行政院國家科學委員會專題研究計畫 成果報告

培養基質、二氧化碳施肥及發光二極體波長對光自營培養帝王花 (Protea cynaroides L.)組培苗之生長及馴化之影響

研究成果報告(精簡版)

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中文摘要： 本研究之目的即在探討二氧化碳施肥對光自營培養帝王花（Protea cynaroides L.）組培苗生長之影響，並探討多孔性材料對其生長發育之效應。本研究依二氧化碳濃度之供應量分為控制組（0 μmol mol-1 CO2）及實驗組（1,000；5,000及10,000 μmol mol-1 CO2），另以洋菜膠、珍珠石及矽砂三種培養基質進行生長測試。研究結果顯示，相較於對照組，供以5,000 及10,000 μmol mol-1 CO2 可明顯增加帝王花組培苗之葉片數量；葉片生長總面積之分析結果，以1,000 μmol mol-1 組較其它組別明顯為多；葉片生長總重量之分析結果，1,000 和 5,000 μmol mol-1 組皆優於對照。在葉綠素含量的結果顯示，10,000 μmol mol-1 組 chlorophyll a, chlorophyll b, 及 total chlorophyll 的含量最高，與其它組別有顯著差異。隨處理的CO2濃度提高，rubisco活性有減少的趨勢；相對的，無CO2供應組其rubisco活性最高。培養基質部分，於珍珠石及矽砂培養之組培苗，其葉片重量較洋菜膠組明顯為多；而洋菜膠組則有較高的葉綠素含量。所有植株於培養天45天後均可於溫室中馴化生長。本研究結果發現二氧化碳施肥可促進帝王花（P. cynaroides L.）組培苗之生長發育，對於提高帝王花繁殖成功率極具參考價值。

中文關鍵詞： 葉綠素含量； 組織培養； 珍珠石； 多孔材料； 1,5-二磷酸核酮糖羧化酶/加氧酶活性

英文摘要： This study was conducted to investigate the effects of CO2 enrichment on the photoautotrophic growth of Protea cynaroides plantlets in vitro. The effects of porous supporting material on their growth and development were also studied. Four CO2 enrichment treatments were used: 0 μmol mol-1 CO2 (control); 1,000 μmol mol-1 CO2； 5,000 μmol mol-1 CO2； 10,000 μmol mol-1 CO2. For the supporting material study, three treatments were used: agar； perlite； silica sand. Results showed that plantlets enriched with 5,000 and 10,000 μmol mol-1 CO2 produced significantly higher number of leaves than plantlets without CO2 enrichment (control). Furthermore, the leaf area (cm2/plantlet) of leaves formed on plantlets enriched with 1,000 μmol mol-1 CO2 was significantly higher than all the other treatments. Moreover, the leaf weight of plantlets enriched with 1,000 and 5,000 μmol mol-1 CO2 were higher than the

control treatment. In addition, the analysis of the chlorophyll content showed that leaves enriched with 10,000 μmol mol-1 CO_2 contained the highest amounts of chlorophyll a, chlorophyll b, and total chlorophyll. These were significantly higher than the chlorophyll contents of all the other treatments. The results of the rubisco activity analysis showed that the higher the CO_2 enrichment of the plantlets, the lower their rubisco activity. Consequently, the highest rubisco activity were detected in plantlets without CO_2 enrichment, whereas those enriched with 5,000 or 10,000 μmol mol-1 CO_2 were found to have the lowest activity. Results from the supporting material study showed that the leaf weight of plantlets cultured on perlite and sand were significantly higher than those grown on agar. In contrast, the chlorophyll contents of plantlets cultured on agar were higher than those on porous supporting materials. After 45 days in culture, all plantlets were successfully acclimatized to the greenhouse. In conclusion, the results of this study demonstrated that CO_2 enrichment significantly improved the vegetative growth of P. cynaroides plantlets in vitro, and is suitable to be used as a means to propagate this difficult-to-propagate plant.

英文關鍵詞： chlorophyll content； in vitro propagation； perlite； porous supporting material； rubisco activity

行政院國家科學委員會補助專題研究計畫 □期中進度報告 ☑期末報告

The effects of growth media, CO_2 enrichment and light-emitting diodes (LEDs) on the growth and acclimatization of photoautotrophically grown *Protea cynaroides* L. plantlets *in vitro*

培養基質、二氧化碳施肥及發光二極體波長對光自營培養帝王花（Protea cynaroides L.)組培苗之生長及馴化之影響

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計畫主持人：吳滄群
共同主持人：林群智
計畫參與人員：

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中　華　民　國　101 年　10　月　28　日

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摘要

本研究之目的即在探討二氧化碳施肥對光自營培養帝王花（*Protea cynaroides* L.）組培苗生長之影響，並探討多孔性材料對其生長發育之效應。本研究依二氧化碳濃度之供應量分為控制組（0 μmol mol^{-1} CO_2）及實驗組（1,000; 5,000 及 10,000 μmol mol^{-1} CO_2），另以洋菜膠、珍珠石及矽砂三種培養基質進行生長測試。研究結果顯示，相較於對照組，供以 5,000 及 10,000 μmol mol^{-1} CO_2 可明顯增加帝王花組培苗之葉片數量；葉片生長總面積之分析結果，以 1,000 μmol mol^{-1} 組較其它組別明顯為多；葉片生長總重量之分析結果，1,000 和 5,000 μmol mol^{-1} 組皆優於對照。在葉綠素含量的結果顯示，10,000 μmol mol^{-1} 組 chlorophyll *a*, chlorophyll *b*, 及 total chlorophyll 的含量最高，與其它組別有顯著差異。隨處理的 CO_2 濃度提高，rubisco 活性有減少的趨勢；相對的，無 CO_2 供應組其 rubisco 活性最高。培養基質部分，於珍珠石及矽砂培養之組培苗，其葉片重量較洋菜膠組明顯為多；而洋菜膠組則有較高的葉綠素含量。所有植株於培養天 45 天後均可於溫室中馴化生長。本研究結果發現二氧化碳施肥可促進帝王花（*P. cynaroides* L.）組培苗之生長發育，對於提高帝王花繁殖成功率極具參考價值。

關鍵字：葉綠素含量；組織培養；珍珠石；多孔材料；1,5-二磷酸核酮糖羧化酶/加氧酶活性

Abstract

This study was conducted to investigate the effects of CO_2 enrichment on the photoautotrophic growth of *Protea cynaroides* plantlets *in vitro*. The effects of porous supporting material on their growth and development were also studied. Four CO_2 enrichment treatments were used: 0 µmol mol^{-1} CO_2 (control); 1,000 µmol mol^{-1} CO_2; 5,000 µmol mol^{-1} CO_2; 10,000 µmol mol^{-1} CO_2. For the supporting material study, three treatments were used: agar; perlite; silica sand. Results showed that plantlets enriched with 5,000 and 10,000 µmol mol^{-1} CO_2 produced significantly higher number of leaves than plantlets without CO_2 enrichment (control). Furthermore, the leaf area (cm^2/plantlet) of leaves formed on plantlets enriched with 1,000 µmol mol^{-1} CO_2 was significantly higher than all the other treatments. Moreover, the leaf weight of plantlets enriched with 1,000 and 5,000 µmol mol^{-1} CO_2 were higher than the control treatment. In addition, the analysis of the chlorophyll content showed that leaves enriched with 10,000 µmol mol^{-1} CO_2 contained the highest amounts of chlorophyll *a*, chlorophyll *b*, and total chlorophyll. These were significantly higher than the chlorophyll contents of all the other treatments. The results of the rubisco activity analysis showed that the higher the CO_2 enrichment of the plantlets, the lower their rubisco activity. Consequently, the highest rubisco activity were detected in plantlets without CO_2 enrichment, whereas those enriched with 5,000 or 10,000 µmol mol^{-1} CO_2 were found to have the lowest activity. Results from the supporting material study showed that the leaf weight of plantlets cultured on perlite and sand were significantly higher than those grown on agar. In contrast, the chlorophyll contents of plantlets cultured on agar were higher than those on porous supporting materials. After 45 days in culture, all plantlets were successfully acclimatized to the greenhouse. In conclusion, the results of this study demonstrated that CO_2 enrichment significantly improved the vegetative growth of *P. cynaroides* plantlets *in vitro*, and is suitable to be used as a means to propagate this difficult-to-propagate plant.

