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Successive media to improve the *in vitro* rhizogenesis of *Nothofagus nervosa* (Phil.) Dim. et Mil.

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Abstract. *In vitro* rooting constitutes a difficult step during the micropropagation process of forest species. The successive media culture technique represents one way to overcome this barrier and includes modifying physical (*e.g.* photoperiod) and chemical (*e.g.* flavonoids) factors during the rooting phases. The aim of this study was to obtain a successive media protocol based on the incorporation of flavonoids during the *in vitro* rooting of *Nothofagus nervosa*. The factors evaluated were the type, concentration, and combination of flavonoids in relation to the rooting phases, the presence of *IBA* in the culture medium, the photoperiod, and the effect of flavonoids on total tissue peroxidase activity. The photoperiod used included a darkness period during the rooting induction stage and the presence of 0.61 μM *IBA* in the culture medium. The results showed that flavonoid incorporation at a concentration of 20 μM accelerated the appearance of roots and improved the quality of the already formed ones. Each type and concentration of flavonoid produced different responses, with *(±)naringenin* giving the best results. The latter caused a peak in the peroxidase activity that was absent in the control treatments. This work allowed identifying an optimized rooting protocol through a successive media culture technique that improved the speed of appearance, as well as the quantity and quality of roots for a single *N. nervosa* clone.

Introduction

The rooting stage often represents a critical and difficult step during the *in vitro* propagation of certain woody species (Davis et al. 1994; Altamura 1996; De Klerk 1996; Kevers et al. 1997). Different micropropagation protocols have been developed for the *Nothofagus* genus (Martínez Pastur and Arena 1995, 1997, 1999; Martínez Pastur et al. 1997). In particular, *N. nervosa* (Phil.) Dim. et Mil. (commonly named 'raulí') showed a good *in vitro* rooting response, but also presented variability in root homogeneity with respect to quantity and quality (Jordan and Velozo 1992; Calderón Baltierra et al. 1994; Jordan et al. 1996; Martínez Pastur and Arena 1996). This variability could be partly attributed to the source of explants, and could possibly be controlled through correct management of plant material, avoiding topophysis and cyclophysis effects (Martínez Pastur et al. 1998). Furthermore it could correspond to internal biochemical changes occuring during the rhizogenesis of the microshoots (Martínez Pastur et al. 2000). These

findings raised the possibility to improve the *in vitro* rhizogenic response of *N. nervosa* by overcoming the variabiltity, using a sequential culture media technique, a two-step culture with media differing in chemical composition (Berthon et al. 1993).

An understanding of the rooting phases (*induction* and *expression*) (Gaspar 1981) is a prerequisite in attempting to formulate successive media culture technique. These phases have been established for *N. nervosa* based on the variation of peroxidase activity of *in vitro* shoots (Calderón Baltierra et al. 1998). Physical and chemical factors change the peroxidase activity during the rooting phases (Druart et al. 1982; Berthon et al. 1993), affecting both the quantity and quality of roots. Among these factors, changes in photoperiod, absence of auxins, and incorporation of chemical compounds, like phenolic acids and flavonoids, have been cited (Berthon et al. 1987; Martínez Pastur and Arena 1996; Calderón Baltierra et al. 1998). The purpose of this work was to optimize a protocol for the *in vitro* rooting quality *of N. nervosa* based on the incorporation of flavonoids in the culture media. We analyzed the type, concentration and combination of flavonoid in the rooting media; the presence of flavonoids during the different rooting phases, the presence of the auxin in the culture medium and the photoperiod conditions. Finally, total tissue peroxidase activity variation of the best successive media treatment during the rooting period was determined.

Materials and methods

In vitro plant material

Nothofagus nervosa plantlets were initiated from seeds in 1994, as previously described (Martínez Pastur and Arena 1996). One clone was selected for its healthy appearance of shoots, low stem callus production, growth homogeneity and *in vitro* multiplication performance. Shoots were subcultured every 21 d to fresh *Broad-leaved Tree Medium* (Chalupa 1983) and divided every 63 d. Multiplication ratio, defined as the number of microshoots obtained from each original microshoot, was 5:1 to 10:1, in agreement with previously reported multiplication ratios (Martínez Pastur and Arena 1996).

Rooting explants and culture conditions

Shoots of 2.5 cm in length with 2–4 leaves and 4–6 buds were used for rooting trials. *Broadleaved Tree Medium* including the modifications proposed by Martínez Pastur and Arena (1996) (half-strength macronutrient salts) was used; 50 ml medium were placed in 350 ml flasks and sterilized for 20 min at 0.1 Mpa and 121 °C. Cultures were incubated at 24 ± 2 °C. Two light conditions were tested: *light* treatments had a 16-hour photoperiod (16 hr light : 8 hr dark), while *darkness/light* treatments were placed 7 d in total darkness followed by 16-hour photoperiod days.

