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IN VITRO PROPAGATION OF JAMELÃO (*SYZYGIUMCUMINI* (L.) SKEELS)

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ABSTRACT

Syzygiumcumini (L.) Skeels is a plant species belonging to the Myrtaceae family, popularly known as jamelão, jamun, or Indian jamelão, widely used in medicine because of its hypoglycemic effects. In vitro cultivation is an alternative to seedling production and conservation of this important genetic resource, highlighting the micropropagation, which allows to obtain high quality plants, with identical genetic characteristics and in a short time. Thus, the study aimed to establish a seed germination system and in vitro multiplication of the jamelão (explants of nodal segments) with different treatments of Stimulate® (biostimulant), and 6-benzylaminopurin and 1-naphthaleneacetic acid, respectively. *S.cumini* seeds treated with 20.0 mL kg^{-1} of biostimulant resulted in higher rates of in vitro germination compared with other concentrations. On the other hand, a combination of 2.0 mg L^{-1} of 6-benzylaminopurin and 0.5 mg L^{-1} of 1-naphthaleneacetic acid favored the increase in the number of leaves, height (cm), and fresh mass (g) of the seedlings, resulting in the most suitable treatment for micropropagation of nodal segments of *S.cumini*. With the results obtained in this work, it can be concluded that the protocol used was efficient for the establishment of a micropropagation system of jamelão.

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INTRODUCTION

Syzygium cumini, popularly known as jamelão, ameixapreta, or amora pretaindiana, is a species native to tropical India (Babu et al., 2019). In Brazil, *S. cumini* occurs in several states of the southeast, central west, northeast, and north regions (Danadio et al., 1998). Jamelão plants are well-known in traditional medicine; their fruit contain high levels of various bioactive compounds like alkaloids, sterols, tannins, flavonoids, saponins (Naaz et al., 2019) and possess several medicinal properties, especially therapeutic effects on diabetes (Saurabh et al., 2019). A few studies have also reported the presence of a high pigment content in the fruit, which has generated interest in *S. cumini* regarding its nutrional effects (Rodrigues et al., 2015). Traditionally, *S. cumini* is propagated by polyembryonic seeds (Cavalcanti, 2010). This procedure inherently leads to variability in the descending plants, which can pose problems for the establishment of commercial orchards (Lorenzi, 2000). On the other hand, in vitro germination has the advantage of producing pathogen-free plants, providing reliable explants for the development of tissue regeneration studies.

A strategy to minimize the effects of uneven sowing is the use of biostimulants consisting of a mixture of several growth regulators in the formulation to improve growth and development. Stimulate® is a commercial plant biostimulant, that has attracted a large volume of research regarding its application on crops in recent years. It induces positive responses during plant development and may increase the absorption of water and nutrients by plants (Correia et al., 2018). Given the difficulties in sexual propagation, it is necessary to study new production methods for *S. cumini*. In this perspective, micropropagation or in vitro propagation are alternatives for obtaining high quality seedlings with desirable characteristics in a short time period and on a commercial scale (Fernandes et al. 2011). In vitro cultivation is considered a viable method for the propagation of several plant species including fruit trees. This method can be utilized in the maintenance and propagation of plant parts (cells or tissues) as well as the regeneration of whole plants thereof in an aseptic environment, representing one of the most prosperous areas of plant biotechnology (Mroginskiand Roca, 1991). Setting up a specific protocol tailored to the requirements of in vitro

cultivation of each culture in question, however, is essential for the process to be successful. In this sense, our study aimed to establish a seed germination system and in vitro propagation of jamelão (explants of nodal segments) with different treatments of Stimulate® (biostimulant), and 6-benzylaminopurin (BAP) and 1-naphthaleneacetic acid (NAA), respectively.

MATERIALS AND METHODS

The experiment was developed at the Plant Biotechnology Laboratory of the Sugarcane Biotechnology and Genetic Improvement Center, Federal University of Grande Dourados, municipality of Dourados, state of Mato Grosso do Sul (MS), Brazil. Explants were obtained from *S. cumini*parent plant fruit collected in the rural region of Guia Lopes da Laguna, MS (21°26′56″S; 56°6′16″W).

