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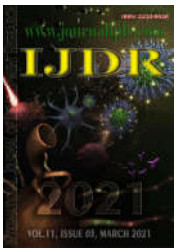

RESEARCH ARTICLE OPEN ACCESS

CALLUS INDUCTION AND QUALITY IN FOLIAR EXPLANTS OF *ALLOPHYLUS EDULIS* (A.ST.-HIL., CAMBESS. & A. JUSS) RADLK, A NATIVE FOREST SPECIES WITH MANY USES

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ABSTRACT

Allophylus edulis, known as cocum, is a medicinal species native to Brazil and used for its anti-diarrheal, anti-inflammatory and antihypertensive properties. The species also produces high quality wood, which can be used in the production of charcoal, is considered a valuable ornamental species, being useful for urban afforestation, in addition to the recovery of degraded areas. Despite the considerable increase in the use of native species, few studies have focused on the *in vitro* cultivation of these species. Considering the importance and complexity of the success of micropropagation, the aim of the present study was to examine the effects of 6-benzylaminopurine (BAP) and naphthalene-acetic acid (NAA) on the *in vitro* callogenesis of *A. edulis* leaf explants. Only the control treatment failed to produce 100% of callus, and the highest intensities of callogenesis, fresh mass and dry mass were obtained with 2.5 and 5.0 mg L^{-1} of BAP and 2.0 mg L^{-1} of NAA. The treatments produced calluses and consistencies of different colors. Both 2.0 mg L^{-1} of NAA and 2.5 mg L^{-1} of BAP must be added to the culture medium to promote the formation of calluses of *A. edulis* with morphological characteristics suitable for organogenesis.

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INTRODUCTION

Allophylus edulis (A.St.-Hil., Cambess. &A.Juss.) Radlk belongs to the Sapindaceae, which is subordinate to the Sapindales, and angiosperm order which that includes 12 families, 460 genera, and 5670 species (Buerki *et al*., 2010). The tree is native to Brazil, where it is commonly known as chal-chal, pigeon fruit, vacunzeiro, and red myrtle (Lorenzi, 2002). The species is also known as paraó-fruit in southeastern Brazil, vacum in southern Brazil (Somner *et al*., 2013), and cocum in central-west Brazil and along the border between Brazil and Paraguay (Alves *et al*., 2008). The fruits of the species are red, sweet, and edible, whereas their flowers are melliferous (Franco and Fontana, 2011). Previous studies have reported that*Allophylusedulis* fruits possess antioxidant, anticholinesterase, and cytotoxic activities (Umeo *et al*., 2011), and *Allophylus edulis* leaves are commonly used for their antidiarrheal (Alves *et al*., 2008), anti-inflammatory, and

It is also possible to find reports of the species genotoxic activity (Yajia *et al*., 1999) and insect-repelling properties (Castillo *et al*., 2009). Furthermore, the identification of alkaloids, flavonoids (anthraquinones and naphthoquinones) and essential oils (steroids and triterpenoids) in *Allophylusedulis* (Tirloni *et al*., 2015) demonstrates that the antimicrobial and antioxidant activities of ethanolic leaves extracts are not toxic to rats. The species produces good-quality wood that can be used for several purposes, including carpentry and the production of charcoal, mats, and posts (Seneme *et al*., 2006). It is considered ornamental and can be used for urban tree planting, owing to the nature of the species root system, the speciesadaptability, and its medium size, which allows planting along sidewalks and central avenues, even where there are electricity and telephone networks. The species is also recommended for use in the recovery of degraded areas, especially during the initial and medium stages of habitat recovery in Mixed Ombrophylous Forest, Deciduous Seasonal Forest,

Despite considerable increases in the use of native species, relatively few studies have focused on the *in vitro* cultivation of such species. The establishment of *in vitro* plant cultures includes a variety of procedures, including callogenesis, which is considered a requirement for a variety of morphogenetic routes, such as somatic embryogenesis (Pinheiro *et al*., 2012), resistant strain selection (Esmaiel *et al*., 2012), and genetic transformation (Cidade *et al*., 2006). The regeneration of plants *in vitro* is considered complex because of the many external and internal factors, which include the genotype, source, and physiological conditions of the plant material, the presence of plant growth regulators, especially auxin and cytokinins, in the culture medium, the characteristics of the culture medium, and environmental conditions (Hussein and Aqlan, 2011). Therefore, in-depth studies are needed to elucidate the mechanisms that underlie the regeneration of plants from isolated cells, organs, or plant tissues (Junghans and Souza, 2013). The objective of the present study was to examine the effects of 6-benzylaminopurine (BAP) and naphthalene-acetic acid (NAA) on the *in vitro* callogenesis of *Allophylus edulis*leaf explants.

