SLOW GROWTH *IN VITRO* CULTURE FOR CONSERVATION OF *HANCORNIA SPECIOSA* GOMES

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Resumo

Crescimento lento em cultura in vitro para a conservação de Hancornia speciosa Gomes. *Hancornia speciosa* Gomes é uma espécie frutífera endêmica do Cerrado e dos tabuleiros costeiros do Nordeste, com grande potencial econômico, nutricional, ecológico e medicinal. O objetivo deste estudo foi avaliar o efeito do sorbitol e sacarose como reguladores osmóticos no crescimento *in vitro* de mangabeira visando à conservação por crescimento lento. Os explantes foram obtidos de plântulas germinadas *in vitro* e inoculados em meio MS suplementado com sacarose (15 e 30 g L^{-1}) e sorbitol (0; 5; 10 e 20 g L^{-1}). O delineamento experimental foi inteiramente casualizado, com 20 repetições, em esquema fatorial 4 x 2 (concentrações de sorbitol x sacarose). As avaliações foram realizadas aos 30, 60, 90 e 120 dias de incubação. As variáveis analisadas foram número de nós/brotação, número de folhas, abscisão foliar, coloração das folhas e sobrevivência dos explantes. Os dados foram avaliados estatisticamente através da análise de modelo linear generalizado. Os resultados indicaram diferença significativa entre os reguladores osmóticos e o tempo de cultivo para todas as variáveis. O sorbitol apresentou efeito redutor de crescimento mais acentuado que a sacarose. O uso de 30 g L^{-1} de sacarose combinado com 10 ou 20 g L^{-1} de sorbitol reduziu o crescimento de forma crítica, deixando evidente que o estresse hídrico provocado não foi tolerado pelas plantas, interferindo negativamente no seu desenvolvimento. O tratamento com 15 g L^{-1} de sacarose combinado com 5 g L^{-1} de sorbitol promoveu os melhores resultados, permitindo a conservação das plantas por 120 dias.

Palavras-chave: Apocynaceae; Regulador osmótico; Fontes de carbono.

Abstract

Hancornia speciosa Gomes is a fruit species endemic to the Cerrado and coastal plains of Northeast of Brazil, with great economic, nutritional, ecological, and medicinal potential. This study aimed to evaluate the effect of sorbitol and sucrose as osmotic regulators on the *in vitro* growth of mangabeira, aiming at conservation by slow growth. The explants were obtained from *in vitro* germinated seedlings and inoculated in MS medium supplemented with sucrose (15 and 30 g L^{-1}) and sorbitol (0, 5, 10 and 15 g L^{-1}). The experimental design was completely randomized with 20 repetitions in a 4 x 2 factorial arrangement (sorbitol x sucrose concentrations). The evaluations were performed at 30, 60 90 and 120 days of incubation. The analyzed variables were number of nodes/budding, number of leaves, leaf abscission, leaf color and survival of explants. The data were statistically analyzed by generalized linear model analysis. The results indicated a significant difference between the osmotic regulators and the culture time for all variables. Sorbitol showed a more pronounced growth-reducing effect than sucrose. The use of 30 g L^{-1} sucrose combined with 10 or 20 g L^{-1} sorbitol reduced the growth in a critical way, making it clear that the water stress caused was not tolerated by the plants, negatively interfering in its development. Treatment with 15 g L^{-1} sucrose combined with 5 g L^{-1} sorbitol promoted the best result, allowing the conservation of plants for 120 days.

Keywords: Apocynaceae; Osmotic regulators; Carbon sources.

INTRODUCTION

The mangabeira (*Hancornia speciosa* Gomes), belonging to the family Apocynaceae, is a fruit species endemic to Brazil and occurs in the Atlantic Forest and Cerrado (SANTOS *et al*., 2015). It has great socioeconomic, ecological and medicinal importance. It stands out for its flavor, pleasant aroma and nutritional value of its fruits, and for its various food products which can be consumed both *in natura* and in processed form (pulp, ice cream, jellies, etc.). It plays an important role in ecosystem services from an environmental point of view, sustaining ecological relations since its seeds are dispersed by the fauna, and serving as food for the human population and for the fauna, in addition to being appropriate for restoring degraded areas.

The species presents propagation difficulties due to the recalcitrance of the seeds, characterized by rapid desiccation of the embryo (SOARES *et al*., 2015). In addition to factors which hinder its natural reproduction, environmental degradation has intensified the reduction of its remaining areas due to real estate expansion, the practice of sugarcane and coconut monocultures and the unsustainable use of resources which contribute to the loss of genetic diversity of the species, threatening it with extinction (SÁ *et al.*, 2011). Thus, there is a need to develop strategies for sustainable use of these resources that enable their restructuring in the natural environment and the conservation of its genotypes.

In vitro conservation of plants based on tissue culture techniques enables storage in the medium or long term, and has been used in several species of economic and medicinal importance, especially those which are vegetatively propagated (SÁ *et al.,* 2011). Long term conservation is possible through cryopreservation techniques, in which plants are kept at ultra-low temperatures and therefore there is total cell growth suppression to avoid plant deterioration (FLORES *et al.,* 2013). On the other hand, storage by slow growth consists in slowing down the metabolism of the plants and increasing the subculture intervals as much as possible or extending it, without altering the genetic uniformity or plant quality (KAMIŃSKA *et al*., 2016). The advantages of inducing slow plant metabolism are reduced costs, labor and physical space for plant maintenance, in addition to reducing the contamination risks during subcultures (OZUDOGRU *et al*., 2017).

