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In vitro morphogenesis of different mangabeira (*Hancornia speciosa* gomes) varieties from the savanna of the Goiás

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Abstract: *Hancornia speciosa* is a fruit tree that is in the process of domestication. This time the propagation *in vitro* when well developed can promote conditions for the production of quality seedlings for this fruit. *In vitro* culture technologies are of great importance for conservation programs of genetic resources and genetic improvement of mangabeira. The objective of this study is to establish a methodology the micropropagation of *Hancornia speciosa*, Gomes, of three varieties (*cuyabensis*, *gardneri* and *pubescens*) by *in vitro* germination of seeds under different growth regulators. Fruits were collected at School of Agronomy of the Federal University of Goiás, transported to the Laboratory of Biotechnology, Federal Institute Campus Urutaí - Goiás, inoculated for germination in test tubes containing MS growth medium with 50% of salts concentrations, with five different concentrations of IBA. It was observed that the *in vitro* germination of *cuyabensis*, *gardneri*, and *pubescens* were 66.00 %, 64.05 % and 76 %, respectively. The beginning of germination occurred from three days for *gardneri* variety, six days for *pubescens* variety, and eight day for *cuyabensis* variety, extending to the 16 days for *gardneri* variety, and 28 days for the other varieties. Explants of different varieties differ in their manifestations when cultured *in vitro*. The aseptic method was effective for the control of fungal agents.

Keywords: Savannah, fruits, mangaba, micropropagation

Introduction

Mangabeira (*Hancornia speciosa* Gomes) is a fruit tree of tropical climate, native to Brazil and found in various regions of the country, from the Coastal Tablelands and Coastal plains of the Northeast, which is more abundant, to the Cerrado of the Midwest, North and Southeast regions (Soares et al., 2007).

Micropropagation is the alternative technique most frequently used for the purpose of obtaining a large number of uniform plants, regardless of the season (Borthakur et al., 1998). *In vitro* seed germination often allows a greater germinability than in nurseries, probably because the *in vitro* conditions are best suited for germination processes and early seedling development (Noleto & Silveira, 2004).

Hancornia speciosa is in domestication phase and thus the aspects related to growing needs more studies involving the vegetative propagation, selection of promising genotypes, development and adaptation of cultural practices,

studies on plant phenology, and aspects related to pre- and post-harvest. *In vitro* propagation technologies when well developed and adapted to Mangabeira are relevant for conservation and breeding programs of genetic resources (Ledo et al., 2007).

Development of mechanisms to assist and encourage the development of micropropagation protocols of native fruit trees is important, due to the few studies with Cerrado mangrove in our country.

The objective of this study was to establish a micropropagation methodology for three varieties of Cerrado's Mangabeira (*Hancornia speciosa* Gomes), varieties *cuyabensis*, *gardneri*, and *pubescens* through germination and *in vitro* development under different indole butyric acid concentrations.

Methods

It was carried out the study with plants of three varieties (*pubescens*, *cuyabensis* and *gardneri*), of which six fruits were collected. The

fruits were placed in boxes with ID, and stored at room temperature for 24 hours, until the transport to the Biotechnology Laboratory of the Federal Institute Goiano Campus Urutaí-GO (LABIOTEC) located on the geographical coordinates latitude 17°27'49" S, longitude 48°18'06" W, and altitude of 807 meters

The pulping was carried out with the fruit kneading under running water. The seeds were placed to dry in the shade for 24 hours on filter paper, and only the seeds with no injuries or mechanical damage were selected.

Aseptic procedures were performed washing with distilled and autoclaved water. Inside the laminar flow chamber, the selected seeds were immersed in sodium hypochlorite 2,5 % concentration, for 30 minutes, under constant agitation, followed by four rinses with distilled and autoclaved water to remove excess hypochlorite.

We conducted the incubation in a laminar flow chamber in test tubes of 1.5 cm in diameter by 15 cm length, containing 10 ml of growth medium. The test tubes already inoculated were sealed with Polyvinyl Chloride (PVC) film, placed in Styrofoam holder and identified, and then moved to a growth room where they remained with the incidence of indirect light for 24 hours and temperature 26 ± 2 °C.

