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In vitro propagation of *Rubus macrocarpus* Benth. and *Rubus bogotensis* Kunth, as an *ex situ* conservation strategy

Propagación *in vitro* de *Rubus macrocarpus* Benth. y *Rubus bogotensis* Kunth, como estrategia de conservación *ex situ*

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Abstract

This research aimed to evaluate techniques for *in vitro* multiplication and rooting and *ex vitro* adaptation of wild blackberry plants (*Rubus bogotensis* Benth. and *Rubus macrocarpus* Kunth). In *in vitro* phases, the influence of culture media was evaluated based on Murashige & Skoog (MS) composition, with or without a 50% reduction in salts, vitamins, and myo-inositol and supplemented or not with benzylaminopurine (BAP), indole acetic acid (IAA), L-cysteine, agar, peat, perlite, and vermiculite. The *ex vitro* phase was based on a soil, rice husks, peat, and perlite substrate management. After two months of evaluation of the *in vitro* phases, it was determined that the MS medium at 50% salts, vitamins, and myo-inositol and supplemented with sucrose (15000 $mg.l^{-1}$) and agar (5000 $mg.l^{-1}$) was the most suitable for obtaining the highest values of apical length (cm), shoot number, rooting percentage, and root number. In *ex vitro* adaptation, the acclimation subphase, evaluated during three months, allowed to determine survival rates of 83.33% and 75% for *R. macrocarpus* and *R. bogotensis,* respectively. The average increase in stem length (cm) was 2.35 cm and 1.30 cm, respectively. In the greenhouse subphase, evaluated for four months, 100% plant survivorship was obtained, and 2.75 cm and 5.31 cm increases in average stem length (cm) for *R. macrocarpus* and *R. bogotensis* were determined.

Keywords: Blackberry, culture medium, high Andean forest, plant growth regulator, wild species.

Resumen

La presente investigación tuvo como objetivo evaluar técnicas para la multiplicación *in vitro,* enraizamiento *in vitro* y adaptación *ex vitro* de plantas de moras silvestres (*Rubus macrocarpus* Benth y *Rubus bogotensis* Kunth). En las fases *in vitro*, se evaluó la influencia de medios de cultivo basados en la composición Murashige & Skoog (MS), reducidos o no en un 50% en sus sales, vitaminas y myo-inositol y suplementados o no con bencilaminopurina (BAP), ácido indol acético (AIA), L-cisteina, agar, turba, perlita y vermiculita. La fase *ex vitro* se fundamentó en el manejo del sustrato conformado por tierra, cascarilla de arroz, turba y perlita. Al término de los 2 meses de evaluación de las fases *in vitro*, se determinó que el medio MS al 50% en sales, vitaminas y myo-inositol y suplementado con sacarosa (15000 $mg.l^{-1}$) y agar (5000 $mg.l^{-1}$), fue el más indicado para la obtención de los mayores valores de la media en las variables longitud apical (cm), número de brotes, porcentaje de enraizamiento y número de raíces. En la adaptación *ex vitro*, la subfase aclimatación evaluada durante 3 meses, determinó porcentajes de sobrevivencia del 83.33% y 75% para *R. macrocarpus* y *R. bogotensis.* El incremento en longitud promedio del tallo (cm) fue de 2.35 cm y 1.30 cm, respectivamente. En la subfase de invernadero, evaluada durante 4 meses, se registró 100% de sobrevivencia en las plantas y un incremento en longitud promedio del tallo (cm) para *R. macrocarpus* de 2.75 cm y para *R. bogotensis* de 5.31 cm.

Palabras clave: Bosque altoandino, especie silvestre, fitorregulador, medio de cultivo, mora.

