

## Micropropagation on multiplication of native greenberries in South of Brazil

Micropropagação na multiplicação de amoreiras-verdes nativas do Sul do Brasil

Micropropagación en la multiplicación de moras verdes originarias del sur de Brasil

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### Abstract

*Rubus brasiliensis* and *Rubus erythroclados* are commonly known as greenberries. This work aimed to test in vitro micropropagation protocols to greenberries. The multiplication experiment was conducted using BAP at concentrations 1 mg L<sup>-1</sup>, 2 mg L<sup>-1</sup> and 3 mg L<sup>-1</sup>. The rooting experiment was carried out in ½ MS medium without the addition of hormones, using 0.1 mg L<sup>-1</sup> NAA or 1.0 mg L<sup>-1</sup> IBA. The highest rate of viability of explants was obtained with *R. brasiliensis* propagation using 1.0 mg L<sup>-1</sup> of BAP. Rooting explants showed survival rates higher than 85%. Rooting rates in *R. erythroclados* was 87% while in *R. brasiliensis* it was 38%, The highest rates were obtained using 1.0 mg L<sup>-1</sup> IBA in *R. erythroclados*. The acclimatization reached survival rates of 75% when rooted using NAA and ½ MS and 86% when rooted with IBA. In vitro culture shows to be a viable method to propagate greenberries.

**Keywords:** *Rubus brasiliensis*; *Rubus erythroclados*; In vitro cultivation; Domestication.

### Resumo

*Rubus brasiliensis* e *Rubus erythroclados* são comumente conhecidas como amoreiras-verdes. Este trabalho teve como objetivo testar protocolos de micropropagação in vitro para amoreiras-verdes. O experimento de multiplicação foi conduzido utilizando BAP nas concentrações de 1 mg L<sup>-1</sup>, 2 mg L<sup>-1</sup> e 3 mg L<sup>-1</sup>. O experimento de enraizamento foi realizado em meio ½ MS sem adição de hormônios, utilizando 0,1 mg L<sup>-1</sup> de ANA ou 1,0 mg L<sup>-1</sup> de AIB. A maior taxa de viabilidade dos explantes foi obtida com a propagação de *R. brasiliensis* com 1,0 mg L<sup>-1</sup> de BAP. Explantes de enraizamento apresentaram taxas de sobrevivência superiores a 85%. A taxa de enraizamento em *R. erythroclados* foi de 87% enquanto em *R. brasiliensis* foi de 38%. As maiores taxas foram obtidas com 1,0 mg L<sup>-1</sup> de AIB em *R. erythroclados*. A aclimação atingiu taxas de sobrevivência de 75% quando enraizada com ANA e ½ MS e 86% quando enraizado com IBA. A cultura in vitro mostra-se um método viável para a propagação de amoreiras-verdes.

**Palavras-chave:** *Rubus brasiliensis*; *Rubus erythroclados*; Cultivo in vitro; Domesticação.

### Resumen

*Rubus brasiliensis* y *Rubus erythroclados* se conocen comúnmente como morera verde. Este trabajo tuvo como objetivo probar protocolos de micropropagación in vitro a morera verde. El experimento de multiplicación se realizó utilizando BAP en concentraciones de 1 mg L<sup>-1</sup>, 2 mg L<sup>-1</sup> y 3 mg L<sup>-1</sup>. El experimento de enraizamiento se llevó a cabo en medio ½ MS sin la adición de hormonas, utilizando 0.1 mg L<sup>-1</sup> NAA o 1.0 mg L<sup>-1</sup> IBA. La mayor tasa de viabilidad de explantes se obtuvo con la propagación de *R. brasiliensis* usando 1.0 mg L<sup>-1</sup> de BAP. Los explantes de enraizamiento mostraron tasas de supervivencia superiores al 85%. La tasa de enraizamiento en *R. erythroclados* fue del 87% mientras que en *R. brasiliensis* fue del 38%, las tasas más altas se obtuvieron con 1.0 mg L<sup>-1</sup> de AIB en *R. erythroclados*, la aclimatación alcanzó tasas de supervivencia del 75% al enraizar con NAA y ½ MS y 86% cuando se arraiga con IBA. El cultivo in vitro demuestra ser un método viable para propagar morera verde.