Light was supplied with standard 20-watt cool white fluorescent tubes (57 μmol m^{-2} s^{-1} *PPD*).

Flavonoid type, concentration and time of application during the rooting phases in the successive media

A multifactor assay with 3 factors and 36 treatments was used. The factors studied were: (a) flavonoid type (*rutin*, *quercetin* and *(±)naringenin*); (b) flavonoid concentration (5, 10, 20 and 30 μM); and (c) time of flavonoid incorporation during the different rooting phases (*induction* stage - 0 to 7th day; *expression* stage - 7th to 28th day; and both stages - 0 to 28th day). The criterion utilized to define the duration of the *induction* stage was to include the first maximum and the following minimum of the total peroxidase activity (for further information about rooting phase definition see Calderón Baltierra et al. (1998)). As variability in the peroxidase activity may be found, a 7-day period considered as the *induction* stage was used to assure the inclusion of the maximum and the minimum of the peroxidase activity in the following experiments. *IBA* (0.61 μM) was added to each treatment (Martínez Pastur and Arena 1996) and flasks were maintained under the above-mentioned *light* treatment. A control treatment containing the same culture medium, under the equal photoperiod conditions, but lacking flavonoids was also used.

Flavonoids were dissolved in hot water and aliquots were added to the media before adjusting the pH. A subculture scheme on the 7th day was used to change media as treatments required. This methodology was used for the following experiments as well.

Flavonoid, light conditions and presence of IBA during the expression rooting phase in the successive media

A multifactor assay with 3 factors and 12 treatments was used. Factors studied were: (a) flavonoid type (*rutin*, *quercetin* and *(±)naringenin*); (b) light conditions (*light* and *darkness/light* treatment); and (c) presence or absence of *IBA* during the expression phase (7 – 28th day). The specific flavonoid concentration used in this assay and its time of incorporation during specific rooting phases were deduced from the best results obtained in the previous assay. Four controls (with or without *IBA* during the expression rooting phase, under *light* or *darkness/light* regimes) were included to compare the different treatments, as these were not conducted simultaneously.

Flavonoid combinations in the successive media

A unifactorial assay with seven treatments was used. Combinations of different concentrations of the three flavonoids, *rutin - quercetin - (±)naringenin* were evaluated (in μM): 20-0-0, 0-20-0, 0-0-20, 10-10-0, 10-0-10, 0-10-10, 6.6-6.6-6.6 respectively. Flavonoids were added to the media and subcultures were done as above-mentioned. These treatments received 0.61 μM *IBA* and were then main-

tained under *darkness/light* photoperiod. Two control treatments, consisting of basic medium with 0.61 μM *IBA* (a) in *light* condition and (b) in *darkness/light* condition, were also used.

Data collection and statistical analysis

Every 2 d throughout the experiment and up to day 28, the rooting percentage (*R*), length (*LR*) and number of roots (*NR*) per shoot were obtained and evaluated. A rooting index (*RI*) was utilized to characterize the rooting quality (for further information see Martínez Pastur et al. (2000)), given by the following equation:

$$RI(mm) = NR \times LR \times R \times R_{11}$$

with R_{11} being the rooting percentage on day 11 for each statistical unit (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 overall rooting response to treatments, since all treatments produced their maximum rooting effect on day 11 (Martínez Pastur and Arena 1996). This index highlights the importance of all the variables together.

Comparisons with control treatments were as follows:

$$\text{Average value} = \frac{\text{Mean parameter value of treatment flask}}{\text{Mean parameter value of control}}$$

The results were evaluated by an analysis of the variance using the Fisher test, and media separation was done using the Tukey test. All tests were performed at a 5% significance level. Each treatment had 5 repetitions (flasks) with 6 shoots each. The data were averaged per flask before doing the analysis.

Tissue peroxidase activity during rhizogenesis in presence of flavonoids

Tissue peroxidase activity (μg Po / mg soluble protein) was determined in microshoots from treatments with the most successful flavonoid combination and microshoots from control treatments (*light* and *darkness/light* conditions without flavonoids). Samples of 100 mg tissue were homogenized with 5 ml 0.2 M citrate-phosphate buffer, pH 7.5, containing 0.1% Triton X-100. The homogenates were kept at 4 °C for 20 min and then were centrifuged at 10500 g for 20 min. An aliquot of the enzyme preparation from the supernatant was used to react with a mixture of guaiacol (8 mM) and hydrogen peroxide (8 mM) in 0.1 M acetate buffer, pH 4.6. The activity of peroxidases was spectrophotometrically measured as absorbance at 470 nm against acetate buffer as a blank. Peroxidase activity (average values of three replicates) was expressed as the mass (μg) of horseradish peroxidase type I (*SIGMA*) needed to transform an equivalent quantity of the same substrate (Calderón Baltierra et al. 1998). Protein tissue content was determined by the Bradford method (Bradford 1976): The homogenate (100 μl) was thoroughly mixed with 2

ml Coomassie Brilliant Blue G-250 stain reactant and after 5 min incubation at room temperature, absorbance was measured at 595 nm. Protein content (average values of three replicates) was given in terms of the mass (mg) of commercial bovine serum albumin (*BSA*), which was used as a standard.

