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Impact of *in vitro* CO_2 enrichment and sugar deprivation on acclimatory responses of *Phalaenopsis* plantlets to *ex vitro* conditions

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ABSTRACT

We assessed the effect of growth at either 400 μmol mol^{-1} (ambient) or 1000 μmol mol^{-1} (elevated) CO_2 and 0 g L^{-1} (deprivation) or 30 g L^{-1} (supplementation) sugar on morphological traits, photosynthetic attributes and intrinsic elements of the CAM pathway using the CAM orchid *Phalaenopsis* 'Amaglade'. The growth of shoot (retarded) and root (induced) was differently affected by CO_2 enrichment and mixotrophic regime (+sugar). The Fv/Fm ratio was 14% more in CO_2-enriched treatment than at ambient level during *in vitro* growth. At elevated level of CO_2 and sugar treatment, the content of Chl(*a* + *b*), Chl *a*/*b* and Chl/Car was enhanced while carotenoid content remained unaltered. During *in vitro* growth, gas-exchange analysis indicated that increased uptake of CO_2 accorded with the increased rate of transpiration and unchanged stomatal conductance at elevated level of CO_2 under both photo- and mixotrophic growth condition. At elevated level of CO_2 and sugar deprivation, activities of Rubisco (26.4%) and PEPC (74.5%) was up-regulated. Among metabolites, the content of sucrose and starch was always higher under CO_2 enrichment during both *in vitro* and *ex vitro* growth. Our results indicate that plantlets grown under CO_2 enrichment developed completely viable photosynthetic apparatus ready to be efficiently transferred to *ex vitro* condition that has far-reaching implications in micropropagation of *Phalaenopsis*.

1. Introduction

Micropropagation is an established technique for rapid propagation of uniform plants. However, the leaves formed during *in vitro* may never attain photosynthetic competence. Therefore, to maintain positive carbon balance, sugar is supplemented as the source of carbon. Exogenous supply of sugar increases starch and sucrose reserves in micropropagated plants that can favor transfer to *ex vitro* conditions, improve acclimatization and speed up physiological adaptations. Nonetheless, addition of sugar to the culture media was negatively correlated with growth (Kwa et al., 1995), photosynthesis and expression of enzymes of the carbon assimilation pathway (Kilb et al., 1996). However, Kozai et al. (1991) concluded that the carboxylation capacity is restricted due to the lower CO_2 concentrations and photon flux densities in the culture vessel.

Phalaenopsis is the monopodial epiphytic CAM orchid with succulent leaves. It has gained a cosmopolitan popularity in recent years due to high economic value as cut flowers and potted plants. Leaves of *Phalaenopsis* are produced alternately where the younger leaves are exposed to complete irradiance and mature leaves gradually shaded by the formation of juvenile leaves above them. This strongly curtails the absorbed photon flux density (PFD) of the leaves situated at the lower half of the stem and seriously hampers the process of carbon assimilation. It is feasible to enhance the assimilate production by increasing the level of irradiance but in shade-loving orchid such as *Phalaenopsis*, it may trigger a photo-inhibition in the leaves (Ali et al., 2005). In such a scenario, elevation of CO_2 could be an alternative to optimize the photosynthetic rates of source leaves. It has been observed that the plants of *Oncidium goldiana* (shade-loving CAM orchid) accumulated massive dry matter when exposed to the elevated levels of CO_2 (Li et al., 2002). However, contradictions exist among the studies on increased CO_2 concentration for CAM plants where CO_2 enrichment has a very little effect on *Kalanchoe daigremontiana* (Holtum et al., 1983) and *Ananas comosus* (Ziska et al., 1991), while nocturnal CO_2 uptake increases in *Opuntia ficus-indica* (Cui et al., 1993), and reduces in *Portulacaria afra* (Huerta and Ting, 1988). Thus, the effects of CO_2 enrichment in the medium on the growth and development of *in vitro* plantlets are still an important topic of research in micropropagation of CAM plants. Therefore, the aim of the present investigation was to identify the more suitable treatments *in vitro* for the best acclimatization of *Phalaenopsis* plantlets to *ex vitro* conditions.

