Notas Científicas

In vitro conservation of blackberry genotypes under minimal growth conditions and subsequent large-scale micropropagation

Hugo Teixeira Gomes[1], Patrícia Monah Cunha Bartos[1], Maíra Teixeira de Andrade[1], Raphael Ferreira Almeida[1], Luciana Florencio de Lacerda[1] and Jonny Everson Scherwinski-Pereira[2]

[1]Universidade de Brasília, Departamento de Botânica, Avenida L3 Norte s/nº, CEP 70910-900 Brasília, DF, Brazil. E-mails: hugotgomes@hotmail.com, patricia.monah@gmail.com, mairatandrade@gmail.com, raphael.botanicaunb@gmail.com, luciana_lacerdaa@yahoo.com.br [2]Embrapa Recursos Genéticos e Biotecnologia, Avenida W5 Norte (Final), CEP 70770-917 Brasília, DF, Brazil. E-mail: jonny.pereira@embrapa.br

Abstract – The objective of this work was to evaluate the micropropagation of blackberry (*Rubus* spp.) cultivars, after in vitro conservation under minimal growth conditions. Nodal segments of the 'Guarani', 'Caingangue', 'Ébano', and 'Xavante' genotypes were conserved under minimal growth conditions at 20ºC, for 15 months. Microshoots were regenerated and multiplied by up to five successive subcultures, when they were rooted and acclimatized. After 30 days of acclimatization in a greenhouse, rooted plantlets showed no significant losses. Blackberry cultivars can be conserved in vitro for 15 months, without subcultures and, after this time, they can be micropropagated on a large-scale, maintaining the regenerative potential and multiplication.

Index terms: *Rubus*, ex situ conservation, plant genetic resources, plant propagation, slow-growth.

Conservação in vitro de cultivares de amoreira-preta em condições de crescimento mínimo e subsequente micropropagação em larga escala

Resumo – O objetivo deste trabalho foi avaliar a micropropagação de cultivares de amoreira-preta (*Rubus* spp.), após a conservação in vitro em condições de crescimento mínimo. Segmentos nodais dos genótipos 'Guarani', 'Caingangue', 'Ébano' and 'Xavante' foram conservados sob crescimento mínimo à temperatura de 20ºC, por 15 meses. Os microbrotos foram regenerados e multiplicados por até cinco subcultivos sucessivos, quando então foram enraizados e aclimatizados. Após 30 dias de aclimatização em casa de vegetação, as plantas enraizadas não apresentaram perdas significativas. As cultivares de amoreira-preta podem ser conservadas in vitro por 15 meses, sem subcultivos e, após esse período, podem ser micropropagadas em larga escala, mantendo-se o potencial regenerativo e a multiplicação.

Termos para indexação: *Rubus*, conservação ex situ, recursos genéticos de plantas, propagação de plantas, crescimento lento.

Blackberry plants are traditionally multiplied by classical vegetative propagation methods, such as layering and cuttings. However, the successful application of these methods is limited because they require a large area for planting, a great deal of manpower, and intensive weed control, aside from the fact that these species are highly susceptible to diseases and pests (Vujović et al., 2012). Thus, the propagation of blackberry through in vitro cultivation techniques, such as micropropagation from nodal segments, has been increasingly studied. The use of this technique eliminates seasonal limitations found in conventional methods and the contamination by viruses and pathogens, and it requires only a small amount of starting material, physical space, and skilled labor to be performed (Fira et al., 2014).

Conservation under minimal growth conditions is a technique for in vitro cultivation aimed at reducing the cellular metabolism, in order to increase the time between subcultures. This method is achieved mainly by reducing the temperature of the cultivation, supplementing the culture medium with osmotic agents, and reducing the concentration of the compounds of the nutrient medium (Engelmann, 2011). Several authors have shown the efficiency of in vitro multiplication in the production of blackberry plantlets, such as Wu et

al. (2009) and Fira et al. (2014). However, the literature still lacks studies that present strategies for in vitro conservation of germplasm of this species, or for the analysis of regeneration and multiplication rates of cultivars after a significant period during which they were kept under minimal growth conditions.