After the 24 hours, we placed the inoculated material in a growth room with a photoperiod of 16 hours of light at a light intensity of $30 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, for approximately 30 days. In this period, daily assessments were performed until 50 % germination were noted, after this, weekly assessments were made. The weekly assessments determined the Oxidation Index (OX), Infection Rate (IR), identifying the type of contaminant agent, Fungi (F), and Bacteria (B); Root Protrusion Index (RPI), and Germination Index (GI) were determined. The germination rate was evaluated daily, registering the number of germinated seedlings; were considered as germinated seedlings those that emitted the root protrusion. Regarding the development of the seedling, the number of roots (NR), root length (RL) was determined; Shoot Length (SL); Leaf pair (PL) at 30 days after sowing (DAS) were determined.

For the *in vitro* seedling development weekly assessments were taken until the 60 days after planting after inoculation (DAI) for the evaluation of root protrusion index (Figure 5), number of roots, root length; shoot emission index (SEI); shoot length; pair of leaves for the varieties *pubescens*, *gardneri*, and *cuyabensis*. The length of shoot and root were measured using a digital caliber with 1.00 mm of precision.

Results and Discussion

The analysis of variance (Table 1) for the germination index (GI) and day after inoculation (DAI) did not reveal difference for the studied parameters, and structural levels analyzed for the three botanical varieties (*cuyabensis*, *gardneri*, and *pubescens*). However, the variety *cuyabensis* had an average GI of 66 % within 16.40 days. The

treatment T2 (1.0 mg L⁻¹ of IBA), had 80 % of the seeds germinated in 14 days, while the T3 (2.0 mg L⁻¹ of IBA) had 60 % in 12.0 days. The lowest mean was observed for the treatment T1 (0.00 mg L⁻¹ of IBA), this treatment had the lowest germination rate (50 %) and the highest number of days for the germination occurred (20 DAI). This variation results in a range of 62.5 % between the lowest and the highest value for the observed germination index.

The beginning of germination occurred on the third after inoculation for the var. *gardneri*, sixth DAI for variety *pubescens*, and eighth DAI for *cuyabensis*, extending to the 16th for *gardneri*, and 28th for the other varieties.

It was also found that between the beginning of root protrusion and rupture of the seed coat by the plumule five days passed and that in greater proportion of the treatments the issuance of taproot and fibrous root performed an 180° spin upon the occurrence of the aerial part, checked after the issuance of the root set.

The variety *gardneri* (Table 1) had average value for the germination index of 64 %, and ge number of 4.8 days. Treatment with higher GI was the T5 (4.0 mg L⁻¹ of IBA), with 80 % of seeds germinated in six days. The lower germination index was observed for this variety when using the dosage of 2.0 mg.L⁻¹. This occurs because the "mangabeira" ("mangaba" fruit tree) still is a non-domesticated species and the concentration of growth regulator (IBA) in this proportion may have contributed to the synergistic action between variety and dosage. The smallest index and days until germination for this variety in 4.8 days, among the varieties, studied since *pubescens* obtained an average of 9.8 days for 76 % of germination.

Variety *pubescens* presented 100 % of GI on the treatment T1 (0.00 mg L⁻¹ IBA) and T4 (3.0 mg.L⁻¹). It is noteworthy that the micropropagation of the *Hancorniaspeciosais* feasible, provided it is established criteria and methodologies of studies for the protocols, as shown for the *pubescens* variety whose indexes reached a level of 100 % of germination. Melo et al. (2008), confirms the important contribution of the *in vitro* germination of seeds of native species to achieve uniformity of production since these species are still under domestication phase.

The difficulty of maintaining germplasm *in vivo* (Cid, 2010) contribute to the development of protocols to assist the conservation of germplasm *in vitro* of "mangabeira" seeds, as these according to Silva et al. (2001) are recalcitrant. The processes inherent to germination *in vitro* of this native fruit tree can provide information that can be strategic alternatives for the conservation of plant resources, reducing the loss of genetic heritage by a decrease in indigenous areas.

