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Peroxidase and polyamine activity variation during the *in vitro* rooting of *Berberis buxifolia*

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Abstract *Berberis buxifolia* is a Patagonian shrub with great economic potential for tinctorial, pharmacological, and food industries. Clonal propagation is possible through *in vitro* culture and is also useful for metabolite production. However, this species is difficult to root, and to improve this, more knowledge of rhizogenesis processes is needed. Polyamines and peroxidases are useful biochemical markers during analysis of rooting phases for correlation with tissue morphological changes. Therefore, endogenous polyamine (putrescine, spermidine, spermine) changes, peroxidase activity evolution, and morphological development were studied to characterise the *in vitro* rhizogenesis of microshoots of *B. buxifolia* and, thus, to define the rooting phases. Polyamine and peroxidase changed significantly during the rooting period, and had opposite behaviours which were directly related to the IBA media content. The lower polyamine concentration and the higher peroxidase activity were found in a treatment with a dark period during the first four days and with IBA in the culture medium. Putrescine was the most abundant polyamine found in *B. buxifolia* tissues, 14- to 18-fold more than spermidine and spermine, respectively. Therefore, these compounds were used to define the rooting phases: an induction phase (0 to 4–7 days) followed by an expression phase (4–7 to 28 days). The observed changes in the biochemical markers could be correlated with microscopic and macroscopic tissue observations in the microshoots, and the time course of rooting percentage. Successive culture media can be developed including polyamines, or other compounds and environmental conditions, which positively modify the studied biochemical markers behaviour.

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INTRODUCTION

Berberis is well represented in Patagonia with 16 species of shrubs native to the region (Orsi 1984; Bottini et al. 1993). *B. buxifolia* (calafate) is an evergreen, spiny, erect shrub up to 4 m high, often growing in coastal scrub, forest margins and clearings, moist areas in grass steppe, and along streams and rivers (Moore 1983). Interest in the species has increased because of its usefulness for industrial (marmalade and jam production), medicinal, and tinctorial applications (Pomilio 1973; Orsi 1984; Fajardo Morales et al. 1986; Fajardo Morales 1987; Bottini et al. 1993; Arena & Martínez Pastur 1995).

Berberis buxifolia can be propagated by conventional (Arena & Martínez Pastur 1994) and *in vitro* (Arena et al. 2000; Arena & Martínez Pastur 2001) techniques, and the rooting phase is the crucial step during the development of protocols. It is necessary to know more about the rooting process (rooting phases, and physical and chemical factors affecting it) in order to improve the quality of *in vitro* root formation (Druart et al. 1982; Berthon et al. 1987; Gaspar et al. 1991; De Klerk 1996; Kevers et al. 1997). Survival rate during acclimatisation also needs to be improved (Arena et al. 2000). During development of a protocol for *in vitro* propagation, only external morphological changes of microshoots (celerity, root quality, and rooting percentage) have been studied to evaluate the influence of different factors on rhizogenesis. However, early morphological events have also to be studied through a histological analysis, to follow tissue changes in the microshoots before the appearance of the roots.

One approach to improve the rooting of microshoots is through the use of successive culture media (Berthon et al. 1987; Ripetti et al. 1994), but knowledge of the rooting phases (induction and expression) is necessary to establish this culture strategy. These phases have been identified through variation of some biochemical markers in endogenous microshoots. Peroxidase activity was proposed to define rooting phases (Gaspar 1981), and has been applied in several species (Berthon et al. 1987; Hausman 1993; Jouvé et al. 1994; Calderón Baltierra et al. 1998). However, other compounds could also be used as rooting markers, and polyamines have been the most studied in recent times (Baraldi et al. 1995; Hausman et al. 1995; Heloir et al. 1996; Gaspar et al. 1997). Thus, a positive relationship between polyamine accumulation and the initial stages of adventitious root formation has been reported in many systems (Friedman et al. 1982, 1985; Tiburcio et al. 1989; Torrigiani et al. 1989; Biondi et al. 1990; Altamura 1994). Although the involvement of polyamines in root formation is generally accepted, the specific role they play in this process remains to be elucidated and information about the exact rooting phase in which they are involved is very scarce.

