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ARTICLE

Callus induction and pro-embryogenic mass formation in *Myrciaria dubia*, an important medicinal and nutritional plant

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Abstract: *Caçari is a fruit tree that is native to the Amazon region of Brazil. The fruit is considered to have the highest vitamin C content of any edible fruit (13.757 mg 100 g^{-1} dry weight). The objective of this study was to evaluate the effects of 2,4-D and 6-BAP alone or in combination on in vitro callus induction and pro-embryogenic mass formation in caçari stem segments in different culture media as a precursor for caçari micropropagation. The experiment consisted of two culture media (MS and WPM) and combinations of four concentrations of 2,4-D (0, 1, 2 and 4 mg L^{-1}) and BAP (0, 0.25, 0.5 and 1 mg L^{-1}). The results showed that the highest percentage of callus formation (99%) was obtained in WPM supplemented with 4 mg L^{-1}, 2,4-D and 1 mg L^{-1} BAP, and this combination resulted in 93% pro-embryogenic mass formation.*

Keywords: *Camu-camu, somatic embryogenesis, culture medium, growth regulators*

INTRODUCTION

The Amazon contains a wide diversity of species with economic, medicinal and nutritional potential. Among those species, caçari (*Myrciaria dubia* (Kunth.) (McVaugh)) stands out. It is a native fruit tree that grows spontaneously on the banks of lakes and rivers of the Amazon and is considered to have the highest content of vitamin C of any edible fruit, with variations between 7.355.20 (Chagas et al. 2015) to 13.756.79 (Ribeiro et al. 2016) mg vitamin C per 100 g $pulp^{-1}$ depending on the stage of maturation (Grigio et al. 2021a). In addition to their high vitamin C concentration, the fruits show high levels of potassium, anthocyanins, flavonoids, carotenoids and phenolic compounds and are considered functional foods (Castro et al. 2018, Grigio et al. 2021b, Grigio et al. 2021c). This fact has attracted the attention of the food, cosmetics and pharmaceutical industries.

This species is usually propagated by seeds. However, *M. dubia* seeds are highly recalcitrant; this can be a limiting factor for propagation since the seeds cannot be stored for long periods. In addition, genetic variability among *M. dubia* plants may occur. This variability is undesirable in commercial plantations since the objective is to preserve the uniformity of the orchard, maintain the

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genetic characteristics of the species and especially maximize the genetic gain of new materials that surpass the mean value of the commercial control (Dias et al. 2018). Thus, vegetatively propagate of this species in order to reduce the segregating effect and to reproduce selected high-yielding materials, like the recent study by Lima et al. (2020). However, despite the vegetative propagation technique having a high success rate, it is limited due to the possibility of infestation by pathogens, Therefore, the use of biotechnological techniques with the possibility of preserving the germplasm and producing it on a large scale in a short period of time, such as somatic embryogenesis, is the best alternative (Kamle and Baek 2017, Naaz et al. 2019, Silva-Cardoso et al. 2019).

To initiate the embryogenic process, most species require auxins, usually 2,4- dichlorophenoxyacetic acid (2,4-D) or 1-naphthaleneacetic acid (NAA), either alone or in combination with a cytokinin. For a great number of species, 2,4-D is sufficient to trigger cell differentiation and embryo formation, as it is rapidly metabolized, leading to cell wall thickening and irreversible differentiation in somatic embryos (Corredoira et al. 2019). Some recent studies have shown success with somatic embryogenesis in forest and fruit species of the family Mytarceae: in *Acca sellowiana* (Pavei et al. 2018, Xiao et al. 2020). Qin et al. (2021) observed the effects of supplementation and the identification of genes and entire genomes of microRNAs. For *Psidium guajava* (Kamle and Baek 2017) mention that it is necessary to advance in genome sequencing to proceed with studies for *Syzygium cumini* (L.), and for the first-time advances in somatic embryo aturation were observed in *Plinia peruviana* (Silveira et al. 2020). However, there is no information in the literature about the callus induction mechanism in *M dubia*.

In this context, the objective of this study was to evaluate the effects of different concentrations of 6-benzylaminopurine (BAP) combined with different concentrations of 2,4-D in different culture media (MS and WPM) on *in vitro* callus induction and pro-embryogenic mass formation in stem segments of *M. dubia*.