No oxidation index was observed in "mangabeira" seeds inoculated *in vitro* in any of the treatments. Oxidation is a phenomenon that can occur in seeds when interacting with aseptic agent,

NaOCl. The activity on “mangaba” seed may be inhibited by some existing physiological process in the seed coat of this fruit tree. However, the development of studies that can explain these mechanisms that may be acting in this situation, so

we can help to minimize the oxidation processes in seeds, observed in other species.

Table 1. Mean, maximum and minimum values; standard deviation and coefficient of variation for the germination rate (%) and days after inoculation (DAI) for the three varieties of mangaba (*cuyabensis*, *gardnerii*, and *pubescens*) from the Germplasm Bank of the Federal University of Goiás, subject to different concentrations of auxin. Urutai-GO, 2013.

Varieties	Treatment (doses AIB mg L ⁻¹)	Germination (%)	DAI
<i>cuyabensis</i>	1 (0.0)	50.00a	20.00a
	2 (1.0)	80.00a	14.00a
	3 (2.0)	60.00a	12.00a
	4 (3.0)	70.00a	18.00a
	5 (4.0)	70.00a	18.00a
	Mean		66.00
Minimum		50.00	12.00
Maximum		80.00	20.00
Standard Deviation		11.40	3.29
CV (%)		0.17	0.20
<i>gardnerii</i>	1 (0.0)	70.00a	6.00a
	2 (1.0)	70.00a	6.00a
	3 (2.0)	30.00a	3.00a
	4 (3.0)	70.00a	3.00a
	5 (4.0)	80.00a	6.00a
	Mean		64.00
Minimum		30.00	3.00
Maximum		80.00	6.00
Standard Deviation		19.49	1.64
CV (%)		0.30	0.34
<i>pubescens</i>	1 (0.0)	100.00a	11.00a
	2 (1.0)	50.00a	9.00a
	3 (2.0)	70.00a	9.00a
	4 (3.0)	100.00a	10.00a
	5 (4.0)	60.00a	10.00a
	Mean		76.00
Minimum		50.00	9.00
Maximum		100.00	11.00
Standard Deviation		23.02	0.84
CV (%)		0.30	0.09

CV%: coefficient of variation; Means followed by the same letter in the columns do not differ statistically by Tukey test.

The variance analysis of growth characteristics of *in vitro* development of *H. speciosa* var. *cuyabensis* revealed significant difference between treatments with different doses of IBA, only to the number and length of roots characteristic (Figure 1, 2). It is noted that the treatments that have obtained the highest averages were Treatments 4 and 5, with 34.14 and 29.28 roots on average, respectively.

As for the average root length, these values represented 57.57 mm and 42.00 mm in the Treatments 4 and 1, respectively. The lowest values can be checked in Treatments 1 and three as for the NR and for growth the Treatments 5 and 3. The study of *in vitro* germination about the “mangabeira” and the other factors related to the root emission and root length are relevant because they can contribute to the success of this fruit tree during acclimatization phase, as this step can become a

limiting factor in the process of micropropagation (Grattapaglia & Machado, 1998). It was observed that the treatments with better effects on the number and length of roots were those who had in their composition higher doses of IBA (4.00 mg L⁻¹).

As for the other variables, SL and PL, although there are significant differences between the treatments, the highest average was also noticed in the T4 with averages of 94.85 mm, and 5.85 mm, respectively. These data lead to the explanation that the auxin IBA, although it is a stimulant of initiation and formation of meristem tissue that will act in the elongation of the tissues that were begging the roots development in early stages (Hartmann & Kester, 1990). It can also assist in the development of shoots of seedlings established *in vitro* as presented in this study with *H. speciosa* var. *cuyabensis*.