The aim of this work was to determine the utility of peroxidases and polyamines as *in vitro* rooting markers in *Berberis buxifolia* by studying (a) peroxidase activity and endogenous polyamine (putrescine, spermidine, spermine) variations during the rooting process and (b) morphological changes in microshoots during the rooting process and its relation to the previous biochemical markers, and (c) evaluating the usefulness of the studied variables to define the rooting phases.

MATERIALS AND METHODS

Plant material and rooting conditions

Berberis buxifolia in vitro shoots 2.5 cm long with elongated internodes and 5–10 expanded leaves were used as explants. Basal medium and culture conditions for rooting were described elsewhere by Arena et al. (2000). The pH 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. Cultures were maintained in a growth chamber at 24 ± 2°C using cool-white fluorescent lamps (57 µmol m^{-2} s^{-1} photosynthetically active radiation), under 16:8 hours light:dark (Light treatment) or dark during the first four days and then 16:8 hours light:dark (Dark treatment). All the assays were ended at Day 28. The rooting percentage was recorded every two days.

Total tissue peroxidase activity during rhizogenesis

Tissue peroxidase activity (ng Po/µg soluble protein) was determined in microshoots from three treatments: (a) with no auxin in the culture medium under Light treatment (Control), (b) same photoperiod with 1.25 µm of indol-3-butyric acid (IBA) in the culture medium (LI), and (c) with 1.25 µm IBA under Dark treatment (DI). Treatments were chosen following Arena et al. (2000), and to differentiate the effect of auxin and photoperiod at the beginning of the rooting period. Samples were collected during the first 16 days of rooting of the cultures. One hundred mg FW tissue were homogenised with 5 ml 0.1 *M* phosphate buffer, pH

7.0, containing 0.001% Triton X-100 and 0.01 m*M* leupeptin. The homogenates were kept at 4°C for 20 min and then were centrifuged at 3000 *g* for 20 min. An aliquot of the supernatant was used to react with a mixture of guaiacol (5 m*M*) and hydrogen peroxide (4 m*M*) in 0.1 *M* phosphate buffer, pH 6.0. The activity of peroxidases was measured spectrophotometrically at 470 nm against phosphate buffer as a blank. Peroxidase activity (mean values from 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). Tissue protein content was determined by the Bradford method (Bradford 1976): homogenate (100 µl) was thoroughly mixed with 2 ml Coomassie Brilliant Blue G-250 stain reactant and absorbance was measured at 595 nm. Protein content (mean values from three replicates) was expressed in terms of the mass (mg) of commercial bovine serum albumin (BSA), which was used as a standard.

Endogenous tissue polyamine content during rhizogenesis

Microshoots from Control, LI, and DI treatments were collected during the first 16 days of the rooting period. Tissue samples (200 mg FW) were homogenised with 5% perchloric acid, kept 30 min on ice, and centrifuged at 5000 rpm for 10 min. Supernatants were then derivatised using the dansylation method described by Smith & Meeuse (1966) and 1.6 hexanediamine was used as internal standard. Standards of putrescine, spermidine, and spermine 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 aluminum sheets 20 × 20 cm, silica gel plates 60 F 254) using *n*-hexane:ethyl acetate (1:1) as the 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 of 365 nm and emission at 510 nm.

Histological analysis

Microshoot samples from *LI* treatment were collected every two days during the rooting period. These samples were fixed in 5:5:90 (v/v/v) formalin/glacial acetic acid/ethanol 96% v/v for 7 days. Shoots were then kept in 70% v/v ethanol until the cuts were made. Shoots were dehydrated through a graded *n*-butanol series during 24 hours and embedded in paraffin. Ten-micrometer transverse sections were made and stained with safranin-fast green (Jensen 1962). Twenty observations were made per section, and three samples (basal stem pieces) were evaluated for each day.

