6-BENZYLAMINOPURINE AND ACTIVATED CHARCOAL AFFECT *In vitro* SHOOT MORPHOGENESIS OF *Berberis buxifolia* LAM

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SUMMARY

An important factor affecting the micropropagation is the quality and rate of shoot multiplication. The objectives of this work are to study the effect of concentration and *BA* continuous presence, and the addition of activated charcoal during two periods of culture on *Berberis buxifolia* morphogenesis processes during *in vitro* shoot multiplication. The addition of *BA* in the three subcultures favored the multiplication index compared with a pulse of *BA* in the first subculture, specially for 0.55 and 1.10 μM *BA*. Lower *BA* concentrations (0.55 to 1.10 μM) were more favorable for shoot multiplication than 2.22 μM BA, allowing to obtain the highest multiplication index. The percentage of shoots longer than 5 mm was maximum with activated charcoal while the multiplication index were significantly lower than those obtained without activated charcoal. In the first culture period, the multiplication index were higher than in the second culture period. However, the percentage of shoots longer than 5 mm was significantly higher in the second culture period than in the first culture. The results obtained in this work show that the morphological processes observed during *in vitro* shoot multiplication of *B. buxifolia* can be controlled by the concentration and continuous presence of *BA* and the addition of activated charcoal.

Key words. *Berberidaceae*, tissue culture, micropropagation, multiplication, growth regulators, activated charcoal, culture period.

6-BENCILAMINOPURINA Y CARBON ACTIVADO AFECTAN LA MORFOGENESIS DE LOS BROTES *In vitro* DE *Berberis buxifolia* LAM

RESUMEN

Un factor importante que afecta la micropropagación es la calidad y la tasa de la multiplicación de los brotes. Los objetivos de este trabajo son estudiar el efecto de la concentración y presencia continua de *BA* y el agregado de carbón activado durante dos períodos de cultivo en los procesos de morfogénesis de *Berberis buxifolia* durante la multiplicación *in vitro* de los brotes. La adición de *BA* en los tres subcultivos favoreció el índice de multiplicación comparado con un pulso de *BA* en el primer subcultivo, especialmente para 0,55 y 1,10 μM de *BA*. Concentraciones más bajas de *BA* (0,55 a 1,10 μM) fueron más favorables para la multiplicación de los brotes que 2,22 μM de BA, permitiendo obtener el mayor índice de multiplicación. El porcentaje de brotes más largos que 5 mm fue máximo con carbón activado mientras que el índice de multiplicación fue significativamente más bajo con respecto a los obtenidos sin carbón activado. En el primer período de cultivo, el índice de multiplicación fue más alto que en el segundo período de cultivo. Sin embargo, el porcentaje de brotes más largos que 5 mm fue significativamente más alto en el segundo período de cultivo con respecto al primer período de cultivo. Los resultados obtenidos en este trabajo muestran que los procesos morfológicos observados durante la multiplicación *in vitro* de *B. buxifolia* pueden ser controlados por la concentración y presencia continua de *BA* y por el agregado de carbón activado.

Palabras clave. *Berberidaceae*, cultivo de tejidos, micropropagación, multiplicación, reguladores del crecimiento, carbón activado, período de cultivo.

Abbreviations. 6-benzylaminopurine *(BA)*; activated charcoal *(AC)*; Murashige and Skoog medium *(MS)*.

