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In Vitro Multiplication of Nodal Segments of "Murici" (Byrsonimacydoniifo A. Juss.): The Use of Growth Regulators and Photoautotrophic Stimulation Lia A. Juss.)

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Abstract Micropropagation is an alternative method for the *in vitro* cultivation of "murici" (*Byrsonimacydoniifolia* A. Juss.), a plant from the Cerrado (Brazilian savanna) that has a high agronomic potential and is grown for its fruit but has limitations in terms of its propagation. Thus, the objective of the present study was to evaluate the use of growth regulators and photoautotrophy stimulation, testing different ways to seal the explant test tube and the use of sucrose for the *in vitro* cultivation of murici. In assay I, the medium was supplemented with BAP at concentrations of 0.0, 11.1, 22.2 and 44.4 µM and NAA at concentrations of 0.0, 6.71, 13.43 and 26.85 µM. In assay II, 4.9 µM IBA was added to the culture medium, with or without sucrose, with the use of 3 different seals: a plastic cap, plastic film and cotton plug. After 60 days of cultivation, the following traits were evaluated: the callus percentage, leaf number, shoot length and length and number of roots. It was determined that the concentrations of 11.1 µM BAP and 13.43 µM NAA were the most suitable. The type of seal did not influence the growth of *B. cydoniifolia*A. Juss., but the medium supplemented with sucrose was effective for the growth of this species.

Keywords: auxins, cytokinins, rooting, fruit of the Cerrado

Introduction

The genus *Byrsonima* (Malpighiaceae) is composed of a wide variety of species that are known as "murici". Murici is a fruit-bearing plant of the Cerrado (Brazilian savanna) with a high agronomic potential because it produces fruit that can be consumed fresh and can be used in the production of pharmaceutical drugs (Carvalho and Nascimento, 2008; Isaac *et al*., 2008, Nogueira *et al*., 2004). Similar to other native species, *Byrsonimacydoniifolia*

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A. Juss.has limitations with regard to its propagation, which is aggravated by the irregularity of its germination, a process that is normally low, slow and has a marked lack of uniformity. This slow growth is due to the thick endocarp that hinders the growth of the embryo, making seedling production unfeasible (Carvalho and Nascimento, 2008; Nogueira *et al*., 2008; Sautu *et al*., 2007).

As an alternative, asexual propagation via micropropagation through direct organogenesis establishes the differentiation of the shoots and roots during the growth of the explant. However, it is necessary to use growth regulators that can stimulate the formation of shoots and roots for the induction of the dedifferentiation and redifferentiation processes responsible for the formation of tissues and organs, and the most suitable type of explant must also be selected (Kielse *et al*., 2009). Thus, compared to seeds, the multiplication of clones in the desired quality are obtained with explants, such as nodal segments, which are the most appropriate for *in vitro* culture (Assis*et al*., 2011).

In general, the shoots are induced in a culture medium supplemented with cytokines, and these shoots are later rooted in a medium containing auxin (Nicioli *et al*., 2008). Therefore, cytokinins are used to break the apical dominance of the shoots and increase the rate of multiplication, whereas auxins, although they do not promote the proliferation of axillary shoots, can assist in growth *in vitro* by inducing the formation of roots (Kielse *et al*., 2009, Grimaldi *et al*., 2008).

Different species have variable requirements for factors, including the concentration of salts and growth regulators in culture medium and the temperature and photoperiod, during micropropagation. However, the addition or omission of carbon sources and the use of caps to close the incubation flasks are of equal importance, as these factors may significantly influence the *in vitro* growth (Fuente *et al*., 2005; Rodrigues MeloandAlonfa, 2006; Skrebsky *et al*., 2006; Santana *et al*., 2008). The processes by which plant shoots and roots develop *in vitro* are anatomically and physiologically different from seedlings grown in soil. This difference is observed because the *in vitro* cultures are typically heterotrophic, and seedlings grown heterotrophically do not efficiently absorb light, water and nutrients (Scaranari, 2008; Dosseau *et al*., 2008).

According to Santana (2008), an improvement in the gas exchange between the atmosphere inside the culture flask and the external environment has a positive effect on growth and on the stimulation of photoautotrophy*in vitro*. The type of seal used is one of the factors influencing the microenvironment within the culture vessel, potentially interfering with the gas exchange between the inside of the flask and ambient air (Santana *et al*., 2008).

