PONTIFICIA UNIVERSIDAD CATOLICA DE CHILE
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Señora
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Fundación para la Innovación Agraria - FIA
Presente

Ref.: Respuesta observaciones Informe Técnico Final
Proyecto FIA-PI-C-2005-1-A-67

Estimada Sra. Muchnik,

Cumplo con enviar copia de respuesta enviada por correo electrónico el día 12 de enero de 2010. Con respecto a las observaciones al Informe Técnico Final del Proyecto "**Unidad especializada de propagación in vitro en especies ornamentales de difícil multiplicación**" Código: FIA-PI-C-2005-1-A-67.

1. **Realizar presentación de resultados a FIA.**
   Estamos en condiciones de presentar, adicionalmente al informe escrito, los resultados a FIA, en la oportunidad que Uds. estimen conveniente, a partir del mes de enero o marzo de este año, dada las fechas de funcionamiento de la Universidad.

2. **Enviar publicación presentada a Acta Horticulturae.**
   Adjunto última revisión de artículo enviado y aceptada en noviembre de 2009 para publicación en fecha aún no definida por la International Horticultural Society. Editoras encargadas de la publicación son la Dra. Audrey Gerber (audrey@gerbermail.com.au) y la Dra. Lynn Hoffman (ewh@sun.ac.za), a quien le puede solicitar más detalles sobre dicha publicación.

3. **Realizar actividad de difusión de presentación de resultados en fecha a acordar con FIA.**
   Quedamos a disposición de FIA para acordar fecha de la presentación de estos resultados, de tal forma que participen las personas que estuvieron involucradas en la ejecución, si así se define como necesario.

Sin otro particular, se despide atentamente de Ud.,

Dr. Eduardo Olate
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ADVANCES AND STRATEGIES FOR MICROPROPAGATION OF PROTEACEAE SPECIES

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Abstract

The objective of this research was to develop *in vitro* protocols for species of the *Proteaceae* family. Three model species were selected according to their different growth characteristics: *Protea* 'Lady Di', *Banksia coccinea* and *Leucospermum* 'High Gold'. These three species are considered difficult for vegetative multiplication both *ex vitro* and *in vitro*. For *Protea* 'Lady Di' and *Leucospermum* 'High Gold' severe pruning was made on stock plants to induce juvenile lateral shoots. The produced buds of *Protea* were also partially etiolated by different shading systems. Shoots of *B. coccinea* stock plants were applied with BAP to stimulate the production of lateral shoots *in vitro*. All the explants were transported in a cooler with their bases in water which demonstrated to be absolutely necessary for the explants to survive *in vitro*. At the laboratory the explants were defoliated and disinfected using a solution of 5% NaOCl plus nonionic detergent, with different contact times for each species, and two consecutive rinses with distilled sterilized water. The explants were cultivated on MS media supplemented with 30 g L^{-1} of saccharose and 6 g L^{-1} of agar, and pH adjusted to 5.7 before sterilization. The effect of different types and concentrations of plant growth regulators on shoot production, multiplication and induction of adventitious roots were evaluated in *L.* 'High Gold'. Significant difference was found for the method of partial etiolation in *Protea* 'Lady Di' depending on the season of the year. In the case of *L.* 'High Gold' a 90% lateral bud development was recorded when cultivated on a medium supplemented with 1 mg L^{-1} TDZ. The maximum rate of multiplication for this cultivar was 3.9 compact shoots per explant after four weeks of culture. The shoot elongation of *L.* 'High Gold' was achieved when the explants were subcultured every four weeks on a MS medium supplemented with 1.0 mg L^{-1} BA for at least four cycles to obtain a rate of multiplication of 2.6. Finally, these buds were cultivated in medium supplemented with different concentrations of IBA (0, 1 and 2 mg L^{-1}) to induce adventitious roots, but to date no positive results could be achieved.

Abbreviations: TDZ (thiadiazuron); NAA (1-naphthalene-acetic acid); KIN (kinetin); BAP (benzyl-amino-purine); MS (Murashige and Skoog); WPM (Woody plant medium); GA_3 (gibberellic acid); 2iP (6-(γ,γ-dimethylallylamino)purine); IBA (indole-3-butiric acid).

