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ORIGINAL PAPER

In vitro growth and single-leaf photosynthetic response of *Cymbidium* plantlets to super-elevated CO_2 under cold cathode fluorescent lamps

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Abstract To examine the effectiveness of super-elevated (10,000 μmol mol^{-1}) CO_2 enrichment under cold cathode fluorescent lamps (CCFL) for the clonal propagation of *Cymbidium*, plantlets were cultured on modified Vacin and Went (VW) medium under 0, 3,000 and 10,000 μmol mol^{-1} CO_2 enrichment and two levels of photosynthetic photon flux density (PPFD, 45 and 75 μmol m^{-2} s^{-1}). Under high PPFD, 10,000 μmol mol^{-1} CO_2 increased root dry weight and promoted shoot growth. In addition, a decrease in photosynthetic capacity and chlorosis at leaf tips were observed. Rubisco activity and stomatal conductance of these plantlets were lower than those of plantlets at 3,000 μmol mol^{-1} CO_2 under high PPFD, which had a higher photosynthetic capacity. On the other hand, plantlets on Kyoto medium grown in 10,000 μmol mol^{-1} CO_2 under high PPFD had a higher photosynthetic rate than those on modified VW medium; no chlorosis was observed. Furthermore, growth of plantlets, in particular the roots, was remarkably enhanced. This result indicates that a negative response to super-elevated CO_2 under high PPFD could be improved by altering medium components. Super-elevated CO_2 enrichment of in vitro-cultured *Cymbidium* could positively affect the efficiency and quality of commercial production of clonal orchid plantlets.

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Keywords Super-elevated CO_2 · CCFL · In vitro growth · Single-leaf photosynthesis · Medium components · *Cymbidium*

Abbreviations

CCFL	Cold cathode fluorescent lamps
VW	Vacin and Went
PPFD	Photosynthetic photon flux density

Introduction

The genus *Cymbidium* comprises about 50 species distributed in tropical and subtropical Asia and Oceania. Almost all cultivated cymbidiums are hybrids, thin-leaved and with a C_3 mode of photosynthesis (Hew et al. 1989). Most attractive *Cymbidium* hybrids have become commercially important as potted plants and cut flowers. *Cymbidium* was the first orchid genus to be propagated using shoot-tip culture (Morel 1960). Since then, Wimber (1963) formulated and described a shoot-tip-based protocol for in vitro propagation of *Cymbidium*. These methods for mass propagation of *Cymbidium* clones are now commercially used.

Over two decades, several studies have been conducted on photoautotrophic culture in many species, including *Cymbidium* hybrids, which has several advantages over photomixotrophic culture: minimal microbial contamination, increased photosynthetic rate, growth and rooting *in vitro* and survival percentages ex vitro (reviewed in Kozai 1991; Kozai et al 1987). Under photoautotrophic culture, CO_2 and light are the most important factors directly affecting the growth and photosynthetic capacity of plantlets because they should produce complex organic compounds from CO_2 as a

carbon source using energy from light. Many studies have shown that an increase in CO_2 concentration and photosynthetic photon flux density (PPFD) can enhance photosynthesis and growth of in vitro plantlets under photoautotrophic culture (Kozai 1991). A report on the effect of super-elevated CO_2 on the in vitro growth of CAM orchid *Mokara* 'White' plantlets showed that higher root dry mass was obtained for 10,000 μmol mol^{-1} CO_2 (super-elevated CO_2) under high light intensity (200 μmol m^{-2} s^{-1}, Hew et al. 1995).

On the other hand, C_3 plants growing in elevated CO_2 showed a decline in photosynthetic capacity (Gunderson and Wullschleger 1994; Sage 1994; Drake et al. 1997), which may reduce plant growth. Several factors to explain this phenomenon have been proposed: limitation by sink capacity (Paul and Foyer 2001), limitation by nitrogen availability (Pettersson and McDonald 1994; Stitt and Krapp 1999), suppressing gene expression of photosynthetic enzymes by leaf carbohydrates (Van Oosten and Besford 1996; Rolland et al. 2002), and accelerated leaf senescence (Ludewig and Sonnewald 2000). It is well known that the magnitude of the decline in photosynthetic capacity due to CO_2 enrichment is variable and depends on the plant species. As for *Cymbidium*, Tanaka et al. (1999) demonstrated that high CO_2 (3,000 μmol mol^{-1}) stimulated the growth of in vitro plantlets and did not cause downregulation of photosynthetic capacity. However, it is not clear whether higher levels of CO_2 would have further positive effects on the in vitro growth of *Cymbidium* plantlets, this being the focus of this study.

The growth and photosynthetic responses to elevated CO_2 will also depend on the availability of plant nutrients, in particular nitrogen. For example, a number of studies that have examined the interaction of elevated CO_2 and nitrogen supply indicates that the decline of photosynthetic capacity to elevated CO_2 was more marked in plants supplied with low levels of nitrogen than those supplied with high levels of nitrogen (Stitt and Krapp 1999). Thus, the growth and photosynthetic response of in vitro *Cymbidium* plantlets to super-elevated CO_2 also may be strongly affected by ionic concentration and/or composition of the medium. However, there are only a few reports on the effect of medium ionic composition for photoautotrophic culture (high CO_2 enrichment); moreover, no report for super-elevated CO_2 enrichment is available (Kozai et al. 1988; Yang et al. 1995).