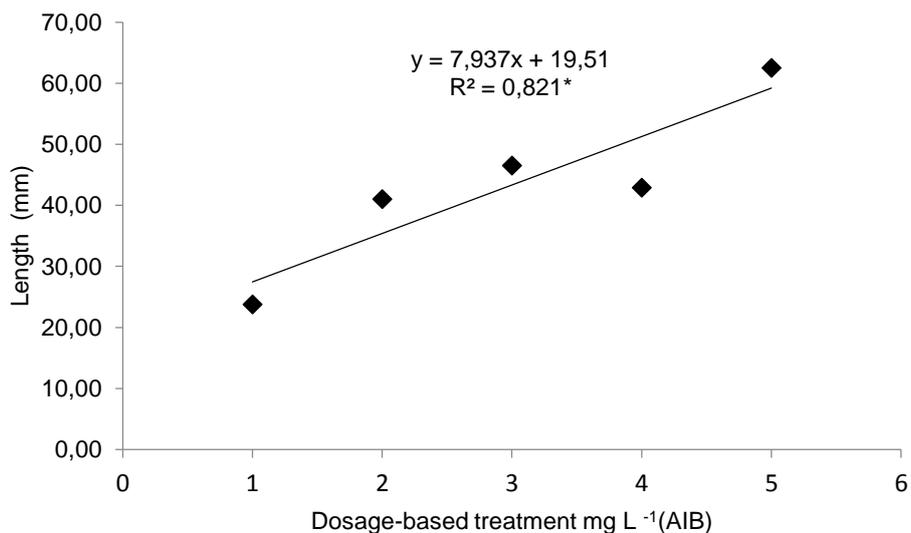


Figure 1. Linear regression between the different of root length and doses of IBA in *H. speciosa* seedlings variety *cuyabensis* 30 DAS *in vitro*.

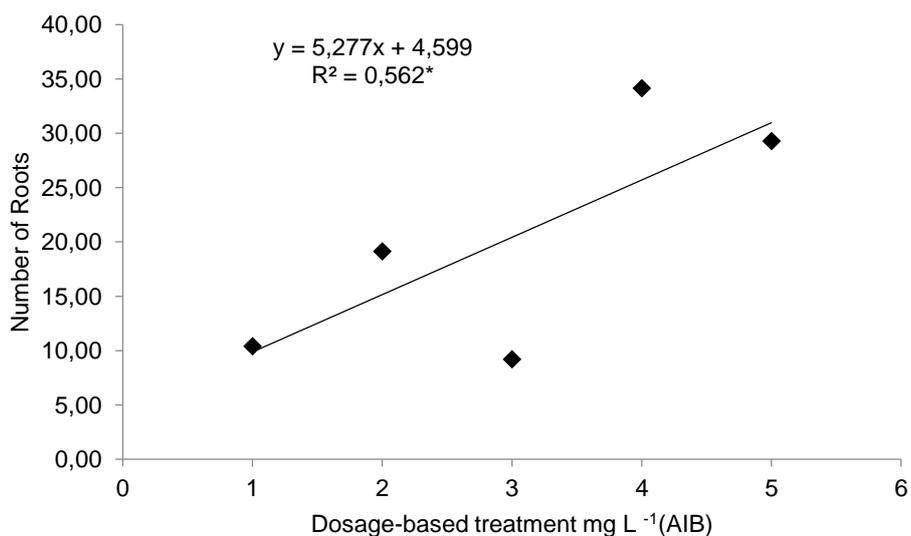


Figure 2. Linear regression between the different number of roots and doses of IBA in *H. speciosa* seedlings variety *cuyabensis* 30 DAS *in vitro*.

It is necessary to note others observe when the “mangabeira” was subjected to the lowest dosage of IBA in T1, as the RL, SL, and PL variables remained significant (42.0 mm, 83.4 mm, and 4.6 mm, respectively). Therefore, it leads to evidence that this variable behavior may occur because the species is still in domestication phase.

