Advances In Micropropagation of *Nothofagus alessandrii* Espinoza, a Chilean Endangered Species

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Nothofagus alessandrii is one of the 11 endangered woody plant species in Chile. Its propagation is normally carried out sexually. The use of micropropagation systems might be a better way to increase the propagation rate. Initial studies comparing both bud- and embryo-culture methods show that it is feasible to achieve the production of plantlets by using either a woody plant medium or others, supplemented with 0.5 mg litre^{-1} BAP or less. The rooting process of the new developed shoots has to be induced by dipping the single shoots in IBA solutions in the last stage of in vitro propagation.

INTRODUCTION

Nothofagus alessandrii Espinoza is one of the 11 *Nothofagus* species growing in Chile (Rodríguez et al., 1983). Its natural distribution is discontinuous from latitude 35° 05'S to 35° 50'S in a very narrow area of the coastal cordillera, occurring at altitudes ranging from 160 to 440 m. The species covers a total area of 825 to 845 ha. (Landaeta, 1981; Villa,1986) within the forest type known as "Bosque maulino".

According to Donoso and Landaeta (1983)*N. alessandrii*, known in Chile as "Ruíl", is seriously threatened by both ecological and anthropogenic factors.

Since the Spanish settlement in the late 16th century, Ruil forests were intensively exploited to be used for firewood and charcoal production. Of all *Nothofagus* species it is considered the most primitive because of its floral structure. Owing to the apparent inability of the species to increase its range naturally and to its endemism in a very restricted area, the survival of Ruil is critical. Therefore, it was declared by CONAF, the National Forest Corporation, as one of the 11 endangered woody plant species of Chile (Benoit, 1989). At present it is the most important species within the National Forest Reserve "Los Ruiles" in the VII Region of Chile (Villa, 1986). It is being propagated by seeds in several official and private nurseries.

Nevertheless, little work has been done with vegetative propagation systems. Rooting of cuttings achieved by Mebus (1993) was very low (25%). For that reason, and in order to increase the regeneration potential of the species, tissue culture propagation was started in our laboratory, as it was done before with other Fagaceae like *Castanea* (Vieitez and Vieitez, 1980; Rodríguez, 1982), *Quercus* sp. (Vieitez et al, 1985; San José, 1986; Bennett and Davies, 1986; Johnson and Walker, 1990), and *Fagus* (Ahuja, 1984).

MATERIAL AND METHODS

Plant Material. Experiments were started using both dormant buds from mature trees and embryos from freshly harvested seeds. Initial sterilization of the explants was done by a four-step procedure consisting of:

a) Washing in distilled water with a few drops of Tween 20.
b) Shaking in a Captan 80 solution (1.5 g litre^{-1}) for 30 min.
c) Dipping in 70% ethanol (5 sec).
d) Sterilizing in NaOCl, 10% commercial bleach, for 10 min.

After that, buds were peeled and placed onto the culture medium. Mature embryos were extracted from the seed and sown individually.

Culture Media and Treatments.

Bud-Culture Experiments. Initially bud explants were cultivated on four different basal media: Murashige and Skoog Medium (MS), Woody Plant Medium (WPM), Aspen Culture Medium (ACM), and Sommer Culture Medium (SCM) (George and Sherrington, 1984). All the media were supplemented with 0.5 mg litre^{-1} BAP, 20 mg litre^{-1} adenine sulphate, 0.1 mg litre^{-1} thiamine, 0.5 mg litre^{-1} nicotinic acid, 0.5 mg litre^{-1} pyridoxine, 100 mg litre^{-1} myo-inositol and 20 g litre^{-1} sucrose. In all cases Difco-Bacto agar was used (8 g litre^{-1}) and pH was adjusted to 5.6 prior to autoclaving for 20 min at 121C. Culture tubes containing 10 ml of medium were used. Incubation was done under dark conditions and a temperature of 20±2C during the first month, changing to a 16-h photoperiod and 3500 lux during the following 30 days.

Afterwards, surviving shoots were randomly transferred to an ACM supplemented with the following hormonal combinations: 0.5 mg litre^{-1} BAP, 0.5 mg litre^{-1} 2iP, 0.5 mg litre^{-1} BAP + 0.1 mg litre^{-1} NAA, or 0.5 mg litre^{-1} 2iP + 0.1 mg litre^{-1} NAA, keeping all the other components constant. Incubation was done under light conditions.

Embryo-Culture Experiments. The following experiments were carried out.

- ACM and WPM supplemented with 0.5 mg litre^{-1} BAP or without BAP. All other components were kept constant as for bud-culture experiments.
- Same media (ACM and WPM) were supplemented with either 0.1 mg litre^{-1} GA_3 or 0.1 mg litre^{-1} GA_3 + 0.5 mg litre^{-1} BAP.
- Finally, the cytokinin source was compared in the following treatment: control (no cytokinin), 0.1 or 0.2 mg litre^{-1} either BAP or kinetin.

All the experiments were incubated under light conditions. Evaluation was performed after 35 days in culture. Where possible, data were submitted to ANOVA and means contrasted by Tukey's Honestly Significant Difference (HSD) with a 5% significance level.

RESULTS AND DISCUSSION

Bud-Culture Experiments. Initial establishment of sterile cultures was very difficult due to high levels of contamination ranging from 65% to 80% of the cultures. Nevertheless, on all media a few surviving explants were achieved. As Table 1 shows, survival rates ranged from 5% to 20% after a 60-day culture period, shoot development on all media was poor (0 to 2 shoots per explant) and reached up to 5 mm in length. Only WPM medium showed some callus formation.