The objective of this study was to evaluate the large-scale micropropagation of blackberry genotypes, after their in vitro conservation under minimal growth conditions.

The source of the explants was obtained from the in vitro Germplasm Collection of Embrapa Recursos Genéticos e Biotecnologia, Brasília, DF, Brazil. The starting explants used were nodal segments of approximately 1.0 cm length, containing two axillary buds, of the following blackberry genotypes already established in vitro: – 'Guarani', 'Caingangue', 'Ébano', and 'Xavante'. The explants for the start of the experiments were placed in test tubes (25x150 mm) containing MS culture medium (Murashige & Skoog, 1962) supplemented with 20 g L^{-1} sucrose and solidified with 6.0 g L^{-1} agar. In vitro conservation was carried out starting from the inoculation of these explants in test tubes (25x150 mm) containing 10 mL MS culture medium, with salt concentrations reduced by half and supplemented with 30 g L^{-1} sucrose; the test tubes were then closed with plastic stoppers, and sealed with transparent plastic film. During this stage, the explants were cultivated up to 15 months in a growth chamber at 20±2ºC, in light-dark periods of 12/12 hours, and luminous radiation of 30 mmol m^{-2} s^{-1} supplied by cold-white fluorescent lamps. After this period, the following variables were evaluated: shoot height (cm), multiplication rate (number of internodes plus apex), number of dead leaves, and number of roots. The experimental layout was completely randomized, composed of 10 replicates per treatment, with each parcel consisting of an explant.

Regeneration and propagation were carried out from the cultivation of nodal segments of approximately 1.0 cm length with two axillary buds, which were randomly obtained from shoots at the end of the conservation phase. Nodal segments were transferred to glass flasks of 250 mL capacity containing 30 mL of MS medium, supplemented with 30 g L^{-1} sucrose, and 1.0 mg L^{-1} 6-benzylaminopurine (BAP; Sigma-Aldrich Co., St. Louis, MO, USA); the flasks were then closed with plastic stoppers, and sealed with transparent plastic film. In a growth chamber at 25±2ºC, in light-dark period of 16/8 hours, and luminous radiation of 30 mmol m^{-2} s^{-1}, the explants were cultivated using five successive 30-day subcultures each. After each subculture, the average height (cm) and number of new shoots were evaluated, in addition to the multiplication rate of the explants (number of internodes plus apex). The experimental design was completely randomized, consisting of five replicates per treatment, with parcels formed by five explants. The treatments were arranged in a 4x5 factorial arrangement, with the four genotypes ('Guarani', 'Caingangue', 'Ébano' and 'Xavante') and five subcultures.

After the multiplication stage, shoots of the 'Ébano' genotype coming from the end of the fifth multiplication subcultivation, randomly chosen due to the similar behavior for in vitro rooting of the genotypes, were selected for the in vitro rooting experiment. For the in vitro rooting evaluation, nodal segments of approximately 1.0 cm length and two axillary buds, obtained from these shoots, were vertically inoculated in 250 mL capacity glass flasks containing 30 mL of nourishing medium (½MS) with 30 g L^{-1} sucrose; the flasks were sealed with plastic lids and transparent film. Indole-3-butyric acid (IBA; Sigma-Aldrich Co.), or the same concentrations of α-napthaleneacetic acid (NAA; Sigma Aldrich Co.) were added to this basic culture medium at 0.0, 0.55, and 1.1 mg L^{-1}. The propagules were cultivated for 30 days in a growth chamber at 25°C±2°C, with a 16-hour photoperiod and 30 mmol m^{-2} s^{-1} light radiation provided by cool white fluorescent lamps. The following variables were evaluated: percentage of rooting and number of roots. The experimental design was completely randomized, consisting of five replicates per treatment, and each parcel was composed of four explants. Before autoclaving at 121°C for 20 min at 100 kPa, all media were solidified using 7 g L^{-1} agar (Sigma-Aldrich Co., St. Louis, MO, USA), and pH was adjusted to 5.7±0.1.