Statistical analysis

Results of total peroxidase activity and polyamine content were evaluated by an analysis of variance using the Fisher test. Orthogonal contrast was done through the factorisation of the square sum of the treatments. In the peroxidase analysis, two orthogonal contrasts were carried out analysing the dark and auxin effects. In the polyamine analysis, eight orthogonal contrasts were carried out analysing the polyamine type, and dark and auxin effects. These analyses were complemented by non-orthogonal contrasts in order to elucidate the dark effect. Means of each day for all the treatments were separated using Tukey test. All tests were performed at $P < 0.05$ significance level. Each treatment for the biochemical analysis had three repetitions, consisting of one flask with 6 shoots each. The analysis for the rooting studies had 30 shoots.

RESULTS

Total tissue peroxidase activity during rhizogenesis

DI treatment produced the highest total peroxidase activity (38 ng/µg Po/Prot) (Table 1). Peroxidase contents varied along the time course of the rooting period, and significant differences were found in the orthogonal contrasts. In the first orthogonal analysis, dark (DI) was significantly higher than light (LI) or (Control). However, a non-orthogonal contrast between LI and DI was necessary in order to elucidate the effect of darkness, and no differences were found between treatments ($P = 0.248$). In the second orthogonal analysis, the effect of IBA in the light treatments was analysed (Control versus LI). Significant differences were found, peroxidase activity increasing with increased auxin in the culture medium. Irrespective of the treatments, total peroxidase activity increased till the end of the sampling period, and for both DI and LI treatments

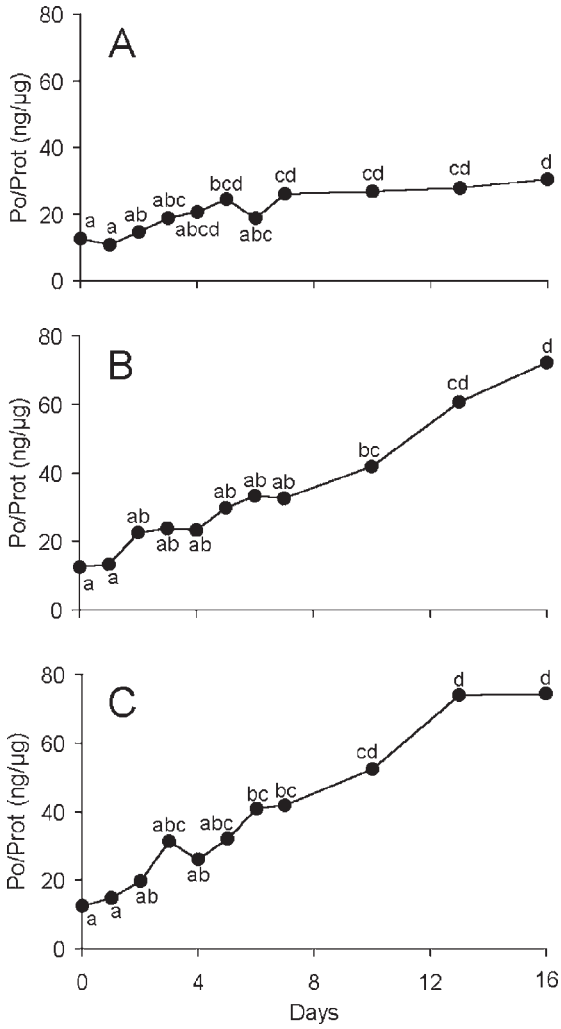

Fig. 1 Total tissue peroxidase activity change over the first 16 days of rooting period in microshoots of *Berberis buxifolia*. **A**, Control, 16:8 hours light:dark without IBA ($F = 10.94$, $P = 0.0000$); **B**, LI, 16:8 hours light:dark with IBA ($F = 16.97$, $P = 0.0000$); **C**, DI, dark during the first four days and then 16:8 hours light:dark with IBA ($F = 19.15$, $P = 0.0000$). For each treatment, different letters show significant differences at $P < 0.05$ by Tukey's test.

the initial enzyme activity increased by almost 6 times (Fig. 1). Enzyme activity peaks were found during the first days: treatment maxima were observed on Day 3 (31 and 24 ng/µg Po/Prot for DI and LI treatments, respectively) and minima on Day 4 (26 and 23 ng/µg Po/Prot for DI and LI treatments, respectively); for the Control, maximum was on Day 5 (24 ng/µg Po/Prot) and minimum on Day 6 (19 ng/µg Po/Prot) (Fig. 1). However, no significant differences were detected among these days and the correlative ones.

