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In vitro Cultivation of *Hancornia speciosa* Gomes : The Physical Constitution of the Culture Medium, Sucrose Concentrations and Growth Conditions

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Abstract

The effect of constitution of the culture medium, different concentrations of sucrose, and different growth conditions on the germination of seeds and growth of explants of "mangaba" (*Hancornia speciosa* Gomes) were evaluated in two *in vitro* assays. In assay one, the physical constitution of a medium (solid and liquid) and different concentrations of sucrose (0, 15, 30, 45, 60, 75, and 90 g/l) were tested, and seed germination was found to be variable. In assay two evaluation of the effect of different growth conditions (with and without agitation) on the *in vitro* growth of explants. Present results showed that "mangaba" had the highest percentage of germination and potentiality when inoculated in a liquid medium with (15 g/l) or without sucrose. The medium without agitation resulted in a better growth of explants the average length number of buds.

Introduction

Mangaba (*Hancornia speciosa* Gomes) is a tropical tree species - a native to Brazil. It is found in the coastal plains and lowlands of the northeast and in the Cerrado, which occupies the midwest, north, and southeast regions. It provides primary materials for the juice and ice cream industries. It is also a rubber-producing plant (Bastos et al. 2007, Lédo et al. 2007, Soares et al. 2007a).

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The harvest of its products is obtained mainly through extraction. According to Vieira et al. (2006), the physical and mechanical characteristics of the rubber obtained from mangaba latex are suitable for several technological, industrial, and commercial applications (Soares et al. 2007a).

The species propagates sexually; however, this method is not satisfactory because the plant has highly recalcitrant seeds. In addition, the pulp of the fruit inhibits germination (Bastos et al. 2007, Soares et al. 2009). Therefore, its seeds should be sown right after removal of the pulp or up to 48 hrs thereafter after the harvesting of fruits because the germination decreases rapidly after the fourth day (Soares et al. 2007b). In general, the percentage of germination is low, and the emergence and growth of seedlings is slow (Vieira et al. 2006).

In this context, micropropagation using tissue culture techniques is useful for propagating different species that, like mangaba, have a low rate of germination. Even though the process involves different stages following a defined protocol for any species, the protocol might be optimized to obtain high quality plants with low production costs (Lédo et al. 2007, Reis et al. 2008).

Cultivation media seek to meet the needs of the species that is being grown and to provide the nutrients necessary for *in vitro* growth. A medium is composed of essential and optional components required for plant growth, which vary according to the species, cultivar, or explant that is used and must be experimentally defined for each particular case. Moreover, all the nutrients in a cultivation medium should be present in optimum concentrations to ensure the growth of explants (Bassan et al. 2006, Fick et al. 2007). Solutions of salts and sugars are also used for cultivation media, and these solutions not only exert a nutritive effect but also influence cellular growth and morphogenesis through their osmotic properties (Braun et al. 2010).

Likewise, physical characteristics of culture media play an important role in the success of the *in vitro* establishment of plants (Murashige 1977). There are certain species whose explants develop better in a liquid medium, while the explants of some species develop better in a solid medium, and still others respond best to a liquid medium in which seeds are anchored with a support.

Even though agar is widely used due to its effectiveness as a gelling agent, liquid medium systems have gained importance due to ease of preparation. In addition, the greater contact between explants and culture medium ensures absorption of water and nutrients, if it is combined with a suitable aeration systems. Furthermore, liquid culture media with aeration systems make automation of the micropropagation process possible and can be used on a commercial scale (Faria et al. 2006, Silva et al. 2007, Mengarda et al. 2009).

The objective of this study was to evaluate the effect of the physical constitution of the medium, different sucrose concentrations, and growth conditions on the germination of seeds and growth of explants of mangaba in two *in vitro* assays.

Materials and Methods

The plant material was obtained from fruits of *H. speciosa* Gomes, which were collected in September of 2010 at the Gameleira farm located in the municipality of Montes Claros de Goiás - GO 592 m in altitude. The assays were conducted in the Laboratory of Plant Tissue Cultivation of IF Goiano - Câmpus Rio Verde, GO.

