BIOCELL
2000, 24(1): 65-71

ISSN 0327 - 9545
PRINTED IN ARGENTINA

Calcium and Boron for *in vitro* Rooting of *Nothofagus nervosa*

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Key words: *Nothofagus nervosa*, rooting, micropropagation, calcium, boron

ABSTRACT: Several mineral compounds influence adventitious rooting, and those containing boron and calcium, play an important role. They are associated to the enzyme regulation, cell division, cellular metabolism (*induction stage*) and roots enlargement (*expression stage*). The aims of this work were to study the variation of the endogenous calcium and boron concentrations along the rooting, and determine their optimal concentrations in the rooting basal medium of *Nothofagus nervosa*. Significant variation in endogenous calcium concentrations was detected along the rooting phase, strongly correlated with rhizogenesis phenomenons and linked to total peroxidase variation as cited elsewhere. Calcium and boron concentrations in the basal medium significantly affected the rooting. Treatment containing 60 $mg \cdot L^{-1}$ Ca and 1 $mg \cdot L^{-1}$ B produced root formation in fewer days than other combinations. The variation of these nutrient concentrations in the medium is a useful tool to improve microcutting rooting. Roots are obtained earlier and in a shorter period of time, finally resulting in more homogeneous microplants with a better developed rooting system.

Introduction

Several mineral compounds influence adventitious root formation (Abu-Qaoud *et al.*, 1991). A reduction of macronutrient salts in basal medium is used to stimulate rooting (Chalupa, 1983; Arrillaga *et al.*, 1991; Rahman *et al.*, 1992). In *Nothofagus* half strength macronutrient salts increases rooting with better quality of the radical system (Martínez Pastur and Arena, 1995; 1996).

Peroxidase was suggested as a marker for distinct phases during adventitious root formation in forest species (Ben-Efraim *et al.*, 1990), as well as in *N. nervosa* (Calderón Baltierra *et al.*, 1998). Recently, variation in the peroxidase concentration was correlated with several hormones, enzymes, phenols and chemical compounds (Hausman *et al.*, 1994; 1995; Heloir *et al.*, 1996; Gaspar *et al.*, 1997; Kevers *et al.*, 1997a; 1997b).

Boron and calcium are some of the main nutrients playing an important role in the rooting process. Some possible functions in the plant metabolism have been ascribed to boron, e.g. it could be an enzymatic inhibitor of polyphenoloxidases, catalases and peroxidases, a participant in lignification and in xylematic differen-

tiation with phenols, a cellular enlargement modifier and a factor in the orientation of cellular division (Street and Opik, 1976; Marschner, 1993). Calcium is a component of cellular walls, cell membranes and also is present as free calcium in cytosol and apoplasm (Burchert *et al.*, 1990; Cleland *et al.*, 1990; Marschner, 1993). Several possible functions are known to involve calcium, e.g. it has been found in association with the peroxidase structure (Van Huystee and Esnault, 1992), acting as an auxin second messenger (Gaspar *et al.*, 1991; Bush, 1993; Weeb *et al.*, 1996), participating in the regulation of mitosis (Weisenberg, 1972; Hepler *et al.*, 1990), regulating several enzymes (Burchert *et al.*, 1990; Gaspar *et al.*, 1991), contributing to the control of apoplastic pH during cellular enlargement (Cleland *et al.*, 1990; Gaspar *et al.*, 1991; Marschner, 1993), and participating in the mechanism of transport of certain compounds through cell membranes (Gaspar *et al.*; 1991; Bush, 1993).

This work was intended firstly, to analyse the variation of endogenous concentrations of calcium and boron in microcuttings of *Nothofagus nervosa* during the *in vitro* rooting process and then, correlate these values with known changes of total peroxidase activity that define different phases throughout this process (Calderón Baltierra *et al.*, 1998) to finally determine the possible correlation of calcium and boron concentrations with the radical morphogenesis processes; and secondly, to determine the concentrations of these nutrients in the culture medium for optimal *in vitro* rooting response of *N. nervosa*.