Endogenous tissue polyamine content during rhizogenesis

Endogenous putrescine concentration in tissue was higher (200 to 300 nmol/g FW) than the other polyamines (spermine and spermidine) (11 to 21 nmol/g FW) in the assayed treatments (Table 2). In the orthogonal contrasts, there were significant differences in polyamine content along the time course of the rooting period. In the first orthogonal analysis, putrescine versus spermine and spermidine concentrations were significantly different. In the second analysis, spermidine versus spermine, significant differences were not found. The effect of light (Control and LI) on putrescine concentration was significant, and putrescine concentration was less in the DI treatment. A non-orthogonal contrast between LI and DI was necessary to elucidate the effect of darkness, and no significant differences were found between treatments ($P = 0.247$). In the orthogonal analysis which analysed the effect of IBA in the light treatments (Control versus LI), differences were not significantly different for putrescine concentration. The other orthogonal contrasts did not show significant differences (Table 2).

Significant differences in putrescine content were not revealed during the first two days of rooting. However, considerable changes were observed thereafter, in LI reaching the maximum at Day 4 (906 nmol/g FW), while Control was maximum at Day 7 with 633 nmol/g FW and DI showed a first peak at Day 1 with 263 nmol/g FW. In the Control and LI treatments, the peaks in the putrescine concentration were significantly different from the correlative ones, and not significant different in DI treatment. Later, putrescine concentration decreased until the end of the experiment, except for DI, which showed a new peak at Day 13 (339 nmol/g FW) which was significantly different from Day 16 (Fig. 2). Spermidine and spermine concentration increased at Day 1, or at least stayed equal, and abruptly decreased at Day 2. Both polyamines reached the maximum concentration at Day 4, except for spermine in DI, to decrease again until the end of the experiments (Fig. 2). These polyamine concentration peaks were significantly different from the correlative days for spermidine in LI and for spermine in Control and LI.

