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Note

Callus Induction in Baru (*Dipteryx alata* Vog.) Explants

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Abstract

Baru (*Dipteryx alata* Vog.) is a species with great economic and environmental potential; it has popular acceptance, besides being a very productive species. Alternative propagation methods are important for species maintenance and exploration. Thus, micropropagation emerged as an alternative technique, providing genetic stability and the production of a large number of seedlings. The aim of the present investigation was to develop a callus induction protocol for *in vitro* baru explants. The tested explants were nodal, internodal and foliar segments. The explants were disinfected for 30 seconds in 70% alcohol (v/v) and 2 minutes in sodium hypochlorite (1.25% active chlorine). This was followed by triple washing. The inoculation was carried out in test tubes containing 15 mL MS medium (30 g L^{-1} sucrose, 6 g L^{-1} agar and 100 mg L^{-1} ascorbic acid) supplemented with 2.0 mg L^{-1} naphthalene acetic acid (NAA). The solution also contained 0.0, 2.5 or 5.0 mg L^{-1} of 6-benzylaminopurine (BAP) with the pH adjusted to 5.8. In the incubation phase, the explants were cultured for seven days in the dark and then subjected to a photoperiod of 16 hours (43 µmol m^{-2} s^{-1}) at 25 ± 2 °C. The treatments were studied with 2.5, 5.0, 7.5 or 10.0 mg L^{-1} BAP additions to the MS. Callus formation, contamination and oxidation evaluations were undertaken. The results obtained when using 2.0 mg L^{-1} NAA concluded that such a treatment should be used to induce callogenesis from nodal explants, while for the tested baru leaf explants, the best results for callus formation were given by the combination of 2.0 mg L^{-1} NAA with 2.5 mg L^{-1} of BAP to.

Keywords: Cerrado; *Dipteryx alata* Vog.; micropropagation; plant growth regulators

Introduction

The Cerrado is the second largest Brazilian biome. It covers 22% of the national territory, has a wide diversity of environments, whereas fauna and flora diversity are high. The fruit species are particularly important. They have numerous uses, but little is known about them and many have not been studied or exploited (Mendonça *et al*., 2008).

Among these species, baru (*Dipteryx alata* Vog.), which belongs to the family Fabaceae, subfam. Faboideae, is thought to have great economic and environmental potential because it has many diverse uses. Its fruits and seeds can be used in human (fresh or processed) and animal diet. The fruit has high crude protein (29.59%), ethereal extract (40.27%) and fiber (19.04%) contents and is a source of minerals (zinc, iron, and calcium). The seeds are used as an antirheumatic treatment and protect against oxidative stress (Lorenzi 1992; Lemos *et al*., 2012; Fetzer *et al*., 2018).

Pott and Pott (2003) highlighted the destruction of these remarkable food resources caused by negative anthropic actions. A consequence of this is that the demand for information on native plants has increased, so that forest conservation and replenishment can be improved. Arakaki *et al*. (2009) reported that anthropic disturbance can be prevented, so that communities can find sustainable production solutions that do not compromise the biome. They suggest that Agroforestry System (AFS) programs could be an alternative, because they integrate rural areas, and include baru in their portfolio of plants as an example of a native species that produces quality wood.

Pott and Pott (2003) pointed out that baru could be seen as a supplier species or as a component of a consortium or system, because it can potentially produce fruits, wood, medicinal products and forage. It can also improve apiculture. Candil *et al*. (2007) concluded that if there were incentives and the production was viable, baru could contribute to rural tourism by enhancing the

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characterization of products intrinsic to local communities, which would increase local land and property values and improve sustainability.

According to Lorenzi (1992), the production of seedlings (mostly seminal propagated) is a limiting factor on the cultivation of this species, despite the high (90%) germination rate. This means that there is a long juvenile period. There is also concern about the genetic variability of the progenies with regards to the agronomic traits that are important for commercial fruit growing. Vieira *et al*. (2016) suggested that it is necessary to prioritize activities, such as germplasm collection, characterization of genetic variability, agronomic evaluation of the native species, as well their *in situ* and *ex situ* conservation, in order to preserve these species, most of which are threatened by the expansion of agriculture in the Cerrado region.

