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In vitro propagation of *Rubus geoides*

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Abstract *Rubus geoides*, a native herb of Patagonia, has fruit with a bitter-sweet taste that can be eaten fresh or in marmalades and jams. The aim of this work was to develop a protocol for the *in vitro* propagation of *R. geoides*. The proposed protocol allowed a multiplication rate of 1:3.5 to be obtained at day 63 with Murashige & Skoog (MS) medium with 1.10 μ*M* 6-benzylaminopurine added. Most (80%) of the shoots rooted on MS with half-strength macronutrient salts and 2.46 μ*M* indol-3-butyric acid added and an initial period of darkness of 7 days. The root systems were of suitable quality to begin the acclimatisation stage. These results are the first for *R. geoides* from adult plants obtained in the field.

Keywords *Rubus geoides*; Patagonia; micropropagation; multiplication; rooting

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INTRODUCTION

In recent years, increasing interest has been shown in intensive agriculture in Argentinean Patagonia (Riádigos & Martínez 1994; Arena & Martínez Pastur 1995; Martínez et al. 1995; INTA EEA Santa Cruz 1996) because of its diversity in soils and climates and, mainly, to the possibility of producing crops out of season with the Northern Hemisphere. The industrial demand for berries worldwide is well known. Likewise, in Argentina, berries are currently of increasing importance for the local food industry as well as for the fresh market. Andean Patagonia is the most important zone for producing *Rubus* and *Ribes* berries in Argentina, particularly the Andean region at 42°S. The culture of red raspberries (*Rubus idaeus*) is the oldest among the small fruit species in Argentinean Patagonia (Martínez & Riádigos 1993), and counts for the largest culture area (100 ha in the Andean region at 42°S) (Riádigos pers. comm.). However, the search for native *Rubus* species for introduction into commercial culture is of no less importance, with the plant resources of Tierra del Fuego having the potential for diversified development of the current Patagonian agricultural production.

Rubus geoides Sm. (frutilla silvestre) is the only native *Rubus* species found in Argentinean Patagonia, with a large distribution from Neuquen to Tierra del Fuego (Grondona 1984). In Tierra del Fuego, *R. geoides* often grows in moist open to partly shaded areas among rocks, grassland, shrub communities, forest clearings and margins, between 0 and 550 m a.s.l. (Moore 1983). *R. geoides* is a perennial herb, with slender, creeping, and somewhat woody stems, branched and rooted at several nodes. Its purple to red fruits are in groups, have a bittersweet taste (Grondona 1984), and can be eaten fresh or in marmalades and jams.

There are several reports on the *in vitro* propagation of *Rubus* species (Welander 1987; McPheeters et al. 1988; Snir 1988; Hoepfner 1989; Hoepfner & Nestby 1991; Jin et al. 1992; Bobrowski et al. 1996; Erig et al. 2002; Martinussen et al. 2004). *R. geoides*

can also be propagated by stolons (Vater & Arena 2003). The aim of this work was to develop a protocol for the *in vitro* propagation of *R. geoides*.

MATERIALS AND METHODS

Plant material

The plant material was obtained from plants growing in clearings near forest margins at Ushuaia (54°48′S, 68°15′W) Tierra del Fuego, Argentina. At the end of the winter, 10 mature plants were chosen, and were placed in an incubation chamber (24 ± 2°C and 80% relative humidity) for sprouting. When the shoots grown from stolons were 5 mm long, the apices were obtained and used as plant material.

Culture initiation and growing conditions

Plant material was surface sterilised with NaOCl (1.0% w/v active Cl_2) for 5 min and then rinsed three times with sterile, distilled water. Explants 2–3 mm long were cultivated on Murashige & Skoog (MS) medium (1962) with 1.10 µ*M* 6-benzylaminopurine (BA), and transferred to fresh medium every 21 days for 6 months until the cultures became stabilised. For all experiments, media were supplemented with sucrose (3.0% w/v), regional agar (0.7% w/v), and dispensed into 350-ml glass flasks containing 50 ml medium. The pH was adjusted to 5.7 ± 0.05 with KOH 0.1*N* before autoclaving, which was performed for 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} s^{-1} photosynthetically active radiation).

Shoot multiplication

In the first experiment (experiment A), with MS as the basal medium, three concentrations of BA were tested (0.55, 1.10, 2.22 µ*M*). The explants (shoots of 2–3 mm long with 3–4 leaves) were transferred to fresh medium every 21 days without subdividing. The experiment was finalised at day 63 when data were collected. The multiplication was evaluated by total shoot number, multiplication rate (number of total new shoots per explant), total number of expanded leaves per explant, total number of expanded leaves per new shoot, explants with vitrification, explants with callus, shoot length, and leaf length.

Rooting of microcuttings

After 8 months in the multiplication medium, shoots of 8–10 mm long with 4–6 expanded leaves were used for rooting experiments. MS medium with half-strength macronutrient salts was used as the basal rooting medium.

