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Somatic Embryogenesis of *Myrciaria dubia* (Kunth.) McVaugh

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Abstract. Myrciaria dubia **(caçari or camu-camu) is a species with great relevance because of its high levels of bioactive compounds and antioxidant activity. The species is propagated mainly by seed, which leads to high genetic diversity. Producing plants that maintain their characteristics on a large scale at a low cost is a challenge for fruit species, especially in the Myrtaceae family. Therefore, this study aimed at assessing the effects of different plant growth regulators at various concentrations for the induction of somatic embryogenesis from caçari nodal segments and leaf disk explants. Two independent experiments were performed using nodal segments and leaf disks from plants grown in the greenhouse. In the first experiment, the combined effect of auxin and cytokinin at different concentrations was evaluated: 2,4-dichlorophenoxyacetic acid at 0, 1, 2, and 4 mg·L^{-1}; and benzylaminopurine at 0, 0.25, 0.5, and 1 mg·L^{-1}). In the second experiment, the application of different plant growth regulators (benzylaminopurine, kinetin, thidiazuron, and isopentenyl adenine) and their concentrations (0, 2, 4, and 6 mg·L^{-1}) were evaluated. In both experiments, the basic culture medium was woody plant medium. Callus formation via nodal segments and leaf disks occurred in the first 30 d from cultivation and proved to be responsive to induction by 2,4-dichlorophenoxyacetic acid and benzylaminopurine. Auxin proved to be essential for somatic embryogenesis induction in nodal segments using 1 and 4 mg·L^{-1} of 2,4-dichlorophenoxyacetic acid alone. The results of this experiment will help advancing protocols for regeneration of somatic embryos and elucidating the physiological, molecular, and genetic mechanisms involved in the process of somatic embryogenesis for *M. dubia*. The development of an efficient protocol for in vitro clonal propagation of this species also lays the groundwork for further optimization of the system for genetic transformation.**

Myrciaria dubia (Kunth) McVaugh is a plant in the Myrtaceae family, commonly known as camu-camu or caçari and is widely distributed in the Amazon region. Because of the high content of bioactive chemical compounds in the plant and fruit constituent parts (seed, peel, and pulp), it is currently considered one of the most valuable Amazonian native fruit-bearing trees in the world.

Caçari fruit have high nutritional value and bring several benefits to human health through a range of by-products such as popsicles, jellies, and yogurts (Grigio et al. 2019, 2021a, 2022). The plant's mineral compounds (potassium, calcium, magnesium, and sodium) (Sousa et al. 2015), phenolic compounds (ellagin, anthocyanins, flavonoids, carotenoids), antioxidant activity, and mainly vitamin C content (7355.20–13,756.79 mg·100 g^{-1} of pulp) currently make this species the largest known source of natural vitamin C worldwide (Chagas et al. 2015; Grigio et al. 2021b; Ribeiro et al. 2016).

Moreover, the bioactive compounds can minimize the risk of incidence of some chronic diseases because they demonstrate antigenotoxic and anti-inflammatory properties (Fidelis et al. 2020; Silva et al. 2019), neuroprotective effects (Azevêdo et al. 2015), antihyperglycemic and antihypertensive activity (Fidelis et al. 2020; Fujita et al. 2015), chromosome damage prevention (Carmo et al. 2019), antimalaria potential, and schistosomicidal activity (Carmo et al. 2020). As a consequence of such benefits, the demand for this species has increased significantly, leading to the need for the development of cultivation practices—in particular, more efficient propagation methods for multiplication, conservation, and regeneration of this species.

Multiplication of selected clones has been reported as one of the limitations in caçari propagation (Chagas et al. 2015). Currently, the most commonly used method for the propagation of caçari is through seeds, which generate wide genetic diversity (Chagas et al. 2015), thus affecting the plant cycle and its chemical attributes, such as vitamin C content (Grigio et al. 2022). Therefore, propagation by stem cuttings has been the selected method for maintaining the parent plants' genetic characteristics, such as uniformity, reduced size, and early fruit production (Hartmann et al. 2011). However, this technique has not been efficient in the multiplication of many selected genotypes because of the high cost of large-scale multiplication, including operations that are time-consuming and require large physical areas. In addition, rooting of cuttings shows low success.

Somatic embryogenesis is a widely used technique for large-scale in vitro propagation of many species (Von Arnold et al. 2002). The process of somatic embryo induction and plant regeneration is influenced by several factors, such as species, explant source, culture medium, and type and concentration of auxins and cytokinins (Araujo et al. 2016; Araújo et al. 2021; Gulzar et al. 2020; Wójcikowska and Gaj 2017).

Somatic embryogenesis has been applied and documented successfully for clonal propagation of fruit-bearing species such as *Acca sellowiana* (Cangahuala-Inocente et al. 2007, 2014; Pavei et al. 2018), *Psidium guajava* L. (Akhtar 2013; Bajpai et al. 2016; Kamle et al. 2014; Nasim 2010), *Eugenia involucrata* (Golle et al. 2020) and *Syzygium cumini* (L.) (Naaz et al. 2019).

The only study of in vitro culture of *M. dubia* reported 99% callus and 93% proembryogenic mass (PEM) formation (Araújo et al. 2021). The best results in the study were obtained using woody plant medium (WPM) (Lloyd and McCown 1980) culture medium supplemented with 4 mg·L^{-1} 2,4-dinitrophenoxyacetic acid (2,4-D) and 1 mg·L^{-1} benzylaminopurine (BAP). However, no somatic embryo induction was observed. WPM has lower values of salts and macronutrients, being the usual method used in the micropropagation of woody plants (Phillips and Garda 2019).