Acclimatization was accomplished by removing the explant shoots from their culture media, and planting them in 200 mL plastic cups containing Bioplant commercial substrate, after washing the roots with running water to remove culture medium residues (Gomes et al., 2015). The explants were cultivated for 30 days in a greenhouse covered with transparent polyethylene film (150 µ), with 75±5%, relative humidity, at 27±4°C, and 450-500 µmol m^{-2} s^{-1}

luminosity. The plants were watered every 6 hours at 6 L m^{-2} h^{-1}, with the nozzles at a distance of about 1.5 m. After cultivation, the survival rate of plantlets was evaluated.

For statistical analysis, we used the variance analysis, and the averages were compared by the Tukey´s test, at a 5% probability, calculated by the Sisvar 4.4 program (Ferreira, 2011). Data obtained by count were processed to $(x + 1)^{0.5}$.

The blackberry genotypes showed different responses to the in vitro conservation process, both for multiplication rate and number of dead leaves (Table 1). For shoot height, no differences were observed among the genotypes. According to Silva & Scherwinski-Pereira (2011), in the in vitro conservation under minimal growth conditions, an excessive elongation of the propagules is unwanted because besides promoting the filling of the cultivation test tubes, it also causes the depletion of the nutrient media, and may lead plant material to death. The maintenance of the shoots in culture medium with the salts reduced by half, associated with the decreasing of the temperature to 20°C, was sufficient to lower the growth rates and to achieve survival rates close to 100%, after 15 months in minimal growth conditions. As to the multiplication rate, 'Xavante' developed a number of buds per explant lower than the results found for 'Guarani', 'Caingangue' and 'Ébano' (Table 1). As to the number of dead leaves, 'Caingangue' and 'Ébano' showed lower senescent leaves per shoot than 'Xavante' and 'Guarani'. In contrast, for the number of roots, no significant differences were found among the studied genotypes. Reed (1993) also observed that in in vitro germplasm conservation of *Rubus* under minimal growth induction, the morphogenesis and development of the cultivations significantly varied among the different genotypes analyzed. These results agree with those by Engelmann (2011), who states that different genotypes have different potentials for the absorption and metabolization of the compounds of the nutrient medium, thereby presenting different responses to the in vitro cultivation. Therefore, for gene bank purposes, it is essential to standardize and simplify the culture conditions for plant conservation, not only for the involved high costs, but also for the in vitro collections that often maintain several accessions of the same species (Reed, 2003; Engelmann, 2011).

Table 1. Shoot height, multiplication rate, number of dead leaves and of roots in blackberry (*Rubus* spp.) cultivars, after 15 months under in vitro conservation at 20°C[(1)].

Cultivar	Shoot height (cm)	Multiplication rate	Number of dead leaves	Number of roots
Guarani	3.5ab	17.1a	4.8a	3.1a
Caingangue	3.3ab	14.0a	1.2b	2.4a
Ébano	4.2ab	17.3a	1.6b	2.8a
Xavante	2.6b	10.2b	4.7a	3.4a

[(1)]Means (n=10) followed by equal letters do not differ by Tukey's test, at 5% probability.

Regarding micropropagation through five subcultures of shoots conserved in vitro, there was no significant interactions among the genotypes and different subcultures analyzed for shoot height, number of shoots per explant, and multiplication rate (Table 2). Among the studied genotypes, 'Ébano' showed the highest results for shoot height. For this variable, the lowest results were observed for 'Guarani'. As to the number of shoots, 'Ébano' and 'Xavante' showed the best results. For multiplication rate, 'Ébano' showed the highest results (Table 3). Additionally, shoot formation and multiplication rate generally had significantly higher averages in the last subcultivation, thus showing that carrying out five subcultures in the micropropagation of blackberry is possible, without affecting the multiplication rates thereof. This fact contradict Vujović et al. (2012), who claim that during in vitro propagation of nodal segments, a decrease of potential shoot growth can be seen in the last subcultures due to successive cuttings, and to the cumulative effect of cytokinins, which, over time, can cause damage to the tissues in cultivation. Thus, the high-multiplication rates obtained in our study show that large-scale propagation of blackberry can be successfully performed, after short and medium-term storage under minimal growth conditions, which is a rare, or nonexistent result in the literature, especially regarding the time of in vitro conservation evaluated without subcultures.

