

Somatic Embryogenesis Induction in Leaf and Root Explants of *Allophylus edulis* (A.St.-Hil., Cambess. and A. Juss.) Radlk.

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Abstract

Allophylus edulis (A.St.-Hil., Cambess. and A. Juss.) Radlk., commonly known as cocum, belongs to the Sapindaceae family. This species is of great medicinal interest, with studies showing that its fruits have antioxidant, anti-cholinesterase, and cytotoxic activity. In addition, it is used in traditional medicine as an antidiarrheal, anti-inflammatory and antihypertensive. The objective of this study was to perform somatic embryogenesis *in vitro* from leaf and root explants of *Allophylus edulis*, using different 6-benzylaminopurine (BAP) concentrations combined with naphthalene acetic acid (NAA). All treatments exhibited 100% callus formation, except for the treatment without supplementation of growth regulators. The calluses developed in treatments from leaf explants showed up to two colors (brown and brown/cream), and the highest fresh and dry mass was observed in the treatment with 0.5 mg L⁻¹ of BAP with 0.1 mg L⁻¹ of NAA. There was no shoot formation from the leaf explants. The callogenesis in treatments from root segments showed callus formation with up to three colors (brown, brown/cream, and cream/green), and the highest fresh and dry mass was obtained when cultivated with 2.0 mg L⁻¹ of BAP combined with 0.1 mg L⁻¹ of NAA. These auxin and cytokinin concentrations also showed a higher number of shoots. The interaction between auxin and cytokinin is recommended to obtain somatic embryogenesis in root segments and callus with morphological characteristics suitable for organogenesis.

Keywords: *in vitro* morphogenesis; plant biotechnology; plant regulators

Introduction

The species *Allophylus edulis* (A.St.-Hil., Cambess. and A.Juss.) Radlk., is part of the native Brazilian flora from the family Sapindaceae, and is subordinate to the order Sapindales, which encompasses 12 families, 460 genera and 5670 species (Buerki *et al.*, 2010). *Allophylus edulis* is commonly known by several different common names in Portuguese, “chal-chal”, “fruto-do-pombo”, “vacunzeiro” and “murta vermelha” (Lorenzi, 2002). In some southern regions, it is known as “vacuum” (Somner *et al.*, 2013), while in the Midwest and on the border with Paraguay it is called “cocum” (Alves *et al.*, 2008). *Allophylus edulis* has red, sweet, edible fruits (Franco and Fontana, 2011), and occurs naturally in parts of South America and in the Brazilian territory, where its distribution ranges from the Northern to the Southern region (Pasqualli *et al.*, 2015).

This species is of great medicinal importance, as its leaves are used in popular medicine as an antidiarrheal (Alves *et al.*, 2008), anti-inflammatory and antihypertensive (Korbes, 1995), for their genotoxic activity (Yajia *et al.*, 1999), and as an insect repellent (Castillo *et al.*, 2009). Its fruits also have antioxidant, anti-cholinesterase and cytotoxic activities (Umeo *et al.*, 2011). Studies have shown that the antimicrobial and antioxidant activity of ethanolic extracts of *A. edulis* leaves were not toxic in rats (Tirloni *et al.*, 2015).

In vitro cultivation allows the development of new techniques, such as the genetic transformation of plants, and is of great importance for species that present difficulties in propagating by other vegetative or seeding methods (Ferreira *et al.*, 2015). *In vitro* cultivation techniques are essential to produce doubled haploids, obtain virus-free plants and produce secondary metabolites or recombinant proteins in plant cells in suspension (Dagla, 2012). The

presence of growth regulators or plant hormones in the culture medium are essential for plant regeneration *in vitro* and are responsible for inducing physiological responses such as sprouting, rooting and cell elongation (Cid, 2010). Growth regulators are synthetic substances with hormone-like effects (Hinojosa, 2005), with the main classes being cytokinins and auxins (Hussain *et al.*, 2012). Cytokinins promote cell division and are related to adenine (Cid, 2010), while auxins are important for the induction of somatic embryogenesis (Fehér, 2005).

Somatic embryos have great advantages, such as unlimited production of clones with elite characteristics, basic functional and molecular research, synthetic seed production and conservation of genetic resources by cryopreservation (Aslam *et al.*, 2011). Somatic embryos originate from a single cell or somatic cell groups that grow and differentiate into embryos when grown in a suitable culture (Rocha *et al.*, 2016). They can originate via direct or indirect routes. The direct route occurs when the embryo originates from the matrix tissues without forming calluses, while the indirect route occurs when the embryo develops from a callus (Rizvi *et al.*, 2013). Several factors influence the ability to obtain somatic embryos, such as the source of explants, genotype of the donor plant, and the type and concentrations of growth regulators (Ahmadi *et al.*, 2014).