Such limitations create an opportunity to advance the multiplication process via somatic embryogenesis and to develop a feasible protocol for the commercial micropropagation of caçari. Therefore, our study aimed at assessing the effect of different plant growth regulators at various concentrations on the induction of somatic embryogenesis in caçari using nodal segments and leaf disks as explants.

Material and Methods

Plant material. Two independent experiments were conducted. Both experiments involved the use of stem nodal segments ~1.0 cm in length and leaf disks with a 0.7-cm diameter as explants, which were extracted from caçari plants germinated in a greenhouse. The explants were taken to the Ornamental Horticulture Laboratory of the University of Florida in Homestead, FL, and submitted to a precleaning process with Oxidate 2.0 (1 mL·L^{-1}) for 30 min. The material was then transferred to a laminar flow chamber for disinfestation using ethanol 70% for 1 min, sodium hypochlorite 3% with six drops of Tween 20 for 15 min, and streptomycin (200 mL·L^{-1}) for 30 min, followed by three washes with distilled autoclaved water. After disinfestation, stem nodal segments and leaf disks were placed in petri dishes.

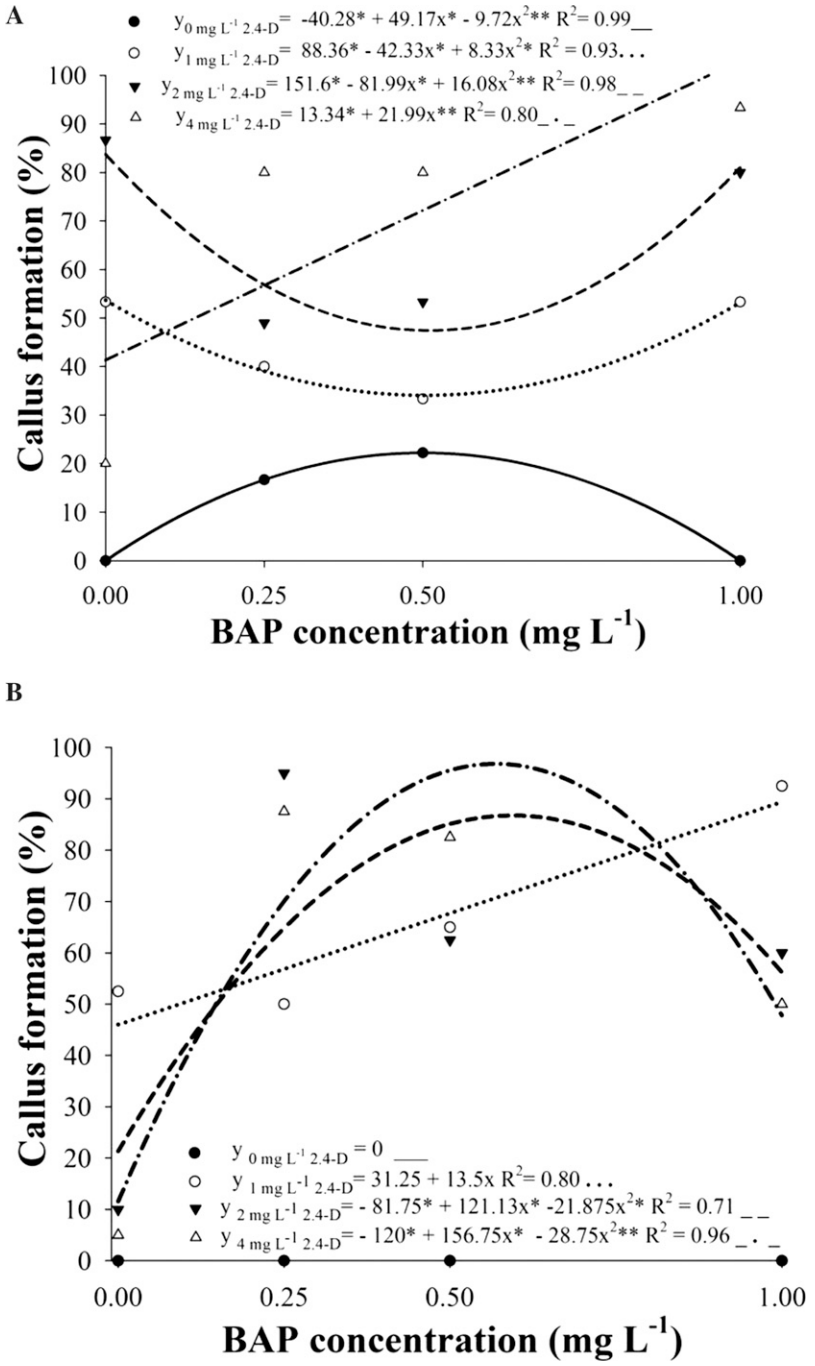

Fig. 1. Percentage of callus formation in caçari stem nodal segments (**A**) and leaf disks (**B**) in benzylaminopurine (BAP) concentrations within each 2,4-dichlorophenoxyacetic acid concentration.

Induction of somatic embryogenesis. For Expt. 1, treatments consisted of 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}). For Expt. 2, treatments consisted of combinations of four plant growth regulators [BAP, kinetin, thidiazuron (TDZ) and isopentenyl adenine (2iP)] at different concentrations (0, 2, 4, and 6 mg·L^{-1}). The culture medium for the two experiments consisted of WPM (Lloyd and McCown 1980) supplemented with 3.5 g·L^{-1} phytagel, 30 g·L^{-1} sucrose, 100 mg·L^{-1} myoinositol, and 100 mg·L^{-1} casein hydrolysate, with the addition of 4 mg·L^{-1} 2,4-D to all treatments. The pH was adjusted to 5.7 before autoclaving at 121 °C for 30 min. The experimental design was randomized in a 4 × 4 factorial design, with five repetitions and four explants per replication for each treatment.