On the other hand, light is also an important factor. Recently, the use of cold cathode fluorescent lamps (CCFLs) as a radiation source for plants have been attempted because of its attractive features, such as a small diameter (1.6–3.0 mm), long life (−50,000 h) and low heat generation (Tanaka et al. 2009). In that study, it was reported that *Cymbidium* in vitro plantlets under CCFL showed enhanced photoautotrophic growth when compared with plants under conventional heat fluorescent lamps.

The aim of the present study was to achieve more efficient and higher quality commercial production of clonal orchid plantlets. To achieve this, we examined (1) the effects of super-elevated CO_2 (10,000 μmol mol^{-1}) enrichment under CCFL on the in vitro growth and photosynthesis, and (2) the effects of N concentration and ionic composition of medium under super-elevated CO_2 enrichment on the in vitro growth and photosynthetic rate of *Cymbidium* hybrid.

Materials and methods

Plant materials

The explants used in this study were shoots with 2–3 leaves obtained from a mass of protocorm-like bodies (PLBs) of *Cymbidium* Music Hour 'Maria' derived from shoot-tip culture. It is a sympodial orchid hybrid with C_3 photosynthesis (Hew et al. 1989). Twelve shoots were cultured in each culture vessel for 3 months, and seven culture vessels (Experiment 1) or two culture vessels (Experiment 2) were used for each treatment. To acclimate the plantlets, groups of 24 in vitro plantlets cultured in two culture vessels were transferred to sphagnum (Pacific Wide (NZ) Ltd., Christchurch, New Zealand) in cell trays (BUMNONG Co., Ltd., Jeonbuk, Korea) with 50 holes.

Culture vessels

Conventional glass bottles (volume 900 cm^3) with TPX® caps were used. For aeration, a hole (4 mm in diameter) was made in the middle of the cap and each hole was covered with a Milliseal® (Japan Millipore Co., Ltd., Tokyo, Japan).

Culture medium

Modified Vacin and Went (VW, Vacin and Went 1949) sugar-free liquid medium supplemented with 1 mL Nitch microelements (Nitsch and Nitsch 1967), 0.1 mg L^{-1} 1-naphthaleneacetic acid (NAA, Nacalai Tesque, Japan) and 0.1 mg L^{-1} kinetin (Wako Pure Chemicals, Japan) was used as the basal medium. To examine the effects of differences in nitrogen concentration and ionic composition of medium under super-elevated CO_2 enrichment with high PPFD, the basal medium supplemented with different concentrations of NH_4NO_3 (0, 100, 200 and 400 mg L^{-1}), and Kyoto medium for *Cymbidium* (Tsukamoto et al. 1963) supplement with 0.1 mg L^{-1} NAA and 0.1 mg L^{-1} kinetin were also used in Experiment 2. The pH of the medium was adjusted to 5.3 before autoclaving at 121°C for 17 min. Rockwool (RW; 12 joined blocks, Grodan A/S, Denmark) substrate was sterilized in a dry sterilizer at 150°C for 1 h prior to being placed

in a conventional glass bottle. Then, 108 mL of sterilized liquid medium was poured over the RW.

Culture conditions

The culture conditions were temperature 25 ± 1°C, photoperiod 16 h a day, PPFD 45 and 75 μmol m^{-2} s^{-1} (conventional CCFL light unit; NK system, Osaka, Japan), CO_2 enrichment conditions: 0, 3,000 and 10,000 μmol mol^{-1} (super-elevated CO_2 enrichment).

In vitro experiments were conducted under various CO_2 concentrations by placing the vessels in different transparent acrylic desiccated chambers in which the CO_2 concentration inside the chamber was controlled with an infrared CO_2 controller (ZEP 9, Fuji Electric Co., Ltd., Japan) and CO_2 gas inlet line (Tanaka et al. 1992). CO_2 was injected in the chamber from a pure source thorough a solenoid valve and a micro needle valve. To prevent the occurrence of air stratification inside the chambers, a tube axial DC fan was fitted in the center of a false floor.

Measurement of growth

The number of leaves, plant height, stem diameter (measured at base of the shoot), number of roots, root length, SPAD value of leaves, shoot and root fresh weight and shoot and root dry weight of *Cymbidium* plantlets cultured in vitro was recorded after 90 days, while the number of leaves, plant height, stem diameter, number of roots, root length, SPAD value of leaves, shoot and root fresh weight and shoot and root dry weight of ex vitro plants were recorded after 30 days.

The SPAD value of leaves was measured with a chlorophyll meter (SPAD-502, Minolta Co., Ltd., Osaka, Japan) in the second leaf, counted from the top downward, of plantlets.

Measurement of photosynthesis

The photosynthetic character of leaves was measured in the second leaf counting from the top downward of plantlets. The net photosynthetic rate, stomatal conductance and intercellular CO_2 concentration in a single leaf after culturing for 90 days were measured at a photon irradiance of 300 μmol m^{-2} s^{-1} using by a portable infrared gas analyzer (LI-6400, Li-COR, Lincoln, NE, USA). The CO_2 concentration of the reference air entering the leaf chamber was adjusted with a CO_2 mixer control unit such that the "sample" air exiting the chamber contained 400 μmol mol^{-1} of CO_2. The chamber temperature was controlled by maintaining the Peltier block temperature at 25°C. The relative humidity of the reference air was kept at 65–70% as best as possible. The air flow rate was 200 mL/min. For determining the photosynthetic light curve response, the photon flux density, which was provided from a red LED light source built into the top of the leaf chamber was changed from 0 to 300 μmol m^{-2} s^{-1}.