Results and discussion

Flavonoid type, concentration and time of application during the rooting phases in the successive media

Significant differences were found in the root number, root length, and rooting index depending on the presence of flavonoids during the different rooting phases (*induction*, *expression*, or both) (Table 1). The best rooting results were obtained in treatments incorporating the flavonoid during the expression phase ($7–28^{th}$ day). The number of roots increased 20% compared to the control, while root length decreased by 6%. Nevertheless, the rooting index markedly increased 120% compared to the control. There were also differences in the root length and rooting index depending on the flavonoid concentration, irrespective of the rooting phase. The best results were obtained in the flavonoid concentration range of 10 to 30 μM, particularly the 20 μM concentration, which produced an excellent quality of roots and a higher rooting index (43 mm compared to 27 and 35 mm obtained with other concentrations, and 120% higher rooting index than the control) (Table 1).

The type of flavonoid applied also produced differences in the rooting percentage, rooting index, and number and length of roots. *Rutin* produced the highest rooting percentage and roots of better quality, followed by *(±)naringenin*, and *quercetin* (Table 1).

The rooting evolution in media containing flavonoids showed a typical sigmoid-like curve (Fig. 1A and 1C). In control media, however, this same response was only observed until day 11, after which it changed, no longer presenting a sigmoid-like curve, probably due to some factor limiting the rooting process. Overall, the presence of flavonoids allowed the shoots to reach their maximal rooting expression. The rooting percentage increased markedly until day 15, with a maximum rate of root initiation on day 11 (Fig. 1B and 1D). The addition of 20 μM flavonoid during the expression stage produced the best responses as assessed by the rooting index (Table 1).

These results showed that media containing flavonoids, at a concentration of 20 μM and applied during the expression stage markedly improved, by the end of the assay, all the parameters studied as well as the evolution of rhizogenesis. Thus, such rooting conditions were chosen for subsequent assays.

Flavonoid, light conditions and presence of IBA during the expression-rooting phase in the successive media

There were no significant differences in the variables under study with regard to

Table 1. Influence of time incorporation, concentration and type of flavonoid on the rooting percentage (*R*), root number (*NR*), length of roots (*LR*) and rooting index (*RI*) on the *Nothofagus nervosa* rooting successive culture media. Values between brackets represent differences with the control treatment (presence of *IBA* in both phases and *light* treatment without flavonoids) (control value = 1).

	R (%)	NR (n)	LR (mm)	RI (mm)
Flavonoid phase incorporation				
Induction + Expression	91.9(1.21)a	4.6(0.99)a	18.4(0.92)b	30.9(1.72)a
Induction	93.5(1.23)a	5.4(1.19)b	16.3(0.83)a	30.6(1.54)a
Expression	94.7(1.24)a	5.6(1.21)b	19.0(0.94)b	41.1(2.21)b
Flavonoid concentration				
5 μM	93.9(1.23)a	5.1(1.09)a	16.8(0.83)a	27.0(1.54)a
10 μM	91.8(1.21)a	5.2(1.12)a	16.8(0.82)a	35.4(1.92)ab
20 μM	96.3(1.26)a	5.5(1.20)a	17.7(0.87)a	43.2(2.20)b
30 μM	91.5(1.20)a	5.0(1.10)a	20.4(1.04)b	31.2(1.66)ab
Flavonoid type				
(±)naringenin	90.6(1.21)a	4.6(1.16)a	19.5(0.86)b	26.1(2.27)a
quercetin	93.3(1.07)ab	4.5(1.14)a	19.0(0.75)b	27.3(0.63)a
rutin	96.3(1.40)b	6.5(1.08)b	15.3(1.07)a	49.2(2.58)b

The different letters in each column and for each factor show significant differences at $P<0.05$ by Tukey's test. F values and significance: (I) MAIN EFFECTS. Rooting phases (*A*): R = 0.884 (0.415), NR = 8.244 (0.000), LR = 6.322 (0.002), RI = 4.159 (0.018); Flavonoid concentrations (*B*): R = 1.636 (0.184), NR = 0.857 (0.465), LR = 6.707 (0.000), RI = 4.099 (0.008); Flavonoid type (*C*): R = 3.448 (0.034), NR = 33.424 (0.000), LR = 16.559 (0.000), RI = 19.294 (0.000). (II) INTERACTIONS: *AxB*: R = 1.503 (0.181), NR = 0.924 (0.479), LR = 1.080 (0.377), RI = 5.144 (0.000); *AxC*: R = 0.876 (0.480), NR = 1.033 (0.392), LR = 4.059 (0.004), RI = 0.636 (0.638);*BxC*: R = 1.661 (0.135), NR = 0.845 (0.537), LR = 2.084 (0.058), RI = 2.201 (0.046); *AxBxC*: R = 1.136 (0.336), NR = 0.637 (0.807), LR = 0.966 (0.484), RI = 0.976 (0.475).