After culture establishment, the explants were maintained in the dark in a growth chamber at 26 ± 2 °C for 60 d for callus formation and under multiplication for two subculturing periods of 30 d in the same culture medium as described earlier. After 120 d, explants were transferred to fresh culture medium without 2,4-D, where they remained for another 30 d in the same environmental conditions.

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Table 1. Percentage of callus formation in caçari from stem nodal segments and leaf disks at 30 and 60 d under different 2,4-dichlorophenoxyacetic acid (2,4-D) and benzylaminopurine (BAP) concentrations.

		Callus formation (%)[i]			
		Stem nodal segments		Leaf disks	
2,4-D (mg·L^{-1})	BAP (mg·L^{-1})	30 d	60 d	30 d	60 d
0	0	0.0 ± 0 a[ii]	0.0 ± 0 a	0.0 ± 0 a	0.0 ± 0 a
0	0.25	6.66 ± 14.90 a	20.00 ± 18.25 a	0.0 ± 0 a	0.0 ± 0 a
0	0.5	13.33 ± 18.25 a	20.00 ± 29.81 a	0.0 ± 0 a	0.0 ± 0 a
0	1	0.0 ± 0 a	0.0 ± 0 a	0.0 ± 0 a	0.0 ± 0 a
1	0	53.33 ± 18.25 a	53.33 ± 18.25 a[iii]	50.00 ± 35.35 a	55.00 ± 31.59 a
1	0.25	40.00 ± 14.90 a	40.00 ± 14.90 a	45.00 ± 20.91 a	50.00 ± 17.68 a
1	0.5	33.33 ± 0 a	33.33 ± 0 a	60.00 ± 22.36 a	70.00 ± 11.18 a
1	1	53.33 ± 29.81 a	53.33 ± 29.81 a	90.00 ± 13.69 a	95.00 ± 11.18 a
2	0	53.33 ± 0 b	86.66 ± 18.25 a	10.00 ± 13.69 a	10.00 ± 13.69 a
2	0.25	33.33 ± 0 a	40.00 ± 14.90 a	95.00 ± 11.18 a	95.00 ± 11.18 a
2	0.5	53.33 ± 18.25 a	53.33 ± 18.25 a	60.00 ± 13.69 a	65.00 ± 13.69 a
2	1	80.00 ± 18.25 a	80.00 ± 18.25 a	60.00 ± 13.69 a	60.00 ± 13.69 a
4	0	6.66 ± 14.90 a	20.00 ± 18.25 a	5.00 ± 11.18 a	5.00 ± 11.18 a
4	0.25	80.00 ± 18.25 a	80.00 ± 18.25 a	85.00 ± 22.36 a	90.00 ± 22.36 a
4	0.5	80.00 ± 18.25 a	80.00 ± 18.25 a	80.00 ± 20.91 a	85.00 ± 22.36 a
4	1	93.33 ± 14.90 a	93.33 ± 14.90 a	50.00 ± 30.61 a	50.00 ± 30.61 a
Coefficient of variation (%)	—	31.91		29.36	

[i] Average ± standard deviation (n = 128).
[ii] Means followed by the same letter in a row do not differ significantly from each other by Tukey's test ($P > 0.05$).
[iii] Treatment that had formed somatic embryos from stem segment explant.

Cytochemical analysis. After 30 and 60 d, the percentage of callus and PEM formation were evaluated, respectively; after 150 d, somatic embryo formation was assessed. PEM formation was evaluated via cytochemical analysis, which was performed using 100 mg of callus tissue from each treatment, subjected to double acetocarmine/Evans blue staining according to the methodology described by Steiner et al. (2005). Stained cells were mounted on slides and photographed using a digital camera coupled to an Olympus BX 60 light microscope equipped with a 10× objective. A red, green, blue image analysis was performed using the histogram tool in Adobe® Photoshop® CS3 version 10.0.

Statistical analysis. Data were tested for normality according to the Shapiro-Wilk test, and for homogeneity of variances according to the Bartlett test ($P < 0.05$). With normality and homogeneity assumptions met, data were submitted to two-way analysis of variance by the F test ($P < 0.05$), followed by the Tukey test ($P < 0.05$) for group means. The quantitative data were subjected to polynomial regression ($P < 0.05$) using R software 4.2.2 (R Foundation for Statistical Computing, Vienna, Austria).

Results

Induction of somatic embryogenesis in caçari using 2,4-D and BAP. Callus formation from stem nodal segment cultures was observed at 60 d in cultures containing 2 mg·L^{-1} of 2,4-D and no BAP, and it was significantly greater than callus formation at 30 d ($P < 0.05$) (Table 1).

The use of 4 mg·L^{-1} of 2,4-D resulted in the greatest percentage of callus formed from stem nodal segments, and this growth was directly proportional to the increase in BAP concentrations, up to 1 mg·L^{-1} (Fig. 1A). However, callus formation was not significantly different when using either 0.25 or 0.5 mg·L^{-1} of BAP ($P > 0.05$) (Table 1).