The objective of this study was to perform *in vitro* somatic embryogenesis from leaf and root explants of *Allophylus edulis* using different concentrations of 6-benzylaminopurine (BAP) combined with naphthalene acetic acid (NAA).

Materials and Methods

Study site description and plant material

The experiment 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). *In vitro*-germinated *Allophylus edulis* individuals at 60 days of age were the source of explants used in the experiment. The seedlings were removed from test tubes one by one in a horizontal laminar flow chamber. Then, the leaves and roots were excised to obtain leaf explants of approximately 1.0 cm², maintaining the central vein and root segments of approximately 1.0 cm.

Explants inoculation and the treatments of growth regulators

Leaf explants were inoculated with the abaxial portion in contact with the culture medium, while the root segments were inoculated horizontally. Both explants were inoculated into test tubes with 15 mL of MS culture medium (Murashige and Skoog, 1962), solidified with 6.0 g L⁻¹ agar, with an additional 30 g L⁻¹ sucrose and supplemented with different concentrations of BAP (control, 0.0; T1, 0.25; T2, 0.5; T3, 1.0; and T4, 2.0 mg L⁻¹) combined with NAA (0.1 mg L⁻¹). The pH was adjusted to 5.8 ± 0.1 before autoclaving at 121 °C and pressure of 1.05 kg cm⁻² for 20 minutes.

After inoculation, the plant material was kept in a growth room at 25 ± 2 °C for 7 days in the absence of light

and was subsequently kept under a 16-hour photoperiod (45 μmol m⁻² s⁻¹) supplied by white fluorescent lamps. Forty-five days after the experiment commenced, the percentage of explants with callus, fresh and dry mass of the callus, consistency (friable or compact), color, number of shoots and percentage of explants with shoots were evaluated. The experiment followed a completely randomized design and consisted of five treatments with five replicates each. Each replicate was composed of five test tubes.

Statistical procedures

The data were analyzed using an 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. The numerical data (fresh mass, dry mass, and number of shoots) were transformed into square root of x + 0.5, while the percentage of callus explants were not transformed. Data analysis was performed using the software GENES (Cruz, 2013).

Results

After 45 days of inoculation, a significant difference was observed for all evaluated variables in the different types of explants used in the experiment (leaf and root), except for consistency where all treatments presented as 100% compact. Callus induction also provided 100% callus formation, except for the control treatment (T0), where calluses were not formed (data not shown). The BAP and NAA combinations used in the treatments resulted in callogenesis with up to two colors in the same callus in leaf explants, and up to three colors for root explants. In T3 treatments with leaf explants, calluses were only brown colored (100%); in T1 and T2, 82.4% of calluses were brown and 17.6% were brown/cream colored, and; T4 were brown and brown/cream colored (71.4 and 28.6%, respectively) (Fig. 1).

T1 and T2 in the root segments treatments presented with more colors, i.e., both had calluses with up to three colors: brown, brown/cream and brown/green, with T1 being 64%, 32% and 4%, respectively and T2 with 28%, 48% and 20%, respectively. T3 and T4 had calluses with up to two colors, with T3 having brown and brown/cream calluses (20% and 80%, respectively). However, the T4 treatment was the only one to present calluses with a cream/green color (76%), while 24% of the calluses had brown coloring (Fig. 2).

The BAP and NAA combinations in the treatments cultivated with leaf and root explants provided significant differences at the 1% level, showing an interaction between the concentrations used and the variables fresh and dry mass (Table 1). The leaf explants treatments differed statistically among each other, with T2 exhibiting the highest mean (1.262) when compared to those of the other treatments. The mean of T2 was not statistically different from that of T4, which had the second highest value (1.092). However, the mean of T2 did differ significantly from that of T1 (0.962) and T3 (0.986), which presented the smallest averages among treatments. The means of T1 and T3 did not differ between each other or that of T4. The overall mean of the fresh mass for leaf explants is 1.075 (coefficient of variation; CV = 10.5%).

Significant differences were observed for the dry mass in the treatments from leaf explants. T2 had the highest mean (0.765) that was not statistically different from that of T4, with the second highest value (0.710), but did differ significantly from that of T1 (lowest mean; 0.661) and T3 (second lowest mean; 0.674) at a 1% probability level. The means of treatments T1 and T3 did not differ from each other and were statistically equivalent to that of T4. The overall mean for the dry mass of the treatments cultivated from leaf explants was 0.702 (CV = 5.4%).