MATERIAL AND METHODS

The experiment was performed at the Tropical Research and Education Center (TREC), University of Florida, located in Homestead, Florida. Stem segments of *M. dubia* plants of approximately 1.0 cm in length from plants grown for 5 months in a greenhouse at 29 °C were used as explant sources. The seeds came from the Serra da Prata experimental field of Embrapa Roraima. The stem segments were taken to the ornamental horticulture laboratory and submitted to a precleaning process with 1% Alconox detergent for 10 minutes. The material was then transferred to a laminar flow chamber for disinfestation using 6% sodium hypochlorite for 1 minute with 6 drops of Tween 20 followed by three washes with distilled and autoclaved water for 3 min. After disinfestation, the explants were placed in Petri dishes for the different treatments, which consisted of two culture media, MS or WPM, and combinations of different concentrations of 2,4-D (0, 1, 2 and 4 mg L^{-1}) and BAP (0, 0.25, 0.5 and 1.0 mg L^{-1}). In each treatment, 7 g L^{-1} agar, 30 g L^{-1} sucrose, 100 mg L^{-1} myo-inositol, and 100 mg L^{-1} casein hydrolysate were added. The pH was adjusted to 5.7 using NaOH before autoclaving at 121 °C for 30 min.

The experiment used a completely randomized design with a triple factorial scheme consisting of two culture media (MS and WPM), four concentrations of 2.4-D (0, 1, 2 and 4 mg L^{-1}) and four concentrations of BAP (0, 0.25, 0.5 and 1.0 mg L^{-1}). Each treatment consisted of 5 Petri dishes containing 5 explants each.

After culture establishment, the explants were maintained in the dark in a growth chamber at a temperature of 26± 2 °C for 60 days for callus formation. At 30 days after culture establishment, the explants were transferred to fresh culture media for continuous callus multiplication. At 60 days after culture establishment, the explants were transferred to fresh culture media without 2,4-D, where they remained for another 30 days in a growth chamber under a 16 h photoperiod at 28 ± 2 °C with a PFD of 52.5 W m^{-2} s^{-1}. The experiment lasted for a total of 90 days. Subsequently, the percentages of callus and pro-embryogenic mass formation were evaluated. The data were subjected to analysis of variance. The means of the qualitative data were subjected to Tukey's test, and the quantitative data were subjected to polynomial regression ($p < 0.05$) using R software (R Core Team 2020).

RESULTS AND DISCUSSION

The analysis of variance showed a significant effect of the triple interaction among the factors evaluated on callus formation and pro-embryogenic mass formation (Table 1). The analysis of the culture media containing the different

concentrations of 2,4-D and BAP (Table 1) revealed a higher percentages of callus formation in the WPM culture medium than in the MS culture medium, particularly at higher concentrations of 2,4-D (2 and 4 mg L^{-1}). When 2,4-D was not added to the culture medium, significant differences between culture media were observed only with 0.5 mg L^{-1} BAP, with 0% callus formation in the MS culture medium and 60% callus formation in the WPM culture medium. When using 1 mg L^{-1} of 2,4-D, no significant differences in callus or mass formation were observed among the treatments. However, the WPM culture medium presented higher means for callus and mass formation compared with those in the MS culture medium.

At a concentration of 2 mg L^{-1} 2,4-D, significant differences were reported only in the combinations with 0.25 and 0.5 mg L^{-1} BAP, resulting in 66.7% and 66.7% callus formation in the MS culture medium and 80.0% and 93.3% callus formation in the WPM culture medium, respectively. The highest mean values for callus formation were observed for all the treatments at the concentration of 4 mg L^{-1} 2,4-D. When 4 mg L^{-1} 2,4-D was combined with 0.25 and 1 mg L^{-1} BAP, 100% callus formation was reported in the WPM culture medium.

Table 1. Percentage of callus formation in *M. dubia* stem segments as a function of the culture medium at each BAP and 2,4-D level

2,4-D (mg L^{-1})	**BAP** (mg L^{-1})	**MS**	**WPM**
		Callus formation (%)	
0	0	0.0 a	0.0 a
0	0.25	13.3 a	26.6 a
0	0.5	0.0 b	60 a
0	1	13.3 a	33.3 a
1	0	26.6 a	26.6 a
1	0.25	93.3 a	93.3 a
1	0.5	66.6 a	86.6 a
1	1	60.0 a	73.3 a
2	0	46.6 a	53.3 a
2	0.25	66.6 b	80.0 a
2	0.5	66.6 b	93.3 a
2	1	86.6 a	93.3 a
4	0	40.0 b	73.3 a
4	0.25	73.3 b	100 a
4	0.5	93.3 a	93.3 a
4	1	73.3 b	100 a
CV (%)		23.74	

* Means followed by the same letter in the same row do not differ significantly from each other by the Tukey test at the 5% probability level.