In experiment B, the effect of indol-3-butyric acid (IBA) concentration (0.00, 0.61, 1.23, and 2.46 µ*M*) on rooting was analysed until day 28 when the trial finished. In experiment C, the effect of different periods of darkness (0, 7, 14, and 21 days) was analysed during the rooting induction period, using a medium with 2.46 µ*M* IBA. After the induction period, cultures were grown on normal photoperiod (16:8 light:darkness) until day 28 when the trial finished.

Rooting was evaluated every 2 days until day 28 when the trial finished. Other variables for explant were evaluated: shoot length, presence or absence of callus, primary root number, primary root length, secondary root number, total root number (primary root number + secondary root number), total primary root length (primary root number × primary root length), and rooting index. The rooting index was used to characterise the rooting quality, given by the following equation:

$$RI\ (mm) = R \times R_{17} \times PRN \times PRL$$

where R and R_{17} were expressed as values between

Table 1 Effect of 6-benzylaminopurine (BA) concentration on total shoot number (SN), multiplication rate (MR), total number of expanded leaves per explant (TNLE), total number of expanded leaves per new shoot (TNLS), explants with vitrification (V), explants with callus (C), shoot length (SL), and leaf length (LL) for *in vitro* shoot multiplication of *Rubus geoides* at day 63. Significant values by Fisher test: SN = 0.0000; MR = 0.0002; TLNE = 0.1085; TLNS = 0.2847; V = 0.0101; C = 0.1942; SL = 0.0000; LL = 0.1050. In each column, mean values followed by the same letter are not significantly different at $P < 0.05$ level by Tukey test.

BA (µ*M*)	SN (*n*)	MR (*n*)	TNLE (*n*)	TNLS (*n*)	V (%)	C (%)	SL (mm)	LL (mm)
0.55	2.43b	2.50b	24.23	5.42	0.00b	0.00	5.62	11.49a
1.10	7.64a	3.53ab	26.76	4.84	0.00b	0.00	6.61	8.23b
2.22	7.50a	4.60a	32.33	4.80	23.81a	9.52	5.67	10.96a

0 and 1. R is the percentage of rooting at day 28, whereas R_{17} is the percentage of rooting at day 17, and accounts for the appreciation of the magnitude and earliness of the overall rooting response to treatments. Day 17 was chosen because by this time most of the treatments attained the maximum rooting celerity. PRN is the primary root number, whereas PRL is the primary root length.

Acclimatisation stage

The rooted shoots were placed in plastic pots containing sterile soil covered with glass jars for maintenance of high environmental moisture condition. The plastic pots were placed into the greenhouse and the plants were irrigated with a salt solution of MS reduced to a quarter.

Statistical analysis

For detecting faulty assumptions, the normality of the data was tested by the standard Skewness test whereas the variance homogeneity was checked by the Bartlett test. Then, the statistical validity of the results was obtained through the analysis of variance by Fisher and Tukey multiple range tests, using a completely random design. Thirty explants were tested in each treatment for all experiments (six explants per flask and five flasks or experimental units). Significance level was $P < 0.05$.

RESULTS AND DISCUSSION

Shoot multiplication

When analysing the effect of BA concentration (experiment A), significant differences were found for total shoot number, multiplication rate, explants with vitrification, and explant leaf length (Table 1). Increasing BA concentration showed higher total shoot number and multiplication rate, but diminished the quality of the formed shoots, with higher percentage of explants with vitrification and callus. For this reason, a concentration of 1.10 µ*M* BA was selected as the best treatment (Fig. 1). A multiplication rate of 1:3.5 with five leaves per new shoot was taken, without vitrified and callus explants. These results are not in agreement with those found for other *Rubus* species (Hoepfner 1989; Erig et al. 2002; Martinussen et al. 2004), where higher BA concentrations (2–9 µ*M*) were used.

Rooting of the microcuttings

When analysing the effect of IBA concentration (experiment B), significant differences were found

Fig. 1 Shoot multiplication of *Rubus geoides* on Murashige & Skoog medium with 1.10 µ*M* 6-benzylaminopurine at day 63.

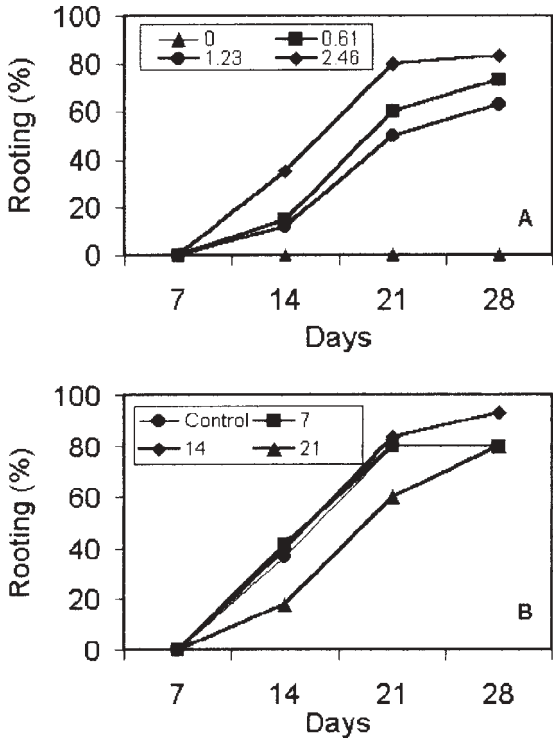

Fig. 2 Analysis of the rooting celerity with **A**, different concentrations of indol-3-butyric acid in the culture medium; and **B**, different periods of darkness.

on rooting, shoot length, presence or absence of callus, primary root number, total root number, total primary root length, and rooting index (Table 2). Without IBA, no rooting was observed. Although there were no significant differences between treatments with IBA for rooting, 2.46 µ*M* IBA gave the highest number of roots per shoot, the longest roots and a root index of 24.2.

