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Nota Técnica

In vitro germination and shoot proliferation of *Dipteryx alata* Vogel (Fabaceae) under conventional and natural ventilation

Germinação in vitro de *Dipteryx alata* Vogel (Fabaceae) e proliferação de brotos sob sistema de ventilação convencional e natural

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ABSTRACT

Dipteryx alata Vogel is a native species of the Cerrado domain with economic potential for use as a timber and food source. The present study aimed to establish an in vitro germination and shoot proliferation protocol for this species for future rooting and acclimatization investigations. Fruits were collected in October 2013. For asepsis and germination, the seeds were divided into two groups: in the first the seed coat was removed and in the second, maintained. Both groups were submitted to asepsis with detergent and 70% alcohol, followed by different concentrations of sodium hypochlorite (NaClO) (2.5% active chlorine) (v/v): 00%; 10%; 50%; and 100%. Two experiments were conducted for in vitro shoot proliferation: conventional lids or natural ventilation. Nodal segments from plants germinated in vitro (two months old) were inoculated with different concentrations of 6-Benzylaminopurine (BAP) (0.00 µM; 4.44 µM; and 11.10 µM) combined with naphthalene acetic acid (NAA) (0.00 µM; 2.69 µM; and 5.37 µM). The seeds were successfully decontaminated, with those exhibiting an intact seed coat and 50% NaClO found to be superior. High shoot proliferation was observed under natural ventilation, with 4.44 µM BAP (average of 4.03 shoots), a 101.50% increase in relation to controls and 43.93% when compared to the best conventional system treatment. On the other hand, this last system produced taller shoots under 2.69 and 5.37 µM of NAA (average of 19.42 and 18.18 mm, respectively), and 2.69 µM of NAA combined with 4.44 µM of BAP (average of 18.46 mm). By contrast, natural ventilation resulted in taller shoots for 5.37 µM of NAA combined with 11.10 µM of BAP (average of 12.81 mm).

Keywords: Baru; Cerrado; Plant growth regulators; In vitro culture

RESUMO

Dipteryx alata Vogel é uma espécie nativa do domínio Cerrado, a qual possui potencial econômico de uso madeireiro e alimentício. Objetivou-se estabelecer um protocolo de germinação e proliferação de brotos in vitro desta espécie, visando futuros estudos de enraizamento e aclimatização. Frutos foram coletados em outubro de 2013. Para assepsia e germinação, as sementes foram divididas em dois grupos: no primeiro, o tegumento foi retirado, e no segundo este foi mantido. Os dois grupos foram submetidos à assepsia com detergente e álcool 70%, e em seguida a distintas concentrações de hipoclorito de sódio (NaClO) (2,5% de cloro ativo) (v/v): 00%; 10%; 50%; e 100%. Para proliferação de brotos in vitro, dois experimentos foram conduzidos: tampas convencionais ou ventilação natural. Segmentos nodais oriundos de plantas germinadas in vitro (dois meses de idade) foram inoculados em diferentes concentrações de 6-Benzilaminopurina (BAP) (0,00 µM; 4,44 µM; e 11,10 µM) combinadas a ácido naftaleno-acético (ANA) (0,00 µM; 2,69 µM; e 5,37 µM). A descontaminação de sementes foi realizada com sucesso, observando-se superioridade de sementes com presença de tegumento e 50% de NaClO. Alta proliferação de brotos foi observada em ventilação natural, com 4,44 µM de BAP (média de 4,03 brotos), um aumento de 101,50% em relação ao controle e 43,93% em relação ao melhor tratamento do sistema convencional. Por outro lado, este último sistema propiciou brotos com maiores alturas em 2,69 e 5,37 µM de ANA (média de 19,42 e 18,18 mm respectivamente), assim como em 2,69 µM de ANA combinado a 4,44 µM de BAP (média de 18,46 mm). Por outro lado, o sistema de ventilação natural mostrou brotos mais altos em 5,37 µM de ANA combinado a 11,10 µM de BAP (média de 12,81 mm).