Determination of total Rubisco activity

Rubisco (EC 4.1.1.39) was extracted and determined as described by Ueno and Sentoku (2006). The leaf blades (0.1 g fw) were ground with a pestle in a mortar (on ice) containing a little sea sand, 25 mg polyvinylpyrrolidone (30 MW, Wako) and 1 mL of grinding medium. The grinding medium contained 50 mM *N*-(2-hydroxyethyl) piperazine-*N*′-ethanesulfonic acid (Hepes, Nacalai Tesque)-KOH (pH 7.5), 0.2 mM EDTA (Wako), 2.5 mM $MgCl_2$ (Nacalai Tesque), 2.5 mM $MnCl_2$ (Nacalai Tesque), 5 mM dithiothreitol (DTT, Roche, Switzerland), 0.2% (v/v) Triton X-100 (Nacalai, Tesque) and 0.7% (w/v) BSA (Nacalai Tesque). Homogenates were filtered through gauze and the filtrates were centrifuged at 10,000*g* for 5 min at 4°C. Rubisco was preincubated in the presence of 10 mM $NaHCO_3$ (Nacalai Tesque) and 10 mM $MgCl_2$ for 15 min to obtain maximum activation. Carboxylase activity of Rubisco was assayed spectrophotometrically in a 1-mL reaction mixture at 25°C. The reaction mixture contained 50 mM Hepes–KOH (pH 8.0), 2.5 mM DTT, 1 mM EDTA, 5 mM ATP (Roche), 5-mM phosphocreatine (Roche), 0.16 mM NADH (Roche), 2 U phosphoglyceric phosphokinase (Wako), 2 U creatine phosphokinase (Roche), 2 U glyceraldehyde-3-phosphate dehydrogenase (Sigma, USA), 10 mM $NaHCO_3$, 20 mM $MgCl_2$, 0.6 mM ribulose-1,5-bisphosphate (Sigma) and 10 μL extract. Chlorophyll levels were determined by the method of Arnon (1949).

Statistical analysis

Means were separated by ANOVA and significant differences assessed by Tukey's multiple range test at $P = 0.05$. The Student's *t* test was employed for data in Tables 4 and 5 because these experiments had only two treatments.

Results

Experiment 1: effects of super-elevated CO_2 enrichment on the in vitro and ex vitro growth and photosynthesis

In vitro growth under super-elevated CO_2 enrichment with CCFL

All growth parameters of plantlets cultured at 3,000 and 10,000 μmol mol^{-1} CO_2 under both PPFD were greater than those cultured under non-CO_2 enrichment under both

Table 1 Effects of super-elevated CO_2 enrichment and high PPFD on the *in vitro* growth of *Cymbidium* plantlets under CCFL

PPFD ($\mu mol\ m^{-2}\ s^{-1}$)	CO_2 concentration ($\mu mol\ mol^{-1}$)	No. of leaves	No. of roots	Plant height (cm)	Root length (cm)	Stem diameter (mm)	Fresh weight (mg)		Dry weight (mg)		Root/shoot ratio (%)	Chlorophyll content[C] (SPAD value)
							Shoot	Root	Shoot	Root		
45	Ambient[A]	5.8c[B]	1.7e	7.0c	0.4b	3.6d	376.2c	31.2d	30.8d	1.4e	0.04c	45.7c
	3,000	7.3ab	4.2d	10.8b	2.6a	4.8c	786.4b	602.5c	66.7c	27.4d	0.39b	50.5ab
	10,000	7.2b	4.8c	11.6a	2.8a	5.0c	823.8b	735.1b	75.5b	36.1bc	0.48a	45.5c
75	Ambient	5.9c	1.7e	6.6c	0.4b	3.7d	347.6c	36.1d	30.6d	2.5e	0.08c	48.6bc
	3,000	7.1b	5.3b	10.6b	2.6a	5.4b	819.8b	812.1ab	76.4b	39.8b	0.52a	52.8a
	10,000	7.6a	5.9a	11.9a	2.7a	5.8a	951.4a	870.2a	95.3a	47.0a	0.49a	45.7c

[A] Non-CO_2 enrichment

[B] Different letters within a column indicate significant differences at $P = 0.05$ by Tukey's multiple range test

[C] Chlorophyll content in the second leaf, counted from top downward, of the plantlets