Introduction

In the generation of scientific knowledge and research, as fundamental elements to assure biodiversity conservation (Josse C. *et al.,* 2009), the José Celestino Mutis Botanical Garden of Bogotá (JBB) employs plant tissue culture as an *ex situ* conservation strategy of promising native high Andean and paramo species. Two prioritized species within this line of research are *Rubus macrocarpus* Benth. and *Rubus bogotensis* Kunth (Rosaceae). The distribution of the giant blackberry and the *granizo* blackberry, common names used for these two species, is found among 2100 and 3200 m.a.s.l. like other species of the genus *Rubus*, they have potential nutritional, medicinal, industrial, or ecological uses (Guzmán-Castañeda *et al.,* 2007) and, due to their wild condition, they could be useful for the improvement of economically important commercial crops. Andrade, Córdoba, Criollo, & Lagos (2013), mention that since wild species could be a source of genetic variability, they represent an alternative for solving problems related to plant sanitation, based on their desirable characteristics such as pest and disease resistance. Apart from being promising species, *R. macrocarpus* and *R. bogotensis* show propagation difficulties through traditional methods, mainly from seed. The germination process is very slow (beginning two or three months after planting) and occurs at very low percentages, especially for *R. macrocarpus* (1%) (Córdoba, Guzmán-Castañeda, Pérez-Martínez, Zúñiga-Upegui, & Pacheco, 2010). Owing to these limitations, the development and adaptation of *in vitro* propagation techniques is proposed as an alternative for *ex situ* blackberry conservation. This research aimed to evaluate methodologies for *in vitro* multiplication and rooting (from vitroplants available in the laboratory) and for the *ex vitro* adaptation (acclimation and greenhouse) of the plant material generated under laboratory conditions.

Material and methods

This research was carried out in the laboratory of plant tissue culture of the Scientific Subdirection at the JBB. The starting materials were *in vitro Rubus macrocarpus* and *Rubus bogotensis* seedlings obtained from *in vitro* seed planting and germination. The following describes the phases developed for this *in vitro* plant material:

Multiplication phase

Seedlings with an adequate general development, related mainly with vigor, height, and color, were selected. The leaves and necrotic tissue were eliminated; individual shoots, with an average length of 0.4 cm to 0.5 cm, and one to two axillary buds were cut and planted in the treatments described in Table 1.

Table 1. Culture media evaluated in the *in vitro* multiplication of *R. macrocarpus* and *R. bogotensis.*

Component ($mg.l^{-1}$)	TM1	TM2	TM3
MS salts	50%	100%	100%
Myo-inositol	50	100	100
Thiamine	0.005	0.1	1
Nicotinic acid	0.25	0.5	—
Pyridoxine	0.25	0.5	—
Glycine	1.0	2.0	—
L-Cysteine	—	—	100
BAP	—	—	1.5
IAA	—	—	0.75
Sucrose	15000	15000	15000
Agar	5000	5000	5000
pH	5.8	5.8	5.8

Culture media composition was based on mineral salts, vitamins, and myo-inositol in MS medium (Murashige & Skoog, 1962) at 50% (50% MS) or 100% (MS), with or without supplementation (L-Cysteine, BAP or IAA). Apical length (cm) and shoot number were evaluated every seven days, during eight weeks. Increases in these variables were determined based on the average initial and final measurements. The experimental unit was composed of one explant in a culture flask. For *R. macrocarpus*, 15 repetitions per treatment were used, for a total of 45 weekly measurements for each variable. For *R. bogotensis*, 12 repetitions per treatment were used (36 weekly measurements for each variable).

Rooting phase

The shoots or stem cuttings generated in the previous phase were transferred to four treatments (Table 2). After eight weeks, the seedlings were removed from the flasks and the number of rooted shoots and roots were counted. The experimental unit corresponded to one seedling in a culture flask. For *R. macrocarpus*, 15 repetitions per treatment were evaluated, for a total of 60 plants. For *R. bogotensis*, a total of 48 plants were managed (12 repetitions per treatment). For elaborating the multiplication and rooting phase media, the nutrients were weighed on an analytical precision balance and dissolved in micro-filtered water; glass containers with 100 ml-capacity were used to distribute the treatments; pH was adjusted to 5.8 and sterilization was carried out in an autoclave at 15 pound-force per square inch ($15\ lb.in^{-2}$) for 15 minutes, with an approximate steam temperature of 121.5°C. For preparing rooting media TE3 and TE4, 50000 mg of substrate were placed into sterilized glass containers, followed by 30 ml of

MS with 100% salts, vitamins, and myo-inositol mix (pH adjusted). The explants were kept in the incubation room, under a natural photoperiod (12.12^{-1}), with a temperature range between 19ºC and 27°C, and relative humidity of 60% to 80%. Light intensity was between 1500 lux and 5000 lux.