The plant's metabolic activity can be reduced through changes in cultivation conditions (temperature, photoperiod and light intensity) or in the growth medium components (organic and inorganic nutrients, osmotic regulators and growth retardants) during the incubation period (RODRIGUES *et al*., 2018). These approaches can be applied in combination or not with another technique (SILVA *et al*., 2016). Such factors directly interfere with breathing, water loss and ethylene production, and therefore the metabolism and growth of plants (OZUDOGRU *et al*., 2010).

The main osmotic regulators used to limit the *in vitro* growth of explants by inducing osmotic stress are mannitol and sorbitol, which modify the hydric potential of the culture medium (SILVA *et al*., 2016). Another osmotic agent used is sucrose, which when added to the culture medium acts by capturing the excess intracellular water by osmotic gradient, causing the culture growth to occur more slowly (ARRIGONI-BLANK *et al*., 2014).

Several studies have been developed using the *in vitro* conservation technique for slow growth, investigating species such as *Saccharum officinarum* L. (LEMOS *et al*., 2002), *Passiflora giberti* N. E. Brown. (FARIA *et al*., 2006), *Pfaffia tuberosa* (FLORES *et al*., 2013), *H. speciosa* (SANTOS *et al*., 2011; SÁ *et al*., 2011) and *Rubus* sp. (SILVA *et al*., 2016). Despite the promising results, it is necessary to expand the research since there is no standard conservation protocol that can be used for all species because the effects can vary according to the species, explant type, and genotype, among other aspects (SÁ *et al*., 2011).

In this context, the objective of this work was to evaluate the effects of osmotic regulators, sorbitol and sucrose on the *in vitro* growth of *H. speciosa* aiming at conservation by slow growth.

MATERIAL AND METHODS

The studies were conducted at the Biotechnology Laboratory of Conservation of Native Species of the Universidade Federal do Rio Grande do Norte. Nodal segments obtained from seedlings germinated *in vitro* from fruits from the native population of mangaba trees of the municipality of Nísia Floresta-RN were used as the explant source.

The explants were excised approximately 1.0 cm long containing a yolk and the leaves were removed, leaving only part of the petiole to protect the yolks. The nodal segments were excised under aseptic conditions and inoculated in test tubes 20 mm x 180 mm, with 20 mL of culture medium. The basal medium was the standard MS (MURASHIGE; SKOOG, 1962) supplemented with 1 mg L^{-1} of indole-3-acetic acid (IAA) and 1 mg L^{-1} of 6-benzilaminopurine (BAP). The pH of the culture medium was adjusted to 5.8 ± 0.2, followed by the addition of agar (7 g L^{-1}) and autoclaving at a temperature of 121°C and 1 atm pressure for 20 minutes. After sterilization, the explants were inoculated onto the medium and maintained in the growth room under a 16-hour photoperiod and light intensity of 60 $\mu mol.m^{-2}s^{-1}$ at 25 ± 2°C.

Different sorbitol concentrations (0; 5; 10 and 20 g L^{-1}) combined with 15 and 30 g L^{-1} sucrose, were evaluated to confer different osmotic potentials to the media (ψs= - 0.109; - 0.217; - 0.177; - 0.285; - 0.244; - 0.353; - 0.380; or - 0.489) (Table 1). MS medium plus 30 g L^{-1} sucrose was considered as a control. The statistical design was completely randomized with 20 repetitions in a 4 x 2 factorial scheme (sorbitol x sucrose concentrations), with each experimental plot composed of one test tube containing an explant, totaling eight treatments (Table 1).

Table 1. Treatments used for *in vitro* conservation of *H. speciosa* using sorbitol and sucrose.
Tabela 1. Tratamentos utilizados para a conservação *in vitro* de *H. speciosa* usando sorbitol e sacarose.

Concentration of osmotic agents (g L^{-1})			
Treatments	Sorbitol	Sucrose	**ψs (MPa)
1	0	15	- 0.109
2*	0	30	- 0.217
3	5	15	- 0.177
4	5	30	- 0.285
5	10	15	- 0.244
6	10	30	- 0.353
7	20	15	- 0.380
8	20	30	- 0.489

* Control treatment; ** Osmotic potential.

The evaluations were performed at 30, 60, 90 and 120 days after inoculation. The parameters observed were: number of nodes per sprout (NNS), total number of leaves (NL), leaf abscission (NLABS), survival of explants and leaf coloration (LC). The following scoring scale was assigned for this last variable: 1-total green leaves; 2-light green leaves; 3-yellow leaves (beginning of senescence) (FARIA *et al*., 2006).

The data obtained were submitted to five generalized linear model analyses for repeated measurements, one for each response variable (R.V.), referring to the nodal explants (number of nodes per sprout, total number of leaves, number of leaves that suffered leaf abscission, leaf coloration and survival of explants). The data for the nodal explants were fitted with the explanatory variables (E.V.), Sorbitol, Sucrose and Time (30, 60, 90 and 120 days), using the IBM SPSS 20.0 software program. The R.V. with binomial distribution (survival) was adjusted with the Logit connection function. As R.V. NNS, NL and NLABS constitute a discrete quantitative variable (Poisson distribution), they were fitted to the log-linear model (Poisson regression model). Furthermore, the cumulative Logit function was fitted for the R.V. leaf staining with Multinomial distribution. The significance level of 5% was used in the models.