Similar results were observed by Brijwal et al. (2015), who verified that the WPM medium was more effective than other media for leaf explants of *Berberis aristata*, a medicinal and woody plant, with 97% callus formation. Conversely, Ahmed et al. (2011), when evaluating the effect of different culture media on callus induction in *Phyla nodiflora* L. Greene, reported that MS medium combined with 0.6 mg L^{-1} of 2,4-D produced more embryogenic calli (94.5%) than WPM medium.

The WPM culture medium was developed for woody crops such as *M. dubia* and has more diluted nutrient concentrations, mainly of nitrogen and potassium, than other culture media such as MS and JADS. This fact confirms the efficiency of the WPM culture medium for the *in vitro* propagation of woody species. Sources of N help to stimulate the proliferation of pro-embryogenic masses and their transformation into different embryonic stages (Carlsson et al. 2017).

Figure 1 shows the percentage of callus formation in *M. dubia* explants when subjected to different concentrations of BAP and 2,4-D with MS culture medium (Figure 1A) and WPM culture medium (Figure 1B). In the evaluation of the interaction between the BAP and 2,4-D concentrations in the MS culture medium (Figure 1A), an increase in the percentage of callus formation was observed as the concentrations of the growth regulators increased. The best result was obtained at a concentration of 4 mg L^{-1} 2,4-D combined with 0.90 mg L^{-1} BAP, which resulted in 99.9% callus formation. Conversely, lower percentages of callus formation were observed when no 2,4-D was added to the culture medium. When using only BAP, a small increase in callus formation was observed with the increase in cytokinin concentrations up to 0.73 mg L^{-1}, resulting in 37% callus formation.

At concentrations of 2 mg L^{-1} and 1 mg L^{-1} 2,4-D, an increase in the percentage of callus formation was observed as the BAP concentration increased to 0.9 and 0.64 mg L^{-1}, resulting in means of 95.3% and 93.07% callus formation, respectively. Concentrations of BAP higher than 0.64 mg L^{-1} reduced callus formation. No callus formation was observed when no growth regulators were added to the culture medium. Conversely, higher callus induction percentages were observed for the explants established in the culture media containing the highest concentrations of growth regulators, revealing that they are needed for callus induction in *M. dubia* explants.

By evaluating the interaction between the concentrations of BAP and 2,4-D with WPM culture medium (Figure 1B), similar behavior to that in the MS culture medium was observed. With increasing concentrations of 2,4-D and BAP, the

results for callus induction improved. However, the results observed in the WPM culture medium were better than those observed in the MS medium, and the WPM medium generally provided higher callus formation. Higher callus induction was obtained from WPM culture medium combined with 4 mg L^{-1} 2,4-D and 0.95 mg L^{-1} BAP, which resulted in 100% callus formation. When 2,4-D was not added to the culture medium, lower percentages of callus formation (34%) were obtained at 0.87 mg L^{-1} BAP. In treatments without BAP or 2,4-D, the results were much lower than those obtained in the treatments that had cytokinin, demonstrating the need for the interaction of auxin and cytokinin in callus induction for *M. dubia* explants.

Similarly, Navroski et al. (2012) observed that when using lower concentrations of BAP, callus formation was reduced in stem segments of *Satureja hortensis* L., and a concentration of 19.45 µM resulted in more callus formation. The effects of BAP and 2,4-D on *Sorghum bicolor* explants indicated that 4 mg L of 2,4-D combined with 1 mg L of BAP were more efficient to callus induction (Espinoza-Sánchez et al. 2018). Similar results for the formation of friable calli were obtained in *Berberis aristata* which presented greater callus proliferation with the combination of 2,4-D (4.53 µM), BAP (2.22 µM) and NAA (2.68 µM), which resulted in 97% callus formation in leaf explants (Brijwal et al. 2015).