Table 2. Culture media evaluated for *in vitro* rooting of *R. macrocarpus* and *R. bogotensis*.

Component ($mg.l^{-1}$)	TE1	TE2	TE3	TE4
MS salts and vitamins	50%	100%	100%	100%
Myo-inositol	50	100	100	100
Peat	—	—	16600	—
Perlite	—	—	16600	—
Vermiculite	—	—	16600	50000
Sucrose	15000	15000	15000	15000
Agar	5000	5000	—	—
pH	5.8	5.8	5.8	5.8

Ex vitro adaptation phase

Two subphases related to acclimation and greenhouse growth were evaluated. The following describes the methodology applied for each one:

Acclimation. The *in vitro* plants, with caulinar, foliar, and rizogenic development, from MS medium with 50% salts, vitamins, and myo-inositol (50% MS), were transplanted to pots with a mix of soil, rice husks, peat, and perlite substrate in equal proportions. The pots were located in pans with a transparent plastic cover, with relative humidity controlled. The covers were gradually lifted since day 30 after the transplant and fully removed two months later. Irrigation, by capillarity, was managed twice a week. Twenty four seedlings from each species were randomly chosen in order to measure survivorship (%) and stem growth length (cm) every three weeks. The total evaluation time was three months, during which five measurements were taken. The corresponding increases were determined based on the average initial and final stem growth length (cm) values.

Greenhouse. The plant material was transplanted into black plastic bags containing the same acclimation substrate (soil, rice husks, peat, and perlite mix). A vertical tutor was placed for each seedling in order to provide support. The bags were transferred to the greenhouse, where daily irrigation through aspersion and foliar and edaphic fertilization was managed every five weeks. Survivorship (%) and stem growth length (cm) were evaluated during four months. The first evaluation was done two weeks after greenhouse establishment. Subsequently, measurements were made every 15 days, for a total of eight data recordings for 75 *R. macrocarpus* and 45 *R. bogotensis* seedlings. The increase in seedling height was determined based on the initial and final stem length (cm) values measured in each subphase.

Statistical analysis

A completely randomized experimental design was used for each of the phases developed. For data analysis, an analysis of variance (ANOVA) was used and a test for difference of means was calculated through Duncan's multiple range test ($p< 0.05$), using the statistical software SAS® 9.0. A descriptive analysis was carried out for apical height increase and shoot number in the multiplication and *ex vitro* adaptation phases.

Results and discussion

Multiplication phase

The results for apical length (cm) and shoot number in the last week evaluated allowed to establish that apical length (cm) for *R. macrocarpus* was the only variable which showed significantly differences (P= 0.026) among culture media treatments, where TM1 culture media (50% MS) showed the highest mean value (2.23). The effect of culture media on the studied variables for both blackberry species is shown in Table 3. Despite the ANOVA results, differences in culture media, related especially with vigor, were observed after finalizing the evaluation. Seedlings from TM3 (MS + thiamine 1 $mg.l^{-1}$ + L-cysteine 100 $mg.l^{-1}$ + myo-inositol 100 $mg.l^{-1}$ + BAP 1.5 $mg.l^{-1}$ + IAA 0.75 $mg.l^{-1}$), did not develop satisfactorily, some of them presented oxidation and withering. The opposite was observed for seedlings planted in the other treatments, mainly in TM1 (50% MS), where large and bright leaves and firm and vigorous stems were observed.

Table 3. Effect of culture media on multiplication phase of *R. macrocarpus* and *R. bogotensis* (56 days after planting).

Culture media	Apical length (cm)	
	R. macrocarpus	***R. bogotensis***
TM1	2.2333 [a]	1.8250 [a]
TM2	1.8000 [ab]	1.6500 [a]
TM3	1.5600 [b]	1.1917 [a]
Culture media	**Shoot number**	
	R. macrocarpus	***R. bogotensis***
TM1	5.0000 [a]	6.8000 [a]
TM2	4.0000 [a]	6.0833 [a]
TM3	3.4667 [a]	5.0833 [a]

* Means with different letters in the same column differ at p<0.05 (Duncan notation).