Keywords: chlorophyll content; *in vitro* propagation; perlite; porous supporting material; rubisco activity

1. Introduction

Protea cynaroides L. (King Protea), which belongs to the Proteaceae family, is endemic to South Africa. It is an important cut flower in the international floriculture industry. Over the years, it has continued to fetch high prices in the flower market, while remaining a highly sought after cut flower. Currently, the majority of cut flowers sold in the market are still harvested from the wild. Overharvesting from the wild has led to dramatic decreases in the wild stocks of *P. cynaroides*. In addition to overharvesting, poor and indiscriminant harvesting techniques have caused severe damage to the natural environment. Furthermore, cut flowers harvested from the wild are of inconsistent quality and fluctuations in the supply are common occurrences. In order to provide high quality cut flowers and interrupted supply to the market, *P. cynaroides* plantations are increasingly being established. However, in the commercial production of *P. cynaroides* cut flowers, growers are faced with propagation problems, which are exacerbated by the lengthy time needed for the establishment of plants in the field. In addition to the fact that the King Protea is an extremely slow-growing plant, their nutritional requirements are unique, and difficult to manage (Littlejohn et al., 2002). Plants belonging to the Proteaceae family usually require sandy, well-drained soils with a low pH (3.5 to 5.8). Of particular importance is the phosphorous content of the soil. Normal application rates of phosphorous are toxic to *P. cynariodes*, which often result in death of the plant (Silber et al., 2001). In general, these plants have low mineral requirements and are therefore not tolerant to salt concentrations that would appear normal to other plants (Montarone and Allemand, 1995).

In order to maintain genetic uniformity, vegetative propagation using stem cuttings is the preferred method for growers to establish plants in the field. However, inconsistent rooting of cuttings and the prolonged time needed for root formation to take place are the main problems faced by growers. Rooting of *P. cynaroides* cuttings usually take six months, while low rooting percentages are a frequent occurrence. Furthermore, newly established plants in the field need 3 years to start producing its first inflorescence, while the first marketable inflorescences are usually only produced after 4 – 5 years. Recently, several methods of *in vitro* propagation of *P. cynaroides* explants have been studied. For example, due to the poor germination rates of *P. cynaroides* seeds in soil, investigations into the *in vitro* germination of excised zygotic embryos were conducted. Results showed that the use of alternating temperatures (12°C/21°C) significantly improved germination percentage, irrespective of growth regulators or light conditions (Wu and du Toit, 2010). In addition, zygotic embryos and cotyledon explants were found to possess high pre-determined embryogenic competency where direct induction of somatic embryos were achieved on *P. cynaroides* cotyledon and zygotic explants cultured on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) without the addition of growth hormones (Wu et al., 2007a). The source explants used in these studies were derived from zygotic embryos and cotyledons, which varies in genotype. To ensure genetic uniformity, the use of vegetative explants is required, and thus micropropagation of vegetative materials such as apical or axillary buds is preferred. *In vitro* establishment of nodal explants taken from stem cuttings has been studied with the aim to stimulate the growth of axillary buds (Wu and du Toit, 2004). Results showed that oxidative browning of nodal explants caused by the leaching of phenolic compounds was a major obstacle to successful explant establishment, causing the death of the explants.

This difficulty was overcome by soaking the nodal explants in antioxidant solution (1500 mg L^{-1} citric acid and 100 mg L^{-1} ascorbic acid) for 1 h before planting. This pre-treatment strongly inhibited oxidative browning and led to the successful sprouting of axillary buds when cultured on MS medium supplemented with 30 mg L^{-1} GA_3 (Wu and du Toit, 2004). Microshoots were subsequently micrografted onto seedling-derived roots (Wu et al., 2007b). Although 3,4-dihydroxybenzoic acid was found to be a regulator of root formation in stem cuttings *in vivo* (Wu et al., 2007c), their effects on *in vitro* rooting of microshoots have not been investigated.

Photoautotrophic propagation of explants is defined as propagation in sugar-free medium (Zobayed et al., 2004). Sucrose, which is the most commonly used carbon source in plant tissue culture, promotes plantlet growth but depresses 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. Carbohydrate accumulation is often found to be vital in root formation. In *P. cynaroides*, a correlation was found between high carbohydrate accumulation and rooting of stem cuttings (Wu et al., 2006). 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; (4) reduction in microbial contamination. Recent studies that have successfully cultured plants under photoautotrophic conditions include: *Dendrobium candidum* (Xiao et al., 2007), *Momordica grosvenori* (Zhang et al., 2009), *Noppalea cochenilifera* (Houllou-Kido et al., 2009) and *Uniola paniculata* (Valero-Aracama et al., 2007).

Of particular importance is that growing plantlets photoautotrophically on medium without sucrose enables the development of fully functional photosynthetic apparatus (Pospíšilová et al., 1999). However, simply removing sugar from the medium without increasing the light intensity and CO_2 concentration inside the culture vessel would not raise the net photosynthetic rate (Xiao et al., 2010). Therefore, a combination of CO_2 enrichment and increased light intensity is usually supplied to plantlets growing photoautotrophically (Pospísilová et al., 1999). A frequently observed response to elevated CO_2 is the increase in photosynthesis and the production of leaves with a higher C:N ratio (Curtis, 1996), while improvements in stomatal conductance and increases in chlorophyll content are also often seen. Kadleček et al. (1998) reported increases in chlorophyll *a* and chlorophyll *b* contents of *Nicotiana tabacum* explants. Similarly, higher chlorophyll content and stomatal conductance were found in CO_2-enriched *Cymbidium* plantlets (Norikane et al., 2010). Common methods of increasing CO_2 concentration are by natural ventilation and forced ventilation. Natural ventilation is carried out by attaching a gas permeable film on the lip or wall of culture vessels and increasing the CO_2 concentration around the culture vessels to elevate the *in vitro* CO_2 concentration (Tichá, 1996). A number of studies have reported the benefits of elevated CO_2 concentrations through natural ventilations on plantlet growth and quality: *Gerbera jamesonii* (Liao et al., 2007), *Myrtus communis* (Lucchesini et al., 2006) and *Saccharum* spp. (Xiao et al., 2003).

Forced ventilation is achieved by directly supplying CO_2 into the vessels (Solárová and Pospíšilová, 1997). Xiao and Kozai (2004) found that growth period of forced-ventilated *Zantedeschia elliottiana* plantlets was reduced by 50%, while the leaf area, shoot and root dry weight were, respectively, 5.2, 4.6 and 3.8 times higher than those

cultured under a conventional micropropagation system. In addition, the net photosynthetic rate and chlorophyll concentration of *Gerbera jamesonii* plantlets were, respectively, 9.2 and 2.2 times greater than those cultured photomixotrophically (Xiao et al., 2005). Moreover, the survival percentage during *ex vitro* acclimatization was 95% compared to 60% for photomixotrophic cultures. Similarly, increased 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), *Ficus benjamina* (Matysiak and Nowak, 1998), and *Rubus idaeus* (Deng and Donnelly, 1993) plantlets during acclimatization to *ex vitro* conditions.

Supporting materials that have been used instead of those with gelling properties such as agar, include: vermiculite, perlite, sand and paper pulp. The common characteristic of these supporting materials is their high porosity. The use of supporting materials with high porosity improves the root zone environment due to the higher oxygen concentration available (Fujiwara and Kozai 1995). Therefore, development of the root system is promoted, and water and nutrient absorption of the plantlets are enhanced. As a result, overall growth of plantlets is improved (Afreen-Zobayed et al., 1999). Findings by Yu et al. (2000) showed that the rooting percentage of *Carica papaya* explants cultured in vermiculite (90%) was significantly higher than those grown on agar (62.2%). Furthermore, Afreen-Zobayed et al. (2000) found that the root dry weight of sweet potato plantlets cultured on vermiculite were at least 2 times greater than those grown on agar. In addition, due to the improved anatomical characteristics and physiological functions of the root systems formed in porous supporting material, higher survival percentage of plantlets were observed during acclimatization of plantlets to the greenhouse (Kirdmanee et al. 1995; Nguyen et al., 1999; Yu et al., 2000).

From the literature review above, it is clear that photoautotrophic micropropagation and porous supporting materials are beneficial to the growth of explants *in vitro*. For slow-growing *P. cynaroides* explants, improving their growth and development *in vitro* is of utmost importance. Therefore, this study was conducted to investigate the effects of CO_2 enrichment and supporting materials on the growth of *P. cynaroides* plantlets *in vitro*.