photoperiod conditions (Table 2). However, the rooting index was slightly superior in *darkness/light* treatments, but did not show a significant improvement over control values (*darkness/light* treatment and presence or absence of *IBA* in the expression stage). The *light* condition treatment produced a 23% increase in the rooting index compared to the control treatments (*light* treatment and presence or absence of *IBA* in the expression stage), but it did not surpass the values obtained in the *darkness/light* treatments.

Significant differences were found in the root percentage, number of roots, and rooting index in the presence or absence of *IBA* during the expression phase, under different *light* conditions (Table 2). *IBA* supplemented at the expression stage yielded a 10% increase in the rooting index compared to the control treatments. In fact, although the main function of auxins during the rooting phase may be to act on tissue differentiation, they also participate on cell division and cell enlargement, both needed to develop a functional root system (Gaspar et al. 1990, 1993; Blakesley et al. 1991; Hausman 1993).

Finally, differences were found in the number of roots and rooting index, depending on the type of flavonoid present during the expression phase (Table 2). *(±)Naringenin* presented a root number 6% higher and a rooting index 11% lesser

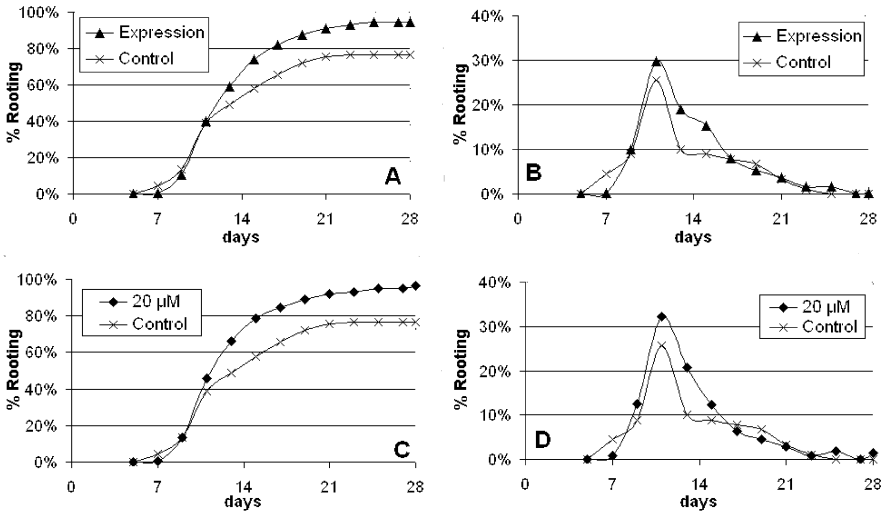

Figure 1. Evolution of the rooting percentage (%) during rhizogenesis in successive media incorporating flavonoids during the expression phase (*A* and *B*) and at a concentration of 20 µM (*C* and *D*) compared with a control (0.61 µM *IBA* and *light* treatment without flavonoids). Values represent the factor level means of the ANOVA analysis. *A* and *C* represent the accumulated values, *B* and *D* represent non-accumulated values

Table 2. Light conditions, presence of *IBA* and flavonoid type on the rooting percentage (*R*), root number (*NR*), length of roots (*LR*) and rooting index (*RI*) on the *Nothofagus nervosa* rooting in successive culture media. Values between brackets represent differences with the control treatment (presence of *IBA* in the induction phase and *light* treatment without flavonoids) (control value = 1).

	R (%)	NR (n)	LR (mm)	RI (mm)
Light conditions				
Light	97.2(1.01)a	6.1(1.19)a	18.4(0.91)a	66.1(1.23)a
Darkness/light	97.8(1.00)a	5.6(0.95)a	16.3(1.02)a	68.4(1.00)a
0.61 µM *IBA during*				
Induction	95.0(1.01)a	4.9(1.04)a	17.5(1.01)a	54.3(1.14)a
Induction + Expression	100.0(1.00)b	6.8(1.10)b	17.2(0.92)a	80.2(1.09)b
Flavonoid type				
(±)naringenin	96.7(1.00)a	7.4(1.06)c	17.6(0.83)a	79.7(0.89)b
quercetin	97.5(1.00)a	6.6(1.12)b	16.2(1.02)a	70.7(1.43)b
rutin	98.3(1.01)a	3.6(1.04)a	18.3(1.05)a	51.4(1.03)a