The interaction between plant regulator concentrations used in treatments with root segments showed a significant difference among treatments ($p < 0.01$). The highest means obtained for the fresh mass in the treatments from root segments were recorded in treatments T4 and T2 (on average 0.989 and 0.893, respectively) and the lowest means were found in T3 and T1 (0.854 and 0.810, respectively). The mean of group T4 (0.989) did not differ statistically from that of T2 (0.893). However, the mean of T4 did differ statistically from that of T1, which presented the lowest mean (0.810), and of T3 (0.854), though these did not differ from each other and were statistically equivalent to the mean of T2. The overall mean for the fresh mass for root segments was 0.887 (CV = 5.8 %).

The dry mass of calluses cultivated from root segments exhibited significant differences between treatments. T4 had the highest mean dry mass value (0.677) when compared with the other treatments and differed statistically from that of T1 with the lowest value (0.673).

However, the mean of T4 was not statistically different from the mean of T2 or T3 (with respective means of 0.638 and 0.637). The means of T2 and T3 were intermediate to those of T1 and T4. The mean of T1 was statistically equivalent to that of T2 and T3. The concentrations used in treatments with root segments showed significant differences, with the overall average for the dry mass treatments being 0.641 (CV = 3.2%).

The treatments from leaf explants did not show shoot formation (data not shown), indicating that the interaction of BAP with NAA did not contribute to its development. However, shoot formation of treatments from root segments differed ($p < 0.01$) (Table 01). The highest mean values for the number of shoots in the treatments cultivated with root segments were observed in treatments T4 and T2 (1.545 and 1.250, respectively). These means did not differ statistically from each other but did from that of the treatments with the lowest means, namely T3 and T1 (0.590 and 0.626), which were statistically equivalent. The mean number of shoots was 1.003 (CV = 22.5%).

Only treatments developed from root segments showed shoot formation, as seen in the percentage of explants with shoot development. The interaction of BAP with NAA produced a difference amongst all treatments in the percentage of explants with shoots, such that T4 exhibited a higher percentage of root segments with 44%, compared with that from T1 (8%), T2 (25%) and T3 (20%) at a 1% probability level. The treatment with the lowest shoot rate was T1 with only 8%.

Table 1. Different treatments effect on on fresh mass, dry matter and number of shoots of leaf and root explants of *Allophylus edulis*

| Treatments | Leaf | | Root | | |
|------------|----------------|--------------|----------------|--------------|------------------|
| | Fresh mass (g) | Dry mass (g) | Fresh mass (g) | Dry mass (g) | Number of shoots |
| T1 | 0,962 b | 0,661 b | 0,810 b | 0,613 b | 0,626 b |
| T2 | 1,262 a** | 0,765 a** | 0,893 ab | 0,638 ab | 1,250 a |
| T3 | 0,986 b | 0,674 b | 0,854 b | 0,637 ab | 0,590 b |
| T4 | 1,092 ab | 0,710 ab | 0,989 a** | 0,677 a** | 1,545 a** |
| MG | 1,075 | 0,702 | 0,887 | 0,641 | 1,003 |
| CV (%) | 10,5 | 5,4 | 5,8 | 3,2 | 22,5 |

Note: ** significant at the 1% probability level ($p < 0.01$) by the F test. BAP levels (T1: 0.25 mg L⁻¹, T2: 0.5 mg L⁻¹, T3: 1.0 mg L⁻¹, T4: 2.0 mg L⁻¹) combined with 0.1 mg L⁻¹ of ANA, 45 days after inoculation

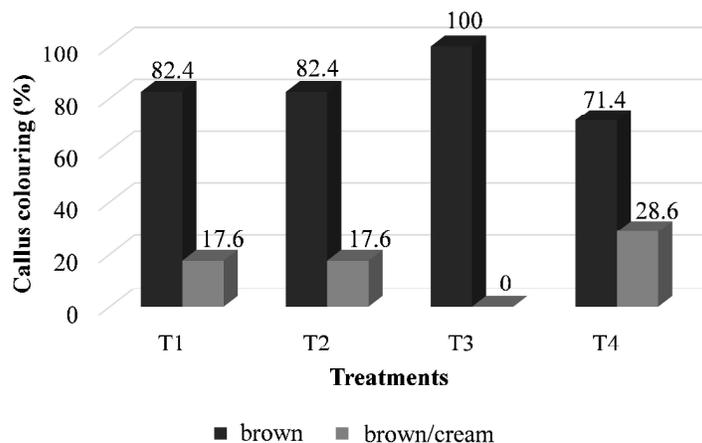


Fig. 1. Callus colouring on leaf explants of *Allophylus edulis* according to BAP levels (T1: 0.25 mg L⁻¹; T2: 0.5 mg L⁻¹; T3: 1.0 mg L⁻¹; T4: 2.0 mg L⁻¹) combined with 0.1 mg L⁻¹ of NAA, 45 days after inoculation (in percentage)