INTRODUCTION

More than 1,400 species have been recognized in the Proteaceae family (Rebelo, 1995). Its main centers of origin are Australia and South Africa, and minor centers of origin

are South America and the islands of eastern New Guinea and to a lesser extent Southeast Asia and Madagascar (Criley, 2001). The genera that have achieved greater importance in the market are *Protea*, *Leucadendron* and *Leucospermum* (Sedgley, 1998) next to the genera *Banksia* (Sedgley, 1998) and *Grevillea* (Criley, 2001). In addition to that there is an increasing interest of growers of other geographical areas in addition to the traditional ones for commercial production of Proteaceae. Among them Chile, Ecuador, Colombia, Peru, and China could be listed. Therefore, there is also an increasing interest in propagation material and new cultivars from this family. It is however already evident that some of the genera and species of Proteaceae may not be easy to propagate vegetatively (Criley, 2001; Tynan et al., 2001; Reuveni et al., 2003). For example, *Banksia* is widely distributed in commercial plantations in Australia, but its production is largely based on material derived from seed and the variability between plants is very high (Sedgley, 1998). Species such as *B. ashbyi* (Reuveni et al., 2003), *B. coccinea* and *B. menziesii* (Tynan et al., 2001) are propagated by seed, because vegetative propagation by cuttings can not be achieved. However, *B. ericifolia* and *B. spinulosa* var. *collina*, can be propagated vegetatively without much difficulty (Tynan et al., 2001). One way to reverse this problem is to use forced juvenile shoots (Reuveni et al., 2003). In the case of the genus *Protea* its great phenotypic and genotypic variability makes its response to vegetative propagation highly variable as well. This is even more the case on those species with a broad geographical distribution such as *P. cynaroides*, *P. nerifolia* and *P. magnifica* in comparison with those species with smaller ranges as *P. compacta* (Vogts, 1989 cited by Criley, 2001). On the other hand, the vegetative propagation of *Leucospermum* species can be accomplished relatively easily (Criley, 2001).

The cultivars of Proteaceae in general can be derived either from the selection of superior individuals within a population or from the selection of hybrids derived from crosses and selection of individuals obtained from seeds from a wild population (Criley et al., 2001). It is widely recognized that plant micropropagation can be a very useful tool to achieve clonal propagation and it plays a key role to bulk up unique individuals and/or hybrids selected from breeding programs. Nevertheless, perennial plants, either woody or herbaceous, may be recalcitrant species to this type of propagation because of their complex and seasonal cycles of life (McCown, 2000). The species of the family Proteaceae, woody and semi-woody, are no exception to that.

Of all the Proteaceae species cultivated commercially, the *Protea* genus seems to be the most difficult to propagate *in vitro*. The most recurrent problems on Proteaceae have been high levels of microbial contamination, oxidation and necrosis of the explants in the stabilization phase *in vitro* (Thillerot et al., 2006). For example in *Banksia ashbyi* the culture time to achieve the formation of buds from callus is about 6 months (Reuveni et al, 2003), and there are no reports indicating lateral sprouting or further phases of multiplication *in vitro* for this genera.

In vitro protocols for one species of the genus *Leucospermum* have been partially developed, but the rooting rate is still very low, together with problems in the acclimatization phase (Thillerot et al., 2006).

In general, impediments are generated in one or more stages of the micropropagation sequence, leading to different responses even among species of the same genus, which can be categorize into: microbial contamination and exudation of phenols that causes necrosis, preventing the *in vitro* stabilization of shoot tips explants; delayed sprouting response of axillary buds of the initial explants; and the inhibition of the

induction and formation of adventitious roots producing a very low or no survival of the plants at the stage of acclimatization. All the above indicates the need to generate a strategy to identify possible solutions for each case and how to address future development of new *in vitro* protocols for new species or cultivars that may have desirable characteristics for the Proteaceae global market.

