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# Role of polyamines during in vitro rhizogenesis of *Nothofagus nervosa* using successive culture media

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**Abstract** An in vitro rooting protocol for producing *Nothofagus nervosa* micro-plants using successive steps during tissue culture is reported. Qualitative and quantitative changes in putrescine, spermidine, and spermine tissue contents, during the rooting process and their correlation with other biochemical markers and morphological changes, provided the rationale for their use during the study of a successive in vitro culture protocol for *N. nervosa*, as well as the use of their inhibitors. The polyamine tissue content was maximum before root emergence (spermine raised to 100 nmol/g FW to later decreased to ca. 50 nmol/g FW in the following days), but it was lower in improved rooting media (197 nmol/g FW compared to 264 nmol/g FW of the control medium). It was concluded that polyamines qualitatively and quantitatively improve in vitro rhizogenesis, and the best successive culture media included 10 μM spermine during the expression phase.

**Resumen** En este trabajo se presenta un protocolo de enraizamiento in vitro para la obtención de micro-plantas de *Nothofagus nervosa* usando medios sucesivos durante el cultivo de tejidos. Los cambios cualitativos y cuantitativos de putrescina, espermidina y espermina en los tejidos durante el enraizamiento, y su correlación con otros marcadores bioquímicos y cambios morfológicos, justifican su uso en el desarrollo de medios sucesivos de enrizamiento para N. nervosa, así como el uso de

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sus inhibidores. La concentración de poliaminas en los tejidos fue máxima antes de la emergencia de las raíces (espermina alcanzó los 100 nmol/g PF para decrecer hasta 50 nmol/g PF en los días siguientes), siendo su concentración menor en los mejores medios de enraizamiento (197 nmol/g PF comparado con 264 nmol/g PF del medio control). Se puede concluir que las poliaminas mejoran cualitativamente y cuantitativamente el enraizamiento in vitro, siendo el mejor medio sucesivo aquel que incluyó 10 μM de espermina durante la fase de expresión del enraizamiento.

**Keywords** Rooting markers · Rhizogenesis · Polyamine inhibitors · Root system quality

**Palabras clavas** Marcadores del enraizamiento · Rizogénesis · Inhibidores de poliaminas · Calidad del sistema radical

## Introduction

In vitro propagation protocols do not typically include the use of successive culture media techniques within a morphogenic phase definition to obtain a given morphological event such as shoot or root production. However, understanding internal biochemical changes that occur during rhizogenesis can make possible an improved rooting response of the microshoots by using a two-step culture medium differing in chemical composition (Berthon et al. 1993). This particular approach helped improve rhizogenesis in several woody species (Berthon et al. 1987; Martínez Pastur et al. 2003). To successfully formulate successive culture media it is of utmost importance to know the timing and characteristics of the *induction* and *expression* phases that underlie the rooting phenomenon (Gaspar 1981). It is known that physical and chemical factors differently influence rooting during these phases (Druart et al. 1982; Berthon et al. 1993) by affecting the quantity and quality of roots. Among these factors, changes in photoperiod, absence of auxins, and incorporation of chemical compounds, like phenolic acids, flavonoids or polyamines have been cited (Berthon et al. 1987, 1993; Hausman et al. 1995a, 1995b; Martínez Pastur and Arena 1996; Calderón et al. 1998; Martínez Pastur et al. 2000, 2003; Arena et al. 2003, 2005). A positive relationship was demonstrated between high polyamine content and the beginning of adventitious root formation (Friedman et al. 1982; Torrigiani et al. 1989; Biondi et al. 1990) thus emphasizing a possible involvement of these substances in rooting (Baraldi et al. 1995; Gaspar et al. 1997). Studies using polyamines in the absence or presence of their specific inhibitors in the culture media as a tool for understanding the underlying processes have already been performed in several species (Tiburcio et al. 1987; Hausman et al. 1994, 1997; Kevers et al. 1997; Arena et al. 2005). *Nothofagus nervosa* (Phil.) Dim. et Mil. is the most important timber Patagonian forest species, and was selected because it has many in vitro rooting studies (Martínez Pastur and Arena 1996; Calderón et al. 1998; Martínez Pastur et al. 2000, 2003, 2005). The objective of this work was to follow endogenous polyamine (putrescine, spermidine, spermine) evolution during the rooting process to provide adequate support to use them and eventually their inhibitors in a successive culture protocol to optimize the in vitro rooting of *Nothofagus nervosa*.

