

Full Length Research Paper

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

H. C. Wu<sup>1\*</sup> and E. S. du Toit<sup>2</sup>

<sup>1</sup>Department of Natural Biotechnology, College of Science and Technology, Nanhua University, No. 55, Section 1, Nanhua Rd., Dalin Township, Chiayi 62248, Taiwan, Republic of China.

<sup>2</sup>Department of Plant Production and Soil Science, Faculty of Natural and Agricultural Sciences, University of Pretoria, Pretoria 0002, South Africa.

Accepted 18 May, 2012

***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  $\text{NH}_4\text{H}_2\text{PO}_4$  were tested:  $0 \text{ mg L}^{-1} \text{ NH}_4\text{H}_2\text{PO}_4$ , and a high P concentration of  $1400 \text{ mg L}^{-1} \text{ NH}_4\text{H}_2\text{PO}_4$ . Both growth media were also supplemented with gibberellic acid ( $\text{GA}_3$ ) ( $30 \text{ mg L}^{-1}$ ), 6-benzylaminopurine (BAP) ( $2 \text{ mg L}^{-1}$ ), ethylenediaminetetraacetic acid (EDTA) ( $50 \text{ mg L}^{-1}$ ) and indolebutyric acid (IBA) ( $0.5 \text{ mg L}^{-1}$ ). 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  $1400 \text{ mg L}^{-1} \text{ NH}_4\text{H}_2\text{PO}_4$  were not significantly different from those grown on  $0 \text{ mg L}^{-1} \text{ NH}_4\text{H}_2\text{PO}_4$ . 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

\*Corresponding author. E-mail: [hcwu@mail.nhu.edu.tw](mailto:hcwu@mail.nhu.edu.tw). Tel: +886 5 272 1001. Ext: 5441. Fax: +886 5 242 7195



**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-Jacov and Jacobs, 1986; Thillerot et al., 2006), however, further multiplication of these explants has not been achieved. In addition, no information on the effects of high P concentrations 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<sup>-1</sup> ascorbic acid and 1500 mg L<sup>-1</sup> citric acid for 1 h. The nodal explants were then transferred to half-strength MS medium supplemented with gibberellic acid (GA<sub>3</sub>) (30 mg L<sup>-1</sup>), 6-benzylaminopurine (BAP) (2 mg L<sup>-1</sup>), myo-inositol (100 mg L<sup>-1</sup>), ethylenediaminetetraacetic acid (EDTA) (50 mg L<sup>-1</sup>), sucrose (20 g L<sup>-1</sup>), activated charcoal (3 g L<sup>-1</sup>), and Gelrite (3 g L<sup>-1</sup>). 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<sup>-2</sup> s<sup>-1</sup> photosynthetically active radiation (PAR).

### Explant multiplication

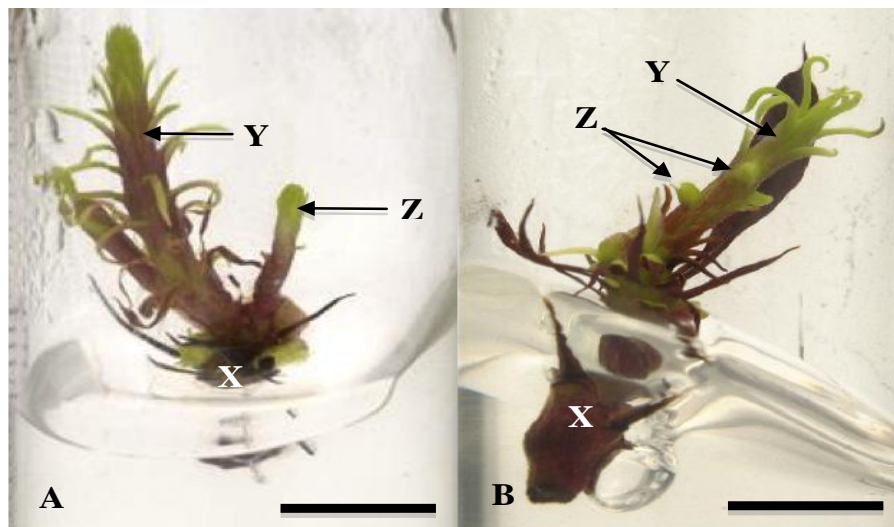
For the multiplication stage, elongated axillary buds (microshoots) of established explants (Figure 1) were cut into shorter sections and subcultured to multiplication media to induce axillary shoot growth. Two types of microshoot explants were used: microshoot sections with one node or two nodes. A basal MS medium consisting of modified macro-nutrients, full strength micro-nutrients and vitamins was used: NH<sub>4</sub>NO<sub>3</sub> (23 mg L<sup>-1</sup>), KNO<sub>3</sub> (51 mg L<sup>-1</sup>), MgSO<sub>4</sub>·7H<sub>2</sub>O (370 mg L<sup>-1</sup>), KH<sub>2</sub>PO<sub>4</sub> (0 mg L<sup>-1</sup>), KI (830 mg L<sup>-1</sup>), and CaCl<sub>2</sub>·4H<sub>2</sub>O (440 mg L<sup>-1</sup>). KH<sub>2</sub>PO<sub>4</sub> was substituted with NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> in the growth medium. Two concentrations of NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> were tested: 0 mg L<sup>-1</sup> (control) and 1400 mg L<sup>-1</sup>. The following were also included in the medium: GA<sub>3</sub> (30 mg L<sup>-1</sup>), BAP (2 mg L<sup>-1</sup>), EDTA (50 mg L<sup>-1</sup>), indole-butyric acid (IBA) (0.5 mg L<sup>-1</sup>), sucrose (30 g L<sup>-1</sup>) and Gelrite® (3 g L<sup>-1</sup>). 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<sup>-2</sup> s<sup>-1</sup> 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<sup>-1</sup> NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> and (B) 1400 mg L<sup>-1</sup> NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>. 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<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> 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<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> 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<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> 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<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>, the mean length of new shoots were similar to those cultured on media without NH<sub>4</sub>H<sub>2</sub>PO<sub>4</sub>. 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<sub>2</sub>PO<sub>4</sub> 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<sub>4</sub>H<sub>2</sub>PO<sub>4</sub> 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 <sub>4</sub> H <sub>2</sub> PO <sub>4</sub> (mg L <sup>-1</sup> )	Explant type	Survival rate (%)	Mean length of new axillary shoot (mm)
0	One node	40 <sup>a</sup>	2.6 ± 1.12 <sup>a</sup>
	Two nodes	100 <sup>b</sup>	13.8 ± 6.38 <sup>b</sup>
1400	One node	60 <sup>a</sup>	5.4 ± 1.73 <sup>a</sup>
	Two nodes	100 <sup>b</sup>	17.4 ± 5.38 <sup>b</sup>

