Growth of *in vitro* Oncidesa plantlets cultured under cold cathode fluorescent lamps with super-elevated CO$_2$ enrichment

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Abstract. As interest in how to increase biomass production through biotechnological means gains traction, focus is turning towards the use of photoautotrophic micropropagation under elevated levels of carbon dioxide (CO$_2$) to maximize plant growth and productivity. The effect of super-elevated CO$_2$ with cold cathode fluorescent lamps (CCFLs) on the photoautotrophic growth of *Oncidesa in vitro* has been studied using a gas-permeable film culture vessel, the ‘Vitron’. The growth of *Oncidesa* (formerly *Oncidesa* Gower Ramsey ‘U-1’) plantlets on Vacin and Went (VW) medium was stimulated by 10 000 μmol mol$^{-1}$ CO$_2$. In particular, increasing the photosynthetic photon flux density (PPFD) from 45 to 60 μmol m$^{-2}$ s$^{-1}$ under 10 000 μmol mol$^{-1}$ CO$_2$ in the growth chamber remarkably increased the number of leaves and roots, and shoot and root fresh and dry weights compared with plantlets under the same level of CO$_2$ under low PPFD (45 μmol m$^{-2}$ s$^{-1}$). However, there was a remarkable decrease in photosynthetic capacity, and chlorosis and browning of leaves. In stark contrast, plantlets grown on Kyoto medium at 10 000 μmol mol$^{-1}$ CO$_2$ under high PPFD had a higher photosynthetic rate than plantlets grown on VW medium, and no chlorosis or browning was observed. Furthermore, shoot growth was remarkably enhanced. Therefore, super-elevated CO$_2$ (10 000 μmol mol$^{-1}$) enrichment and growth under CCFLs can positively affect the efficiency and quality of commercial production of clonal *Oncidesa* plantlets.

Keywords: CCFL; *Oncidesa* (formerly *Oncidesa* Gower Ramsey ‘U-1’); photoautotrophic growth; single-leaf photosynthesis; super-elevated CO$_2$.

Introduction

The use of plant tissue culture as a way to increase plant biomass in a short span of time is an attractive application of biotechnology practised by many plant scientists. The ability to induce greater biomass by increasing the carbon dioxide (CO$_2$) concentration through photoautotrophic micropropagation—which has proven benefits in terms of productivity (Kozai *et al.* 2005)—has wide practical applications for ornamental and other horticultural crops.

*Oncidium* is an epiphytic and terrestrial orchid. This genus comprises about 400 species distributed mainly in tropical and subtropical South and Central America (Endress 1996). Many hybrid *Oncidium* have been produced, the most attractive having become commercially important as potted plants and cut flowers. Fast (1973) first reported that *Oncidium* plantlets could be induced...
by shoot tips, and several effective protocols for clonal propagation have been developed since then (Arditti 2008) and are now used for commercial micropropagation. However, relatively high production costs continue to hamper the expansion of hybrid Oncidium production since it may take almost one year for plantlets to reach the acclimatization stage. Since orchids such as Oncidium are inherently slow growers, there are considerable energy costs spent on controlling air temperature and lighting in the culture room. To overcome these limitations, despite costs spent on controlling air temperature and lighting in the culture room. To overcome these limitations, despite several studies existing on efficient multiplication and regeneration of Oncidium plantlets (e.g. Chen and Chang 2000; Chen et al. 2001; Jheng et al. 2006), it is also important to enhance the growth of plantlets regenerated in vitro.

Over two decades, many studies have been conducted on photoautotrophic culture in various species, including Oncidium hybrids (He et al. 2003), which has several advantages over photomixotrophic culture: promotion of growth and photosynthesis, high survival percentage ex vitro, elimination of physiological and morphological disorders, and less microbial contamination (reviewed in Xiao et al. 2011). Under photoautotrophic culture, CO₂ is one of the most important factors directly affecting the growth and photosynthesis of plantlets because they should produce complex organic compounds from CO₂ as a carbon source using energy from light. Therefore, it is necessary for enhancing photoautotrophic growth to provide a sufficient optimal concentration of CO₂. Tanaka (1991) developed several film culture vessels that had high gas and light permeability. The photoautotrophic growth of several orchid plantlets was stimulated in film culture vessels (Norikane and Tanaka 2010), several studies existing on efficient multiplication and regeneration of Oncidium plantlets (e.g. Chen and Chang 2000; Chen et al. 2001; Jheng et al. 2006), it is also important to enhance the growth of plantlets regenerated in vitro.