## Materials and methods

### Plant material and rooting conditions

In vitro *Nothofagus nervosa* shoot cultures were used, which were maintained in the culture conditions described by Martínez Pastur and Arena (1996) during two years. The shoot culture was originated from seeds, and one clon was selected for its healthy and rapid growth. In vitro grown shoots, 2.5 cm in length having elongated internodes and 2–4 expanded leaves, were employed as explants. The pH of the medium (Broadleaved Tree medium proposed by Chalupa 1983) was adjusted to 5.7–5.8 with 0.1 N KOH. Fifty ml of medium were dispensed into 350 ml flasks, and autoclaved at 0.1 MPa for 20 min (120°C). Tissue cultures were maintained in a growth chamber at 24 ± 2°C, for the first seven days in darkness, to be followed by a 16 h photoperiod using cool-white fluorescent lamps (57 μmol $m^{-2}s^{-1}$). Assays ended at day 28, and rooting (percentage, length and root number) were recorded every two days.

### Polyamine tissue content measurement during rhizogenesis

In experiment one, a multifactor assay with three factors was conducted. The factors were: polyamine type (putrescine, spermidine or spermine), days through the rooting period (0, 1, 2, 3, 5, 7, 9, 11, 13, 15, 20 or 28), and incorporation of indole-3-butyric acid (IBA) in the culture medium (with 0.61 μM or without). During the sampling rooting period 200 mg FW of microshoots were collected. Tissue samples were homogenized with 5% perchloric acid, kept 30 min on ice and centrifuged at 5,000 rpm for 10 min. Supernatants were then derivatized using the dansylation method described by Smith and Meeuse (1966) and 1.6 hexanediamine was used as an internal standard. Putrescine, spermidine, and spermine standards were dansylated simultaneously. The dansylated derivatives were extracted with 1 ml ethylacetate. Polyamines were separated and identified by TLC, performed on high resolution silica gel plates (J. T. Baker IB 2-F TLC aluminium sheets 20 × 20 cm, silica gel plates 60 F 254) using n-hexane: ethyl acetate (1:1) as developing solvent system. Dansylated polyamines were identified by comparing the Rf values of dansylated standards. Silica plates were observed under UV light and bands corresponding to the polyamines in the samples and standards were scraped off the plates and eluted with 1 ml ethyl acetate. Their fluorescence was then measured with a spectrofluorometer (Aminco Bowman), with an excitation wavelength at 365 nm and emission at 510 nm.

### Polyamine incorporation during the rhizogenesis phases

In experiment two, a multifactor assay with two factors and nine treatments was conducted for each polyamine (putrescine, spermidine or spermine). The factors assayed for each polyamine were three polyamine concentrations (1, 10 or 100 μM) and three times where polyamines were added to the medium, which was defined according to the rooting phases (Calderón et al. 1998) (induction stage, days 0–7; expression stage, days 7–28; and both stages, days 0–28). When a rooting phase of each treatment did not include polyamine, a mixture of polyamine inhibitors was added to

the media. The inhibitors were added to avoid the endogenous polyamine effect over the shoot growth. When the inhibitors were incorporated, the mixture included a final media concentration of 25 μM of cyclohexylamine (CHA), aminoguanidine (AG), α-difluoromethylornithine (DFMO), and methylglyoxal-bis-guanylhydrazone (MGBG). The treatments of the multifactor assay were compared with a control treatment in the absence of polyamines or inhibitor mixture. Polyamines and the inhibitor mixtures were filter-sterilized using a 0.22 μm filter unit, and then added to the autoclaved rooting media under sterile conditions. Micro-shoots were sub-cultured at day 7, even if the same treatment were to be continued.