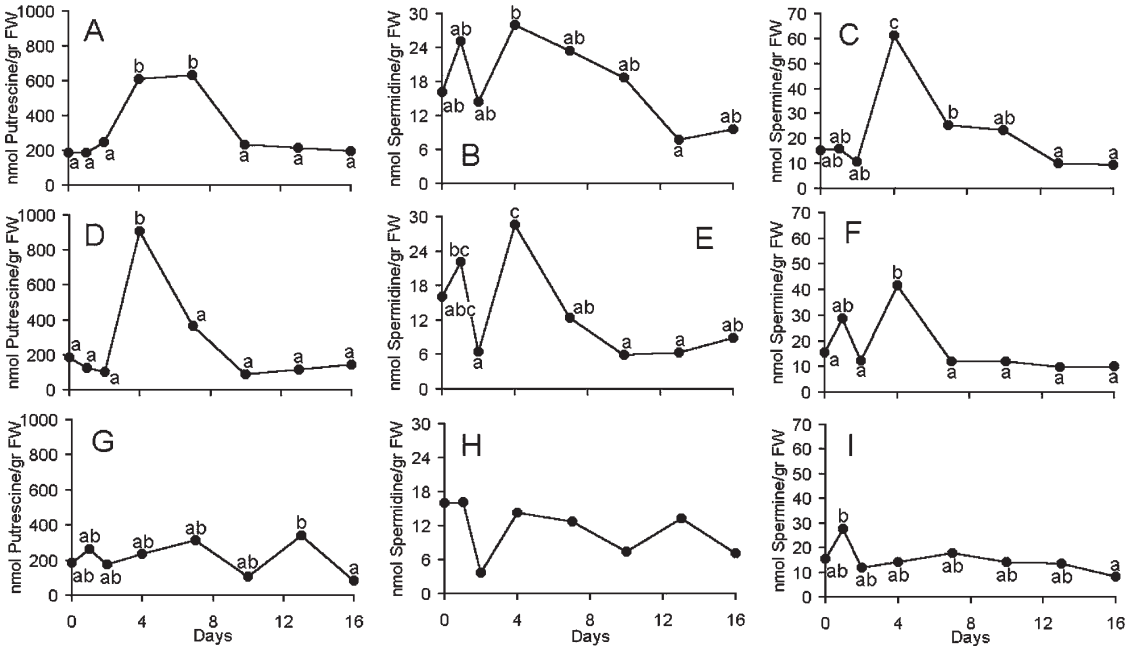

Fig. 2 Endogenous tissue polyamine concentration change over the first 16 days of rooting period in microshoots of *Berberis buxifolia*. **A–C**, Control, 16:8 hours light:dark without IBA (A: $F = 12.89$, $P = 0.0000$; B: $F = 3.68$, $P = 0.0146$; C: $F = 33.30$, $P = 0.0000$); **D–F**, LI 16:8 hours light:dark with IBA (D: $F = 13.64$, $P = 0.0000$; E: $F = 8.39$, $P = 0.0002$; F: $F = 7.84$, $P = 0.0003$); **G–I**, DI, dark during the first four days and then 16:8 hours light:dark with IBA (G: $F = 3.48$, $P = 0.0184$; H: $F = 3.23$, $P = 0.0248$; I: $F = 2.37$, $P = 0.0722$). A, D, G, putrescine; B, E, H, spermidine; C, F, I, spermine. For each treatment, different letters show significant differences at $P < 0.05$ by Tukey's test.

Table 1 Peroxidase levels, analysis of variance, and orthogonal contrast analysis for different treatments during the rooting period of *Berberis buxifolia*. Control = 16:8 hours light:dark without IBA; LI = 16:8 hours light:dark with IBA; DI = dark during the first four days and then 16:8 hours light:dark with IBA.

Treatment	Average values Po/Prot (ng/μg)			
Control	21.09			
LI	33.25			
DI	38.20			
	Orthogonal contrast analysis			
Source	d.f.	*S.S.*	*F*	*P*
Treatment	2	5115.9	8.55	0.0004
Control, LI versus DI	1	2675.3	8.93	0.0035
Control versus LI	1	2440.6	8.15	0.0053
Error	96	28736.7		
Total	98	33852.6		

Determination of rooting phases

According to Gaspar et al. (1992), it is necessary to detect maximum and minimum peroxidase activity to define the end or the beginning of the rooting phases. In this study, significant differences were not detected in the total peroxidase activity peaks, to allow the definition of the rooting phases. However, Gaspar et al. (1997) highlighted the fact that polyamine concentration changes during rooting showed similar behaviour to IAA (indol-3-acetic acid) and opposite behaviour to the peroxidases. Thus, in this work the rooting phases were defined through the variations of the endogenous polyamine concentration, which presented significant differences in the peaks during the rooting process.

The induction stage ended at different days depending on the treatments (Fig. 2). In the Control the induction stage ended at Day 4–7 according to putrescine analysis, and at Day 4 in spermidine and spermine analysis, when maximum concentrations were detected. In LI treatments the induction stage ended at Day 4. In DI treatments, significant differences were not detected in the polyamine concentration peaks, and rooting stage definition was not possible. However, it is possible that this phase ends early, at Day 1, when the first concentration maxima were detected, considering that the best rooting conditions induce an advance in the rooting phase periods (Calderón Baltierra et al. 1998). During rooting, peroxidase activity did not show well-defined maximum and minimum activity peaks, but they were opposite to the maximum and minimum polyamine concentrations. Hence, the remaining time period defined the expression stage.