In vitro germination and initial seedling development: Fruit was pulped and seeds were sieved in the laboratory to remove mucilage. Subsequently, seeds were left under running water for 24 hours to eliminate all surface impurities. After this period, they were weighed for the correct determination of biostimulant concentration to be applied in different treatment regimes. Seeds then underwent disinfection under a horizontal laminar flow hood. This protocol consisted of a one-minute treatment by 70% ethyl alcohol (v/v) and a 20minutes treatment in sodium hypochlorite solution (NaClO) at 2.5% active chlorine, followed by three washes with autoclaved distilled water. The next step comprised the immersion of seeds for 30 minutes in Stimulate® (Stoller® - CMS-470, 300 – Itapavussu, Cosmópolis - SP) solutions at different concentrations (0.0, 5.0, 10.0, 15.0, 20.0, 25.0, and 30.0 mL kg^{-1} of seed). Following biostimulant treatment, seeds were inoculated into vials individually containing 30.0 mL of MS medium (30.0 g L^{-1} sucrose, 6.0 g L^{-1} agar) (Murashige and Skoog, 1962). The pH of the culture medium was adjusted to 5.8 ± 0.1 prior to autoclaving at 120 °C for 20 minutes. The culture was then transferred to the growth room, where it was incubated for 7 days in the dark at 25 °C. At the completion of this period, seedlings were exposed to a 16hours photoperiod (43 µmol m^{-2} s^{-1}) at 25 ± 2 °C. The completely randomized experimental design included seven treatments with each treatment consisting of five replicates of six bottles of sample, making up 30 bottles per treatment. The following variables were evaluated on the 30th day of cultivation: degree of fungal and bacterial contamination, stage of germination, seedling length (cm), and the number of leaves. Data were subjected to regression analysis using the Sisvar statistical program (Ferreira, 2000).

Micropropagation

Approximately 1.0 cm long nodal segment-type explants from seedlings obtained from in vitro germinated seeds were inoculated into test tubes containing 15.0 ml of MS medium (30,0 g L^{-1} sucrose, 6,0g L^{-1} agar) (Murashige and Skoog, 1962) supplemented with different concentrations of BAP (0.0 mg L-1, 0.25 mg L^{-1}, 0.5 mg L^{-1}, 1.0 mg L^{-1}, and 2.0 mg L^{-1}) and NAA (0.0 mg L^{-1} and 0.5 mg L^{-1}), respectively. The pH of the culture medium was adjusted to 5.8 ± 0.1 prior to autoclaving at 120 °C for 20 minutes.

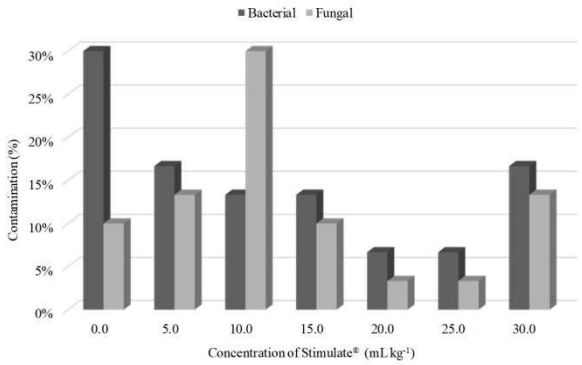

Figure 1. Rates of fungal and bacterial contamination during the *in vitro* germination of *Syzygiumcumini* seeds in culture medium with different Stimulate® concentrations

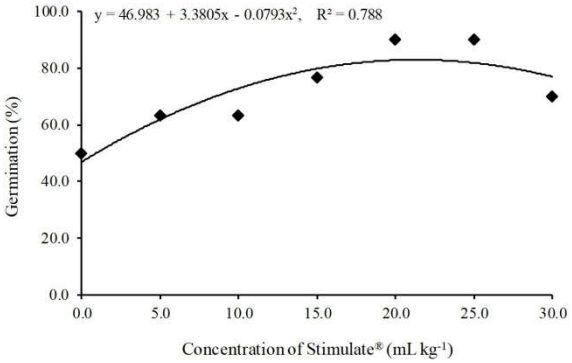

Figure 2. Rates of *in vitro* germination of *Syzygiumcumini* seeds in culture medium with different concentrations of Stimulate®

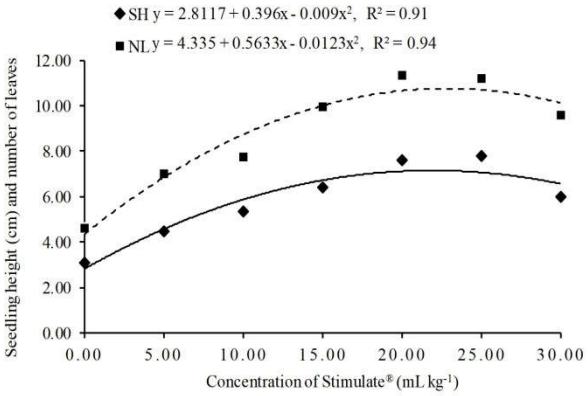

Figure 3. Effects of different concentrations of Stimulate® (in mL kg^{-1} of seeds) on the development of *Syzygiumcumini seedlings* after *in vitro* germination, expressed by variables seedling height (SH) (cm) and the number of leaves (NL)

The culture was then transferred to the growth room, where it was incubated for seven days in the dark. At the next step seedlings were transferred to a photoperiod of 16 hours at 25 ± 2 °C. The experimental design was completely randomized with a 5 × 2 factorial scheme consisting of ten treatments with twenty replicates each, and each experimental plot consisting of a tube containing an explant. The following variables were evaluated on the 45th day of cultivation: degree of fungal and bacterial contamination, seedling height (cm), number of leaves, fresh and dry mass (g). Data were subjected to analysis of variance. When significance was at 5% measured by an F-test, means were compared by the Bonferroni t-Test for treatments containing different concentrations of NAA, and regression analysis for treatments with different concentrations of BAP using the statistical program Sisvar (Ferreira, 2000).