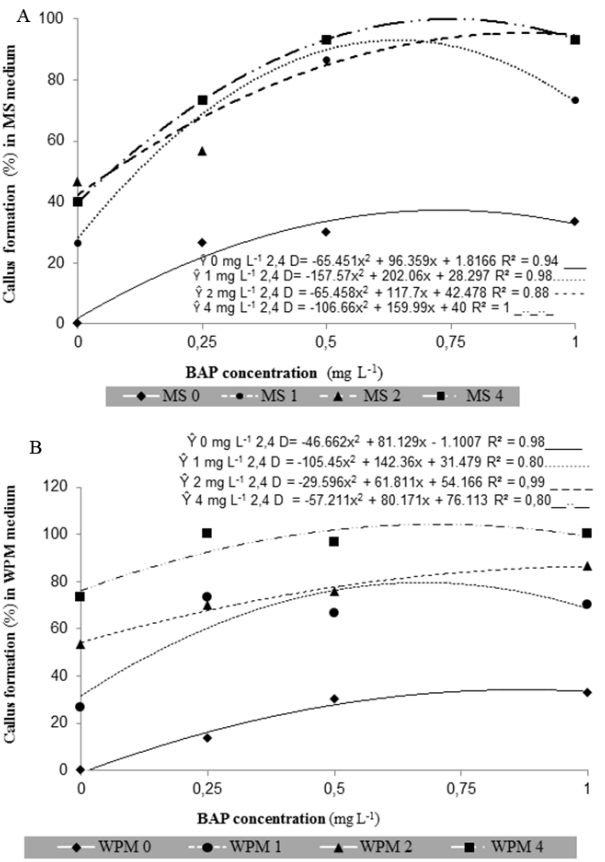

Figure 1. Percentage of callogenesis in *M. dubia* stem segments as a function of the BAP and 2,4-D concentrations in the MS (1A) and WPM (2A) media.

The results reported by Silveira et al. (2020) corroborate the present results. The authors reported no callus induction in leaflets of *Plinia peruviana* in the absence of 2,4-D. Instead, higher means for callus induction were observed at a concentration with 10 µM of 2,4-D, when the explants were maintained in the dark.

A satisfactory percentage of callus and pro-embryogenic mass formation was obtained in the present study. This result is important since it can be used to improve the process of *in vitro M. dubia* propagation via somatic embryogenesis (SE). In the first stage of the SE is the callus induction, which include the dedifferentiation of cells, activation of cell division and reprogramming of cell physiology, metabolism, and gene expression patterns (Zimmerman 1993). The results revealed the formation of pro-embryogenic masses at the highest concentrations of 2,4-D in combination with BAP. Pro-embryogenic masses were not formed at lower concentrations of these growth regulators (Figure 2).

For the formation of pro-embryogenic mass (PEM), a triple interaction among the tested factors was observed, showing that the culture media and the concentrations of BAP and 2,4-D used in this experiment are directly related to PEM formation. The WPM culture medium presented a higher percentage of PEM formation than the MS medium. PEM formation also presented the best results when high concentrations of BAP and 2,4-D were combined in the WPM medium (Table 2). When 2,4-D was not added to the culture medium, a significant difference was observed only when 0.5 mg L^{-1} of BAP was used, which resulted in 46.6% PEM formation. At the concentration of 1 mg L^{-1} 2,4-D, no differences were reported between the two media; however, the highest mean values were obtained in the WPM culture medium.

At a concentration of 2 mg L^{-1} 2,4-D, differences among treatments were observed only with 1 mg L^{-1} of BAP, which resulted in 60% PEM formation in the MS culture medium and 86.66% PEM formation in the WPM culture medium. At the 4 mg L^{-1} concentration of 2,4-D, when combined with 0.25, 0.5, and 1 mg L^{-1} BAP, the treatments presented the