Table 2 Polyamine endogenous concentration, analysis of variance, and orthogonal contrast analysis for different treatments during the rooting period of *Berberis buxifolia*. Put, putrescine; Spd, spermidine; Spm, spermine; C, 16:8 hours light:dark without IBA; LI, 16:8 hours light:dark with IBA; DI, dark during the first four days and then 16:8 hours light:dark with IBA.

Treatment	Average values nmol/g FW
Put C	312.49
Put LI	253.28
Put DI	212.18
Spd C	17.87
Spd LI	13.35
Spd DI	11.31
Spm C	21.40
Spm LI	17.67
Spm DI	15.38

Source	d.f.	Orthogonal contrast analysis *S.S.*	*F*	*P*
Treatment	8	2961590.0	24.57	0.0000
Put C, Put LI, Put DI versus Spd C, Spd LI, Spd DI, Spm C, Spm LI, Spm DI	1	2837981.0	188.32	0.0000
Spd C, Spd LI, Spd DI versus Spm C, Spm LI, Spm DI	1	567.8	0.04	0.8463
Put C, Put LI versus Put DI	1	79990.5	5.30	0.0222
Put C versus Put LI	1	42071.3	2.79	0.0963
Spd C, Spd LI, Spm C, Spm LI versus Spd DI, Spm DI	1	9.3	0.00	0.9801
Spd C, Spm C versus Spd LI, Spm LI	1	409.3	0.03	0.8692
Spd C versus Spm C	1	149.2	0.01	0.9208
Spd LI versus Spm LI	1	223.8	0.01	0.9031
Error	207	3119330.0		
Total	215	6080920.0		

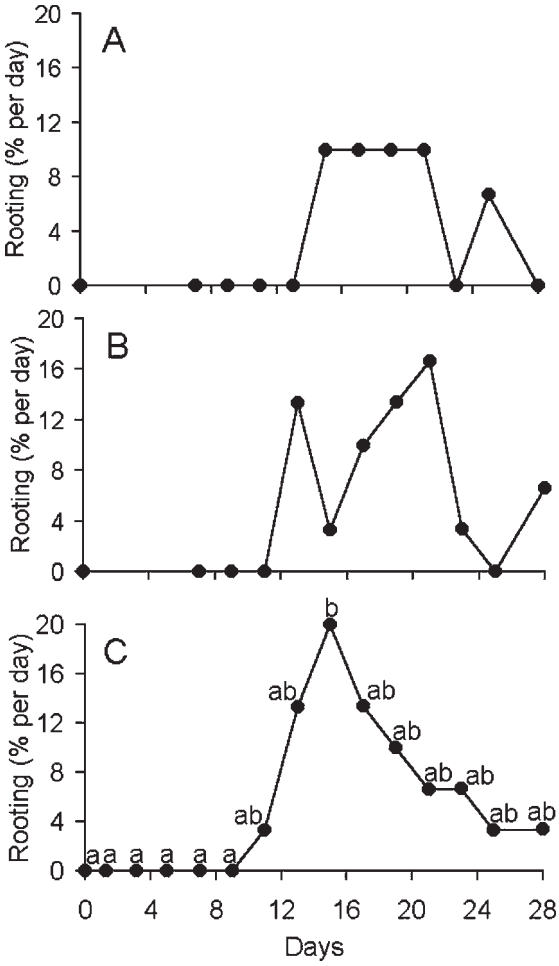

Fig. 3 Non-accumulated rooting percentage in microshoots of *Berberis buxifolia* over 28 days. **A**, Control, 16:8 hours light:dark without IBA ($F = 2.14$, $P = 0.0116$); **B**, LI, 16:8 hours light:dark with IBA ($F = 2.57$, $P = 0.0020$); **C**, DI, dark during the first four days and then 16:8 hours light:dark with IBA ($F = 2.32$, $P = 0.0057$). For each treatment, different letters show significant differences at $P < 0.05$ by Tukey's test.