The data for the variety *gardneri* used on analysis of variance for germination characteristics showed the existence of significant differences only for the variable RL with means 62.0 mm, 48.0 mm, 46.42 mm, 42.6 mm, and 31.5 mm. The best average was noted on the T5 with 4.00 mg L⁻¹ of

IBA. The amplitude generated between the highest and lowest average of NR and RL was 50.81 %. Although there was no significant difference between treatments (1, 3 and 4), it can be seen that the amplitude obtained from the T5 (higher value) and T1 (statistically similar value) was 77.41 %, amplitude itself justify the choice by T5 as an option to carry out further studies with *in vitro* germination of “mangaba” variety *gardneri*.

For the characteristics NR, SL, and PL, there were no significant differences between the different doses of IBA. The highest average for this characteristics was observed in T3 with 18.33 mm,

92.57 mm, and 6.0 mm, respectively. The highest rates for SL and PL occurred in Treatment 1, while the highest rates for NR were found in T5 (4.00 mg L⁻¹ of IBA). Therefore, to obtain seedlings with more robust root systems, maybe the inoculation of “mangaba” seeds in growth medium with greater IBA ratios is interesting, while the objective of the study is to gain nodal segments from axillary buds could be used lower doses of the growth regulator (RC) as mentioned above. The development of seed *in vitro* germination protocols is interesting to further studies with this species.

When analyzing the variety *pubescens* (Figure 3, 4), as observed for *cuyabensis*, significant differences for the variables NR and RL were noted. The highest averages were seen in Treatments 5 for

both NR and RL. The lowest values, although there were no significant were found in Treatment 1. The RPI and SEI, the levels are in line having the same performance proportional. As for the SL and PL, Treatments 5 and 1 obtained the highest average. The development of the leaves of seedlings *in vitro* is important, therefore, to submit these materials to the stage of acclimatization (Phase IV of micropropagation) there is the transition condition, turn a heterotrophic plant into an autotrophic plants, i.e. photosynthesize and thus produce carbon necessary for their survival. As these leaves have stomata but are still somewhat functional, production of leaves would be indicative of a promising protocol for germination and *in vitro* development of species, especially woody.

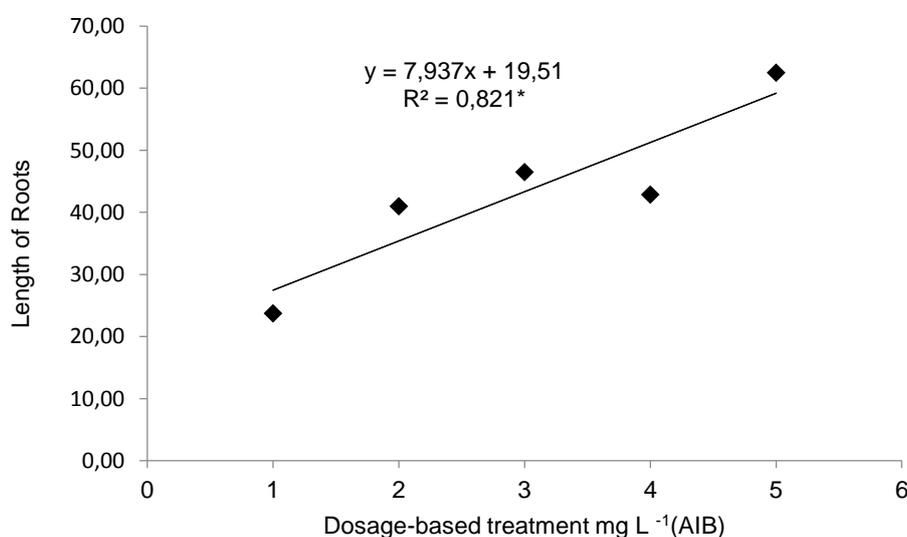


Figure 3: Linear regression between the different of root length and doses of IBA in *H. speciosa* seedlings variety *pubescens* 30 DAS *in vitro*.