The dark period (experiment C) significantly affected shoot length, primary root length, total primary root length, and rooting index (Table 3). Although there were no significant differences between treatments for rooting, an initial 7-day period of darkness gave the longest roots and a root index of 24.1. The beneficial effect of reduction of salts in the culture medium as well as an initial period of darkness, was also found for several cultivars of *Rubus idaeus* (Welander 1987). The percentages of rooting obtained for *R. geoides* were comparables to those cited for several clones of *R. idaeus* (Hoepfner 1989; Hoepfner & Nestby 1991; Jin et al. 1992; Bobrowski et al. 1996).

Rooting began at days 11 and 13 when different IBA concentrations (Fig. 2A) and a different period of initial darkness (Fig. 2B) were tested, respectively. The rooting followed an ever smoother sigmoid curve, with a growth expansion component (until day 15) and a growth decline component (from day 15) (2.46 µ*M* IBA in experiment B and 7 days of initial darkness in experiment C) (Zeide 1993).

Acclimatisation stage

After 3 months from the start of the acclimatisation stage, 94% of the plants were still alive; 50% of them showed a vigorous aspect with big leaves and good development.

Final considerations

These results are the first antecedents for the *in vitro* culture of *R. geoides* from adult plants obtained in the field. The proposed protocol obtained a multiplication rate of 1:3.5 at day 63 with MS medium added with 1.10 µ*M* BA. Most (80%) of the microshoots rooted on MS medium with half

Table 2 Effect of indol-3-butyric acid (IBA) concentration on rooting (R), shoot length (SL), presence or absence of callus, primary root number (PRN), primary root length (PRL), secondary root number (SRN), total root number (TRN), total primary root length (TPRL), and rooting index (RI) for *in vitro* shoot rooting of *Rubus geoides* at day 28. Significant values by Fisher test: R = 0.0000; SL = 0.0000; C = 0.0000; PRN = 0.0001; PRL = 0.5837; SRN = 0.8060; TRN = 0.0172; TPRL = 0.0007; RI = 0.0000. In each column, mean values followed by the same letter are not significantly different at $P < 0.05$ level by Tukey test.

IBA (µ*M*)	R (%)	SL (mm)	C (%)	PRN (*n*)	PRL (mm)	SRN (*n*)	TRN (*n*)	TPRL (mm)	RI (mm)
0.00	0.00b	–	–	–	–	–	–	–	0.00c
0.61	70.00a	9.62b	4.76c	2.24b	8.86	2.47	4.71b	20.02b	5.61b
1.23	63.33a	10.73b	78.94b	2.73b	7.67	3.05	5.79ab	22.04b	3.83bc
2.46	83.33a	12.56a	100.00a	4.96a	8.42	2.68	7.64a	39.59a	24.19a

Table 3 Effect of different periods of darkness on rooting (R), shoot length (SL), presence or absence of callus (C), primary root number (PRN), primary root length (PRL), secondary root number (SRN), total root number (TRN), total primary root length (TPRL), and rooting index (RI) for *in vitro* shoot rooting of *Rubus geoides* at day 28. Significant values by Fisher test: R = 0.4186; SL = 0.0000; C = 0.2726; PRN = 0.1268; PRL = 0.0001; SRN = 0.4191; TRN = 0.1758 NS (not significant); TPRL = 0.0072; RI = 0.0001. In each column, mean values followed by the same letter are not significantly different at $P < 0.05$ level by Tukey test.

	R (%)	SL (mm)	C (%)	PRN (*n*)	PRL (mm)	SRN (*n*)	TRN (*n*)	TPRL (mm)	RI (mm)
Days									
0	80.00	8.25b	87.50	2.45	10.87ab	0.95	3.41	21.63ab	10.95bc
7	80.00	12.79b	91.66	3.58	13.79a	1.16	4.71	37.66a	24.10a
14	93.33	21.71a	100.00	3.39	8.15b	0.50	3.89	25.69ab	16.72ab
21	80.00	25.54a	95.83	2.58	7.08b	0.58	3.16	15.39b	6.15c

strength of macronutrient salts added with 2.46 µ*M* IBA and an initial 7-day period of darkness. The root systems were of suitable quality to begin the acclimatisation stage. These results allowed us to obtain nearly 500 plants from one explant in one year. The development of this *in vitro* propagation protocols allows the extensive use of selected genotypes for the creation of mother plant stocks. However, the development of the *in vitro* protocol of *R. geoides* will play a crucial role in the introduction of this species to commercial culture and satisfies the requirements for producing a great number of plants in a short time.

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