MATERIAL AND METHODS

Study site description and plant material: The present study was conducted at the Plant Biotechnology Laboratory of the Center for Biotechnology and Genetic Improvement of Sugarcane at the Federal University of Grande Dourados (UFGD).

Young vegetative branches were collected from ~ 4-year-old *Allophylusedulis* specimens that were located on the UFGD campus. After collection, the branches were taken to the laboratory, where they were left in running water for 24 h. Afterward, leaves were excised from the branches and were sterilized in a horizontal laminar flow chamber. During the sterilization procedure, the leaves were soaked in 70% (v/v) ethyl alcohol for 2 min, soaked in sodium hypochlorite (2.5% chlorine active, v/v) for 6 min, and then washed three times with distilled and autoclaved water. Explants (~1.0 cm^2) were obtained, maintaining the central vein.

Explants inoculation and the treatments of growth regulators: The explants were inoculated, with the abaxial portion in contact with medium, into test tubes that contained 10 mL MS culture medium (Murashige and Skoog, 1962), which was amended with 6.0 g L^{-1} agar, 30 g L^{-1} sucrose, and 100 mg L^{-1} ascorbic acid. The medium was also supplemented with different concentrations of BAP (6-benzylaminopurine) and NAA (naphthalene-acetic acid; Table 1). When preparing the medium, the pH was adjusted to 5.8 ± 0.1 before autoclaving (121°C and 1.05 kg cm^{-2} for 20 min). After inoculation, the plant material was kept in a growth room at 25 ± 2°C for 7 d in the dark and then maintained under a 16-h photoperiod (45 µmol m^{-2} s^{-1}) provided by white fluorescent lamps. At 30 d after inoculation, callus induction (percentage explants with callus), fresh and dry callus mass, callogenesis intensity (0–5, based on amount of callus formed), callus consistency (friable or compact), and callus color were evaluated.

Statistical procedures: A completely randomized design was used and the data were analyzed by analysis of variance and, when significant, the means were compared using a Tukey's test, at a 1% ($p < 0.01$) level of error probability, using the software GENES (Cruz, 2013).

RESULTS

Significant differences were observed for all of the analyzed variables, with interactions between plant regulator inclusion and BAP concentration. Even though all of the treatments, except the control treatment (T0), yielded 100% callus formation (data not shown), there were significant differences ($p \leq 0.05$) in callogenesis intensity (Figures 1 and 2). For example, the callogenesis intensity of explants that were cultured with both auxin and cytokinin (T2 and T3)

Table 1. Treatments with different concentrations of 6-benzylaminopurine (BAP) and naphthalene-acetic acid (NAA) for callus induction in *Allophylus edulis* leaf explants

Treatment	NAA (mg L^{-1})	BAP (mg L^{-1})
T0	0.0	0.0
T1	2.0	0.0
T2	2.0	2.5
T3	2.0	5.0

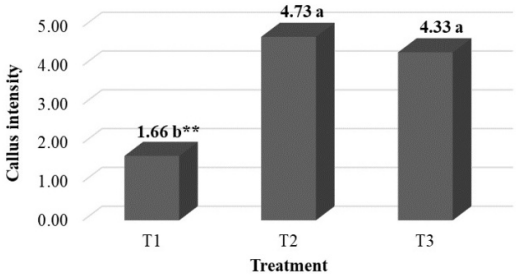

Figure 1. Intensity of callogenesis in different treatments in explants of*Allophylus edulis*.Average intensity of callus from leaf explant *Allophylus edulis* depending on the BAP levels and NAA, 30 days after inoculation (0-absent, 1-poor, 2-poor moderate; 3-moderate, 4-high moderate and 5-High). ** significant at the 1% probability level ($p < 0.01$) by the F test

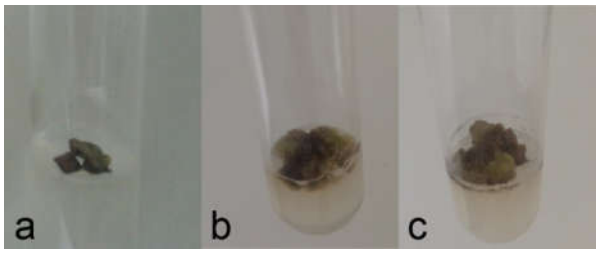

Figure 2. Callus appearance after 30 days of inoculation.Aspect of callus intensity in leaf explants of *Allophylus edulis* grown at different concentrations of BAP (a – 0.0, b – 2.5, c – 5.0 mg L^{-1}) combined with 2.0 mg L^{-1} of NAA, 30 days after inoculation.

