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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 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^{-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

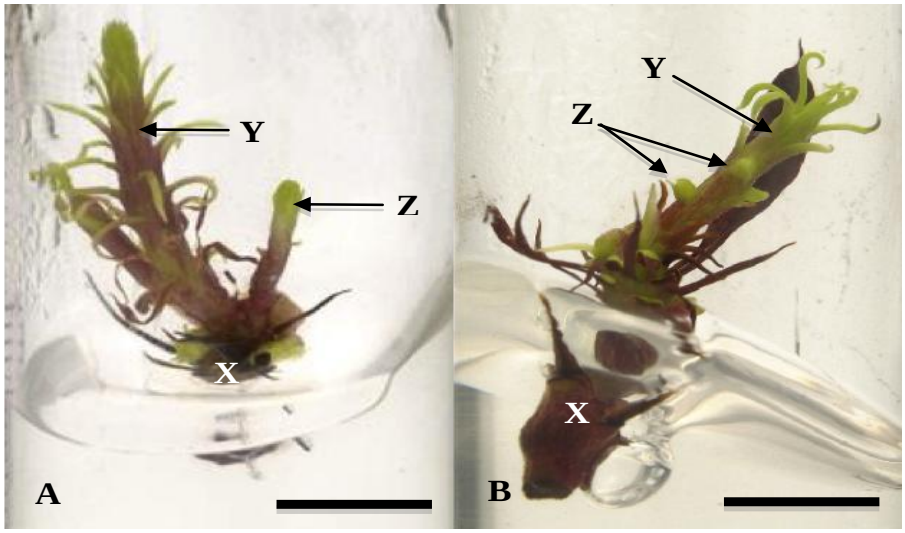

Figure 2. Modified MS medium with (A) 0 mg L^{-1} $NH_4H_2PO_4$ and (B) 1400 mg L^{-1} $NH_4H_2PO_4$. X, Microshoot with two nodes from established explant; Y, formation of new axillary shoot after 60 days in culture; Z, formation of new buds and shoots on new axillary shoot. Bar = 1 cm.

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.

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