Ciencia e Investigación AGRARIA

Cien. Inv. Agr. 38(1):107-115. 2011
www.rcia.uc.cl

ENVIRONMENT AND ECOLOGY

RESEARCH PAPER

In vitro rooting of *Beilschmiedia berteroana*, endemic to the South Central area of Chile

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Abstract

M.E. Uribe, M. Sandoval, A. Méndez, F. Mora, and C. Delaveau. 2011. *In vitro* rooting of *Beilschmiedia berteroana,* endemic to the South Central area of Chile. Cien. Inv. Agr. 38(1): 107-115. *Beilschmiedia berteroana* ("belloto del sur") is a screrophyllous tree of the family Lauraceae that is endemic to the Central-South region of Chile. It is an endangered species; considered a second priority in the "Extinction Danger" category. This species is known for its timber, fruits and lots of mucilages, also has great potential for its size and ornamental evergreen foliage. This study had as a main objective to develop an *in vitro* rooting protocol of microshoots, obtained from the proliferation phase, by adding auxin: indole 3-butyric acid (IBA), naphthaleneacetic acid (NAA) and indole 3-acetic acid (IAA) in order to contribute to the recovery of the species. Results are discussed in the rooting phase, where this species is induced or maintained in different trials with exogenous auxin. Rooted microshoots were obtained when 250 µM of IBA were applied for 24 hours, obtaining a 40% rate of rooting, whereas no response was observed with the hormone-free treatment. Therefore, it can be concluded that *Beilschmiedia berteroana* can be vegetatively propagated *in vitro* through microcutting induced by IBA or a combination of exogenous auxin (IBA + NAA and IBA + IAA).

Key words: Auxin, *Beilschmiedia berteroana*, endangered species, Lauraceae.

Introduction

Belloto del sur, *Beilschmiedia berteroana* (Gay) Kosterm. is a threatened species considered a second priority in the "Extinction Danger" category (Benoit, 1989; CONAMA, 2007). It is an endemic evergreen tree from Central Chile that belongs to the family Lauraceae (Rodríguez *et al*., 1983). It has been reported the presence of only 8 subpopulations inhabiting between the Metropolitan Region (Melipilla province) (34°10'S), to the Bio Bio Region in the Ñuble province (36°43'S) (Hechenleitner *et al*., 2005). It is estimated that the whole population of this species is formed by no more than 2,000 indi-

Received October 15, 2009. Accepted February 12, 2011.
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viduals; almost all the forests surrounding the subpopulation to the North of La Rufina were destroyed in a fire in 1999 (Hechenleitner *et al.,* 2005).

This species is protected in the Los Bellotos del Melado and Roblería del Cobre de Loncha National Reserves; the largest known subpopulation lives in Roblería del Cobre de Loncha (Hechenleitner *et al*., 2005; Ricci *et al*., 2007). Belloto del sur grows in the altitudinal range from 60 m to 1,800 m in Andes, Central Valley and Coast Range. In Andes, it can be found in rocky sidehills close to watercourses, associated to vegetation formed by a mixture of sclerophyllous -forest and Roble-Hualo forest vegetal species (Hechenleitner *et al*., 2005). San Martín *et al.* (2002) indicated that the patterns of vegetative growth, flowering and fruiting of this species are adapted to temperate climate conditions. This species is interesting because of timber, fruits and large amounts of mucilages; it additionally has a great ornamental potential because of its height and evergreen foliage (Rodríguez *et al*., 1983).

The micropropagation techniques have been widely used as a powerful tool for propagation of plants which are difficult or impossible to obtain through conventional techniques (Fay and Clemente, 1997; Thorpe, 2007; García-Gonzáles, 2010). A fast production of selected material and availability of pathogen-free microplants is induced by an adequate management of the process, whose usefulness extends to numerous activities, among them, safeguarding biodiversity, as the germplasm from species close to extinction and with conservation problems are multiplied (Teixeira, 2003; Uribe and Cifuentes, 2004; Jordan *et al*., 2005; Hamidoghli *et al*., 2007; Uribe *et al*., 2008).