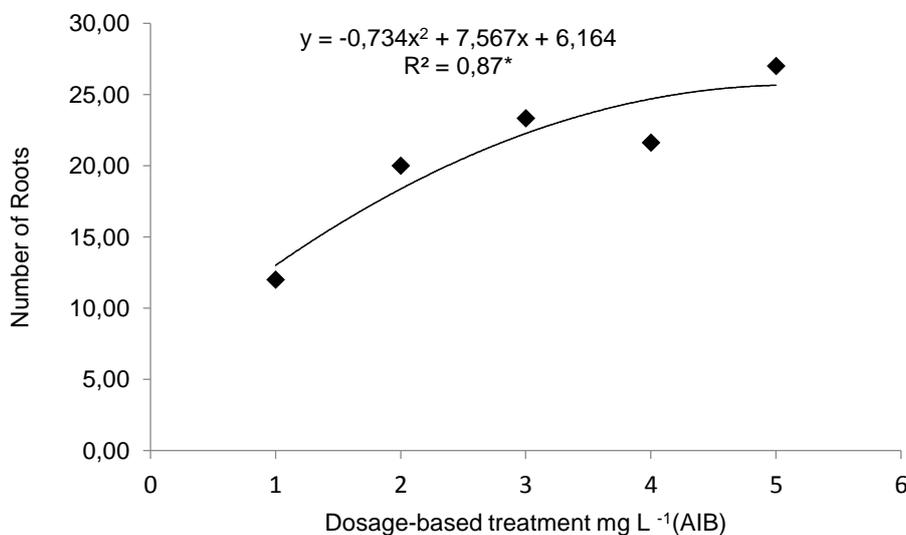


Figure 4: 2nd Order Polynomial Regression between the different number of roots and doses of IBA in *H. speciosa* seedlings variety *pubescens* 30 DAS *in vitro*.

In the study noted that *H. speciosa* when germination *in vitro* has the occurrence of protrusion and emission of epigene roots, soon after the opening of the cotyledons and plumule, perform a rotating of 180° to hypogeal position and that time with the issuance of taproot and fibrous root. This phenomenon takes place with simultaneous development of the first pair of leaves. *H. speciosa* has mostly remaining integument associated to a cotyledon structure already in opening stage, or to the hood of taproot.

Oxidation was not present for “mangaba” seeds inoculated *in vitro* in any of the treatments (Table 2). Oxidation is a phenomenon that can occur in seeds when interacting with aseptic agent, NaOCl, and some existing physiological process in its integument maybe inhibits that action on “mangaba” seed. Cola et al. (2010), in his studies of *in vitro* establishment of *Eugenia uniflora*, found the lowest percentage of oxidized seeds in treatments with long time (20 min) on seeds submitted to NaOCl (16.2 %), and the worst result, with higher oxidation in treatments T1 (25.00 %) and T3 (20.00 %). The concentration of aseptic agent and duration

of treatment are critical in establishing seed germination *in vitro*, since the aseptic treatments may be ineffective or harmful when too smooth or too aggressive, respectively (Gomez, 2007).

The disinfection treatments for 5 minutes which the seeds were submitted caused depigmentation. This problem is more evident in the treatments of 10 minutes in sodium hypochlorite solution (0.60% NaOCl) in both MS growth medium tested.

For contamination (Table 2), it is noticed that the occurrence of this event was not verified only in the treatment four (T4) for the varieties *gardneri* and *pubescens*. Noteworthy that the contamination percentage values ranged from 0.00 % to 30.00 % for *gardneri*, and *pubescens*, while for *cuyabensis* these values range 20.00 % between the highest and the lowest value observed. All the contamination contaminants were of bacterial origin.

In this study, the analyzes lead to the inference that there was aseptic protocol efficiency for elimination of fungal agents, as infection rates were 5.00 % (*gardneri*-T3 and *pubescens*-T1), not occurring for *cuyabensis*.

Table 2. Mean values (%) for the occurrence of oxidation (Oxid) and contamination (Contam.) in seeds inoculated *in vitro* of three varieties of *Hancorniaspeciosa* (*cuyabensis*, *gardneri* and *pubescens*) from the Germplasm Bank of Agronomy School of Federal University of Goiás (EA / UFG), subjected to different concentrations of auxin. Urutai, GO, 2013.

