HORTSCIENCE 48(10):1293–1297. 2013.

Carbon Dioxide Enrichment during Photoautotrophic Micropropagation of *Protea cynaroides* L. Plantlets Improves In Vitro Growth, Net Photosynthetic Rate, and Acclimatization

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Additional index words. chlorophyll content, ex vitro condition, King Protea, micropropagation, superelevated CO_2

Abstract. **The effects of CO_2 enrichment on the in vitro growth and acclimatization of *Protea cynaroides* L. plantlets were investigated. Three CO_2 enrichment concentrations were used: 0 (control), 1000, 5000, and 10000 $\mu mol \cdot mol^{-1}$. Plantlets in the control treatment were cultured on half-strength Murashige and Skoog (MS) medium supplemented with sucrose, whereas those enriched with different CO_2 concentrations were grown on sucrose-free MS medium. Compared with the control, significant improvements were observed in the growth of plantlets enriched with CO_2 irrespective of the concentration. Plantlets enriched with 5000 $\mu mol \cdot mol^{-1}$ CO_2 produced the highest number of leaves and the largest leaf area. In addition, the photosynthetic ability of plantlets enriched with CO_2 was enhanced, which resulted in significant increases in shoot growth and dry matter accumulation. In particular, the shoot dry weight of plantlets cultured in 5000 $\mu mol \cdot mol^{-1}$ CO_2 and 10000 $\mu mol \cdot mol^{-1}$ CO_2 were, respectively, 2.1 and 4.2 times higher than those without CO_2 enrichment. During acclimatization, the survival percentage, rooting percentage, and leaf number of plantlets grown in elevated CO_2 were, respectively, up to 4.5, 1.8, and 2.7 times higher than plantlets without CO_2 enrichment. The improvements in survival percentage and ex vitro growth of these plantlets were the result of their enhanced photosynthetic ability in vitro, which resulted in the production of high-quality plantlets. Significant improvements in the overall growth of *P. cynaroides* plantlets were achieved through the use of photoautotrophic micropropagation with CO_2 enrichment.**

Protea cynaroides L., known as King Protea, is a well-known cut flower in the international floriculture industry. The most characteristic feature is its attractive flowerhead, which typically consists of hundreds of flowers. In vitro propagation of *P. cynaroides* explants is often faced with difficulties such as phenolic browning and slow vegetative growth. In recent years, several advancements in propagating *P. cynaroides* explants have been achieved. These include the initiation of cultures through nodal explants (Wu and du Toit, 2004) and apical tips (Thillerot et al., 2006), and the multiplication of microshoots (Wu and du Toit, 2012). In addition, inverse correlations between root growth and endogenous concentrations of phenolic compounds have been established in *P. cynaroides* explants cultured under red light-emitting diodes (Wu and Lin, 2012). Despite these advancements, shoot growth and leaf development of *P. cynaroides* explants in vitro remain very slow and inconsistent. Furthermore, attempts to promote vegetative and root growth during ex vitro acclimatization have achieved limited success. Thillerot et al. (2006) reported very slow growth of *P. cynaroides* explants with no root growth after 2 months in ex vitro conditions.

The use of photoautotrophic propagation to improve overall explant growth has been successful in numerous plant species. As described by Kozai and Kubota (2005), the main advantages of photoautotrophic propagation are: 1) stimulation of photosynthesis; 2) reduction of anatomical and physiological disorders; 3) higher survival percentage in ex vitro environment; and 4) reduction in microbial contamination. Sucrose, which is the most commonly used carbon source in plant tissue culture, promotes plantlet growth but suppresses photosynthesis. The objective of photoautotrophy is to reduce the dependency of cultures on sugar and promote growth and carbohydrate accumulation through photosynthesis and inorganic nutrient uptake. Of particular importance is that growing plantlets photoautotrophically on medium without sucrose enables the development of fully functional photosynthetic apparatus (Pospíšilová et al., 1999). A frequently observed response to elevated CO_2 is an increase in photosynthesis (Kubota et al., 2001; Xiao and Kozai, 2006), whereas increases in chlorophyll content are also reported. Findings by Xiao et al. (2005) showed that the net photosynthetic rate and chlorophyll concentration of *Gerbera jamesonii* plantlets were, respectively, 9.2 and 2.2 times greater than those cultured photomixotrophically. In addition, Xiao and Kozai (2004) found that the growth period of CO_2-enriched *Zantedeschia elliottiana* plantlets was reduced by 50%, whereas the leaf area and shoot and root dry weight were, respectively, 5.2, 4.6, and 3.8 times higher than those cultured under a conventional micropropagation system.