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INTRODUCTION

An important factor affecting the micropropagation is the quality and rate of shoot multiplication. It is well known the role of the cytokinins on shoot multiplication (George and Sherrington 1984). In those species that produce berberines, as *Berberis buxifolia*, growth regulators like *BA*, promote the synthesis of such substances and the release to the culture medium (Jayakumaran *et al.,* 1992, Hara *et al.,* 1993) and can affect the morphogenesis processes (Uno and Preece 1987, Jayakumaran *et al.,* 1992). This phenomenon could be controlled through the reduction or elimination of *BA* levels or by the addition of *AC* (Fridborg and Eriksson 1975, Fridborg *et al.,* 1978, Ebert *et al.*,1993) during the shoot multiplication. The addition of *AC* to media has been shown to have a beneficial effect on the growth of the explants. However, in a few instances the incorporation of *AC* proved to be inhibitory to growth (Weatherhead *et al.,* 1979). *AC* has frequently improved plant tissue culture by adsorption of growth inhibitors, prevention of unwanted callus growth, promotion of morphogenesis, root formation and elongation (Fridborg and Eriksson 1975, Druart and De Wulf, 1993). The beneficial effects of *AC* are attributed to the removal of inhibitory substances from the media, produced either on autoclaving the media or by the tissue itself (Ebert *et al.*, 1993). The *AC* adsorbs phenols (Fridborg *et al.,* 1978; Weatherhead *et al.,* 1978), 5-hydroxymethylfurfural produced by sucrose autoclaving (Weatherhead *et al.,* 1978), agar impurities and ethylene (Druart and De Wulf, 1993). *AC* could also adsorb medium components such as vitamins (Weatherhead *et al.,* 1978), cytokinins and auxins (Fridborg *et al.*, 1978, Ebert *et al.*, 1993), ascorbic acid, nicotinic acid and thiamine (Weathe-rhead *et al.,* 1978, 1979). For these reasons, the objectives of this work are to study the effect of concentration and *BA* continuos presence, and the addition of *AC* during two periods of culture on *Berberis buxifolia* morphogenesis processes during *in vitro* shoot multiplication.

B. buxifolia, commonly named "calafate" is a small fruit species of Patagonia (Moore, 1983). Its economical importance is due to its fruits can be eaten fresh or in marmalades and jams (Orsi, 1984, Bottini *et al.,* 1993), while it is an important source of alkaloids like berberines and anthocyanins too (medicinal and tinctorial application) (Pomilo 1973, Fajardo Morales *et al.,* 1986, Fajardo Morales 1987). There are a few reports on *in vitro* and cutting propagation of *Berberis* (Knox and Hamilton 1982, Uno and Preece 1987, Karhu and Hakala 1990, 1991, Arena and Martínez Pastur 1998, Arena *et al.*, 1998). *B. buxifolia* can be propagated by seeds and rhizomes too (Arena and Martínez Pastur 1994, 1995, Arena *et al.,* 1999).

MATERIALS AND METHODS

1) *Plant material:*

The plant material was obtained from plants growing in clearings near forest margins at Ushuaia (54° 48' SL, 68° 15' WL) Tierra del Fuego - Argentina. At the end of the winter, ten mature plants of 8-10 years old were chosen, and its rhizome systems were obtained. The rhizomes were cut in portions of 10 cm long and were placed in an incubation chamber (24 ±2°C and 80% relative humidity) for bud sprouting. When the shoots grown from the rhizomes were 3-5 cm long, the apice and uninodal segments were obtained and used as plant material.

2) *Culture initiation and growing conditions:*

Plant material was surface sterilised with NaOCl (1.0% w/v active Cl_2) for 5 minutes. Thereafter it was rinsed three times with sterile, distilled water. Explants of 5-10 mm long were placed in tubes containing Murashige and Skoog medium (1962) (*MS*), with half strength of macronutrient salts and peptone (0.1% w/v), and cultured for 21 days to favor the appearance of contamination. The uncontaminated explants were kept on *MS* medium with 2.22 μM *BA* for 18 to 22 months. For all the experiments the media were supplemented with sucrose (3.0% w/v), regional agar (0.7% w/v) and dispensed into 20 mm x 120 mm glass tubes containing 10 ml. The pH was adjusted to 5.7 ±0.05 with KOH 0.1 N before autoclaving, which was performed 20 min at 0.1 MPa. Cultures were grown in a growth chamber at 24 ±2°C with a photoperiod (16:8, light:darkness) using cool-white fluorescent lamps (57 μmol m^{-2} photosynthetically active radiation).