Palavras-chave: Baru; Cerrado; Reguladores de crescimento; Cultivo in vitro

1 INTRODUCTION

The Cerrado domain is home to a wide variety of native species with economic potential. These include *Dipteryx alata* Vogel (Fabaceae), commonly known as baru, which occurs in central Brazil and is highly valued for its nuts and potential as a timber source (SANO *et al*., 2006). Its edible nuts are highly nutritious (PAGLARINI *et al*., 2018) and can be eaten fresh or used in a variety of foods, such as cookies and cakes. The species is also important because of the use of its wood, considered high quality, compact and dense (1.1g/cm^3) (SANO *et al*., 2006).

Given the high deforestation rates observed in recent decades (BRANDÃO *et al*., 2017), studies on the propagation of Cerrado plant species are particularly important. Although *Dipteryx alata* shows high reforestation potential, morphological variables (such as number of leaves and plant height) are directly related to the survival of its

seedlings in the field (SANTOS *et al*., 2018). As such, studies aimed at in vitro culture protocols for *Dipteryx alata* are important in obtaining a larger number of quality seedlings.

In addition to enabling large-scale production, plants obtained by micropropagation are disease-free, which prevents losses in the field (SHAHZAD *et al*., 2017) he made several valuable predictions about the nutrients' requirement for in vitro culture conditions, which could possibly induce cell division, proliferation and embryo induction. Tissue culture has now become a well-established technique for culturing and studying the physiological behaviour of isolated plant organs, tissues, cells, protoplasts and even cell organelles under precisely controlled physical and chemical conditions. Micropropagation is one of the most important applications of plant tissue culture. It provides numerous advantages over conventional propagation like mass production of true-to-type and disease-free plants of elite species in highly speedy manner irrespective of the season requiring smaller space and tissue source. Therefore, it provides a reliable technique for in vitro conservation of various rare, endangered and threatened germplasm. Micropropagation protocols have been standardized for commercial production of many important medicinal and horticultural crops. Somatic embryogenesis is an extremely important aspect of plant tissue culture, occurring in vitro either indirectly from callus, suspension or protoplast culture or directly from the cells. This is due to the use of substances that promote explant asepsis (TRIGIANO, 2016), including sodium hypochlorite (NaClO), which is widely used for its effectiveness in the removal of disease-causing microorganisms from in vitro plant cultures (HESAMI *et al*., 2017).

Different plant growth regulators (PGRs) are used in micropropagation, including 6Benzylaminopurine (BAP) for shoot multiplication (SANT'ANA *et al*., 2018) and naphthalene acetic acid (NAA) for shoot growth or etiolation (KUMAR *et al*., 2016). However, it is important for these regulators to be correctly balanced to obtain satisfactory responses capable of influencing other stages of micropropagation, such as rooting or acclimation (KUMAR *et al*., 2016).

Conventional in vitro micropropagation (also known as heterotrophic systems) is characterized using sealed flasks. Despite being the most widely used system, it has several disadvantages, including buildup of moisture and ethylene inside the flask, which can cause anatomical abnormalities, small leaves, senescence and death during acclimatization (SHIN *et al*., 2014) and its potential benefits appear to be promising. Nevertheless, little is known regarding the effects of such culture conditions on plant physiology during ex vitro acclimation. In this study, plantlets were grown under three different microenvironmental conditions: (1. Natural ventilation, also termed mixotrophic cultivation by some authors (ŠEVČÍKOVÁ *et al*., 2018), is used to enhance micropropagation protocols and involves using flasks sealed with membranes that enable gas exchange, but prevent the entry of microorganisms (SILVA *et al*., 2017). This exchange can help improve the number and quality of seedlings produced by increasing the number of shoots and leaves, as well photosynthetic pigments (SALDANHA *et al*., 2012).

Given that the Cerrado is subject to constant deforestation and considering the economic potential of *Dipteryx alata*, propagation protocols for the species are important. As such, this study aimed to establish a germination and proliferation protocol for *Dipteryx alata* shoots for future rooting and acclimatization investigations.

2 MATERIAL AND METHOD

Dipteryx alata fruits were collected in October 2013 from the fruit species collection of the Federal University of Goiás (UFG). The seeds were removed and stored at a low temperature (10ºC) for 30 days.