Therefore, the objective of this study was to evaluate the use of growth regulators and stimulate photoautotrophy by testing different types of seals and the use of sucrose for the *in vitro* growth of *B. cydoniifolia* A. Juss.

Materials and methods

The study was conducted at the Laboratory of Plant Tissue Culture of the Goiano Federal Institute – Rio Verde Campus. A voucher specimen of the plant material is deposited in the Jataiense Herbarium at the Federal University of Goiás – Jataí Campus under number 5646. The primary explants were obtained using *B. cydoniifolia* A. Juss. seedlings pre-established *in vitro*, which were inoculated in a 50% woody plant medium (WPM) and cultivated without growth regulators for 60 days *in vitro*. The nodal segments containing a pair of lateral buds with an average length of 1.0 cm were excised.

Assays I and II: Use of BAP and NAA for the multiplication of B. cydoniifolia A. Juss. from nodal segments

The nodal segments were inoculated in test tubes (25 x 150 mm) containing 20 mL of WPM medium (Lloyd &McCown, 1980) with 50% of the salt concentration. The culture medium was supplemented with 6-benzylaminopurine (BAP) at concentrations of 0.0, 11.1, 22.2 and 44.4 µM and naphthalene acetic acid (NAA) at concentrations of 0.0, 6.71, 13.43 and 26.85 µM. Agar was added at the concentration of 3.5 g L^{-1} (Dinâmica) to solidify the medium, and the pH was adjusted to 5.7±0.3 before autoclaving at 120 °C for 20 minutes. The test tubes containing the inoculated explants were sealed with plastic caps (polypropylene) and maintained in a growth chamber at a temperature of 25±2 °C, relative humidity of 45%, 16-hour photoperiod and photosynthetic active radiation of 45-55 µmol m^{-2} s^{-1}, which was obtained using white fluorescent lamps. At 60 days of cultivation, the percentage of callus tissue, the number of lateral shoots, the average number of expanded leaves, the shoot length and the rooting percentage were evaluated. For each assay, the experimental design was completely randomized, containing 4 treatments and 20 repetitions, each consisting of 1 test tube, for a total of 80 experimental units for each plant growth regulator. The numerical data were statistically evaluated using an analysis of variance with the application of the F-test at a 5% probability. The means were analyzed by regression using the SISVAR software (Ferreira, 2011).

Assay III: Stimulating photoautotrophic behavior - growth of shoots and roots

The nodal segments were inoculated in test tubes (25 x 150 mm) containing 20 mL of WPM medium with 50% of the salt concentration and supplemented with 4.9 µM indolebutyric acid (IBA), with or without 30 g L^{-1} of sucrose. For the solidification of the medium, 3.5 g L^{-1} agar (Dinâmica) was added, and the pH was adjusted to 5.7±0.3 before autoclaving at 120 ℃ for 20 minutes. The test tubes containing the explants were inoculated and closed with three types of seals: a plastic cap (polypropylene), plastic film (PVC film) or cotton plug. These test tubes were subsequently maintained in a growth chamber at a temperature of 25±2 ℃, relative humidity of 45%, 16-hour photoperiod and photosynthetic active radiation of 45-55 µmol m^{-2} s^{-1} using white fluorescent lamps. After 60 days of cultivation, the percentage of callus tissue, the number of expanded leaves per seedling, the number of roots and the average lengths of the shoots and roots were evaluated. The experimental design was completely randomized in a 2 x 3 factorial arrangement (sucrose concentration x types of seal), and each treatment contained 20 replicates, each consisting of 1 test tube, for a total of 120 experimental units. The numerical data were statistically evaluated using an analysis of variance, testing the means with the Tukey's test at a 5% probability using the SISVAR software (Ferreira, 2011).

Results and discussion

Assay I: Use of BAP for the multiplication of B. cydoniifoliaA. Juss. from nodal segments

The use of BAP in the culture medium was significant, and the quadratic regression model exhibited the best fit. The use of BAP in the culture medium induced callus formation in the lower region of all of the inoculated explants (Figure 1 A). With respect to the number of leaves, there was an increase with the addition of BAP up to 22.2 µM, though the number of leaves decreased when this concentration was exceeded (Figure 1 B). Root formation was only observed in the treatment using no growth regulator with an average of 16.66% (Figure 1 C). Regarding the number of buds and the average length of the shoots, there was no significance difference at the concentrations tested. The maximum values for the BAP concentration were 7 µM for callus formation, 4.10 µM for the number of leaves and 6.75 7 µM for the rooting percentage.