### Data collection and statistical analysis

In experiment two, data were recorded at 0, 1, 2, 3, 5, 7, 9, 11, 13, 15, 20 and 28 days. Parameters evaluated were: rooting percentage (*R*), number of roots (*RN*), length of roots (mm) (*RL*) and presence of secondary roots (*SR*). Using some of these parameters, a rooting index (*RI*) was calculated as described previously (Martínez Pastur et al. 2003):

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

where $R$ and $R_{11}$ were percentage values between 0 and 1; $R_{11}$ was the rooting percentage at day 11, as a value of celerity (new roots emergence per day) of the rooting process, chosen because in this day most of the treatments attained the maximum rooting.

Both experiments were subjected to an analysis of variance using a Fisher test. Media separation was done using a Tukey test at $P \leq 0.05$ significance level. In experiment one (analysis of the polyamine content), each treatment for the biochemical analysis had three replications, consisting of one flask with six shoots each. In experiment two (analysis of polyamine incorporation), each treatment had six replications (flasks) with 6 microshoots each, and the data were averaged per flask.

## Results

### Endogenous tissue polyamine content during rhizogenesis

When a multifactor ANOVA was performed, endogenous tissue polyamine content significantly varied with the polyamine type and along the rooting period, while IBA treatments did not significantly differ (Table 1). Endogenous spermidine content (125 nmol/g FW) was significantly higher than putrescine (59 nmol/g FW) and spermine contents (46 nmol/g FW) (Table 1). These tissue concentrations varied through the rooting period (Fig. 1), with a maximum content between days 5–7 in all cases. In the absence of IBA in the culture media, endogenous tissue putrescine content in shoots exhibited a peak at day 6 (163 nmol/g FW), being higher than the one, which appeared at days 5–6 in the IBA medium (94–98 nmol/g FW). Spermidine showed a similar pattern profile, with a maximum content corresponding to the control medium at day 6 (335 nmol/g FW) and with a lower peak for the IBA medium treatment at days 5–6 (243–239 nmol/g FW). Finally, spermine also presented a similar behavior, showing a maximum in the absence of IBA in the culture

**Table 1** Endogenous tissue polyamine concentration related to polyamine type, IBA concentration and days along rooting period in microshoots of *Nothofagus nervosa*

| Polyamine type | Polyamine concentration (nmol/g FW) | IBA concentration (μM) | Polyamine concentration (nmol/g FW) | Days along rooting period | Polyamine concentration (nmol/g FW) |
|---|---|---|---|---|---|
| Putrescine | 58.71 b | 0.00 | 82.87 a | 0 | 45.65 b |
| Spermidine | 125.19 a | 0.61 | 70.57 a | 1 | 56.81 cd |
| Spermine | 46.25 b | | | 2 | 50.47 d |
| | | | | 3 | 48.43 d |
| | | | | 4 | 48.10 d |
| | | | | 5 | 141.75 ab |
| | | | | 6 | 195.88 a |
| | | | | 7 | 115.70 bc |
| | | | | 9 | 64.08 cd |
| | | | | 11 | 56.14 cd |
| | | | | 13 | 46.61 d |
| | | | | 15 | 56.09 cd |
| | | | | 20 | 72.74 cd |
| | | | | 28 | 75.59 cd |

Significance of the main effects: Polyamine type ($F$: 48.17, $P$: 0.000), IBA concentration ($F$: 3.03, $P$: 0.083), Days along rooting period ($F$: 11.27, $P$: 0.000) and Interactions (Polyamine type × IBA concentration = $F$: 0.28, $P$: 0.759, Polyamine type × Days along rooting period = $F$: 2.39, $P$: 0.000, IBA concentration × Days along rooting period = $F$: 1.66, $P$: 0.073, Polyamine type × IBA concentration × Days along rooting period = $F$: 1.27, $P$: 0.188). Different letters means significant differences in each factor at $P < 0.05$

medium at day 7 (264 nmol/g FW), and an earlier maximum on the IBA medium at day 6 (197 nmol/g FW). Endogenous tissue spermine content decreased abruptly after reaching the maximum, while great variability was observed with the other polyamine concentrations over the remaining 20 days of testing (Fig. 1). A tendency to drop to initial values at the end of the assay for spermine and putrescine was observed. However, in the spermidine peaks, 78% or 48% of this polyamine content were retained by the end of the assay in the presence or absence of IBA in the culture medium, respectively. The maximum rooting celerity of the microshoots appeared on day 11, being higher in the IBA treatment, i.e. 40% of the microshoots were rooted compared to 17% in the control treatment. Nevertheless, the final rooting percentages did not present significant differences (96% and 93%, respectively) (data not shown).