MATERIALS AND METHODS

A series of different *in vitro* experiments were conducted in the laboratories of the School of Agriculture and Forestry of the Pontificia Universidad Catolica de Chile, Santiago de Chile. Stock plants were selected from a commercial orchard 125 km west from Santiago (Flores del Fynbos Farm) and maintained as stock plants. In additional to regular crop management preventive and recurrent fungicide treatments against *Phytophthora* and other fungi was performed on stock plants of *Banksia coccinea* (6 years old), *Leucospermum* 'High Gold' (3 years old) and *Protea* 'Lady Di' (4 years old). Severe pruning to induce new shoot growth was performed on stock plants of *L.* 'High Gold' and *P.* 'Lady Di'. All the material was collected during the morning, placed into moist floral foam and transported in a cooler at about 15°C. The *in vitro* initiation was performed always on the same day of collection. The plant material was defoliated leaving attached only 1-2 mm of petiole. Disinfection consisted on a washing solution of 5% NaOCl (v/v) plus a drop of Tween-20® with constant agitation during 10 minutes followed by two rinses with sterile distilled water. The explants were cultured into test tubes or flasks containing 15 or 30 ml of MS medium (Murashige and Skoog, 1962) respectively, supplemented with 30 g L^{-1} sucrose, and 6 g L^{-1} agar (Phytotechnology ® A111). The cultivation period was 4 weeks in a growth chamber at 20±1°C with a photoperiod of 16/8 hours and fluorescent light of 40-50 µmol m^{-2} s^{-1}.

Banksia

In addition, in the case of *B. coccinea* stock plants, BAP was applied on a 30 day-cycle at 11,000 ppm to the aerial part of the plant. A different experiment was also conducted on this species to test the effect of the presence of the leaf on the *in vitro* initiation. For that purpose shoot explants with intact (whole leaf), half and totally removed leaves were initiated *in vitro*. All culture media included 4.54 µM TDZ.

Protea

Either a selection of new young shoots produced by *P.* 'Lady Di' stock plants were covered with brown paper bags or the complete plants were covered with a black shade net (65% mesh) for a 5 week period. Different disinfection methods also were evaluated including: distilled water only plus a drop of Tween20 ® for 3 min; 70% ethanol solution; and soaking of the sterilized explants into a sterile solution containing 1500 mg L^{-1} citric acid and 100 mg L^{-1} ascorbic acid for 1 hour. There also were included treatments with no sterilization at all to elucidate whether the disinfection methods used, i.e. ethanol and/or NaOCl, produced or not severe oxidation on the explants used (Table 1). All culture media included 4.54 µM TDZ.

Leucospermum

In the case of *L.* 'High Gold' different types of plant growth regulators were evaluated as a supplement to 100% MS medium, including TDZ, BAP, KIN and NAA, all

in concentrations of 1.0 mg L^{-1}. Compact shoots formed from the *in vitro* initiation were subcultured onto MS medium supplemented with 4.54 µM TDZ, with or without supplemental GA_3, in a concentration of 5.8 µM. The produced elongated shoots were then subcultured after 28 days onto MS or WPM supplemented with either BAP or 2iP in a concentration of 4.4 µM. The buds formed from the elongation phase were subcultured onto MS medium supplemented with 4.4 µM BAP after 4 weeks of culture. The rate of increase was assessed after 4 cycles of 6-8 weeks for each culture. From this phase onward, cultures were carried out in a growth chamber at 25±1°C with a photoperiod of 16/8 hours and fluorescent light of 40-50 µmol m^{-2} s^{-1}. Shoots resulting from the previous multiplication phase were subcultured onto MS medium without any plant growth regulator for 4 weeks followed by a subculture on MS medium supplemented with 0, 1 and 2 mg L^{-1} IBA. The formation of adventitious roots was evaluated after 4-6 weeks of culture.

Results were analyzed statistically by means of ANOVA using SAS software (SAS 1999) and multiple comparisons were performed using Scheffé-test. All the experiments were replicated at least twice.

RESULTS AND DISCUSSION

Partial etiolated shoots were evaluated as initial explants to prevent exudation of phenols during *in vitro* initiation of apical buds of *P.* 'Lady Di'. During winter shoots shaded with brown paper bags and using subapical segment explants achieved 100% of initiation (Table 2). In summer the best treatment was the control (no shading) with 88% initiation, but without significant difference with explants collected from plants shaded with shading net, achieving 73% of the explants to initiate successfully.