The different letters in each column and for each factor show significant differences at $P<0.05$ by Tukey's test. F values and significance: (I) MAIN EFFECTS. Light conditions (*A*): R = 0.083 (0.777), NR = 2.422 (0.126), LR = 3.376 (0.072), IE = 0.078 (0.784); Presence of *IBA* (*B*): R = 6.750 (0.012), NR = 27.548 (0.000), LR = 0.086 (0.774), IE = 9.064 (0.004); Flavonoid type (*C*): R = 0.250 (0.779), NR = 36.607 (0.000), LR = 1.194 (0.312), IE = 3.789 (0.029). II) INTERACTIONS: *AxB*: R = 0.083 (0.777), NR = 0.077 (0.785), LR = 0.052 (0.823), IE = 0.187 (0.672); *AxC*: R = 0.583 (0.561), NR = 4.462 (0.016), LR = 1.112 (0.337), IE = 3.421 (0.041); *BxC*: R = 0.250 (0.779), NR = 10.323 (0.000), LR = 1.334 (0.273), IE = 2.232 (0.118); *AxBxC*: R = 0.583 (0.562), NR = 0.466 (0.631), LR = 0.284 (0.754), IE = 1.472 (0.239).

than control treatments, which included the absence of *IBA* in the expression phase and *light* treatment without flavonoids.

The improvement of the rooting quality in media with flavonoid was lesser in this second assay (*light* conditions and presence of *IBA*) compared to the first one (rooting phase incorporation and flavonoid concentration). In fact, by incorporating new factors that positively affected the rooting process (*light*/*darkness* condition and 0.61 μM *IBA*), the possibility of improving the rooting quality was narrowed.

The rooting in media containing flavonoids occurred earlier and at an increased rate compared to the control treatments. Fig. 2A shows the evolution of the rooting process of microshoots in the control treatment - including *IBA* in the *induction* stage under *light* treatment - compared with the best treatment obtained - 20 μM *(±)naringenin* applied during the expression stage, *IBA* at both stages and with *darkness*/*light* condition. The rooting response of the other control treatments during the process had values between both curves.

Treatments including flavonoids showed an earlier and higher root initiation peak on days 9–11 than the control treatments on day 11 (Fig. 2B). Moreover, the root initiation with flavonoid treatments was homogeneous and synchronized in time, with a peak of rooted microshoots of 93% (until day 15) and a second peak of 7%, while controls showed an erratic response with several peaks until the end of the experiment. As flavonoids helped roots appear earlier, these had more time to grow and develop a better quality root system by the end of the rooting process, as could be seen through the higher rooting index values.

The treatment presenting the best results included 20 μM flavonoids applied in the *expression* stage, *IBA* in both rooting stages and *darkness*/*light* regime. Nevertheless, responses were different depending on the flavonoid type. After comparing the rooting evolution of each flavonoid treatment with control treatments, it was concluded that *(±)naringenin* produced the best results compared to the other two flavonoids, showing a maximum of 150% of rooting ratio on day 9 between the treatments and the control results (Figure 3).

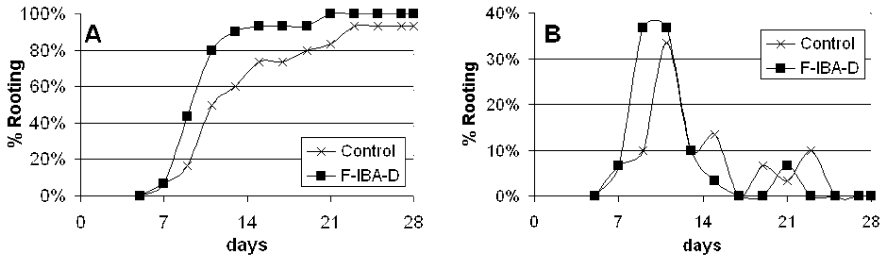

Figure 2. Evolution of the rooting percentage (%) during rhizogenesis in successive media incorporating 20 μM flavonoid in the expression phase, with 0.61 *IBA* in both phases and *darkness*/*light* condition (*F-IBA-D*) compared with a control (*light* treatment, without *IBA* in both phases and without flavonoids). *A* represents accumulated values; *B* represents non-accumulated values

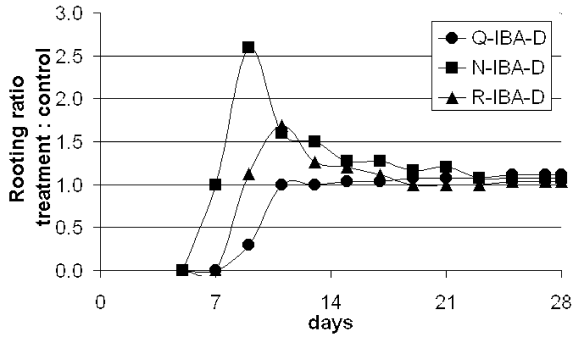

Figure 3. Comparison of the evolution of rooting during rhizogenesis between treatments containing different flavonoid types (*R = rutin*, *Q = quercetin*, *N = (±)naringenin*) with 0.61 *IBA* in both phases and *darkness/light* condition (*D*) and a control (0.61 μM *IBA* and *light* treatment) (control value = 1)