Abbreviations: CAM, crassulacean acid metabolism; C_A, CO_2 ambient; C_E, CO_2 enriched; PEPC, phosphoenolpyruvate carboxylase; Rubisco, ribulose 1,5-bisphosphate carboxylase.

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2. Material and methods

2.1. Plant materials and growth conditions

Phalaenopsis plantlets (2.5 cm in shoot length and 0.8 g in weight) were derived from flower stalk cultures and multiplied on a hyponex medium (6.5 N:6 P:19 L 1 g + 20 N:20 P:20 K 1 g L^{-1}) containing 2 g L^{-1} peptone, 0.5 g L^{-1} activated charcoal, 8 g L^{-1} agar and without any addition of hormone in 500 ml culture vessels (Phytohealth, SPL, Korea). Medium was sterilized by autoclaving at 121 °C and at 15 pounds pressure in an autoclave. Ten plantlets in each vessel and twenty vessels for each treatment were used. Cultures were maintained at 25 ± 2 °C under a 60 μmol m^{-2} s^{-1} photosynthetic photon flux (PPF) and 16 h photoperiod using fluorescent light. The air (sterile) flow rate was adjusted to 0.17 vvm by attaching a gas-permeable microporous filter (1.5 cm^2) on top of the vessel (Mill-Seal, Millipore, Tokyo; pore size 0.5 μm). In the sugar treatments, medium was either supplemented with 30 g L^{-1} sugar or withheld. For CO_2 enrichment treatment, air was mixed with pure CO_2 using EYELA gas control unit F140 in small growth cabinet and was continuously monitored by Sensonix (0–3000 μmol mol^{-1}, Japan). For *ex vitro* acclimatization, *Phalaenopsis* plantlets were transplanted in culture box (55 cm × 45 cm × 25 cm) containing sphagnum moss. During first 20 days of acclimatization, the plantlets were kept in a growth chamber at 95 ± 2% RH, 26 ± 2 °C air-temp., 90 μmol m^{-2} s^{-1} PPFD and 14 h photoperiod). The plantlets were transferred to the green house (70 ± 20% RH, 27 ± 3 °C air-temp., 150 μmol m^{-2} s^{-1} PPFD and 14 h photoperiod) after 20 days of acclimatization and grown for 20 days.

2.2. Growth measurements

Dry weights of root and shoot, length of root and shoot, leaf area and leaf number were determined after 90 days of *in vitro* growth and at the end of *ex vitro* acclimatization. The plant samples (leaves and roots) were dried at 65 °C for 3 days to measure dry weight. Shoot length was measured from the shoot base to the first node from shoot apex. Young fully emerged leaves were selected for estimation of chlorophyll content, chlorophyll fluorescence and enzymatic activities.

2.3. Chlorophyll fluorescence and photosynthetic measurements

Chlorophyll fluorescence was recorded with a pulse-amplitude-modulation fluorometer (Model PAM 2000, Heinz-Walz, Effeltrich, Germany) at different intervals and calculated according to Kooten and Snel (1990). Minimal fluorescence was determined after 30 min dark adaptation at 250 μmol m^{-2} s^{-1} PPFD and maximal fluorescence under saturated pulse at 2400 μmol m^{-2} s^{-1} PPF. Diurnal changes of net CO_2 uptake, stomatal conductance and transpiration were recorded every 2 h to determine various phases of CAM photosynthesis between 06:00 and 24:00 h on ten randomly selected plantlets for each treatment using LI-6400, LI-COR, Lincoln, NE.