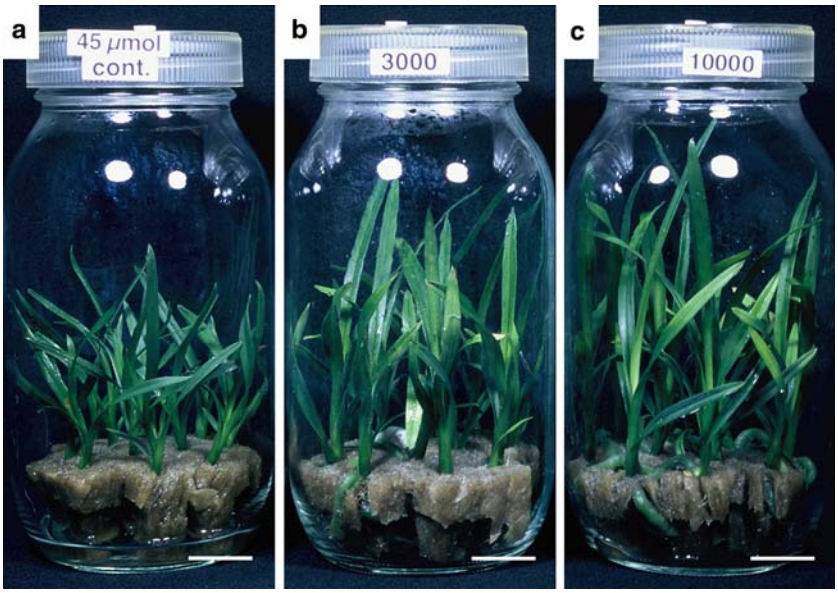

Fig. 1 *Cymbidium* plantlets grown at 0, 3,000, 10,000 $\mu mol\ mol^{-1}$ CO_2 enrichment under low PPFD. **a** Non-CO_2 enrichment under low PPFD, **b** 3,000 $\mu mol\ mol^{-1}$ CO_2 enrichment under low PPFD, **c** 10,000 $\mu mol\ mol^{-1}$ CO_2 enrichment under low PPFD. *Bar* 2 cm

PPFD (Table 1; Fig. 1). Under low PPFD, the following parameters of plantlets cultured at 10,000 $\mu mol\ mol^{-1}$ CO_2 was larger than those at 3,000 $\mu mol\ mol^{-1}$ CO_2: number of roots, plant height, fresh root weight, shoot and root dry weights and root/shoot ratio. On the other hand, the number of leaves, root length, stem diameter, fresh shoot weight did not differ between 3,000 and 10,000 $\mu mol\ mol^{-1}$ CO_2. The SPAD value of leaves at 10,000 $\mu mol\ mol^{-1}$ CO_2 was lower than that at 3,000 $\mu mol\ mol^{-1}$ CO_2. Under high PPFD, the parameters of plantlets cultured at 10,000 $\mu mol\ mol^{-1}$ CO_2 was larger than those at 3,000 $\mu mol\ mol^{-1}$ CO_2: number of leaves and roots, plant height, stem diameter, fresh shoot weight and shoot and root dry weights. On the other hand, root length, shoot dry weight and root/shoot ratio did not differ between 3,000 and 10,000 $\mu mol\ mol^{-1}$ CO_2. The SPAD value of leaves at 10,000 $\mu mol\ mol^{-1}$ CO_2 was lower than that at 3,000 $\mu mol\ mol^{-1}$ CO_2. The parameters of plantlets cultured at 10,000 $\mu mol\ mol^{-1}$ CO_2 under high PPFD were larger than those at 10,000 $\mu mol\ mol^{-1}$ CO_2 under low PPFD: number of leaves and roots, stem diameter, fresh shoot and root weights and dry shoot and root weights. On the other hand, plant height, root length, root/shoot ratio and SPAD values did not differ between low and high PPFD. Chlorosis was observed in all leaf tips of plantlets at 10,000 $\mu mol\ mol^{-1}$ CO_2 under high PPFD except for new leaves; browning at the tips of some roots was observed.

Photosynthetic rate of plantlets grown under super-elevated CO_2 enrichment with CCFL

The single-leaf photosynthetic rate of plantlets cultured at 3,000 and 10,000 $\mu mol\ mol^{-1}$ CO_2 under both PPFD levels was greater than that at non-CO_2 enrichment under both PPFD levels (Fig. 2). Under low PPFD, the single-leaf photosynthetic rate of plantlets did not differ between

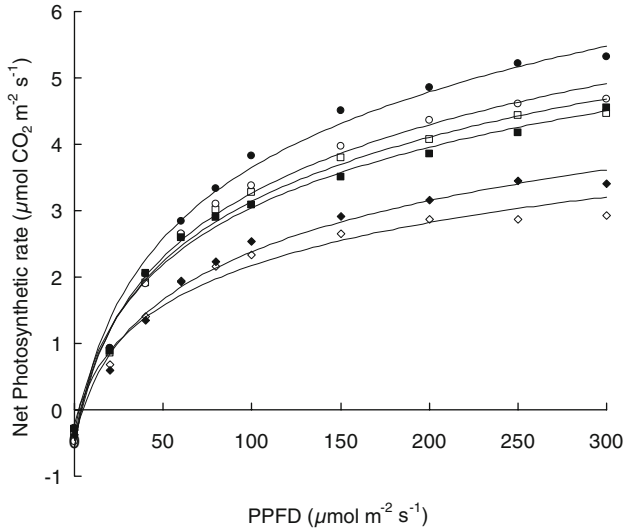

Fig. 2 Photosynthetic light-response curve for single leaves of *Cymbidium* plantlets grown at 0, 3,000 and 10,000 μmol mol^{-1} CO_2 enrichment under low and high PPFD. *Opened diamonds* non-CO_2 enrichment under low PPFD, *closed diamonds* non-CO_2 enrichment under high PPFD, *opened circles* 3,000 μmol mol^{-1} CO_2 enrichment under low PPFD, *closed circles* 3,000 μmol mol^{-1} CO_2 enrichment under high PPFD, *opened squares* 10,000 μmol mol^{-1} CO_2 enrichment under low PPFD, *closed squares* 10,000 μmol mol^{-1} CO_2 enrichment under high PPFD

3,000 and 10,000 μmol mol^{-1} CO_2. On the other hand, under high PPFD, a single-leaf photosynthetic rate of plantlets cultured at 10,000 μmol mol^{-1} CO_2 was lower than that at 3,000 μmol mol^{-1} CO_2.