The aim of the present study was to achieve more efficient and higher-quality commercial clonal orchid plantlets, in this case, an Oncidium hybrid (Oncidesa), by super-elevated CO₂ enrichment under CCFLs on two different media.

Methods

Plant materials

The explants used in this study were shoots with 2–3 leaves obtained from a mass of protocorm-like bodies of Oncidesa (formerly Oncidium Gower Ramsey ‘U-1’; Royal Horticultural Society (RHS) 2013) derived from shoot-tip culture. This is a sympodial orchid hybrid, thin-leaved and with a C₃ mode of photosynthesis (Hew and Yong 1997). Twenty-five shoots were cultured in each culture vessel for 3 months, and two culture vessels were used for each treatment.

Culture medium

Vacin and Went (VW) (Vacin and Went 1949) sugar-free liquid medium was used as the basal medium. To examine the effect of basal medium under super-elevated CO₂ enrichment, Kyoto (Tsukamoto et al. 1963) sugar-free
liquid medium was also used. VW and Kyoto media are two of the most commonly used media in orchid biotechnology (Teixeira da Silva et al. 2005). The pH of the media was adjusted to 5.3 with 1 N NaOH or HCl before autoclaving at 121 °C for 17 min.

Preparation of the ‘Vitron’ rockwool system
The film culture vessel ‘Vitron’ (122 mm × 122 mm × 140 mm) consists of a three-dimensional injection-moulded polypropylene frame covered by a heat-sealed OTP film (Otsuka Techno Co. Ltd, Tokushima, Japan) on all sides, except the top (Giang and Tanaka 2004). OTP is a multi-layer film consisting of three layers: the outer layer of TPX (4-methyl-1-pentane polymer) and the inner layer of CPP (a polypropylene) which are bonded together by a middle layer of polyolefin resins. The top seal film (OTP) is affixed to the top of the vessel after removing the paper backing to expose the adhesive. The medium substrate was rockwool (a 25 joined block, 5 × 5, of Grodan® Rockwool Multiblock™ AO 18/30, Grodania A/S, Denmark) with 180 mL of liquid medium. The rockwool was previously sterilized in a dry sterilizer (150 °C, 1 h), and placed in the ‘Vitron’. Then, sterilized liquid medium was poured evenly over the rockwool.

Culture conditions
The culture conditions were 25 ± 1 °C, a 16-h photoperiod, and a photosynthetic photon flux density (PPFD) of 45 and 60 µmol m⁻² s⁻¹ (R/B ratio: 80 % red (~660 nm) + 20 % blue (~450 nm), a conventional CCFL light unit; NK System, Osaka, Japan). CO₂ enrichment was 380 (ambient/control), 3000, 5000 or 10 000 µmol mol⁻¹. Experiments were conducted under each CO₂ concentration by placing the vessels in different transparent acrylic desiccation chambers (Fig. 1) in which the CO₂ concentration inside the chamber was controlled with an infrared CO₂ controller (ZEP 9, Fuji Electric Co., Ltd, Japan) and a CO₂ gas inlet line (Tanaka et al. 1992). CO₂ was injected into the chamber from a pure source through a solenoid valve and a micro-needle valve. To prevent air stratification inside the chambers, a tube axial DC fan was fitted to the centre of a false floor and a conventional CCFL light source was installed on the roof of the chamber (Fig. 1).