2. Materials and Methods

2.1. Experiment 1: Effects of CO_2 enrichment on the photoautotrophic growth of *P. cynaroides* plantlets *in vitro*

2.1.1. Plant material and culture conditions

In vitro established *P. cynaroides* plantlets (terminal microshoots), which have been maintained in culture for over six months, were used in this study. Unrooted plantlets with six leaves were collected and placed onto growth medium. Modified glass desiccators (20 L) (dimensions: 25 cm I.D.; 20 cm H) consisting of a customized inlet and outlet connector at the lid were used as culture vessels (Fig. 1). The inlet connector of each desiccator was connected to a CO_2 gas cylinder (60 L) via silicone pipes (i.d. 60 mm). The outlet connector was also connected to silicone pipes, which allowed CO_2 to exit the culture room. Gas cylinders containing a mixture of different

concentrations of CO_2 were obtained from Jing De Gas Inc. Four CO_2 enrichment treatments were used: 0 µmol mol^{-1} CO_2 (control), 1,000 µmol mol^{-1} CO_2, 5,000 µmol mol^{-1} CO_2, and 10,000 µmol mol^{-1} CO_2. The CO_2 flow rate was controlled with an air flow meter (New-Flow Technologies Inc., Taiwan) and set at 0.5 L h^{-1}. A timer connected to an electronic valve was used to regulate the flow of CO_2 (Fig. 1). Gas flow was set every six hours for 15 min each. In all treatments, half-strength Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) supplemented with 0.5 mg L^{-1} 6-benzylaminopurine (BAP), 0.01 mg L^{-1} naphthalene acetic acid (NAA), 5 mg L^{-1} silver nitrate, 100 mg L^{-1} meso-inositol, fresh banana (70 g L^{-1}), activated charcoal (2 mg L^{-1}), 100 mg L^{-1} ascorbic acid (filter-sterilized), and agar (9 mg L^{-1}), were used. In the control treatment, where a completely sealed desiccator was used, sucrose (30 g L^{-1}) was included in the growth medium. The pH of all growth media was adjusted to 5.5 before autoclaving at 121℃ and 104 KPa for 25 min. Two liters of growth medium was dispensed into the desiccators. Desiccators were placed in a culture room with the temperature and photoperiod adjusted to 25℃±2 and 16 h, respectively. The photosynthetic photon flux (PPF) was adjusted to 70 µmol sec^{-1} m^{2} in all treatments.

2.1.2. 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 PVP (polyvinylpyrrolidone), 0.1% (v/v) Triton X-100 and 5 mM dithiothreitol (DTT; added at 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 (CPK), 15 U phosphoglycerate kinase, 6U glyceraldehyde-3-phosphate dehydrogenase (GADPH), 5 mM ATP and 5 mM DTT (added at last). An aliquot of 0.05 g of leaf sample 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 a UV-Vis spectrometer (GENESYS 10S UV-Vis, Thermo Fisher Scientific Inc., USA), followed by mixing with 50 µL of 0.2 mM NADH. A background rate of NADH oxidation was determined by recording the absorbance at 340 nm (A_{340}) every 20 seconds for 3 min. An aliquot of 50 µL of 0.5 mM ribose-5-phosphate was then added for reaction. After a delay of 3s, the decreasing rate of NADH was monitored at A_{340} every 20 seconds for 3 min. Rubisco activity was calculated by converting the net decreasing rate at A340 to a rate of NADH oxidation according to Usuda et al (1984).

2.1.3. Chlorophyll content determination

The concentration of chlorophyll was determined according to Wintermans and 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 1,000 G of centrifugation at 4°C for 15 min, an aliquot of 1 mL of supernatant was analyzed with a UV-Vis spectrometer (Helios Alpha, Thermo Fisher Scientific Inc., USA) 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. The concentration of chlorophyll *a* and *b* was calculated with Eq. 1 and Eq. 2, respectively. Total chlorophyll concentration was calculated with Eq. 3.

$$[\textbf{Chlorophyll a}]\left(\frac{\textbf{mg}}{\textbf{gFW}}\right) = \frac{(13.7A_{665}-5.76A_{649})\times 50}{1000\times 0.05} \quad (1)$$

$$[\textbf{Chlorophyll b}]\left(\frac{\textbf{mg}}{\textbf{gFW}}\right) = \frac{(25.8A_{649}-7.6A_{665})\times 50}{1000\times 0.05} \quad (2)$$

$$[\textbf{Total chlorophyll}]\left(\frac{\textbf{mg}}{\textbf{gFW}}\right) = \frac{(6.1A_{665}-20.04A_{649})\times 50}{1000\times 0.05} \quad (3)$$

2.1.4. Acclimatization

Plantlets grown *in vitro* were transferred to the greenhouse for acclimatization. Plantlets were grown in plastic pots containing a mixture of bark and peat (1:1; v:v). The temperature of the greenhouse was maintained at 27℃±2.

2.1.5. Statistical analysis

One plantlet per culture vessel was used in each treatment, with ten replications. A completely randomized design was used in all treatments. Data for number of leaves, leaf area, leaf dry weight, rubisco activity, and chlorophyll content were collected after 45 days in culture. Data were analyzed using Duncan's Multiple Range test to compare treatment means using SAS (SAS Institute Inc., 1996).

2.2. Experiment 2: Effects of supporting material on the growth of *P. cynaroides* plantlets *in vitro*

2.2.1. Plant material and culture conditions

P. cynaroides plantlets germinated from embryos were used as explants. Surface-sterilization and *in vitro* excision of the embryos were done according to Wu et al. (2007b). Plantlets with three true leaves and roots were placed into liquid half-strength MS medium containing 0.01 mg L^{-1} NAA, 0.5 mg L^{-1} BAP, 5 mg L^{-1} silver nitrate, 100 mg L^{-1} meso-inositol, 70 g L^{-1} fresh banana, 30 g L^{-1}sucrose, 2 g L^{-1} activated charcoal, and 100 mg L^{-1} ascorbic acid (filter-sterilized). The pH of all growth media was adjusted to 5.5 before autoclaving at 121℃ and 104 KPa for 25 min. Three supporting material treatments were used: Agar (9 g L^{-1}), perlite (13 g), and sand (255 g). Growth medium, which were dispensed into glass culture vessels (dimensions: i.d., 15 cm; h, 25 cm), were used in the following amounts: agar (50 mL), perlite (80 mL), and sand (50 mL). The culture vessels were placed in a culture room with the temperature and photoperiod adjusted to 25℃±2 and 16 h, respectively. The photosynthetic photon flux (PPF) was adjusted to 50 μmol sec^{-1} m^2 in all treatments.

2.2.2. Acclimatization

Plantlets grown *in vitro* were transferred to the greenhouse for acclimatization. Plantlets were grown in plastic pots containing a mixture of bark and peat (1:1; v:v). The temperature of the greenhouse was maintained at 27°C±2.

2.2.3. Statistical analysis

One plantlet per culture vessel was used in each treatment, with ten replications. A completely randomized design was used in all treatments. Data for number of leaves, leaf area, leaf dry weight, and chlorophyll content (SPAD value, Minolta, Japan) were collected after 45 days in culture. Data were analyzed using Duncan's Multiple Range test to compare treatment means using SAS (SAS Institute Inc., 1996).

3. Results and Discussion

3.1. Experiment 1: Effects of CO_2 enrichment on the growth of *P. cynaroides* plantlets *in vitro*

Results showed that *P. cynaroides* plants enriched with 5,000 µmol mol^{-1} CO_2 and 10,000 µmol mol^{-1} CO_2 produced significantly higher number of new leaves than those without CO_2 enrichment (Fig. 2). In contrast, the number of new leaves produced by plantlets enriched with only 1,000 µmol mol^{-1} CO_2 was similar to those in the control treatment. Similar results were reported in sweet potato where significantly higher number of leaves was formed in cultures that were enriched with CO_2 (Zobayed et al. 1999). In *Cymbidium* explants, findings by Norikane et al. (2010) also showed that explants enriched with 3,000 µmol mol^{-1} CO_2 or 10,000 µmol mol^{-1} CO_2 produced significantly higher number of leaves. The leaf area (cm^2/leaf) of plantlets enriched with 1,000 µmol mol^{-1} CO_2 were significantly higher than all the other treatments, which suggests that although fewer leaves were produced, larger leaves were formed in this treatment (Fig. 3). Similarly, the highest leaf weight (mg/plantlet) was found in plantlets enriched with 1,000 µmol mol^{-1} CO_2 (Fig. 4). Zobayed et al. (1999) also reported significantly higher leaf weight and leaf area in sweet potato plants that were enriched with CO_2. Similarly, the leaf weight and leaf area of banana plantlets were also significantly higher when CO_2 enrichment was used (Navarro et al., 1994). Furthermore, in our study, although the leaf weight of plantlets enriched with 5,000 µmol mol^{-1} CO_2 was similar to those enriched with 1,000 µmol mol^{-1} CO_2, this may have been due to the thickness of the leaves. Fig. 5 illustrates the chlorophyll content of leaves in the different treatments. Overall, results showed that leaves of plantlets enriched with 10,000 µmol mol^{-1} CO_2 contained the highest amounts of chlorophyll *a* and chlorophyll *b*. These results are in agreement with studies by Norikane et al. (2010), which reported higher chlorophyll content in leaves of CO_2-enriched *Cymbidium* plantlets. However, contrasting results were found in banana plantlets, where significantly lower chlorophyll content was detected in explants that were enriched with CO_2 (Navarro et al., 1994).