The remaining plantlets, randomly transferred onto ACM with different hormone combinations, survived up to 83%, showing better responses in the presence of NAA in combination with cytokinin (Table 2). Auxin also improves shoot development. No root formation could be achieved during the incubation period. Further experiments

(not shown here) resulted in more and longer shoots, although rooting has to be induced separately by dipping single shoots in auxin solution as indicated by Vieitez et al. (1985) and Bennett (1987) with *Quercus robur* and *Q. shumardii*, respectively.

Table 1. Effect of culture medium on growth response of *Nothofagus alessandrii* dormant buds after 60 days.

Medium	Survival (%)	Shoot number	Shoot length (mm)	Callus formation (%)
MS	20	0	0	0
WPM	15	1	3	5
ACM	5	1	3	0
SCM	5	2	5	0

Table 2. Organogenic response of *Nothofagus alessandrii* as affected by hormonal treatment.

Treatment	Survival (%)	Shoot Number	Shoot Length (mm)	Callus formation (%)	Root number
BAP*	64	1.1	5.5	55	0
2iP*	64	0.7	7.0	64	0
BAP+NAA**	83	1.7	9.2	39	0.1
2iP+NAA	75	1.3	8.8	56	0

* 0.5 litre^{-1} ; ** 0.1 litre^{-1}.

Embryo-Culture Experiments. The use of mature embryos proved to be by far a better culture system to micropropagate *Nothofagus alessandrii*, achieving up to 95% sterile explants which could be grown into plants or even induced to multiple shoot formation. The use of ACM, differing from WPM only in some micronutrients, does not seem to lead to different results, as may be seen in Table 3. Nevertheless, the addition of BAP to the culture medium clearly increases the callus formation and the number of shoots, although the shoot length is not affected significantly by the addition of BAP. In contrast, as could be expected, the root development (number and length of roots) is affected by the addition of BAP to the culture medium.

A further experiment using the same basal media (ACM and WPM) supplemented with gibberellic acid (GA_3) alone or combined with BAP, showed differences in both callus and shoot development when seedlings were cultured on WPM (Table 4).

Root development is the same on both media. The addition of BAP combined with GA_3 increases the callus formation and the shoot number, but does not improve shoot growth within the incubation period. As expected, root development is

Table 3. Morphogenic response of *Nothofagus alessandrii* embryos to culture medium.

Treatment	Callus formation score**	Shoot number	Shoot length (mm)	Roots number	Root length (mm)
ACM-BAP	1.2 c	1.4 c	18.0 a	3.5 b	13.0 ab
ACM+BAP*	2.9 a	3.6 b	18.5 a	0.5 a	2.6 a
WPM-BAP	1.0 c	1.2 c	20.0 a	4.9 b	16.0 b
WPM+BAP	2.4 b	7.2 a	20.9 a	0.0 a	0.0 a
H.S.D. 5% (Tukey)	0.3	1.4	4.6	1.2	4.0

* 0.5 mg litre^{-1}; ** 1=min., 4=max. callusing.

Table 4. Effect of culture medium and hormones on morphogenesis of *Nothofagus alessandrii* seedlings after 35 days.

Source of variation	Callus score*	Shoot length (mm)	Shoot number	Root length (cm)	Root number
Medium					
ACM	2.0 a **	12.1 a	2.1 a	0.6 a	0.1 a
WPM	1.9 b	10.8 b	1.8 b	0.8 a	0.1 a
Hormones					
0.1 GA_3	1.3 b	11.8 a	1.3 b	1.1 a	0.2 a
0.1 GA_3+0.5 BAP	2.7 a	11.2 a	2.6 a	0.2 b	0.1 a

* 1=min., 4=max. callusing.

** Treatment means for medium and hormones followed by same letter do not differ significantly within the columns (P=5%, Tukey).

affected by the combination of GA_3 + BAP although the root number does not differ significantly.

Lowering the concentration and source of cytokinin (Table 5) as compared to previous experiments, does not bring additional advantages. In most of the parameters measured, the control (no cytokinin) was better than any other treatment. The use of Kinetin instead of BAP does not significantly affect the shoot development. The same occurs with the root number and root length. This would indicate, that after the initial growth phase of the explanted embryos, the addition of exogenous cytokinins to the culture medium would inhibit the further development of the seedling, but if cytokinins are used, BAP should be preferred.

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Table 5. Cytokinin effects on in vitro growth of *Nothofagus alessandrii* seedlings.

Treatment	Shoot formation (%)	Shoot number	Shoot length (mm)	Root number	Root length (mm)
Control	100	0.9 a *	16.6 a	2.5 a	59.9 a
0.1 BAP	53	0.6 ab	8.3 ab	0.7 a	23.2 b
0.2 BAP	67	0.6 ab	10.7 ab	0.7 a	23.5 b
0.1 KIN	40	0.3 b	3.7 b	0.9 a	13.0 b
0.2 KIN	38	0.3 b	3.6 b	1.1 a	5.6 b
H.S.D. 5% (Tukey)	-	0.4	6.5	n.s.	21.1

* Treatment means for medium and hormones followed by same letter do not differ significantly within the columns (P=5%, Tukey).

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