Effects of the physical constitution of the culture medium and different concentrations of sucrose on the in vitro germination of Hancornia speciosa: The removal of the fruit pulp was performed manually with the help of a number 30 aluminum mesh sieve. To remove the excess of fruit flesh from seeds, a solution of 5% sodium hydroxide was used for 5 min. Subsequently, the seeds were manually washed under running water to remove the tegument and submersed for 10 min in a container with running water with three drops of Tween (80%). Then, they were immersed in a bowl with 70% (v/v) alcohol for 1 min and in a solution of sodium hypochlorite (20%) for 20 min. A triple wash was performed inside a laminar flow with autoclaved distilled water.

The seeds were cultured in bottles (12 cm × 5 cm) containing 15 ml of WPM culture medium (Lloyd and McCown 1980) at half of the original concentration; moreover, the media prepared differed in their physical constitution (liquid and solid) and in the sucrose concentration (0, 15, 30, 45, 60, 75 and 90 g/l). For the preparation of the solid medium, 3.5 g/l of agar (Dinâmica®) was used. The pH was adjusted at 5.7 ± 0.3 before autoclaving. The inoculated bottles were maintained under a photoperiod of 16 hrs at 25 ± 3°C, a relative humidity of 45 - 46%, and an active photosynthetic radiation of 45 - 55 μmol m^2/s.

Daily counts were performed to determine the complete stabilization of the germination percentage. The germination rate index (GRI) and the average time to obtain 50% of germination (T50) were also assessed. The GRI was calculated according to Maguirre (1962), and the T50 was measured according to Resende et al. (2009). Seeds displaying radicle protrusion were considered to have germinated.

The experiment was set up using a completely randomized design, in a 2 × 7 factorial design (culture medium × sucrose concentration); similarly, each treatment was replicated 20 times, one per bottle, comprising a total of 280

experimental units. The numerical data were statistically evaluated through analysis of variance with the application of the F test at a level of probability of 5%, and the means were analyzed through linear regression using SISVAR software (Ferreira 2008).

Effects of different growth conditions on explants of H. speciosa: Nodal segments from *in vitro* established seeds were used as the source of explants. Each segment was 2 cm in length and had two buds. The culture medium was WPM (50%) with two different physical constitutions, liquid and solid. Agar was used to solidify the medium (3.5 g/l), while the liquid medium was maintained both with and without agitation. Bottles (12 cm × 5 cm) containing 15 ml of culture medium were used. The agitation of the medium was performed with an orbital shaker (Nova Técnica NT 712) at a rotation speed of 90 rpm.

The pH of the medium was adjusted to 5.7 ± 0.3 before autoclaving. The inoculated bottles were kept under a photoperiod of 16 hrs, at 25 ± 3ºC, a relative humidity of 45 - 46%, and an active photosynthetic radiation of 45 - 55 μmol m^2/s. After 30 days, average length of the shoot (cm), the number of buds, and the number of leaves were recorded.

The experiment was set up using a completely randomized design, with 3 treatments replicated 25 times; each repetition consisted of a bottle containing 3 explants, comprising a total of 225 experimental units. The numerical data were statistically evaluated through analysis of variance, and the means were tested using a Tukey's test at a level of probability of 5%, using SISVAR software (Ferreira 2008).

Results and Discussion

The seeds produced seedlings in all the treatments. The first radicle protrusions were observed 15 days following inoculation, and at 25 days, the emergence of the epicotyl was observed. The latter, the cotyledonary nodes and the first pair of leaves were ~~a~~ purple-yellow colored, which is characteristic of this plant. The same observation was made by Lédo et al. (2007).

