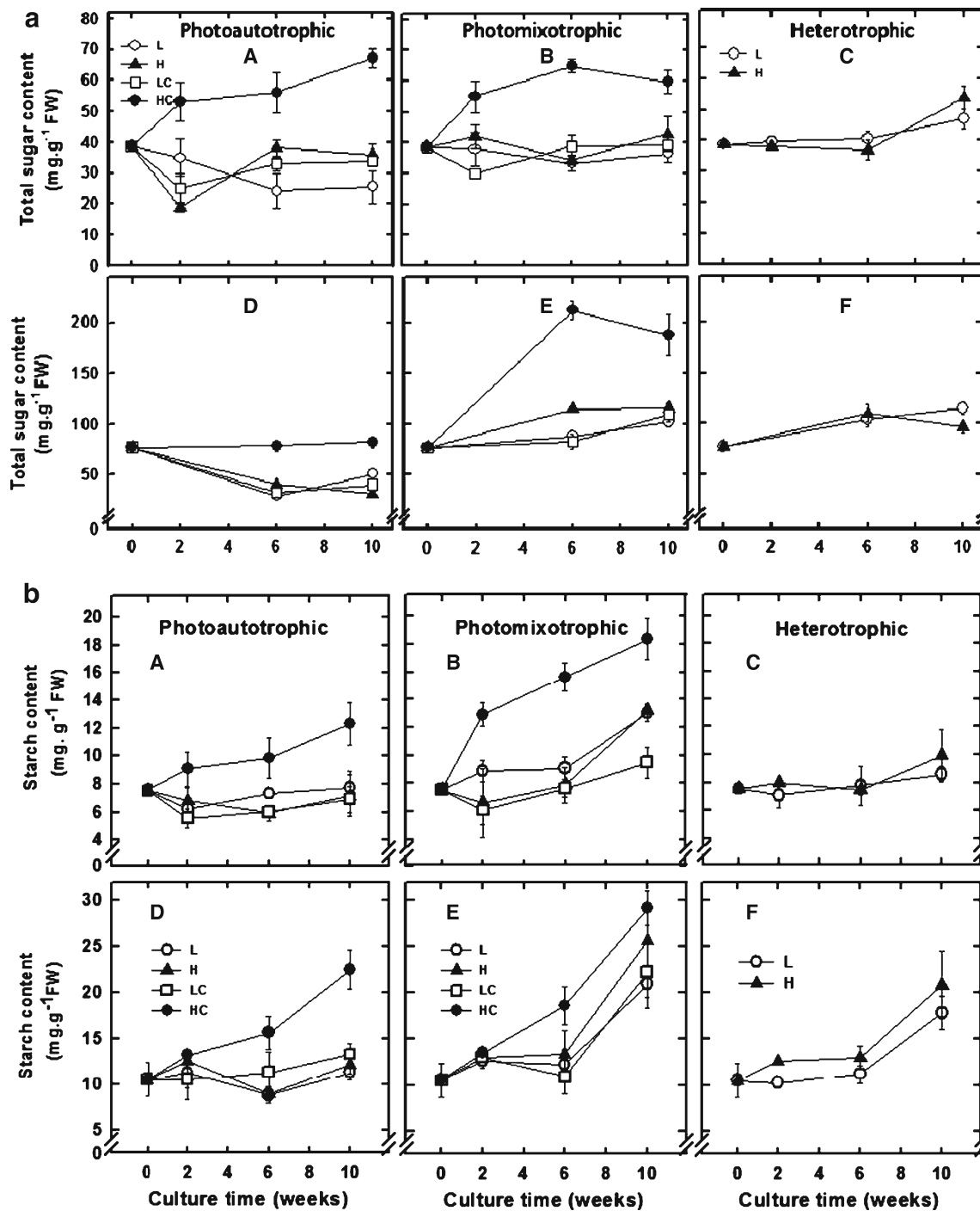


Figure 6. Changes in total sugar (a) and starch (b) of leaves (A–C) and roots (D–F) of *Doritaenopsis* hybrid plantlets under photoautotrophic, photomixotrophic, and heterotrophic conditions over 10 wk

culture. Vertical bars represent mean ± standard error of plantlets from three replicate vessels.

contents increased after net photosynthesis was increased during the night (Figs. 2 and 6). Photosynthetically active cells and tissues *in vitro* have two major carboxylation pathways for CO₂ fixation. The first is autotrophic fixation of CO₂ mediated by Rubisco; the second is non-autotrophic carbon fixation mediated by the enzyme phosphoenolpyruvate (PEP) carboxylase, which catalyses the irreversible β-

carboxylation of PEP by HCO₃⁻ (Bender 1987; Kumar *et al.* 1988). Under low light, CO₂ enrichment caused the highest activity of PEP carboxylase in the photoautotrophic and photomixotrophic plantlets (data not shown). A similar observation was reported for *Platycerium coronarium* callus culture (Kwa *et al.* 1997). The nonphotosynthetic fixation of CO₂ through PEP carboxylase has been previously observed

in shoot cultures of eastern white cedar (Nour and Thorpe 1994).

The accumulation of sugars may be attributed to the specific activation of sucrose synthesis by up-regulation of sucrose phosphate synthase (SPS) in the MHC treatment group. Sucrose synthesis in higher plants occurs through the action of SPS and sucrose synthase (SS). SPS activity is influenced by different culture conditions, whereas SS activity increases with increased metabolic carbohydrate demand. The activity of SPS was enhanced by CO₂, which accelerates the biosynthesis of sucrose (Signora *et al.* 1998). The *in vitro*-grown plantlets of *Doritaenopsis* showed accumulation of starch and an increase in sucrose particularly in the AHC and MHC (CO₂ enriched) treatments. However, monosaccharide content decreased over time. The trend for sucrose content increase correlated with the length of the cultivation period, whereas glucose and fructose contents decreased gradually during culture.

Plantlets grown under various conditions were planted in the pots and acclimatized for 10 wk. Most plants were successfully acclimatized, however the plantlets grown in AHC and MHC showed the most vigorous and rapid growth in greenhouse (data not shown)

In conclusion, the results of the studies on growth parameters, photosynthesis, and carbohydrate analyses suggest that photoautotrophic growth *in vitro* could be promoted significantly by increasing the CO₂ concentration and light intensity in culture vessels. Plant tissues that were cultured in CO₂-enriched conditions, such as AHC and MHC showed higher CO₂ absorption throughout the culture period compared with other treatments, and CO₂ absorption was remarkably high in the photoautotrophic treatments. The findings of the present study will be helpful to enhance acclimatization of *Doritaenopsis* plantlets to *ex vitro* conditions.

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