Moreover, plantlets produced photoautotrophically have been shown to acclimatize better in ex vitro conditions. Low photosynthetic ability of plantlets cultured in vitro has been found to be the cause of low survival rate and poor growth during acclimatization (Kubota et al., 2001). Xiao et al. (2005) showed that during acclimatization, the survival percentage of photoautotrophically grown plantlets was 95% compared with 60% for photomixotrophic cultures. Similarly, high CO_2 concentration accompanied by increased irradiance and decreased relative humidity during in vitro culture promoted survival rate and growth of *Eucalyptus camaldulensis* (Kirdmanee et al., 1995) plantlets during acclimatization in ex vitro conditions.

P. cynaroides plantlets grown in conventional photomixotrophic micropropagation exhibit slow and inconsistent growth. In addition, little is known about the acclimatization of *P. cynaroides* explants and their growth in an ex vitro environment. Photoautotrophic micropropagation has great potential to improve the in vitro growth and acclimatization of these explants. This study was conducted to investigate the effects of CO_2 enrichment on the photoautotrophic growth of *P. cynaroides* plantlets and their acclimatization in ex vitro conditions.

Materials and Methods

Plant material and culture conditions. Unrooted terminal microshoots (length: 20 mm; fresh weight: ≈50 mg) with two leaves taken from in vitro-established *P. cynaroides* plantlets were used in this study. The plantlets were previously established on half-strength MS (Murashige and Skoog, 1962) basal medium supplemented with 2 $mg \cdot L^{-1}$ 6-benzylaminopurine (BAP), 0.5 $mg \cdot L^{-1}$ naphthalene acetic acid (NAA), 2 $mg \cdot L^{-1}$ activated charcoal, 30 $g \cdot L^{-1}$ sucrose, and 9 $g \cdot L^{-1}$ agar.

Received for publication 22 July 2013. Accepted for publication 21 Aug. 2013.

Financial support in the form of a research grant (NSC 100-2313-B-343-001) from the National Science Council of Taiwan (R.O.C.) is gratefully acknowledged.

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In this study, the microshoots were grown under three CO_2 enrichment conditions: 1000 μmol·mol^{-1}, 5000 μmol·mol^{-1}, and 10000 μmol·mol^{-1} (superelevated CO_2). In the control treatment (photomixotrophic culture), where no CO_2 enrichment was used, microshoots were cultured in glass vessels (250 mL) plugged with a silicone stopper. Culture vessels were placed in a growth room with ambient CO_2 levels. This setup was similar to the conditions commonly used to propagate *P. cynaroides* plantlets. In the treatments with enriched CO_2 (photoautotrophic culture), 6-L modified glass desiccators (25 cm I.D.; 20 cm H) consisting of an inlet and outlet connector were used as culture vessels. The inlet of each desiccator was connected to gas cylinders (60 L), which contained different CO_2 concentrations (Jing De Gas Inc., Taichung, Taiwan, China) through a silicone pipe (60 mm I.D.). Before entering the desiccator, the air was directed through a flow rate meter (New-Flow Technologies Inc., Taiwan, China) set at 0.5 L·h^{-1} and a gas-permeable microporous filter (Millipore, Tokyo, Japan; pore size 0.45 μm). In all treatments, half-strength MS basal medium supplemented with 0.5 mg·L^{-1} BAP, 0.01 mg·L^{-1} NAA, 5 mg·L^{-1} silver nitrate, 100 mg·L^{-1} meso-inositol, 2 mg·L^{-1} activated charcoal, 100 mg·L^{-1} ascorbic acid (filter-sterilized), and 9 g·L^{-1} agar were used. Sucrose (30 g·L^{-1}) was included in the growth medium of the control treatment, whereas the CO_2 enrichment treatments contained no sucrose. Each culture vessel of the photomixotrophic and photoautotrophic cultures contained 50 mL and 1 L of growth medium, respectively. The pH of all growth media was adjusted to 5.5 before autoclaving at 121 °C and 104 KPa for 25 min. All cultures were placed in a growth room with the temperature and photoperiod adjusted to 25 °C ± 2 and 16 h, respectively. The photosynthetic photon flux was adjusted to 70 μmol·sec^{-1}·m^{-2}.