In all the treatments, the seeds generated well-formed, vigorous plants without morphological alterations and without the formation of calluses. Visually, plants grown in the solid medium were more vigorous and rigid. The green color of the stem and leaves were large accentuated, and there was a profuse growth of adventitious roots (Fig. 1A). At the beginning of germination, next to the hypocotyls, a bulge of yellowish color was noted from where the roots emerged (Fig. 1B).

In contrast, plants grown in the liquid medium initially displayed an appearance of over-watering, and the leaves and stems were initially a light green color and progressively acquired a darker color (Fig. 1C). The hypocotyl was clear, almost white, with few adventitious roots and without the previously mentioned bulge (Fig. 1D). In addition, the seedlings were more fragile.

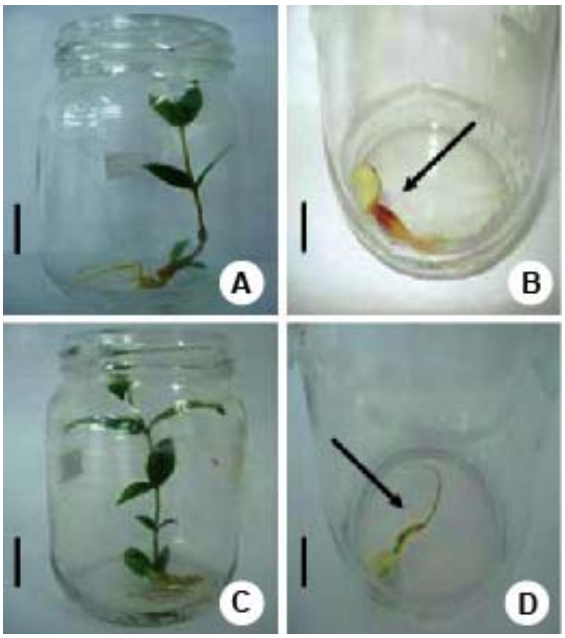

Fig. 1. Seedlings of *Hancornia speciosa* at 35 days of cultivation: (A) solid medium with no sucrose. (B) solid medium with 15 g/l of sucrose. (C) liquid medium with no sucrose. (D) liquid medium with 30 g/l of sucrose. The arrows indicate the bulge of the hypocotyl. Bar = 10 mm.

A quadratic regression model was the most appropriate to explain the development of mangaba seedling generation. There was an effect of both the consistency of the medium and the sucrose concentration (Fig. 2).

A higher percentage of germination (45%) was observed in the liquid medium (Fig. 2A). As the concentration of sucrose increased, there was a reduction in the percentage of germination. A higher average value was obtained in the medium without sucrose and in the medium supplemented with only 15 g/l sucrose. The values were 60 and 62%, respectively.

For the germination rate index, a behavior similar to that of the germination percentage was observed, i.e., the highest average value (0.28) was observed in the liquid medium (Fig. 2B). As the concentration of sucrose increased, a reduction in the vigor of seeds was observed. The germination rate was greater

when there was no sucrose in the culture medium; the average value of this treatment was 0.39 (Fig. 2B).

Seeds cultured in the solid medium reached 50% of germination (T50) in 2.91 days, which represents a fast germination rate (Fig. 2C). Regarding the different sucrose concentrations, the lowest average values of T50 were obtained with the medium supplemented with 90 g/l of sucrose (1.39).