Materials and Methods

Plant material

Young *Nothofagus nervosa* shoots 2.5 cm long with 2-4 leaves and 4-6 buds were employed as explants. The basal medium proposed by Martínez Pastur and Arena (1996) was used for rooting. This medium has the same salt moiety given for broadleaved trees (Chalupa, 1983), and the pH was adjusted to 5.7 - 5.8 with 0.1 N KOH. Fifty ml of medium were dispensed into each of 350 ml flasks, and then autoclaved at 0.1 MPa for 20 min. Cultures were kept in a growth chamber at 24 ± 2°C using cool-white fluorescent lamps (57 µmol m^{-2} s^{-1} photosyntetically active radiation) with a 16 h photoperiod. Trials and sampling were performed in laboratories of the Centro Austral de Investigaciones Científicas (CADIC).

Calcium and boron tissue contents during in vitro rooting

Microcuttings (1-2 g FW) were collected every two days along rooting. Samples were dried at 60°C and ground in a porcelain mortar. Then, they were prepared in a single step using a mixture of nitric acid and percloric acid (2:1) according to Johnson and Ulrich (1959). Calcium and boron concentrations in these extracts were measured using inductively-coupled plasm atomic emision spectrophotometer (ICPS Shimadzu 1000 III) (LANAQUI - CERZOS - Universidad Nacional del Sur). Three replicates of samples (0.1 g DW) were taken throughout the rooting period, and distilled water was used as a control.

Calcium and boron in the rooting medium

A bifactorial design was used resulting in 16 treatments. Four concentrations of boron (0, 1, 5 and 10 $mg \cdot L^{-1}$) and calcium (0, 60, 300 and 600 $mg \cdot L^{-1}$) were tested. Indol butyric acid (0.125 $mg \cdot L^{-1}$) was added to all media. Each treatment was given to six explants *per* flask. Nutrient concentration of media in each flask was averaged before performing statistical analysis (n = five *per* treatment). Rooting was evaluated every two days during a 28 day period. The percentage of mortality (M), rooting ratio (R), root number (RN), root length (RL) and percentage of explants with secondary roots (SR) were recorded. A rooting index was defined as:

$$RI\ (mm) = RN \times RL \times R \times R_{11}$$

with R_{11} as the rooting ratio at day 11 (R and R_{11} were expressed as values between 0 and 1). The inclusion of R_{11} in this equation accounts for the appreciation of the magnitude and earliness of the rooting response to treatments, since all treatments produced their maximum rooting effect at this time.

Statistical analysis

Data of endogenous calcium and boron concentrations were studied by an analysis of correlation. The statistic validity of the other results was obtained through analysis of variance by Fisher and Tukey multiple range tests. For all tests the significance was $P<0.05$.

Results and Discussion

Calcium and boron tissue content during the in vitro rooting phases of microcuttings

Average values of endogenous calcium (3110.6 ppm/DW) and boron (22.9 ppm/DW) are well within the known ranges in higher plants (Marschner, 1993). Boron and calcium are positively (0.716) and significantly (0.030) correlated (Fig. 1). However, no correlation was found between variation in boron and calcium concentrations and previously known variation in total peroxidase activity in the same plant cultured under the same conditions.

Nutrient concentrations were largest at the beginning of the trial (days 1 to 3) followed by the smallest values during days 5 to 7 and successive peaks and falls during days 9 to 28 (Table 1).

Peroxidases were previously suggested to differentiate different phases during rooting, and total tissue peroxidase activity was suggested as a marker (Calderón Baltierra *et al.*, 1998). The peak of peroxidase activity

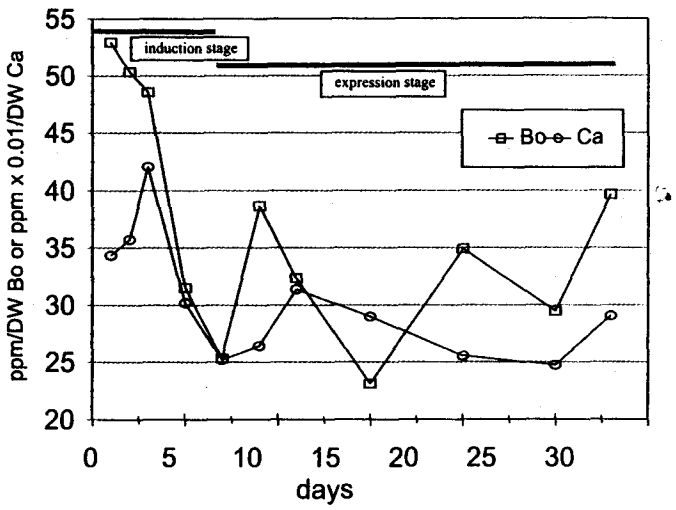

FIGURE 1. Endogenous variation of calcium (ppm x 0.01/DW) and boron (ppm/DW) along the rooting phases of *Nothofagus nervosa* microcuttings. Phases were defined by Calderón Baltierra *et al.*, 1998.

