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Efficient Micropropagation through Receptacle Culture in *Telopea Speciosissima* R. Br. (Proteaceae)

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Abstract

A new, efficient and reproducible *in vitro* propagation system of *Telopea speciosissima* has been established. Excised receptacle disks were cultured for inducing adventitious shoots on modified woody plant media (WPM) supplemented with 6-benzyladenine (BA) and 3-indole-butyric acid (IBA) at various concentrations. Efficient shoot regeneration was obtained when explants were cultured on media containing 5 - 10 mg l^{-1} BA and 0 - 0.2 mg l^{-1} IBA. Most of the shoots were regenerated directly from the epidermis of the lateral side, and in some cases, from the cut surface of the receptacle disk explants. Nodal explants taken from the regenerated shoots were cultured on the modified WPM supplemented with a wide range of BA concentrations. The number of shoots produced from the nodal explants increased linearly until 0.3 mg l^{-1} BA, where it then plateaued. The shoot length and leaf number were negatively correlated with increased BA concentration. Shoots were rooted by dipping their base into a solution of 150 mg l^{-1} IBA for 10 minutes and then inserting them into Kanuma soil.

Introduction

Telopea speciosissima, commonly called waratah, is characterized by its spectacular red inflorescence, comprised of numerous paired florets (90 - 260) spirally arranged around an elongated cone (Whelan and Goldingay, 1989). This shrub can be propagated from lignotuberous materials (Worral, 1976) or by seeds, but the latter form has the disadvantage of a long juvenile period and a large spectrum of variability in flower color and size. Lignotuber buds or shoots are a likely source of clonal propagules, but their occurrence is restricted to particular genotypes. In the field, we have consistently noted that the base of the receptacle sometimes bears shoots after blooming, indicating that the receptacle has inherent capacity to regenerate shoots (Hasegawa *et al.*, 1998). *In vitro* micropropagation of *T. speciosissima* has previously been described by Seelye *et al.* (1986) and Offord *et al.* (1990) using shoot tip explants from greenhouse-grown plants, and later by Offord *et al.* (1992) who examined the effects of genotype and plant growth regulators on shoot proliferation. In all of these studies, culture protocols were based on stimulating axillary shoot growth. However, our preliminary attempts to establish nodal cultures were unsuccessful due to excessive microbial contamination. In the present paper, we propose a new protocol for *Telopea* micropropagation via receptacle culture. The protocol has the advantage of starting materials, since the propagation can be started from an elite cut flower even though the mother stock is not available.

Materials and Methods

Receptacle culture

Inflorescences (raceme) from 16-year-old potted plants of *T. speciosissima* were harvested on April 15, 1999. The racemes (**Fig. 1A**) were washed with running tap water, sonicated in 5% Hibitane solution (antibacterial liquid soap ICC) for 10 minutes, and then immersed for a few seconds in 70% ethanol. This was followed by surface-disinfection under continuous agitation in a 10% aqueous solution of sodium hypochlorite (Antiformin, 5.25% active chlorine; Wako Pure Chemical, Osaka, Japan) for 10 minutes, and finally rinsed thoroughly with sterile, distilled water. All florets were removed from the receptacle. A total of 16 receptacles were used in this experiment. Then the receptacle