Micropropagation is an alternative method for plant regeneration, as there are efficient protocols provided for vegetative propagation. Even more, it needs to be carried out under controlled conditions, so that genetically and physiologically uniform seedlings that meet sanitary quality standards can be produced (Eldessoky *et al*., 2014).

The *in vitro* development of baru (*Dipteryx alata* Vog.) has already been studied. Araruna *et al*. (2017) evaluated the effect of different salt concentrations in the culture media. During the initial development of baru seedlings, the stem tips developed better in the MS culture medium (Murashige and Skoog, 1962), when no salts were added, compared to different concentrations of salt. Also, the seedliong developed better when they were cultivated on MS media rather than WPM medium (Lloyd and McCown, 1980). Silva *et al*. (2016) tested various activated carbon concentrations in different culture media and found out that the MS medium plus 3.0 g L^{-1} of activated carbon could potentially improve *in vitro* baru establishment.

The objective of the present study was to develop a callus induction protocol for *in vitro* foliar, nodal and internodal baru explants, by comparing different concentrations and combinations of naphthalene acetic acid (NAA) and 6-benzylaminopurine (BAP).

Materials and Methods

Description of the study site

The experiment was conducted at the Plant Biotechnology Laboratory of the Center for Biotechnology and Genetic Improvement of Sugarcane, located at the Faculty of Agricultural Sciences, Federal University of Grande Dourados, Dourados-MS.

The experiment had two phases; during the first phase the different explant types and growth regulators were investigated, and during the second phase the *in vitro* protocol was adapted so that it could be used with leaf segments.

First phase of the experiment

Young plants, derived from *in vitro* germination of baru seeds, were placed in flasks containing 50 mL of MS medium, and 6 g L^{-1} of agar, but no sucrose, and these were used as matrices. The flasks were kept in a growth room at 25 ± 2 °C and with a photoperiod of 16 hours (43 μmol m^{-2} s^{-1}) light.

Twenty-one days after germination (DAG) in the horizontal flow chamber, the plants underwent disinfection in alcohol 70% (v/v) followed by 2 minutes in sodium hypochlorite (1.25% active chlorine) with three drops of neutral detergent. Then, they were triple washed with distilled water.

The seedlings were sectioned using a scalpel, to obtain three types of explants: nodal and internodal segments, inoculated in the horizontal position,, and leaf segments that were approximately 1 cm^2 in size.

The inoculation of the explants was carried out in test tubes containing 15 ml MS medium (30 g L^{-1} sucrose, 6 g L^{-1} agar and 100 mg L^{-1} ascorbic acid) supplemented with two regulators, which were 2.0 mg L^{-1} of naphthalene-acetic acid (NAA) and 0.0, 2.5 or 5.0 mg L^{-1} 6-benzylaminopurine (BAP). The pH was adjusted to 5.8 before the samples were autoclaved at 121 °C for 20 minutes.

During the incubation phase, the material was submitted to seven days of darkness and then transferred to a photoperiod of 16 hours (43 μmol m^{-2} s^{-1}) light, at 25 ± 2 °C. The percentage of callus formation, contamination and oxidation were evaluated on a weekly basis until their cessation. The regulator combinations were randomly distributed between explant types (Table 1).

Second phase of the experiment

The second phase only used leaf segments, but the same methodology used in phase one was adopted, except that the growth regulator concentrations were altered. Therefore,, inoculation of the explants was carried out test tubes containing 15 mL of MS medium (30 g L^{-1} sucrose, 6 g L^{-1} agar and 100 mg L^{-1} ascorbic acid) supplemented with 2.0 mg L^{-1} of NAA, combined with 0.0, 2.5; 5.0, 7.5 or 10.0 mg L^{-1} of BAP (Table 2).

Statistical procedures

A completely randomized design was used in both phases of the experiment. The experimental data were submitted to analysis of variance followed by Tukey's test at the $P < 0.05$ probability level. The SISVAR statistical program (Ferreira, 2011) was used for the analyses.