These observations relate to the descriptive analysis regarding increase in the studied

variables (comparison between average initial and final values). As can be seen from figures 1 and 2, *R. macrocarpus* and *R. bogotensis* explants in medium TM1 reached the highest increase values for apical length (cm) and shoot number.

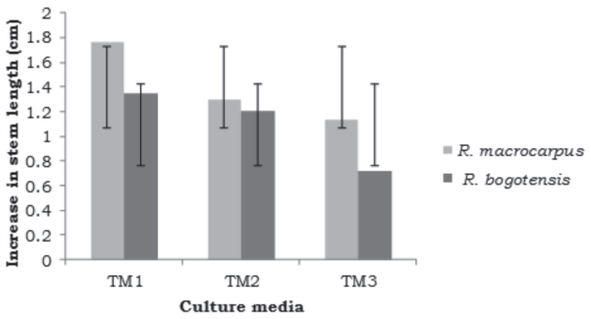

Figure 1. Increase in stem length (cm) for R. macrocarpus and R. bogotensis. Bar= standard error.

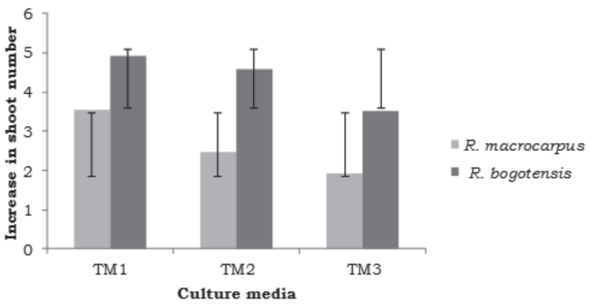

Figure 2. Increase in shoot number for R. macrocarpus and R. bogotensis. Bar= standard error.

Considering the combined use of phytohormones (6 BAP + GA_3; 6 BAP + IAA or BAP + GA_3) for species of the genus *Rubus*, such as *Rubus glaucus* Benth., is appropriate for achieving the greatest length and shoot number in explants (Sigarroa-Rieche & García-Delgado, 2011; Marulanda, Carvajalino, & Vento, 2000), it can be noted that for the wild species evaluated here, growth regulators are not required to produce complete plants from microcuttings. The latter is associated with a 50% reduced concentration in salts, vitamins and myo-inositol in the MS medium, which was determined to be valid for this phase. The advantage in explant growth and development, which is seen with the use of TM1, is accompanied by a cost reduction in the *in vitro* production of plant material.

Stem elongation is a key parameter which is strongly affected by culture exposition time (Uribe-Moraga & Cifuentes, 2004). Sigarroa-Rieche & García-Delgado (2011), mention that hormonal combinations of BAP (1.5 mg/l) + IAA (0.75 $mg.l^{-1}$) and BAP (1.5 $mg.l^{-1}$) + IBA (0.75 $mg.l^{-1}$) did not provide satisfactory results in *Rubus glaucus* Benth., plant length and its multiplication coefficients, in addition to 81% to 84% callus formation. In this research, callogenesis was not observed for *R. macrocarpus* and *R. bogotensis* under any of the treatment influence, which is considered to be an advantage, since callus appearance tends to weaken the existing shoots (Marulanda *et al.,* 2000).

Shoot number is an equally important variable in the multiplication phase, since with greater shoot numbers, a greater multiplication phase is obtained (Solis, Olivera, & La Rosa, 2011). Villa et al., 2005 (cited in Sigarroa-Rieche & García-Delgado, 2011), evaluated the *in vitro* multiplication of blackberry (*Rubus* sp.) and determined that a greater shoot number (3.99) is obtained in a culture medium with 1 $mg.l^{-1}$ BAP. This value was greater for *R. macrocarpus* and *R. bogotensis* seedlings evaluated in this study, with an average of 5.0 and 6.8 shoots per explant in a salts and vitamins-reduced medium without phytoregulators (TM1). López, (1998), cited in Uribe-Moraga & Cifuentes, (2004), mentions that, in many species, bud development is favored by the use of salt-reduced culture media.