In the rooting of in vitro cultivations originating from the multiplication stage, we found that there was a significant interaction between the various types and concentrations of auxin, in relation to the percentage of rooting and the number of roots per explant (Table 2). For the rooting percentage, the best results were obtained when the cultures were maintained in media without auxins (control), or with the IBA auxin,

Table 2. Shoot height, number of shoots per explant, and multiplication rate, per subcultivation of blackberry (*Rubus* spp.) cultivars, during large-scale micropropagation[1].

Cultivar	Subcultures					Mean
	1	2	3	4	5	
			Shoot height (cm)			
Guarani	1.3	1.2	1.2	1.2	1.1	1.2c
Caingangue	1.3	1.8	1.6	1.3	1.3	1.5b
Ébano	1.0	1.8	2.3	1.6	1.6	1.7a
Xavante	1.8	2.0	1.2	1.4	1.5	1.6ab
Mean	1.3C	1.7A	1.6AB	1.3C	1.4BC	-
			Number of shoots per explant			
Guarani	2.9	3.4	2.0	2.2	4.0	2.9cb
Caingangue	2.0	3.0	2.4	1.8	2.8	2.4c
Ébano	4.5	4.5	3.0	3.6	5.9	4.3b
Xavante	3.8	4.2	2.1	2.4	5.2	5.2a
Mean	3.1B	4.0AB	2.5C	2.5C	4.3A	-
			Multiplication rate			
Guarani	18.4	16.6	11.2	9.4	21.9	15.5c
Caingangue	10.3	15.6	10.9	9.8	13.4	12.0d
Ébano	22.4	27.8	15.1	23.3	28.8	23.5a
Xavante	19.2	25.5	8.2	14.8	29.1	19.4b
Mean	16.5B	21.9A	12.7C	14.4BC	23.2A	-

[1]Means followed by equal letters, lowercase in the columns and uppercase in the lines, do not differ by Tukey's test, at 5% probability.

Table 3. Effect of auxin types and concentrations on the rooting percentage and number of roots per explant, during in vitro rooting of the 'Ébano' genotype[1].

Auxin	Dose (mg L^{-1})	Rooting (%)	Percentage of plants	
			Less than 3 roots per explant	More than 3 roots per explant
Control	-	62.5a	0.0b	100.0a
NAA	0.55	0.0b	-	-
NAA	1.1	0.0b	-	-
IBA	0.55	100.0a	0.0b	100.0a
IBA	1.1	100.0a	100.0a	0.0b

[1]Means followed by equal letters do not differ by Tukey's test, at 5% probability.

irrespective of the concentration used. However, NAA auxin, regardless of the concentration used, promoted inhibition of root formation. As to the number of roots, it was found that the use of 1.1 mg L^{-1} IBA in the culture medium resulted in a decrease of the quantity of roots formed. In the treatment without auxin, and in the treatment based on the use of 0.55 mg L^{-1} IBA, 100% of the propagules had more than three roots per explant. In the in vitro rooting of micropropagated shoots of *Rubus* hybrids, Gupta & Mahalaxmi (2009) also found that increasing the concentration of IBA from 0.5 mg L^{-1} to 1.0 mg L^{-1} caused a decrease in the number of roots formed, although Najaf-Abadi & Hamidoghli (2009) found, in in vitro root formation of *Rubus* sp. shoots, that the number of roots increased significantly with increased concentrations of IBA in the nutrient medium.

As to the acclimatization of the rooted plantlets in IBA, no significant losses were observed after 30 days of cultivation in greenhouse, irrespective of the concentration used. Gupta & Mahalaxmi (2009) and Najaf-Abadi & Hamidoghli (2009), also found high rates of survival – over 90% – in the process of acclimatization of micropropagated plantlets of *Rubus* sp. rooted with IBA auxin. Therefore, the obtained data show that blackberry genotypes can be maintained under minimal growth conditions, by reducing the temperature to 20°C in the growth room, for up to 15 months, without subcultures. This method, besides being effective for in vitro blackberry conservation, provides subsequent plant regeneration and enables the regenerated plants to be multiplied on a large scale by micropropagation.

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Received on September 26, 2016 and accepted on April 27, 2017