2.1 Seed asepsis and germination

A total of 240 seeds were used; the integuments were removed from half of these and maintained in the other half. After being separated into individual flasks, the seeds were immersed in Tween 80™ solution (20µL per 1.0 L of water) for 10 minutes and then washed under running water for 5 minutes. Under aseptic conditions, the seeds

were immersed in 70% alcohol (v/v) for 5 minutes, then separated into four groups and disinfected in different concentrations of NaClO (2.5% active chlorine): 0% (autoclaved control of distilled water); 10%; 50%; and 100%. After 20 minutes, the seeds were submitted to triple rinsing, autoclaved and then inoculated in flasks (200 mL) containing 30 mL of WPM medium (LLOYD; MCCOWN, 1980) supplemented with 3% sucrose (p/v) and 0.25% Gelsan™ sanitizer (p/v). The flasks containing the culture medium and seeds were sealed using conventional lids or polyvinyl chloride (PVC) film. The seeds were placed in a growth chamber (16 h of light and 8 h of dark, 25°C ± 1°, light intensity of 37 μE/m^2/s) and assessments conducted after 45 days to determine the percentage of decontaminated seeds and the germination percentage. Decontaminated seeds were those that had no microorganisms growing on them or in the culture medium. A completely randomized design was used in a 2 x 4 factorial scheme (seeds with or without integuments x 4 NaClO concentrations), resulting in eight treatments with 10 repetitions each. Each experimental unit consisted of three flasks containing one seed each.

2.2 Shoot proliferation

Plants established in vitro 60 days after seeding were used. Under aseptic conditions, 20 mm nodal segments were inoculated in flasks (200 mL) containing 30 mL of WPM medium (LLOYD; MCCOWN, 1980) supplemented with 3% sucrose (p/v) and 0.25% Gelsan™ sanitizer (p/v) and their respective treatments, consisting of different concentrations of naphthalene acetic acid – NAA (0.00 μM; 2.69 μM; and 5.37 μM) combined with different concentrations of 6-Benzylaminopurine – BAP (0.00 μM; 4.44 μM; and 11.10 μM). Two experiments were conducted, the first with conventional (polypropylene) lids and the second using seals that enabled gas exchange (denominated natural ventilation). The gas exchange system used in the seals was developed by Saldanha *et al*. (2012) and consisted of a 5 mm-wide hole covered with two layers of micropore tape and one layer of polytetrafluoroethylene (PTFE) tape.

The flasks containing the explants were placed in a growth chamber (16 h of

light and 8 h of dark, 25°C ± 1°, light intensity of 37 µE/m2/s). Assessments were carried out ninety days after inoculation to determine the number of shoots, shoot height and number of leaves. Shoot height was measured from base to tip. A completely randomized design was used in both experiments, with a 3 x 3 factorial scheme (three levels of NAA x 3 levels of BAP), nine treatments and three repetitions. Each experimental unit consisted of one flask containing one nodal segment.

2.3 Statistical analysis

The data were submitted to analysis of variance (ANOVA) and means compared using Tukey's test ($\alpha<0.05$). Data that did not meet the ANOVA assumptions were submitted to Box-Cox transformation. The analyses were performed using R software (R CORE TEAM, 2017) (version 4.0.0) and graphs constructed with the GraphPad Prism program (RADUSHEV, 2007).