3) *Shoot Multiplication:*

A design with the following factors was chosen: (a) the effect of concentration and continuos presence of *BA* (0.55, 1.10, 2.22 μM in the three subcultures, a pulse of 0.55 to 2.22 μM in the first subculture and two subcultures on a BA-free medium), (b) the presence of 0.5% w/v *AC* in the last two subcultures and (c) the culture period (first and second). *MS* was employed as basal medium. The explants (shoots of 3 mm long with four leaves) were transferred to fresh medium every 21 days without subdividing. The first period of culture was

finalized at day 63 when the data were collected. Consecutively, explants with the same characteristics as the original ones were obtained from the shoots grown in each treatment and then were cultivated in the same media (the defined treatments) for other period of 63 days (second period of culture). The multiplication was evaluated through: mortality (*M*), total axillary shoot number and discriminated by length (shorter or equal than 5 mm, longer than 5 mm), shoot number with elongated internodes, shoot leaf number, shoots with expanded leaves, multiplication rate (*MR*), explants with vitrification, browning and callus, dry weight of callus (*DWC*) and multiplication index (*MI*). The multiplication index was defined as:

$$MI = [a \times S] + \left[\left(b \times \frac{MR}{100}\right) \times \left(10^{(S \times 0.01)}\right)\right] + \left[\frac{\left(10^{\left(\frac{MR}{100}\right)}\right)}{(c \times DWC) + \left(10^{DWC}\right)}\right]$$

and $S = ((100\text{-}M)\text{-}(100\text{-}Mmax))$

where **a**, **b** and **c** are the parameters of the equation (a = 0.1068639, b = 14.99743, c = 152.1752) and *Mmax* =58%.

4) *Statistical analysis*:

The statistic validity of the results was obtained through the analysis of variance by Fisher and Tukey multiple range tests. Thirty explants were tested in each treatment for all the experiments. Significance level was $P \leq 0.05$.

RESULTS AND DISCUSSION

The concentration and continuos presence of *BA* affected significantly the most of the studied variables (Table 1 and 2). The addition of *BA* in the three subcultures favored the total axillary shoot number, multiplication rate and multiplication index (Table 2) compared with a pulse of *BA* in the first subculture, specially for 0.55 and 1.10 μM *BA*. These results are not coincident with those found for other species, where secondary media free from cytokinin were used for increasing the bud sprouting (von Arnold and Tillberg, 1987, Nielsen *et al.*, 1995). However, the elimination of *BA* during the

Table 1. The effect of concentration and *BA* continuous presence, the addition of *AC* and culture period on mortality (*M*), vitrification (*V*), browning (*B*), explants with callus (*C*) and dry weight of the callus (*DWC*) on shoot multiplication of *B. buxifolia*.

	Subc.	M (%)	V (%)	B (%)	C (%)	*DWC* (mg)
BA (μM)						
0.55	1°	17.8b	2.1b	11.7a	4.7e	0.0b
0.55	1° to 3°	11.7b	9.3b	7.5a	18.4bcd	0.2b
1.10	1°	34.2a	1.4b	14.0a	11.4de	0.1b
1.10	1° to 3°	14.7b	7.0b	14.8a	31.6b	0.9b
2.22	1°	46.7a	5.0b	14.8a	24.4bc	0.6b
2.22	1° to 3°	41.2a	22.8a	11.1a	60.7a	5.9a
Activated Charcoal						
No		25.2a	11.3a	12.8a	36.5a	2.3a
Yes		30.2a	4.6b	11.8a	13.9b	0.2b
Culture Period						
First		23.1b	7.4a	20.1a	34.6a	1.3a
Second		32.1a	8.5a	4.5b	15.7b	1.3a

Significant values by Fisher test: **BA:** M=0.0000; V=0.0000; B=0.6659; C=0.0000; DWC=0.0000. **AC:** M=0.1288; V=0.0046; B=0.7602; C=0.0000; DWC=0.0000. **CP:** M=0.0048; V=0.6272; B=0.0000; C=0.0000; DWC=0.8906. **Interactions (BAxACxCP):** M=0.5181; V=0.4379; B=0.7400; C=0.0068; DWC=0.0048. In the same column and for each factor, mean values followed by the same letter are not significantly different at P<0.05 level by Tukey multiple range test.

