



## *In vitro* conservation of *Amburana cearensis* (Fabaceae)

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**ABSTRACT:** *Slow-growth tissue culture allows in vitro conservation of plant genetic resources and is a complementary technique to conventional preservation methods. The objective of this study was to evaluate the effect of the culture medium supplemented with sucrose, mannitol and sorbitol osmotic agents on the induction of in vitro slow growth of A. cearensis, seeking to establish alternative techniques for ex situ conservation of the species. Plants with age of 10 days were inoculated in woody plant medium (WPM) supplemented with different concentrations of sucrose (87.64, 131.46, 175.28 or 262.92 mM) combined with mannitol or sorbitol at varying concentrations (0.0, 43.8, 65.73, 87.64 or 131.46 mM), solidified with 0.7% agar. The following parameters were evaluated: survival percentage, number of senescent leaves, aerial part length, root length, aerial part dry mass, root dry mass; and number of shoots. The conservation of A. cearensis was viable for up to 300 days in WPM supplemented with 262.92 mM of sucrose or the combination of 87.64 mM of sucrose with 43.80 mM of mannitol, because besides achieving a survival percentage greater than 55.0%, the plants conserved in these media presented normal development and the best appearances, with predominance of uniform green leaves during the storage process.*

**Key words:** *slow growth, osmotic agent, growth retardant, cumaru, conservation of germplasm.*

## Conservação *in vitro* de *Amburana cearensis* (Fabaceae)

**RESUMO:** *A cultura de tecidos, através do crescimento mínimo, possibilita a conservação in vitro dos recursos genéticos vegetais, tornando-se uma técnica complementar aos métodos convencionais de conservação. O objetivo deste estudo foi avaliar o efeito do meio de cultura suplementado com sacarose, manitol e agentes osmóticos sorbitol na indução de crescimento mínimo in vitro de A. cearensis, buscando estabelecer técnicas alternativas para conservação ex situ da espécie. Plantas com 10 dias foram inoculadas em meio de cultura WPM suplementado com diferentes concentrações de sacarose (87,64; 131,46; 175,28 e 262,92 mM) combinado com manitol ou sorbitol nas concentrações (0,0; 43,8; 65,73; 87,64 e 131,46 mM), solidificados com 0,7% de ágar. Foram avaliados a porcentagem de sobrevivência, número de folhas senescentes, comprimento da parte aérea, comprimento da raiz, massa seca da parte aérea, massa seca da raiz e número de brotos. A conservação de A. cearensis mostrou-se viável até 300 dias, em meio de cultura WPM suplementado com 262,92 mM de sacarose ou na combinação de 87,64 mM de sacarose com 43,80 mM de manitol. Assim sendo, além de apresentarem porcentagem de sobrevivência acima de 55,0%, as plantas conservadas nesses meios apresentaram um desenvolvimento normal e com melhores aparências com predomínio de folhas verdes uniformes ao longo do processo de armazenamento.*

**Palavras-chave:** *crescimento mínimo, agente osmótico, retardante de crescimento, cumarú, conservação de germoplasma.*

## INTRODUCTION

*Amburana cearensis* (Allemão) A.C.Smith. (Fabaceae) is a species native to the Brazilian semiarid region, especially the Caatinga (seasonally dry shrubland) biome (PEREIRA et al., 2017), popularly known as 'imburana de cheiro', 'amburana de cheiro' or

'cumarú' (CAMPO et al., 2013). Different parts of this plant, such as leaves, stems and seeds, are widely used in folk medicine and have been confirmed to have multiple medicinal applications (SÁ et al., 2014; LATALIZA et al., 2019; PEREIRA et al., 2017). Besides this, *A. cearensis* is known for its wood quality and as an ornamental plant and source of forage (CAMPOS et al., 2013).

The growing economic demand for cumarú as a source of wood and medicinal substances is mainly satisfied by extractivism that can be classified as predatory, posing a threat to its survival. As a result, *Amburana cearensis* has been classified as endangered according to the International Union for the Conservation of Nature (IUCN, 2019).

Therefore, urgent measures are necessary to preserve the species. The application of tissue culture techniques has enabled *in vitro* active germplasm banks of various species, such as *Curcuma longa* and *Zingiber officinale* (JAIN et al., 2018), *Polygonum multiflorum* Thunb (HUANG et al., 2014), *Solanum lycopersicum* L. (AL-ABDALLAT et al., 2017) and *Salix tetrasperma* Roxb. (KHAN et al., 2018). These have advantages over *ex vitro* germplasm banks, among them maintenance of many accessions in a small physical space and freedom from bad weather in open fields or conditions in covered spaces or greenhouses. Although, the *in vitro* multiplication of *A. cearensis* has been studied (CAMPOS et al., 2013) to enable large-scale production of seedlings, to date no information is available on the *in vitro* conservation of this species.

One of the ways to conserve plant matter is by slow growth in culture media, a method of *ex situ* conservation that reduces the plants' metabolism, by chemical and/or physical modifications in the medium or culture conditions (AL-ABDALLAT et al., 2017; ATTIA et al., 2018; MUÑOZ et al., 2019).