Ex vitro growth of the plantlets grown under super-elevated CO_2 enrichment with CCFL

The number of roots, plant height, root length, stem diameter, fresh shoot and root weights, dry shoot and root weights and SPAD value were lowest in non-CO_2 enrichment conditions under both PPFD levels (Table 2). On the other hand, the number of leaves of plantlets under non-CO_2 enrichment under low and high PPFD was not different with those at 3,000 μmol mol^{-1} CO_2 under low PPFD and at 3,000 and 10,000 μmol mol^{-1} CO_2 under high PPFD, respectively. Plant height, shoot and root dry weights of plantlets at 10,000 μmol mol^{-1} CO_2 under low and high PPFD were greater than those at 3,000 μmol mol^{-1} CO_2 under low and high PPFD, respectively. All other parameters were not significantly different between 3,000 and 10,000 μmol mol^{-1} CO_2 under low and high PPFD, respectively. Only root dry weight of plantlets at 10,000 μmol mol^{-1} CO_2 under high PPFD was greater than that at 10,000 μmol mol^{-1} CO_2 under low PPFD.

The leaf tips of plantlets cultured at 10,000 μmol mol^{-1} CO_2 under high PPFD were withered and tips died after transfer to sphagnum (Fig. 3). Root tip browning occurred in all roots which had differentiated and developed in vitro.

Stomatal conductance, intercellular CO_2 concentration and total Rubisco activity of plant leaves grown under super-elevated CO_2 enrichment with CCFL

Stomatal conductance at 3,000 μmol mol^{-1} CO_2 under low and high PPFD was higher than that under non-CO_2 enrichment and 10,000 μmol mol^{-1} CO_2 under low and high PPFD, respectively (Table 3). Intercellular CO_2 concentration at non-CO_2 enrichment under both PPFD was higher than that at 3,000 and 10,000 μmol mol^{-1} CO_2 under low and high PPFD, respectively (Table 3). On the other hand, there was not much difference in intercellular CO_2 concentration between 3,000 and 10,000 μmol mol^{-1} CO_2 under low and high PPFD. Total

Table 2 Effects of super-elevated CO_2 enrichment under high PPFD with CCFL on the subsequent growth of *Cymbidium* plantlets for 30 days after transferring to sphagnum

PPFD (μmol m^{-2} s^{-1})	CO_2 concentration (μmol mol^{-1})	No. of leaves	No. of roots	Plant height (cm)	Root length (cm)	Stem diameter (mm)	Fresh weight (mg)		Dry weight (mg)		Chlorophyll content[C] (SPAD value)
							Shoot	Root	Shoot	Root	
45	Ambient[A]	6.8b[B]	3.0c	7.5c	3.0b	4.3b	533.5c	484.6b	50.1d	15.0d	50.2b
	3,000	7.8ab	4.7b	11.8b	5.7a	5.4a	1,018.4b	1,719.2a	112.0c	65.5c	56.2a
	10,000	7.9a	5.6ab	13.3a	5.3a	6.0a	1,181.5ab	1,804.1a	136.5ab	80.7b	56.2a
75	Ambient	6.9ab	2.9c	6.6c	2.6b	4.4b	474.9c	430.8b	46.0d	15.5d	50.3b
	3,000	7.8ab	6.0a	10.9b	4.8a	5.8a	1,101.8ab	1,576.6a	128.6bc	72.8bc	56.9a
	10,000	7.9a	6.5a	13.3a	5.3a	6.0a	1,241.2a	1,774.3a	153.1a	97.9a	57.4a

[A] Non-CO_2 enrichment

[B] Different letters within a column indicate significant differences at $P = 0.05$ by Tukey's multiple range test

[C] Chlorophyll content in the second leaf, counted from top downward, of the plantlets

Fig. 3 *Cymbidium* plantlet cultured at super-elevated CO_2 under high PPFD in vitro and acclimatized for 30 days after transfer to sphagnum

Rubisco activity was not very different among non-CO_2 enrichment, 3,000 and 10,000 μmol mol^{-1} CO_2 under low PPFD. On the other hand, under high PPFD, total Rubisco activity at 10,000 μmol mol^{-1} CO_2 was lower than that at non-CO_2 enrichment and 3,000 μmol mol^{-1} CO_2 (Table 3).

Experiment 2: the effects of differences in nitrogen concentration and ionic composition of medium under super-elevated CO_2 enrichment with high PPFD on in vitro growth and photosynthetic rate

The growth and photosynthetic rate of in vitro plantlets grown under different levels of supplemental NH_4NO_3 in medium

Plant height and root length at 100 mg NH_4NO_3 supply were greater than those with non-NH_4NO_3 supply (control, Table 4). Root length and dry shoot weight at 200 mg NH_4NO_3 supply were greater than those of the control. The number of leaves and root length at 400 mg NH_4NO_3 supply was greater than those of the control. All other growth parameters did not differ between the control and 100, 200 or 400 mg NH_4NO_3 supply. There was no difference in the single-leaf photosynthetic rate of plantlets regardless of the NH_4NO_3 supply (Table 4). Plantlets grown without any NH_4NO_3 showed leaf tip chlorosis.

The growth and photosynthetic rate of in vitro plantlets grown on Kyoto medium

The following parameters of plantlets cultured on Kyoto medium were greater than those cultured on modified VW medium: plant height, root length, fresh shoot and root weights, dry shoot and root weights and root/shoot ratio (Table 5). The number of leaves and stem diameter did not differ between both media. On the other hand, the number of roots and SPAD value of leaves on Kyoto medium were lower than those on modified VW medium. Single-leaf photosynthetic rate of plantlets on Kyoto medium was higher than that of plantlets on modified VW medium (Table 5). No chlorosis was observed in leaves and no browning in roots of plantlets cultured on Kyoto medium (Fig. 4).