Chlorophyll content determination. The concentration of chlorophyll was determined according to Wintermans and De Mots (1965). Fifty milligrams [fresh weight (FW)] of sample was ground in 2 mL of phosphate buffer solution (pH 7.0). The solution was then centrifuged at 25 °C. An aliquot of 40 μL of supernatant was transferred into a centrifuge tube, mixed with 960 μL of absolute alcohol, and placed in the dark at 4 °C for 30 min. After 1000 *g* of centrifugation at 4 °C for 15 min, an aliquot of 1 mL of supernatant was analyzed with an ultraviolet-Vis spectrometer (Helios Alpha; Thermo Fisher Scientific Inc., Pittsburgh, PA) at 649 nm (A_{649}) and 665 nm (A_{665}), which were calibrated with a 95% absolute-alcohol blank. Three repeats were carried out for the determination.

Rubisco activity determination. An extraction cocktail was prepared by mixing the following reagents: 100 mM HEPES-KOH (pH 8.0), 10% glycerol, 2% (w/v) insoluble polyvinylpyrrolidone, 0.1% (v/v) Triton X-100, and 5 mM dithiothreitol (DTT; added last). The reaction cocktail consisted of 50 mM HEPES-KOH (pH 8.0), 10 mM KCl, 1 mM EDTA, 20 mM $MgCl_2$, 5 mM creatine phosphate, 20 mM $NaHCO_3$, 3 U creatine phosphokinase, 15 U phosphoglycerate kinase, 6U glyceraldehyde-3-phosphate dehydrogenase, 5 mM ATP (Adenosine-5′-triphosphate), and 5 mM DTT (added last). Leaf sample (0.05 g) was ground to powder with 0.5 g of acid-washed sand in liquid N_2. Two milliliters of extraction cocktail was then added for extraction. After 12,000 *g* of centrifugation at 4 °C for 10 min, an aliquot of 50 μL of supernatant was mixed with 850 μL of reaction cocktail and transferred to a cuvette. The solution in the cuvette was placed in a water bath (25 °C) for 10 min and then put into an ultraviolet-Vis spectrometer (GENESYS 10S ultraviolet-Vis; Thermo Fisher Scientific Inc.) followed by mixing with 50 μL of 0.2 mM NADH (Nicotinamide adenine dinucleotide, reduced form). A background rate of NADH oxidation was determined by recording the absorbance at 340 nm (A_{340}) every 20 s for 3 min. An aliquot of 50 μL of 0.5 mM ribose-5-phosphate was then added for reaction. After a delay of 3 s, the decreasing rate of NADH was monitored at A_{340} every 20 s for 3 min. Rubisco activity was calculated by converting the net decreasing rate at A_{340} to a rate of NADH oxidation according to Usuda (1984).

