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Polyamines and inhibitors used in successive culture media for *in vitro* rooting in *Berberis buxifolia*

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Abstract Although *Berberis buxifolia* has been reported to propagate *in vitro*, rooting is difficult to achieve during subcultures, with reduced quantity and quality of roots, as is the case with many other woody species. Several cofactors of rhizogenesis have been proposed, and the role of polyamines in rooting has recently acquired relevance. Polyamines can stimulate or inhibit microshoot rooting depending on the type and concentration of polyamine and rooting phase, while polyamine inhibitors can improve rooting in some cases. A study of the *in vitro* rooting of *B. buxifolia* using polyamines and a mix of polyamine inhibitors in a two-step culture medium is described here, in which a new successive rooting medium was successfully implemented. While polyamine inhibitors sometimes improved rooting, nutrient medium containing a low polyamine concentration (i.e., 1 μ*M*) enhanced rooting compared with the control medium. The best microshoot rooting response resulted from the addition of 1 μ*M* spermidine during the expression phase in the absence of inhibitors in both rooting phases. Overall, incorporating polyamine in the successive media enhances the quality and quantity of roots in *B. buxifolia*, thus confirming their role during the induction and expression of the radicle primordia differentiation.

Keywords calafate; micropropagation; Patagonia; rhizogenesis; polyamine inhibitors; root system quality; *Berberis buxifolia*

INTRODUCTION

Berberis buxifolia Lam. is a woody species that grows in Patagonia where it is known as calafate. This woody shrub is appreciated for its yellow flowers and for the marmalade made with its fruits. Also these fruits possess a violet pigment (anthocyanins), while that of their rhizomes and roots is yellow (berberines). Both are used as tinctures. These compounds possess the anti-inflammatory, antipyretic, and antinociceptive properties that are well known for the genus (Pomilio 1973; Fajardo Morales et al. 1986; Fajardo Morales 1987; Kupeli et al. 2002).

Micropropagation is a technique that allows selection and cloning of elite field specimens of *B. buxifolia* for subsequent domestication. Micropropagation also provides the possibility of controlling growth by modifying the biochemical and physical environment, which in turn could influence secondary metabolism, i.e., the adjustment of culture conditions could lead to optimised alkaloid production. Rooting differentiation and growth are often critical and difficult to perform during *in vitro* propagation of woody species (De Klerk 1996; Kevers et al. 1997). To overcome this difficulty, a rooting culture technique using successive media was successfully employed (Martínez Pastur et al. 2003).

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Among rooting differentiation and growing factors, polyamines (putrescine, spermidine, and spermine) have acquired relevance in relation to rhizogenesis during the last decade (Baraldi et al. 1995; Hausman et al. 1995a; Heloir et al. 1996; Gaspar et al. 1997). Addition of polyamines in the culture media, and the removal of their effects through specific inhibitors, have already been proposed for several species (Tiburcio et al. 1987; Hausman et al. 1994, 1995a,b, 1997; Kevers et al. 1997) according to their specific role in each rooting phase (Arena et al. 2003).

Different macro and micropropagation protocols have been developed for the *Berberis* genus (Knox & Hamilton 1982; Uno & Preece 1987; Karhu & Hakala 1990, 1991; Arena & Martínez Pastur 1994). *B. buxifolia* can be easily multiplied *in vitro*, but difficulties during rooting have appeared (Arena et al. 2000, 2003; Arena & Martínez Pastur 2001). As a consequence, the survival rate during acclimatisation has been acceptable (Arena et al. 2000), although the *in vitro* plantlets then died. Moreover, *B. buxifolia* exhibits great variability in rooting response, with declining quantity and quality of roots during subcultures (data not shown). Previously biochemical markers were defined (endogenous peroxidases and polyamines) for different rhizogenesis phases (Arena et al. 2003) together with histological analysis. The optimisation of auxin concentration and photoperiod were also studied. Polyamines were good indicators for the rooting phases, giving useful information for successive media formulation.