For leaf disks, high percentages of callus formation were observed when 1, 2, and 4 mg·L^{-1} of 2,4-D were used combined with BAP at concentrations of 0.25, 0.5, and 1 mg·L^{-1}. However, there were no statistical differences between assessments at 30 and 60 d ($P > 0.05$) (Table 1). The use of 1 mg·L^{-1} of 2,4-D showed a linear increase in callus formation as BAP concentration in the culture medium increased, and 92.5% of calluses formed were obtained at the greatest BAP

Table 2. Level of color of red and blue images of caçari callus from stem nodal segments and leaf disks double-stained with acetocarmine and Evans blue as a function of 2,4-dichlorophenoxyacetic acid (2,4-D) and benzylaminopurine (BAP) concentrations.

		Level of color (byte)[i]			
		Stem nodal segments		Leaf disks	
2,4-D (mg·L^{-1})	BAP (mg·L^{-1})	Red	Blue	Red	Blue
0	0	0.0 ± 0 a[ii]	0.0 ± 0 a	0.0 ± 0 a	0.0 ± 0 a
0	0.25	0.0 ± 0 a	0.0 ± 0 a	0.0 ± 0 a	0.0 ± 0 a
0	0.5	0.0 ± 0 a	0.0 ± 0 a	0.0 ± 0 a	0.0 ± 0 a
0	1	0.0 ± 0 a	0.0 ± 0 a	0.0 ± 0 a	0.0 ± 0 a
1	0	39.90 ± 21.22 a	24.38 ± 22.19 b	52.16 ± 13.45 a	62.91 ± 10.62 a
1	0.25	32.35 ± 27.70 a	26.62 ± 25.33 a	43.28 ± 11.44 a	53.70 ± 13.89 a
1	0.5	27.96 ± 14.54 a	22.73 ± 11.86 a	67.07 ± 9.27 a	38.06 ± 9.04 a
1	1	34.22 ± 8.47 a	37.54 ± 8.60 a	90.06 ± 22.89 a	70.13 ± 27.41 a
2	0	48.86 ± 14.10 a	33.05 ± 16.85 b	52.26 ± 4.61 a	65.14 ± 7.67 a
2	0.25	26.55 ± 9.18 a	23.47 ± 9.31 a	71.08 ± 13.30 a	55.35 ± 14.46 a
2	0.5	21.20 ± 6.08 a	15.08 ± 5.98 a	55.80 ± 12.51 a	61.77 ± 20.03 a
2	1	41.20 ± 1.93 a	33.09 ± 5.70 a	84.88 ± 19.46 a	81.90 ± 32.68 a
4	0	29.90 ± 11.29 a	26.00 ± 14.08 a	67.70 ± 14.66 a	115.25 ± 13.12 b
4	0.25	55.63 ± 10.02 a	44.56 ± 14.25 a	66.54 ± 16.49 a	78.27 ± 25.41 a
4	0.5	68.12 ± 7.37 a	75.03 ± 5.14 a	33.41 ± 9.90 a	37.10 ± 10.69 a
4	1	41.90 ± 7.20 a	36.73 ± 6.59 a	38.28 ± 16.55 a	50.70 ± 19.04 a
Coefficient of variation (%)	—	22.46		14.78	

[i] Average ± standard deviation (n = 128).
[ii] Means followed by the same letter in a row do not differ significantly from each other by Tukey's test ($P > 0.05$).

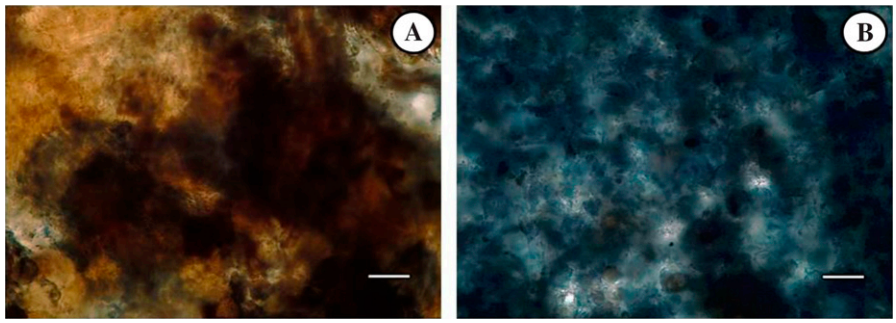

Fig. 2. Callus of caçari from stem nodal segments cultured on 1 mg·L^{-1} 2,4-dichlorophenoxyacetic acid showing reaction to acetocarmine stain (red) (**A**) and to Evans blue stain (blue) (**B**). Bar = 50 μm.

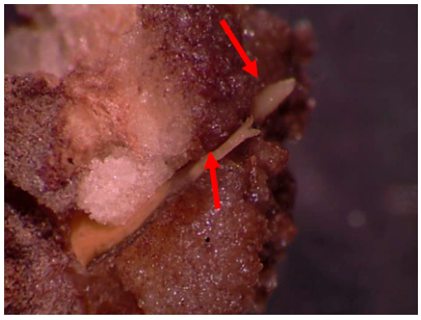

Fig. 3. Somatic embryo of caçari developed from stem nodal segments in vitro with 1 mg·L^{-1} 2,4-dichlorophenoxyacetic acid.

concentration (Fig. 1B). Concentrations of 2 and 4 mg·L^{-1} of 2,4-D showed a quadratic effect, with greater percentages of callus formed from leaf disks at BAP concentrations of 2.77 and 2.73 mg·L^{-1}, providing 85.9% and 93.7% callus formation, respectively (Fig. 1B).