Microscopic and macroscopic observations in microshoots during the rhizogenesis process

The rooting phases defined through peroxidase activity and endogenous polyamine concentration change closely agreed with microscopically observed tissue differentiation and with the macroscopic observation of root appearance. On the first and second days, tissues did not show morphological changes. From Day 3, some cells of the parenchyma and cambial zone were stained more densely and their nuclei and nucleoli became prominent. Numerous periclinal divisions were observed from Day 4. On Day 5, the first root primordium was observed near the cambial zone.

Roots emerged at Day 11 in DI, Day 13 in LI, and Day 15 for the Control treatment (Fig. 3). Rooting percentage in DI was more concentrated in time than in LI and Control treatments, where roots appear more discontinuously. At the end of the assay, Control had 47% rooting, LI reached 67% rooting, and DI attained the highest rooting values with 80%.

DISCUSSION

Tissue peroxidase activity during rhizogenesis

Peroxidases are the most traditional biochemical markers used in rooting studies (Berthon et al. 1987; Gaspar et al. 1992; Jouvé et al. 1994; Ripetti et al. 1994; Calderón Baltierra et al. 1998). A maximum in peroxidase activity at the beginning of the induction stage has been observed at Day 7 (50 and 80 µg/mg Po/Prot for adult and juvenile material, respectively) in *Sequoiadendron giganteum* (Berthon et al. 1987), at Day 2 (140 µg/mg Po/Prot) in *Juglans regia* (Ripetti et al. 1994), at Day 1 (151 µg/mg Po/Prot) in *Eucalyptus globulus* (Calderón Baltierra 1994), and at 42 hours (130 µg/mg Po/Prot) in *Lupinus mutabilis* (Jouvé et al. 1994). The time taken to reach maximum activity and its intensity value could be related to the rooting capacity of the species. *In vivo* and *in vitro* propagation of *B. buxifolia* is considered difficult (Arena et al. 2000; Arena & Martínez Pastur 2001). This probably explains the low maximum enzyme activity compared with the other species. Lag time in the appearance of the maximum, as well as its extension, could be related to the assayed rooting conditions. In *B. buxifolia*, maximum peroxidase activity was highest and earliest under better rooting conditions, DI compared with LI, and both compared with the Control (Fig. 1; Table 1). Similar behaviour was observed in *Nothofagus* (Calderón Baltierra et al. 1998) where a dark period at the beginning of root formation advanced the maximum peroxidase activity (Days 1 and 2 in DI treatment, Days 2 and 3 in LI treatment, for *N. nervosa* and *N. antarctica*, respectively) and increased the peroxidase activity (46 and 51 µg/mg Po/Prot in DI treatment, 36 and 16 µg/mg Po/Prot in LI treatment, for *N. nervosa* and *N. antarctica*, respectively).

Endogenous tissue polyamine content during rhizogenesis

Polyamines have been implicated in the regulation of several developmental phenomena in plants (Evans & Malmberg 1989). Putrescine was the most abundant polyamine found in *B. buxifolia* tissues, 14- to 18-fold more than spermidine and spermine, respectively. This agrees with observations in other species, such as *Pyrus communis* (Baraldi et al. 1995) and a *Populus* hybrid (Gaspar et al. 1997). The total endogenous polyamine levels in *B. buxifolia* found in this work during adventitious rooting were higher than in *Pyrus communis* (Baraldi et al. 1995) and lower than in *Juglans regia* (Heloir et al. 1996).

Changes in polyamine tissue concentration during *in vitro* rooting have also been reported for other species such as *P. communis* (Baraldi et al. 1995) and *Populus* (Hausman et al. 1994; Gaspar et al. 1997). However, in *J. regia*, Heloir et al. (1996) found no significant variations in spermidine content. The appearance of the maximum and its intensity is related to rooting conditions. In *B. buxifolia*, the maximum was lowest under better rooting conditions (DI treatment compared with LI treatment and both with Control treatment). Lower celerity and rooting percentage in the Control treatment could be due to the higher polyamine tissue content compared with those of IBA treatments.