Plant rooting is of great importance in the case of plants that are difficult to reproduce sexually, or in those plants where the resource is degraded (Caro *et al*., 2003; Martínez-Pastur *et al.,* 2005). In this context, there is information on *in vitro* rooting in Chilean microprogated species such as *Nothofagus alessandrii*, *N. glauca* and *N. leonii* (33, 93.3 and 56.7% respectively) (Cardemil, 2000). Studies performed by Martínez-Pastur and Arena (1997), in *N. pumilio,* reported rooting rates between 60 to 70% in microshoots obtained from secondary forest shoots and an adult tree, respectively. In *Gomortega keule*, root formation occurred in presence of 24.6 µM IBA in approximately 46% of the sub-cultivated nodal segments after a three months period (Jordan *et al*., 2005). Studies performed by Martínez-Pastur *et al*. (1998) in *Nothofagus obliqua* showed that it is possible to obtain acceptable rooting percentages in microshoots from apical and basal portions (48.6 and 27.0%, respectively). An induction of adventitious roots was made in *Drymis winteri* in a MS medium, from stem apices from 6-month-old plants, obtaining rooted plants in MS medium, with half diluted macronutrients, in presence of 26.58 µM NAA and 0.5 mg L^{-1} of calcium pantothenate (rooting percentages are not provided) (Jordan, 1999). In regard to the species *Beilschmiedia berteroana*, there are no reports on *in vitro* rooting; only one study covers the *in vitro* propagation of this species, using terminal and axillary buds with nodal segment from young plants as seedlings (Calderón-Baltierra and Rotella, 1998).

In vitro rooting represents a difficult stage during the micropropagation process of forest species; therefore, the aim of this work was to determine a protocol of *in vitro* rooting of belloto del sur, studying the influence of auxins on the rhizogenic potential of the species influence and whether they affect positively the obtaining of complete plants.

Materials and methods

The material used corresponded to seedlings maintained in a nursery during 3 years, obtained from germinated seeds. The source plants were treated with a broad spectrum systemic fungicide (Benomil, Bayer® and Mancozeb, Bayer®) in a 10 g L^{-1} concentration applied every seven days during seven consecutive weeks. After this period, lateral branches were isolated and the material was washed under tap water in order to eliminate dust remains completely. Subsequently, they were submerged in a fungicide solution (Captan, Bayer® 2.5 g L^{-1}) during 2 hours. Then,

they were rinsed in sterile distilled water and the nodal segments longer than 1.5 cm were cut with at least two internodes.

The surface asepsis of the nodal segments was initiated under sterile conditions in laminar flow cabinet and continuous agitation by immersion in alcohol at 70% (v/v) during 5 minutes, followed by four successive rinses with sterile distilled water for 2, 5, 10 and 15 minutes, respectively. Then, they were submerged in a solution of commercial chlorine at 50% (v/v) with 2.5% of active chlorine during 15 minutes. After that, four washes with sterile distilled water were applied during 5 minutes each.

In the establishment phase, two seedlings were placed per 5.5 cm diameter x 6.5 cm high-glass recipient, with 30 ml from the MS culture medium (Murashige and Skoog, 1962), with complete macronutrients and diluted at 25% (MS and $MS_{1/4}$) and DKW (Driver and Kuniyuki, 1984), with total macronutrients or diluted at 50% (DKW and $DKW_{1/2}$). The media were supplemented with sucrose (30 g L^{-1}) and 0.8% of bacteriological agar (Merck®). The pH was fitted at 5.8 and sterilized in autoclaving during 20 minutes at 1 pressure atmosphere and 121 ºC of temperature.

Thirty repetitions were made by treatment. The quantification was made at 45 days, when the percentages of viables, non-viable and contaminated seedlings were evaluated.