## Polyamine and inhibitor mixture incorporation during the rhizogenesis phases

When an ANOVA was performed the polyamines and the inhibitor mixture under study produced different responses in the quality and quantity of roots in the range of 1–100 μM, following their addition to different rooting phases (Table 2). Putrescine concentration significantly influenced the rooting, rooting length, and consequently the rooting index, and 100 μM putrescine yielded the best performance. When added to different rooting phases, putrescine significantly affected rooting and secondary root percentages. Nevertheless, these differences did not significantly affect the rooting index. The best putrescine incorporation treatment for rooting (root quantity and quality) was the incorporation of 100 μM during the induction phase, but this treatment had a lower secondary root percentage.

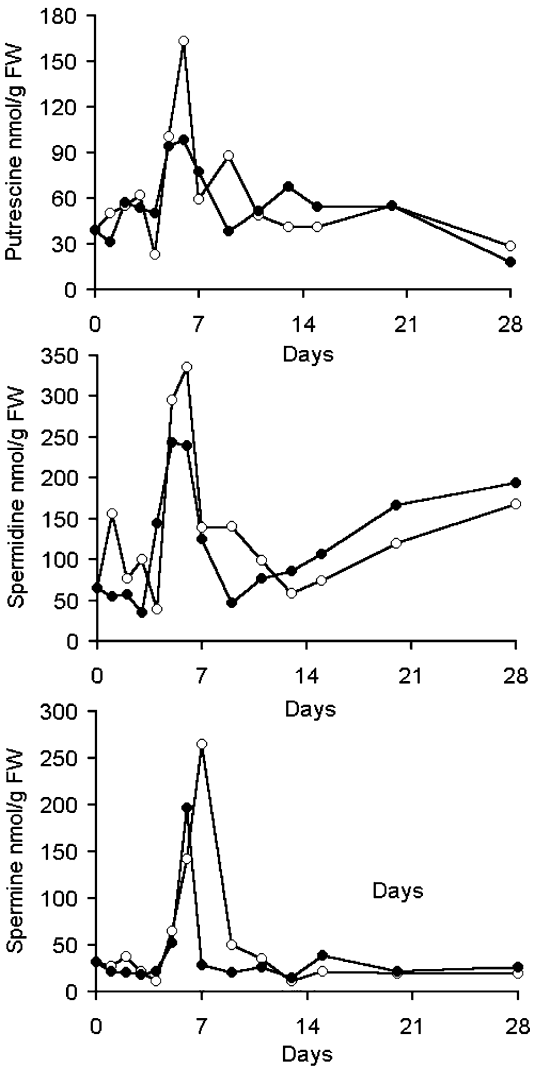


**Fig. 1** Endogenous tissue polyamine concentration ($n$ = 3 flasks with six shoots each) changes over the rooting period in microshoots of *Nothofagus nervosa*. (●) media with IBA; (○) media without IBA

Spermidine concentration influenced the rooting length and rooting index, and 10 μM gave the best results. When spermidine was added in different rooting phases it did not significantly affect the parameters under evaluation. In the case of spermine, concentration significantly affected root number, length, and rooting index and 10 μM produced the highest values. When spermine was added in different rooting phases it significantly affected the rooting index, with the expression phase the most advantageous time for its use.