Flavonoid combinations in the successive media

Significant differences were found in the root number and rooting index depending on the flavonoid combination present in the successive media (Table 3). As expected, according to previous results reported by Martínez Pastur and Arena (1996), the lowest values were found in the light control treatment (*ca.* 3 roots per microshoot). When combinations of flavonoids were added to the culture media, *ca.* 5 roots per microshoot were obtained. However, the best results were still produced by treatments containing only one flavonoid (*ca.* 5–6 roots per microshoot), with the exception of a medium including 10 μM *quercetin* and 10 μM *(±)naringenin*, which also produced *ca.* 6 roots per shoot. The highest rooting index value (59 mm) was produced by media with *(±)naringenin*. It is interesting to note that some rooting values were lower (rooting index between 7 to 39 mm) to those corresponding to control treatments, thus indicating that some flavonoid combinations are harmful for the rhizogenesis process (Table 3,4). The root percentage was not markedly variable between treatments, with values *ca.* 93% to 100%. The length of roots also did not change in a significant manner, with the highest values obtained under light conditions (Table 3).

Only the rooting index showed marked differences between treatments and control. The highest rooting index values were obtained with media containing only 20 μM *(±)naringenin*, and was 1.4 and 2.7 times superior to corresponding *darkness/light* and *light* control treatments, respectively (Table 4). These results could be partially attributed to the *darkness/light* treatment which produced a 23% increase in the rooting index with respect to the light treatment (data not shown), and partially to the flavonoid *(±)naringenin*, which markedly improved it more than 1.4 times with respect to the *darkness/light* control (Table 4).

At the end of the rooting period, different responses were observed for the parameters under study. The treatment including 20 μM*(±)naringenin* showed comparable results to the *darkness/light* control treatment and markedly different results from the *light* control treatment (Figure 4,5). This flavonoid treatment

Table 3. Flavonoid combinations and the rooting percentage (*R*), root number (*NR*), length of roots (*LR*) and rooting index (*RI*) on the *Nothofagus nervosa* rooting successive culture media.

			R (%)	NR (n)	LR (mm)	RI (mm)
***Rutin* (μM)**	***Quercetin* (μM)**	***Naringenin* (μM)**				
20	0	0	100.0a	5.5b	21.7a	35.9ab
0	20	0	100.0a	5.2ab	17.8a	26.6ab
0	0	20	100.0a	5.5b	15.7a	58.7b
10	10	0	100.0a	4.6ab	19.4a	38.1ab
10	0	10	96.7a	4.9ab	20.7a	28.3ab
0	10	10	100.0a	5.6b	17.6a	6.7a
6.6	6.6	6.6	93.3a	4.9ab	20.7a	44.8ab
Light control treatment			100.0a	3.3a	22.0a	21.8ab
Darkness/light control treatment			100.0a	5.3ab	16.4a	39.1ab

The different letters in each column and for each factor show significant differences at $P<0.05$ by Tukey's test. F values and significance: R = 0.900 (0.526), NR = 2.320 (0.040), LR = 1.301 (0.274), RI = 2.716 (0.019).

Table 4. Flavonoid combinations compared with control treatments (control value = 1) for the rooting percentage (*R*), root number (*NR*), length of roots (*LR*) and rooting index (*RI*) on the *Nothofagus nervosa* rooting successive culture media.

			R (%)	NR (%)	LR (%)	RI (%)
***Light* control treatment**						
***Rutin* (μM)**	***Quercetin* (μM)**	***Naringenin* (μM)**				
20	0	0	1.00a	1.66a	0.98a	1.63ab
0	20	0	1.00a	1.57a	0.81a	1.21ab
0	0	20	1.00a	1.66a	0.71a	2.67b
10	10	0	1.00a	1.38a	0.88a	1.73ab
10	0	10	0.97a	1.45a	0.94a	1.29ab
0	10	10	1.00a	1.69a	0.80a	0.30a
6.6	6.6	6.6	0.93a	1.48a	0.94a	2.03ab
***Darkness/light* control treatment**						
20	0	0	1.00a	1.04a	1.32a	0.88ab
0	20	0	1.00a	0.99a	1.08a	0.65ab
0	0	20	1.00a	1.04a	0.96a	1.44b
10	10	0	1.00a	0.87a	1.18a	0.94ab
10	0	10	0.97a	0.92a	1.26a	0.70ab
0	10	10	1.00a	1.06a	1.07a	0.16a
6.6	6.6	6.6	0.93a	0.93a	1.26a	1.10ab