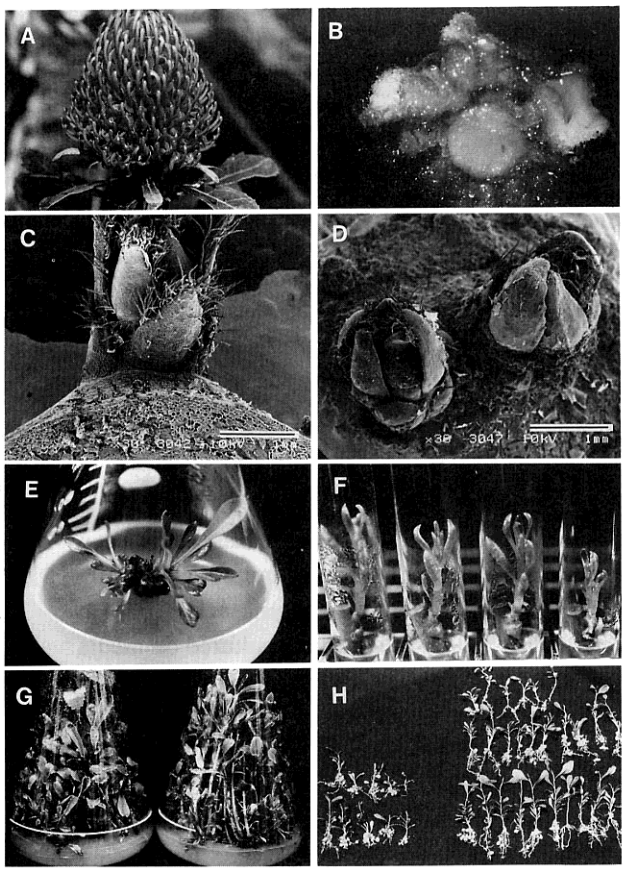

Fig. 1 Micropropagation of *Telopea speciosissima* through an initial receptacle culture and subsequent nodal culture. (A) An inflorescence of *T. speciosissima*. (B) Callus formation on the receptacle explant surface 14 days after incubation. (C) A SEM micrograph showing an adventitious bud arising directly from the lateral portion of the receptacle explant. Bar = 1 mm. (D) A SEM micrograph showing adventitious buds formed on the cut surface of the receptacle explant. Bar = 1 mm. (E) Multiple shoots initiated from a receptacle explant after two months. (F) Shoot initiation from nodal segments cultured on medium containing 0.3 mg l^{-1} BA for 30 days. (G) Proliferating shoot mass after two months of culture on plant growth regulator-free medium. (H) Rooted shoots under *ex vitro* conditions with either 1% Oxyberon powder (left) or 150 mg l^{-1} IBA solution for 10 minutes (right) after two months of treatment.

(50 - 90 mm in length) was transversely cut into 5 - 6 mm thick disks, and randomly placed with the cut side down onto a modified WPM basal salts (SIGMA, ref. M-6774), augmented with the iron component to 200 μM and supplemented with WPM vitamins, 2% sucrose, 0.2% gellan gum and plant growth regulators. Two plant growth regulators, BA at 2, 5, 10 or 15 mg l^{-1} and IBA at 0.2 or 2 mg l^{-1} were tested either separately or in combination together with a plant growth regulator-free control to select the optimum combination for proliferation. The pH was adjusted to 5.7 with 1 N HCl or NaOH prior to autoclaving at 115 ℃ for 15 min. Each of the 100 m*l* Erlenmeyer flasks containing 30 m*l* of medium was capped with a silicone plug. Each treatment consisted of five flasks with two explants per flask. Cultures were incubated at 25 ℃ under a 16 h photoperiod provided by cool-white fluorescent lamps (35 μmol m^{-2} s^{-1}). Explants were subcultured every 4 weeks onto the same medium. After 8 weeks of incubation, data on the percentage of surviving explants, the explants with shoot regeneration, the average number of shoots per regenerating explant, the number of explants forming calli and the shoot length were recorded. The explants with adventitious shoots were transferred to a medium without plant growth regulators.

Nodal culture from regenerated shoots

Shoots (10 cm in length) derived from receptacle cultures were cut into single-node pieces of 1 - 2 cm long and cultured on the aforementioned modified WPM for receptacle culture. Five concentrations of BA (0.15, 0.3, 0.6, 0.9, 1.2 mg l^{-1}) were tested together with a plant growth regulator-free control to determine the optimum level for multiple shoot formation. Aliquots of 12 m*l* medium were dispensed into 25 x 150 mm borosilicate glass tubes capped with a polypropylene closure. Cultures were incubated in a growth chamber at 25 ℃ under continuous light with cool white fluorescent lamps (80 μmol m^{-2} s^{-1}). Shoot cultures were maintained by subculture on the same medium at 4 - 5 week intervals. After 8 weeks, the cultures were evaluated for three growth parameters (shoot number, shoot length and leaf number). To promote shoot elongation, newly-formed axillary shoots were transferred to culture vessels with 200 m*l* of the plant growth regulator-free WPM and cultured under the same conditions.