Rooting phase

Rooting percentage per treatment was determined 56 days after planting, and prior to the statistical analysis of root number. For *R. macrocarpus* and *R. bogotensis*, TE3 provided the best root number percentages (100%). For *R. bogotensis*, TE1 also provided 100% rooting. Significant differences were seen for root number between treatments. In *R. macrocarpus* (P= 0.0062), TE1, TE3, and TE4 media formed a homogenous group, where TE3 showed the highest mean value. For *R. bogotensis* (P= 0.0271), TE1 and TE3 displayed the highest mean root number values (Table 4). Accordingly, TE1 (MS al 50%) and TE3 (agar was replaced by inert substrate composed of peat, perlite, and vermiculite) are the most adequate media for obtaining the highest rooting percentages and root number. However, under TE1, plants showed greater vigor, color, and foliar development; thus, it is considered to be the most indicated medium for the *in vitro* rooting phase.

Salt reduction has been shown to affect root induction (Flores, Chacón, Jiménez, & Ortiz,2012), since it generates physiological stress in plants, which leads to root production in search for potential far away nutrient sources, accelerating radicular development (Salisbury & Ross, 1994). Auxins (NAA, IBA, IAA, and 2-4 D) induce *in vitro* root formation (Pedroza-Manrique & Bejarano-Tibocha, 2008). In Flores *et al.,* (2012), 50% MS medium, supplemented with 0.5 $mg.l^{-1}$ IBA, achieved the greatest root length averages in *Rubus adenotrichus* Schltdl. shoots. However, Vaca & Landázuri (2013), and

Alev & Arslan (2006), throughout their studies in roses, determined that MS medium reduced 50% in salts and vitamins or nitrates, without hormonal supplements, favored root number. These results, in which no phytoregulators were used for *in vitro* rooting, agree with those obtained in this study for *R. macrocarpus* and *R. bogotensis*.

Table 4. Percentage and root number in *R. macrocarpus* and *R. bogotensis* (56 days after planting).

Culture media	Rooting percentage	
	R. macrocarpus	R. bogotensis
TE1	86.67 %	100 %
TE2	46.67 %	83.00 %
TE3	100 %	100 %
TE4	66.67 %	83.00 %
Culture media	**Root number**	
	R. macrocarpus	***R. bogotensis***
TE1	2.1333 [a]	2.2500 [a]
TE2	0.8000 [b]	1.2500 [b]
TE3	3.0667 [a]	2.2500 [a]
TE4	2.2667 [a]	1.5000 [ab]

Although, with the use of inert substrate instead of agar (TE3) similar effects were obtained in rooting percentage and root formation, it was unfavorable for plant vigor, probably because this medium contained 100% salts, vitamins, and myo-inositol from the MS medium. Nevertheless, an inert substrate, together with 50% salts, vitamins, and myo-inositol from MS, could be a cost reducing alternative that can also be used in the shoot multiplication phase, according to Martínez-Hernández, Alonso, Osorio, Gallardo, López, & Mata, M. (2009). The use of simple culture media is related with greater current knowledge regarding the other factors that affect *in vitro* culture (Pedroza-Manrique & Bejarano-Tibocha, 2008), and is determined by endogenous auxin and cytokine concentrations present in the explants, which depend on the species and the explant type (Pedroza & Micán, 2006). In this manner, *in vitro* cellular processes are favored by synergic concentrations between endogenous and exogenous phytohormones (Salisbury & Ross, 1994).

Ex vitro adaptation phase

Acclimation. Acclimation is one of the most care-requiring phases in *in vitro* propagation, therefore, environmental factors, such as relative humidity, light intensity (Martínez-Montiel *et al.,* 2011), irrigation frequency, and substrate quality (Marulanda *et al.,* 2000) must be controlled. In this study, relative humidity in the transparent-covered pans was maintained around 90% and the average daytime temperature for acclimation was 24°C. Under these conditions and after three months of acclimation, survival percentages for *R. macrocarpus* and *R. bogotensis* were 83.33% and 75%, respectively. With the substrate and environmental conditions described, increases of 2.35 cm and 1.30 cm in stem length were obtained in 12 weeks of evaluation (Figure 3). The survivorship results found in this study support those reported by Flores *et al.,* (2011), for *R. adenotrichus*, where the maximum survival achieved in four weeks of acclimation was 78.6% for plants grown in MS medium with 50% salts and supplemented with 0.250 mg.l^{-1} IAA.