Net photosynthetic rate. The net photosynthetic rate, P_n (μmol·h^{-1} per plantlet), was calculated in accordance with the method described by Fujiwara et al. (1987) using the following equation: $P_n = K \cdot E \cdot V(C_{out} - C_{in})/n$, where K is the conversion factor of CO_2 from volume to moles (0.0407 mol·L^{-1} at 26 °C), E is the number of air exchanges of the vessel (h^{-1}) estimated according to Kozai et al. (1986), V is the air volume of the vessel (L), C_{in} and C_{out} are the CO_2 concentrations (μmol·mol^{-1}) inside and outside the culture vessel, and n is the number of plantlets per culture vessel. The CO_2 concentrations were measured using a CO_2 analyzer (CGP-1; DKK-TOA Corp., Japan) when the concentrations were stable during the photoperiod at Day 45.

Acclimatization. Plantlets cultured in vitro were transferred to a greenhouse for acclimatization. The plantlets were grown in plastic pots containing a mixture of bark and peat (1:1). Throughout the acclimatization process, all plants received natural daylight with an approximate photoperiod of 12 h. The temperature of the greenhouse was maintained at 27 °C ± 2. During the study, the plants were given 50 mL of distilled water every 5 d with no fertilization.

Statistical analysis. One plantlet per culture vessel was used in each treatment with 10 replications. A completely randomized design was used in all treatments. This study was repeated three times. Data for number of leaves, leaf area, shoot length, shoot fresh and dry weights, chlorophyll content, Rubisco activity, and net photosynthetic rate were collected from the in vitro study after 45 d in culture. For the acclimatization study, the in vitro study was repeated without undergoing destructive analysis, and the plantlets were transferred directly to the greenhouse. The survival percentage, rooting percentage, and leaf number of plantlets were collected after 60 d. Where appropriate, data were analyzed using Duncan's multiple range test and χ^2 to compare treatment means using SAS (SAS Institute Inc., 1996). Values were considered significant at $P \leq 0.05$.

Results and Discussion

Leaf and shoot growth. Results showed that CO_2 enrichment enhanced the growth and development of leaves and shoots on *P. cynaroides* plantlets after 45 d in culture (Tables 1 and 2). In the control treatment where no CO_2 enrichment was used, plantlets produced the lowest number of leaves. In contrast, a significantly higher number of leaves was formed by plantlets enriched with CO_2, irrespective of the concentration. This is in agreement with results of studies that also reported increases in leaf formation in explants enriched with CO_2 (Dave and Purohit, 2004; Norikane et al., 2010). In the present study, the highest number of leaves was observed in *P. cynaroides* plantlets enriched with 5000 μmol·mol^{-1} CO_2, which produced 1.8 times more leaves than the control. Furthermore, the average leaf area of plantlets cultured under elevated CO_2 conditions was significantly increased, which was up to 1.7 times higher than plantlets with no CO_2 enrichment. Similar results were reported in *Limonium latifolium* (Xiao and Kozai, 2006) and *Momordica grosvenori* (Zhang et al., 2009) plantlets.

Increases in dry matter accumulation and improvements in overall growth of plantlets enriched with CO_2 have been well documented (Gouk et al., 1997; Mortensen, 1987). In particular, increases in the dry weight of roots are often reported in plantlets treated with CO_2 enrichment (Gouk et al., 1999; Norikane et al., 2010). Root formation was, however, absent in all plantlets in our in vitro study. This was expected because *P. cynaroides* plants are notoriously known to be difficult to root in vitro (Wu and Lin, 2012). Nevertheless, our results demonstrated that compared with the control, CO_2 enrichment enhanced shoot growth and shoot dry mass accumulation, as illustrated by significant increases in shoot length, shoot FW, and shoot dry weight (Tables 1 and 2). Similar increases in shoot growth have been reported in plantlets exposed to high CO_2 enrichment (Hew et al., 1995; Tanaka et al., 1999). Our results showed that the shoot dry weight of plantlets cultured in 5,000 μmol·mol^{-1} CO_2 and 10,000 μmol·mol^{-1} CO_2 were, respectively, 2.1 and 4.2 times higher than those without CO_2 enrichment. This suggests that there was a sizable translocation of photosynthates from the source leaves to the shoots of these plantlets. In the absence of roots, it appears that plantlets enriched with CO_2 developed a large sink capacity as a result of shoot growth and elongation, which used the photosynthates for further development. These findings are particularly noteworthy because one of the main challenges facing conventional photomixotrophic micropropagation of *P. cynaroides*