The internal biochemical changes occurring during rhizogenesis (Arena et al. 2003) raised the possibility of improving the *in vitro* response through the use of a two-step culture medium differing in chemical composition (Berthon et al. 1993). Thus, the aim of this work was to optimise a protocol for the *in vitro* rooting of *B. buxifolia* mainly based on the incorporation of polyamines and a mix of polyamine inhibitors in a successive media culture protocol. In doing this, the type, concentration, and combination of polyamines and inhibitors in the rooting media were analysed.

MATERIALS AND METHODS

In vitro plant material and rooting conditions

Berberis buxifolia in vitro shoots 2.5 cm long with elongated internodes and 5–10 expanded leaves were employed as explants, after 3 years on *in vitro* multiplication (18–20 subcultures). Murashige & Skoog (1962) medium with half strength of macronutrient salts containing 1.25 μ*M* indole-3-butyric-acid (IBA) was employed as basal medium (Arena et al. 2000) supplemented with 0.7% w/v regional agar. 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. Culture vessels containing 6 microshoots each were maintained in a growth chamber at 24 ± 2°C, in the dark during the first four days followed by a regime of 16 h photoperiod under cool-white fluorescent lamps (57 μmol m^{-2} s^{-1} photosynthetically active radiation).

Polyamine incorporation into the successive media

In a first experiment, for each polyamine (putrescine, spermidine, and spermine), a multifactor assay with 2 factors and 12 treatments was conducted. The factors studied were: polyamine concentration (0, 1, 10, and 100 μ*M*), and rooting phase when polyamines were added to the medium (induction stage, Days 0 to 7; expression stage, Days 7 to 28; and both stages, Days 0 to 28) (Arena et al. 2003). In the absence of polyamines, a mixture of polyamine inhibitors (Inh) containing cyclohexylamine (CHA), aminoguanidine (AG), α-difluoromethylornithine (DFMO), and methylglyoxal-bis-guanylhydrazone (MGBG) was added to the media to a final concentration of 25 μ*M* each.

In a second experiment, the best concentration and rooting phase incorporation was selected from the first experiment for each polyamine: 1 μ*M* putrescine (Put) in the induction phase, 1 μ*M* spermidine (Spd) in the expression phase, and 100 μ*M* spermine (Spm) in the induction phase. Hence, the polyamine combination in the successive media was tested in a factorial assay with 14 treatments. Their seven possible combinations were tested (Put, Spm, Spd, Put-Spd, Put-Spm, Spm-Spd, Spm-Put-Spd) including a treatment with the polyamine inhibitor mixture (Inh) in the absence of polyamines in each rooting phase. In addition, four treatments with polyamines (Put, Spm, Spd, Put-Spm) in the absence of inhibitor mixture were tested to quantify the polyamine influence over the rooting process. Three control treatments were included, two using inhibitor mixture (Inh) with no polyamine addition (Inh in induction phase, and Inh in expression phase), and one containing neither polyamines nor the inhibitor mixture.

Polyamines and the inhibitor mixture were filter-sterilised using a 0.22 μm filter unit while adding them to the autoclaved rooting media under sterile