TABLE 1.

Concentration of calcium and boron in *Nothofagus nervosa* tissue microcuttings during *in vitro* rooting.

Days	Boron (ppm/DW)	Calcium (ppm/DW)
1	52.94a	3435.5ef
2	50.38a	3574.1f
3	48.58a	4207.8g
5	31.54a	3020.3cd
7	25.35a	2520.9ab
9	38.70a	2642.5abc
11	32.38a	3140.6de
15	23.11a	2899.3bcd
20	34.92a	2554.1ab
25	29.49a	2474.0a
28	39.67a	2907.1bcd

Significance of the main effects: Boron (0.061), Calcium (0.000).

TABLE 2.

Effect of calcium (mg•L^{-1}) and boron (mg•L^{-1}) concentrations in basal medium for rooting of *Nothofagus nervosa* microcuttings, on mortality (*M*), percentage of rooting at day 28 (*R*), percentage of rooting at day 11 (R_{11}), number (*RN*) and length of roots (*LR*), percentage of explants with secondary roots (*SR*) and rooting index (*RI*). Values represent means for each factor.

		M (%)	R (%)	R_{11} (%)	RN (n)	RL (mm)	SR (%)	RI (mm)
Calcium (mg•L^{-1})								
0		1.42a	94.77bc	82.73b	6.39c	3.64b	12.81b	19.27c
60		1.42a	97.73c	86.90b	6.94c	3.88b	3.33a	23.29c
300		3.92a	84.76b	60.71a	5.12b	3.04b	0.83a	9.83b
600		32.97b	67.42a	45.38a	2.92a	1.71a	0.00a	2.30a
Boron (mg•L^{-1})								
0		24.73b	81.93a	72.58a	5.42a	3.50b	5.76a	16.42a
1		9.16a	84.18a	67.73a	5.39a	3.25ab	6.00a	15.79a
5		2.88a	88.82a	68.86a	5.19a	3.12ab	3.66a	12.61a
10		2.97a	89.76a	66.54a	5.36a	2.40a	1.54a	9.87a
Calcium (mg•L^{-1})	Boron (mg•L^{-1})							
0	0	5.71	94.28	85.23	6.38	4.82	16.38	27.35
0	1	0.00	96.66	86.66	6.23	4.40	14.00	23.03
0	5	0.00	88.15	74.76	6.42	2.87	14.66	12.80
0	10	0.00	100.00	84.28	6.52	2.46	6.19	13.90
60	0	5.71	93.80	81.42	6.76	4.03	6.66	21.93
60	1	0.00	100.00	96.66	7.63	4.51	6.66	31.74
60	5	0.00	100.00	88.09	6.74	3.80	0.00	22.51
60	10	0.00	97.14	81.42	6.61	3.18	0.00	16.98
300	0	0.00	93.80	67.14	6.02	3.48	0.00	15.00
300	1	6.66	89.99	56.66	4.97	2.63	3.33	7.73
300	5	9.04	67.14	51.90	4.25	3.90	0.00	9.70
300	10	0.00	88.09	67.14	5.24	2.17	0.00	6.89
600	0	87.49	45.83	56.54	2.51	1.66	0.00	1.40
600	1	30.00	50.08	30.95	2.74	1.46	0.00	0.65
600	5	2.50	100.00	60.71	3.36	1.92	0.00	5.40
600	10	11.90	73.80	33.33	3.06	1.81	0.00	1.72

Significance of the main effects: Calcium: M (0.000), R (0.000), R11 (0.000), RN (0.000), RL (0.000), SR (0.000), RI (0.000); Boron: M (0.000), R (0.311), R11 (0.819), RN (0.939), RL (0.017), SR (0.197), RI (0.055); Calcium x Boron: M (0.000), R (0.000), R11 (0.227), RN (0.518), RL (0.060), SR (0.752), RI (0.115).

found by those authors at day 2 could be related to the maximum boron concentration of 52.9 ppm/DW on day 1 and the largest calcium concentration of 4207.8 ppm/DW on day 3. On the other hand, the peroxidase minima at days 5 and 13 found by Calderón Baltierra *et al.* (1998) could be related to minima measured in calcium (2520.9 ppm/DW) and boron (25,3 ppm/DW) at day 7 (Table 1).