The rooting tests were made with microshoots obtained from the second subculture, longer than 1.5 cm, testing the effect from different concentrations and types of auxins. One hundred microshoots of 1.5 of length with at 4 leaves were used, to which an incision was made in the lower portion in order to allow the absorption of the rooting hormones by the seedling. A first test consisted on checking the effect of IBA 24.6 µM (Merck®), for 5 minutes of induction. The effect of AIB applied on a solution of 250 µM during 12 and 24 hours was checked in a second test. Finally, two hormonal combinations were applied: IBA (49 µM) plus NAA (5.37 µM, Merck®) and IBA (49 µM) plus indole-3-acetic acid (1 µM, Merck®). The hormones used in combination were added to the basal medium in order to evaluate the responses generated after 30 days.

The microshoots, once induced in the corresponding hormone, were introduced in $DKW_{1/4}$; basal medium supplemented with sucrose 20 g L^{-1} and bacteriological agar 0.8% (manifestation medium), fitting the pH to 5.8. Then, they were incubated in a growth chamber under controlled conditions, at 25 ± 1ºC of temperature, 55% of relative humidity, under a photoperiod of 16 hours of cold light and luminous intensity of 40 µmol m^{-2} s^{-1}, for a minimum period of 30 days.

Ten microshoots with their respective control treatment were used in all the tests, and the following parameters were evaluated at the end of the period: callus percentages, radicular primordia and rooted microshoots.

Statistical analysis

The methodology of Generalized Linear Model was used for the statistical analysis, according to the formula by Nelder and Wendderburn (1972). The variables of interest were considered binary; therefore, the binomial distribution (absence or presence of an event or variable) and the canonical link function (logit) were considered in the GLM fit, according to Mora *et al*. (2007). The procedure GENMOD by SAS was used for the analysis of deviance (ANDEVI) with the option of generalized orthogonal contrasts in order to determine the differences between treatments (Dos Santos and Mora, 2007).

Results and discussion

In the establishment stage, the analysis of deviance indicated significant differences among the media used for the variables evaluated (Table 1). The high percentage of establishment in both culture media (Table 2), at over 70% and a pathogenous contamination that does not exceed 10% in the cases, where the saline concen-

Table 1. Analysis of deviance (ANDEVI) performed for each binary variable during establishment period *in vitro* of *Beilschmiedia berteroana*.

		Survival		Necrosis		Fungal Contamination		Bacterial Contamination	
	GL	D	χ^2	D	χ^2	D	χ^2	D	χ^2
Intercept		371.5	-	174.8	-	313.0	-	58.4	-
Middle	3	340.4	31.08**	165.9	8.9*	284.3	28.63**	49.3	9.11*
Mean (%)		68.09		9.05		21.15		1.71	

GL: degrees of freedom; χ^2: Chi-square; D: deviance; **: significant to P≤0.01; *: significant to P≤0.05.

Table 2. Analyses of generalized orthogonal contrasts performed for each binary variable measured in the establishment period, from the micropropagation of *Beilschmiedia berteroana*.

Media	Survival	Necrosis	Fungal contamination	Bacterial contamination
T_1	58.8 bc	5.0 b	35.0 a	1.3 ab
T_2	73.0 b	17.0 a	10.0 b	0.0 b
T_3	50.6 c	11.2 ab	32.6 a	5.6 a
T_4	90.0 a	3.0 b	7.0 b	0.0 b

T_1: MS; T_2: MS¼; T_3: DKW; T_4: DKW½.

tration was reduced, indicated that the treatment applied to the source plants before the extraction of the initial material, provided an excellent alternative to introduce contamination-free tissues. These results are similar to those from other studies made on native species, as *Nothofagus pumilio,* which presented 80% of contamination-free seedlings (Martínez-Pastur and Arena, 1997), as well as the results obtained by Cardemil (2000) in *Nothofagus glauca, N. alessandrii* and *N. leonii*.

Establishing pathogen-free material in this stage is the base to obtain a successful i*n vitro* culture (Pérez, 1998); which shows partly the good sanitary status of the mother plant and the effectiveness of the surface asepsis applied (Figure 1).