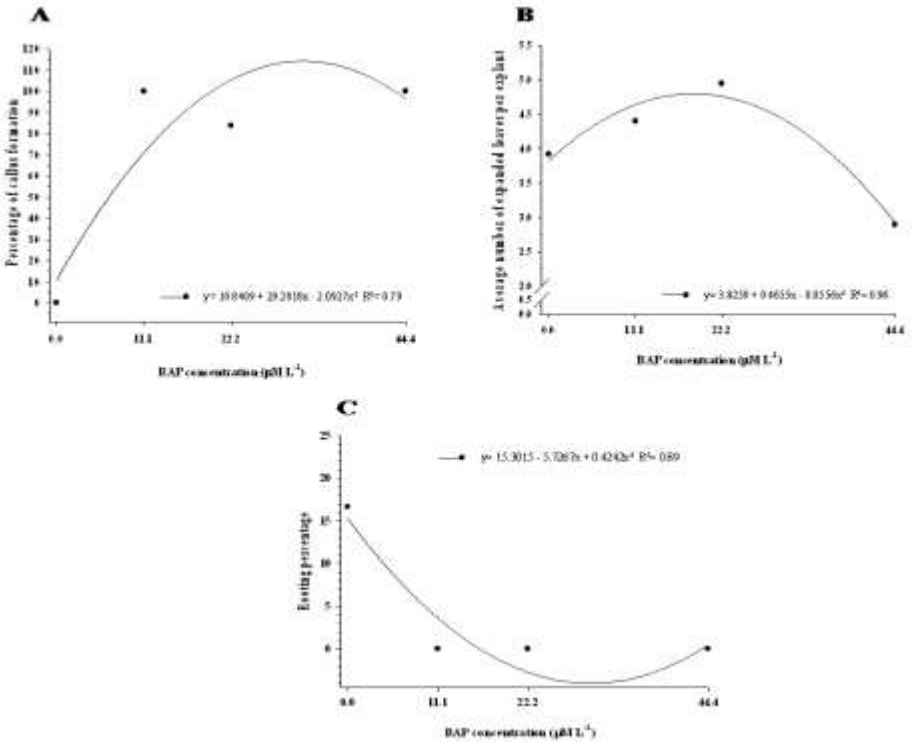

Fig. 1. *In vitro* cultivation of *B. cydoniifolia* A. Juss. plants using different BAP concentrations (µM) and evaluated at 60 days: (A) percentage of callus formation; (B) number of expanded leaves per explant; (C) rooting percentage in relation to the BAP concentration (µM). Rio Verde-Goiás state (GO), 2012.

With the exception of the treatment without the addition of growth regulator (control, Figure 2 A), a visual inspection revealed that calli formed at the base of the explant (Figure 2) in all of the treatments. Calli formed at the base when using concentrations of 11.1 µM, but there was a greater growth of the shoot (Figure 2 B). It was also determined that as the BAP concentration was increased in the culture medium (from 22.2 to 44.4 µM), there was a change in the color of the explant leaves, which became red (Figure 2 C-E).

The callus formation was more pronounced when a concentration of 44.4 µM was used (Figure 2 E).