The quality of the callus induced via stem nodal segments and leaf disks in the different treatments was evaluated by cytochemical analyses, using a double-staining technique with acetocarmine and Evans blue, to assess their potential for conversion into PEMs (Table 2).

For callus induced via stem nodal segments, the double-staining analysis showed a statistical difference across the cells assessed in the treatments with 1 and 2 mg·L^{-1} of 2,4-D without BAP ($P < 0.05$) (Table 2). The level of red staining is significantly more intense than the blue staining (Fig. 2A and B), showing the potential for PEM formation.

Somatic embryo induction and development was observed from stem nodal segments in cultures with 1 mg·L^{-1} of 2,4-D alone (Fig. 3).

When comparing the cytochemical analyses between stem nodal segments and leaf disks, calli from stem nodal segments showed a greater potential for PEM formation, although there were no significant differences between treatments across the calli double-stained with acetocarmine and Evans blue ($P > 0.05$) (Table 2).

Induction of somatic embryogenesis in caçari using cytokinin concentrations. Callus induction from stem nodal segments and leaf disks at 30 and 60 d after in vitro culture with four cytokinins (BAP, kinetin, TDZ, and 2iP) under different concentrations (0, 2, 4, and 6 mg·L^{-1}) showed no statistical difference for the percentage of callus formed ($P > 0.05$) (Table 3).

Callus induction from both stem nodal segments and leaf disks started in the first 30 d after in vitro culture establishment. The greatest percentages of callus induction, ranging from 53.3% to 100%, were obtained using stem nodal segments as explants, regardless of the plant growth regulator and concentration used (Table 3). The greatest callus induction from leaf disks was 60% at 60 d, which is less responsive than stem nodal segments (Table 3).

Callus formation in stem nodal segments showed ascending linear behavior when explants were cultured with TDZ and kinetin, reaching up to 100% (Fig. 4A). When 2iP was used at 2.16 mg·L^{-1}, callus formation was reduced to 68.1%. Callus formation in stem nodal segments cultured with BAP showed a significant reduction as BAP concentration increased (Fig. 4A). Callus formation from leaf disks with BAP, kinetin, and 2iP showed a positive linear increase as concentrations of these cytokinins increased. However, for all treatments, the maximum callus formation was 65% (Fig. 4B).

When TDZ was used up to 2.78 mg·L^{-1} in leaf disks, a small increase (35.6%) in callus formation was noted. Subsequently, well-defined somatic embryos formed and developed (Fig. 5). Somatic embryo formation was observed only in cultures with 4 mg·L^{-1} of 2,4-D, without any cytokinin (Table 3), thus validating the results from Expt. 1.

The quality of the callus induced from stem nodal segments and leaf disks was

Table 3. Percentage of callus formation in caçari stem nodal segments and leaf disks at 30 and 60 d under different cytokinin types and concentrations.

		Callus formation (%)[ii]			
		Stem nodal segments		Leaf disks	
Cytokinin[i]	Concentrations (mg·L^{-1})	30 d	60 d	30 d	60 d
BAP	0	86.66 ± 29.81 a[iii]	100.00 ± 0 a[iv]	20.00 ± 27.38 a	20.00 ± 27.38 a
BAP	2	80.00 ± 18.25 a	80.00 ± 18.25 a	20.00 ± 11.18 a	30.00 ± 11.18 a
BAP	4	80.00 ± 18.25 a	80.00 ± 18.25 a	15.00 ± 13.69 a	25.00 ± 17.68 a
BAP	6	53.33 ± 18.25 a	53.33 ± 18.25 a	35.00 ± 22.36 a	45.00 ± 27.38 a
Kinetin	0	60.00 ± 14.90 a	66.67 ± 0 a	5.00 ± 11.18 a	10.00 ± 13.69 a
Kinetin	2	60.00 ± 14.90 a	60.00 ± 14.90 a	45.00 ± 32.59 a	50.00 ± 25 a
Kinetin	4	100.00 ± 0 a	100.00 ± 0 a	40.00 ± 41.07 a	45.00 ± 11.18 a
Kinetin	6	80.00 ± 0 a	80.00 ± 18.25 a	55.00 ± 17.68 a	60.00 ± 13.69 a
TDZ	0	53.33 ± 29.1 a	53.33 ± 29.81 a	5.00 ± 11.18 a	5.00 ± 11.18 a
TDZ	2	100.00 ± 0 a	100.00 ± 0 a	25.00 ± 17.67 a	40.00 ± 13.69 a
TDZ	4	100.00 ± 0 a	100.00 ± 0 a	20.00 ± 20.91 a	45.00 ± 11.18 a
TDZ	6	100.00 ± 0 a	100.00 ± 0 a	20.00 ± 11.18 a	25.00 ± 13.69 a
2iP	0	80.00 ± 18.25 a	80.00 ± 18.25 a[iv]	10.00 ± 13.69 a	20.00 ± 27.38 a
2iP	2	66.67 ± 0 a	73.33 ± 14.90 a	40.00 ± 13.69 a	40.00 ± 13.69 a
2iP	4	73.33 ± 27.88 a	73.33 ± 24.88 a	45.00 ± 41.07 a	60.00 ± 22.36 a
2iP	6	100.00 ± 0 a	100.00 ± 0 a	50.00 ± 17.69 a	65.00 ± 13.69 a
CV (%)	—	13.74		45.38	

[i] 2iP = isopentenyl adenine; BAP = benzylaminopurine; *CV* = coefficient of variation; TDZ = thidiazuron.
[ii] Average ± standard deviation (n = 128).
[iii] Means followed by the same letter in a row do not differ significantly from each other by Tukey's test ($P > 0.05$).
[iv] Treatments that had formed somatic embryos from stem segment explant.