is the inability to achieve rapid and consistent leaf and shoot growth. It is generally agreed that, of all plants in the Proteaceae family, those belonging to the *Protea* genus are the most difficult to propagate in vitro (Olate et al., 2010; Rugge, 1995). Growth of *P. cynaroides* buds has been achieved in vitro through the use of growth medium specially formulated for Proteaceae (Thillerot et al., 2006; Wu and du Toit, 2012). However, development of these buds was particularly slow, where sizeable growth was only obtained after at least 60 d in culture. In addition, phenolic browning, which is often reported to be a problem during micropropagation of *P. cynaroides* explants, was not observed in the present study. This could be the result of the addition of ascorbic acid, which is an antioxidant previously used to reduce phenolic browning in *P. cynaroides* explants (Wu and du Toit, 2004), in the growth medium of the plantlets.

Chlorophyll content, Rubisco activity, and net photosynthetic rate. Leaf analysis results revealed that plantlets cultured in superelevated CO_2 contained the highest concentration of chlorophyll (Table 3). Similar findings were reported by Gouk et al. (1999) and Hew et al. (1995), in which plantlets cultured under superelevated CO_2 were found to contain higher chlorophyll content than plantlets grown in ambient CO_2. Our results also showed that the total chlorophyll content of plantlets enriched with 1000 $\mu mol \cdot mol^{-1}$ CO_2 and 5000 $\mu mol \cdot mol^{-1}$ CO_2 was similar to the control plantlets. Xiao and Kozai (2006) reported that the concentration of cytokinin supplemented in the growth medium is a factor affecting chlorophyll content. Their results revealed that a relatively high concentration of BAP concentration ($0.5 mg \cdot L^{-1}$) reduced the chlorophyll content in statice plantlets cultured on sugar-free medium enriched with 1500 $\mu mol \cdot mol^{-1}$ CO_2. It was proposed that a high BAP concentration induces stress on the physiological and morphological development of plantlets, which may cause a decrease in chlorophyll content (Xiao and Kozai, 2006). It is possible that the relatively low chlorophyll content of the *P. cynaroides* plantlets enriched with similar levels of CO_2 in our study was the result of the high BAP concentration ($0.5 mg \cdot L^{-1}$) used in the growth medium. However, the relationship between cytokinin concentration and chlorophyll content of plantlets grown under superelevated CO_2 needs to be studied.

Rubisco activity in leaves of plantlets cultured under different CO_2 concentrations did not differ significantly (Table 3). However, compared with the control treatment, a slight decline in Rubisco activity was detected in plantlets enriched with 5,000 $\mu mol \cdot mol^{-1}$ and 10,000 $\mu mol \cdot mol^{-1}$ CO_2. Numerous studies have shown Rubisco activity to be lower in plantlets enriched with CO_2 (Bowes, 1991; Gouk et al., 1997; Graham and Nobel, 1996; Norikane et al., 2010; Tanaka et al., 1999). Rubisco is a major controlling factor of photosynthesis in plantlets cultured under ambient CO_2 and saturating irradiance (Evans, 1986). According to Bowes (1991), the decline in Rubisco activity may be attributed to a decrease in Rubisco protein, its activation state, or its specific activity. Furthermore, higher CO_2 concentrations cause an increase in the proportion of Rubisco in the activated state, resulting in improved efficiency in the use of resources (Graham and Nobel, 1996).