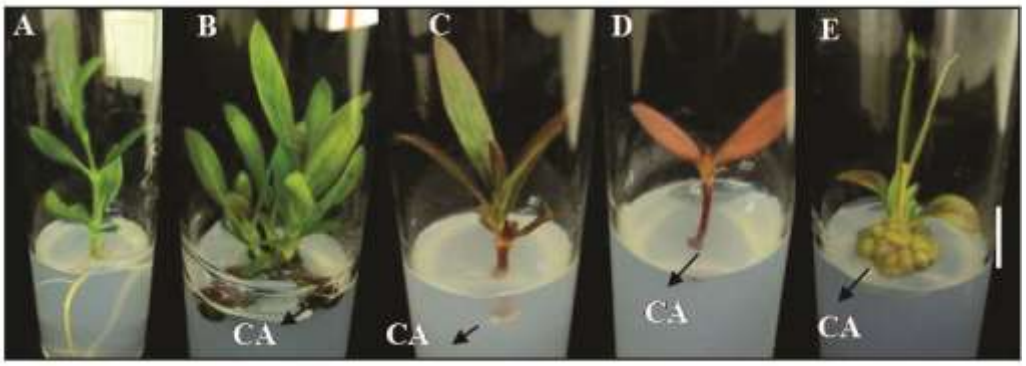

Fig. 2. *In vitro* growth of *B. cydoniifolia* A. Juss. plants from nodal segments: (A) seedling in culture medium without growth regulators; (B) seedling with several shoots in the culture medium supplemented with 11.1 µM BAP; (C) seedling showing leaves with reddish ends and without the formation of roots in culture medium containing 22.2 µM BAP; (D) seedling with a reddish color and without the formation of roots in the culture medium supplemented with 44.4 µM BAP; (E) seedling showing callus formation in the culture medium supplemented with 44.4 µM BAP. CA: callus. Rio Verde-GO, 2012. Scale bar: 10 mm. Photo: Cíntia de Oliveira Martendal.

Therefore, the use of 22.2 µM BAP in the medium for *B. cydoniifolia* A. Juss.provided the best results for the formation of expanded leaves and buds with a lower percentage of callus formation. However, because the addition of BAP did not influence the induction of the number of shoots, it is recommended that 11.1 µM BAP be used to maintain the explants' morphological similarities with the mother plant.

According to Nogueira (2003), BAP concentrations greater than 17.7 µM were not effective for the induction of axillary buds on nodal segments of "pequeno-murici" (*B. intermedia* A. Juss). Furthermore, when testing concentrations of 4.44 and 8.88 µM BAP, Soares *et al.* (2007) found that more developed shoots were developed and that these were accompanied by callus formation at the base of explants of the "mangaba" tree(*Hancorniaspeciosa* Gomes). The use of 8.88 µM BAP promoted a greater number of buds in blackberry plants (*Rubus sp.*), cultivar Brazos, when tested using different culture media and BAP concentrations (VILLA *et al.*, 2010). However, Souza et al. (2008) suggest using low BAP concentrations for the *in vitro* multiplication of Surinam cherry (*Eugenia uniflora* L.).

Assay II: Use of NAA for the multiplication of B. cydoniifolia A. Juss. from nodal segments

With regard to NAA, it was observed that the values of the traits, except for the average number of buds, were greater the higher the concentration of the growth regulator. In general, high NAA concentrations (26.85 µM) induced responses in all of the traits evaluated; however, there was callus formation at the base of the explant. The maximum concentration for the use of NAA was 1.17 µM for callus formation, 3.64 µM for the number of shoots, 1.01 µM for the number of expanded leaves, 0.66 µM for the shoot length and 10.68 µM for the rooting percentage. Visually, it was observed that the shoots were well-formed with expanded leaves and were compromised only by the formation of buds (Figure 3).

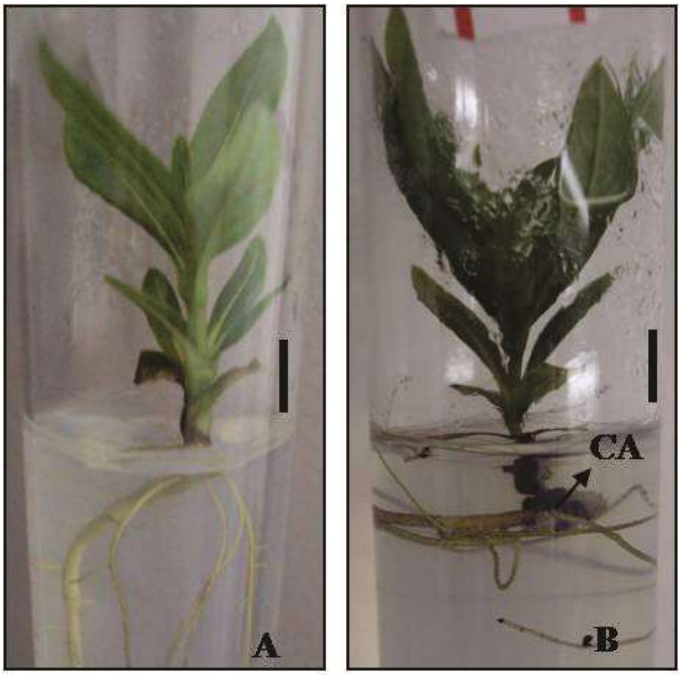

Fig. 3. The *in vitro* growth of *B. cydoniifolia* A. Juss. plants from nodal segments: (A) seedling supplemented with 13.43 µM NAA; (B) seedling supplemented with 26.85 µM ANA. CA: callus. Rio Verde-GO, 2012. Scale bar: 10 mm. Photo: C ńtia de Oliveira Martendal.