On the other hand, the results suggest that the belloto del sur tissues established *in vitro* may be susceptible to the salts concentration of the medium, as the best results were obtained in these media ($MS_{1/4}$ and $DKW_{1/2}$), with statistically significant differences between them, reaching the best survival result (90%) in medium $DKW_{1/2}$. Media with low salt concentration have been used successfully in the propagation of *Nothofagus nervosa* and various Fagaceae (Vieitez *et al*., 1993; Jordan *et al*., 1996).

It is observed in medium $DKW_{1/2}$, that there is a low percentage of seedlings loss due to their brownishing. Literature reports that this brownishing may be caused by enzyme oxidases, like polyphenoloxidases and tyrosinases, which are released when the tissues are wounded. The growth inhibition of seedlings, on the other hand, occurs by phenols oxidation and the subsequent formation of quinonic compounds, which are highly active (Espejo *et al*., 1990; Marks and Simpson, 1990; Seemann, 1993; Alvarado, 1998).

During the phase of root induction, the *in vitro* microshoots rooted when indole 3-butyric acid or an auxin combination were applied to the $DKW_{1/4}$ (Figure 2). Therefore, the results from the analysis of deviance indicate that there are significant differences among the tests for the variables radicular primordia and rooted microshoots (Table 3). In this regard, both IBA (49 µM) + NAA (5.37 µM) applied

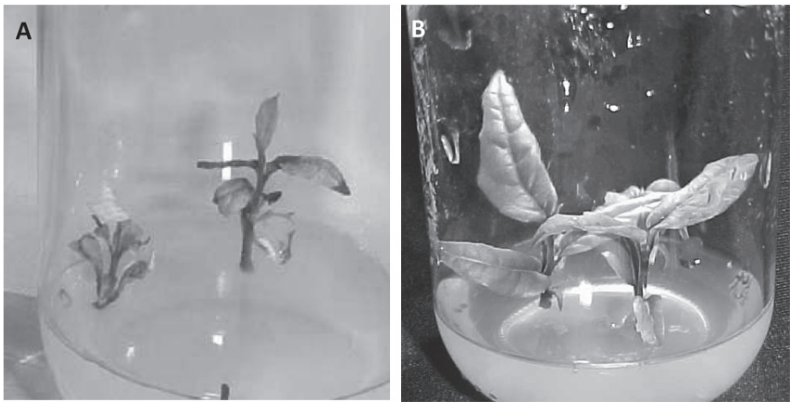

Figure 1. Establishment of microshoots of *Beilschimiedia berteroana* after 45 days of incubation on culture media. A) Microshoots in medium $MS_{1/4}$. B) Microshoots in medium $DKW_{1/2}$.

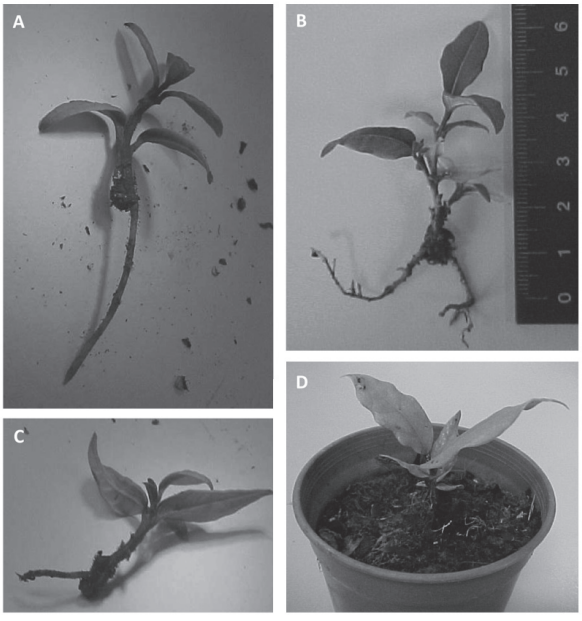

Figure 2. Microplants of *Beilschimiedia berteroana* grown under the treatments A) IBA (49 µM) + NAA (5.37 µM), B) IBA 250 µM during 24 hours, C) IBA 24.6 µM, and D) Conditioned plant (T_2).