RESULTS

The models used for statistical analysis of number of nodes per sprout (NNS), number of leaves (NL), leaf abscission (NLABS), leaf coloration and survival were significant ($p<0.05$) for the different sorbitol and sucrose concentrations and time, with the exception of NLABS and survival which were not influenced by the use of sorbitol and sucrose, respectively. There was an effect of the interaction between sorbitol and sucrose as a function of time only for the NNS, NL and NLABS variables (Table 2).

Table 2. Parameter estimates of the models fitted to express the relationship between sorbitol and sucrose concentrations as a function of time in *H. speciosa.*

Tabela 2. Estimativas dos parâmetros dos modelos ajustados para exprimir a relação entre as concentrações de sorbitol e sacarose em função do tempo, em *H. speciosa*.

	NNS *Gama* (1)	NL *Gama* (2)	NLABS *Gama* (3)	Leaf coloration *Multinomial* (4)	Survival *Logistic* (5)
Intercept	0.000*	0.000*	0.000*	-	0.000*
Sorbitol	0.008*	0.003*	0.462^{ns}	0.000*	0.000*
Sucrose	0.000*	0.001*	0.022*	0.049*	0.538^{ns}
Time	0.000*	0.000*	0.000*	0.000*	0.000*
Sorbitol*Sucrose	0.335^{ns}	0.110^{ns}	0.053^{ns}	0.244^{ns}	0.000*
Sorbitol*Time	0.000*	0.000*	0.000*	0.101^{ns}	0.000*
Sucrose*Time	0.540^{ns}	0.000*	0.000*	0.101^{ns}	0.001*
Sorbitol*Sucrose*Time	0.000*	0.000*	0.000*	0.050^{ns}	0.632^{ns}
Observações	160	160	160	160	160
Quase Likelihood Independence (QIC)	163.955	173.241	98.686	-	546.959

* The significance level of 0.05 was used in the tests.

* Foi utilizado nos testes o nível de significância de 0,05.

Different behaviors were verified for each dependent variable for the interaction between sorbitol and sucrose, independent of the culture time. A decrease in the multiplication rate was also observed according to the increase in sorbitol concentrations. The reduction in sucrose concentration promoted the highest explant multiplication rates (Figure 1A). The highest averages were obtained by 15 g L^{-1} sucrose (5.38 nodes) (T_1), 5 g L^{-1} sorbitol + 15 g L^{-1} sucrose (4.43 nodes) (T_3) and 10 g L^{-1} sorbitol + 15 g L^{-1} sucrose (4.94 nodes) (T_5).

The addition of sorbitol to the culture medium promoted a decrease in the number of leaves. The use of 15 g L^{-1} sucrose, without sorbitol (10.6 leaves/explant) or associated with 10 g L^{-1} sorbitol (8.89 leaves/explant), provided higher means of number of leaves, while the lowest mean was obtained in 10 g L^{-1} sorbitol + 30 g L^{-1} sucrose (T_6) (5.07 leaves/explant) (Figure 1B).

A differentiated behavior was observed in relation to leaf abscission. The reduction in sucrose concentration favored abscission, and the use of 10 g L^{-1} sorbitol + 15 g L^{-1} sucrose (T_5) significantly increased leaf abscission (3.3 leaves) (Figure 1C). The addition of 10 g L^{-1} sorbitol to the culture medium differed significantly with higher numerical value for leaf coloration, indicating a greater number of yellow leaves. The treatment with 15 g L^{-1} sucrose, in the absence of sorbitol reached the lowest value, indicating a greater number of leaves with a more intense green.

It was also observed that the isolated effect of 15 g L^{-1} sucrose (T_1) and the interaction between 5 g L^{-1} sorbitol and 15 g L^{-1} sucrose (T_3) presented higher averages of dark green leaves when compared to the control. The plants from the treatments with 5 g L^{-1} of sorbitol and 30 g L^{-1} sucrose (T_4) and 10 g L^{-1} sorbitol and 15 g L^{-1} sucrose (T_5) concentrations showed higher means of light green leaves; while 10 g L^{-1} sorbitol + 30 g L^{-1} sucrose (T_6), 20 g L^{-1} sorbitol + 15 g L^{-1} sucrose (T_7) and 20 g L^{-1} sorbitol + 30 g L^{-1} sucrose (T_8) presented yellow leaves, indicating greater leaf senescence, regardless of cultivation time (Figure 1D).

There was also a decrease in the survival rate according to the increase in sorbitol concentrations since the addition of sucrose did not interfere with the survival of mangabeira nodal explants. The survival rate was 100% for the treatments with 15 g L^{-1} sucrose, with or without 5 g L^{-1} sorbitol, while the survival rate in 20 g L^{-1} sorbitol + 15 g L^{-1} sucrose (T_7) was 40% (Figure 1E).