In terms of rubisco activity, our results showed higher activity in leaves of plantlets in the control treatment compared to those enriched with CO_2 (Fig. 6). Similar results were found in Cymbidium plantlets (Norikane et al. (2010) where the lowest rubisco activity were recorded in leaves exposed to 10,000 $µmol^{-1}$ CO_2. These results are also

in agreement with Campbell et al. (1998), where lower rubisco activity were found in soybean plantlets exposed to higher CO_2 concentrations. In addition, our results showed very similar rubisco activity between plantlets cultured in ambient CO_2 (control) and those enriched with 1,000 μmol^{-1} CO_2. Vegetative growth of *P. cynaroides* plantlets enriched with different concentrations of CO_2 after 45 days in culture are shown in Fig. 7. As shown in Fig. 2, these results show a higher number of leaves formed in plantlets enriched with 5,000 and 10,000 μmol^{-1} CO_2, compared to the control treatment. After 45 days in culture, plantlets were transferred to the greenhouse for acclimatization (Fig. 8).

3.2. Experiment 2: Effects of supporting material on the growth of *P. cynaroides* plantlets *in vitro*

Results showed that plantlets grown in agar produced significantly higher number of new leaves, compared to those cultured in perlite and sand (Table 1). It is probable that plantlets produced a lower number of leaves in perlite and sand may be due to the lack of a root system in these plantlets. As a result, with these plantlets being grown in porous supporting materials, and without direct contact with the liquid growth medium, poor absorption of nutrients could be the primary reason for their lack of vegetative growth. In contrast, the leaf dry weights of plantlets grown on porous supporting material (perlite and sand) were significantly higher than those in agar. However, no significant differences in leaf area (cm^3) were found between plantlets in the different treatments. These results are in contrast with those reported by Xiao and Kozai (2006) where significantly higher leaf areas were found in plantlets cultured in porous supporting material, compared to those grown in agar. On the other hand, our results similar to those reported by Afreen-Zobayed et al. (2000) where significantly lower leaf weight were found in leaves of sweet potato plantlets cultured in agar compared to those in porous supporting material. In our study, although the thickness of the leaves were not measured, it is likely that the differences in leaf weight may have been due to leaf thickness. In terms of chlorophyll content (SPAD value), leaves of plantlet cultured in agar were significantly higher than those grown in sand (Table 1). No significant differences were found in chlorophyll content between plantlets grown in agar and perlite. Growth of *P. cynaroides* plantlets *in vitro* are shown in Fig. 9. Results of this study indicate that without the presence of roots in the plantlets, a direct contact between the shoot and liquid media used in the porous supporting material treatments is particularly important. In other studies such as those in statice plantlets, where root formation was present, plantlets cultured on porous supporting material performed better than those on agar (Xiao and Kozai, 2006).

4. Conclusion

Results of this study clearly showed the beneficial effects of CO_2 enrichment on the growth and development of *P. cynaroides* plantlets *in vitro*. The number of leaves formed, leaf area, leaf weight, and chlorophyll content of plantlets enriched with either 1,000, 5,000 or 10,000 $\mu mol^{-1} CO_2$ were found to be significantly higher than those without elevated CO_2. These results provided a better understanding of how CO_2 enrichment improves the growth of *P. cynaroides* plantlets, which are known to be difficult to propagate *in vitro*. The results of this investigation have contributed a step closer toward mass producing *P. cynaroides* explants in an *in vitro* environment.

During the course of this research, a scientific paper was published in a peer-review journal (Wu and du Toit, 2012) [Appendix A].

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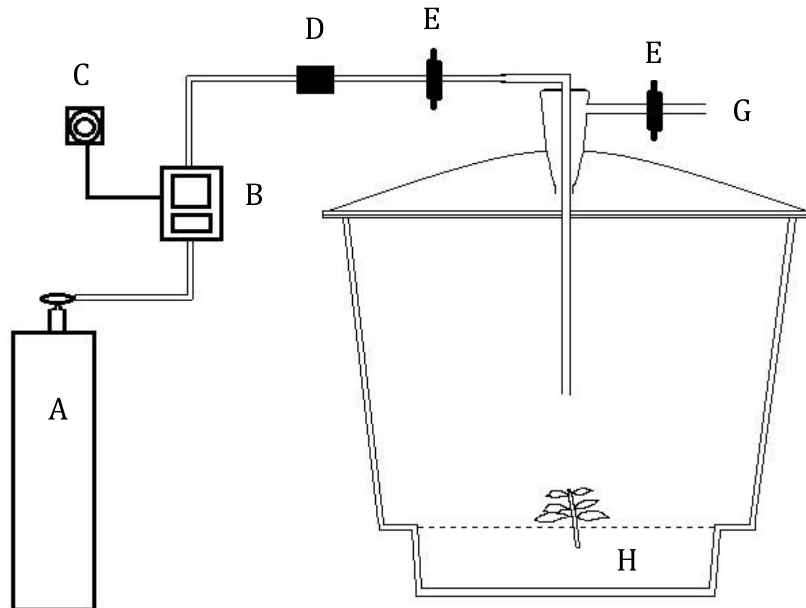

Fig. 1. Modified desiccator used as culture vessel. A. CO_2 gas cylinder; B. Air flow meter; C. Timer; D. Valve; E. Filter; F. Inlet; G. Outlet; H. Growth medium.

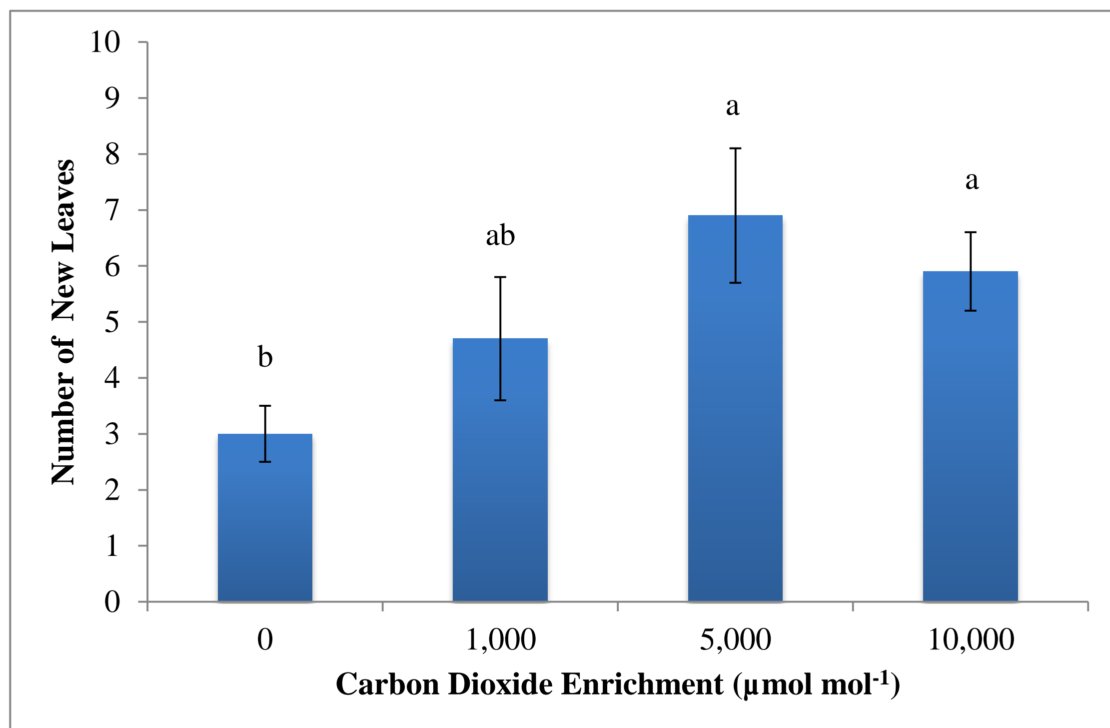

Fig. 2. Effect of CO_2 enrichment on leaf formation in *P. cynaroides* plantlets after 45 days in culture. Different letters indicate significant difference at $P \leqq 0.05$ based on Duncan's Multiple Range test.