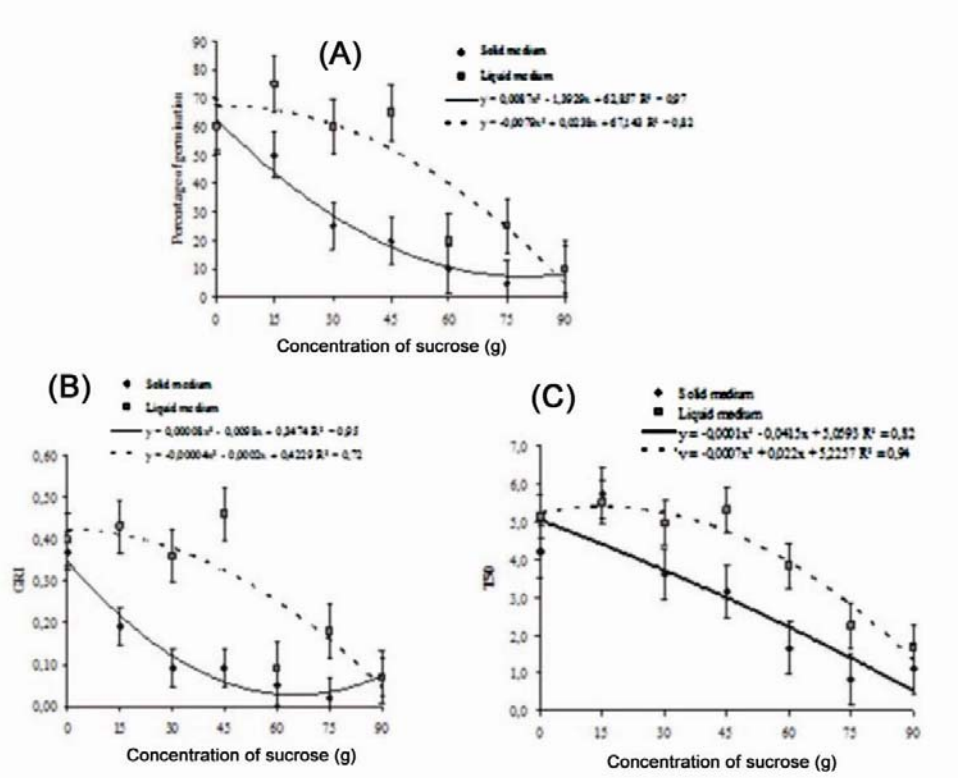

Fig. 2. Percentage of germination (A), germination rate index (GRI) (B), and average time to obtain 50% of germination (T50) (C) of *Hancornia speciosa* Gomes seeds in different types of culture media and different sucrose concentrations.

The results of T50 differed from those found for GRI and germination percentage. Despite ~~a~~ rapid germination, the combination of the solid medium and high concentrations of sucrose negatively affected the germination percentage and the vigor of seeds. This outcome might be due to the formation of nutrient gradients that may occur in the solid media; in contrast, the growth of roots and shoots in the liquid medium was more homogenous (Faria et al. 2006).

Seeking to optimize the culture media for different microorganisms or plants, several authors described experiments similar to the one described here. For instance, Faria et al. (2006) reported similar results in *Oncidium baueri* Lindl.

(Orquidaceae), where use of a liquid medium resulted in greater growth of shoots, roots, and buds. Coelho et al. (2011) observed a higher percentage of *in vitro* germination of sucupira-branca (*Pterodon pubescens* (Benth.) Benth.) seeds inoculated on to a 50% MS liquid medium.

Regarding the sucrose concentrations, our results are in agreement with those obtained by Soares et al. (2009), where the greatest percentage of *in vitro* germination of mangaba was found in an MS-50% and WPM medium supplemented with 15 g/l of sucrose. In addition, Pinheiro et al. (2001) found a greater percentage of germination of mangaba seeds when inoculated onto a liquid MS medium with less than 20 g/l of sucrose. Likewise, Reis et al. (2008) obtained a higher percentage of germination and a faster GRI *in vitro* with *Melissa officinalis* L. seeds inoculated into an MS-25% medium supplemented with 15 g/l of sucrose.

Similarly, beet seeds pre-soaked in gibberellic acid and inoculated into an MS medium supplemented with 15 g/l of sucrose showed a greater percentage of germination. Yet, more vigorous seedlings were obtained from seeds kept in media supplemented with 15 or 30 g/l of sucrose (Braun et al. 2010). Moreover, Silva et al. (2005) tested different sucrose concentrations with squash seeds (*Cucurbita pepo* L., Cucurbitaceae) and observed higher indices of germination in a culture medium supplemented with 20 or 30 g/l of sucrose.