Finally, the rooting evolution was analyzed (Fig. 2). Rooting in the absence of exogenous polyamine incorporation and with inhibitors mixture in the media began at day 9 and ended at day 23 reaching to 83.3%, with a maximum celerity at day 11 recording 33% rooting at this single observation day. This rooting evolution clearly

**Table 2** Rooting parameters in *Nothofagus nervosa* microshoots evaluated in response to polyamine type, concentration and rooting phase incorporation

| Polyamine | Factor | *R* (%) | *RN* (*n*) | *RL* (mm) | *SR* (%) | *RI* (mm) |
|---|---|---|---|---|---|---|
| Putrescine | *Concentration (μM)* | | | | | |
| | 1 | 66.66b | 3.27a | 8.80c | 3.33a | 5.64b |
| | 10 | 71.38b | 4.07a | 14.07b | 10.27a | 13.80b |
| | 100 | 91.11a | 3.85a | 19.32a | 6.66a | 29.13a |
| | *Rooting Phase Incorporation* | | | | | |
| | Induction | 86.94a | 3.88a | 15.63a | 2.50b | 18.48a |
| | Expression | 81.11a | 3.83a | 15.61a | 13.33a | 17.20a |
| | Induction + Expression | 61.11b | 3.48a | 10.95a | 4.44ab | 12.89a |
| Spermidine | *Concentration (μM)* | | | | | |
| | 1 | 95.55a | 4.09a | 23.17a | 24.44a | 67.49a |
| | 10 | 97.77a | 4.51a | 22.32a | 20.00a | 68.56a |
| | 100 | 92.22a | 4.50a | 16.84b | 10.00a | 44.18b |
| | *Rooting Phase Incorporation* | | | | | |
| | Induction | 92.22a | 4.33a | 22.25a | 18.88a | 65.52a |
| | Expression | 95.55a | 4.69a | 20.14a | 16.66a | 57.22a |
| | Induction + Expression | 97.77a | 4.09a | 19.92a | 18.88a | 57.48a |
| Spermine | *Concentration (μM)* | | | | | |
| | 1 | 95.55a | 3.61b | 19.33ab | 17.77a | 43.34b |
| | 10 | 100.00a | 4.30ab | 20.76a | 25.55a | 66.86a |
| | 100 | 100.00a | 5.10a | 17.54b | 17.77a | 64.37a |
| | *Rooting Phase Incorporation* | | | | | |
| | Induction | 98.88a | 4.27a | 19.74a | 23.33a | 59.02ab |
| | Expression | 97.77a | 4.70a | 19.97a | 27.77a | 69.64a |
| | Induction + Expression | 98.88a | 4.04a | 17.92a | 10.00a | 45.90b |

*R* = rooting percentage, *RN* = root number, *RL* = root length, *SR* = secondary roots; *RI* = rooting index

Significance of the main effects: *C* = polyamine concentration, *P* = rooting phase incorporation, *I* = interaction. (a) Putrescine: *R* (*C* = 0.0006, *P* = 0.0004, *I* = 0.0189), *RN* (*C* = 0.1749, *P* = 0.6022, *I* = 0.2984), *RL* (*C* = 0.0000, *P* = 0.0395, *I* = 0.0458), *SR* (*C* = 0.2584, *P* = 0.0280, *I* = 0.9790), *RI* (*C* = 0.0015, *P* = 0.6275, *I* = 0.0340). (b) Spermidine: *R* (*C* = 0.1643, *P* = 0.1643, *I* = 0.1605), *RN* (*C* = 0.3746, *P* = 0.2153, *I* = 0.0501), *RL* (*C* = 0.0004, *P* = 0.2705, *I* = 0.0937), *SR* (*C* = 0.0558, *P* = 0.9104, *I* = 0.1153), *RI* (*C* = 0.0126, *P* = 0.5646, *I* = 0.2886). (c) Spermine: *R* (*C* = 0.1332, *P* = 0.8756, *I* = 0.2764), *RN* (*C* = 0.0027, *P* = 0.2559, *I* = 0.0697), *RL* (*C* = 0.0257, *P* = 0.1546, *I* = 0.0028), *SR* (*C* = 0.4857, *P* = 0.0559, *I* = 0.9392), *RI* (*C* = 0.0129, *P* = 0.0234; *I* = 0.1453). Different letters means significant differences in each polyamine, concentration and rooting phase at $P < 0.05$