**Palabras clave:** *Rubus brasiliensis*; *Rubus erythroclados*; Cultivo in vitro; Domestificación.

## 1. Introduction

In Brazil, 11 species of the genus *Rubus* are described, six of these occur in the Santa Catarina state and are associated with the Atlantic Forest Biome (Reflora, 2019; Reitz, 1996). The species *Rubus brasiliensis* Mart. and *Rubus erythroclados* Mart. are commonly known as whiteberries or greenberries, alluding to the color of the fruits that remain green even when ripe (Bueno, 2015). The fruits have high levels of sugar and a pleasant peculiar flavor, which gives market potential compared to other small fruits (Bueno, Biasi, & Tofaneli, 2018). Information about the ethno-knowledge of these species in the Santa Catarina South Plateau indicates that although they are much appreciated for their sweet taste and were less acidity than blackberries their consumption is made only in fresh fruits mode, when the fruits are found at random, without the habit of collection for processing (Couto Waltrich, Boff & Boff, 2017).

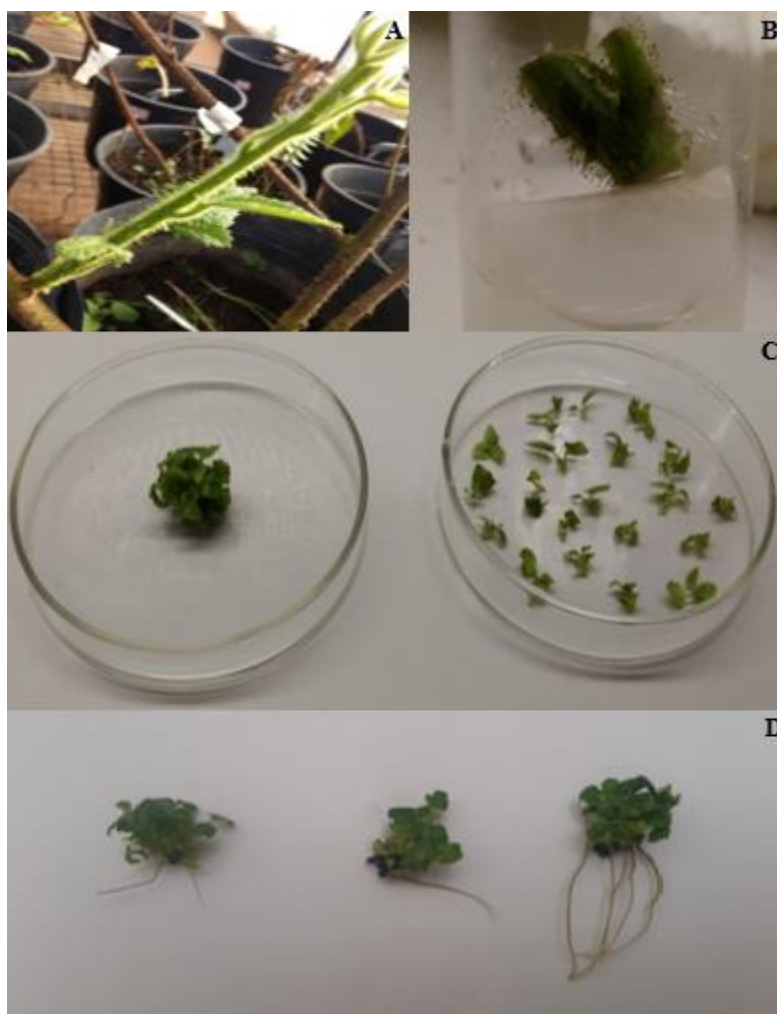
Greenberries are not domesticated or cultivated commercially yet, although their potential as an alternative for cultivation and economic exploitation by family farmers, like blackberries. Currently, around 500 ha of blackberries originating from genetic material native from United States are cultivated in the south and southeast of Brazil (Antunes, et al., 2014). This fruit consumption is associated with nutraceutical properties, such as anticarcinogenic compounds (Magalhães, Maciel & Orsolin, 2017), which are present also in greenberries.

Blackberries are multiplied by rooting steam cuttings, root cuttings and tissue culture using *in vitro* micropropagation (Antunes & Raseira 2004; Villa, et al., 2008; Villa, et al., 2010). For greenberries, vegetative propagation studies indicate that multiplication via cuttings is inefficient, whereas *in vitro* cultivation requires adjustments and further studies (Bueno, 2015). The *in vitro* propagation of *R. erythroclados* was evaluated by Bueno et al. (2018) who found low rooting rates and paralyzed studies, which highlights the necessity of retake work on development of specific protocols for these species. Micropropagation can provide better seedling uniformity and vigor, increased mass production and better health (Dutra, et al., 2010), in addition to allowing molecular biology work to conserve plant genetic resources (Bakhtiar, Mirjalili, & Sonboli, 2016). Success in berry tissue culture involves adjusting the types and concentrations of growth regulators in the culture medium, especially the cytokinin and auxin types (Villa, et al., 2006). The objective of this work was to define an *in vitro* micropropagation protocol for greenberries in order to contribute to the domestication of *R. brasiliensis* and *R. erythroclados*.