Measurement of growth parameters
The number of leaves and roots, plant height, pseudobulb volume, root length, shoot, pseudobulb and root fresh weights, shoot, pseudobulb and root dry weights, pseudobulb formation frequency and the soil plant analysis development (SPAD) value of leaves of plantlets grown in vitro were recorded after 90 days. The pseudobulb formation frequency was calculated as a percentage of the plantlets that formed a pseudobulb. Pseudobulb volume (Vₚ) was calculated as an ellipsoid:

\[ Vₚ = (2\pi/3)HBW \]

where H, B and W are pseudobulb height, breadth and width, respectively (Winkler et al. 2009). 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 light response curve and net photosynthetic rate were measured in the second leaf, counting from the top downwards, of plantlets, in which a pseudobulb was not formed, after culturing for 90 days. It was measured in at least five plants using a portable infrared gas analyser (LI-6400, Li-COR, Lincoln, NE, USA). For obtaining the photosynthetic light response curve, the photon flux density that was provided from a red LED light source built into the top of the leaf chamber was changed from 300 to 0 µmol m⁻² s⁻¹. The net photosynthetic rate was measured at 3000 µmol m⁻² s⁻¹ (saturating or near-saturating PPFD). The CO₂ concentration and temperature in the leaf chamber were adjusted to maintain 400 µmol mol⁻¹ and 25 °C, respectively. The relative humidity in the leaf chamber was kept as close to 65–70 % as possible. The air flow rate was 200 mL min⁻¹.
Statistical analysis
Means were separated by ANOVA and significant differences were assessed by Tukey’s multiple range test and a Student’s t-test at \( P = 0.05 \).

Results and Discussion
Elevated CO₂ increases the dry mass of plants (Mortensen 1987). Assimilate partitioning to the roots under elevated CO₂ has also been shown for a wide range of herbaceous species (Farrar and Williams 1991). Regarding the micropropagation of orchids, high CO₂ enrichment in Cymbidium (Tanaka et al. 1999; Teixeira da Silva et al. 2007) and Phalaenopsis (Norikane and Tanaka 2010) or super-elevated CO₂ enrichment in Mokara Yellow (Hew et al. 1995) and Cymbidium (Norikane et al. 2010) increased the dry weight, especially in roots, playing a role as a sink. In our present study, the enhanced root growth of plantlets in the ‘Vitron’ was also observed with an increase in CO₂ concentration from 380 (non-CO₂ enriched) to 10 000 \( \mu \text{mol mol}^{-1} \) under both low and high PPFD; the maximum number of roots, root length, and root fresh and dry weights were obtained when plantlets were grown under 10 000 \( \mu \text{mol mol}^{-1} \), regardless of PPFD level (Table 1). The enhanced root growth of in vitro plantlets as a result of super-elevated CO₂ enrichment might enhance ex vitro growth through the acquisition of essential resources that would increase the carbohydrate sink that would accumulate in the root and be utilized when these plantlets are transferred to the greenhouse. On the other hand, our study showed that an increase in CO₂ concentration remarkably increased plantlet root and shoot weights; maximum shoot fresh and dry weights were obtained when plantlets were grown under 10 000 \( \mu \text{mol mol}^{-1} \) CO₂ with low or high PPFD, respectively (Table 1). Plantlets grown at 10 000 \( \mu \text{mol mol}^{-1} \) CO₂ under both levels of PPFD also had larger and heavier in vitro-formed pseudobulbs than those at other CO₂-enrichment conditions, although the frequency of formation did not differ greatly (Table 1). It therefore seems that the plantlets had the heaviest shoot weight as a result of the accumulation of carbohydrates in the pseudobulb as a direct consequence of photosynthesis. Young plants of sympodial thin-leaved Oncidium usually produce a new shoot from an axillary bud at the second node under the pseudobulb (Tanaka et al. 1986), so, for the shoot to develop, the pseudobulb acts as a source of photosynthate (Hew and Ng 1996). Therefore, increasing pseudobulb weight and volume by super-elevated CO₂ enrichment influences the formation and rapid development of the new shoot.