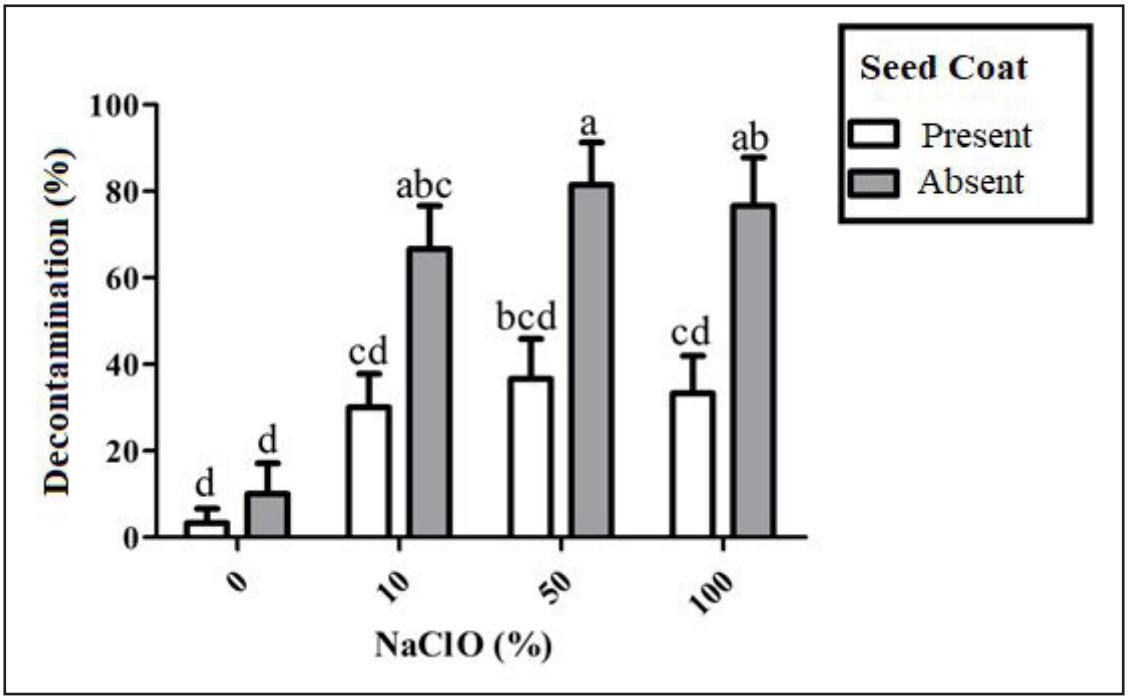
3 RESULTS AND DISCUSSION

3.1 Seed asepsis and germination

With respect to asepsis in seeds submitted to decontamination, there was no interaction between an intact seed coat and NaClO concentrations. Seeds with a seed coat showed high average decontamination at concentrations above 10% NaClO. Within this group, seeds submitted to 50% sodium hypochlorite exhibited a seven-fold higher increase in average decontamination when compared to controls.

High NaClO concentrations can be cytotoxic to some species, which may cause physiological disorders (SEVERING *et al*., 2018). Endophytes inhabit plant tissue without causing disease, promoting plant health through antagonistic action against pathogens. A disturbance in the physiological status of an explant may provoke a response from these endophytes (SELIM *et al*., 2012; SUDHA *et al*., 2016).

Figure 1 – Decontamination of *Dipteryx alata* seeds submitted to different concentrations of sodium hypochlorite (NaClO) combined with two different seed coat conditions



Source: Authors (2019)

In where: *Means followed by different letters differ significantly according to Tukey's test (α = 0.05).

In the present study, NaClO likely caused an imbalance in the seeds whose integuments were removed, thereby eliciting a response from the endophytes.

However, further research is needed to better understand the relationship between types of endophytes and physiological stress in *Dipteryx alata*.

The efficient decontamination of *Dipteryx alata* was likely due to the sensitivity of the microorganisms on the surface to NaClO. This decontaminating agent releases hypochlorous acid in aqueous solution, which can diffuse through the plasma membrane and microbial cell wall and inactivate bacterial metabolism, causing the death of these microorganisms (SEVERING *et al*., 2018).

All the NaClO concentrations from 10% onwards contributed to *Dipteryx alata* germination (Table 1). Although microorganisms were observed in all the treatments

(Figure 1), the contamination rates did not influence seed germination, since microorganisms were present in germinated seeds. Additionally, the status of the seed coat did not interfere in germination for the species under study. Silva *et al*. (2016) evaluated the in vitro germination of *Dipteryx alata* and found a germination rate of up to 88.88% when submitted to NaClO in MS medium supplemented with 0.3 % activated carbon (p/v), similarly to the present study.

