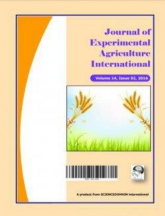

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Germination *In vitro* De Jabuticabeira *Myrciaria jaboticaba* (Vell.) Berg

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Authors' contributions

This work was carried out in collaboration among all authors. Author SKS designed the study, wrote the protocol and wrote the first draft of the manuscript. Author NPCL guided all research. Authors ODCJ and DSG helped in the execution of the experiment and in the bibliographical researches. Author MVA helped with bibliographic searches. Author KTEF helped in the execution of the experiment. All authors read and approved the final manuscript.

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ABSTRACT

Aims: The present work aimed to determine the influence of antibiotic use on seed germination and development of jabuticabeira (*Myrciaria jaboticaba*) seedlings grown *in vitro.*
Study Design: The experiment was conducted in a completely randomized design, where the treatments were composed of two types of culture medium and three forms of antibiotic use.

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Place and Study duration: The experiment was carried out at the Laboratory of Cell Biology and Culture of Vegetable Tissues (LABCULTIVE), at the Department of Biological Sciences (DCB), at the Agricultural Sciences Center (CCA), Federal University of Paraíba (UFPB), from November 2016 until May 2017.
Methodology: The fruits of jabuticabeira were harvested from a matrix plant and the seeds were removed manually, with subsequent elimination of the pulp and removal of the tegument. They underwent a disinfestation procedure in 70% alcohol and sodium hypochlorite and grown in culture medium.
Results: The highest germination average was obtained when the seeds were soaked for 24 hours in autoclaved water + antibiotic and when placed in liquid medium. In all analyzed variables the liquid medium provided better means. There was no statistical difference in any of the variables analyzed in relation to the use of the antibiotic in the imbibition and the non-use of the antibiotic.
Conclusion: The seeds of *Myrciaria jaboticaba* have greater germination and better development in the liquid culture medium; the presence of the antibiotic in the culture medium probably caused phytotoxicity, thus compromising the germination.

Keywords: Antibiotic; germination; Myrciaria jaboticaba; polyembryony; recalcitrance.

1. INTRODUCTION

The jabuticabeira (*Myrciaria jaboticaba*) originates from the Atlantic Forest, more precisely from the Center-South of Brazil, belongs to the family *Myrtaceae* and to the genus *Myrciaria* [1]. One of the forms of multiplication of the jabuticabeira is via seminiferous, however, it can also be propagated by grafting [2] and air layering [3], yet, both are less used methods due to the difficulty of rooting [2].

The jabuticaba seeds besides initiating their germination late, also present uneven germination, causing a setback in the species' perpetuation, damaging mainly the production of seedlings. According to Donadio [4], the jabuticabeira seeds germination can begin from 10 to 40 days after sowing, depending on the conditions in which they are found.

In vitro culture is a technique that has been used in large scale in the production of seedlings of several fruit species. By using this technique, the seedlings develop in aseptic conditions, free of pathogens, being therefore a market that has been growing exponentially, since the producers look for seedlings that do not compromise the good formation of the orchard.

One of the most widely used tissue culture techniques is micropropagation, since it allows large-scale rapid multiplication of plants with superior agronomic characteristics [5], however, it is necessary to avoid microbial contamination through preventive measures, so that success in *in vitro* propagation. In some cases, there is a need to add antibiotics to the culture medium for the microorganisms control [6,7], since competition between the explants and microorganisms occurs by the components of the culture medium, which can lead to the plant material death [8].