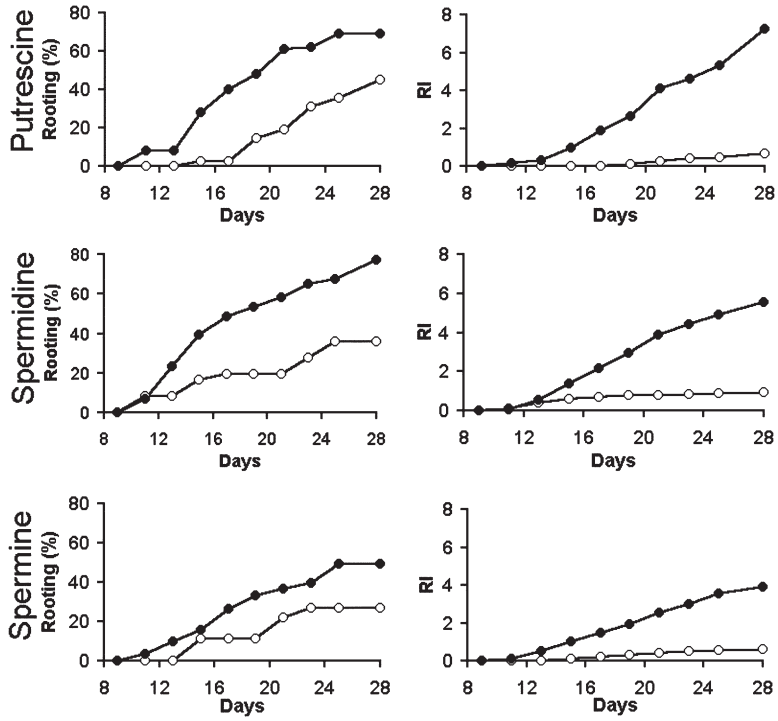

Fig. 1 Rooting percentage and rooting index (RI) evolution for the polyamine incorporation in the *in vitro* successive media of *Berberis buxifolia* microshoots through the rooting process. ●, assayed treatment; ○, control treatment. Putrescine: 1 μ*M* in induction phase, and a mixture of polyamine inhibitors in expression phase; Spermidine: 1 μ*M* in expression phase, and a mixture of polyamine inhibitors in induction phase; Spermine: 100 μ*M* in induction phase, and a mixture of polyamine inhibitors in expression phase.

air. All microshoots were subcultured at Day 7, even if the same treatment were to be continued.

Data collection and statistical analysis

Data were recorded every 2 days until Day 28 when assays were ended. Variable parameters evaluated were: good quality microshoot percentage (Q), rooting percentage (R), number of roots (RN), and length of roots (mm) (RL). The quality of the microshoots depended on the degree of explant oxidation (colour of the leaves) and on its healthy appearance (well development and expanded leaves). With these variables, a rooting index (RI) was calculated as described elsewhere (Arena et al. 2000):

$$RI\ (mm) = Q \times R \times R_{21} \times RN \times RL$$

where Q, R, and R_{21} were percentage values between 0 and 1; R_{21} is the rooting percentage at Day 21, as a value of celerity of the rooting process; Day 21 was chosen because at this day most of the treatments attained the maximum rooting celerity.

All treatments were subjected to an analysis of variance using a Fisher test. Media separation was done using the Tukey test. All tests were performed at $P \leq 0.05$ significance level. Each treatment had 6 replications (flasks) with 6 microshoots each. The data were averaged per flask before the analysis.

RESULTS

The assayed polyamines produced different responses on the quality and quantity of roots following their addition in different concentrations and rooting phases (Table 1). Putrescine concentration influenced the rooting number and, consequently, the rooting index, with 1 μ*M* being the best concentration tested. This low concentration also favoured all the studied variables as could be observed when comparing the corresponding response with its control treatment medium containing no polyamine. Higher putrescine concentrations (10 to 100 μ*M*) produced negative effects on the quality and quantity of roots of the explants. As a whole, putrescine did not present a different behaviour when it was added in different rooting phases, with the exception of the rooting length, for which significant differences were found. Nevertheless, this parameter did not significantly affect the rooting index. The putrescine treatment that greatly affected the rooting index was 1 μ*M* supplied in the induction phase, resulting in maximal rooting (44%), root number (2.2 roots per shoot), and rooting index (2.9 mm). The rooting evolution in media containing putrescine was significantly higher than the control treatment without polyamines (Fig. 1). The presence of putrescine allowed the shoots to

reach their maximum rooting expression. Moreover, rooting in culture medium containing putrescine was precocious in comparison with the corresponding control treatment. The rooting percentage markedly increased until Day 21, with a maximum rate of root initiation on Day 15. Rooting index was higher, and separated from the control treatment until the end of the assay.

With the exception of rooting percentage, spermidine concentration significantly affected all the studied variables (Table 1), and 1 μ*M* spermidine produced the best rooting index value (2.5 mm). As was observed in the case of putrescine, higher polyamine concentrations adversely affected the studied variables. One micro-molar spermidine in the media in different rooting phases significantly affected rooting percentage (58% in the expression phase compared with 40% in the other treatments). This treatment greatly affected the rooting index when spermidine was added in the expression phase, i.e., maximum rooting (49%) and rooting index (2.9 mm) values were obtained. In the presence of spermidine rooting evolution was also significantly higher than that resulting from the control treatment (Fig. 1). Also, rooting percentage markedly increased until Day 19, with a maximum rate of root initiation on Days 13–15, while rooting index showed augmented differences until the end of the assay.