The optimum method of disinfection varied for each species. *L.* 'High Gold' presented a sterilization rate of 87% using a 3% NaOCl solution and 5 min of agitation. In *P.* 'Lady Di' we were able to identify two disinfection methods, i.e. sterilization solution of 1.0% NaOCL followed by immersion into an antioxidant solution of 100 mg L^{-1} ascorbic acid and 1,500 mg L^{-1} citric acid, and culture in complete darkness or reduced light, with 75% of success in both cases (Table 1).

The form of transport of the shoots significantly affected the *in vitro* results on all the species. For instance, in *B. coccinea* the use of a cooler and moist flower foam as a medium for transporting the shoots to the laboratory consistently showed positive effects by achieving up to 77% initiation of the explants compared to a complete loss of the explants *in vitro* if no cooler or water supply was supplied (data not shown). In *Banksia* it was possible to achieve up to a 67% sterilization of the explants and 88.3 % delayed sprouting when a 5% NaOCl solution was used in conjunction with a stirring time of 5 minutes and complete defoliation of explants (Table 3).

In *L.* 'High Gold' the highest percentage of sprouting of lateral buds was achieved when shoots were culture on MS medium supplemented with 4.54 µM TDZ, reaching an average of 68% (Fig. 1 and 2). The rate of formation of compact shoots was 3.9 shoots/explant and the best treatment for multiplication was MS medium supplemented with 4.4 µM BAP. The formation rates were significantly different than those cultured on WPM supplemented either with 4.4 µM BAP or 2iP or MS supplemented with 4.4 µM 2iP. The buds formed during the stage of multiplication and subcultured onto MS medium without any plant growth regulators or onto MS medium supplemented with various IBA concentrations did not form any roots.

CONCLUSIONS

In vitro culture of Proteaceae is possible, but remains a challenge, with results being very dependant on the genera tested. Using juvenile, vegetative material it is important during the initiation phase, as to avoid problematic phenolic exudation (*in vitro* blackening), mainly in those more woody species like *Protea.* The season of the year can be important (*Banksia coccinea*) or less important (*Leucospermum* 'High Gold') to achieve *in vitro* initiation. Environmental and/or hormonal pre-treatments to the stock plants are crucial to all three species/cultivars tested to improve chances of success. The use of stronger cytokinins like TDZ as well as a further exploration of various environmental culture conditions (temperature, light intensity) hold promise and warrant a continuation of experimental research.

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Tables

Table 1. Effect of different initiation treatments on phenolic exudation and survival of *Protea* 'Lady Di' explants after four weeks of *in vitro* culture.

Treatment	Sterilization		Anti oxidant solution	Photoperiod			Phenolic exudation (%)	Survival (%)
	NaOCl (1.0%)	Etanol (70%)		16 hrs	Darkness	Reduced light		
1				X			0	0
2					X		25	0
3			X		X		25	0
4	X		X		X		2	75
5		X	X		X		100	0
6	X		X			X	25	75

Table 2. Effect of two shading treatments to stock plants on the survival of *Protea* 'Lady Di' after four weeks of *in vitro* initiation. Same letters on the same column indicate no statistical difference ($P > 0.05$, Scheffé's test).

Shading treatment	Explant type	Survival (%)	
		Summer	Winter
Shading net	Subapical	73.3 a	100.0 a
	Nodal segments	73.3 a	---
	Apical	33.3 b	26.7 c
Paper bag	Subapical	0.0 c	80.0 b
	Nodal segments	0.0 c	---
	Apical	0.0 c	40.0 bc
Control	Subapical	80.0 a	0.0 d
	Nodal segments	33.3 b	---
	Apical	66.7 a	0.0 d

Table 3. Effect of the type of explant used during the *in vitro* initiation of *Banksia coccinea* on the survival and growth after six weeks of culture. Same letters on the same column indicate no statistical difference ($P > 0.05$, Scheffé's test).