The darkening of the explants has been related to the release of phenolic compounds during the excision of the plant, which may inhibit its development and lead to death [9] In addition, some plant materials, usually those with woody characteristics, have a common problem that is the oxidation [10]. Direct contact with the culture medium can affect the development of the explant, so it is used "bridges" that act as a link between the explant and the liquid culture medium, that is without addition of gelling agents, such as agar and the phytagel, which also provides the decrease in the production costs of the culture media. The present work aimed to determine the influence of antibiotic use and consistency of the culture medium on the germination and development of jabuticabeira (*M. jaboticaba*) cultivated *in vitro.*

2. MATERIALS AND METHODS

The experiment was developed in the Laboratory of Cell Biology and Culture of Vegetable Tissues (LABCULTIVE), in the Department of Biological Sciences (DCB), Center for Agrarian Sciences (CCA), Federal University of Paraíba (UFPB), located in the municipality of Areia-PB, in the Brejo Paraibano microregion with latitude: 6°57' 55"S, longitude: 35°42'53" W and an average altitude of 507 m.

2.1 *Myrciaria jaboticaba* Seed Preparation

The jabuticabeira's fruits were harvested from a matrix plant located in the Macacos site, located

in the rural area of the city of Areia - PB. They were washed in running water to remove excessive impurities, leaving only those with adequate phytosanitary characteristics and no physical damage.

The seeds were manually removed from the fruits and the pulp was removed by washing them with running water, with subsequent drying of the seeds at room temperature in the shade.

After two days the tegument was removed and the seeds underwent a disinfestation procedure, washed three times with autoclaved distilled water, then immersed in 70% alcohol shaking for 30 seconds, and then washed three times in autoclaved distilled water, followed by immersion in 0.63% sodium hypochlorite solution, in the latter, there was mechanical agitation for 20 minutes and finally they were washed three more times with autoclaved distilled water.

2.2 Culture Media Preparation

The culture medium used was the ½MS [11]. The culture medium pH was adjusted to 5.8 before inclusion of 2.0 g L^{-1} of activated carbon and 7.0 g L^{-1} of Sigma® agar, the latter has been used only in treatments 2, 4 and 6. The culture media were then autoclaved at 120°C and 1.5 atm for 20 minutes.

2.3 Treatments

The treatments were arranged as follows:

Treatment 1 - After asepsis, the seeds were put to soak for 24 hours in autoclaved distilled water. Afterwards, they went through the disinfestation process again, following the methodology described in item 2.1, and in this case, after mechanical agitation for 20 minutes, the washing with autoclaved distilled water occurred in the laminar flow chamber, as well as the seeds transfer to tubes (Vinyl polychloride) with filter paper and 5 ml of liquid culture medium;

Treatment 2 - The methodology used was identical to the previous treatment, but the seeds were transferred to test tubes containing semi-solid culture medium with 5 mL;

Treatment 3 - After asepsis, the seeds were put to soak for 24 hours in autoclaved distilled water using an antibiotic capsule amoxicillin 500 mg L^{-1} in imbibition. Then, the seeds passed again through the disinfestation process, according to treatment 1;

Treatment 4 - The methodology used in this treatment was identical to the previous treatment, but the seeds were transferred to test tubes containing 5 mL of semi-solid culture medium;

Treatment 5 - After asepsis, the seeds were put to soak for 24 hours in autoclaved distilled water. Afterwards, they went through the disinfestation process according to treatment 1, in which case the liquid culture medium contained an amoxicillin 500 mg L^{-1}antibiotic capsule;

Treatment 6 - The methodology used in this treatment was identical to the previous treatment, but the seeds were transferred to test tubes containing 5 mL of semi-solid culture medium.

All cultures were kept in the growth room in the presence of light with photoperiod of 16 hours and temperature of 25 ± 2 °C.

2.4 Experimental Design and Evaluations

The experiment was conducted in a completely randomized design, in a factorial scheme 2x3 (Culture media x Antibiotic conditions), totaling 6 treatments with 5 replicates. Each repetition consisted of the average of 10 tubes.

Evaluations were carried out daily, where the percentage of germination that was obtained after the beginning of the test installation was evaluated, by calculating the number of normal seedlings obtained according to the Rules for Seed Analysis [12], the percentage of oxidation, polyembryony and contamination.