Table 1 Polyamine incorporation in the *in vitro* successive media of *Berberis buxifolia* microshoots analysing type, concentration, and rooting phase incorporation. R, rooting percentage; RN, root number; RL, root length; RI, rooting index. Significance of the main effects: *C*, polyamine concentration; *P*, rooting phase incorporation; *I*, interaction. Putrescine: *R* (*C* = 0.0969, *P* = 0.1294, *I* = 0.2309), *NR* (*C* = 0.0129, *P* = 0.0863, *I* = 0.2817), *LR* (*C* = 0.5707, *P* = 0.0410, *I* = 0.0208), *RI* (*C* = 0.0018, *P* = 0.0947, *I* = 0.5426). Spermidine: *R* (*C* = 0.0775, *P* = 0.0078, *I* = 0.0044), *NR* (*C* = 0.0001, *P* = 0.6376, *I* = 0.3766), *LR* (*C* = 0.0006, *P* = 0.5087, *I* = 0.0011), *RI* (*C* = 0.0463, *P* = 0.1518, *I* = 0.0003). Spermine: *R* (*C* = 0.3750, *P* = 0.6767, *I* = 0.0113), *NR* (*C* = 0.7646, *P* = 0.9293, *I* = 0.2911), *LR* (*C* = 0.6145, *P* = 0.1073, *I* = 0.3159), *RI* (*C* = 0.2986, *P* = 0.3967; *I* = 0.0331). Different letters means significant differences in each polyamine, concentration and rooting phase incorporation at $P < 0.05$.

Polyamine	Factor	R (%)	RN (*n*)	RL (mm)	RI (mm)
Putrescine	Concentration (μ*M*)				
	0	51.45	1.93ab	5.11	1.89b
	1	61.11	2.20a	5.88	4.85a
	10	49.02	1.71b	5.41	1.40b
	100	42.85	1.71b	5.09	1.44b
	Rooting Phase Incorporation				
	Induction	53.57	2.07	5.55ab	3.45
	Expression	43.71	1.83	6.01a	2.11
	Induction + Expression	56.05	1.77	4.56b	1.63
Spermidine	Concentration (μ*M*)				
	0	56.06	2.21a	6.52a	2.06ab
	1	48.41	1.59b	5.55a	2.55a
	10	40.21	1.55b	5.55a	1.38ab
	100	39.62	1.27b	2.95b	0.78b
	Rooting Phase Incorporation				
	Induction	40.27b	1.59	5.57	1.40
	Expression	58.15a	1.75	5.14	2.34
	Induction + Expression	40.00b	1.63	4.72	1.34
Spermine	Concentration (μ*M*)				
	0	37.07	1.65	5.52	1.13
	1	47.88	1.75	4.57	1.04
	10	45.06	1.79	5.21	1.75
	100	46.37	1.84	5.09	2.07
	Rooting Phase Incorporation	42.60	1.79	5.91	1.93
	Induction	46.99	1.74	4.52	1.30
	Expression	42.76	1.74	4.86	1.27
	Induction + Expression				

Spermine did not significantly influence the studied variables when either the concentration or the rooting phase addition was analysed (Table 1). The best treatment was 100 μ*M* spermine added in the induction phase, where the root number (2.0 roots per shoot), and rooting index (2.0 mm) were greatest. Significant differences were found in rooting evolution in media containing spermine when the rooting index was analysed in comparison with the control treatment (Fig. 1). In the presence of spermine, the rooting index exhibited higher values than the corresponding control until the end of the assay.