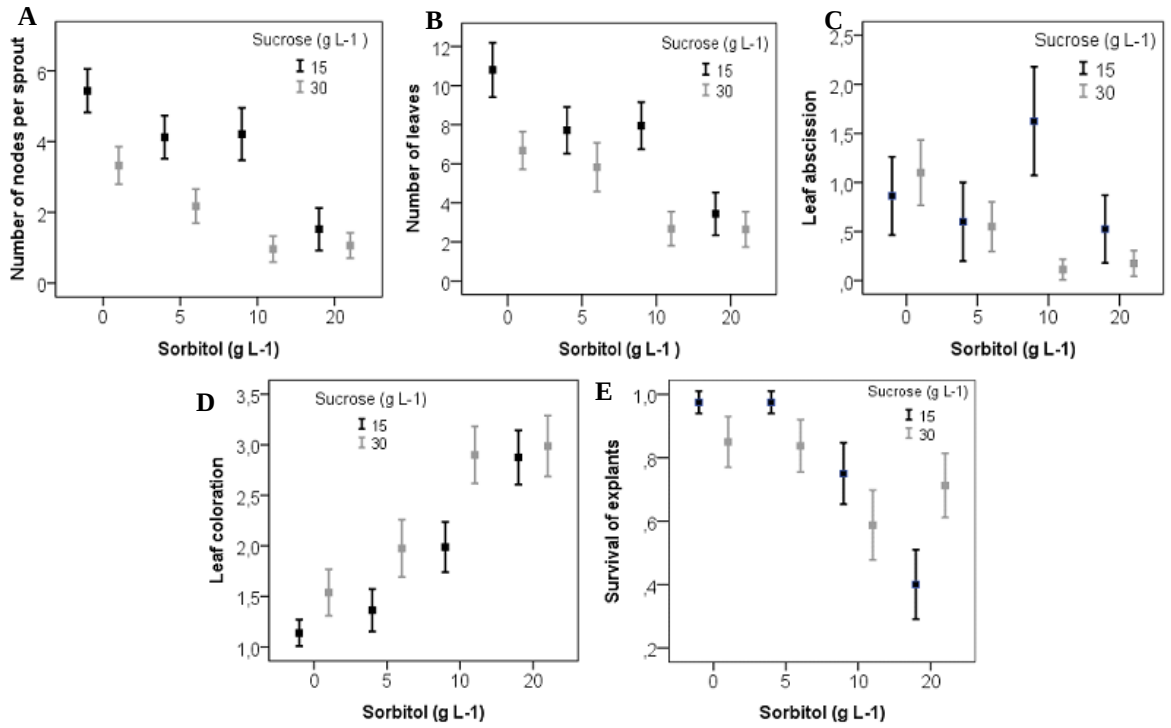

Figure 1. Variation of number of nodes per sprout (A), number of leaves (B), leaf abscission (NLABS) (C), leaf coloration (D) and survival of explants (E), as a function of sorbitol and sucrose concentrations, independent of culture time.

Figura 1. Variação do número de nós por brotação (A), número de folhas (B), abscisão foliar (NFABS) (C), coloração das folhas (D) e sobrevivência dos explantes (E), em função da concentração de sorbitol e de sacarose, independente do tempo de cultivo.

Regarding *in vitro* cultivation time, there was an increasing behavior for all analyzed variables, except for survival which reduced its rates throughout the 120 days of cultivation (Figure 2A-E). All comparisons for the number of nodes per adventitious sprout (NNS) indicated statistical difference ($p<0.05$), except for 20 g L^{-1} sorbitol + 15 g L^{-1} sucrose (T_7) in which the temporal sequence was linear (Figure 2F). The use of 15 g L^{-1} sucrose, without sorbitol, induced greater knot formation by adventitious sprouting, with mean production of 7.84 knots at the end of 120 days.