For seed vigor analysis, the germination speed index (IVG) was evaluated and calculated according to the formula proposed by Maguirre [13] where: IVG = G1 / D1 + G2 / D2 + ... Gn / Dn. When the seedlings were 5 to 13 cm in length, the length of the largest root, shoot length, number of leaves and number of roots were evaluated. The data obtained were submitted to analysis of variance when a significant effect was detected for the F test, the Tukey test was applied at a 5% probability level using the statistical software SAS University 3.4.

3. RESULTS AND DISCUSSION

3.1 Germination

The germination of Sabará jabuticabeira seeds cultivated *in vitro* was initiated on the fourth and fifth day after sowing using the liquid and semi-solid culture medium, respectively (Fig. 1), which

is a very expressive precocity when compared to the studies on the *ex vitro* germination as found by Santos et al. [14], when germination of the Sabará jabuticaba seeds occurred in 20 days after sowing in substrate composed of vegetable soil + vermiculite. In the work done by Wagner Júnior et al. [15], germination of Sabará and Cambinho jaboticaba seeds with a diameter of less than 6 mm using Plantmax® substrate started at 25 and 27 days, respectively. According to Wagner et al. [16] the germination of Paulista and Cabinho jabuticaba seeds placed in individual Petri dishes containing Germitest paper started seven days after sowing when exposed to 24 and 32°C and when treated with fungicide solution (Benlate 500 - 15 g L^{-1}). Alexandre et al. [17] evaluating the effect of maturation stage and substrate in *ex vitro* conditions on Sabará jabuticabeira, observed that the germination started 18 days after sowing. The anticipation of the germinative process is related to the removal of the integument, since this structure involves the embryo and it must break the integument to start the germinative process, however, as this structure was removed from the seeds, germination occurred more quickly.

There was no statistical difference regarding the use or not of the antibiotic in the semi-solid medium, but the same did not occur in the liquid medium, since when using antibiotic in the culture medium, the germination average was lower when compared to other means (Table 1). This fact probably occurred due to the fact that, when coming in contact with the medium, some explants may have their development affected, due to the restrictions in the absorption rate of the nutrients in the semi-solid medium, as well as the antibiotic may have provided phytotoxicity, inhibiting the seed development.

The highest germination average (84%) was obtained by using liquid culture medium and seeds imbibed in antibiotics. In the work done by Coellho et al. [18] with sucupira-branca, which is also a woody species, using liquid culture medium, the germination obtained was 95% when the tegument was removed from the seed and 80% when the tegument was sectioned.

3.2 Number of Roots and Leaves, Length of the Largest Root and Shoot, Germination Speed Index

All variables presented higher averages when using liquid medium, since the use of "bridges" prevented the direct contact of the explant with the culture medium and consequently the explant had a better development (Table 2). In relation to the type of medium, there was significance for length of the largest root, with averages of 3.82 and 3.55 cm in the liquid and semi-solid medium, respectively, whereas for the shoot length there was significance only when liquid medium was used. Wagner Júnior et al. [15] using Sabará seeds with 6mm and larger than 8mm in diameter, seeded in Plantmax substrate, obtained plant height averages of 2.18 cm and 2.73 cm, respectively. Maldonado 2014 [19] obtained the size of the seedlings of gabirobeira grown *in vitro* for 60 days of 17.67 mm.