In Cymbidium, an increase in PPFD (using CCFLs) under 10 000 \( \mu \text{mol mol}^{-1} \) CO₂ enrichment further enhanced

| Table 1. Effects of CO₂ concentration and PPFD on the in vitro growth of Oncidium plantlets under CCFL. Chlorophyll content in the second leaf, counted from the top downwards, of the plantlets. Different letters within a column indicate significant differences at \( P \leq 0.05 \) by Tukey’s multiple range test. n = 50. Non-CO₂ enrichment. |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| PPFD (mmol m⁻² s⁻¹) | CO₂ concentration (mmol mol⁻¹) | No. of leaves | No. of roots | Plant height (cm) | Pseudobulb volume (cm³) | Root length (cm) | Fresh weight (mg) | Dry weight (mg) | Pseudobulb formation frequency (%) | Chlorophyll content (SPAD value) |
|-----------------|-----------------|-------------|-------------|-----------------|-----------------|-----------------|-----------------|----------------|-------------------------------|-----------------|
| 45              | Ambient         | 6.36        | 5.86        | 1.56            | 5.7             | 5.7             | 293.1           | 36.0           | 8.1                           | 16.8            |
| 3000            | 6.45            | 5.86        | 1.56        | 5.7             | 5.7             | 293.1           | 36.0           | 8.1                   | 16.8            |
| 5000            | 7.18            | 6.86        | 1.56        | 5.7             | 5.7             | 293.1           | 36.0           | 8.1               | 16.8            |
| 10 000          | 3000            | 7.18        | 6.86        | 1.56            | 5.7             | 5.7             | 293.1           | 36.0           | 8.1                           | 16.8            |
|                 | 5000            | 7.18        | 6.86        | 1.56            | 5.7             | 5.7             | 293.1           | 36.0           | 8.1                           | 16.8            |
|                 | 10 000          | 7.18        | 6.86        | 1.56            | 5.7             | 5.7             | 293.1           | 36.0           | 8.1                           | 16.8            |
the in vitro growth of both shoots and roots (Norikane et al. 2010). A similar trend was observed in Oncidesa plantlets in the present study; the number of leaves and roots, and shoot and root fresh and dry weights of the plantlets grown at 10 000 μmol mol⁻¹ CO₂ under high PPFD increased remarkably, although plant height, root length, and pseudobulb volume, fresh and dry weights and formation frequency did not differ between both PPFD levels under 10 000 μmol mol⁻¹ CO₂ (Table 1). Thus, we concluded that super-elevated CO₂ enrichment (10 000 μmol mol⁻¹ in the ‘Vitron’) with high PPFD (provided by CCFLs) also has a positive effect on the growth of both shoots and roots in Oncidesa.

High CO₂ and super-elevated CO₂ enrichment tend to cause foliar symptoms such as chlorosis, necrosis or bleaching in several plant species (Mortensen 1987; Wheeler et al. 1993; Mackowiak and Wheeler 1996; Sicher 2008; Croonenborghs et al. 2009). Leaf yellowing is attributed to photoinhibition, nutrient deficiency, premature senescence and other causes (Cook et al. 1998; Sicher 1998, 2008). Norikane et al. (2010) also indicated that chlorosis could be observed in Cymbidium plantlets grown at 10 000 μmol mol⁻¹ CO₂ under high PPFD in all leaf tips except for new leaves; furthermore, the leaf tips of these plantlets withered and died after transferring them to the greenhouse for acclimatization and growth ex vitro. In the present study, Oncidesa plantlets at 10 000 μmol mol⁻¹ CO₂ under high PPFD showed remarkably enhanced growth and reduced chlorophyll content (SPAD value) compared with plantlets at the same CO₂ concentration under low PPFD (Table 1), and severe chlorosis in the whole leaf blade and browning in part of the leaf blade were observed from a late stage of culture, although no such symptoms were observed in plantlets grown at 10 000 μmol mol⁻¹ CO₂ under low PPFD, nor at 3000 and 5000 μmol mol⁻¹ CO₂ under both PPFDs (Fig. 2). This may negatively affect the ex vitro growth of plantlets that were cultured at 10 000 μmol mol⁻¹ CO₂ under high PPFD.