## 2. Methodology

This study was based on the quantitative research method (Pereira, et al., 2018) and was developed at the EPAGRI Lages Biotechnology Laboratory, based on the *in vitro* culture methodology described by George (1993). The vegetative material used in this study came from parent plants of *Rubus brasiliensis* and *R. erythroclados* kept in a greenhouse ( $26 \pm 2^\circ \text{C}$ ) with intermittent irrigation (Figure 1A). The explants, consisting of nodal segments containing an axillary bud, without leaf and approximately 3 cm, were collected and immediately immersed in water. Asepsis was performed in a laminar flow chamber with 70 % alcohol for 1 minute, followed by 30 minutes in 2 % sodium hypochlorite with drops of Tween® 20 and under constant stirring. After triple washings in water, the nodal segments were sectioned at 1 cm and transferred to paper bridges in test tubes containing 5 ml of liquid culture medium (Figure 1B). The culture medium consisted of MS medium (Murashige & Skoog, 1962) supplemented with  $1.0 \text{ mg L}^{-1}$  benzylaminopurine (BAP),  $0.125 \text{ mg L}^{-1}$  salicylic acid (AAS),  $0.25 \text{ mg L}^{-1}$  gibberellic acid ( $\text{GA}_3$ ),  $30 \text{ g L}^{-1}$  sucrose,  $0.30 \text{ g L}^{-1}$  of reduced glutathione, pH 5.8. The segments remained in the dark for 72 hours in a growth room and afterwards a 16h photoperiod ( $24 \pm 2^\circ \text{C}$ ,  $50 \mu\text{mol m}^{-2}$ ). After 40 days, cultures were subcultured in test tubes containing basal medium (MS,  $30 \text{ g L}^{-1}$  of sucrose,  $0.25 \text{ mg L}^{-1}$   $\text{GA}_3$ , 2.6 g of phytagel and pH 5.8) supplemented with  $1.0 \text{ mg L}^{-1}$  of BAP.

**Figure 1.** In vitro cultivation and propagation of Greenberries. A: Mother plant kept in greenhouse. B: In vitro inclusion. C: Multiplication. D: Rooting ( $\frac{1}{2}$  MS;  $\frac{1}{2}$  MS + 0.1 mg L<sup>-1</sup> NAA;  $\frac{1}{2}$  MS + 1.0 mg L<sup>-1</sup> IBA).



Source: Authors.

The multiplication experiment was carried out in culture flasks containing 30 ml of basal medium in concentrations of BAP at 1 mg L<sup>-1</sup>; 2 mg L<sup>-1</sup> and 3 mg L<sup>-1</sup>, which constituted the treatments. After 60 days, survival and multiplication rates (Figure 1C) were analyzed. The rooting experiment was carried out in culture flasks containing 30 ml of MS medium with half the concentration of salts ( $\frac{1}{2}$  MS), supplemented with 20 g L<sup>-1</sup> of sucrose, 2.6 g L<sup>-1</sup> of Phytigel and pH adjusted to 5.8. Treatments were established with addition of 0.1 mg L<sup>-1</sup> of  $\alpha$ -naphthalene acetic acid (NAA) or 1.0 mg L<sup>-1</sup> of indolbutyric acid (IBA) to the medium without growth regulator. After 38 days, survival rates, rooting, length and number of roots were analyzed (Figure 1D).

Acclimatization was carried out by transferring 60 rooted plants to alveolate trays of 128 cells, containing commercial substrate composed of carbonized husks of *Pinus* sp, vermiculite, chalky and fertilized with N, P and K, sand and rice husks in proportion 1: 1: 1 (v/v/v), arranged inside plastic trays and covered by transparent plastic to maintain humidity.

The experiments were carried out independently for each species of greenberries (*R. brasiliensis* and *R. erythroclados*), in a randomized block design, with three replicates of 25 replicates each. The results were submitted to analysis of variance and test of separation of means (Tukey,  $p < 0.05$ ) using the R environment (R Core Team, 2015).

### 3. Results and Discussion

The explant survival of in vitro cultures of greenberries did not differ between the tested BAP concentrations (Table 1). The highest rate of viability of explants in *R. brasiliensis* was obtained using 1.0 mg L<sup>-1</sup> of BAP. In the species *R. erythroclados*, the treatments did not differ from each other (Table 1).