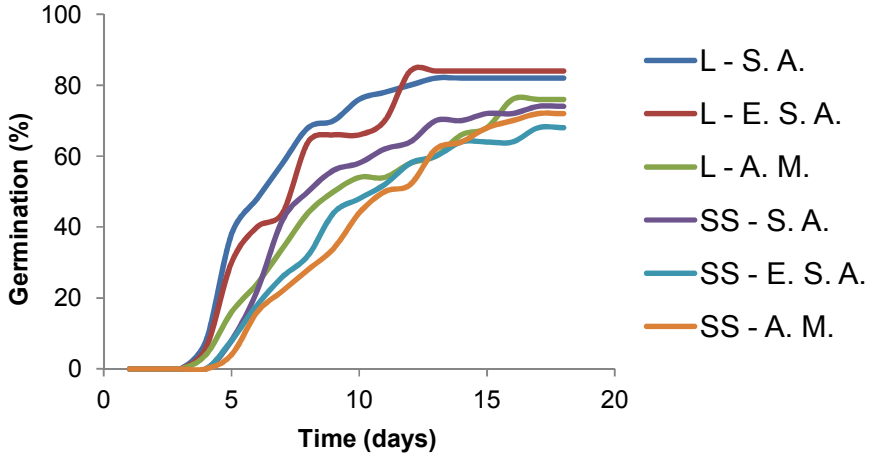

Fig. 1. Germination curve of the six treatments. L - liquid medium; SS - semi-solid medium; S.A. - without use of the antibiotic; E.S.A. - imbibition of seeds in the antibiotic; A.M. - antibiotic in culture medium

Table 1. Germination of the seeds of jabuticaba (*Myrciaria jaboticaba*) according to the type of culture and use of antibiotic or not. S.A.: without use of the antibiotic; E.S.A: seed embedding in the antibiotic; A.M.: antibiotic in culture medium

Type of medium	Germination (%)		
	S.A.	E.S.A.	A.M.
Liquid	82 aA	84 aA	62 aB
Semi-Sólid	74 aA	68 bA	74 aA
CV(%)	15,60		

Averages followed by distinct letters, uppercase in the row and lowercase in the column differ from each other by the Tukey test at 5% probability

Table 2. Number of roots and leaves, length of the largest root and shoot, germination speed index of *Myrciaria jaboticaba* as a function of the two medium types

Type of Medium	Number of roots	Number of leaves	Length of largest root (cm)	Length of shoot (cm)	IVG
Liquid	0,80 a	5,60 a	3,82 a	5,69 a	0,568 a
Semi-Sólid	0,65 a	4,72 a	3,55 a	4,25 b	0,475 b
CV(%)	33,23	36,55	38,60	36,79	23,45

Averages followed by distinct letters differ from each other by the Tukey test at 5% probability.

Table 3. Number of roots and leaves, length of the largest root and shoot, germination speed index (IVG) of *Myrciaria jaboticaba* due to antibiotic use or not. S.A. = No use of antibiotic; E.S.A. = Soaking seeds in the antibiotic; A.M. = Antibiotic in the culture medium.

Use of the antibiotic	Number of roots	Number of leaves	Length of largest root (cm)	Length of shoot (cm)	IVG
S.A.	0,84 a	6,58 a	4,51 a	5,58 a	0,606 a
E.S.A.	0,71 a	5,64 a	3,84 ab	5,63 a	0,530 ab
A.M.	0,63 a	3,27 b	2,69 b	3,71 a	0,429 b

Averages followed by distinct letters differ from each other by the Tukey test at 5% probability

Regarding the number of roots and number of leaves, there was no statistical difference in relation to the type of medium. The germination speed index (IVG) for seeds in liquid culture medium was significant, with an average of 0.568.

The number of leaves was significant when the antibiotic was not used, obtaining an average of 6.58, and when the seeds were soaked in the antibiotic, with an average of 5.64 (Table 3). In a study carried out by Wagner Júnior et al. [15] Sabará jabuticaba seeds classified between 6-8 mm, they obtained leaf number 1.78 after 46 days of cultivation. Sasso [2] when using Sabarája buticabeira stem as explant, they obtained the number of leaves of 4.2. Maldonado [19] obtained the number of leaves of gabirobeira grown *in vitro* for 60 days of 1.4. Santos et al. [20] observed that the addition of rifampicin in the culture medium was phytotoxic at concentrations of 0.5 and 1.0 g L^{-1}.