Significant improvements were found in the net photosynthetic rate of *P. cynaroides* plantlets enriched with CO_2 (Table 3). In contrast, plantlets not enriched with CO_2 (control) showed a negative net photosynthetic rate. Low photosynthesis in photomixotrophic micropropagation is usually associated with the presence of sucrose in the growth medium, low light intensity, and infrequent air exchanges in the culture vessel (Zhang et al., 2009). It is generally agreed that the presence of sucrose in the growth medium suppresses photosynthesis of plantlets in vitro, which limits photoauthotrophic development and impedes growth of plantlets (Desjardins et al., 1995; Kozai, 1991). Furthermore, with the availability of sucrose in the growth medium, plantlets tend to depend on sucrose and less on CO_2, particularly when the CO_2 concentration in the culture vessel is low (Hew et al., 1995; Kubota et al., 2001). Moreover, it is known that plantlets cultured in sucrose-containing growth medium usually have poorly developed mesophyll with small palisade cells, which affects the availability of CO_2 and causes the reduction of photosynthesis (Gouk et al., 1997). It is likely that the overall poor growth of *P. cynaroides* plantlets under conventional photomixotrophic micropropagation as previously reported (Thillerot et al., 2006) is the result of the combination of these aforementioned factors. In the present study, plantlets enriched with CO_2 showed positive photosynthetic rates, which improved with increasing CO_2 concentrations (Table 3). The stimulation of photosynthetic activity of plantlets in vitro by CO_2 enrichment has been widely reported (Kubota

Table 1. Effects of CO_2 enrichment on the in vitro growth of *P. cynaroides* plantlets after 45 d in culture.

CO_2 concn ($\mu mol \cdot mol^{-1}$)	No. of leaves	Avg leaf area (mm^2/plantlet)	Shoot length (cm)	Shoot wt (mg/plantlet)	
				Fresh	Dry
Ambient[z]	5.0 ± 0.5 d[y]	352.5 ± 21.7 c	4.4 ± 0.6 c	78.0 ± 8.1 c	6.8 ± 1.3 d
1,000	6.7 ± 0.5 c	522.4 ± 44.3 b	5.4 ± 0.7 b	104.1 ± 10.4 b	12.1 ± 1.8 c
5,000	8.9 ± 0.7 a	598.0 ± 44.1 a	5.9 ± 0.6 ab	109.2 ± 10.2 b	14.3 ± 2.1 b
10,000	7.9 ± 0.8 b	576.1 ± 64.3 a	6.2 ± 0.6 a	169.6 ± 12.4 a	28.3 ± 2.6 a

[z]Non-CO_2 enrichment.
[y]Means in the same column with different letters are significantly different (Duncan's multiple range test at $P \leq 0.05$).

Table 2. Analysis of variance summary for number of leaves, leaf area, shoot length, shoot fresh weight, and shoot dry weight of *P. cynaroides* plantlets.

Source of variation	df	Mean square	F value	*P* value
No. of leaves				
Treatment	3	28.1583	72.93	<0.0001
Error	36	0.3861		
Corrected total	39			
Leaf area				
Treatment	3	123,509.9000	58.01	<0.0001
Error	36	2,129.1056		
Corrected total	39			
Shoot length				
Treatment	3	5.9423	15.79	<0.0001
Error	36	0.3762		
Corrected total	39			
Shoot fresh weight				
Treatment	3	15,008.0250	139.29	<0.0001
Error	36	107.7472		
Corrected total	39			
Shoot dry weight				
Treatment	3	842.1253	211.91	<0.0001
Error	36	3.9740		
Corrected total	39			

Table 3. Effects of CO_2 enrichment on the chlorophyll content, Rubisco activity, and net photosynthetic rate (P_n) of *P. cynaroides* plantlets after 45 d in culture.