Table 1 – Germination rates of *Dipteryx alata* seeds submitted to different concentrations of sodium hypochlorite (NaClO) under different seed coat conditions

Seed coat	NaClO (%)	Germination (%)
Absent	00%	03.33b
Absent	10%	93.33a
Absent	50%	96.67a
Absent	100%	83.33a
Present	00%	10.00b
Present	10%	80.00a
Present	50%	77.78a
Present	100%	80.00a

Source: Authors (2021)

In where: *Means followed by different letters differ significantly according to Tukey's test (α=0.05); NaClO = sodium hypochlorite.

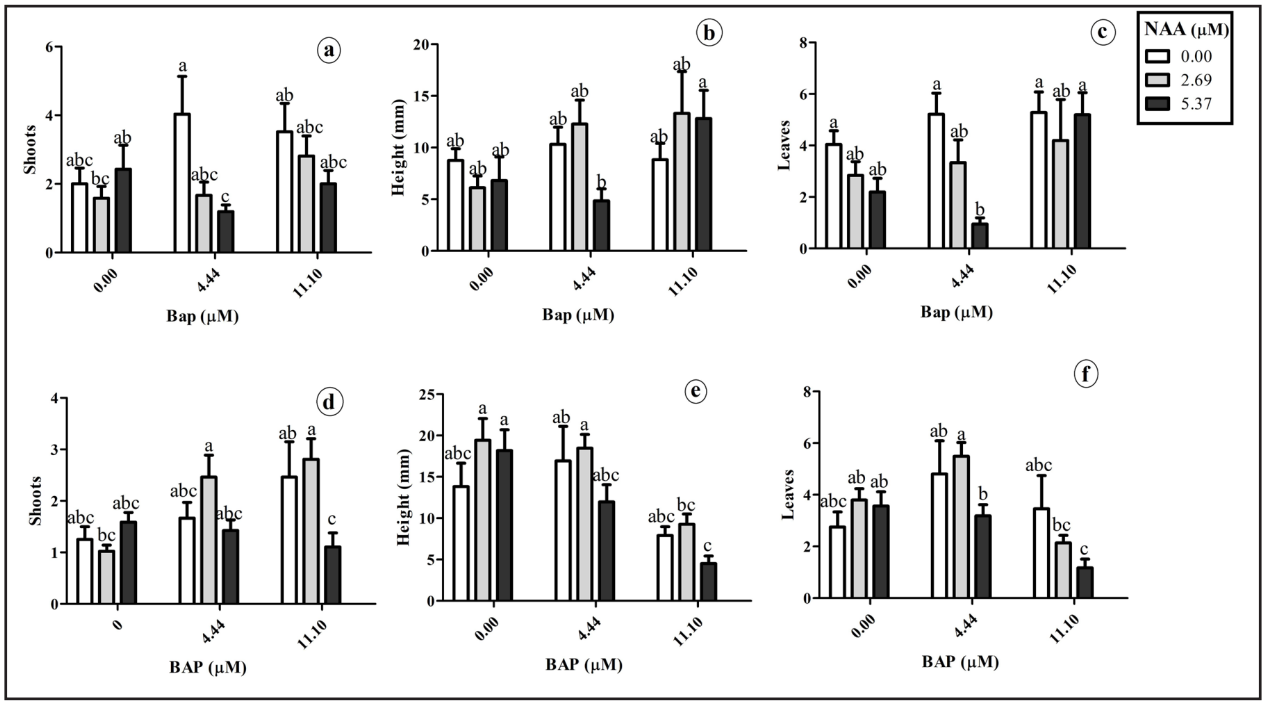
Germination percentages were higher in treatments containing NaClO. In addition to its roles in asepsis, NaClO also breaks seed dormancy (HESAMI *et al*., 2017) via chemical scarification (JESUS *et al*., 2016).

3.2 Shoot proliferation

The proliferation of *Dipteryx alata* shoots was well established, with interaction observed between NAA and BAP. Higher average values were recorded for the number of shoots under natural ventilation (Figure 2). The best treatment for this variable under natural ventilation was 0.00 μM of NAA combined with 4.44 μM of BAP, with explants exhibiting an average of 4.03 shoots, a 101.50% increase in relation to the control treatment (WPM medium without NAA and BAP). By contrast, the best treatment for

this variable under the conventional system was 6.69 µM of NAA combined with 4.44 µM or 11.10 µM of BAP (Figure 2-d).