IBA mg L ⁻¹	<i>cuyabensis</i>			<i>gardneri</i>			<i>pubescens</i>		
	Oxid	Contam.		Oxid	Contam.		Oxid	Contam.	
		Fungus	Bacteria		Fungus	Bacteria		Fungus	Bacteria
T1 (0)	0.00	0.00	30.00	0.00	0.00	20.00	0.00	5.00	20.00
T2 (1)	0.00	0.00	20.00	0.00	0.00	30.00	0.00	0.00	30.00
T3 (2)	0.00	0.00	30.00	0.00	5.00	30.00	0.00	0.00	20.00
T4 (3)	0.00	0.00	10.00	0.00	0.00	0.00	0.00	0.00	0.00
T5 (4)	0.00	0.00	20.00	0.00	0.00	10.00	0.00	5.00	10.00
Mean	0.00	0.00	22.00	0.00	1.00	18.00	0.00	2.00	16.00
Maximum	0.00	0.00	30.00	0.00	5.00	30.00	0.00	5.00	30.00
Minimum	0.00	0.00	10.00	0.00	0.00	0.00	0.00	0.00	0.00

The percentage of “mangaba” seed germination under nursery conditions in general, is low not only due to the presence of inhibitors in the pulp as well as the fact that the seeds are recalcitrant (Lorenzi, 2004). Ledo et al. (2007) in a study about *in vitro* germination of “mangabeira,” found germination rates above 95% in seeds with the removal of the seed coat. In this study, it can be seen rate of 100.0% for Treatments 1 and 5, for some varieties. It is likely that this species has physiological mechanisms that promote seeds germination and *in vitro* development of mangabeira seedlings, with the absence of auxin IBA, as well as

its concentration of 4.00 mg L⁻¹. According to Rohr & Hanus (1987), responses to auxin, are not universal. Some species, especially woody, rooting with difficulty or not even root, despite the presence of auxin and some species even require the use of growth regulators in its roots. The data obtained in this study are higher than those found by Lopes et al. (2011) when evaluating the development (SL and RL) *in vitro* of “mangabeira” of Cerrado with different doses of auxin. Noted that the development of seedlings despite the PR rate of 51.33 % (T1) not all seeds that issued protrusion had root development

(39.50 %) and not all that issued roots had the shoot emission (20.82 %).

The methods of sterilization for native species, especially for the varieties of *H. speciosa* obtained in this research, are not yet established, with necessary adjustments that include a minimum of contamination rate without the need to add chemosterilants agents. The contamination by microorganisms is a major problem for plant in the world so that we can establish and develop protocols for the establishment of *in vitro* culture, the starting point is the knowledge culture is plant pathogens (Cassels, 2001).

The pathogenicity of the bacteria was observed in 100% (Table 2) of the cases, verified by contaminants in *cuyabensis* and 90% for other varieties, with the need for additional studies to investigate the non-effectiveness of aseptic treatment (alcohol + hypochlorite) for different doses of AIB and variety studied.

According to Pereira et al. (2003), microorganisms that occur *in vitro* can be endophytic and can not cause damage to vitroplants, appearing because of some stress. It can compete with seedlings grown *in vitro* by nutrients and carbohydrates of the growth medium, contributing to the decrease of proliferation, development, rate of budding, rooting, leading to micro plant to necrosis and eventual death.

Conclusions

The conditions in which the study was conducted, it can be concluded that the *in vitro* germination for the varieties *cuyabensis*, *gardneri* and *pubescens* was 66.00%, 64.05 and 76% on average, respectively;

The start of germination occurred from the third to DAI for the variety *gardneri*, sixth DAI for variety *pubescens* and eighth DAI for *cuyabensis*, extending to the 16th day to variety *gardneri*, and 28 day for the other varieties.

The aseptic method was effective for controlling fungal agents.

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