Table 3 Stomatal conductance, intercellular CO_2 concentration, and total Rubisco activity of leaves of the plantlets grown at 0, 3,000 and 10,000 μmol mol^{-1} CO_2 enrichment under low and high PPFD

PPFD (μmol m^{-2} s^{-1})	CO_2 concentration (μmol mol^{-1})	Stomatal conductance (mol H_20 m^{-2} s^{-1})	Intercellular CO_2 concentration (μmol mol^{-1})	Total Rubisco activity (μmol (mg Chl)$^{-1}$ h^{-1})
45	Ambient[A]	0.120 ± 0.005[B]	348.5 ± 2.2[C]	116.2 ± 5.2[D]
	3,000	0.149 ± 0.008	334.0 ± 2.4	111.6 ± 9.1
	10,000	0.123 ± 0.004	327.0 ± 3.9	98.3 ± 6.6
75	Ambient	0.136 ± 0.007	347.4 ± 4.4	139.5 ± 16.9
	3,000	0.170 ± 0.004	333.9 ± 2.6	124.9 ± 3.4
	10,000	0.134 ± 0.004	334.3 ± 2.3	72.0 ± 21.9

[A] Non-CO_2 enrichment

[B] SE (n = 6–10)

[C] SE (n = 6–9)

[D] SE (n = 3–4)

Table 4 Effects of different levels of supplemental NH_4NO_3 in modified Vacin and Went medium on the in vitro growth and single-leaf photosynthetic rate of *Cymbidium* plantlets at super-elevated CO_2 enrichment under high PPFD

NH_4NO_3 (mg L^{-1})	No. of leaves	No. of roots	Plant height (cm)	Root length (cm)	Stem diameter (mm)	Fresh weight (mg)		Dry weight (mg)		Root/shoot ratio (%)	Chlorophyll content[B] (SPAD value)	Net photosynthetic rate[C] (μmol CO_2 m^{-2} s^{-1})
						Shoot	Root	Shoot	Root			
0	7.9b[A]	5.2a	12.3bc	2.8c	5.6a	996.5a	854.4a	96.9b	45.3a	0.47a	48.9a	3.8 ± 0.4
100	7.9b	5.1a	13.4a	3.5b	5.4a	1,040.9a	995.2a	111.0ab	55.4a	0.50a	49.3a	4.1 ± 0.1
200	8.0b	5.5a	13.1ab	3.7ab	5.5a	1,038.5a	1,000.7a	114.4a	55.8a	0.49a	47.7a	4.8 ± 0.6
400	8.6a	5.3a	12.1c	4.3a	5.2a	951.0a	866.3a	98.5ab	46.2a	0.47a	46.7a	3.4 ± 0.3

[A] Different letters within a column indicate significant differences at $P = 0.05$ by Tukey's multiple range test

[B] Chlorophyll content in the second leaf, counted from top downward, of the plantlets

[C] SE ($n = 3$–4)

Table 5 Effects of different media on the in vitro growth and single-leaf photosynthetic rate of *Cymbidium* plantlets at super-elevated CO_2 enrichment under high PPFD

Medium	No. of leaves	No. of roots	Plant height (cm)	Root length (cm)	Stem diameter (mm)	Fresh weight (mg)		Dry weight (mg)		Root/shoot ratio (%)	Chlorophyll content[B] (SPAD value)	Net photosynthetic rate[C] (μmol CO_2 m^{-2} s^{-1})
						Shoot	Root	Shoot	Root			
M-Vacin and Went	7.9a[A]	5.2a	12.3b	2.8b	5.6a	996.5b	854.4b	96.9b	45.3b	0.47b	48.9a	3.8 ± 0.4
Kyoto	8.2a	4.5b	13.3a	6.5a	5.4a	1,104.9a	2,028.1a	119.8a	100.7a	0.85a	46.5b	5.0 ± 0.2

[A] Different letters within a column indicate significant differences at $P = 0.05$ by *t*-test

[B] Chlorophyll content in the second leaf, counted from top downward, of the plantlets

[C] SE ($n = 4$)

Fig. 4 *Cymbidium* plantlets grown on different media at super-elevated CO_2 under high PPFD. *Left* modified Vacin Went medium, *right* Kyoto medium

Discussion

In vitro and ex vitro growth of plantlets at super-elevated CO_2

Super-elevated CO_2 enrichment (10,000 μmol mol^{-1}) remarkably enhanced the in vitro growth of *Cymbidium* plantlets under CCFL light source, particularly under high PPFD (Table 1). A similarly positive effect of super-elevated CO_2 (10,000 μmol mol^{-1}) in vitro has been shown in a few reports in other orchid species (Hew et al. 1995; Gouk et al. 1997; Gouk et al. 1999).