Depending on the species, there is no need to supplement culture media with sucrose. Yet, sucrose might help maintaining seedlings *in vitro* for longer periods (Soares et al. 2009; Braun et al. 2010).

To conclude, the liquid medium with the lowest concentration of sucrose promoted the greatest germination of mangaba seeds.

According to the analysis of variance, both the average length of explants and the average number of buds reached higher values in plants cultivated in the liquid medium without agitation. The average length was 2.72 cm in the liquid medium without agitation and 2.16 cm in the liquid medium with agitation. However, those values did not differ from the one obtained with the solid medium, which was 2.45 cm (Fig. 3A). Regarding the average number of buds, the values were 3.01 and 2.42 for the liquid medium without and with agitation, respectively. Similarly, those values did not differ from the one obtained with the solid medium, which was 2.73 (Fig. 3B). Likewise, the average number of expanded leaves showed no difference in value between different cultural conditions.

By visual observation, it was found that inoculated nodal segments generated seedlings in all treatments. No fungal or bacterial contamination was found. Seedlings in the liquid medium with agitation were etiolated and, as a

result, they were more fragile. The leaves and the stems were depigmented (clear green). The leaves were rudimentary, although they completely expanded, and there was no formation of adventitious roots (Fig. 4A and 4B). Moreover, several explants suffered from necrosis (Fig. 4C, D).

In contrast, seedlings obtained from the liquid medium without agitation and from the solid medium were more rigid than were those obtained from the liquid medium with agitation. Leaves and stem displayed a darker green coloration, which is characteristic of the species (Fig. 4E, F). There was a greater growth of leaves and adventitious roots and no necrosis of explants (Fig. 4G, H).

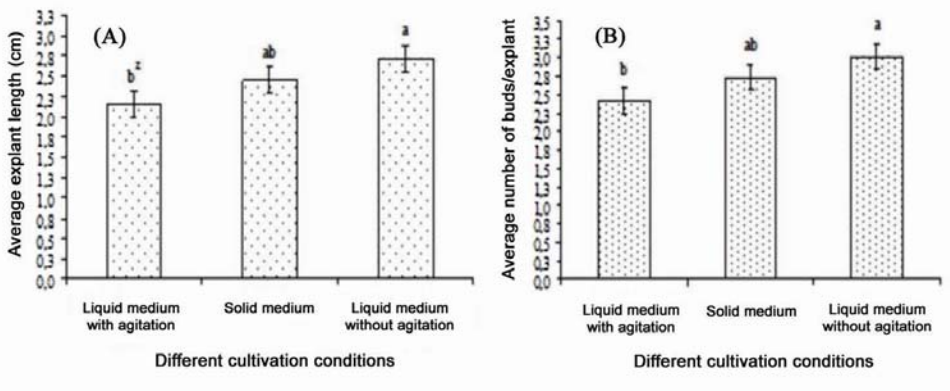

Fig. 3. Average explant length (A) and average number of buds (B) of *Hancornia speciosa* Gomes under different cultivation conditions. Means followed by the same letter do not differ by Tukey test at 5% probability.

In addition, seedlings obtained from the solid medium (Fig. 4I, J) had less leaves and adventitious roots than those obtained from the liquid medium without agitation (Fig. 4K, L). In addition, no necrosis of the explants was observed. According to Bassan et al. (2006), who worked with explants similar to those used in this study, the lack of phenol oxidation in the explants of canafistula (*Peltophorum dubium* Speng) is attributed to the reduced concentrations of phenols in the tissues or to the seminal origin of the explants because the majority of authors consider the age of the explants to be directly correlated to the formation of phenolic compounds in *in vitro* cultures.

Several species require support or agitation to provide oxygen for the metabolism of explants, which ensures the division and differentiation of sprouts (Mengarda et al. 2009). However, when the liquid medium was agitated, the growth of nodal segments of mangaba was unsatisfactory and suffered from necrosis. This outcome decreased the capacity of regeneration of the explants and reduced the number of seedlings obtained at the end of the cultural experiment.