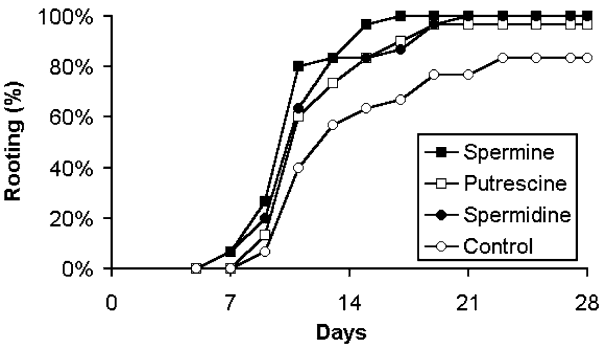


**Fig. 2** Rooting percentage evolution during the in vitro successive media of *Nothofagus nervosa* through the rooting process compared to a control treatment. *Spermine*: 10 μM in the expression phase. *Putrescine*: 100 μM in the induction phase. *Spermidine*: 10 μM in the induction phase

improved with exogenous incorporation of polyamines (Fig. 2). Spermidine advanced root emergence by two days (day 7), compared to putrescine (day 9) and the control treatment. Spermine presented a higher maximum rooting at day 11 (53% of the new shoot rooted at this observation day) and it was produced in a relatively short time (reaching a plateau with 100% rooting percentage at day 17). In comparison, putrescine and spermidine had a lower maximum rooting at day 11 (47% and 43% of shoot rooted at this day, respectively), and the rooting speed was relatively delayed until it finally reached 96.7% and 100% rooting at day 19 and 21, respectively.

## Discussion

### Endogenous tissue polyamine content during rhizogenesis

Polyamines are involved in plant growth development (Evans and Malmberg 1989), and their role in rhizogenesis was previously proposed (Hausman et al. 1995a; Heloir et al. 1996; Bellamine and Gaspar 1998; Arena et al. 2003), although some authors do not agree (Tiburcio et al. 1987; Biondi et al. 1990; Jarvis et al. 1983).

Endogenous tissue polyamine content varied during the rooting process. Spermidine was the most abundant polyamine found in shoots of *N. nervosa*, being almost three-fold the other two polyamine contents. However, in other woody species (*Pyrus communis*, *Populus* hybrid, and *Berberis buxifolia*) putrescine was the most abundant polyamine (Baraldi et al. 1995; Gaspar et al. 1997; Arena et al. 2003). The endogenous total polyamine levels found in the present work during adventitious rooting were higher than in *Pyrus communis* (Baraldi et al. 1995), and lower than in *Juglans regia* (Heloir et al. 1996), and *Berberis buxifolia* (Arena et al. 2005).

Changes in polyamine tissue concentration with time during in vitro rooting have also been reported for other woody species (Hausman et al. 1994; Baraldi et al. 1995; Gaspar et al. 1997; Arena et al. 2003). Here, the appearance of the maximum polyamine content and its magnitude was related to the auxin incorporation, being lowest in the IBA medium for the three studied polyamines. In fact, IBA advanced the presentation of the polyamine peak which was lower than those of the remaining polyamines. In the case of spermidine and putrescine IBA did not markedly affect the time of appearance of their maximum content, but lowered their amplitude. Increased rooting and time celerity could be related to lower polyamine tissue content, as cited previously for *B. buxifolia* (Arena et al. 2003). A higher polyamine concentration could have an inhibitory effect on rooting (Baraldi et al. 1995; Ballester et al. 1999), although its role during the process is highlighted by the presence of the detected maxima over time. Likewise, polyamine tissue concentration fell when the rooting stopped (Baraldi et al. 1995; Arena et al. 2003), as can be seen in Fig. 1 and 2. Another possibility considered was that polyamines stimulated the rooting, and the scarce tissue concentration observed was mainly attributed to its utilization during rhizogenesis (Martin-Tanguy and Carre 1993).