In many studies, it has been reported that elevated CO_2 increased the dry mass of plants (Mortensen 1987). Also increases in the partitioning of assimilate to the roots under elevated CO_2 have been shown for a wide range of herbaceous species (Farrar and Williams 1991). In *Cymbidium*, Tanaka et al. (1999) proved that in vitro plantlets grown under high CO_2 (3,000 μmol mol^{-1}) enrichment under low PPFD (45 μmol m^{-2} s^{-1}) increased the dry weight of roots as a role in the sink. In our present study, the elevated CO_2 enrichment under low PPFD also stim-

ulated root rather than shoot growth, number of roots, root fresh and dry weight and root/shoot ratio of plantlets grown under 10,000 μmol mol^{-1} CO_2 enrichment was higher than that under 3,000 μmol mol^{-1} CO_2 (Table 1; Fig. 1). Similar results have been obtained for CAM orchid *Mokara* 'White' in vitro plantlets under super-elevated CO_2 (Hew et al. 1995). The enhanced root growth of in vitro plantlets as a result of super-elevated CO_2 enrichment might enable enhanced ex vitro growth through the acquisition of essential resources that would increase the carbohydrate sink that would accumulate in the root and would be utilized when these plantlets are transferred to the greenhouse. On the other hand, increasing PPFD of CCFL under super-elevated CO_2 remarkably not only increased root weight, but also increased the number of leaves, plant height, stem length and fresh and dry weight of plantlets when compared with plantlets at non-CO_2 enrichment and 3,000 μmol mol^{-1} CO_2. Therefore, the root/shoot ratio of these plantlets was not different with those of 3,000 μmol mol^{-1} CO_2 enrichment under high PPFD (Table 1). Thus, we concluded that super-elevated CO_2 enrichment with high PPFD certainly had positive effects on the growth of both shoots and roots.

Many studies have shown that high CO_2 and super-elevated CO_2 enrichment tend to induce foliar symptoms of chlorosis or even necrosis in several plant species (Mortensen 1987; Wheeler et al. 1993; Mackowiak and Wheeler 1996; Sicher 2008; Croonenborghs et al. 2009). Leaf yellowing was attributed to photo-inhibition, nutrient deficiency, premature senescence and other causes (Cook et al. 1998; Sicher 1998, 2008). In our study, plantlets grown at 10,000 μmol mol^{-1} CO_2 under high PPFD, which remarkably enhanced growth, reduced chlorophyll content (SPAD value) and chlorosis in all leaf tips except for new leaves and browning of root tips was observed. Furthermore, the leaf tips of these plantlets, in which chlorosis was observed, were withered and died after transfer to the greenhouse for acclimatization and growth ex vitro, although root dry weight of these plantlets was also highest after transfer to sphagnum for 30 days. In addition, their roots, which were differentiated and developed in vitro, displayed tip browning, therefore, growth of roots after transfer to the greenhouse was not observed, although new roots differentiated (Fig. 3). Consequently, chlorosis of leaf tips and browning root tips of plantlets, which were caused by 10,000 μmol mol^{-1} CO_2 with high PPFD, might negatively affect the ex vitro growth of plantlets.

The single-leaf photosynthetic rate of plantlets grown at super-elevated CO_2

In a wide variety of plants, the growth of plants under high CO_2 could lead to the accumulation of carbohydrates in leaves (Long and Drake 1992), which may be caused when the photosynthetic rate exceeds sink capacity. An apparent correlation between starch accumulation and suppression of photosynthesis has long been reported (e.g. Neales and Incoll 1968). An increase in leaf carbohydrates has long been proposed to negatively modulate the expression of photosynthetic genes (Moore et al. 1999). Makino (1994) also described that starch accumulation by CO_2 enrichment hinders CO_2 diffusion in the chloroplast. However, photosynthesis downregulation by elevated CO_2 under moderate light intensity did not occur in many plant species that have a major sink for utilizing or accumulating carbohydrates due to no overaccumulation of sugars in young leaves (i.e. Sage et al. 1989; Usuda and Shimogawara 1998). Similar results were obtained for CAM orchid *Mokara* 'Yellow' in vitro plantlets under 10,000 μmol mol^{-1} CO_2 (Gouk et al. 1999). In our study, the single-leaf photosynthetic rate of plantlets grown at 10,000 μmol mol^{-1} CO_2 enrichment under low PPFD was not different with that of plantlets grown at 3,000 μmol mol^{-1} CO_2 under low PPFD (Fig. 2). On the other hand, the single-leaf photosynthetic rate at 10,000 μmol mol^{-1} CO_2 under high PPFD, in which browning was observed in some root tips, was lower than that at 3,000 μmol mol^{-1} CO_2 under the same PPFD, which may have negative effects on growth rate during acclimatization to ex vitro conditions. Browning of root tips implies that they may have already died. Thus, the overaccumulation of sugar due to limitation of sink capacity may have occurred in the leaves of these plantlets.

The stomatal conductance of plantlets grown at 10,000 μmol mol^{-1} CO_2 under high PPFD was also lower than that of 3,000 μmol mol^{-1} CO_2 under the same PPFD (Table 3). A decrease in stomatal conductance may cause a decrease in the CO_2 supply rate into intercellular spaces. However, in plantlets grown at 10,000 μmol mol^{-1} CO_2 under high PPFD, the intercellular CO_2 concentration was not different with 3,000 μmol mol^{-1} CO_2. Therefore, a decline of photosynthetic rate in leaves of these plantlets might be due to a decrease in fixation rate in mesophyll tissue rather than a decrease in CO_2 supply rate to intercellular spaces associated with lower stomatal conductance.