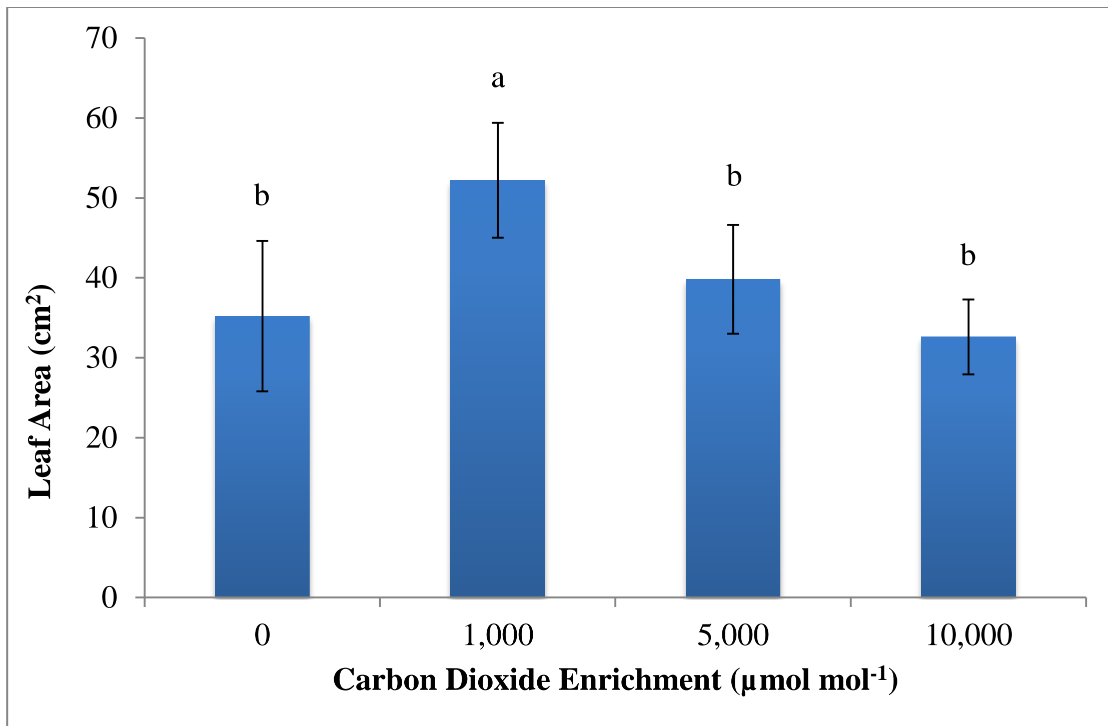

Fig. 3. Effect of CO_2 enrichment on leaf area of *P. cynaroides* plantlets after 45 days in culture. Different letters indicate significant difference at $P \leqq 0.05$ based on Duncan's Multiple Range test.

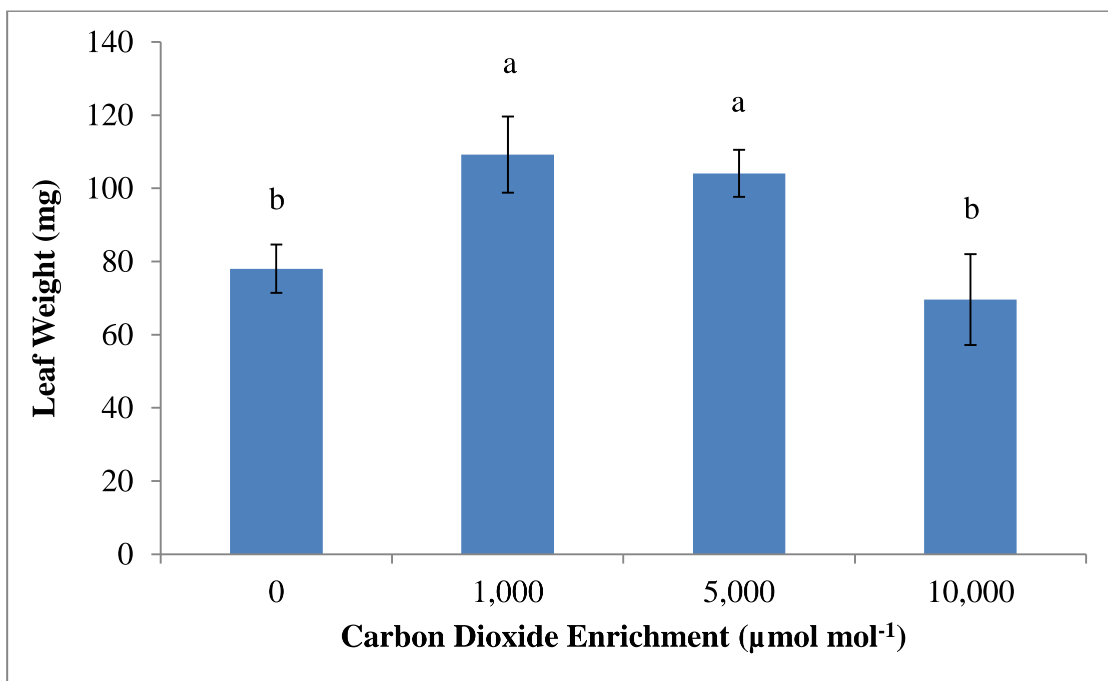

Fig. 4. Effect of CO_2 enrichment on leaf weight of *P. cynaroides* plantlets after 45 days in culture. Different letters indicate significant difference at $P \leqq 0.05$ based on Duncan's Multiple Range test.

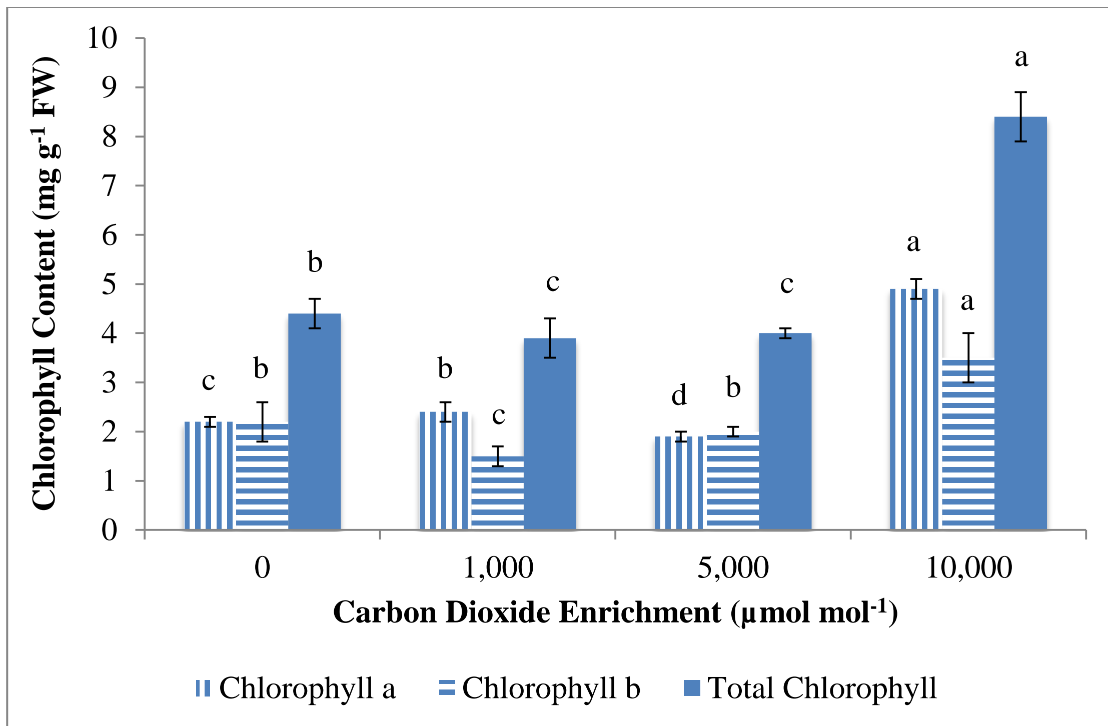

Fig. 5. Effect of CO_2 enrichment on the chlorophyll content of *P. cynaroides* plantlets after 45 days in culture. Different letters indicate significant difference at $P \leqq 0.05$ based on Duncan's Multiple Range test.