Table 1. Treatments used in phase one of baru (*Dipteryx alata* Vog.) callogenesis induction experiment

Treatment	NAA (mg L^{-1})	BAP (mg L^{-1})	Explant	No. of replicates
T1	2.0	0.0	Nodal segment	20
T2	2.0	2.5	Nodal segment	20
T3	2.0	5.0	Nodal segment	20
T4	2.0	0.0	Internodal segment	20
T5	2.0	2.5	Internodal segment	20
T6	2.0	5.0	Internodal segment	20
T7	2.0	0.0	Leaf segment	20
T8	2.0	2.5	Leaf segment	20
T9	2.0	5.0	Leaf segment	20

Table 2. Treatments used in phase two of the baru (*Dipteryx alata* Vog.) callogenesis induction experiment

Treatment	NAA (mg L^{-1})	BAP (mg L^{-1})	Explant	No. of replicates
T1	2.0	2.5	Leaf segment	20
T2	2.0	5.0	Leaf segment	20
T3	2.0	7.5	Leaf segment	20
T4	2.0	10.0	Leaf segment	20

Results

First phase

Twenty days after inoculation, the highest callogenesis percentage occurred in the nodal explants treatments that contained 2.0 mg L^{-1} NAA and 2.0 mg L^{-1} of NAA with 2.5 mg L^{-1} BAP (Fig. 1). Phenolic oxidation did not occur in any of the treatments. There were differences in explant contamination between the treatments and between the different types of explants. The lowest contamination percentages occurred on the leaf segments and the highest on the uninodal explants (Fig. 2).

Second phase

The results for the leaf segments in the first phase showed that they had the lowest callus percentages. Therefore, the second phase objective was to adapt the protocol for leaf segments. The highest callus percentage for the leaf explants was observed during the same period as it was during the first phase (20 DAI) when 2.0 mg L^{-1} NAA was combined with 2.5 mg L^{-1} BAP. Interestingly, oxidation of the explants was higher in the treatments where 2.0 mg L^{-1} NAA was combined with 7.5 mg L^{-1} or with 10.0 mg L^{-1} BAP, but there was no contamination. The results are shown in Fig. 3.

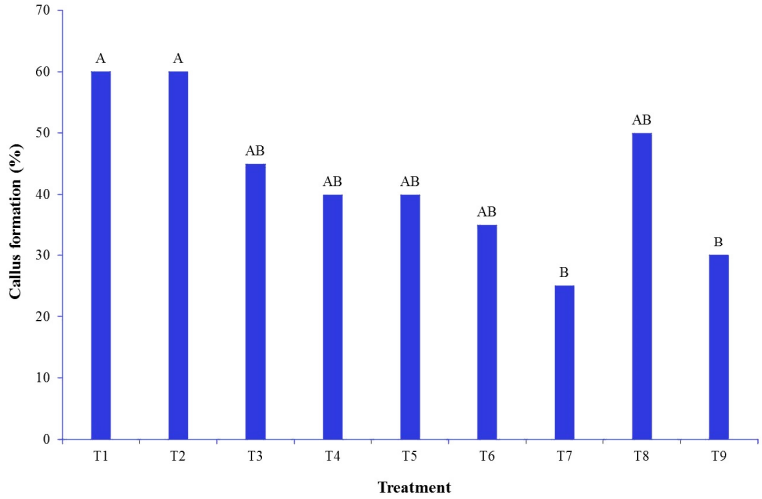

Fig. 1. Callus formation percentages for different types of baru explants cultured *in vitro* at different concentrations and combinations of NAA and BAP. Means followed by the same letter were not significantly different according to Tukey's test at $P < 0.05$. Treatments T1 to T9 are explained in Table 1