The combinations resulting from the best treatment for each polyamine increased the rooting parameter values differences with respect to the controls (Table 2). Significant differences were found in rooting percentage, and therefore in rooting index. Incorporating spermidine in the expression phase presented the best rooting values (42%) and rooting index (1.3 mm), followed by their combinations with spermine. Addition of inhibitor mixture affected the studied rooting parameters. Inhibitors in the induction phase acted negatively, while their incorporation in the expression phase significantly improved rooting (41%) and rooting index (0.5 mm) in comparison with the results from the control treatment containing neither polyamines nor inhibitors (19% and 1.2 mm, respectively). The rooting percentage and rooting index evolution in media containing the best polyamine combination showed a sigmoid-like curve (Fig. 2). However, in control

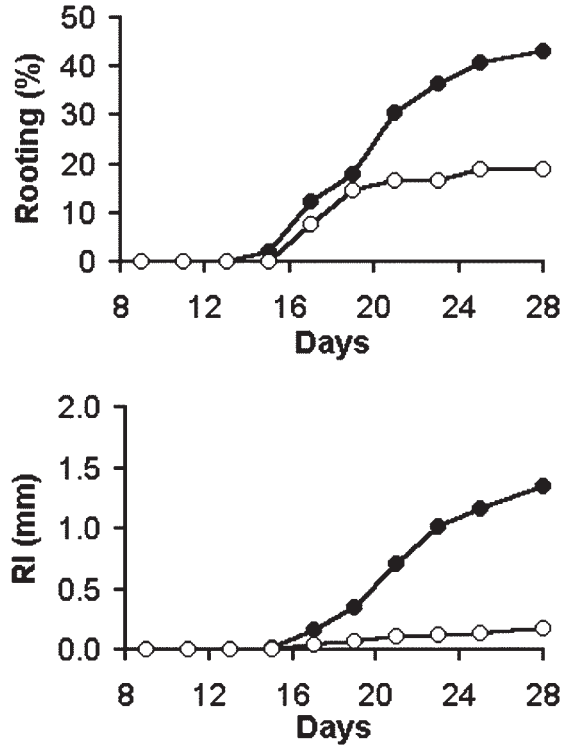

Fig. 2 Rooting percentage and rooting index (RI) evolution for the 1 μ*M* spermidine in the expression phase in the *in vitro* successive media of *Berberis buxifolia* microshoots through the rooting process compared with a control treatment. ●, assayed treatment; ○, control treatment.

Table 2 Polyamine incorporation in the *in vitro* successive media of *Berberis buxifolia* microshoots analysing different combinations of type and rooting phase incorporation. Put, 1 μ*M* Putrescine; Spm, 100 μ*M* Spermine; Spd, 1 μ*M* Spermidine; Inh, 100 μ*M* inhibitors; –, without polyamines nor inhibitors. Significant level $R = 0.0065$; $RN = 0.8467$; $RL = 0.4112$; $RI = 0.0178$.

Treatment		R	RN	RL	RI
Induction Phase	Expression Phase	(%)	(*n*)	(mm)	(mm)
Put	–	11.00ab	1.25	2.50	0.03b
Spm	–	19.58ab	1.58	4.85	0.29ab
–	Spd	42.97a	1.60	3.35	1.34a
Put	Spd	27.08ab	1.33	3.22	0.48ab
Spm	Spd	38.09ab	1.61	3.85	0.90ab
Spm-Put	–	38.09ab	1.35	3.52	0.57ab
Spm-Put	Spd	22.91ab	1.71	3.42	0.14b
Put	Inh	28.64ab	1.27	4.11	0.25ab
Inh	Spd	24.16ab	1.57	4.69	0.42ab
Spm	Inh	30.95ab	1.28	2.64	0.23ab
Spm-Put	Inh	21.42ab	1.46	3.40	0.25ab
Inh	–	10.83b	1.25	3.75	0.06b
–	Inh	41.25ab	1.78	2.96	0.56ab
–	–	18.98ab	1.66	2.97	0.17b

media this same response was only observed until Day 19 in rooting percentage, quite probably due to some factor limiting full expression of the rooting process. The differences in rooting index evolution are markedly higher after Day 19.