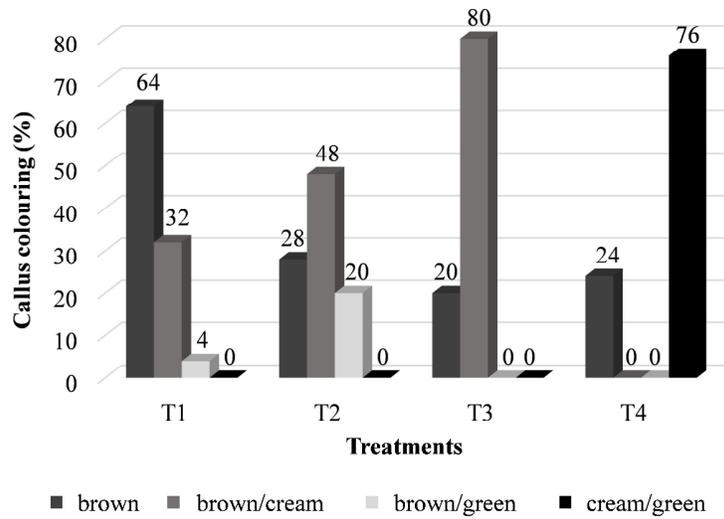


Fig. 2. Callus colouring on root explants of *Allophylus edulis* according to BAP levels (T1: 0.25 mg L⁻¹; T2: 0.5 mg L⁻¹; T3: 1.0 mg L⁻¹; T4: 2.0 mg L⁻¹) combined with 0.1 mg L⁻¹ of NAA, 45 days after inoculation (in percentage)

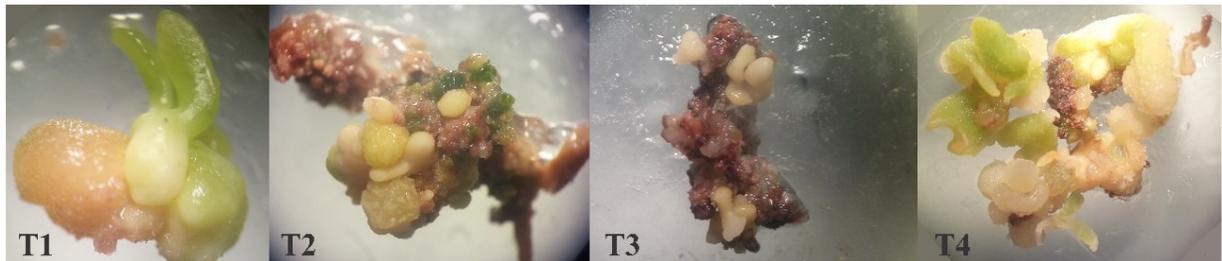


Fig. 3. Shoots aspects in *Allophylus edulis* root segments grown under different concentrations of BAP (T1 - 0.25, T2 - 0.5, T3 - 1.0 and T4 - 2.0 mg L⁻¹) combined with 0.1 mg L⁻¹ of NAA, 45 days after inoculation

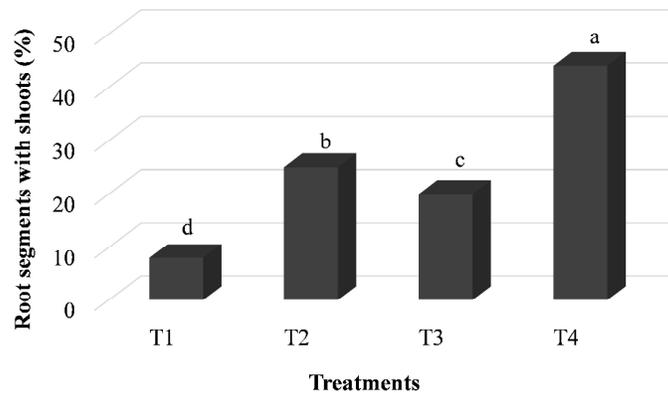


Fig. 4. Percentage of root segments with bud formation at different BAP levels (T1: 0.25 mg L⁻¹; mg L⁻¹, T3: 1.0 mg L⁻¹, T4: 2.0 mg L⁻¹) combined with 0.1 mg L⁻¹ of NAA, 45 days after inoculation. Means followed by distinct letters differ from each other by the F test at the 1% probability level (p<0.01)