According to FettNeto *et al*. (1992) and Blakesley *et al*. (1991), when the auxin concentration in the medium is excessive, callus formation is observed at the base of the explant, compromising the growth of the shoots and rhizogenesis. These authors also report that auxin toxicity during rooting may only be evident during the root elongation phase, which is why it is generally

recommended that two types of culture media be used. First, a culture medium containing auxin should be used to favor root induction followed by the transfer of the explants to an auxin-free medium to stimulate rhizogenesis and root growth. Therefore, the NAA concentration of 26.85 µM was considered toxic. However, as positive results were obtained with the use of 13.43 µM NAA, this concentration was indicated because it did not induce callus formation at the base of the explant (Figure 4 A). The concentrations of 13.43 and 26.85 µM NAA resulted in the highest rooting percentages (56.25 and 70.16%), respectively (Figure 4 E).

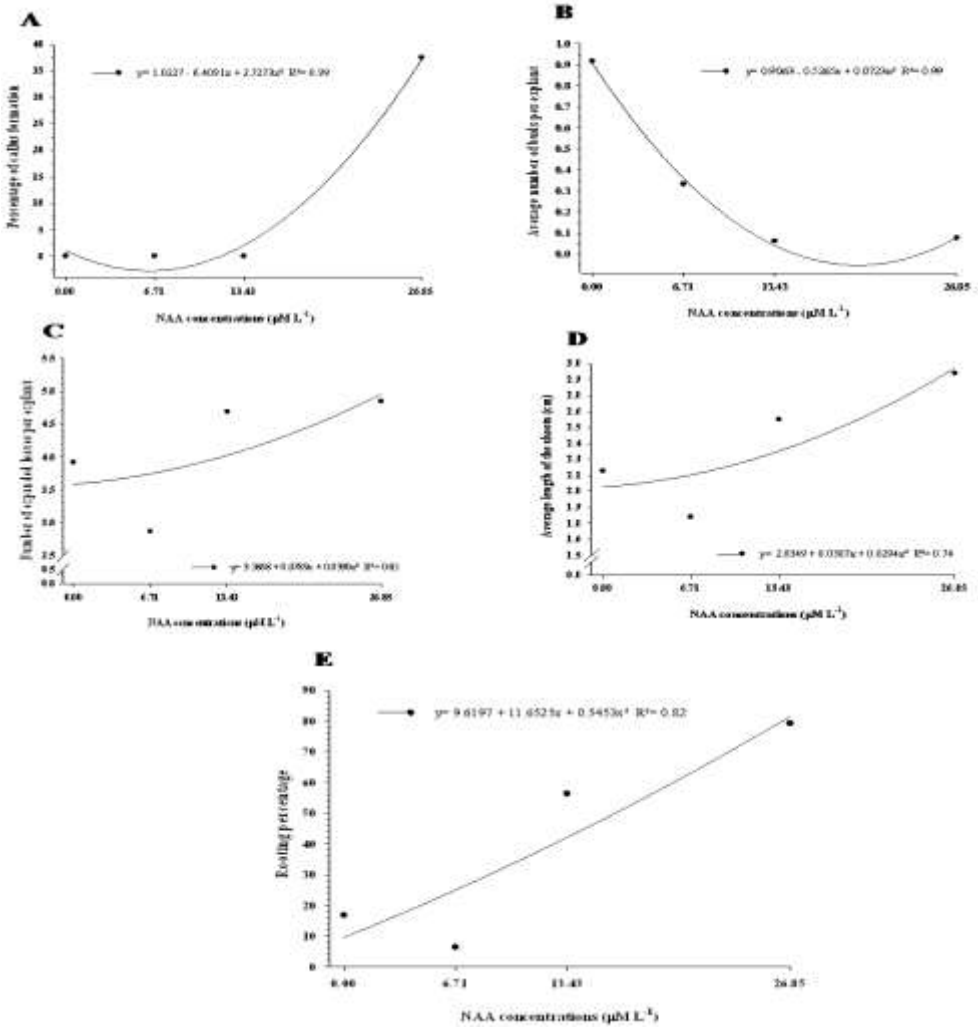

Fig. 4.*In vitro* cultivation of *B. cydoniifolia* A. Juss. plants using different concentrations of NAA (µM) and evaluated at 60 days: (A) percentage of callus formation; (B) average number of buds per explant; (C) number of expanded leaves per explant; (D) average length of the

shoots (cm); (E) rooting percentage. Rio Verde-GO, 2012.