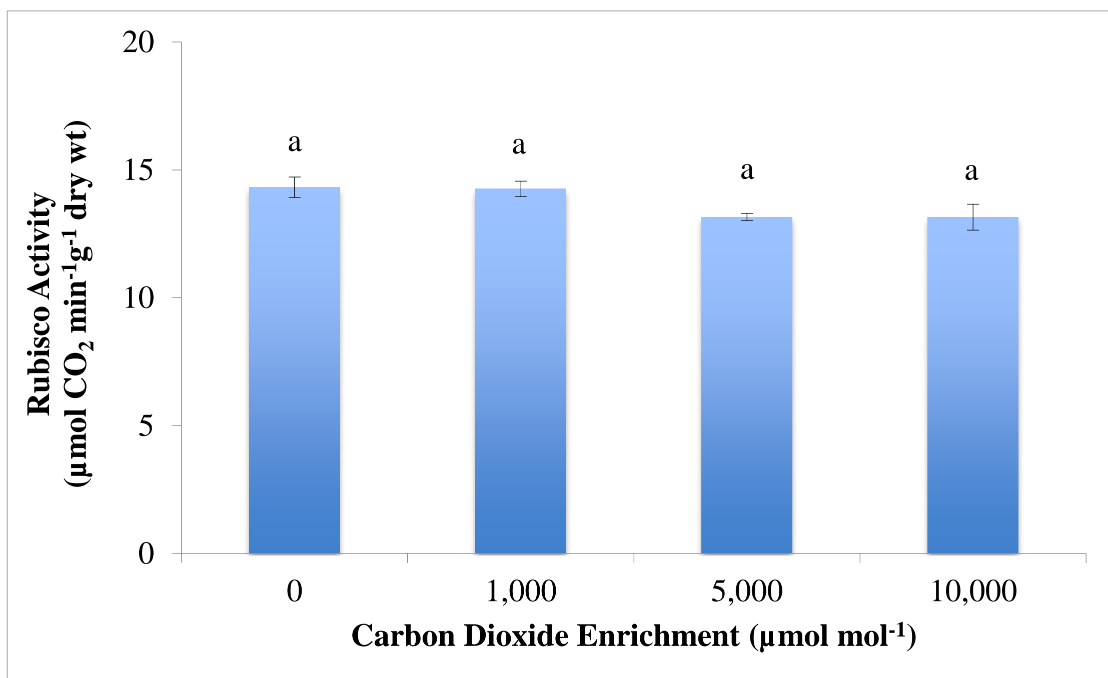

Fig. 6. Effect of CO_2 enrichment on the rubisco activity of *P. cynaroides* plantlets after 45 days in culture. Different letters indicate significant difference at $P \leqq 0.05$ based on Duncan's Multiple Range test.

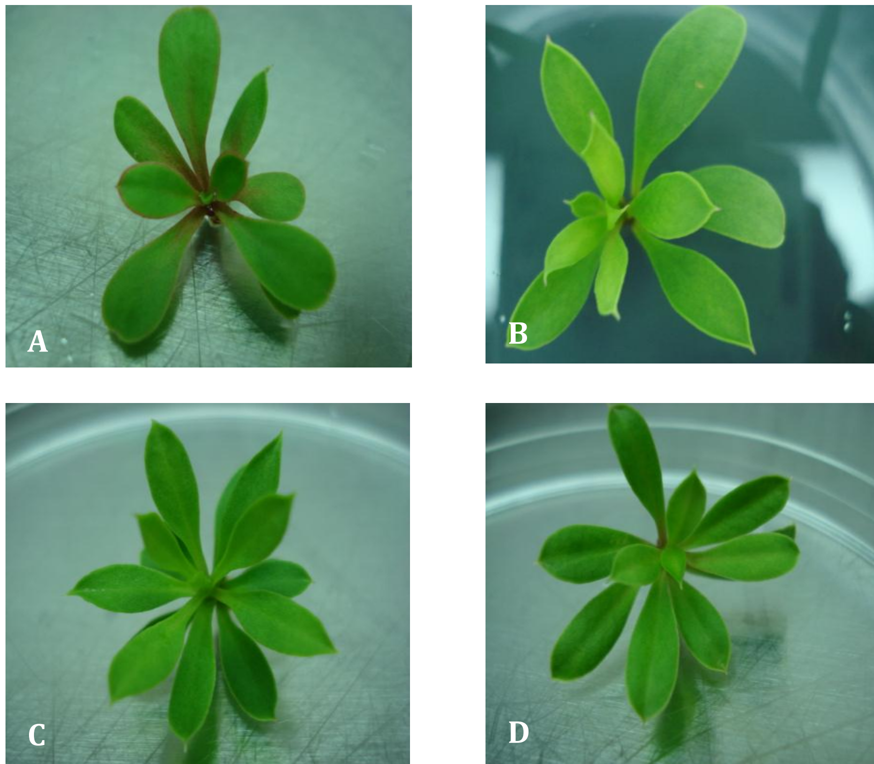

Fig. 7. Growth of *P. cynaroides* plantlets under different CO_2 enrichment concentrations after 45 days in culture. A. 0 µmol mol^{-1} CO_2 (control); B. 1,000 µmol mol^{-1}; C. 5,000 µmol mol^{-1}; CO_2; D. 10,000 µmol mol^{-1} CO_2.

Fig. 8. Acclimatization of *P. cynaroides* plantlets. A. 0 µmol mol^{-1} CO_2 (control); B. 1,000 µmol mol^{-1}; C. 5,000 µmol mol^{-1}; CO_2; D. 10,000 µmol mol^{-1} CO_2.

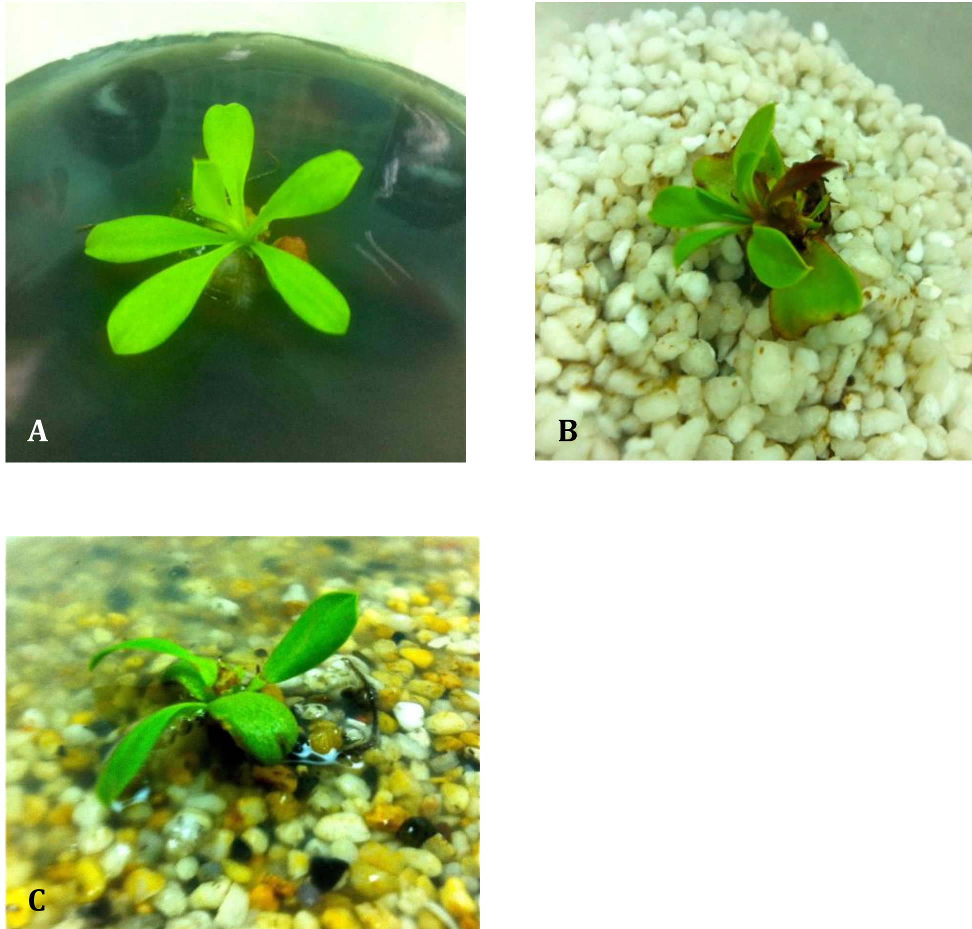

Fig 9. Growth of *P. cynaroides* plantlets on different supporting materials. A. Agar; B. Perlite; C. Sand.

Table 1. Effects of different supporting materials on the growth of *P. cynaroides* plantlets after 45 days in culture. Different letters in each column indicate significant difference at $P \leqq 0.05$ based on Duncan's Multiple Range test.