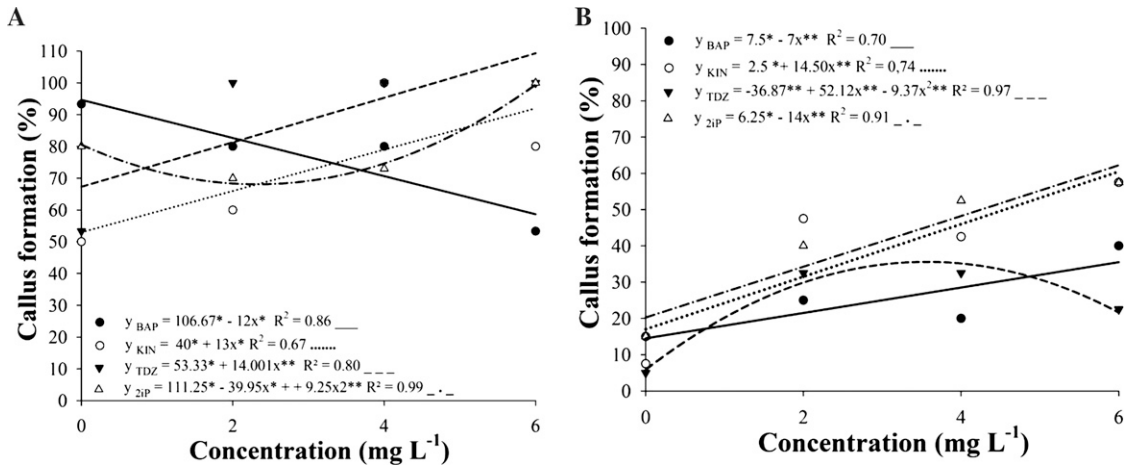

Fig. 4. Percentage of callus formation in caçari stem segments (**A**) and leaf disks (**B**) in the unfolding of growth regulators at different concentrations.

assessed using the same cytochemical analyses described previously (Table 4).

For callus induced from stem nodal segments, only TDZ at 6 mg·L^{-1} and 2iP at 2, 4, and 6 mg·L^{-1} showed significant differences between red and blue ($P < 0.05$), and the greatest values were observed for blue ($P < 0.05$) (Table 4). When leaf disks were used as explants, BAP (4 and 6 mg·L^{-1}), kinetin (6 mg·L^{-1}), and TDZ (2 mg·L^{-1}) did not show significant differences between red and blue ($P > 0.05$). For all treatments with or without significant differences, blue values were superior to those of red, demonstrating that the callus from leaf disks did not show a potential for PEM formation (Fig. 6).

Discussion

For woody species, callus induction has presented great challenges. In *M. dubia*, reports on callus formation and development of somatic embryos are even more scarce in the literature. The low response to callus formation and/or embryogenesis in woody species occurs as a result of the high exudation of phenolics and other compounds (Corredoira et al. 2015).

Our study showed high callus formation in caçari explants from nodal segments cultured with 2,4-D for both experiments. Although the results for leaf disks showed these explants to be more responsive to PEMs, they represented a great advance for stem nodal segments, because plant regeneration by somatic embryogenesis occurs from already differentiated cells (Ikeuchi et al. 2016).

It is known that somatic embryogenesis can be induced directly or indirectly (Pulianmackal et al. 2014; Yang and Zhang 2010). Direct somatic embryogenesis occurs without callus formation, whereas indirect somatic embryogenesis goes through callus formation before somatic embryos are formed. For indirect somatic embryogenesis to occur, PEM formation must occur first; later, formation, maturation, and, consequently, conversion of the somatic embryo must occur (Von Arnold et al. 2002). Thus, the callus formation process in caçari explants used in PEM production was fundamental because it is the intermediate stage between callus and somatic embryos (Halperin 1986). Studies of the in vitro behavior of callus and/or somatic embryos in caçari, including developmental stages, are very limited. Araújo et al. (2021) reported attempts to obtain somatic embryos, although without success. However, callus and PEM formation were observed in nodal segments of caçari at 30 d after in vitro culture establishment.

The use of the double-staining technique with acetocarmine and Evans blue proved to be efficient to observe PEMs, which are cell agglomerates capable of producing somatic embryos, with smaller cells and dense cytoplasm (Xu and Huang 2008). These cells are reactive to acetocarmine and produce a red color when in contact with the dye (Steiner et al. 2005), as shown in Figs. 2A, 6B, and 6C. In contrast, nonembryogenic cells are more elongated and react with Evans blue dye, showing a blue color (Steiner et al. 2005), as in Figs. 2B and 6A. Similar responses were observed in *Byrsonima intermedia* (Silva et al. 2018) and *Araucaria angustifolia* (Farias-Soares et al. 2014), where callus that reacted with acetocarmine could develop into somatic embryos.