The results using liquid medium without agitation indicated that the constant immersion of mangaba explants in the culture medium did not affect the ability to obtain healthy seedlings. The technique was efficient because it produced the best seedlings between all the treatments.

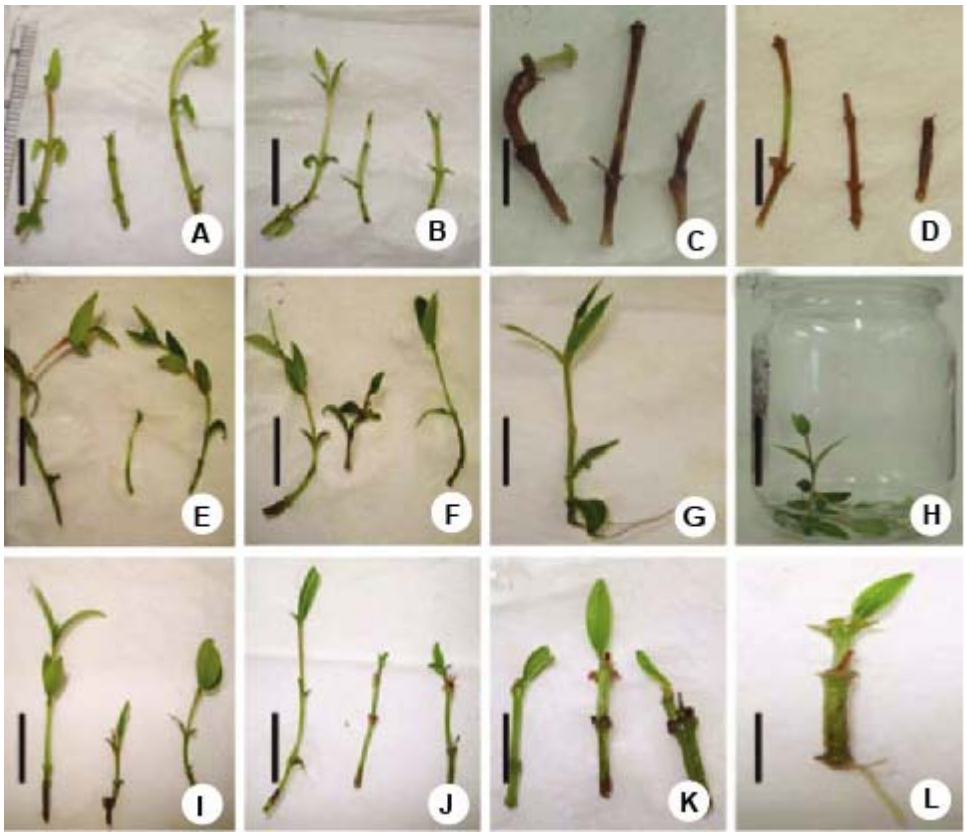

Fig. 4. *In vitro* growth of *Hancornia speciosa* after 30 days of culture. Liquid medium with agitation (A and B). Necrosed explants in liquid medium with agitation (C and D). Liquid medium without agitation (E, F, G, and H). Solid medium (I and J). Seedlings with few expanded leaves or roots in the solid medium (K and L). Bar = 10 mm.

With the elimination of agar from the culture medium, the cultivation process became more flexible and less strenuous. Because liquid media lack agar, they are easy to prepare, and a smaller quantity of medium can be used per experimental unit; thus, the production process is optimized. In commercial terms, this efficiency constitutes a great advantage because it allows mass production of large quantities of healthy seedlings at lower affordable costs. These advantages were also described by Faria et al. (2006) and Pereira and Fortes (2003), who showed the benefits of *in vitro* liquid culture media.

Mangaba seeds showed the best indices of germination percentage and germination rate when inoculated into a liquid medium with no sucrose or 15.0 g/l of sucrose. Likewise, the liquid medium without agitation resulted in a greater growth of mangaba explants.

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