The first peak of peroxidase concentration defined the induction stage of rooting (Calderón Baltierra *et al.*, 1998). Increased calcium and boron concentrations were related to induction, probably through enzyme regulation, cell division and cellular metabolism (Burchert *et al.*, 1990; Gaspar *et al.*, 1991; Van Huystee and Esnault, 1992; Druart, 1997). During expression of the rooting stage, the roots enlarged, and calcium and boron participate in the process (Street and Opik, 1976; Sentenac and Grignon, 1981; Cleland *et al.*, 1990; Gaspar *et al.*, 1991; Marschner, 1993).

The effect of Ca and B concentration in the rooting medium

When analysing the effect of different concentrations of calcium, significant differences were detected on all the studied variables (Table 2). The largest response was a rooting index of 23 mm and was found with 60 mg•L^{-1} of calcium. Boron significantly affected the mortality and root length (Table 2); mortality declined at concentrations greater than 5 mg•L^{-1}, whereas the longest roots were in the treatments with lowest boron concentration. Treatment with 60 mg•L^{-1} calcium and 1 mg•L^{-1} boron gave the best rooting response with a rooting index of 32 mm and 100% of rooted explants.

When analysing variation of the rooting percentage during the culture period, we observed a peak between days 9 and 11, and the maximum rooting percentage was attained at day 23 (Table 3). The increased concentrations of both nutrients delayed the onset of the peak (day 11), while the smaller concentrations presented an earlier peak at day 7. By day 9, treatments with 0-60 mg•L^{-1} of calcium gave the largest rooting celerity (51-59%), while in boron this response (40-45%) was observed at 0-5 mg•L^{-1}. The optimal combination of these nutrients (60 mg•L^{-1} Ca and 1 mg•L^{-1} B) produced roots in fewer days and more concentrated in time than the other treatments (Table 2).

The calcium concentration influenced the rooting percentage more markedly than variations in boron concentrations (Fig. 2). In this point, it is worthwhile to recall the low tissue variation of boron concentration

TABLE 3.

Effect of calcium and boron concentrations over the celerity of non-accumulated rooting (%) of *Nothofagus nervosa in vitro* microcuttings.

Concentration		Days								
mg•L^{-1}		7	9	11	13	15	17	19	21	23
Calcium	0	10.23b	51.78b	20.71a	4.64a	0.71a	0.00a	2.40a	0.83a	1.66a
	60	8.92b	58.57b	19.40a	6.07a	0.83a	0.71a	0.00a	0.71a	2.50a
	300	2.38ab	29.52a	28.80a	11.19ab	1.42a	2.26a	2.26a	0.00a	3.92a
	600	0.00a	14.28a	30.51a	17.60b	2.28a	0.65a	3.16a	1.50a	1.62a
Boron	0	11.90b	45.23a	16.29a	5.38a	0.75a	0.75a	0.00a	0.00a	3.38a
	1	4.16ab	42.26a	21.30ab	10.23a	1.66a	0.00a	2.50a	0.83a	2.50a
	5	3.69ab	40.35a	24.82ab	10.65a	1.33a	0.62a	2.17a	1.42a	2.38a
	10	2.38a	27.85a	36.30b	12.61a	1.42a	2.26a	3.09a	0.71a	1.54a

Significance of the main effects: Calcium: 7 (0,002), 9 (0,000), 11 (0,187), 13 (0,008), 15 (0,649), 17 (0,430), 19 (0,253), 21 (0,717), 23 (0,706); Boron: 7 (0,015), 9 (0,140), 11 (0,009), 13 (0,388), 15 (0,924), 17 (0,429), 19 (0,279), 21 (0,748), 23 (0,881).

during the rooting period. In the absence of boron, rooting progressed until day 13, but did not reach the maximal percentage at the end of the culture period. The optimal concentration of calcium (60 mg•L^{-1}) produced the largest rooting ratio and maximal celerity during the experiment.