to the culture medium during 30 days, and IBA 250 µM applied during 24 hours, produced one or various roots, with lateral roots and shoots and leaves development after 3 to 5 months of culture in manifestation medium, obtaining between 30 and 40% of rooting respectively (Table 4, Figure 2A and B). Regardless belloto del sur has been categorized as a recalcitrant species to propagation (Reyes, 1997), as acceptable rooting percentages were obtained in this study.

Similar rooting results (46%) were obtained in *Gomortega keule* in seedlings from nodal sections from 2-year material, with the application of IBA (24.6 µM) in the culture medium during three months (Jordan *et al*., 2005). In *Quercus rubra,* using a hormonal concentration of IBA of 25 mg L^{-1} during 24 hours (San-Jose *et al*., 1996), a 37.3% of rooting was reached. Higher rooting percentages (59%) were obtained from seedlings of *Quillaja saponaria*, with the addition of IBA (0.49 µM) during one month (Prehn *et al*., 2003).

Table 3. Analysis of deviance (ANDEVI) performed for each binary variable measured in the rooting period *in vitro* of *Beilschmiedia berteroana*.

		C		RP		RM	
	GL	D	χ^2	D	χ^2	D	χ^2
Intercept		27.8769	-	50.0402	-	50.0402	-
Treatment	4	23.0114	4.87 ns	36.9285	13.11*	42.1872	12.3*
Mean (%)		8.0		20.0		20.0	

GL: degrees of freedom; χ^2 : Chi-square; D: deviance; ns: the effect was not significant (P>0.05); *: significant to P≤0.05; C: callu; RP: root primordia; RM: rooted microshoots.

The low rhizogenic response (10%) obtained in the concentration of 24.6 µM of IBA (Table 4, Figure 2C) may be attributed to the reduced immersion time of the microshoots in the growth hormone, which did not allow its absorption by the seedling and the low concentration used.

Similar studies made *ex vitro* in *Gomortega keule* (Peña, 1995), provided similar results with the application of IBA 6000 mg L^{-1} (3.3% of rooting). If only the total of treatments applied in queule are considered, only 0.26% of rooted cuttings were obtained.

However, higher percentages have been observed in other wood-like species. For example, Peralta (2001) obtained 84.38% of rooting in *Nothofagus alpina* with IBA 500 mg L^{-1} for a 7-minute-induction time, from shoots from seedlings cultivated *in vitro*, while San-Jose *et al.* (1996), using the same concentration for 2 minutes, obtained 45% of rooting in *Quercus rubra*.

In regard to the number of radicular primordia obtained, it must be considered that, during the manifestation stage and radicular development, other problems reducing the efficiency of the process become evident. Also, a portion of the centers of radicular initiation creates nodular structures that never evolve to root formations, which might cause a problem to the sites of origin and tissues implied (Hartmann and Kester, 1999).

In general, there is a low percentage of formation of basal callus in all the tests, an important aspect to be mentioned, as the presence of callus partly reduces root production and greatly impedes the transference of the vitroplants to the consolidation field (Ríos *et al*., 2005).

A difference was recorded in the time required for the manifestation of rhizogenic responses among the different test; a faster response was seen when hormonal combinations were used in the culture medium (IBA/NAA and IBA/IAA), in a period of three months. Potentially, it was necessary to wait for five months for results for the treatment IBA 250 µM 24 hours.

The results obtained were powered by the low number of subcultures (2) which had the source material, as the number of subcultures is increased, the response capacity of the tissues is affected, which is reflected in a lower rooting capacity (Ríos *et al*., 2005).

Table 4. Analyses of generalized orthogonal contrasts performed for each binary variable measured in the rooting period, from of *Beilschmiedia berteroana*.