According to Palú et al. [21] high concentrations of antibiotic added to the culture medium can cause phytotoxicity and may be a limiting factor for the development of the explants. Phytotoxic action generally occurs due to disturbances of protein synthesis and inhibitory action in the synthesis of RNAs and ATPs, with interference, in the energy systems of the plant [22]. In relation to the number of roots and length of the aerial part, there was no significant difference as a function of the antibiotic use or not.

The largest mean of the IVG and length of the largest root of *M. jaboticaba* was obtained when the antibiotic was absent in the medium (S.A.), however, it did not differentiate when the seeds imbibition was done with the said product (E.S.A.). Rossa et al. [23] sowed totally cleaned jabuticaba seeds on a substrate composed of Florestal Plantmax® (50% v/v) + sieved organic compound (30% v /v) + vermiculite of medium granulometry (20% v/v), and they obtained IVG of 1.12, when the seeds were with the attached endocarp, they obtained the IVG of 0.98. Santos et al. [14] using semi-solid culture medium in the germination of Sabará jabuticaba seeds they obtained IVG of 0.32. It is worth mentioning that

the higher the IVG, the higher the daily germination speed.

3.3 Percentage of Oxidation, Polyembryony and Contamination

Regarding the type of medium, the percentage of oxidation was higher when semi-solid medium was used, and the percentage of polyembryony was higher when the liquid medium was used (Fig 2). The development of the explant may be influenced by the type of nutrient medium, explant type Golle [24], but also by the addition of some components such as antibiotic and fungicide. Coelho [25] using different concentrations of sodium hypochlorite for disinfestation of jabuticaba seeds and medium with inclusion of ágar, obtained oxidation percentage of 45%.

As regards the use of the antibiotic, the percentage of oxidation was higher when it was used in the culture medium, possibly causing toxic effect and modifying the morphogenetic characteristics of the explant (Fig 3). The percentage of polyembryony was higher when the antibiotic was not used, and there was no contamination in any of the treatments. Coelho [25] obtained a percentage of contamination of approximately

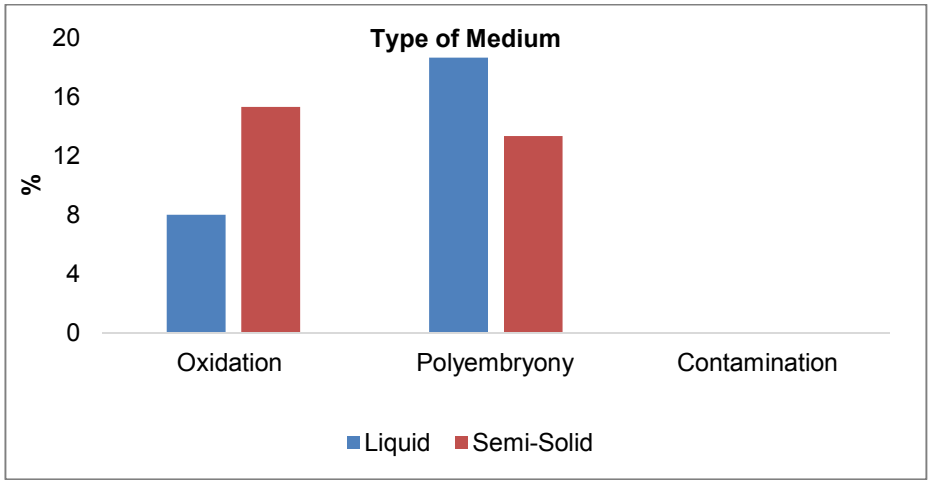

Fig. 2. Percentage of oxidation, polyembryony and contamination of *Myrciaria jaboticaba* as a function of the two types of medium