The different letters in each column and for each factor show significant differences at $P<0.05$ by Tukey's test. F values and significance: R = 0.867 (0.531), NR = 0.675 (0.670), LR = 1.178 (0.346), RI = 2.760 (0.031).

yielded maximal rooting percentage and rooting index (Figure 4), while the darkness/light control treatment rooted earlier, but was lower in intensity (Fig. 4B and 4D). When the evolution of the number of roots was analyzed, the light control

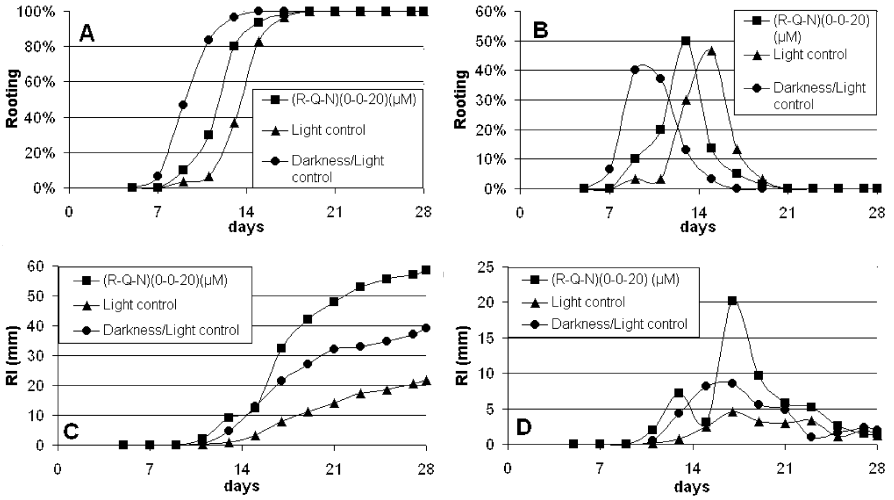

Figure 4. Evolution of the rooting percentage (%) (*A* and *B*) and rooting index (*RI*) (mm) (*C* and *D*) during rhizogenesis in treatments incorporating the best combination of flavonoids (*R = rutin*, *Q = quercetin*, *N = (±)naringenin*) compared with a control (0.61 μM *IBA* and *light* or *darkness/light* conditions). *A* and *C* represent accumulated values, *B* and *D* represent non-accumulated values

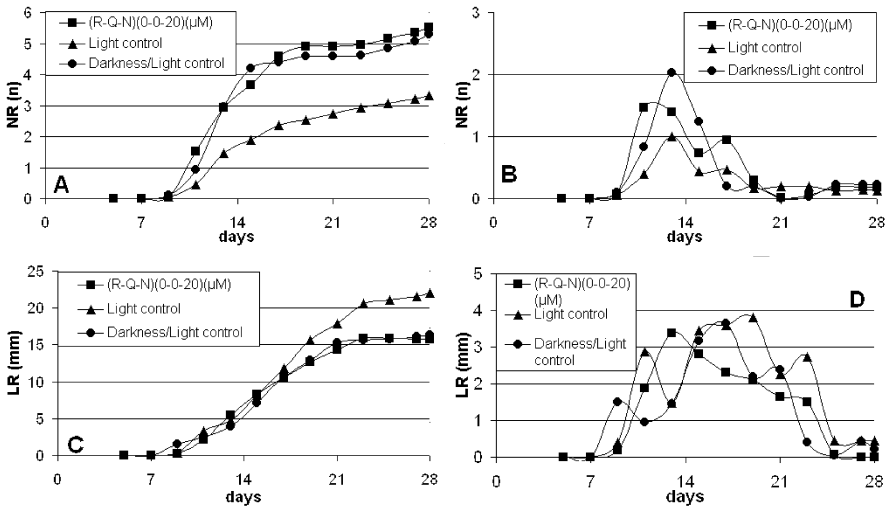

Figure 5. Evolution of the root number (*RN*) (*A* and *B*) and length of roots (*LR*) (mm) (*C* and *D*) during rhizogenesis in treatments incorporating flavonoids (*R = rutin*, *Q = quercetin*, *N = (±)naringenin*) compared with a control (0.61 μM *IBA* and *light* or *darkness/light* conditions). *A* and *C* represent accumulated values, *B* and *D* represent non-accumulated values.

treatment presented the lower values, while the evolution of the length of roots presented the highest values. The darkness/light control treatment showed little differences with respect to the flavonoid treatment (Figure 5).

The response of the *(±)naringenin* treatment presented differences compared with the control treatments, mainly during the first week of root initiation. The presence of this flavonoid produced an intermediate response with respect to both controls, but yielded a higher rooting homogeneity. However, as for the rooting index evolution, there was a marked difference between the *(±)naringenin* and the control treatments, from day 13 until the end of the assay (Fig. 4C). Moreover, the *(±)naringenin* treatment presented a maximum on day 17, duplicating control values, and therefore representing the best response obtained from shoots (Fig. 4D).