2.4. Determination of photosynthetic enzyme activity

2.4.1. Rubisco extraction and assay

Rubisco was extracted at 4 °C by homogenizing frozen leaf samples (0.6 g) in 2 ml of extraction buffer (350 mm HEPES–KOH, pH 8.0, 10 mm $MgCl_2$, 5 mm EDTA, 14 mm β-mercaptoethanol, 3% (w/v) PVP 25, 15% (w/v) PEG 20,000, and 2.5% (v/v) Tween 20), 20 ml of 100 mm PMSF, and 200 mg of polyvinylpolypyrrolidone. After centrifugation (5 min at 20,000 × *g*), 50 μl of supernatant was added to 950 μl reaction medium (100 mM Bicine–KOH, pH 8.0, 20 mM $NaHCO_3$, 5 mM $MgCl_2$, 3.5 mM *p*-creatine, 10 mM $MgCl_2$, 20 mM NaCl, 3.5 mM ATP, 3.5 mM phosphocreatine, 4 U creatine *p*-kinase, 4 U glyceraldehydes phosphate dehydrogenase, 0.4 mM NADH). Reactions were started after 15 min incubation at 25 °C by the addition of 25 μl of 0.5 mM RuBP. Measurements were made using a UV/vis spectrophotometer (UV-1650, Shimadzu, Japan) at 340 nm according to Cheng and Fuchigami (2000) with modifications. Soluble-protein contents of the enzyme extracts and leaves were determined according to Bradford (1976).

2.4.2. PEPC extraction and assay

Liquid nitrogen frozen sample (1.0 g) was grinded with 3.0 ml sample buffer containing 50 mM HEPES–NaOH, pH 7.5, 20 mM $MgCl_2$, 10 mM *iso*-ascorbic acid, 1 mM EDTA and polyvinylpyrrolidone (PVP, 1%, w/v), and centrifuged at 12,000 × *g* for 20 min. The supernatant was filtered by 0.45 μm membrane filter. Assay of PEPC was done according to Van Huylenbroeck et al. (2000). Briefly, 50 μl of the filtrate, 950 μl of enzyme buffer containing 25 mM Tris–HCl (pH 8.0), 5.0 mM $MgCl_2$, 2 mM $NaHCO_3$, 5 mM glucose 6-P, 5 mM PEP, 0.2 mM NADP, and 2 units l-MDH ml^{-1} were added. PEPC activity (nmol mg^{-1} protein min^{-1}) was measured by the reduction of the absorbance at 340 nm.

2.5. Determination of metabolite content

Soluble sugars were extracted twice with 80% ethanol and once with Mili-Q ultrapure water (Millipore, Bedford, MA) 50 mg of dry weight and all three supernatants were pooled and evaporated to dryness. The solid was re-dissolved in Mili-Q water. The samples were filtered through 0.45 μm membrane filter (PVDF syringe filter 13 mm, Whatman) and were kept in a freezer (−20 °C) until analysis. Analyses of extracted sugars were performed using HPLC (Waters 600S controller, Waters 626 pump, Waters, USA) with Coregel 87C carbohydrate column (300 mm × 7.8 mm, Waters Co., Milford, USA; flow rate 1.0 ml min^{-1}) using RI detector (Refractometer Diffrential, Waters 410, Milford, USA). Starch was quantified according to Hibberd et al. (1996).

3. Results

3.1. Growth characteristics

In vitro CO_2 enrichment and sugar deprivation promoted most of the growth parameters during *in vitro* growth and *ex vitro* acclimatization of *Phalaenopsis* plantlets (Table 1). At elevated level of CO_2, leaf number of *in vitro* grown plantlets decreased but during *ex vitro* acclimatization, it was both increased (6.4%) and decreased (2.0%) in response to C_E/(−sugar) [enriched CO_2/(−sugar)] and C_E/(+sugar) [enriched CO_2/(+sugar)] treatments. Remarkable increase in shoot length and shoot dry weight was noticed at C_E/(−sugar) during *in vitro* and *ex vitro* growth of *Phalaenopsis* plantlets (Table 1). However, addition of sugar either at ambient or enriched-CO_2 levels, increased all the root parameters studied, during both the periods of *in vitro* growth and *ex vitro* acclimatization. At the end of *in vitro* growth period, data on root:shoot ratio exhibited that presence of sugar under CO_2 enrichment favored the downward distribution of dry matter, i.e. roots while sugar deprivation at the ambient level of CO_2 translocated the dry matter toward shoots (Table 1).

3.2. Photosynthetic characteristics

To resolve the intrinsic efficiency of PS II of *in vitro* grown *Phalaenopsis*, variable fluorescence/maximum fluorescence (Fv/Fm) was determined (Fig. 1A). It was noted that at treatments C_A/(−sugar) [ambient CO_2/(−sugar)] and C_A/(+sugar) [ambient