Previous works indicate that increasing boron and calcium concentrations could be either favorable or not for the rooting phase. Variations of calcium in the culture medium also stimulated the growth in *N. nervosa* and root celerity presentation in *Malus domestica* (Druart, 1997). On the contrary, boron supplementation in the *in vitro* culture of *Actinidia chinensis* cuttings did not improve rooting percentage (Ono *et al.*, 1995). In the case of *Berberis thunbergii* the addition of both boron and calcium did not improve the *in vitro* rooting either (Kahru and Hakala, 1990).

The variation of tissue calcium and boron concentrations in *N. nervosa* was found to be correlated with known rhizogenesis phenomena. In fact, these nutrients increase during the induction phase - that was previously defined from the total peroxidase activity (Calderón Baltierra *et al.*, 1998) - and decrease during the expression phase. The availability of these nutrients throughtout the *in vitro* culture is very important because they could be limiting the whole phenomenon of rooting. These nutrients appear to improve rooting in *N. nervosa* and modification of their concentration in the original medium would improve the celerity in the root formation, and the *in vitro* rooting ratio of

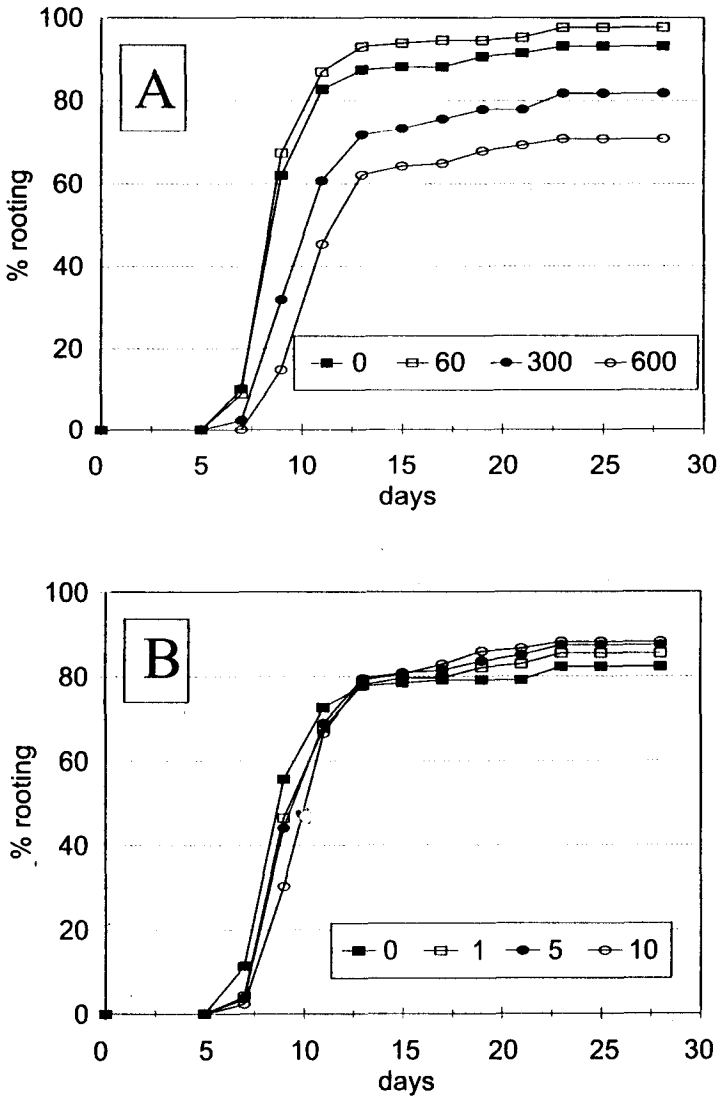

FIGURE 2. Development of rooting in *Nothofagus nervosa* microcuttings as a function of calcium (***A***) and boron (***B***) concentrations (mg•L^{-1}) in the basal medium.

microcuttings of *N. nervosa* as demonstrated here. In fact, roots appear earlier and in greater numbers, giving rise to more homogeneous microplants with well - and better - developed rooting system. Otherwise, explants with high quality roots produced more adequate plantlets to face *ex vitro* conditions. Also, results mentioned above could be of direct application in *ex vitro* rooting of microcuttings propagated *in vitro*.

Aknowledgement

The authors wish to thank Dr. Osvaldo Caso for critical comments and to Miriam Crespo, Ricardo Devalis (CERZOS) and Gustavo Vater (CADIC) for helpful technical assistance. This work was supported by a grant from the National Research Council for Science and Technology (CONICET).

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