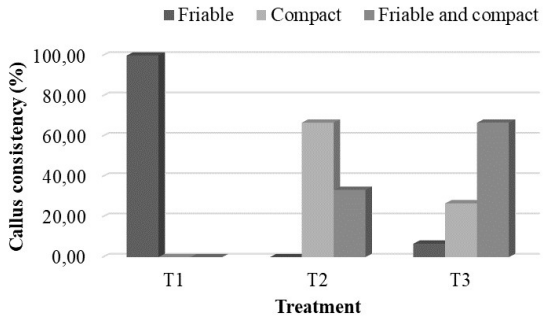

Figure 3. Consistency of callogenesis.Callus consistency (%) in leaf explants of *Allophylus edulis* as a function of BAP levels combined with 2.0 mg L^{-1} of NAA, 30 days after inoculation.

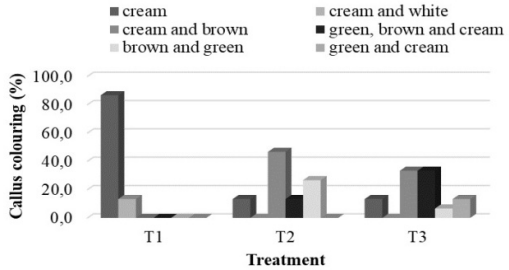

Figure 4. Staining developed in the callogenesis after 30 days of explant inoculation.Callus coloring (%) on leaf explants of *Allophylus edulis* as a function of BAP levels combined with 2.0 mg L^{-1} of NAA, 30 days after

Table 2. Effect of different treatments on leaf explants of *Allophylus edulis* on fresh mass, dry mass 24 hours and dry mass 48 hours

Treatment	Fresh mass (g)	Dry mass (24 h) (g)	Dry mass 48 h (g)
T1	0.075 b**	0.009 b**	0.009 b**
T2	0.653 a	0.077 a	0.079 a
T3	0.509 a	0.066 a	0.065 a
MG	0.412	0.051	0.050
CV%	30.90	26.00	25.78

** significant at the level of 1% probability ($p < 0.01$) * significant at the level of 5% probability of error ($0.01 = < p < 0.05$), by the F test.

differed from that of explants that were only cultured with auxin (T1). In addition, the greatest mean callogenesis intensities (4.73 and 4.33) were produced by the T2 and T3 treatments, respectively, and were statistically similar. Meanwhile, the T1 treatment, which lacked BAP, yielded a lower mean intensity (1.66) than the other treatments.

The presence of BAP affected explant budding intensity and both fresh and dry callus mass. The greatest intensity and masses (fresh and dry) were produced by the T2 and T3 treatments (Table 2). However, even though there were no significant statistical differences between the intensities or masses of the treatment groups, a decreasing trend was observed in the measurements of the T3 group, which may indicate that the combination of 2.5 mg L^{-1} BAP and 2.0 mg L^{-1} NAA was sufficient for cell proliferation. The combinations used in different treatments also affected callus consistency, which ranged from friable to compact (Figure 3). Both the T1 and T3 treatments yielded friable callus. However, only friable callus (100%) was produced when NAA was used alone (i.e., without BAP; T1), and the treatment with the highest concentration of BAP (T3) yielded only 6.7% friable callus. Meanwhile, compact and friable-compact callus was only formed by explants that were treated with a combination of BAP and NAA (T2 and T3; Figure 3). For example, 67.7% of the T2 explants produced compact callus, and 33.3% of the T2 explants produced both compact and friable-compact callus. In contrast, 26.7 and 66.7% of the T3 explants produced compact and friable-compact callus, respectively. The different treatments also yielded explants with up to three different colors (Figure 4). The T1 explants, for example, which lacked BAP, exhibited cream and cream-white callus (86.7 and 13.3%, respectively). Meanwhile, the T2 and T3 explants exhibited greater variation in coloration. More specifically, the T2 explants produced cream (13.3%), cream-brown-green (13.3%), brown-green (27.7%), and cream-brown (46.7%) callus, whereas the T3 explants produced cream (33.3%), cream-brown (33.3%), brown-green (33.3%), cream and cream-green (13.7%), and brown-green (6.7%) callus.