Treatment	No. of new leaves	Leaf weight (mg/leaf)	Leaf area (cm^2/leaf)	Chlorophyll content (SPAD value)
Agar	5.7 a	16.7 b	36.2 a	32.0 a
Perlite	1.0 b	33.0 a	35.7 a	27.7 ab
Sand	0.8 b	26.1 a	35.3 a	23.0 b

APPENDIX

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Full Length Research Paper

In vitro multiplication of *Protea cynaroides* L. microshoots and the effects of high phosphorous concentration on explant growth

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***Protea cynaroides* L. is a slow-growing, difficult-to-propagate plant. Due to problems such as phenolic browning and their sensitivity to the phosphorous nutrient, *in vitro* multiplication of *P. cynaroides* explants have not been successful. The present study was conducted to induce shoot proliferation of established *P. cynaroides* microshoots, and investigate the effects of high phosphorous concentration during explant multiplication. Microshoots with either one or two nodes were cultured on Murashige and Skoog (MS) medium containing modified macronutrients and full strength micronutrients. Two concentrations of $NH_4H_2PO_4$ were tested: 0 mg L^{-1} $NH_4H_2PO_4$, and a high P concentration of 1400 mg L^{-1} $NH_4H_2PO_4$. Both growth media were also supplemented with gibberellic acid (GA_3) (30 mg L^{-1}), 6-benzylaminopurine (BAP) (2 mg L^{-1}), ethylenediaminetetraacetic acid (EDTA) (50 mg L^{-1}) and indole-butyric acid (IBA) (0.5 mg L^{-1}). Results show that, contrary to what is often reported, the presence of a high phosphorous concentration in the growth media did not adversely affect *P. cynaroides* explants. The survival rate and mean axillary shoot length of explants cultured on growth media containing 1400 mg L^{-1} $NH_4H_2PO_4$ were not significantly different from those grown on 0 mg L^{-1} $NH_4H_2PO_4$. No phosphorous toxicity symptoms were observed in explants cultured on media with high phosphorous levels. Results also show that explants with two nodes had a higher survival rate and produced significantly longer axillary shoots than those with one node, irrespective of phosphorous concentration. Multiplication of *P. cynaroides* microshoots was successfully achieved for the first time.**

Key words: King Protea, micropropagation, Proteaceae, shoot proliferation.

INTRODUCTION

Protea cynaroides L. (King Protea), which is a member of the Proteaceae family, is an important cut flower in the floriculture industry. Proteaceae plants are usually found in low-nutrient, acidic soils in their natural environment (Cowling and Holmes, 1991). In particular, phosphorous (P) levels in these soils are very low (Witkowski and Mitchell, 1987). It is well known that plants belonging to the Proteaceae family are sensitive to P nutrition (Silber et al., 2001). It is often reported that high P concentrations are harmful to Proteaceae plants, which result in the development of P toxicity (Hawkins et al., 2008; Montarone and Allemand, 1995; Montarone and Ziegler, 1997; Nichols et al., 1979). In fact, P fertilization is not recommended when growing Proteaceae plants (Littlejohn, 2000).

Regarding *in vitro* propagation, very few researches have focused on the effects of P on the growth of Proteaceae plants. A reduction of P concentrations in the growth medium is usually applied when Proteaceae

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Figure 1. Growth and elongation of axillary bud during establishment of *P. cynaroides* nodal shoot segment.

plants are propagated *in vitro*. In a study by Thillerot et al. (2006), the macro-nutrients, with P in particular, were greatly reduced in the growth medium used to propagate *Leucospermum* (Proteaceae). Similarly, success was achieved by using reduced Murashige and Skoog (MS) (Murashige and Skoog, 1962) macro-nutrients and full strength micro-nutrients to propagate *Protea repens* (Rugge, 1995) and *Telopea speciosissima* (Seelye et al., 1986).

Due to its slow-growing nature and particular nutritional needs, limited success has been achieved in the *in vitro* propagation of *P. cynaroides* explants. Furthermore, phenolic browning of explants is often reported to be a limiting factor that severely affects the survival rates and growth of *P. cynaroides* explants *in vitro* (Thillerot et al., 2006). Previous studies reported the successful of *in vitro* establishment of *P. cynaroides* (Ben-Jaacov and Jacobs, 1986; Thillerot et al., 2006), however, further multi-plication of these explants has not been achieved. In addition, no information on the effects of high P concen-trations during *in vitro* propagation of *P. cynaroides* is available.

The aim of this study was to induce bud proliferation in established *P. cynaroides* nodal explants, and determine the effects of high P concentration on the survival rate and axillary shoot growth of two types of explants.

MATERIALS AND METHODS

Explant establishment

Shoots were taken from 1-year-old *P. cynaroides* plants grown in a greenhouse with the temperature maintained at 22 to 25°C. After removing the leaves, each shoot was cut into 1 cm long segments with one or two nodes, and placed under running water for 2 h. Each nodal segment was then dipped into 70% ethanol for 10 s, and stirred in 0.35% sodium hypochlorite for 6 min. Afterwards, the explants were placed in filter-sterilized antioxidant solution containing 100 mg L^{-1} ascorbic acid and 1500 mg L^{-1} citric acid for 1 h. The nodal explants were then transferred to half-strength MS medium supplemented with gibberellic acid (GA_3) (30 mg L^{-1}), 6-benzylaminopurine (BAP) (2 mg L^{-1}), myo-inositol (100 mg L^{-1}), ethylenediaminetetraacetic acid (EDTA) (50 mg L^{-1}), sucrose (20 g L^{-1}), activated charcoal (3 g L^{-1}), and Gelrite (3 g L^{-1}). The pH of the medium was adjusted to 5 before autoclaving at 104 KPa at 121°C for 20 min. The explants were cultured in glass test tubes containing 10 ml of growth medium, and placed in a growth chamber. A 16-h photoperiod was used with the temperature maintained at 25 ± 2°C. Cool white fluorescent tubes provided 60 µmol m^{-2} s^{-1} photosynthetically active radiation (PAR).

Explant multiplication

For the multiplication stage, elongated axillary buds (microshoots) of established explants (Figure 1) were cut into shorter sections and subcultured to multiplication media to induce axillary shoot growth. Two types of microshoot explants were used: microshoot sections with one node or two nodes. A basal MS medium consisting of modified macro-nutrients, full strength micro-nutrients and vitamins was used: NH_4NO_3 (23 mg L^{-1}), KNO_3 (51 mg L^{-1}), $MgSO_47H_2O$ (370 mg L^{-1}), KH_2PO_4 (0 mg L^{-1}), KI (830 mg L^{-1}), and $CaCl_24H_2O$ (440 mg L^{-1}). KH_2PO_4 was substituted with $NH_4H_2PO_4$ in the growth medium. Two concentrations of $NH_4H_2PO_4$ were tested: 0 mg L^{-1} (control) and 1400 mg L^{-1}. The following were also included in the medium: GA_3 (30 mg L^{-1}), BAP (2 mg L^{-1}), EDTA (50 mg L^{-1}), indole-butyric acid (IBA) (0.5 mg L^{-1}), sucrose (30 g L^{-1}) and Gelrite® (3 g L^{-1}). The pH was adjusted to 5 before autoclaving for 20 min. Microshoots with either one or two nodes were planted into test tubes containing 10 ml medium. The explants were placed into a growth chamber with a 16-h photoperiod. Cool white fluorescent tubes were used as the light source providing 60 µmol m^{-2} s^{-1} PAR at 30 cm above plant height, and the temperature was maintained at 28 ± 2°C.

Statistical analysis

A completely randomized design with 20 explants per treatment was used. Data for survival rate (%) and axillary shoot length were recorded after 60 days in culture. Significant differences in the survival percentage between treatments were tested using Chi-square analysis. Treatment means for axillary shoot length were separated using Tukey's studentized test at 5% level of significance. All statistical analyses were performed using the Statistical Analysis System (SAS) program (SAS Institute Inc., 1996).

RESULTS AND DISCUSSION

After 60 days in the multiplication medium, growth and elongation of new axillary shoots were observed on microshoots in all media treatments (Figure 2). No P

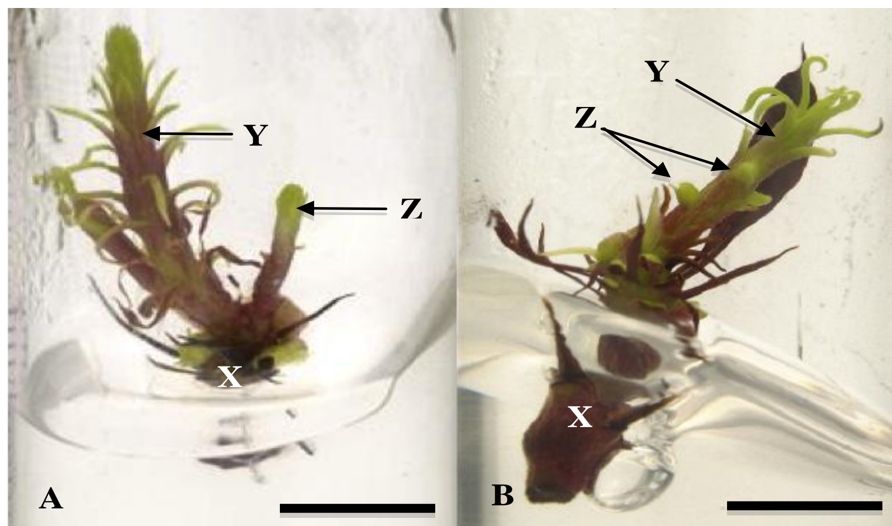

Figure 2. Modified MS medium with (A) 0 mg L^{-1} $NH_4H_2PO_4$ and (B) 1400 mg L^{-1} $NH_4H_2PO_4$. X, Microshoot with two nodes from established explant; Y, formation of new axillary shoot after 60 days in culture; Z, formation of new buds and shoots on new axillary shoot. Bar = 1 cm.

toxicity was observed in any of the media treatments. According to Hawkins et al. (2008), the main effects of P toxicity on Proteaceae plants are necrosis, chlorosis, stunted growth and rosetting. None of these P toxicity symptoms were observed at any time during this investigation.