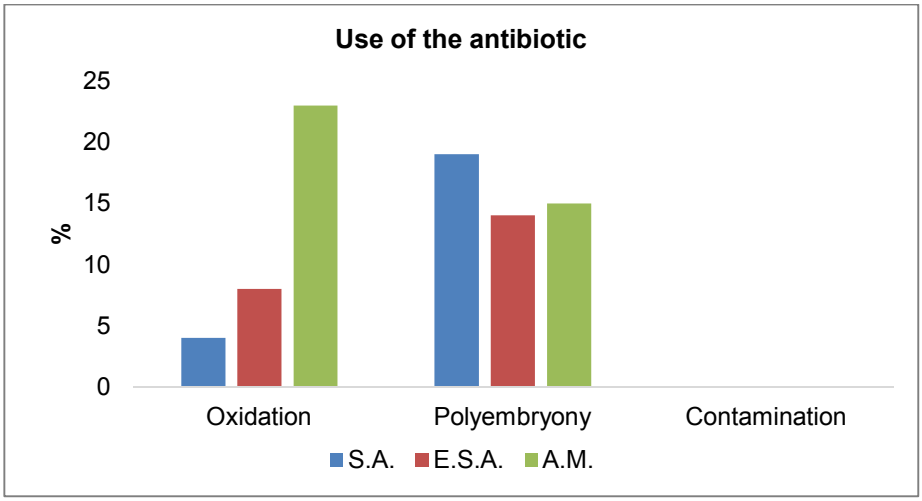

Fig. 3. Percentage of oxidation, polyembryony and contamination of *Myrciaria jaboticaba* due to antibiotic use or not. S.A. = No use of antibiotic; E.S.A. = Soaking seeds in the antibiotic; A.M. = Antibiotic in the culture medium

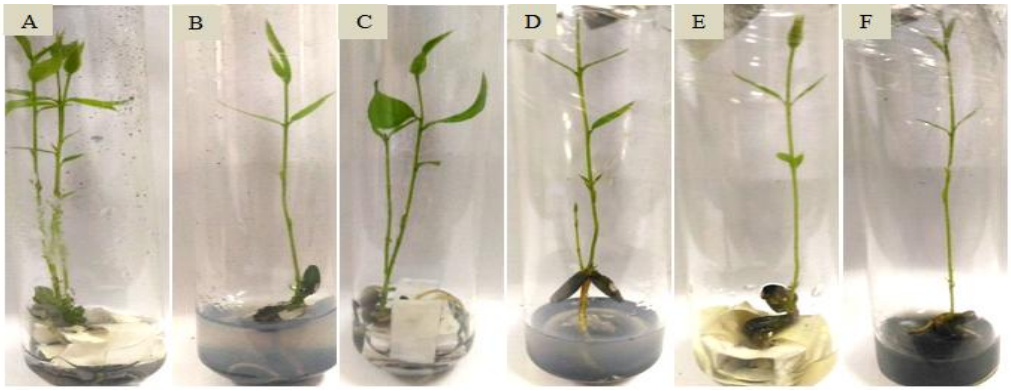

Fig. 4. *Myrciaria jaboticaba* seedlings obtained *in vitro* culture. Liquid medium without antibiotic (A); Semi-solid medium without antibiotic (B); Liquid medium, seeds soaked in the antibiotic (C); Semi-solid medium, seeds embedded in the antibiotic (D); Liquid medium with antibiotic in the medium (E); Semi-solid medium, with antibiotic in medium (F)

25% and 5% when used hypochlorite at 1,75% and 2.25%, respectively. According to Palú et al. [21] one of the factors limiting the use of antibiotics in the medium is the phytotoxicity of these substances, mainly due to the common use of high concentrations.

Fig. 4. Shows seedlings of the six treatments at 45 days after seed inoculation, where germination and seedling development occurred uniformly in all treatments, obtaining normal and healthy seedlings.

4. CONCLUSION

It was possible to obtain seed germination in the fourth and fifth day seeds after sowing in the liquid and semi-solid medium, respectively, however, it was in the liquid medium that the seeds of *Myrciaria jaboticaba* obtained greater germination and the seedlings obtained better development. The presence of the antibiotic in the culture medium can cause phytotoxicity, thus compromising the germination and development of *M. jaboticaba* seedlings.

COMPETING INTERESTS

Authors have stated that there are no competing interests.

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