DISCUSSION

Regarding the callus induction percentage, the inclusion of cytokinin in the culture medium had a positive effect. However, it is possible that the concentrations used interact with the amount of auxin in the explants. Indeed, the induction of callus by *Jatropha curcas* L. leaf segments indicated that there was a significant interaction between BAP and IBA concentration, and this callus was used to produce adventitious shoots (Feitosa *et al*., 2013). Santos *et al*. (2008) obtained high rates of cell masses in nodal and leaf segments of *Coffea canephora* 'Apoatã' (*Robusta coffee*) using 2,4-D, but the interaction of 2,4-D and IBA failed to yield satisfactory results. In the case of Cerrado fruit species, growth regulators are even more important since most require the stimuli generated by growth regulators, which are indispensable for the proper development of the *in vitro* culture. However, the responses vary according to the species, classes, types, and doses of growth regulator used (Pinhal *et al*., 2011). The endogenous auxin concentrations of *Allophylusedulis* leaf explants are probably slightly higher than the endogenous cytokinin concentrations no callus formation was observed in the absence of BAP. Therefore, the exogenous supply of growth regulators, via culture medium, is often necessary for callogenesis (Pêgo *et al*., 2013) and may even increase the cellular totipotency (Garcia *et al*., 2011). Similarly, Cordeiro *et al*. (2004) reported low frequencies of callus formation in *Schizolobiumamazonicum* (paricá) when growth regulators were excluded from the culture medium and that the incidence of callus formation increased with increasing BAP concentrations. However, Brunetta *et al*. (2006) reported that callogensis by *Swietenia macrophylla* (mahogany) hypocotyl segments was relatively high (91%) on media that contained 0.5 mg L^{-1} NAA, regardless of BAP content. Cells can become competent as long as the interaction between endogenous and exogenous factors, such as the concentration and type of growth regulator, is balanced since such conditions can trigger molecular events that affect gene expression and tissue differentiation (Carvalho *et al*., 2015; Rosa *et al*. 2015). Nogueira *et al*. (2007) reported that callus induction is dependent on an intermediate balance of auxin and cytokinin. However, it is unlikely that such an intermediate balance was reached during the present study because none of the treatment groups exhibited shoot or adventitious root formation, probably owing to imbalances in endogenous hormonal levels that, ultimately, promoted callus formation and inhibited adventitious shoot formation.

In the present study, cytokinin was also essential to high callogenesis intensity, and treatments that included BAP resulted in substantially more intense callogenesis. Indeed, Davies (1990) proposed that cytokinins are essential to the processes of cell division and tissue differentiation, and Erig and Schuch (2005) reported that apple foliar explants cultivated with cytokinin and auxin yielded the greatest callus intensities.

Furthermore, in the absence of such regulators, callus formation failed to occur. Considering the fresh callus mass of *Cissus sisyoides* (plant insulin), Rodrigues and Almeida (2010) verified that the BAP concentrations used did not differ among themselves. However, the dry callus mass was greater, although not significantly, in plants treated with 6.0 mg L^{-1} BAP than in those treated with either 2.0 or 4.0 mg L^{-1} BAP. The results of the present study were also similar to those of Cerqueira *et al*. (2002), who reported that, in *Tridax procumbens* (bull-grass) leaf segments, treatment with 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} BAP yielded the greatest fresh callus mass, whereas treatments with either 1.0 mg L^{-1} IBA or 2.0 mg L^{-1} NAA + 2.0 mg L^{-1} IBA yielded the greatest dry callus mass. During *in vitro* culture, the salt and sugar components of culture media affect cell growth and morphogenesis, via osmosis, in addition to culture nutrition (George *et al*., 2013).

The *Allophylusedulis* leaf explants yielded callus of various textures and colors. Interestingly, these parameters have been used as indicators of the regenerative capacity of the plant materials (Rodrigues *et al*., 2006). Carvalho *et al*. (2015) reported that, in callus produced by foliar explants of *Passiflora gibertii* (passion fruit), light and compact coloring indicated greater organogenetic potential. Brunetta *et al*. (2006) reported that treatment with either 4.44 μM BAP + 1.34 μM NAA or 8.88 μM BAP + 5.37 μM NAA caused the epicotyls of *Swietenia macrophylla* (mahogany) to produce green or brown friable callus, only at the epicotyle ends.

Cerqueira *et al*. (2002) reported that the color and consistency of *in vitro*cultured *Tridaxprocumbes* callus was dependent on both the concentration and type of growth regulator used in the culture medium, with the predominant green color and is related to the BAP and NAA regulators. According to George *et al*. (2013), who investigated the effects of media constituents on callus texture and morphology, high-auxin, low-cytokinin media tend to yield friable and moist callus. Therefore, it is plausible that low-auxin, high-cytokinin media would yield compact and dry callus. Callus may contain cells or groups of cells that have active centers responsible for cell division. Under suitable conditions (favorable balance between cytokinin and auxin), such centers are induced and are then able to differentiate into organs. Cells that are capable of responding to specific stimuli are considered competent, cell differentiation and bud or root formation may occur (George *et al*. 2013).

CONCLUSIONS

Exogenous auxin and cytokinin improve the callus formation and callogensis intensity of foliar explants of*Allophylusedulis*. The results of the present study suggest that the addition of both 2.0 mg L^{-1} NAA and 2.5 mg L^{-1} BAP to the culture medium promotes the formation of callus with morphological characteristics that are suitable for organogenesis.

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