Figure 2 – Average values for number of shoots (a; d), shoot height (b; e) and number of leaves (c; f) in *Dipteryx alata* under natural ventilation (a; b; c) or conventional lids (d; e; f)



Source: Authors (2021)

In where: * Means followed by different letters differ significantly according to Tukey's test (α=0.05); BAP = 6-Benzylaminopurine; NAA = Naphthalene acetic acid.

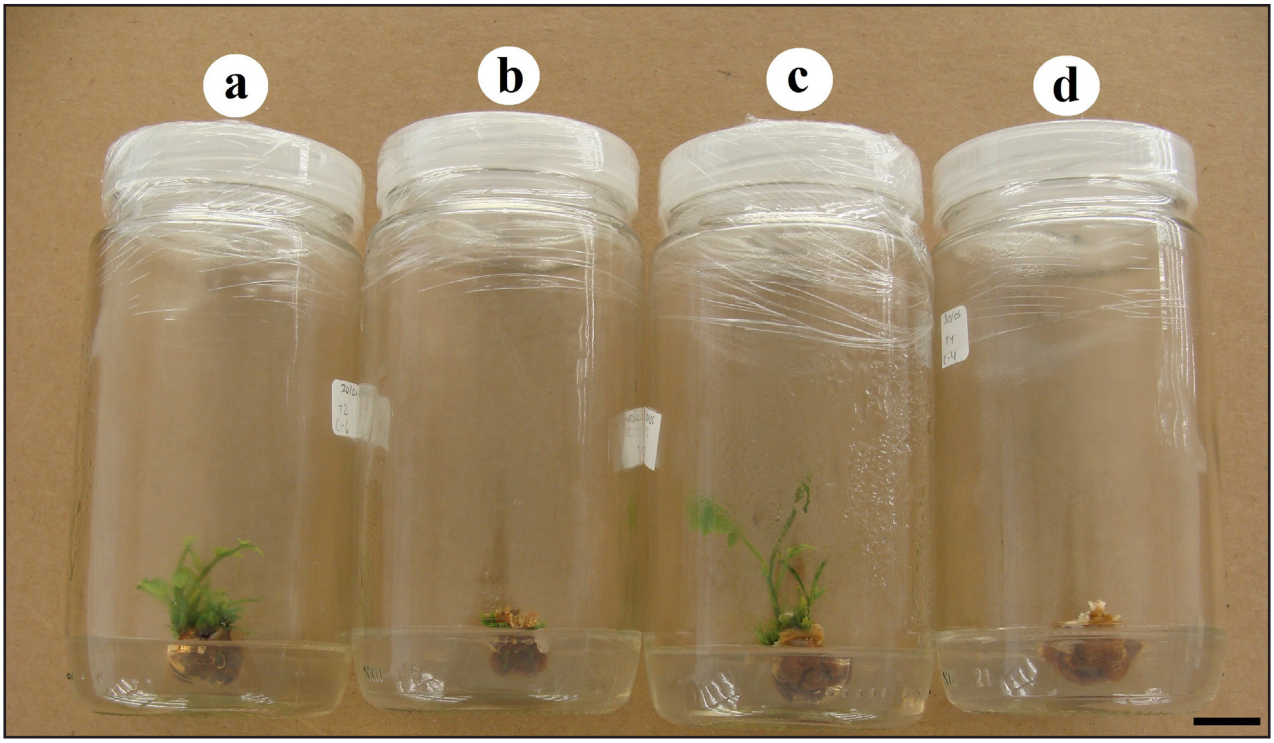
BAP is a cytokinin commonly used in plant tissue cultures, particularly shoot multiplication, and a balanced ratio between this substance and endogenous and exogenous auxins ensures a successful micropropagation protocol. High cytokinin and low auxin levels are typically used for in vitro shoot proliferation; and low cytokinin and high auxin concentrations for etiolation and rooting (KUMAR *et al*., 2016). In a few species, the addition of NAA to the culture medium is not necessary for shoot multiplication. For example, in the micropropagation of Laburnum anagyroides (Fabaceae), shoot multiplication was achieved in 2.22 µM of BAP without NAA (TIMOFEEVA *et al*., 2014).