Discussion

There was no difference in the consistency of the calluses developed in the treatments with different concentrations of BAP combined with NAA. The calluses developed were all compact, in agreement with George *et al.* (2013), who observed that low concentrations of auxin and

high concentrations of cytokinin produce compact calluses. The ability to form compact calluses is among the parameters used to indicate the regenerative capacity of the plant material (Rodrigues *et al.*, 2009). In this study, it was possible to observe compact calluses in all treatments with different concentrations of auxin when using low auxin concentration in comparison to cytokinin, similar to the

results obtained by George *et al.* (2013). The calluses grown from root segments presented with up to three colors, namely brown, brown/cream, brown/green or cream/green. On the other hand, treatments with leaf explants presented two colors (brown and brown/cream). Carvalho *et al.* (2015) observed that calluses of *Passiflora gibertii* with clear and compact colors in leaves were more favorable for organogenesis.

The colors developed in treatments with root segments may be related to shoot formation. The treatment with root segment, where there were more light colored calluses (2.0 mg L⁻¹ of BAP with 0.1 mg L⁻¹ of NAA; 76%, cream/green), differed from the other treatments regarding shoot formation. Under suitable culture conditions where there is balance between growth regulators, groups of cells that have active centers may be induced to differentiate into organs. These cells are called competent because of their ability to react to certain stimuli, resulting in shoot or root formation (George *et al.*, 2013).

The calluses of different treatments, when cultivated with root segments, presented shoot formation. However, when cultivated with leaf explants, they did not develop shoots, indicating that there was an intermediate hormonal balance of the growth regulators. The concentrations of auxin and endogenous cytokinin in the leaves of *A. edulis* probably induced a balance in the growth regulators, which did not occur in root segments, showing that the endogenous concentrations of auxin and cytokinin are different in leaf and root explants of *A. edulis*. These results corroborate with the study of Anwar *et al.* (2015), where they found the endogenous growth hormone content and the type of growth hormone is directly related to each explant source.

For Nogueira *et al.* (2007), callus formation depended on an intermediate hormonal balance of auxin and cytokinin. In many cases, the addition of a plant regulator to the crop is essential for callus formation (Pêgo *et al.*, 2013). This exogenous supplementation of growth regulators can provide the plant cells with a higher totipotency to regenerate (Garcia *et al.*, 2011). The concentrations and type of endogenous and exogenous growth regulators are factors that generate several events at the molecular level, affecting the transcription of genes responsible for cell differentiation (Rosa *et al.*, 2015).

Dibax *et al.* (2010) compared three combinations of NAA and Thidiazuron (TDZ, resulting in a callus formation of 73% when using 0.1 µM NAA with 1.0 µM TDZ, showing that the interaction between auxin and cytokinin stimulates callogenesis. BAP and NAA interactions showed differences among treatments for fresh and dry mass of leaf and root explants. Fresh and dry mass and higher concentrations of BAP were not directly related in treatments with leaf explants, indicating that a hormonal balance between growth regulators is required for greater cell proliferation in *A. edulis* leaf explants. This hormonal balance for callus formation with greater fresh and dry mass can be achieved with different concentrations of BAP combined with 0.1 mg L⁻¹ of NAA.

In the absence of a plant regulator, *Schizolobium amazonicum* (paricá) showed a reduced frequency of callus formation (Cordeiro *et al.* 2004). Cordeiro *et al.* (2004) also

found that callus mass is directly related to the increase of the BAP regulator, as was observed in this study in the treatments cultivated with root segments. As the BAP concentration increased, a callus formation with higher mass was recorded compared to the callus developed in lower concentrations of BAP.

Conclusions

Callus formation in leaf explants of *Allophylus edulis* requires growth regulator supplementation. The combination of 0.5 mg L⁻¹ of BAP with 0.1 mg L⁻¹ of NAA provides calluses with higher fresh and dry mass and up to two colors. It was clear in this experiment that root segments are more suitable for obtaining somatic embryos. The use 2.0 mg L⁻¹ of BAP combined with 0.1 mg L⁻¹ of NAA is advised to form the largest number of shoots.

Conflict of Interest

The authors declare that there are no conflicts of interest related to this article.

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