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

This work was supported by the National Science Council of Taiwan (R.O.C) in the form of a research grant (NSC 100-2313-B-343-001).

## REFERENCES

- Ben-Jaacov J, Jacobs G (1986). Establishing *Protea*, *Leucospermum* and *Serruria* *in vitro*. Acta Hort. 185:39-52.
- Bunn E, Stone B, Williams D, Yan G (2010). *In vitro* conservation of *Synaphea stenoloba* (Proteaceae). Acta Hort. 869:143-156.
- Chin C, Miller D (1982). Some characteristics of the phosphate uptake by *Petunia* cells. HortScience 17:488 (Abstract 199).
- Cowling RM, Holmes PM (1991). Flora and vegetation. In: R. Cowling (ed.), The Ecology of Fynbos, Oxford University Press, Oxford, U.K. pp. 23-61.
- Groves RH, Keratis K (1976). Survival and growth of seedling of three sclerophyll species at high levels of phosphorous and nitrogen. Aust. J. Bot. 24:681-690.
- Grundon NJ (1972). Mineral nutrition of some Queensland heath plants. J. Ecol. 60:171-181.
- Hawkins HJ, Hettasch H, Mesjasz-Przybylowicz J, Przybylowicz W, Cramer MD (2008). Phosphorus toxicity in the Proteaceae – a problem in post-agricultural lands. Hort. Sci. 117:357-365.
- Kunisaki JT (1990). Micropropagation of *Leucospermum*. Acta Hort. 264:45-48.
- Littlejohn G (2000). Cultivation method of fynbos crops. In: ARC – Fynbos Unit (Eds). Fynbos Cultivation. ARC – Fynbos, Private Bag X1, 7607, Elsenburg, South Africa.
- Montarone M, Allemand P (1995). Growing Proteaceae soilless under shelter. Acta Hort. 387:73-84.
- Montarone M, Ziegler M (1997). Water and mineral absorption for two *Protea* species (*P. eximia* and *P. cynaroides*) according to their development stage. Acta Hort. 453:135-144.
- Montarone M, Ziegler M, Dridi N, Voisin S (2003). Comparison of mineral requirements of some cultivars in two Proteaceae genera. Acta Hort. 602:103-111.
- Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant 15:473-493.
- Nichols DG, Beardsell DV (1981). Interactions of calcium, nitrogen and potassium with phosphorous on the symptoms of toxicity in *Grevillea* cv. 'Poorinda Firebird'. Plant Soil 61:437-445.
- Nichols DG, Jones DL, Beardsell DV (1979). The effect of phosphorous on the growth of *Grevillea* 'Poorinda Firebird' in soilless potting-mixtures. Sci. Hort. 11:197-205.
- Prasad M, Dennis DJ (1986). Phosphorous nutrition of *Leucadendron* 'Safari Sunset'. Acta Hort. 185:155-162.
- Rugge BA (1995). Micropropagation of *Protea repens*. Acta Hort. 387:121-127.
- SAS Institute Inc. (1996). The SAS system for Windows. SAS Institute Inc. SAS Campus drive, Cary, North Carolina, USA.
- Seelye JF, Butcher SM, Dennis DJ (1986). Micropropagation of *Telopea speciosissima*. Acta Hort. 185: 281-285.
- Silber B, Mitchnick J, Ben-Jaacov J (2001). Phosphorous nutrition and the rhizosphere pH in *Leucodendron* 'Safari Sunset'. Acta Hort. 545:135-143.
- Tal E, Solomon H, Ben-Jaacov J, Watad AA (1992). Micropropagation of selected *Leucospermum cordifolium*: Effect of antibiotics and GA<sub>3</sub>. Acta Hort. 316:55-58.
- Thillerot F, Choix A, Poupet M, Montarone (2006). Micropropagation of *Leucospermum* 'High Gold' and three cultivars of *Protea*. Acta Hort. 716:17-24.
- Watad AA, Ben-Jaacov J, Cohen S, Tal E, Solomon H (1992a). *In vitro* establishment of *Protea obtusifolia*. Acta Hort. 316:59-62.
- Watad AA, Ben-Jaacov J, Tal E, Solomon H (1992b). *In vitro* propagation of *Grevillea* species. Acta Hort. 316:51-53.
- Witkowski ETF, Mitchell DT (1987). Variations in soil phosphorus in the Fynbos biome, South Africa. J. Ecol. 75:1159-1171.