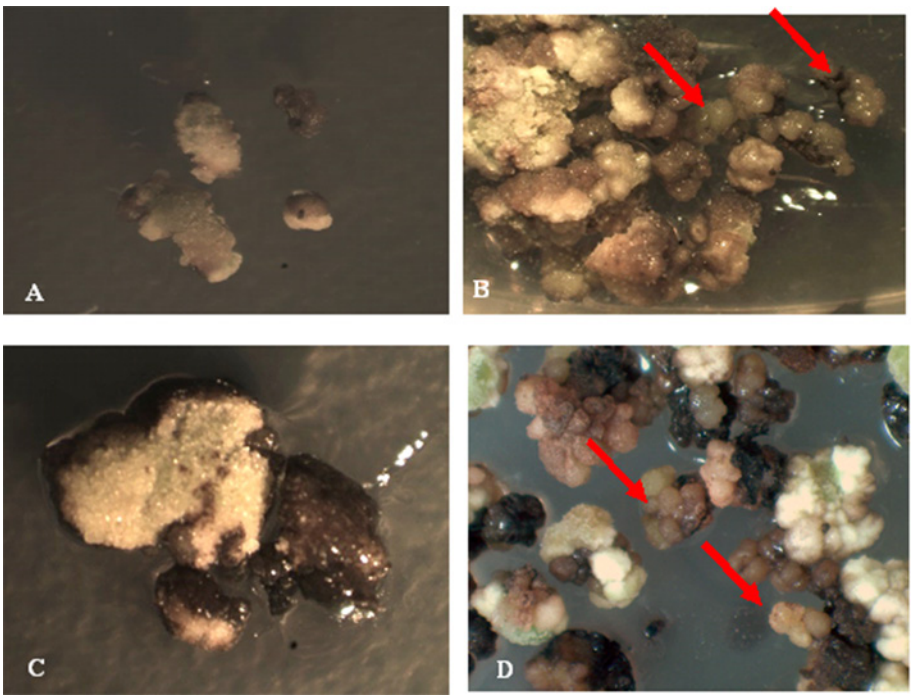

Figure 2. Callus (A and C) and pro-embryogenic mass (PEM) formation with globular somatic embryo production (B and D) in *M. dubia* stems at different concentrations of 2,4-D and BAP. (A) 0.25 mg L^{-1} BAP in MS culture medium, (B) 4 mg L^{-1} 2.4 D + 1.0 mg L^{-1} BAP in MS culture medium, (C) 0.25 mg L^{-1} BAP in WPM culture medium, and (D) 4 m L^{-1} 2.4 D + 1 mg L^{-1} BAP in WPM culture medium.

highest means, with 100% PEM formation. The composition of the culture medium is fundamental for the growth and development of *in vitro* calli. Thus, the WPM medium proved to be the best for the *in vitro* cultivation of *M. dubia* since its nutritional composition favors the *in vitro* cultivation of woody species. The culture medium is essential for the success of somatic embryogenesis (Ramos et al. 2020), and the nutritional balance of macronutrients, micronutrients, vitamins and carbohydrates is essential for morphogenetic transformations in the process of cell dedifferentiation and differentiation (Kulus and Tymoszuk 2020).

M. dubia explants presented excellent PEM formation responses with the addition of 2,4-D and BAP to the WPM culture medium. This finding will make advances in research on the *in vitro* regeneration of *M. dubia* possible. Figure 3 shows the percentage of PEM formation in *M. dubia* explants subjected to different concentrations of BAP and 2,4-D in the MS culture medium (Figure 3A) and the WPM culture medium (Figure 3B).

When evaluating the interaction between the 2,4-D and BAP concentrations in the MS culture medium (Figure 3A), a linear increase in PEM formation was observed with the increase in the concentration of plant growth regulators.

Table 2. Percentage of pro-embryogenic mass formation in *M. dubia* explants in different culture media at each BAP and 2,4-D concentration

2,4 D (mg L^{-1})	**BAP** (mg L^{-1})	**MS**	WPM
		Callus formation (%)	
0	0	0. a	0.0 a
0	0.25	0.0 a	16.6 a
0	0.5	0.0 b	46.6 a
0	1	6.6 a	6.66 a
1	0	10.0 a	23.3 a
1	0.25	86.6 a	93.3 a
1	0.5	66.6 a	80.0 a
1	1	53.3 a	73.3 a
2	0	20. 0 b	46.6 a
2	0.25	26.6 b	73.3 a
2	0.5	60.0 b	86.6 a
2	1	86.6 a	93.3 a
4	0	33.3 b	66.6 a
4	0.25	66.0 b	100 a
4	0.5	86.6 a	100 a
4	1	93.3 a	100 a
CV (%)	23.52		

* Means followed by the same letter in the same row do not differ significantly by the Tukey test at the 5% probability level.

Better results were observed when using 4 mg L^{-1} 2,4-D combined with 0.63 mg L^{-1} BAP, with a mean of 97.6% PEM formation. At the concentration of 2 mg L^{-1} 2,4-D, linear growth was observed with the increase in the BAP concentration, resulting in a mean of 86.6% PEM formation. At concentrations of 0 and 1 mg L^{-1} 2,4-D, an increase in PEM formation occurred when the BAP concentration increased to 0.62 and 0.67 mg L^{-1}, with means of 16.40 and 91% PEM formation, respectively.