Statistical analyses

Values obtained in the receptacle culture were subjected to an analysis of variance procedure for the factorial combination of simple and main effects, using MSTAT-C software (Michigan State Univ., East Lansing, 1988). Data were transformed to meet the assumption of homogeneity for ANOVA. Square root transformation was used as appropriated for the number of surviving explants, explants with calli and explants with shoots, while the number of regenerated shoots and shoot length were transformed with the $\log_{10}$ [(Y+1) x 10] func-

tion. A conversion to percentages was used for the presentation in the data table.

In the nodal culture, the experimental model adopted for data analysis was a randomized complete-block design with five blocks. Data analysis was carried out by a general linear model procedure (SAS Institute, Cary, N.C.). Best-fit linear plateau model for shoot number response was determined based solely on minimizing mean square error and used to fit the critical BA concentration (Anderson and Nelson, 1975; Nelson and Anderson, 1977).

Scanning electron microscope (SEM) observations

The cultured explants were fixed in FAA (formalin : acetic acid : 70% ethanol, 5 : 5 : 90), dehydrated through an ethanol series, which was then replaced by iso-amyl acetate, dried at critical-point, sputter-coated with Pt and examined under a SEM (Hitachi, S-2150).

Rooting

The rooting experiment was carried out using shoots of unequal sizes excised from proliferating nodal cultures. The basal ends of the shoots were treated with either an aqueous solution of 150 mg l^{-1} IBA for 10 minutes or Oxyberon® talc (1% IBA Oxyberon, Shionogi Pharmaceutical Co. Osaka, Japan), and then inserted into a tray containing screened Kanuma soil. Each treatment was composed of 50 microshoots. The trays were covered with a clear vinyl film and kept in a shaded greenhouse with average day/night time temperature of 30/20 ℃.

Results and Discussion

The receptacle explants showed swelling over the surface within 6 - 7 days of culture and greenish and/or reddish calli developed from the cut end, which eventually covered the entire explant (**Fig. 1B**) after two weeks. The first sign of shoot regeneration was the formation of small green protuberances at the surface of the disks within 30 days of culture. SEM observations also revealed that shoots mainly appeared on the periphery of the disk, but occasionally on the cut surface of explants (**Fig. 1C, D**) without an intermediate callus phase. The adventitious shoots continue developing as clusters in annular form (**Fig. 1E**). Our preliminary experiments demonstrated that a higher percentage of shoot formation was obtained from explants derived from the most proximal regions of the receptacle as compared with those from the distal region (data not shown). In this experiment, explants from all regions of the receptacle were used and a relatively low regeneration percentage was obtained.

A combination of 0.2 mg l^{-1} IBA and 5 mg l^{-1} BA was most effective for inducing well-elongated shoots with curved leaves (**Table 1**). The highest concentration of IBA (2.0 mg l^{-1}) combined with high levels of BA (5 - 15 mg l^{-1}) inhibited shoot formation. Regenerated shoots were stunted without leaf expansion on medium containing 10 mg l^{-1} BA alone. Although such explants with stunted shoots were transferred to a plant growth regulator-free medium, the shoots proliferated as abnormal needle-like multiple shoots. The shoots regenerated on media containing 2 mg l^{-1} BA elongated and became morphologically normal after transfer to a plant growth regulator-free medium (**Fig. 1G**). Calli formed on the explants did not regenerate shoots, gradually turned brown, and eventually died after 5 months of culture. The explants often turned