Peroxidases are the most traditional biochemical markers used (Berthon et al. 1987; Gaspar et al. 1992; Ripetti et al. 1994; Jouvé et al. 1994) to define the rooting phases in *N. nervosa* (Calderón et al. 1998). The induction phase (a maximum followed by a minimum) defined by total peroxidase activity ends at days 5–7. The

maximum peroxidase activity in the same culture conditions occurs earlier (day 2) than the spermine maximum (day 6), and in the two cases the minimum occurs at day 7. To define rooting phases in *N. nervosa*, peroxidases and polyamines would both be good biochemical indicators. Moreover, both compounds have an opposite behavior during the induction phase: when the total peroxidase reaches the maximum, the polyamine content gets the minimum; conversely, when the total peroxidase activity declines, the polyamine content reaches its highest values. Optimized rooting media, e.g. containing IBA, accelerates the maximum occurrence of roots (as in spermine treatment) compared with medium without IBA. Tiburcio et al. (1989) and Biondi et al. (1990) also reported the occurrence of a maximum of polyamines prior to root emergence. This polyamine maximum could be explained by the fact that the polyamines are related to growth and cellular division (Hausman et al. 1994). The cell activity determined as cell division and cambium activity reached the maximum in the last days of the induction phase, when the radical nodules are formed, as was previously reported for *N. nervosa* (Martínez Pastur et al. 2005).

## Exogenous polyamine incorporation into the rooting media

Depending on the type, concentration, and rooting phases, exogenously added polyamines can either stimulate or inhibit rooting (Martin-Tanguy and Carre 1993; Hausman et al. 1995a; Sha Valli Khan et al. 1999; Arena et al. 2003, 2005). In woody species, polyamine concentrations hitherto used varied from 0.1 to 2000 μM (Rugini 1992; Martin-Tanguy and Carre 1993; Hausman et al. 1994; Kevers et al. 1997; Arena et al. 2005). In the present study, a low polyamine concentration of 10 μM was enough to stimulate *N. nervosa* microshoot rooting. The incorporation of polyamines to the in vitro rooting media improved the rooting response compared to the original protocol (Martínez Pastur and Arena 1996), as occurred with the addition of other compounds (e.g. flavonoids) (Martínez Pastur et al. 2003). Moreover, our results show that the range of 1–100 μM polyamines did not inhibit microshoot rooting, as observed in other woody species (Baraldi et al. 1995; Ballester et al. 1999; Arena et al. 2005).

Polyamine inhibitors were previously used to either inhibit or modify functionality of endogenous polyamines during microshoot rooting (Hausman et al. 1994; Gaspar et al. 1997; Kevers et al. 1997; Arena et al. 2005). Some polyamines have an outstanding role in a specific rooting phase, and according to some authors closely resemble the auxin effects (e.g. putrescine in the induction phase or spermidine in the expression phase) (Altamura et al. 1991; Rugini 1992; Hausman et al. 1994, 1995a, 1995b; Gaspar et al. 1997; Kevers et al. 1997; Arena et al. 2005). In the present work, the best rooting response appeared when spermine was incorporated during the expression phase, just when the tissue concentration drops, and a medium with polyamine inhibitors during the induction phase.

Thus far, only a few authors have considered that spermine is beneficial during rhizogenesis (Rugini 1992; Sha Valli Khan et al. 1999), e.g. in *B. buxifolia* a slight rooting improvement was produced only if high spermine concentrations were added during the induction phase (Arena et al. 2005). With the other assayed polyamines, putrescine and spermidine, incorporation in the induction phase, and a medium with an inhibitor mixture in the expression phase, was more effective in rooting than the control.

## Conclusions

The profile of the endogenous polyamine content (putrescine, spermidine, or spermine) measured in microshoots of *N. nervosa* during rhizogenesis supports the idea of polyamines as an alternative biochemical marker in rhizogenesis. Polyamine tissue concentration was lower in the optimized rooting media, in which the maximum concentration occurred prior to root emergence. Altogether, the inclusion of polyamines in successive culture media improves the qualitative and quantitative responses of the in vitro rhizogenesis of *N. nervosa.* A proposal for successive culture media with polyamines, which includes the addition of 10 μM spermine during the expression phase, is proposed.

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