Several factors influence the somatic embryogenesis process, including the source of explant, the culture medium, and the type and concentration of plant growth regulators

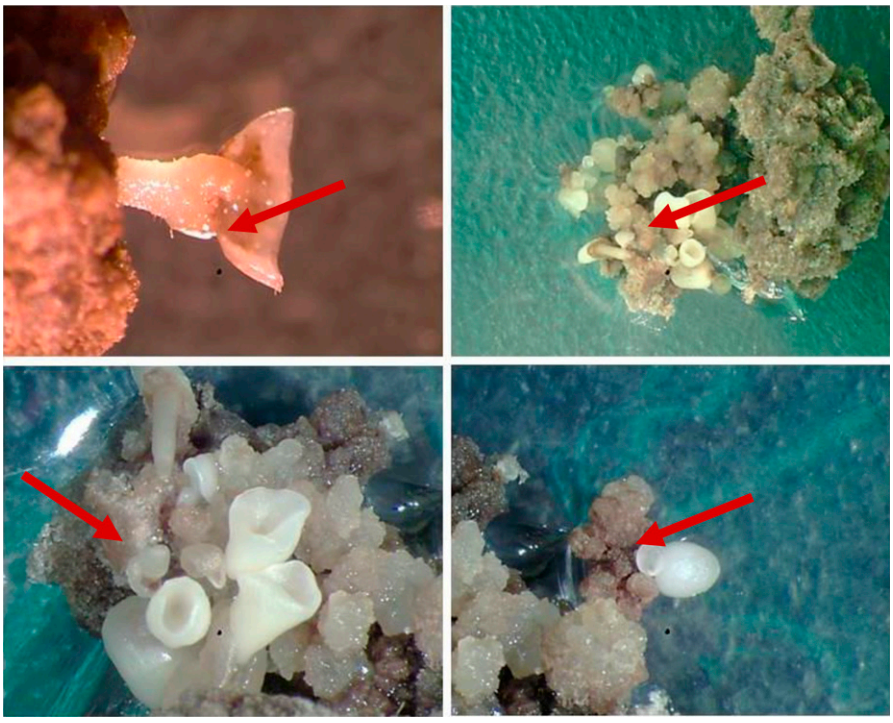

Fig. 5. Somatic embryos in caçari in vitro cultures from stem nodal segments cultured with 4 mg·L^{-1} 2,4-dichlorophenoxyacetic acid.

Table 4. Level of color of red and blue images of caçari callus from stem nodal segments and leaf disks double-stained with acetocarmine and Evans blue for different cytokinin types and concentrations.

		Callus formation (%)[ii]			
		Stem nodal segments		Leaf disks	
Cytokinin[i]	Concn (mg·L^{-1})	Red	Blue	Red	Blue
BAP	0	65.43 ± 22.21 a[iii]	70.26 ± 24.01 a	79.39 ± 5.26 b	112.83 ± 6.59 a
BAP	2	65.02 ± 12.67 a	66.98 ± 15.54 a	68.77 ± 19.62 b	98.72 ± 7.49 a
BAP	4	47.08 ± 18.81 a	41.75 ± 12.54 a	72.95 ± 20.31 a	90.05 ± 23.33 a
BAP	6	37.04 ± 8.95 a	50.88 ± 13.82 a	72.31 ± 24.58 a	89.00 ± 14.58 a
Kinetin	0	58.28 ± 6.22 a	49.30 ± 6.07 a	53.05 ± 8.0 b	85.62 ± 9.99 a
Kinetin	2	26.90 ± 13.36 a	25.00 ± 12.17 a	53.62 ± 9.71 b	80.20 ± 12.53 a
Kinetin	4	41.77 ± 12.55 a	54.82 ± 17.89 a	41.30 ± 10.61 b	73.78 ± 16.30 a
Kinetin	6	37.25 ± 6.59 a	40.30 ± 7.03 a	60.95 ± 11.21 a	84.77 ± 12.02 a
TDZ	0	47.68 ± 6.14 a	45.08 ± 6.40 a	96.26 ± 16.0 b	150.38 ± 13.96 a
TDZ	2	36.09 ± 15.79 a	46.01 ± 16.35 a	49.81 ± 24.32 a	67.98 ± 27.02 a
TDZ	4	62.75 ± 16.55 a	53.13 ± 25.84 a	110.87 ± 8.44 b	149.78 ± 5.39 a
TDZ	6	90.82 ± 26.29 b	129.76 ± 23.22 a	70.22 ± 30.77 b	99.62 ± 20.50 a
2iP	0	87.98 ± 9.90 a	88.90 ± 8.46 a	59.73 ± 20.36 b	111.70 ± 15.80 a
2iP	2	45.06 ± 10.80 b	84.43 ± 11.14 a	59.28 ± 10.78 b	117.28 ± 7.49 a
2iP	4	42.85 ± 18.81 b	72.92 ± 24.26 a	50.84 ± 23.55 b	82.85 ± 23.33 a
2iP	6	49.50 ± 8.95 b	76.45 ± 7.91 a	41.70 ± 13.84 b	70.11 ± 14.56 a
CV (%)	—	13.36		12.30	

[i] 2iP = isopentenyl adenine; BAP = benzylaminopurine; *CV* = coefficient of variation; TDZ = thidiazuron.
[ii] Average ± standard deviation (n = 128).
[iii] Means followed by the same letter in a row do not differ significantly from each other by Tukey's test ($P > 0.05$).

(Araujo et al. 2016; Gulzar et al. 2020; Wójcikowska and Gaj 2017). Our results demonstrate that callus formation and PEM induction varied according to the type of explant used (stem nodal segments and leaf disks) and type and concentration of plant growth regulators—specifically, auxin—without which no somatic embryos formed. This result is in contrast to the observations made by Yang and Zhang (2010), who found auxin was essential for proliferation of PEMs, but inhibited the development of somatic embryos.