**Table 1.** Survival and multiplication rates of greenberries *Rubus brasiliensis* and *Rubus erythroclados* grown in vitro with different concentrations of benzylaminopurine (BAP). Lages/SC, 2020.

Species	BAP (mg L <sup>-1</sup> )	Survival rate (%)	Multiplication rate (N <sup>o</sup> )
<i>Rubus brasiliensis</i>	1,0	88 % <sup>ns</sup>	5,61 ± 1,58 <sup>a</sup>
	2,0	91 %	3,37 ± 0,77 <sup>b</sup>
	3,0	87 %	3,93 ± 1,74 <sup>b</sup>
<i>Rubus erythroclados</i>	1,0	73 % <sup>ns</sup>	4,79 ± 0,97 <sup>ns</sup>
	2,0	85 %	4,39 ± 1,01
	3,0	84 %	5,61 ± 2,14

ns: not significant; averages followed by the same letter do not differ (Tukey, p≤0,05). Source: Authors.

The concentration of 1.0 mg L<sup>-1</sup> of BAP was also the most efficient in the multiplication of the blackberry cultivar Xavante (Toledo & Biasi, 2018) and cv. Ebano (Villa, et al., 2005). On the other hand, for the cultivar Thornless, Najaf-Abadi & Hamidoghli (2009) found that the combination of 2.0 mg L<sup>-1</sup> BAP and 0.5 mg L<sup>-1</sup> GA<sub>3</sub> was efficient. Erig, De Rossi & Fortes (2002) observed that in the Tupy cultivar, the highest multiplication rate was obtained with 0.45 mg L<sup>-1</sup> of BAP. Responses to BAP concentrations are related to the species genotypes, as found by Hunková, Libiakova & Gajdošová (2016) under study with *Rubus* Black Jewel, Tayberry, Tulameen and Black Satin cultivars. Their results point to multiplication rates between 2.34 and 4.08 in medium with 1 mg L<sup>-1</sup> BAP.

Explants of greenberries submitted to rooting showed high survival rates (91 % in *Rubus brasiliensis* and 85 % in *Rubus erythroclados*), with no difference between treatments (p≤0.05). The rooting rate presented by *R. erythroclados* explants (87 %) differed significantly from that presented by *Rubus brasiliensis* explants (38 %) (Table 2).

**Table 2.** Survival, rooting, length and number of roots in greenberries *Rubus brasiliensis* and *Rubus erythroclados* in vitro culture.

Species	Treatment	Survival rate (%)	Rooting rate (%)	Root length (mm)	Root number (n°)
<i>Rubus brasiliensis</i>	IBA	0,98 % <sup>ns</sup>	0,38 % <sup>ns</sup>	9,13 ± 3,76 <sup>ns</sup>	2,25 ± 1,38 <sup>ns</sup>
	MS	0,90 %	0,22 %	9,35 ± 1,06	1,39 ± 0,37
	NAA	0,88 %	0,32 %	6,99 ± 6,21	1,28 ± 1,33
<i>Rubus erythroclados</i>	IBA	0,92 % <sup>ns</sup>	0,87 % <sup>a</sup>	14,91 ± 3,06 <sup>ns</sup>	4,54 ± 0,63 <sup>a</sup>
	MS	0,97 %	0,28 % <sup>b</sup>	21,73 ± 3,18	2,92 ± 0,14 <sup>b</sup>
	NAA	0,92 %	0,46 % <sup>b</sup>	17,84 ± 3,37	2,54 ± 0,55 <sup>b</sup>

ns: not significant; averages followed by the same letter do not differ (Tukey,  $p \leq 0,05$ ). Source: Authors.

The rate of in vitro rooting obtained in this work for the species *R. brasiliensis* was higher than that obtained by Bueno (2015), which was 16 % and 5 % of rooting using stem and root cuttings, respectively.

The present results point to 75 % survival rates for greenberries on acclimatization when rooted using NAA and ½ MS and 86 % when rooted with IBA. The facility on acclimatization of blackberries was described by Toledo & Biasi (2018) in work with cultivar Xavante.

#### 4. Conclusion

Greenberries of the species *Rubus brasiliensis* and *Rubus erythroclados* can be propagated through in vitro micropropagation using MS medium with addition of 1.0 mg L<sup>-1</sup> of BAP and rooted using ½MS culture medium plus 1.0 mg L<sup>-1</sup> IBA. It is necessary to improve the in vitro propagation protocol of Greenberries, especially by testing different concentrations of hormones in the culture medium. Thus, this protocol can contribute to the domestication process and the consequent preservation of these species forgotten until now.

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