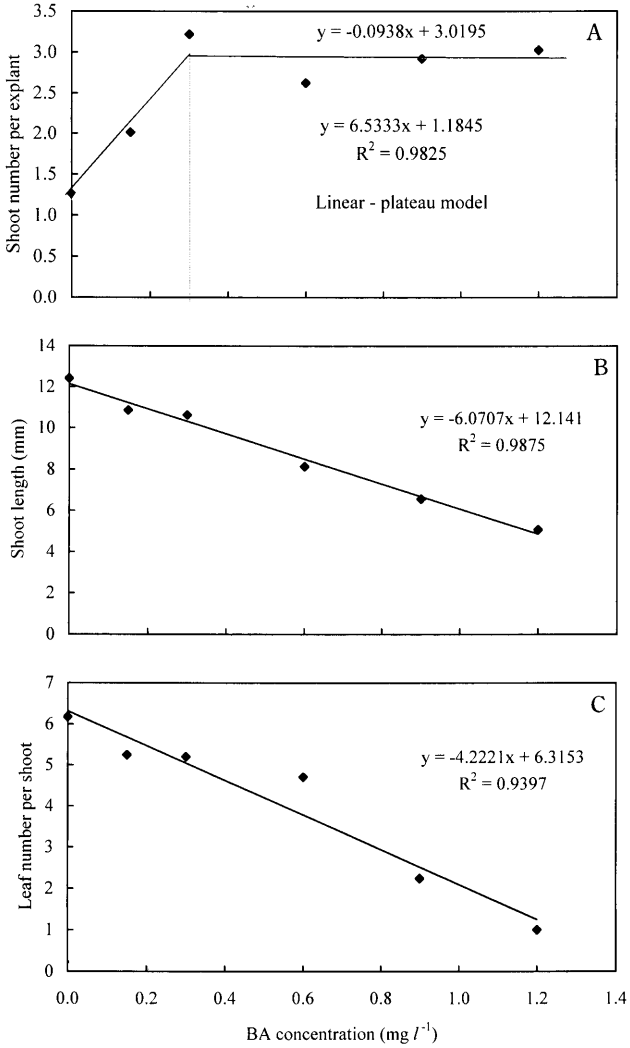

Fig. 2 Effect of BA concentration on (A) number of shoots per explant, (B) shoot length and (C) number of leaves per shoot in culture of nodal explants excised from the receptacle-derived shoots. Data represents the mean of five replications. R^2 values are based on all data and are for the entire model.

brown when they were cut and subcultured onto the same fresh medium. In this case, formed shoots on the explants also browned rapidly.

The first response exhibited by the nodal segments was the enlarging and breaking of the axillary buds within 8 days of incubation, and elongated shoots were formed after 21 days. Callus formation was promoted by 0.6 - 1.2 mg l^{-1} BA, with nodal segment explants becoming engulfed by callus tissues, masking the axillary shoots developed. At lower BA concentrations, callus formation was confined to the basal cut surface of the explants. BA concentration also strongly affected shoot proliferation and growth. The relationship between BA concentration and the number of shoots per explant fitted a linear-plateau model, which initially showed a linear increase up to 0.3 mg l^{-1} BA, after which it reached a plateau with no further changes despite an increase in BA concentration (**Fig. 2A**). On the other hand, a negative linear correlation was found between shoot length and BA concentration (**Fig. 2B**). A similar trend was observed for the number of leaves per shoot (**Fig. 2C**). Both the maximum shoot length and the maximum leaf number per shoot

Table 1. Effect of IBA and BA on explant survival, callus and adventitious shoot formation of *Telopea speciosissima* receptacle disk explants after 8 weeks of culture.

Plant growth regulator (mg l^{-1})		Surviving explants (%)[z]	Explants with callus (%)[z]	Explants with shoots (%)[z]	No. of regenerated shoots[y]	Shoot length (mm)[y]	Shoot appearance
IBA	BA						
0	0	40	40	0	0	0	
	2	100	60	40	45	21.6	Slightly curved leaf
	5	40	40	0	0	0	
	10	100	80	20	19	9.5	Stunted shoots
	15	100	90	10	6	7.8	
0.2	0	80	80	0	0	0	
	2	50	50	0	0	0	
	5	100	40	60	118	17.6	Curved leaf
	10	40	0	40	38	17.2	
	15	0	0	0	0	0	
2.0	0	40	40	0	0	0	
	2	100	90	10	10	15.4	
	5	60	60	0	0	0	
	10	80	80	0	0	0	
	15	90	90	0	0	0	

Scurce of variation	df[x]	Surviving explants[z]		Explants with callus[z]		Explants with shoots[z]		No. of regenerated shoots[y]		Shoot length (mm)[y]	
		Mean square	F value	Mean square	F value	Mean square	F value	Mean square	F value	Mean square	F value
Replication	4	0.01	1.48	0.02	2.14	0.01	1.3	0.21	1.89	0.15	1.8
IBA	2	0.06	8.26**	0.16	17.59**	0.04	5.79**	1.61	14.32**	0.57	7.08**
BA	4	0.03	4.11**	0.01	1.43	0.03	3.84**	1.02	9.11**	0.4	5.01**
IBA × BA	8	0.11	14.85**	0.09	9.19**	0.04	5.70**	1.53	13.65**	0.6	7.50**
Error	56	0.01		0.01		0.01		0.11		0.08	

Each treatment was composed of ten discs.