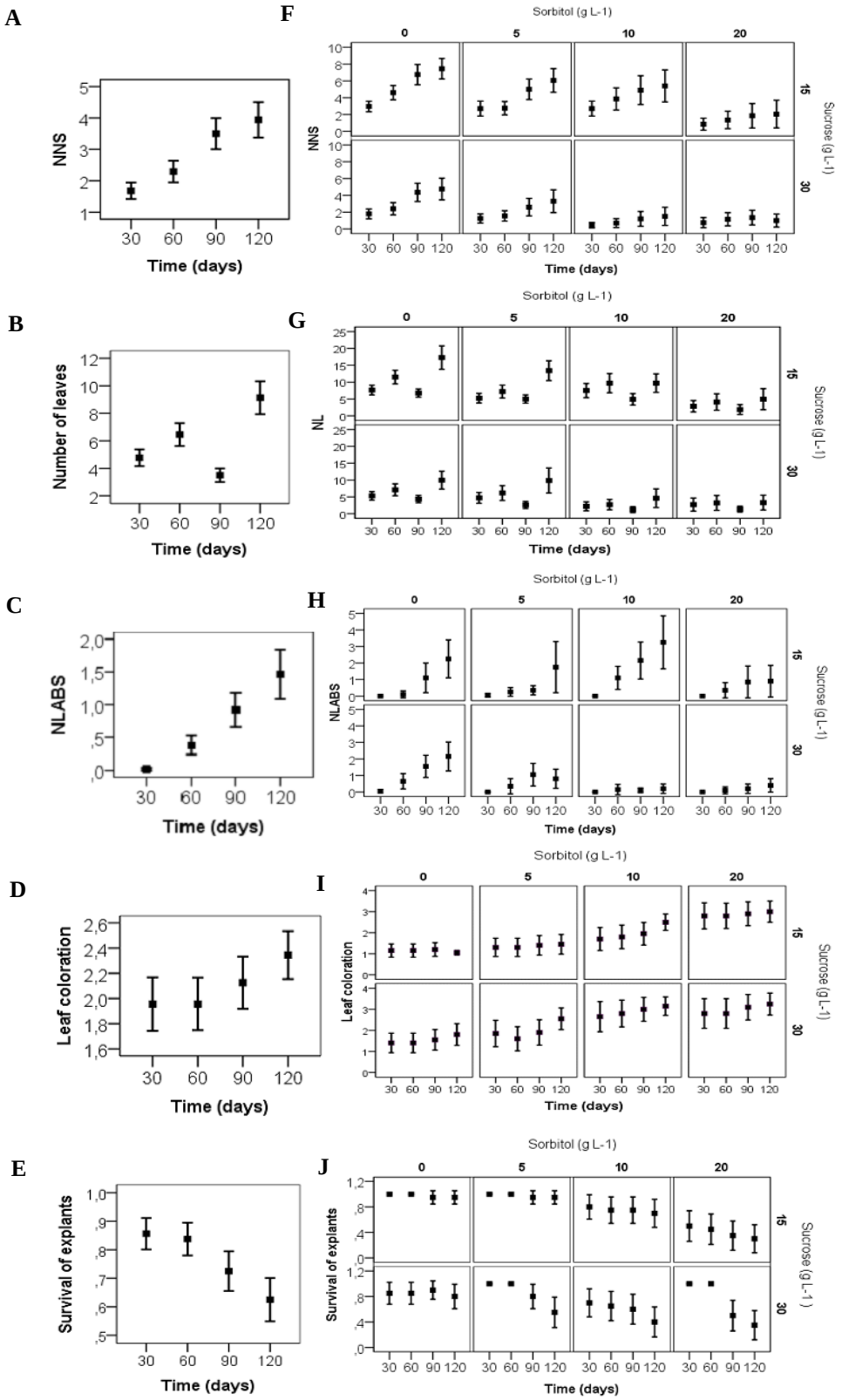

Figure 2. Number of nodes per sprout (NNS), number of leaves (NL), leaf abscission (NLABS), leaf color and survival as a function of culture time (A-E) and for each sorbitol and sucrose concentration as a function of culture time (F-J).

Figura 2. Número de nós por brotação (NNB), número de folhas (NF), abscisão foliar (NFABS), coloração das folhas e sobrevivência dos explantes, em função do tempo de cultivo (A-E) e para cada concentração de sorbitol e sacarose em função do tempo de cultivo (F-J).

The effect of the cultivation time was verified with an increase in the number of leaves at 30 and 60 days. There was distinct behavior at 90 days with the reduction of the number of leaves in all treatments. From then on there was a new addition to the number of leaves (Figure 2B). The use of 15 g L^{-1} sucrose combined with 0 and 5 g L^{-1} sorbitol showed a greater number of leaves at the end of the evaluation (Figure 2F). There was a significant difference between 90 and 120 days for the leaf abscission variable which presented a higher mean leaf abscission of 2.12 and 2.89 leaves, respectively (Figure 2C). Moreover, T5 presented the highest mean abscission at the end of 120 days of culture (Figure 1H) regarding the interaction between osmotic agents as a function of time.

An increase in the number of yellow leaves was observed for leaf coloration over the 120 days of cultivation (Figure 2D). The effect of osmotic agents' interaction was observed after 90 days, whit 15 g L^{-1} sucrose combined with 5 and 10 g L^{-1} sorbitol being significantly different from the others. There was an increase in the number of yellowish leaves in most treatments, except for the T1 and T3, which reached the lowest values for leaf coloration, which indicates a greater number of dark green leaves at the end of 120 days. The highest mean yellow leaves were reached by 10 g L^{-1} sorbitol + 30 g L^{-1} sucrose (T_6) and 20 g L^{-1} sorbitol combined with 15 (T_7) and 30 g L^{-1} sucrose (T_8) (Figure 2I).

There was a reduction in the survival rate along the 120 days of cultivation, but the highest averages were observed at 30 and 60 days (Figure 2E). The treatments with 30 g L^{-1} of sucrose combined with 10 (T_6) e 20 g L^{-1} sorbitol (T_8) and 15 g L^{-1} sucrose + 20 g L^{-1} sorbitol (T_7) significantly reduced the survival rate, with means equal or inferior to 40%. On the other hand, the survival rates when using 15 g L^{-1} sucrose combined with 0 (T_1) and 5 g L^{-1} sorbitol (T_3) were 95% at 120 days of culture (Figure 2J). It was verified that reduced concentrations of sorbitol and sucrose enabled a lower reduction of osmotic potential, increasing survival, and consequently maintaining the viability of the plants at the end of 120 days of cultivation.