Results show that the survival rates of the microshoots with two nodes were significantly higher than those with one node, irrespective of the P concentration (Table 1). Furthermore, microshoots with two nodes also produced significantly longer axillary shoots than those with only one node. Explants with two nodes cultured on media without $NH_4H_2PO_4$ produced axillary shoots that were up to five times longer than those with one node, while those grown on media containing 1400 mg/L $NH_4H_2PO_4$ were three times longer (Table 1).

Similar to the survival rate, no significant differences were found between the mean lengths of the new axillary shoots formed on microshoots with the same number of nodes, despite the $NH_4H_2PO_4$ concentration (Table 1 and Figure 2). These results demonstrate that the number of nodes on the microshoot explant is an important factor affecting explant survival rate, as well as the mean length of new axillary shoot formed. It is probable that the explants with two nodes are physiologically stronger with more actively growing tissues and contain more nutrient reserves to start growing, which gave them a better chance of survival. No root formation was observed on any of the explants in all media treatments.

The results of this study are in disagreement with most reports that generally conclude that a high phosphorous concentration is detrimental to the growth of plants in the Proteaceae family. It further contrasts the common use of standard half strength Murashige and Skoog medium in most *in vitro* propagation of Proteaceae (Bunn et al., 2010; Kunisaki, 1990; Tal et al., 1992; Watad et al., 1992a, b). In certain cases, macro-nutrients were specifically reduced, while micro-nutrients were kept at full strength (Rugge, 1995; Seelye et al., 1986; Thillerot et al., 2006).

Moreover, not only was there no P toxicity observed in any microshoots cultured in media containing 1400 mg/L $NH_4H_2PO_4$, the mean length of new shoots were similar to those cultured on media without $NH_4H_2PO_4$. This could be due to a number of factors: according to Chin and Miller (1982), potassium deficiency in the media causes a decrease in the rate of P absorption. In this study, the normal KH_2PO_4 concentration used in standard MS medium was not added to the modified MS basal medium, therefore it is possible that with a reduction of potassium concentration in the medium, potassium deficiency caused lower P absorption.

High nitrogen concentration may have also played an important role. The use of $NH_4H_2PO_4$ in this study maintained a high ammonium concentration in the medium, which may have reduced the toxic effects of P. This was demonstrated in several Proteaceae studies: Nichols and Beardsell (1981) reported that high levels of nitrogen alleviated P toxicity in *Grevillea* cv. 'Poorinda Firebird'. Similar findings were also reported by Grundon (1972) where increasing the nitrogen levels in nutrient solution cultures helped reduce P toxicity in *Banksia* and *Hakea* species. However, a study by Groves and Keraitis (1976) showed that high nitrogen levels induced P toxicity in *Banksia serrata* seedlings grown in sand culture, whereas Prasad and Dennis (1986) reported that *Leucadendron* 'Safari Sunset' was tolerant to high levels of P, irrespective of the levels of other nutrients.

Table 1. The response of microshoots with either one or two nodes cultured on modified MS media after 60 days in multiplication media.

$NH_4H_2PO_4$ (mg L^{-1})	Explant type	Survival rate (%)	Mean length of new axillary shoot (mm)
0	One node	40^a	2.6 ± 1.12^a
	Two nodes	100^b	13.8 ± 6.38^b
1400	One node	60^a	5.4 ± 1.73^a
	Two nodes	100^b	17.4 ± 5.38^b

For survival rate, values with different letters within the same column are significantly different based on Chi-square analysis ($P \leq 0.05$). For shoot length, values within the same column with different letters are significantly different at $P \leq 0.05$ according to Tukey's studentized test.

From the results of numerous studies mentioned above,it can be established that the inconsistencies in the causes and alleviation of P toxicity in Proteaceae may be due to the fact that different genera and species of Proteaceae react differently to P, where in one species, a certain mineral nutrient may alleviate P toxicity, and in another species, it may aggravate it. Montarone et al. (2003) confirmed in their study that large differences in mineral requirements exist between cultivars belonging to the same Proteaceae genus, with even larger differences between genera.

In conclusion, successful multiplication of microshoots produced from established shoot segments was achieved for the first time. Microshoots with two nodes were the most suitable for multiplication in terms of explant survival and subsequent axillary shoot growth. Of particular importance is that *P. cynaroides* explants were not adversely affected by high P concentrations in the growth medium. The results of this study throw more light on the nutritional requirements of *P. cynaroides*. The successful multiplication of the microshoots is an important step towards mass-production of this difficult-to-propagate species *in vitro.* Further studies are needed to establish the effects of other nutrients on *P. cynaroides* explants cultured *in vitro*, and to induce root formation in microshoots.

ACKNOWLEDGEMENTS

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國科會補助計畫衍生研發成果推廣資料表

日期:2012/10/26

<table>
<tr><td rowspan="3">國科會補助計畫</td><td>計畫名稱: 培養基質、二氧化碳施肥及發光二極體波長對光自營培養帝王花(Protea cynaroides L.)組培苗之生長及馴化之影響</td></tr>
<tr><td>計畫主持人: 吳澔群</td></tr>
<tr><td>計畫編號: 100-2313-B-343-001-　　　　學門領域: 農藝及園藝</td></tr>
<tr><td colspan="2">無研發成果推廣資料</td></tr>
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100 年度專題研究計畫研究成果彙整表

計畫主持人：吳滿群 | **計畫編號：**100-2313-B-343-001-

計畫名稱：培養基質、二氧化碳施肥及發光二極體波長對光自營培養帝王花（Protea cynaroides L.）組培苗之生長及馴化之影響

成果項目			量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）
			實際已達成數（被接受或已發表）	預期總達成數(含實際已達成數)	本計畫實際貢獻百分比		
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	1	2	100%	篇	The photo used on the cover of the August edition of the African Journal of Biotechnology was taken from the author's paper, which was published in that edition: Wu H.C. and du Toit, 2012. In vitro multiplication of Protea

							11(63): 12630-12633.
		研究報告/技術報告	1	1	100%		
		研討會論文	0	1	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		

其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)	A paper published with the support of this research grant was co-authored with Prof. Elsa du Toit of the Department of Plant Production and Soil Science, University of Pretoria, South Africa. Subsequently, the author was invited to give a presentation at the Department of Plant Production and Soil Science, University of Pretoria, South Africa on the 16th April 2012.

	成果項目	量化	名稱或內容性質簡述
科教處計畫加填項目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與（閱聽）人數	0	

國科會補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

■達成目標

□未達成目標（請說明，以 100 字為限）

□實驗失敗

□因故實驗中斷

□其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

論文：■已發表 □未發表之文稿 □撰寫中 □無

專利：□已獲得 □申請中 ■無

技轉：□已技轉 □洽談中 ■無

其他：（以 100 字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以 500 字為限）

The opportunity to conduct this research study was of great importance to the Protea industry as a whole. The results obtained from this study showed that carbon dioxide enrichment of P. cynaroides plantlets significantly improved their growth and morphogenesis. In addition, their chlorophyll content was also significantly increased. During the course of this study, the author attended the International Protea Association conference, in which parts of the results were presented as an oral presentation. Response from the attendees at the conference was very positive, which further solidified the importance of this investigation. This study forms part of a larger effort towards resolving numerous propagation difficulties faced by researchers of Proteaceae plants. Due to the lack of success in the in vitro propagation of Proteaceae plants in previous years, a concerted effort has been put in place through the International Protea Association to bring the propagation of Proteaceae plant to the forefront of modern technology. This research study has provided invaluable knowledge and a better understanding of how P. cynaroides plantlets respond to carbon dioxide enrichment and porous supporting materials during their growth in vitro. The results of this study are a major breakthrough in the in vitro propagation of P. cynaroides plantlets, and have formed an important basis for future studies. Subsequently, efforts to achieve

further advancements in the in vitro propagation of P. cynaroides plants are underway, particularly in the area of improving their growth rates. Preliminary results have been very positive, and show great potential for further investigations. Parts of the results of the current study have been published in a peer-reviewed journal, while preparations are underway for the publication of the remaining results.