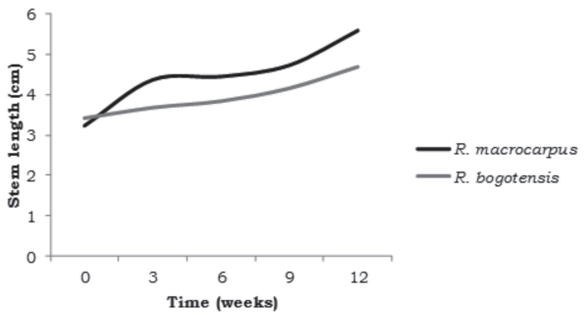

Figure 3. Height growth (cm) of R. macrocarpus and R. bogotensis during three months of acclimation.

Seedling death evaluated in this study was mainly due to dehydration by water stress and, in lesser proportion, to the presence of *Penicillium nalgiovense* (non-pathogen, environmental fungus) in the leaves and stems of the evaluated plant material, which appeared between the third and sixth weeks after transplant. Vitroplants, compared to plants produced in growth chambers or greenhouses, are fragile and vulnerable to environmental conditions, since they are morphologically, anatomically, and physiologically abnormal (Pospíšilová, Tichá, Kadleček, Haisel, & Plzáková, 1999). They have a low epicuticular wax content (Martínez-Montiel *et al.,* 2011), and low stomatal function and photosynthetic activity, due to which the acclimation period is necessary for correcting these abnormalities (Nava et al., 2011).

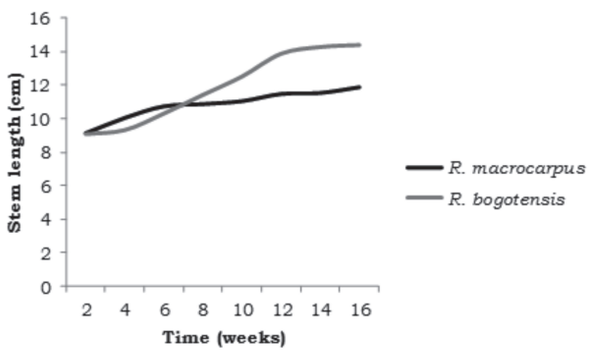

Figure 4. Height growth (cm) of R. macrocarpus and R. bogotensis during four months under greenhouse conditions.

Greenhouse. The environmental conditions under which this subphase was carried out into an average daytime temperature of 20°C and nocturnal temperature of 12°C. Average environmental humidity was 65% (diurnal) and 85% (nocturnal). For the 75 *R. macrocarpus* and 45 R. *bogotensis* seedlings, survivorship was 100% after four months of greenhouse follow-up. The latter indicates that the acclimation phase was adequately managed, since it allowed the formation of vigorous plants that displayed high survival levels when transplanted and transferred to the covered system. Based on the average initial and final stem lengths (cm), there was an increase of 2.75 cm for *R.macrocarpus* and 5.31 cm for *R. bogotensis* (Figure 4).

Conclusions

For *in vitro* multiplication and rooting phases, the best culture medium was MS at 50% salts, vitamins, and myo-inositol, supplemented with 15000 $mg.l^{-1}$ sucrose and 5000 $mg.l^{-1}$ agar. Under this culture medium, endogenous seedling concentrations could autoregulate, favoring caulinar, foliar, and rizogenic formation. In e*x vitro* adaptation, the acclimation and greenhouse subphases were overcome by using plants from 50% MS medium, transplanted in a soil, rice husks, peat, and perlite (1:1:1:1) substrate. The substrate employed and the environmental conditions managed allowed for acceptable survival levels and seedling height.

Considering that there are few reported studies on wild *Rubus* species, the methodology presented here constitutes a basis for developing micropropagation processes of species selected under criteria related mainly with degree of threat, difficulty of propagation under traditional methods, population accessibility, plant material availability, and potential for sustainable use.

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