Somatic embryogenesis in cultures with low 2,4-D concentration (1 mg·L^{-1}) was also observed for other species in the Myrtaceae family, such as *A. sellowiana* (Cangahuala-Inocente et al. 2007, 2014; Fraga et al. 2012; Pavei et al. 2018; Pescador et al. 2012) and *P. guajava* (Akhtar 2013; Kamle et al. 2014; Nasim 2010). For *P. guajava*, low concentration of 2,4-D (1 mg·L^{-1}) resulted in the greatest induction of somatic embryos, whereas greater concentrations of 2,4-D (>1.0 mg·L^{-1}) led to a reduction in frequency and intensity of somatic embryo formation. This response to low concentrations of auxin, leading to somatic embryo formation, was also reported by Verma et al. (2018). In our study, high 2,4-D concentrations (>2 mg·L^{-1}) induced callus formation; however, they did not induce somatic embryo formation. Such a response is important, because it is a starting point for technique improvement, contributing to scientific and technological research, with the possibility of obtaining an efficient protocol for somatic embryogenesis in caçari.

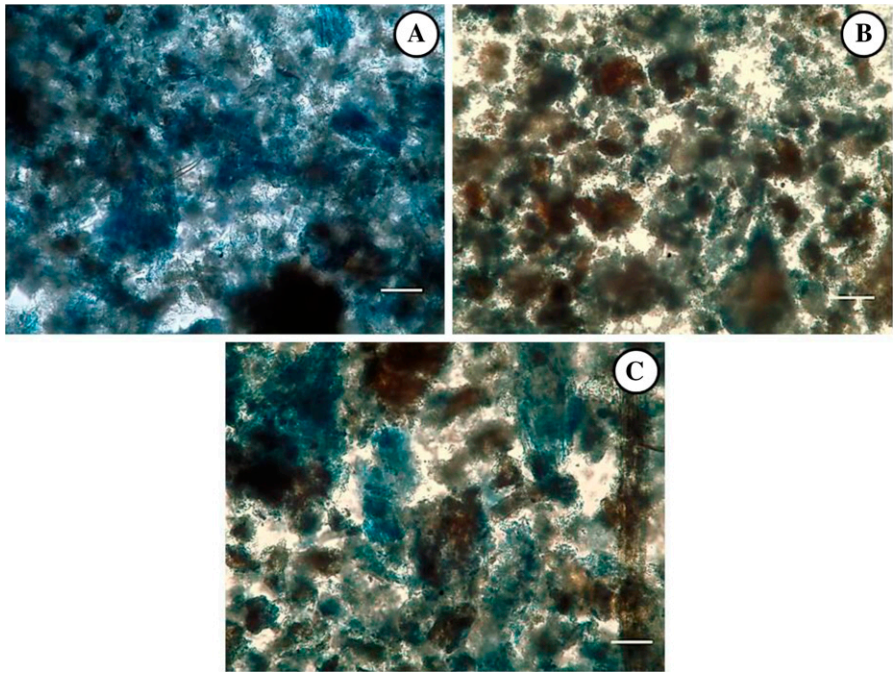

Fig. 6. (**A**) Callus masses from stem nodal segments and leaf disks of caçari reacting to Evans blue stain (blue). (**B, C**) Proembryogenic masses induced from stem nodal segments cultured with 4 mg·L^{-1} 2,4-dichlorophenoxyacetic acid, reacting to acetocarmine stain (red), representative of treatments that will induce somatic embryos. Bar = 50 μm.

Somatic embryogenesis is induced via stress by means of genes that share the signaling response to stress (Gulzar et al. 2020). Excessive stress results in cell collapse and death. However, lower levels of stress cause increases in metabolic activity and induce somatic embryogenesis (Altamura et al. 2016; Gulzar et al. 2020).

Plant growth regulators are some of the main factors that drive the embryogenic response, with auxin as an important factor that induces the signaling for in vitro induction of the somatic embryo (Verma et al. 2018; Wójcikowska and Gaj 2017; Wójcikowska et al. 2013). In our study, auxin efficiency was clear, because the presence of 2,4-D at 4 mg·L^{-1} resulted in the induction of somatic embryos. According to Gulzar et al. (2020), somatic embryo formation is induced during a long exposure to auxin, thus confirming our results and showing that the use of 2,4-D was essential to the signaling of somatic embryo induction.

The induction of somatic embryos exclusively from stem nodal explants was not expected, because success in somatic embryogenesis using leaf disks has been reported previously for several woody species, such as *Caesalpinia echinata* (Werner et al. 2010), *A. angustifolia* (Stachevski et al. 2013), and

E. involucrata (Golle et al. 2020), among others. Although somatic embryo induction from leaf disks has been reported for some species, for caçari these explants were not responsive.

Several factors influence somatic embryogenesis induction, such as genotype (Dias et al. 2018) and the source of explants (Araújo et al. 2021). In our study, somatic embryo induction was observed only in PEMs induced from stem nodal segments, which were more responsive to all plant growth regulators in greater concentrations (range, 4–6 $mg \cdot L^{-1}$). This demonstrates that the somatic embryogenesis process for caçari depends on these factors.

Subsequent studies should address the optimization of the protocol to improve somatic embryo formation and regeneration into plantlets.

Conclusion

We showed that somatic embryo induction in caçari is possible using stem nodal segments and WPM culture medium supplemented with 1 or 4 $mg \cdot L^{-1}$ 2,4-D alone. Callus formation from leaf disks occurred during the first 30 d of in vitro culture. The results of this experiment will help to advance somatic embryogenesis for many species of the Myrtaceae family and to elucidate the physiological, molecular, and genetic mechanisms involved in the process of somatic embryogenesis for *M. dubia*. The development of an efficient protocol for in vitro clonal propagation of this species also lays the groundwork for further optimization of the system for genetic transformation.

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