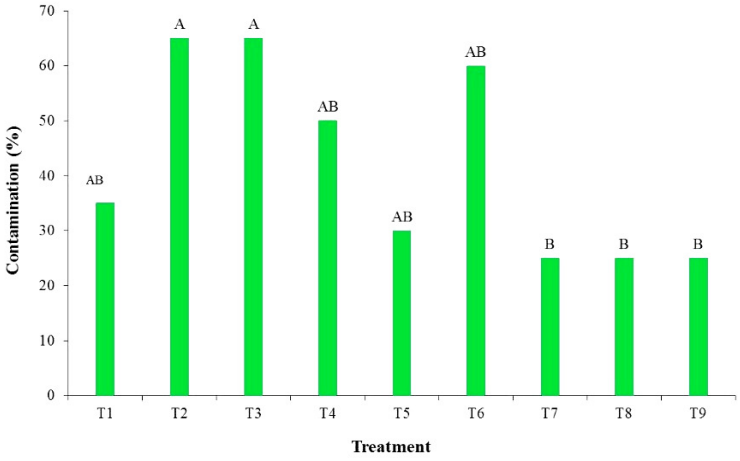

Fig. 2. Percentage contamination of the baru explants cultivated *in vitro* at different concentrations and combinations of NAA and BAP. Means followed by the same letter do not differ according to Tukey's test at $P < 0.05$. Treatments T1 to T9 are explained in Table 1

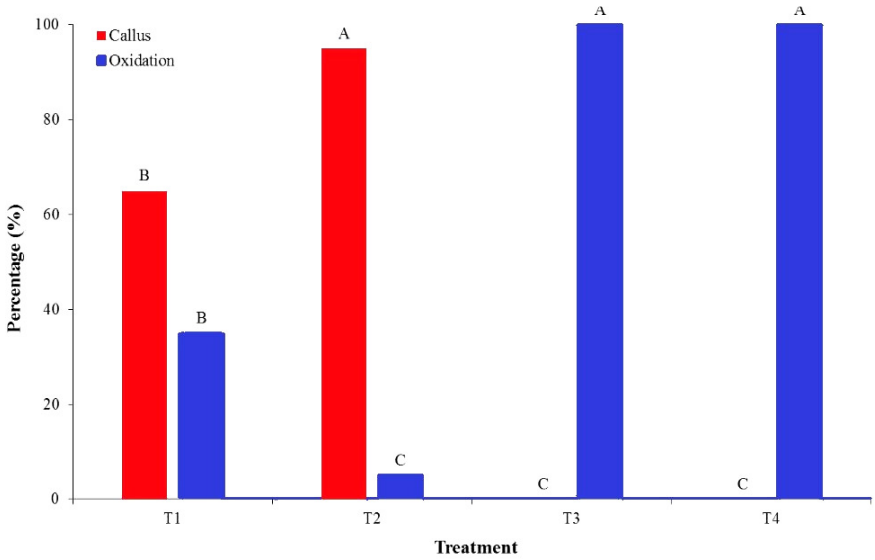

Fig. 3. Percentage callus formation and oxidation for baru leaf explants grown *in vitro* at different concentrations and combinations of NAA and BAP. Means followed by the same letter do not differ according to Tukey's test at $P < 0.05$. Treatments T1 to T4 are explained in Table 2

Discussion

According to Grattapaglia and Machado (1998), excessive amounts of auxin and sucrose may limit callus formation to the median explant region. However, in this experiment, callus formation occurred across all explants. Silva (2012) observed that the callus area expanded when tissue was treated with 2,4-D (dichlorophenoxyacetic acid).

The auxin and cytokinin ratio directly influences callus formation, whereas higher auxin concentrations lead to the formation of roots. In contrast, higher cytokinin concentrations induce the formation of aerial parts. However, when tissues are cultivated in a culture medium containing both regulators, intermediate stages are found and tissue growth occurs in the form of undifferentiated cells (Taiz and Zeiger, 2013).

Pasqual *et al.* (1998) reported that auxins are mostly responsible for callus induction and that the effect of cytokinins, which stimulate cell division and control morphogenesis, in tissue culture, is emphasized when applied along with auxins. This was confirmed by the results produced by the present study, where the highest callogenesis rates were recorded when only NAA was applied and when the BAP concentration was the lowest. This result confirmed the balance between the two types of regulators.

Larson (2014) observed that more callus tissue formed on the nodal segments when the NAA concentration was 1.0 mg L^{-1} NAA and that callus formation was initiated after 45 days of *in vitro* incubation. This plant cell totipotency principle suggests that several types of explants can be used. However, in practice, some tissue types have a higher proportion of meristematic tissue, which means that they have a greater differentiation capacity (Torres *et al.*, 1998). This explains why nodal segments generally perform better than other explants.