Type of initial explant	Survival (%)	Buds produced (N°)	Sprouting (%)
Defoliated	66.7 a	77 a	88.3 a
Half leaf	20.0 b	27 b	48.2 b
Entire leaf	0.0 c	0 c	0.0 c

Figures

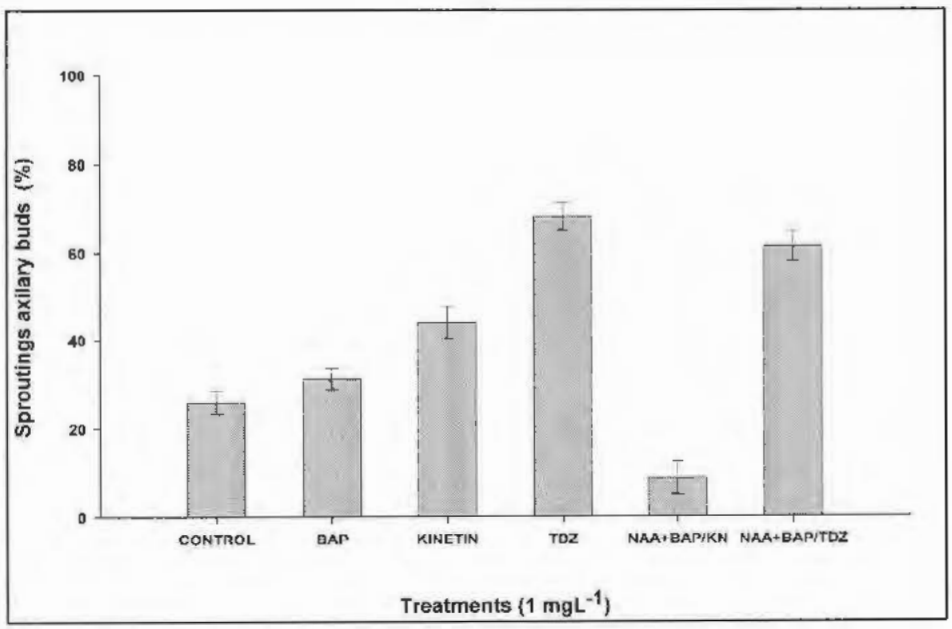

Fig. 1. Effect of different types of plant growth regulators on lateral bud sprouting of *Leucospermum* High Gold' after four weeks of *in vitro* culture using MS medium and light conditions. Bars indicate SE of the mean.

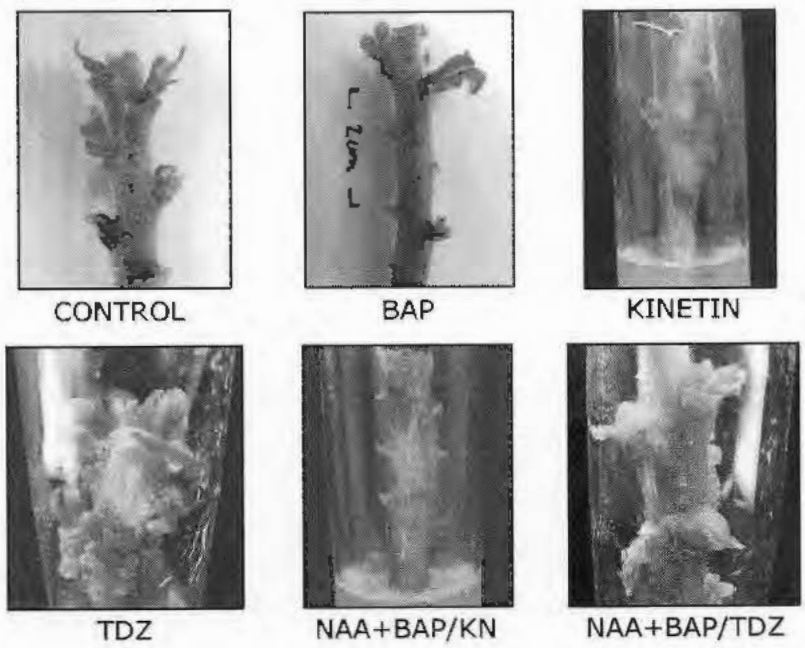

Fig.2. Effect of different types of plant growth regulators on the quality of *in vitro* sprouting of *Leucospermum* 'High Gold' after four weeks of *in vitro* culture. Explants

cultured on MS media supplemented with TDZ or NAA+BAP/TDZ produced the highest rate of lateral normal shoots.