The results mentioned so far agree with those of Berthon et al. (1987, 1993), who concluded that o-dihydric phenols, as flavonoids, given during the expression stage, are effective inducers of rooting, while monohydric phenols or benzoic acids act as inducers during the induction stage. However, it is of interest to mention that flavonoids can produce different responses depending on the plant species, *e.g.* while *rutin* and *quercetin* stimulate rooting, *(±)naringenin* has negative effects on *Sequoiadendron giganteum* (Berthon et al. 1993). Furthermore, flavonoids can act as inhibitors of rooting, through the inhibition of the auxin transport as it was shown in many *in vitro* experiments (Stenlid 1976; Jacobs and Rubery 1988). Finally, it should be pointed out that the tested flavonoid combinations did not improve the rooting of *N. nervosa*.

Tissue peroxidase activity during rhizogenesis in the presence of (±)naringenin

The peroxidase activity showed different maximum and minimum peaks during rooting. The peroxidase response of control treatments during the rooting process agreed with previous reports (Calderón Baltierra et al. 1998), and is consistent with the criterion utilized to define the duration of the *induction* stage.

The *light* control treatment induced an activity peak on day 2 and decreased markedly afterwards, while the *darkness/light* control treatment had an earlier peak of activity (Calderón Baltierra et al. 1998), on day 1. The inclusion of 20 μM *(±)naringenin* in the medium on day 7 produced a peak of peroxidase activity on day 9, which did not appear in control treatments (Figure 6). This *(±)naringenin*-induced peak could be related to the rooting differentiation occurring in the shoots. In fact, peroxidases are known to be involved in several physiological processes. In rooting, they play a role in auxin catabolism (*basic peroxidases*), and during cell wall construction, they contribute to rigidity (*acidic peroxidases*) (Van Der Berg et al. 1983; Gaspar et al. 1985, 1991; Mader 1992; Faivre-Rampant et al. 2000). Thus, it is possible that the activity peak of peroxidases could be part of the biochemical impact of the flavonoid on the root system response, perhaps by participating in the buildup of cell walls in the newly formed roots. However, the localized transport inhibition can result in localized accumulation of auxin, which in turn, can cause increased root primordium formation and/or root elongation.

Conclusions

This work optimized a protocol for micropropagating *Nothofagus nervosa* through a

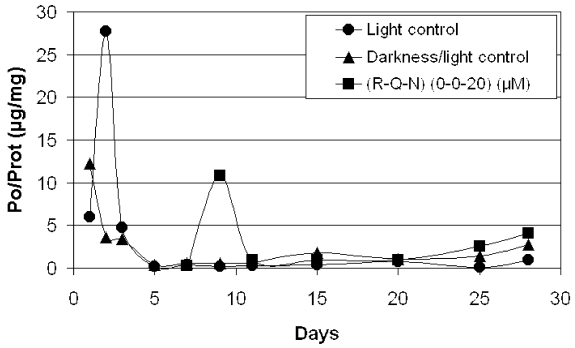

Figure 6. In vitro tissue peroxidase activity (Po/Prot) (µg/mg) during rhizogenesis in the treatment incorporating flavonoids (*R = rutin*, *Q = quercetin*, *N = (±)naringenin*) (0.61 µM *IBA* in *darkness/light* condition) compared with a control (0.61 µM *IBA* and *light* or *darkness/light* conditions)

successive media culture technique, by improving the earliness, quantity, and quality of roots, which would positively affect the survival rate following hardening. This new technique involves a first culture step using half strength *Broadleaved Tree Medium* as the basic medium, with 0.61 µM *IBA* and with one week of dark incubation, as was proposed in the original protocol. This is followed by a second culture step, corresponding to the expression phase of rhizogenesis, which uses the same basic medium but also incorporates 20 µM *(±)naringenin* and a 16-hour photoperiod. This research was carried out with a single clone of *N. nervosa* in order to minimize the genetic factor effect. Therefore, these results must also be tested with other clones to be able to generalize the use of this protocol in this species.

Each flavonoid added to the culture medium produced variable responses, with *(±)naringenin* offering the best results. This flavonoid caused a peak of peroxidase activity and could be partly responsible for the enhancement of physiological processes related to root differentiation during the expression stage.

In general, rooting media described in the literature (Chalupa 1983; George et al. 1987; Bonga 1987; Bellarosa 1989) do not include all the main cofactors that participate in the rooting process. The inclusion of some of these cofactors in media culture could greatly improve the shoot responses, especially in recalcitrant species. In this study, only a few flavonoids were analyzed; this points out the need to study the incorporation, in different rooting stages, of other flavonoids, or similar compounds such as phenolic acids.

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