Table 2. The effect of concentration and *BA* continuous presence, the addition of *AC* and culture period on total axillary shoot number (*SH*), shoot number with elongated internodes (*SE*), shoot leaf number (*SL*), shoots with expanded leaves (*EL*), multiplication rate (*MR*) and multiplication index (*MI*) on shoot multiplication of *B. buxifolia*.

	Subc.	SH (n)	SE (n)	SL (n)	EL (%)	MR	*MI*
BA (μM)							
0.55	1°	3.1c	0.3b	7.9a	80.4[a]	2.4b	9.2b
0.55	1° to 3°	6.3b	0.2b	7.9a	84.4[a]	5.3[a]	15.5a
1.10	1°	2.8c	0.1b	7.9a	78.6[a]	2.2b	5.6c
1.10	1° to 3°	9.1a	0.5a	7.8a	79.0a	6.4a	13.9a
2.22	1°	3.4c	0.0b	7.3ab	75.9[a]	2.7b	3.1d
2.22	1° to 3°	7.0ab	0.1b	6.4b	41.3b	2.1b	3.1d
Activated Charcoal							
No		7.4a	0.2a	6.7b	67.7b	4.5a	10.1a
Yes		3.2b	0.2a	8.3a	78.9[a]	2.5b	6.7b
Culture Period							
First		6.5a	0.2a	7.4a	64.6b	4.1a	10.1a
Second		4.1b	0.2a	7.6a	81.9[a]	2.9b	6.7b

Significant values by Fisher test: **BA:** SH=0.0000; SE=0.0000; SL=0.0000; EL=0.0000; MR=0.0000; MI=0.0000. **AC:** SH=0.0000; SE=0.1727; SL=0.0000; EL=0.0001; MR=0.0000; MI=0.0000. **CP:** SH=0.0000; SE=0.1782; SL=0.2557; EL=0.0000; MR=0.0008; MI=0.0000. **Interactions (BAxACxCP):** SH=0.9499; SE=0.3396; SL=0.0458; EL=0.1144; MR=0.8547; MI=0.0018. In the same column and for each factor, mean values followed by the same letter are not significantly different at P<0.05 level by Tukey multiple range test.

last two subcultures prevented the unwanted callus growth (explants with callus and dry weight of callus) (Table 1), as was cited by Druart and De Wulf (1993). The bud neoformation mainly observed for 2.22 μM *BA* in the three subcultures was also reduced with media free from cytokinin, being coincident with that observed for *Nothofagus leoni* (Martínez Pastur and Arena, 1999). The percentage of shoots longer than 5 mm was higher with the lowest BA concentrations (Figure 1a). A pulse of BA did not enhance the length of the shoots, being not coincident with those results found by van Niewkerk *et al.*, (1987) and Kataeva *et al.*, (1991). Lower *BA* concentrations (0.55 to 1.10 μM) were more favorable for shoot multiplication than 2.22 μM BA, allowing to obtain the highest multiplication index, with the best values of mortality, shoot with elongated internodes, shoot leaf number, shoot with expanded leaves and vitrification (Table 1 and 2). These results are not coincident with those found for *B. thunbergii atropurpurea* (Uno and Preece 1987) and for *B. thunbergii* (Karhu and Hakala, 1991), where were used higher *BA* concentrations (5 to 10 μM) than in *B. buxifolia*.

The addition of *AC* affected the shoot multiplication process, favoring the shoot leaf number, the percentage of shoots with expanded leaves and percentage of shoots longer than 5 mm (Table 2 and Figure 1b). The *AC* inhibited the callus growth (explants with callus and callus dry weight) (Table 1). However, the multiplication rate and multiplication index obtained with *AC* were significantly lower than those obtained without *AC* (Table 2). This could be caused by the *AC* absorption of cytokinins (Fridborg *et al.*, 1978), fact that could favor the elongation of the shoots (Fridborg and Eriksson 1975).