CO_2 concn ($\mu mol \cdot mol^{-1}$)	Chlorophyll content ($mg \cdot g^{-1}$ fresh wt)			Chlorophyll *a*/*b* ratio	Rubisco activity (μmol CO_2/min/mg dry wt)	P_n ($\mu mol \cdot h^{-1}$)
	a	*b*	Total			
Ambient[z]	2.2 ± 0.1 bc[y]	2.2 ± 0.5 b	4.4 ± 0.3 b	1.0 ± 0.2 b	1.4 ± 0.3 a	–0.3 ± 0.02 d
1,000	2.4 ± 0.2 b	1.5 ± 0.3 c	3.9 ± 0.4 b	1.6 ± 0.1 a	1.4 ± 0.3 a	2.3 ± 0.03 c
5,000	1.9 ± 0.1 c	2.0 ± 0.1 bc	3.9 ± 0.1 b	0.9 ± 0.1 b	1.3 ± 0.1 a	13.5 ± 0.19 b
10,000	4.9 ± 0.3 a	3.5 ± 0.5 a	8.4 ± 0.6 a	1.4 ± 0.2 a	1.3 ± 0.5 a	33.4 ± 1.32 a

[z]Non-CO_2 enrichment.
[y]Means in the same column with different letter are significantly different (Duncan's multiple range test at $P \leq 0.05$).

et al., 2001; Xiao and Kozai, 2006). In addition, it is known that carbohydrate accumulates in leaves of plantlets enriched with CO_2, which in some cases hinders CO_2 diffusion in the chloroplast (Makino, 1994), or disrupts the chloroplast by starch overloading (Gouk et al., 1997). This occurs when the photosynthetic rate exceeds sink capacity (Norikane et al., 2010; Tanaka et al., 1999). However, research findings have shown that this does not occur when there is a major sink elsewhere that uses or accumulates carbohydrates (Usuda and Shimogawara, 1998). The results of our study suggest that this may have occurred in the plantlets enriched with super-elevated CO_2, where the relatively fast-growing shoot acted as a major sink for carbohydrate accumulation, as shown by the exceptionally high shoot dry weight (Table 1). This in turn promoted further increases in the net photosynthetic rate and enhanced the overall growth of these plantlets.

Ex vitro acclimatization. The quality of plantlets produced in vitro is one of the key factors affecting survival percentage during acclimatization (Afreen-Zobayed et al., 1999). The effectiveness of a micropropagation system can be measured by the survival percentage of plantlets successfully transferred to ex vitro conditions. One of the main advantages of plantlets grown under CO_2 enrichment is the enhanced photosynthetic ability of leaves, which increases the plantlets' survival and growth rate ex vitro (Kirdmanee et al., 1995). Results of our study showed that the highest survival percentage of plantlets after 60 d of acclimatization was those grown under CO_2 enrichment conditions, irrespective of the CO_2 concentration (Table 4; Fig. 1). The ex vitro survival percentage of CO_2-enriched plantlets was at least four times higher than the control plantlets. Infante et al. (1989) suggested that the presence of sucrose in the growth medium could affect the time course required for cultures to achieve a positive CO_2 balance and photosynthetic dry matter accumulation. This may explain the enhanced survival of plantlets during acclimatization when cultured in the absence of exogenous sugar during in vitro propagation (Kozai et al., 1987). Furthermore, in a previous study where *P. cynaroides* plantlets were transplanted to ex vitro conditions, it was reported that the plantlets showed very slow growth and did not develop any roots after 60 d (Thillerot et al., 2006). Our results showed very high ex vitro rooting percentage during acclimatization by plantlets grown under elevated CO_2. These results clearly demonstrated the importance of CO_2 enrichment in enhancing the ex vitro survival and rooting percentages of in vitro-cultured *P. cynaroides* plantlets. In addition, the vegetative growth of plantlets ex vitro was significantly improved in CO_2-enriched plantlets, as shown by the number of new leaves formed. Compared with the control, at least twice as many new leaves were formed ex vitro by plantlets cultured under CO_2 enrichment conditions (Table 4). Our results showed that the high survival percentage and improvement in the overall growth of *P. cynaroides* during acclimatization was a direct result of CO_2 enrichment and the promotion of photoautotrophic growth in vitro.