These results showed that the high polyamine concentration could have an inhibitory effect on rooting of *B. buxifolia* microshoots, which agrees with the results of Baraldi et al. (1995) and Ballester et al. (1999). Polyamine tissue concentration in the DI treatment was lower than in the LI treatment. A similar finding was reported for *Olea europaea* (Rugini 1992), where a rapid degradation of polyamines occurs in the dark. It has also been shown that the tissue polyamine content is low in completely differentiated tissues or when rhizogenesis or callogenesis ceases to occur (Baraldi et al. 1995). This is in agreement with these results for *B. buxifolia*. However, it is possible that the presence of polyamine stimulates the rooting, and that the low tissue concentration observed is mainly due to its utilisation during the induction stage of the rhizogenesis process (Martin-Tanguy & Carre 1993).

Biochemical variations related to morphological aspects

Peroxidase activity has been used as a marker for distinct phases during adventitious root formation in forest species (Ben-Efraim et al. 1990), and Gaspar et al. (1992) defined the induction stage for rooting as a maximum of total peroxidase followed by a minimum. However, changes in several hormones, enzymes, phenols, polyamines, and chemical compounds have also been correlated with the rooting phases (Hausman et al. 1994, 1995; Heloir et al. 1996; Gaspar et al. 1997; Kevers et al. 1997; Martínez Pastur et al. 2000). In *B. buxifolia* the speed of root appearance could be related to endogenous variation of the main three polyamines and the total peroxidase activity (Fig. 3), which allowed the definition of rooting phases. Also, identified rooting phases were consistent with morphological changes of microshoots, observed during rooting differentiation at both microscopic and macroscopic levels (Fig. 1–3). Similar behaviour was observed in *Nothofagus nervosa* (Martínez Pastur 2000) and in *Eucalyptus globulus* (Calderón Baltierra 1995) where a close correspondence between chemical compounds variation and morphological changes was observed. Densely stained cells and periclinal divisions were observed in *B. buxifolia* from Day 3 during the induction stage. Then, the first primordia were observed at Day 5 inside microshoot tissues when a little thickness appeared in microshoot bases. Roots emerged from the tissues by Day 11.

In plants which produce berberines, such as *B. buxifolia*, plant growth regulators such as auxins promote the synthesis of these secondary metabolites (Jayakumaran Nair et al. 1992). Production and release of berberines into the culture medium can affect the morphogenetic responses (Uno & Preece 1987; Jayakumaran Nair et al. 1992; Arena & Martínez Pastur 2001), especially in rhizogenesis (Arena et al. 2000). As excreted berberine is accumulated the medium turns yellow in colour, which could be associated with the increase of peroxidase activity and the decrease of polyamine concentrations during the expression stage.

The enhancement in peroxidase activity was stimulated by the presence of IBA (Hausman 1993; Gaspar et al. 1996; Table 1). Hausman et al. (1995) observed a correlation between putrescine catabolism, peroxidase activity and IAA during the rooting process in poplar shoots, indicating that in rooting induction this polyamine was an indissociable factor of the two others. Peroxidase activity increased in DI and LI treatments compared with the Control, and, hence, berberine excretion could be associated with an increase of IBA in the culture media. As in our experiments, yellowish media coloration by berberine was observed in all treatments with exception of the controls.

CONCLUSIONS

Changes in presence and intensity in putrescine, spermidine, and spermine content, as well as in peroxidase activity, during the rooting process and their correlation with morphological events occurring in tissues of *B. buxifolia* microshoots justify the use of these compounds as biochemical markers. These markers allow the identification of two rooting phases in *B. buxifolia*, i.e., the induction phase (0 to 4th–7th day) followed by an expression phase (4th–7th to 28th day).

Hence, the development of an optimised protocol could be proposed that uses these markers. This proposal must include a successive culture media with polyamines, or other compounds and environmental conditions, which positively modify the behaviour of biochemical markers. This rooting system would lead to a qualitatively and quantitatively improvement of the root system in this species.

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