[z]Data were $(X+1)^{1/2}$ transformed before analysis.

[y]Data were log (Y+1) × 10 transformed before analysis.

[x]Degrees of freedom

**F_{test} significant at $P \leq 0.05$

Table 2. Rooting of shoots after dipping into 150 mg l^{-1} IBA for 10 min or applying Oxyberon powder at the cut ends.

Measured parameter	Application form	
	150 ppm IBA solution	1% IBA powder*
Number of cuttings treated	50	50
Percentage of rooting	52	18
Number of roots per cutting	6.7	7.7
Longest root length (mm)	48.5	38.8
Proteoid root formation (%)	70	45

*Oxyberon

Data were collected after two months of cutting.

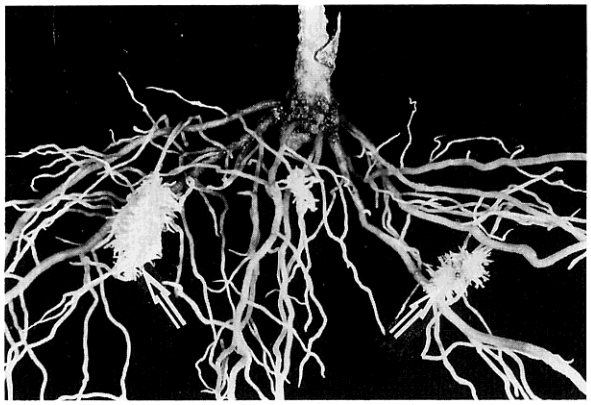

Fig. 3 A rooted cutting of *Telopea speciosissima* showing proteoid roots (arrowed).

were obtained on medium without BA. The shoots produced on media containing 0.3 mg l^{-1} BA or more showed no further development and remained stunted, even after transferring to a plant growth regulator-free medium. A similar result was previously obtained by Offord *et al.* (1992), who recommended a concentration of 1.25 μM BA (≅ 0.28 mg l^{-1}) for shoot proliferation of normal shoots. It is worth noting that a low threshold level of BA was necessary for optimal shoot induction from nodal sections, while at least seven times more cytokinin was needed with receptacle tissue.

Shoots obtained from *in vitro* cultures were rooted and acclimatized *ex vitro*. The results presented in **Table 2** show that IBA applied in liquid form was more effective in promoting rhizogenesis than the powder form (Oxyberon). The treatment with the IBA solution not only increased rooting percentage, but also favored the resumption of vegetative growth, probably due to a well-developed root system (**Fig. 1H**). In both rooting treatments, proteoid root formation was observed (**Fig. 3**). This peculiar structure is generally considered to enhance the ability to absorb phosphorous (Offord *et al.*, 1992; Purnell, 1960) and other soil nutrients in Proteaceae species.

The present study demonstrates, for the first time, that it is possible to induce shoot regeneration from receptacle explants and that suitable shoots for *ex vitro* rooting or to continue the proliferation cycle could be obtained in a relatively short period of time (70 - 75 days). Unfortunately, the propagation efficiency obtained by the receptacle culture is too low and unacceptable for commercial applications. Therefore, complementation with nodal section cultures will be necessary. Theoretically, on the basis of the results of this study, it is estimated that a total of 240 - 400 shoots should be available from one receptacle within 1 year of culture. Assuming that only 50% of the shoots are rooted, the number of plantlets obtained will be reduced by half. Since 10 - 20 adventitious shoots could be developed per explant during the initial receptacle disk culture stage, a thousand rooted plantlets could be obtained.

The micropropagation system reported here is envisaged to be more suitable than those in earlier reports. Our method allows the recognition of desired floral traits prior to propagation with a substantial decrease in microbial contamination during culture establishment, and without causing serious injury to the mother plant. Therefore, it offers a high potential for rapid and mass propagation of *T. speciosissima*.

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