C₃ plants growing in long-term elevated CO₂ showed a decline in photosynthetic capacity (Gunderson and Wullschleger 1994; Sage 1994; Drake et al. 1997), which may reduce plant growth. Our previous study on super-elevated CO₂ enrichment in vitro indicated that Cymbidium...
plantlets grown at 10,000 μmol mol⁻¹ CO₂ under high PPFD showed decreased photosynthetic capacity and total Rubisco activity tended to decline, possible factors explaining the decreasing photosynthetic capacity (Norikane et al. 2010). In the present study the net photosynthetic rate of single leaves of Oncidesa was measured at saturating or near-saturating PPFD (200–300 μmol m⁻² s⁻¹) at the end of the culture period. At low PPFD, even in plantlets grown at 10,000 μmol mol⁻¹ CO₂, a decrease in the net photosynthetic rate of single leaves did not occur, while at high PPFD, plantlets grown at the same high level of CO₂ showed a significant decrease (Fig. 3). The latter value was similar to that of plantlets grown at non-CO₂ enrichment under high PPFD, in which almost no growth and browning of leaves were observed. This reduction may be due to damage of the photosynthetic apparatus rather than the photosynthetic acclimation response to elevated CO₂ (Sage 1994) because browning was observed in plantlets’ leaves (Fig. 2). This might also negatively impact the ex vitro growth of plantlets cultured at 10,000 μmol mol⁻¹ CO₂ under high PPFD. Therefore, super-elevated CO₂ enrichment as a method to improve the culture of Oncidesa in vitro must be further refined for it to be effective.

It is occasionally mentioned that media developed for photomixotrophic culture are not suitable for the photoautotrophic growth and development of plants in vitro (Kozai et al. 1988; Yang et al. 1995). Norikane et al.
(2010) also demonstrated that Cymbidium plantlets on Hyponex-based Kyoto medium, which uses compound fertilizer for plant cultivation, had a higher photosynthetic capacity at 10 000 μmol mol⁻¹ CO₂ under high PPFD than plantlets grown on modified VW medium developed for photomixotrophic culture of orchids; no symptoms such as chlorosis were observed and growth was remarkably enhanced. This was also observed in our present study. The growth of plantlets on Kyoto medium under 10 000 μmol mol⁻¹ CO₂ at high PPFD was enhanced relative to plantlets grown on VW medium; in particular, plant height, and shoot fresh and dry weights increased remarkably, although the number of leaves, root dry weight, pseudobulb formation frequency, volume, and fresh and dry weights did not differ, and the number of roots was slightly fewer (Table 2). Furthermore, the net photosynthetic rate (Fig. 4) and the SPAD value of these plantlets were higher (Table 2) and no chlorosis and browning were observed in all leaf blades (Fig. 5). Similar to a previous study on super-elevated CO₂ (Norikane et al. 2010), our results also indicate that negative responses such as a decrease in photosynthetic capacity, chlorosis and browning, which were observed in plantlets grown at 10 000 μmol mol⁻¹ CO₂ under high PPFD, can be improved by altering medium components. Media composition and the nature of the carbon source strongly affect Cymbidium organogenic outcome (Teixeira da Silva et al. 2006, 2007).

Conclusions

We have shown in this study that super-elevated CO₂ (10 000 μmol mol⁻¹) under high PPFD emitted by CCFLs enhanced the photoautotrophic growth of Oncidesa plantlets in the ‘Vitron’, although the upper ‘threshold limit’ would be 5000 μmol mol⁻¹ CO₂ enrichment under high PPFD before a negative impact on photosynthesis would occur. This would help to maximize the productivity and quality of Oncidesa plantlets cultured in vitro. In addition, CCFLs have several advantages over existing lighting systems used for plant tissue culture (Tanaka et al. 2009). In particular, CCFLs emit much less heat through their unique properties, allowing plants or cultures (culture vessels) to be placed very close to the light source,
making more efficient use of the culture room and intensifying the efficiency of CO₂ enrichment. Our results indicate that there is great hope for using super-elevated CO₂ enrichment under CCFLs for more efficient and high-quality commercial production of clonal orchid plantlets, which is a key objective of orchid biotechnology (Hossain et al. 2013; Teixeira da Silva 2013).

Contributions by the Authors
All authors have made a substantial contribution to the manuscript and the research presented. M.T. and A.N. co-designed the experiment. M.T. and J.A.T.d.S. oversaw the experimental execution. A.N. conducted all research. A.N. and J.A.T.d.S. drafted the paper and made all edits for the revisions. All authors have seen and agreed to the submitted manuscript.

Conflicts of Interest Statement
None declared.

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