Treatments	RP	RM
T1	0.0 b	0.0 b
T2	20.0 ab	40.0 a
T3	0.0 b	10.0 b
T4	40.0 a	20.0 ab
T5	40.0 a	30.0 a

T_1: IBA 250 µM (12 hours); T_2: IBA 250 µM (24 hours); T_3: 24.6 µM IBA; T_4: IBA 49 µM + IAA 1.0 µM (30 days); T_5: IBA 49 µM + NAA 5.37 µM (30 days); RP: root primordia; RM: rooted microshoots.

On the other hand, the hormone-free tests did not provoke any response of rhizogenic induction. This might indicate the absence of preformed structures for the emergence of radicular primordia in the species; structures found easily induced in other species like *Salix* sp., *Populus* sp., *Ribes nigrum* or *Fagus sylvatica* (Haissing, 1972; Fink, 1982*)*, where these primordia may develop roots, depending on the culture conditions. Similar observations were seen in a study made with *Nothofagus nervosa,* where the lack of pre-radicular structures was evident (Martínez-Pastur *et al*., 2005). Therefore, the exogenous application of inducing agents as auxins and other regulators of vegetal growth becomes relevant, as they play an important role in this response (Martínez-Pastur *et al*., 2007).

Acclimation of the rooted plants was made in a peat-perlite mixture (80%: 20%) and maintained inside the culture chamber for one month. Subsequently, they were transferred to the laboratory of tissue culture in fall-winter, under uncontrolled conditions; this is, at temperatures lower than 15 °C and with high humidity. A permanent irrigation with macronutrients from the medium DKW diluted at 25% was made, reaching 50% of survival (Figure 2 D).

The present study is the first report on *in vitro* rhizogenic induction for the species *Beilschmiedia berteroana,* from nodal segments of three-year-old plants, by the application of IBA 250 µM during 24 hours and IBA/NAA in media DKW at a quarter of the normal macronutrient concentrations. Between 30 and 40% of rooted microshoots in IBA 49 µM + NAA 5.37 µM and IBA 250 µM were obtained, respectively. These results will allow a continuous improvement of the rhizogenic process, and will represent the base for the development of further studies.

Acknowledgements

Study financed by Universidad de Concepción, Project DIUC N° 203.412.003-1.0. The authors thank Forestal y Agrícola Monteáguila (Los Ángeles) for providing the vegetal material used in this study.

Resumen

M. Uribe, M. Sandoval, A. Méndez, F. Mora y C. Delaveau. Enraizamiento *in vitro* de *Beilschmiedia berteroana*, endémica de la zona Centro-Sur de Chile. Cien. Inv. Agr. 38(1): 107-115. *Beilschmiedia berteroana* (belloto del sur), es un árbol esclerófilo de la familia Lauraceae endémica de la zona Centro-Sur de Chile. Es una especie amenazada, considerada en segundo lugar de prioridad ubicada en la categoría "En Peligro". La especie es interesante por su madera, frutos y gran cantidad de mucílago; además tiene gran potencial ornamental por su porte y follaje siempreverde. Este trabajo tuvo por objetivo establecer un protocolo de enraizamiento *in vitro* de microtallos provenientes de la fase de proliferación mediante la aplicación de auxinas: ácido indolbutírico (AIB), ácido naftalenacético (ANA) y ácido indolacético (AIA), con la finalidad de contribuir a la recuperación de la especie. Se discuten los resultados obtenidos en la fase de enraizamiento, donde esta especie es inducida o mantenida en diferentes ensayos con auxinas exógenas. Microtallos enraizados se obtuvieron con AIB 250 µM aplicado por 24 horas, donde se logró obtener un 40% de enraizamiento, mientras que el ensayo libre de hormonas no indujo respuesta. Se concluye que *Beilschmiedia berteroana* puede ser propagada vegetativamente *in vitro* a través de microestacas inducidas con AIB o con una combinación de auxinas exógenas (AIB + ANA y AIB + AIA).

Palabras clave: Auxina, *Beilschmiedia berteroana*, especie en peligro, Lauraceae.

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