The maintenance of slow growing mangabeira plants for 120 days was possible when only 15 g L^{-1} sucrose or combined with 5 g L^{-1} sorbitol was used, resulting in greater viability of the explants. The microplants preserved in this way showed normal phenotype with thick stems and wide leaves (Figure 3A-B; 3I-J; 3Q-R). In contrast, toxic and stressing conditions were evidenced in microplants submitted to high concentrations of sorbitol and sucrose, such as 30 g L^{-1} sucrose associated with 10 or 20 g L^{-1} sorbitol, due to a greater reduction in the osmotic potential of the medium, with reduced growth or atrophied sprouting, thin stems and reduced or absent leaves, in addition to higher oxidation incidence (Figure 3P, X, Z). Furthermore, it was observed that the isolated effect of sucrose induced greater callus formation at the base of the explants (Figure 3I; 3M).

It was also found that the sprouting resulting from the media increased with 15 g L^{-1} sucrose, combined with 0 and 5 g L^{-1} sorbitol, obtained more uniform leaf coloration, with a predominance of green leaves at the end of 120 days (Figure 3Q-R).

The mangabeira cuttings showed different behavior regarding the applied osmotic stress when submitted to sorbitol and sucrose (Figure 3A-Z). Sorbitol is more responsive in reducing growth, but it does not maintain the viability of the microplants at the end of 120 days in higher concentrations.

DISCUSSION

Sorbitol and sucrose used to induce osmotic stress during the *in vitro* conservation period promoted growth reduction when used at higher concentrations. Despite the increasing behavior of the number of knots per adventitious sprout over the 120 days, the plants grew more slowly with the reduced osmotic potential of the medium, especially in the presence of sorbitol. This can be explained by the fact that sorbitol is a difficult substance to metabolize, so it is more effective than sucrose in reducing growth (WITHERS; WILLIAMS, 1998).

In this study, the addition of sorbitol reduced the growth *in vitro*, as the presence of sucrose favored sprouting elongation. Other studies also found that the addition of sucrose positively influenced the growth and development of plants *in vitro* (FARIA *et al*., 2006; FLORES *et al*., 2013). In studying the effect of sorbitol and sucrose in nodal segments of mangabeira, Santos *et al*. (2011) obtained less growth *in vitro* of explants with the formation of 3.32 nodes per sprout in the treatment supplemented with 15 g L^{-1} sucrose and 40 g L^{-1} sorbitol, while the use of 15 g L^{-1} sucrose combined with 10 g L^{-1} and 20 g L^{-1} sorbitol promoted higher multiplication rates.

Increased osmotic pressure directly interferes in the plant metabolism, which was reflected in the lower development of sprouts and shoot growth of the *H. speciosa*. These results agree with those obtained by Silva *et al*. (2019), who obtained a greater reduction in the shoots of *Poincianella pyramidalis* in the presence of higher sucrose, sorbitol and mannitol concentrations. This can be justified by the fact that these osmotic agents act by reducing its osmotic potential when added to the culture medium, and therefore promote a reduction in water potential and availability of water and nutrients in the medium, inducing slower growth (HUANG *et al*., 2014).