RESULTS

In vitro germination: Based on the overall mean, 18% of contaminations observed on jamelão seeds during germination were of bacterial, while 14% were of fungal origin (Figure 1). Each treatment had a measurable effect on germination. However, treatments with Stimulate® resulted in comparatively higher germination rates that increased with concentrations reaching a maximum peak at 20 mL kg-1. However, concentrations above 30 mL kg^{-1} promoted a slowdown of germination, probably due to changing the hormonal balance of or being toxic to the embryo (Figure 2). Seedling height and the number of leaves showed a positive correlation with biostimulant concentration up to 25 mL kg^{-1}; values higher than 25 mL kg^{-1} resulted in a reduction in these variables and thus are not recommended (Figure 3).

Micropropagation: No fungal or bacterial contamination was observed throughout the procedure of jamelão nodal segment micropropagation. This was probably due to the good health status of plants from which explants were obtained, and the efficiency of the explant disinfection protocol. Referring to further variables studied, a significant correlation between growth regulator (NAA and BAP) concentrations and the number of leaves, seedling height, and fresh mass was revealed by analysis of variance. Dry mass was shown to be significantly affected by NAA concentration only (Figure 4 A, B and C). Treatments of a combination of 2.0 mg L^{-1} of BAP and 0.5 mg L^{-1} of NAA resulted in the highest mean values for seedling height and fresh mass when compared to a lack of NAA. Regarding the number of leaves, however, the highest applied concentration of BAP resulted in the highest value for this variable regardless of NAA presence. Furthermore, significantly higher mean values were measured for the number of leaves and fresh mass for a combination of 1.0 mg L^{-1} of BAP and 0.5 mg L^{-1} of NAA, in comparison to treatment without auxin. We hereby emphasize that these values resulting from either an interaction of the regulators or from treatment with cytokinin alone, are in a region of the curve featuring the minimum asymptote, further supporting the fact that a combination of these regulators tends to promote better results even though with lower mean values. No significant interaction was shown between dry mass and a combination of growth regulators, and only an isolated effect of NAA was observed with respect to this variable (Figure 5).

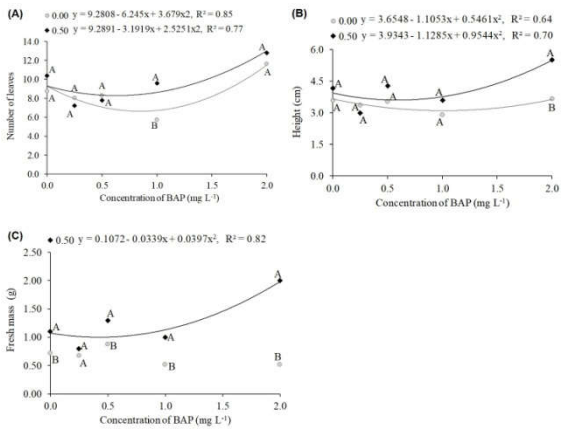

Figure 4. Interactions between concentrations of the growth regulator BAP (0.0 mg L^{-1}, 1.0 mg L^{-1}, and 2.0 mg L^{-1}) and NAA (0.0 mg L^{-1} and 0.5 mg L^{-1}) and variables. (*A*) The number of leaves. (*B*) Height (cm). (*C*) Fresh mass (g) of *Syzygiumcumini* seedlings. Means followed by the same capital letters do not differ significantly by Tukey's test ($P \leq 0.05$).

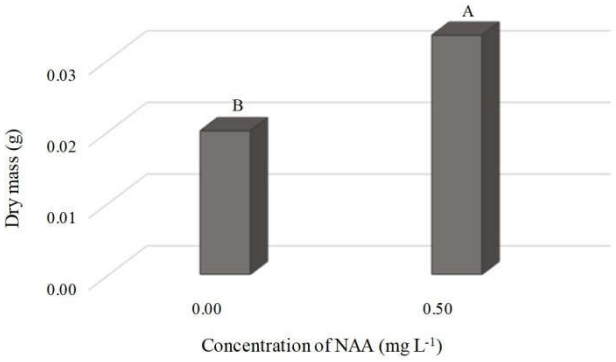

Figure 5. Effects of NAA concentrations (mg L^{-1}) on *Syzygiumcumini*seedling dry mass.Means followed by the different capital letters differ significantly by Tukey's test ($P \leq 0.05$).