Total Rubisco activity of plantlets grown at super-elevated CO_2

Many studies demonstrated that a reduction in the amount and activity of Rubisco occurred in plants grown at elevated CO_2 (reviewed in Bowes 1991). As for *Cymbidium* in vitro plantlets, Tanaka et al. (1999) demonstrated that 3,000 μmol mol^{-1} CO_2 enrichment under low PPFD did not cause a decline in total Rubisco

activity. Our results at low PPFD also showed that even in plantlets grown at 10,000 μmol mol^{-1} CO_2 a significant decrease in total Rubisco activity did not occur (Table 3). However, under high PPFD, the plantlets grown at 10,000 μmol mol^{-1} CO_2, in which a decrease in photosynthetic capacity and chlorosis at leaf tips occurred, total Rubisco activity tended to decline. Rubisco is major controlling factor of photosynthesis in ambient CO_2 and saturating irradiance (Evans 1986; Makino et al. 1985). Therefore, a decline in total Rubisco activity could be a factor in the decrease of photosynthetic capacity of plantlets grown at 10,000 μmol mol^{-1} CO_2 under high PPFD.

Many studies have reported a reduction in leaf nitrogen concentration due to elevated CO_2 (Taub and Wang 2008). Several factors have been proposed to explain this phenomenon: dilution of nitrogen by increased photosynthetic assimilation of C (Stitt and Krapp 1999), decreased nitrogen uptake due to decreased transpiration (McDonald et al. 2002), low nitrogen partitioning to leaves (Kanemoto et al. 2009). Because nitrogen is an essential component of protein and chlorophyll, there is a strong relationship between leaf nitrogen content and total Rubisco activity or chlorophyll content (Evans 1983). Therefore, it is well known that the amount of Rubisco tends to decrease with a decline in leaf nitrogen content (Evans 1989). In our results, total Rubisco activity of plantlets grown at 10,000 μmol mol^{-1} CO_2 decreased under high PPFD condition only. Thus, super-elevated CO_2 might cause an indirect (as leaf nitrogen deficiency) decline in total Rubisco activity, rather than directly effect it.

In vitro growth and photosynthesis response to nitrogen supply at super-elevated CO_2 under high PPFD

A number of reports on elevated CO_2 have indicated that reduced nitrogen supply enhanced the reduction of photosynthetic capacity (e.g. Harmens et al. 2000). In our study, nitrogen (NH_4NO_3) supply to medium had no effect on the photosynthetic rate of plantlets grown at 10,000 μmol mol^{-1} CO_2 under high PPFD, although the shoot dry weight of plantlets at 200 mg NH_4NO_3 was increased when compared with the plantlets of non-NH_4NO_3 supply (control; Table 4). In addition, chlorosis was observed in leaf tips of these plantlets at any NH_4NO_3 level without a significant difference in chlorophyll content (SPAD value). No difference in chlorophyll content of plantlets among non-NH_4NO_3 supply and NH_4NO_3 supply indicated that nitrogen supply might not cause an increase in leaf nitrogen content. Thus, a decline of total Rubisco activity could not have been caused by low medium nitrogen content or medium nitrogen depletion.

In vitro growth and photosynthesis response to different ionic composition of medium at super-elevated CO_2 under high PPFD

A study on photoautotrophic culture showed that the growth and photosynthetic rate of carnation and strawberry plantlets were greater on medium with ionic composition widely used for hydroponic culture than on Murashige and Skoog (1962) medium developed for photomixotrophic culture (Kozai et al. 1988; Yang et al. 1995). In our study, plantlets on Kyoto medium based on Hyponex (N:P:K = 6.5:6.0:19.0) at 10,000 μmol mol^{-1} CO_2 under high PPFD showed enhanced growth as compared to that of modified VW medium developed for photomixotrophic culture of orchids; in particular, root length and root fresh and dry weights increased remarkably (Table 5). Furthermore, the photosynthetic capacity of these plantlets was higher and no chlorosis was observed in leaves and no browning of root tips, although the SPAD value was lower (Fig. 4). The potassium concentration Kyoto medium is double that of modified VW medium. Potassium has been shown to promote adventitious root growth of some horticultural crops (Zhao et al. 1991). In addition, it is known that the plantlets on Kyoto medium were often enhanced root growth rather than shoot growth, may due to the features of Hyponex which is containing higher K^+ and NO_3^- ions than NH_4^+ ion. The enhanced root growth of plantlets on Kyoto medium observed in our study might indicate that there is sizable translocation of photosynthate from the source leaves. It appears that the plantlets on Kyoto medium developed a large sink capacity as a result of the formation of longer and larger roots; therefore, these plantlets might have allowed the utilization of extra carbon fixed. A predominant behavior of roots as a sink could be the cause of higher photosynthetic rate of plantlets and non-occurrence of chlorosis in leaves grown at 10,000 μmol mol^{-1} under high PPFD. Teixeira da Silva et al. (2005) demonstrated that medium constituents of several commonly used tissue culture media have been shown to affect the organogenic outcome of hybrid *Cymbidium*. Our results indicate that the negative responses such as decrease in photosynthetic capacity and chlorosis of leaf tips, which was observed at the plantlets grown at 10,000 μmol mol^{-1} CO_2 under high PPFD, can be improved by altering medium components.

Conclusion

We have shown that super-elevated CO_2 (10,000 μmol mol^{-1}) under high PPFD enhanced the in vitro growth of *Cymbidium* plantlets. This would help in maximizing the productivity and quality of *Cymbidium* plantlets cultured in

vitro. In addition, CCFL has several advantages over the existing lighting system used for tissue culture (Tanaka et al. 2009). Therefore, we will expect that super-elevated CO_2 enrichment under CCFL make possible more efficient and higher quality commercial production of clonal orchid plantlets.

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