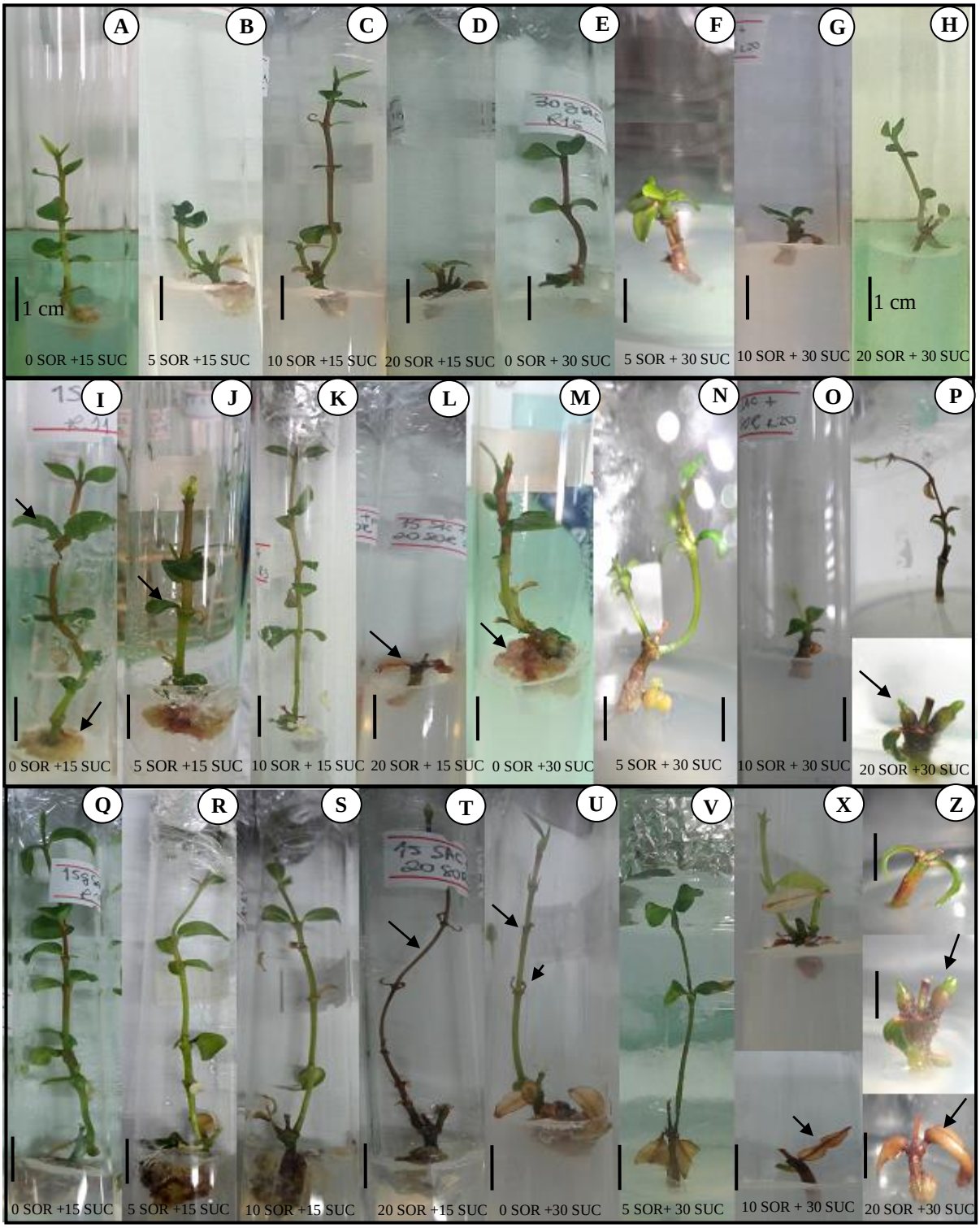

Figure 3. Aspects of the development of *H. speciosa* plants at different concentrations of sorbitol (SOR) and sucrose (SUC) for 30 days (A-H), 60 days (I-P) and 90 days (Q-Z) culture *in vitro.* Bar = 1cm.

Figura 3. Aspectos do desenvolvimento de plantas de *H. speciosa* em diferentes concentrações de sorbitol (SOR) e sacarose (SAC) ao longo de 30 dias (A-H), 60 dias (I-P) e 90 dias (Q-Z) de cultivo *in vitro*. Barra = 1cm.

The reduction in the growth of *H. speciosa* plants reflected in the lower multiplication rate of sprouts is an advantage for *in vitro* conservation, whose objective is to limit the growth of plants and to increase the interval between subcultures as much as possible. However, although the combination of carbohydrates in the

culture medium at high concentrations in this study it induced a slower growth of *H. speciosa* plants, it is not recommended for *in vitro* conservation of this species because it negatively interferes with the vigor of the plants.

It is important to point out that plants kept *in vitro* do not perform photosynthesis activity to supply their energetic demands, and therefore they need the addition of a carbon source to the cultivation medium to ensure their development. The addition of carbohydrates significantly affects the growth and physiological responses of plants *in vitro*, acting as an osmotic regulator, as well as carbon and energy sources of the culture medium (FLORES *et al*. 2013). Depending on the concentration used, the osmoregulators act by removing the excess intracellular water by osmotic gradient, causing the culture growth to occur more slowly (ARRIGONI-BLANK *et al*., 2014), thus making its conservation possible.

Sorbitol acts as a primary photosynthetic light and a translocation and storage element in plants (SINGH *et al*., 2015). According to Lemos and Baker (1998), it can induce shoot initiation and callus formation in the explants, performing other functions in addition to its action as carbon source or osmotic regulator. Sorbitol was more responsive than sucrose in reducing sprouting elongation in this study, as well as in its development. This suggests that sucrose, as a universal carbon source, is easily metabolized by most species, while sorbitol is more restrictive to growth, although it has some effects that allow or even promote cell divisions in plant tissues.

The addition of sucrose in reduced concentration favored an increase in the number of leaves at the end of the evaluation, while sorbitol showed antagonistic effect in response to the decrease in the osmotic potential of the culture medium. Faria *et al.* (2006) found that the use of 30 g L^{-1} sucrose combined with sorbitol at 40 g L^{-1} induced production of a lower number of leaves of *P. giberti*. Therefore, in works of *in vitro* conservation it is important to consider that the greater number of leaves in the plants increases the photosynthetic rate, thus favoring the acclimatization stage.

An increase in the leaf abscission rate was observed with increasing *in vitro* cultivation time in the presence of sorbitol, also contributing to the senescence of microplants. In studies with *H. speciosa*, Santos *et al.* (2011) observed higher leaf abscission in the presence of sucrose, probably due to the availability of carbon source to the culture medium, thus favoring *in vitro* ethylene metabolism and accumulation. In contrast, Sá *et al.* (2011) observed a reduction in leaf abscission with high mannitol concentrations, although its harmful effect on mangaba explants was evidenced.

According to Lemos and Blake (1994), some woody species present foliar abscission, reduced growth, and the explant becomes fragile when submitted to *in vitro* cultivation conditions, making it impossible to continue the process. This occurs because the closed environment in which plants or explants are submitted to generally provides an accumulation of ethylene, responsible for leaf abscission. Even in small quantities, this hormone can be physiologically active and induce adverse effects on plant development, affecting differentiation, development, morphology, and growth; reducing leaf expansion and sprout elongation; and inhibiting the regeneration of new sprouts and causing apical necrosis (ERIG; SCHUCH, 2005).