The best treatment in terms of shoot height for explants under natural ventilation was 5.37 µM of NAA combined with 11.10 µM de BAP (average height of 12.81 mm). When conventional lids were used, the best treatment was 2.69 µM de ANA associated with 0.00 µM of BAP (average of 19.42 mm), which did not differ from 5.37 µM of NAA combined with 0.00 µM of BAP (average of 18.18 mm) or 2.69 µM of NAA with 4.44 µM of BAP (average of 18.46 mm). The mechanism of a physiological interaction between BAP and NAA that regulates plant growth is reported by several authors, who found that a drastic increase in auxins can lower the endogenous level of cytokinins or vice versa (ROZOV *et al*., 2013). This balance is described in different eudicot species, including *Carthamus tinctorius* (Asteraceae) (MENDHE; SHEIKH, 2018) and *Hibiscus cannabinus* (Malvaceae) (SULTANA *et al*., 2016).

The tallest plants were observed for explants submitted to conventional sealing (Figure 3), with no significant interaction between NAA and BAP for this variable. The fact that the tallest shoots were obtained under the conventional system likely occurred due to the buildup of ethylene inside the culture flasks. Although ethylene has been reported as a growth inhibitor in several studies, it can have a contrasting etiolation effect in many species (KHAN *et al*., 2014). Thus, further research involving ethylene quantification is needed to confirm the relationship between this hormone and plant height in *Dipterix alata*.

In the natural ventilation system, five treatments exhibited an average number of leaves greater than four, with no difference between four of these treatments (Figure 2-c). On the other hand, only one treatment under the conventional system obtained a higher average, namely that consisting of 2.69 µM of NAA combined with 4.44µM of BAP (average of 5.49 leaves). As such, the natural ventilation system likely promoted greater phenotypic plasticity for the number of leaves in *Dipteryx alata*.

Figure 3 – *Dipteryx alata* explants submitted to different treatments in WPM medium. a: Natural ventilation + 5.37 µM naphthalene acetic acid (NAA) + 11.10 µM 6-Benzylaminopurine (BAP). b: Natural ventilation and no plant growth regulators. c: Conventional system + 2.69 µM NAA. d: Conventional system and no plant growth regulators. Bar = 1.75 cm



Source: Authors (2019)

Callus formation was observed for all the explants regardless of the treatment. The calluses were compact and yellowish and became oxidized 30 days after inoculation (Figure 3). This corroborates the findings of Rezende *et al*. (2019), who studied different types of *Dipteryx alata* explants and reported a greater incidence of calluses in nodal explants.

No rhizogenesis was observed in the explants, even in those submitted to NAA (Figure 3). Although some woody species root easily in the presence of this growth regulator [such as *Dalbergia nigra* (Fabaceae) (ALMEIDA *et al*., 2021)], others that exhibit secondary growth may have difficulty rooting (FORD *et al*., 2002). In a study

with *Apuleia leiocarpa* (Fabaceae), indole butyric acid (IBA) was efficient in rooting (HAYGERT-LENCINA *et al*., 2017) so asexual propagation is a promising alternative. This study aimed to evaluate in vitro and ex vitro rooting of A. leiocarpa and acclimatization of micropropagated plantlets. The hypothesis was that type of explant, indolebutyric acid (IBA. Thus, additional studies with *Dipteryx alata* are suggested to develop new in vitro rooting protocols using other auxins and different auxins combinations.

4 CONCLUSIONS

The *Dipteryx alata* seed germination and shoot proliferation protocol of *Dipteryx alata* shoots can be established with the factors analyzed exhibiting an influence on germination, decontamination and shoot proliferation. Seeds with an intact seed coat and NaClO concentrations greater than 10% are recommended for germination and decontamination. Shoot proliferation is more efficient with the use of 4.44 µM of BAP associated with natural ventilations seals.

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