Similar results were obtained for the *in vitro* rooting of quince cv. MC when using NAA at the concentration of 10 µM in the culture medium, promoting the highest percentage of rooting (Erig and Schuch, 2004; Soares *et al*. (2007) obtained different results when testing various concentrations of NAA, whereby roots were not formed from buds of *Hancorniaspeciosa* Gomes.

Assay III: Stimulation of photoautotrophic behavior - shoot and root growth

The results showed that there was only an interaction of the effect of the factors sucrose x seal type for the percentage of callus formation. The lowest callus percentages (15 and 10%, respectively) were obtained when plastic film and cotton plugs were used to seal the tubes in the absence of sucrose (Table 1).

The largest number of leaves per plant (1.84) was obtained in the presence of sucrose when using the cotton plug. However, no difference was observed when comparing the seal type in the absence and presence of sucrose (Table 1).

Regarding the number of roots per plant and average root length, the highest results were obtained with the presence of sucrose in the culture medium, regardless of the seal type used. Furthermore, there was no difference between the seal type in the absence and presence of sucrose (Table 1).

It was visually confirmed that the murici plants were well-formed without oxidation or callus formation. The presence of sucrose in the culture medium optimized the muricigrowth and was essential for the formation of the root system (Figure 5). The seedlings grown in the medium without sucrose showed signs of deficiencies that were noticeable by the color, which was a lighter green than normal. It was also observed that the size of the plants was reduced in the medium free of sucrose. According to Fuentes *et al*. (2007), a decrease in the sucrose concentration in the culture medium can improve plant photosynthesis but can also adversely affect growth under the standard conditions used in growth chambers.

Table 1. Percentage of callus formation, number of leaves per seedling, average length of the shoots (cm), number of roots per seedling and average length of roots of *Byrsonimacydoniifolia* A. Juss. in relation to the type of seal in the absence and presence of sucrose after 60 days of *in vitro* cultivation. Rio Verde-GO, 2012

Seal type	Absence of sucrose	Presence of sucrose	
	Percentage of callus formation		Mean
Plastic cap	23.75 A[Z]a[Y]	43.75 Ba	33.75 A
Plastic film	15.00 Ab	58.33 Aba	36.66 A
Cotton plug	10.00 Ab	77.08 Aa	43.54 A
Mean	16.25 b	59.72 a	
	Number of leaves per seedling		Mean
Plastic cap	1.00 Aa	1.56 Aa	1.26 A
Plastic film	0.30 aa	0.71 Aa	0.47 A
Cotton plug	0.66 Ab	1.84 Aa	1.29 A
Mean	0.61 b	1.37 a	
	Average length of the shoots (cm)		Mean
Plastic cap	1.38 Aa	1.62 Aa	1.50 A
Plastic film	1.02 Aa	1.21 Aa	1.10 B
Cotton plug	1.22 Aa	1.46 Aa	1.32 AB
Mean	1.20 a	1.44 a	
	Number of roots per plant		Mean
Plastic cap	0.38 A[Z]b[Y]	1.56 Aa	0.94 A
Plastic film	0.10 Ab	2.14 Aa	0.94 A
Cotton plug	0.72 Ab	2.07 Aa	1.29 A
Mean	0.39 b	1.90 a	
	Average length of the roots (cm)		Mean
Plastic cap	0.27 Ab	1.68 Aa	0.94 A
Plastic film	0.05 Ab	2.03 Aa	0.86 A
Cotton plug	0.25 Ab	1.38 Aa	0.72 A
Mean	0.18 b	1.70 a	

[Z]The means followed by the same upper case letter in each column are not significantly different at a 5% probability according to Tukey's test.
[Y]The means followed by the same lower case letter in each row are not significantly different at a 5% probability according to Tukey's test.

The addition of sucrose to the culture medium was the factor that most influenced the traits studied. Although many authors have reported the potential of species to adapt to photoautotrophic conditions *in vitro*, the presence of sucrose was essential for the good formation of the murici seedlings (*B. cydoniifolia*A. Juss.) under the controlled conditions used in this study.