In the first culture period, the explants with browning, explants with callus, the shoot number,

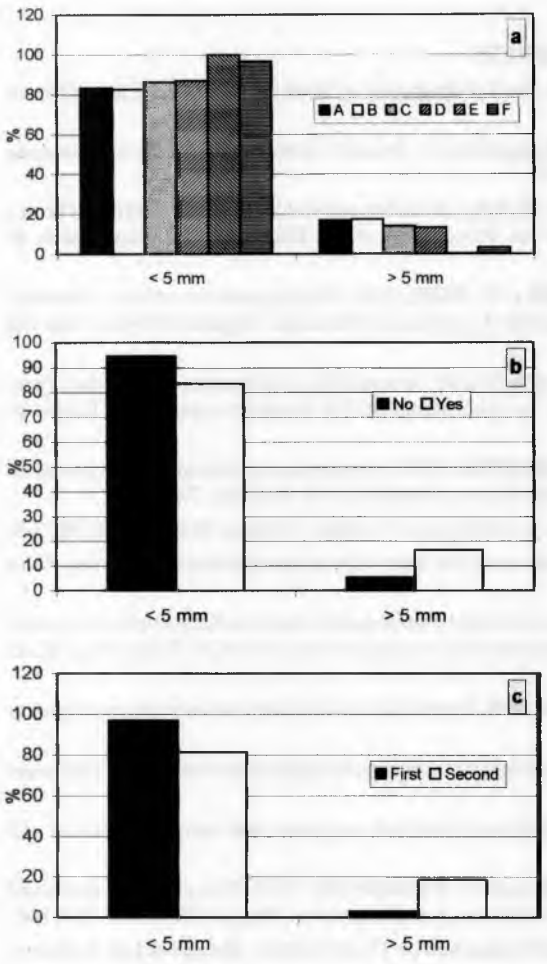

Figure 1. Effect of the concentration and continuous presence in subcultures of BA (**a**), the addition of activated charcoal (**b**) and the culture period (**c**) on percentage of shoots shorter o equal than 5 mm (<5mm) and longer than 5 mm (>5mm).

For Figure 1**a**: A= 0.55 μM BA in the first subculture; B= 0.55 μM BA in the three subcultures; C= 1.11 μM BA in the first subculture; D= 1.11 μM BA in the three subcultures; E= 2.22 μM BA in the first subculture; F= 2.22 μM BA in the three subcultures.

Significant values by Fisher test: **BA:** <5= 0.0000; >5= 0.0000. **AC:** <5= 0.0000; >5=0.0000. **PC:** <5= 0.0000; >5= 0.0000. **Interactions (BAxACxCP):** <5= 0.0002; >5= 0.0002.

the multiplication rate and multiplication index were higher than in the second culture period (Table 1 and 2). However, the mortality and the shoots with expanded leaves were maximum in the second culture period. The percentage of shoots longer than 5 mm was significantly higher in the second culture period than in the first culture period (Figure 1c). The carry-over effect of hormones in shoot multiplication medium, on rooting responses has been reported in several species (Bennett*et al.*, 1994). The high endogenous *BA* concentration in the shoots at the beginning of the first culture period due to the culture of the shoots for several months on a medium with 2.22 μM *BA*, could explain the differences observed between the two culture periods. The results obtained in this work show that the morphological processes observed during *in vitro* shoot multiplication of *B. buxifolia* can be controlled by the concentration and presence of *BA* and the addition of *AC*. Shoots showed marked differences in their growth responses according to these two factors and associated to the culture period. *MS* medium added with 0.55 μM BA in the three subcultures allowed to obtain a multiplication index higher than those obtained with *AC* and all *BA* concentrations tested. These results could be applied to other species that produce alkaloids like berberines.

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