The results regarding leaf color, were similar to those obtained by Faria *et al.* (2006) in studies with *P. giberti*, who found that the sprouts from the use of 15 g L^{-1} sucrose practically maintained the coloration of the leaves, with a predominance of green leaves, while 30 g L^{-1} sucrose led to light green leaves predominated at 90 days, and from then on there was a predominance of leaves with a totally green color.

The increase in yellow leaves in the presence of sorbitol demonstrates its toxic effect by promoting leaf senescence and reducing the viability of plants. This is possibly related to the fact that sorbitol is generally not metabolized by plant tissues, since many species do not have a natural pathway for the biosynthesis of alcohol sugars (THORPE *et al*., 2008). Toxic effects due to high doses of osmoregulators have also been reported in studies with mangaba (SÁ *et al*., 2011; SANTOS *et al*., 2011) and *P. tuberosa* (FLORES *et al*., 2013).

Despite the reduction in leaf senescence with the decrease in the osmotic potential of the environment, sorbitol potentialized this effect at the end of 120 days. This demonstrates that once growth begins, plants become more vulnerable to environmental stresses. Thus, the occurrence of yellowish leaves is often associated with the stress caused by the environment resulting from nutrient scarcity in the culture medium and/or difficulties in gas exchange, which accelerate senescence (RODRIGUES *et al*., 2018).

The decrease in plant survival rate in response to the addition of sorbitol has also been reported for *S. officinarum* (LEMOS *et al*., 2002). Sá *et al.* (2011) observed that despite the positive result in reducing the elongation of *H. speciosa* microplants, the addition of mannitol to the medium negatively affected the survival rate. On the other hand, Silva *et al*. (2019) found that most culture media supplemented with the osmotic agents sorbitol and mannitol produced low survival rates in studies with catingueira (*P. pyramidalis*). In this aspect, an ideal osmotic agent would be non-toxic and non-penetrating, reducing the osmotic potential of the medium (BÜNDIG *et al*., 2016).

Studies conducted with other species have also shown that culture mediums only containing sucrose increased the survival rate of plants preserved *in vitro*. The minimum growth maintenance in plants of *S.*

officinarum L. for 12 months was possible when only 20 g L^{-1} sucrose was used, indicating that the species does not have the necessary mechanisms to metabolize sorbitol (LEMOS *et al.*, 2002). Camillo and Scherwinski-Pereira (2015) found that sucrose is the most appropriate carbohydrate to maintain the quality of *Elaeis guineenses* and *E. oleifera*, while sorbitol and mannitol reduced the survival of plants. The authors also reported that the medium containing 3% sucrose favored normal development of plants maintained *in vitro*, resulting in plants with better aspects and more green leaves throughout the process.

The treatments with 15 g L^{-1} sucrose combined 0 and 5 g L^{-1} sorbitol produced no mortality and no abnormalities (senescence or darkening), with the latter presenting a reduced growth rate. Although no significant reduction in final length was found at the end of the evaluations, the reduction in the growth rate is successful in extending the intervals to reach the complete capacity of the container and increase the period between periodic subcultures without negatively affecting its viability. Santos *et al.* (2011) verified greater viability of mangabeira plants maintained in slow growth for 120 days in media containing only sorbitol at concentrations of 10 or 20 g L^{-1} than in those plus sucrose and sorbitol.

According to Rodrigues *et al.* (2018), the final quality of the plant material maintained *in vitro* by slow growth should be the best possible given the conditions to which the explants are submitted. This is because the maintenance of *in vitro* culture can cause morphological, physiological and anatomical changes that make it difficult to acclimatize *ex vitro* or use the plant material in other cultures (CHANDRA *et al.*, 2010). In addition, the genotype and quality of cultures, as well as the concentration of osmotic agents in the culture medium and the type of culture container, which may differ in gas permeability, are factors that significantly affect the maximum storage period of *in vitro* cultures (OZUDOGRU *et al*., 2017). Therefore, the viability of plants is an important characteristic to be considered in *in vitro* maintenance for long periods and should enable reducing the appropriate metabolism to generate healthy and viable microplants for subsequent cultivation stages.

The use of different culture media enables promising alternatives to approach *in vitro* mangabeira sprouting culture. Depending on the purpose of *in vitro* culture, different growth rates may be necessary to achieve the objectives of the various germplasm maintenance steps (MUÑOZ *et al*., 2019). For example, 15 g L^{-1} or 30 g L^{-1} sorbitol-free sucrose can be applied to obtain rapid growth of plant material before transplanting plants in greenhouses or even under field conditions. For another *in vitro* culture stage, in the case of longer conservation without immediate requirement for multiplication, 15 g L^{-1} sucrose + 5 g L^{-1} sorbitol medium can be used to reduce resource expenditure and to maintain mangabeira plants for longer periods as slow-growing storage. Despite evaluations for only four months, the results obtained herein indicate the viability of using these osmotic agents in the in vitro conservation of this species, but further studies are needed to test the effects for a longer period.

CONCLUSION

- *In vitro* conservation of *H. speciosa* microplants under slow growth conditions for 120 days is viable in MS mediums supplemented with 15 g L^{-1} sucrose and 5 g L^{-1} sorbitol.

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