DISCUSSION

Fungal and bacterial contamination observed during the cultivation period was low. This indicates that the type and concentration of disinfectant, as well as length of jamelão seed exposure to disinfectants preceding in vitro germination of were all efficient. Furthermore, the prevention of contamination was more efficient when the seeds were treated with Stimulate® at 20 mL kg^{-1} and 25 mL kg^{-1}. Conversely, contamination rates intensified parallel with an increasing concentration of biostimulant. Sodium hypochlorite is an antimicrobial agent used for the disinfection of inert surfaces to inactivate/kill microorganisms. However, its efficiency directly depends on the concentration used, the health condition of the parent plant, and the length of explant exposure to the disinfecting agent (Pereira et al., 2015). In addition, the formulation, concentration, and form of application of biostimulant are all factors that may interfere with the efficiency of growth regulator applied on the culture. Although further research is needed on the interaction of biostimulant and contaminants in in vitro cultivations, our findings demonstrate that microorganism growth was affected by the addition of 30 mL kg^{-1} of Stimulate®. An increase in cytokinin concentration leads to higher rates of contamination sabiá (*Mimosa caesalpiniifolia*) explants (Bezerra et al., 2014). Accordingly, our results support the hypothesis that growth regulators may promote an increase in contaminations.

Biostimulants comprise of mixtures of different plant regulators that promote plant hormonal balance. Stimulate®, for example, is composed of 0.005% indolebutyric acid (auxin), 0.009% kinetin (cytokinin), and 0.005% gibberellic acid (gibberellin). Each of these have direct effects on plant physiology (Gonçalves et al., 2018). Seed germination was likely promoted by gibberellin present in the biostimulant used in the present study. Gibberellin has been associated with the synthesis of hydrolytic enzymes that promote the degradation of proteins and starch, and the stimulation of cell elongation causing the primary root to break the seed tegument or structures restricting its growth (Buchelt et al., 2019). Stimulate® bearing synergistic properties may be solely responsible for the best results obtained for seedling height, number of leaves, and fresh and dry mass, respectively, because it comprises the plant hormones cytokinin, auxin, and gibberellin (Gonçalves et al. 2018). Not only can this biostimulant accelerate the development of seedlings and assist in root growth, but it can also increase the absorption and utilization of nutrients by providing hormonal balance to the

plant (Souza et al., 2013; Do Amaral, 2017). Gibberellin causes the primary root to rupture tissues, such as the endosperm, seed and/or fruit tegument, restricting its growth, while cytokinin and auxin complete the action of gibberellin by inducing cell division and differentiation, thereby promoting plant growth and development (Petri et al., 2016). Cytokinins induce the formation of large numbers of sprouts and increase the multiplication rate in many micropropagation systems by promoting cell differentiation, elongation and growth (Salisbury and Ross, 2013). BAP has become one of the extensively used commercially available cytokinins with satisfactory results for micropropagation (De Moura *et al*., 2013). In addition to the stimulation of cell expansion and elongation, auxins play a role in cell division together with cytokinin, and occur at higher levels in plants naturally (Kerbauy, 2013).

Fresh mass is a critical variable for studies on the efficiency and quality of a specific type of treatment on plant development, since it indicates the biomass gain of a seedling during a specified time period (Siqueira, 2014). A sharp rise of fresh mass was observed in our study, when the explants were submitted to treatments combining 2 mg L^{-1} of BAP with NAA. A potential explanation is that plants can absorb more water into their tissues given this concentration of the regulator (Asmar*et al*., 2012). In addition, the presence of BAP in the growth medium may induce a rise in the fresh mass production of cultivated plants (Grattapaglia and Machado, 1998). The greatest accumulation of dry matter was measured for seedlings cultivated in a nutrient medium containing 0.5 mg L^{-1} of NAA. Auxins promote the growth of the radicle and aerial parts of the plant, and are responsible for apical dominance, an essential condition to be considered in in vitro cultivations (Taiz and Zeiger, 2013); therefore, the presence of auxin in this medium must have favored the increase in biomass.

Conclusion

It was demonstrated through our study that it is possible to perform an in vitro propagation of jamelão. The highest rates of in vitro germination were observed for *S. cumini* seeds treated with Stimulate® at 20 mL kg^{-1}. The most favorable combination of plant hormone concentrations to achieve the greatest height, number of leaves, and fresh seedling mass in micropropagation were 2.0 mg L^{-1} of BAP with 0.5 mg L^{-1} ofNAA, respectively. The application of BAP was not necessary to achieve a rise in dry mass.

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