The same author also recorded lower contamination rates in baru leaf explants when *in vitro* germinated seeds were compared. The results showed that each type of explant possesses different susceptibilities to contamination by microorganisms. This means that it is important to develop protocols for each stage of the micropropagation process so that quality regenerated plants can be obtained.

There was still considerable contamination by microorganisms growth in the hereby experiment, irrespective of the contamination results for each treatment. Therefore, the protocol proposed here was not particularly effective at preventing microorganism growth. However, this contamination, which was mostly due to fungi, did not prevent callus formation and did not lead to pathogenic symptoms. Chaibub *et al.* (2009) reported a similar contamination situation when cultivating *D. alata* almonds *in vitro*. They concluded that the fungi causing the contamination were endophytic species.

Controlling tissue contamination is inherent to the success of *in vitro* explant establishment. Therefore, the preservation of explant integrity should be taken into account when developing callus induction methodologies. In particular, attention should be paid to plant hormone concentrations and cultivation techniques (Dutra *et al.*, 2009).

There was no phenolic oxidation observed in the present study. Larson (2014) also reported the same results. This suggests that the use of ascorbic acid (100 mg L^{-1}) efficiently controlled oxidation. It is already known that ascorbic acid has antioxidant activity and can absorb phenolic compounds during *in vitro* propagation. Therefore, it is frequently used in many different protocols.

Soares *et al*. (2007) suggested that callus formation is very common in woody species. The NAA regulator affects cell differentiation (Taiz and Zeiger, 2013). The cytokinins, in turn, play an important role in meristematic function, which induces callus formation (Werner and Schmüulling, 2009; Buechel *et al*., 2010; Su *et al*., 2011). They may have positively affected the baru leaf explant results.

During the first phase of the experiment, callus formation occurred even in the treatment without cytokinin addition, which suggested that baru contains high endogenous concentrations of this regulator. However, when BAP was added, the callus percentage increased. The results suggest that 2.0 mg L^{-1} of NAA, combined with 2.5 mg L^{-1} of BAP, was the most viable regulator ratio for the development of baru leaf explants. According to Bray *et al*. (2000), oxidation is caused by the release of phenolic compounds *in vitro*, by tissue injury, or by the senescence of tropical native species that contain high levels of these compounds.

One of the roles played by cytokinins during plant adaptation to stress is to serve as a protective agent when there is excess of light, which causes the inhibition of photosystem II and the synthesis of reactive oxygen species (ROS). These, in turn, can degradate protein D1. Cytokinin is part of the antioxidant system and is involved in the repair of protein D1 (Cortleven *et al*., 2014). However, the higher the BAP concentration added to the culture medium, the higher the oxidation rate. Oliveira *et al*. (2016) reported a similar result when they studied mangaba (*Hancornia speciosa* Gomes) and suggested that it was due to the toxic effect of the regulator when the BAP concentration was too high. Such a toxic effect then negatively affected the development of the explant and probably led to its degradation. Therefore, the endogenous concentrations of the regulator can negatively affect explant growth at high concentrations. This means that the use of substances such as ascorbic acid, which has an antioxidant role, may positively influence the growth and development of some explants, but in this experiment, the dosage used was not high enough and its effect was suppressed by the toxic BAP concentrations.

The disinfection methodology used in the second phase of the experiment was the same as the one used in the first phase. The absence of phenolic oxidation could be due to the absence or the reduced presence of endogenous inocula and the reduced susceptibility of the leaf segment compared to the other explant types, because in the first stage, the leaf segments had the lowest contamination percentages.

Conclusions

Baru (*Dipteryx alata* Vog.) micropropagation could be successfully performed and considerable callus development was observed. However, the different responses of each explant type must be taken into account, particularly in terms of the growth regulators concentrations applied and their effects on tissue contamination and oxidation levels. The results from the present study suggest that 2.0 mg L^{-1} NAA can be used for callogenesis within nodal explants and 2.0 mg L^{-1} NAA combined with 2.5 mg L^{-1} BAP can be used for baru leaf explants.

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