Table 4. Effects of CO_2 enrichment on the ex vitro acclimatization of *P. cynaroides* plantlets after 60 d.

CO_2 enrichment (µmol·mol^{-1})	Survival (%)	Rooting (%)	New leaves/plantlet (no.)
Ambient[z]	20 a[y]	50 a[y]	3.0 ± 1.4 c[x]
1,000	90 b	89 a	6.0 ± 0.8 b
5,000	90 b	89 a	7.3 ± 0.9 a
10,000	80 b	88 a	8.2 ± 0.4 a

[z]Non-CO_2 enrichment.
[y]Percentages in the same column with different letters are significantly different (χ^2 at $P \leq 0.05$).
[x]Means in the same column with different letter are significantly different (Duncan's multiple range test at $P \leq 0.05$).

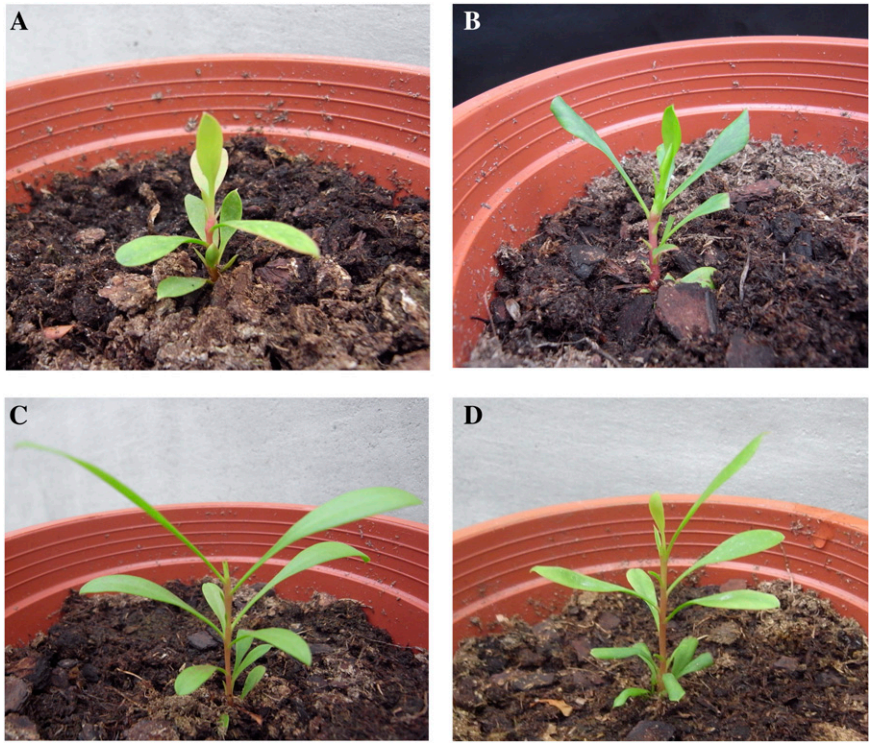

Fig. 1. Acclimatization of *P. cynaroides* plantlets in the greenhouse after 60 d. (**A**) Total of 0 µmol·mol^{-1} CO_2 (control); (**B**) 1,000 µmol·mol^{-1} CO_2; (**C**) 5,000 µmol·mol^{-1} CO_2; (**D**) 10,000 µmol·mol^{-1} CO_2. Bar = 1 cm.

In conclusion, *P. cynaroides* plantlets cultured photoautotrophically under elevated CO_2 on sucrose-free growth medium produced higher number of leaves, larger leaf areas, longer shoots with higher shoot weight, and had greater photosynthetic ability. Overall, these plantlets were of higher quality than those cultured on sucrose-containing growth medium without CO_2 enrichment. Plantlets enriched with CO_2 also survived better during acclimatization with improved root and leaf growth. Significant improvements in the overall growth of *P. cynaroides* plantlets were achieved through the use of photoautotrophic micropropagation with CO_2 enrichment.

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