DISCUSSION

Endogenous polyamine content varied during the rooting process, and different authors have proposed that they have a role in rhizogenesis (Hausman et al. 1995a; Heloir et al. 1996; Bellamine & Gaspar 1998; Arena et al. 2003), but others do not support their participation in rooting (Tiburcio et al. 1987; Biondi et al. 1990; Jarvis et al. 1983). Some workers have reported that adding polyamine can stimulate or even inhibit the rooting phenomenon depending on the polyamine type, concentration, and opportunity for their addition with regard to the different rooting phases (Martin-Tanguy & Carre 1993; Hausman et al. 1995a; Sha Valli Khan et al. 1999; Arena et al. 2003).

The polyamine concentrations assayed vary from 0.1 to 2000 μ*M* in different species (Rugini 1992; Martin-Tanguy & Carre 1993; Hausman et al. 1994; Kevers et al. 1997). In the present study, a low polyamine concentration (1 μ*M*) had a remarkable effect on the rooting indicators. Thus, including this polyamine in the *in vitro* culture of *B. buxifolia* improved the rooting behaviour of this species in relation to that exhibited in a previously proposed medium (Arena et al. 2000). A higher polyamine concentration had an inhibitory effect on the rooting of this species (Arena et al. 2003), as was also cited for other species by Baraldi et al. (1995) and Ballester et al. (1999). It is also interesting to note that rooting could be stimulated by polyamines in the absence of any plant growth regulator (Hausman et al. 1994, 1995a; Faivre-Rampant et al. 2000). Polyamine inhibitors have been widely used (Hausman et al. 1994; Gaspar et al. 1997; Kevers et al. 1997) to inhibit or modify the functionality of polyamines in the rooting phenomenon. However, some authors (Biondi et al. 1990) have considered that polyamine does not play a specific role during rooting. In *B. buxifolia* polyamine incorporation into the culture media could either improve (expression phase) or worsen (induction phase) the rooting response compared with the control treatment.

Putrescine incorporation in the induction phase was more effective when it was assayed together with a polyamine inhibitor mixture in the expression phase (Table 1), if compared with rooting results obtained in the absence of polyamine inhibitors (Table 2). This is in good agreement with the findings reported by Hausman et al. (1994) working with *Populus* species. Since a particular polyamine can stimulate or inhibit the rooting process depending on the particular rooting phase, it is necessary to study each polyamine inhibitor separately. In fact, stimulation of rooting by putrescine inclusion in the medium was possible during the induction phase while added spermidine stimulated rooting during the expression phase. To favour the accumulation of a given polyamine, the incorporation of specific inhibitors was studied (Hausman et al. 1995b).

Among polyamines, putrescine has displayed an outstanding role in rooting, closely resembling auxins in the inductive process (Altamura et al. 1991; Rugini 1992; Hausman et al. 1994, 1995a,b; Gaspar et al. 1997; Kevers et al. 1997). Low putrescine concentrations during the induction phase enhanced the rooting parameters evaluated in the present study, and hence the celerity compared with the controls in *B. buxifolia*. A few authors considered the benefit of spermidine or spermine during rhizogenesis (Rugini 1992; Sha Valli Khan et al. 1999). In *B. buxifolia* low spermidine concentrations were favourable during the expression phase, while spermine produced only a little rooting improvement at high concentrations when added during the induction phase.

The use of polyamine inhibitors in the culture media could improve or inhibit rooting. Some inhibitors can arrest the effect of polyamines through the suppression of their action or production sites. This effect suppression could favour the production of other polyamines, e.g., cyclohexlamine (CHA), which is known to inhibit spermine synthase while promoting putrescine accumulation (Hausman et al. 1995a,b). In the case of *B. buxifolia*, the best treatment includes the addition of spermidine in low concentrations during the expression phase, in the absence of inhibitors during the induction phase. The addition of exogenous polyamines during the induction phase was not necessary, since the endogenous polyamine content is adequate for good root system development (Arena et al. 2003).

It is concluded that incorporating polyamines in successive media definitions improves *B. buxifolia* rhizogenesis *in vitro* from both a qualitative and quantitative point of view. Previous results also emphasise that polyamines play a role during the induction and expression phases involved in root

differentiation. The best treatment included the addition of 1 μ*M* during the expression phase, but it is necessary to assay lower concentrations to adjust to the better combination.

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