The responses of PEM formation in *M. dubia* explants varied depending on the culture medium and on the concentrations of BAP and 2,4-D used in the experiment (Figure 3B). The WPM medium was more efficient for PEM formation than the MS medium, and better results were obtained when the WPM medium was combined with 4 mg L^{-1} 2,4-D and 1 mg L^{-1} BAP, which resulted in 100% PEM formation. At concentrations of 1 mg L^{-1} 2,4-D, linear growth was observed with the increasing BAP concentration. When 2,4-D was not added to the culture medium, the PEM formation results were lower; however, a slight increase in the means was observed with an increase in the BAP concentration to up to 0.75 mg L^{-1}, resulting in 42% PEM formation.

The results with low percentages were due to osmotic stress (Kadokura et al. 2018). Similar results were observed by Ratanasanobon and Seaton (2010) when evaluating the effect of different concentrations of 2,4-D on the induction of somatic embryogenesis in leaf explants of *Chamelaucium spp*. The authors found that higher concentrations (5 µM) of 2,4-D favored tissue differentiation and somatic embryo formation. It is observed in the literature that the use of 2,4 -D individually has greater success (Bajpai et al. 2016, Kamle and Baek 2017). Luis and Scherwinski-Pereira (2014) efficiently induced embryogenic callus formation in *Acrocomia aculeate* by using low concentration of 2,4-D (1.5 mg L^{-1}).

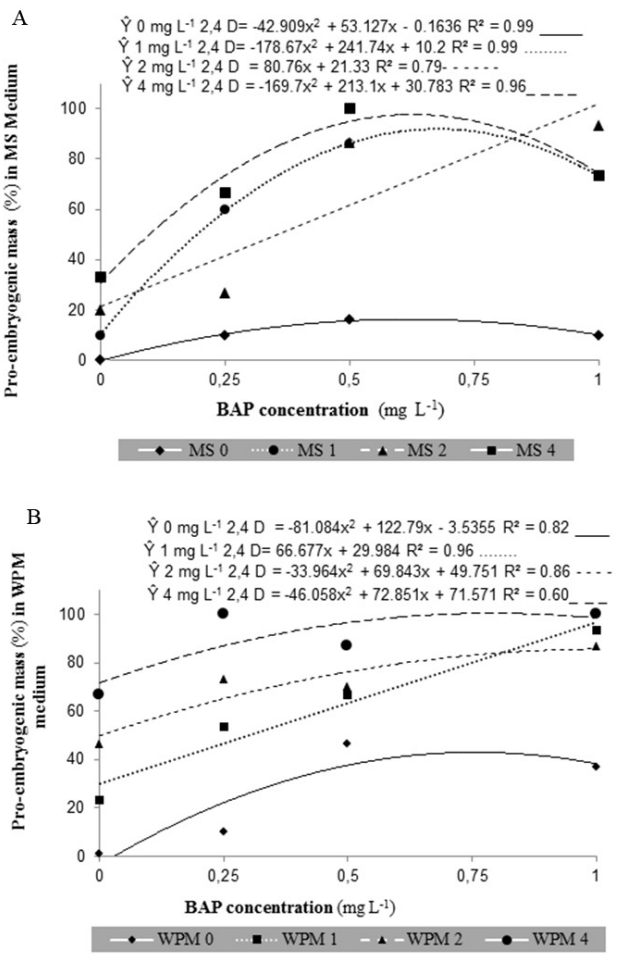

Figure 3. Percentage of pro-embryogenic mass formation in *M. dubia* explants in MS (A) and WPM (B) culture media at different BAP and 2,4-D concentrations.

Aman and Afrasiab (2014) obtained greater somatic embryo formation (100%) in *Rosmarinus officinalis* L. explants, when using 2.25 µM of 2,4-D and 2.25 µM of BAP in WPM culture medium. The authors observed no embryo formation when only 2,4-D was used, requiring the combination of auxin and cytokinin. Conversely, Sharmin et al. (2014), in a study on *Wedeli acalendulacea* Less, testing different concentrations of 2,4-D, verified greater conversion of callus into somatic embryos when using low concentrations of 2,4-D (0.5 mg L^{-1}).

CONCLUSION

This is the first study to show the benefits of 2,4-D for the induction of somatic embryogenesis in *Myrciaria dubia*. Under the tested conditions for the callus induction of somatic embryogenesis in *Myrciaria dubia*, the WPM culture medium should be used in combination with 4 mg L^{-1} 2,4-D and 0.25 mg or 1 mg L^{-1} BAP. This research provides significant results for the development of a feasible and efficient *in vitro* propagation system for *Myrciaria dubia*.

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