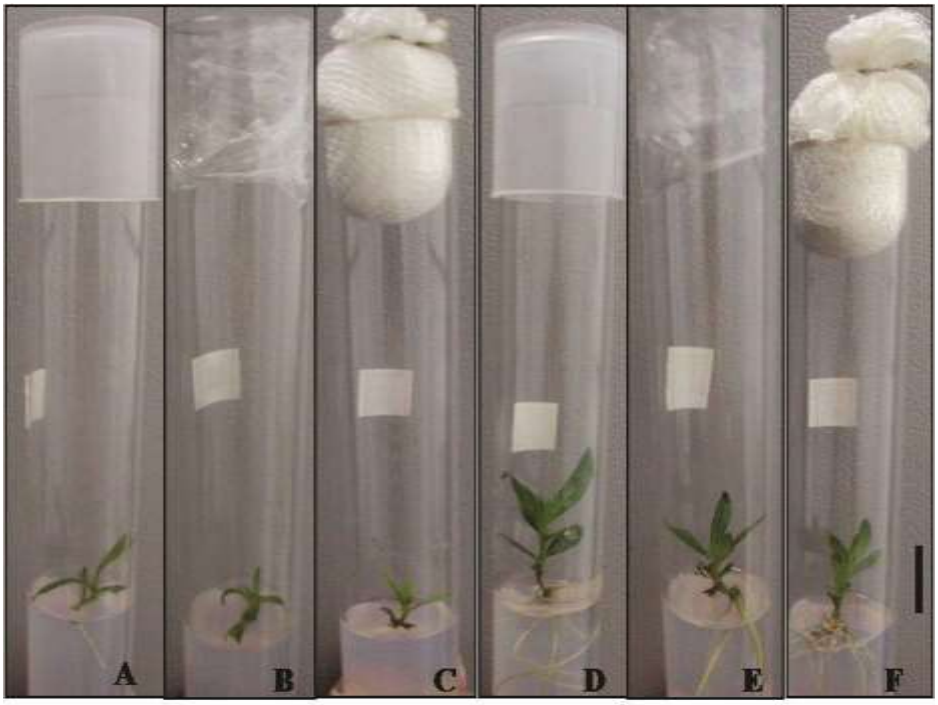

Fig. 5. *B. cydoniifolia*A. Juss. plants from nodal segments at 60 days of *in vitro* cultivation in tubes with different types of seals in the presence or absence of sucrose: (A) sealed with a plastic cap without sucrose; (B) sealed with a plastic film without sucrose; (C) sealed with a cotton plug without sucrose; (D) sealed with a plastic cap with sucrose; (E) sealed with a plastic film with sucrose; (F) sealed with a cotton plug with sucrose. Rio Verde-GO, 2012. Scale bar: 10 mm. Photo: C ńtia de Oliveira Martendal.

Similar results were obtained by Costa *et al*. (2009) when analyzing the *in vitro* propagation of Banana cultivars (Musa spp), indicating that sucrose in the culture medium is used for both elongation and rooting. Hawerroth *et al*. (2010) also reported that sealing the flasks with plastic film resulted in a greater number of buds and leaves per explant of 'Abate Fetel' pears when utilizing high sucrose concentrations.

Different results were obtained by Santana *et al*. (2008) when cultivating *Annonaglabrain vitro*; their results revealed that the rooting of the buds is not dependent on the sucrose concentration in the culture medium in tubes sealed

with cotton or plastic caps. The photoautotrophic rooting of blueberry (Vacciniumspp) performed in a culture medium free of sucrose also increased the rooting percentage and number of roots, also promoting an increase in root length and total fresh weight when combined with sealing the flasks with cotton (Damiani and Schuch, 2009).

Although the type of seal did not influence the *in vitro* growth of *B. cydoniifolia*A. Juss., the use of plastic (polypropylene) or cotton plugs has the advantage of being more economical, allowing the reuse of the material, and is also more practical for sealing the tubes.

Moreover, seals with permeable filters, such as cloth or cotton wool, favor gas exchange, reducing the concentration of ethylene, which results in improved plant growth (Kozai and Nguyen, 2003).

Conclusion

The use of 11.1 µM BAP and NAA, 13.43 µM was appropriate for the *in vitro* cultivation of *B. cydoniifolia* A. Juss. The seal type did not influence the *in vitro* cultivation of *B. cydoniifolia* A. Juss. The presence of sucrose in the culture medium influenced the *in vitro* growth *B. cydoniifolia.*

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