The use of osmotic agents like sucrose, sorbitol and mannitol reduces the water potential of the medium, limiting the water availability and thus restricting the uptake of nutrients and the metabolic activity of the cells (HUANG et al., 2014). This conservation strategy has several advantages, such as longer times between subcultures, reduced costs to maintain the germplasm bank and lower risk of somaclonal variation (CHAUHAN et al., 2016).

A previous study conducted by LEITE et al. (2005) about the state of knowledge regarding *A. cearensis* for genetic conservation in Brazil reported that actions for *ex situ* conservation are highly recommended for specific parts, in places where loss of resources is already so extensive that *in situ* measures by themselves are not sufficient.

Therefore, it is necessary to establish a protocol for *in vitro* conservation of *Amburana cearensis*, as an alternative way of germplasm preservation. The objective of this study was to evaluate the effect of the culture medium supplemented with sucrose, mannitol and sorbitol osmotic agents on the induction of slow growth *in*

*vitro* of *A. cearensis*, seeking to establish alternative techniques for *ex situ* conservation of the species.

## MATERIALS AND METHODS

### *Explant collection and surface disinfection*

The experiments were performed in the Plant Tissue Culture Laboratory (LCTV), belonging to the Experimental Forest Plantation Unit of Universidade Estadual de Feira de Santana, located in the municipality of Feira de Santana, Bahia.

*A. cearensis* seeds were collected in a Caatinga area located near the experimental farm of the Embrapa Semiárido research unit, in Petrolina, Pernambuco, Brazil. Seeds were placed in kraft paper bags and stored in a cold chamber, where they remained until the start of the experiment.

To obtain the plants *in vitro*, seeds were washed in running water for 10 minutes, followed by disinfection in a laminar flow chamber with immersion in 70% ethanol for 1 minute followed by a solution of sodium hypochlorite (NaOCl) [commercial bleach - 2.5% active chlorine] with 2 droplets of neutral detergent for 10 minutes. Seeds were washed four times in sterile distilled water and inoculated in tubes containing 15 mL of culture medium, according to the method described by CAMPOS et al. (2013).

### *Culture media and conditions*

The woody plant medium (WPM) of LLOYD & MCCOWN (1980) was used in all the experiments, solidified with 0.7% agar. The pH of the medium was adjusted to  $5.7 \pm 0.1$  before autoclaving. After that, the culture medium was distributed in test tubes (25 x 150 mm) sealed with plastic lids and sterilized by autoclaving for 15 minutes at temperature of 121 °C and pressure of 1 atm.

### *Slow-growth storage strategy*

The germinated plantlets with 10 days of age were inoculated in new test tubes (25 mm x 150 mm) containing 15 mL of WPM supplemented with different concentrations of sucrose (87.64, 131.46, 175.28 or 262.92 mM) combined with mannitol or sorbitol, at varied concentrations (0.0, 43.8, 65.73, 87.64 or 131.46 mM), totaling 12 treatments, as described in the table 1.

The survival percentage (% SURV) was evaluated every two months to ascertain the interaction of the factors culture time x concentration of osmotic agent. At 300 days after the start of the experiment, the following variables were recorded:

Table 1 - Osmotic treatments used for *in vitro* conservation of *Amburana cearensis* (Fabaceae) using woody plant medium (WPM).

Medium names	Concentration of osmotic agents (mM)		
	Sucrose	Sorbitol	Mannitol
M1	87.64	0	0
M2	131.46	0	0
M3	175.28	0	0
M4	262.92	0	0
M5	87.64	0	43.80
M6	131.46	0	65.73
M7	175.28	0	87.64
M8	262.92	0	131.46
M9	87.64	43.80	0
M10	131.46	65.73	0
M11	175.28	87.64	0
M12	262.92	131.46	0

number of senescent leaves, aerial part length (mm), aerial part dry mass (mg), root dry mass (mg) and number of shoots.

The cultures were kept in a growth room under controlled conditions of temperature ( $25 \pm 2$  °C), photoperiod (16/8 h) and photosynthetically active radiation ( $60 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ ) supplied by cool white fluorescent lamps. The experimental design was completely randomized with six repetitions per treatment, each composed of five experimental units.

#### Data analysis

For statistical analysis, the survival percentage data were transformed into arcsine ( $\sqrt{x/100}$ ) for normalization and homogenization of the variances and then submitted to analysis of variance (ANOVA), and the means value treatments were compared by the Scott-Knott test at 5% probability. All the statistical procedures were performed with the SAS 9.3 software (SAS Institute, Cary NC).

## RESULTS AND DISCUSSION

To retard the *in vitro* survival and vegetative development of *A. cearensis*, we investigated the effect of the sorbitol, mannitol and sucrose (osmotic sucrose) in the culture medium (Table 1). The survival percentage was significantly higher (100.0%) in the first four months (120 days of storage) (Figure 1). However, the percentage of surviving plants decreased significantly ( $p \leq 0.05$ ) after 120 days. This reduction was greater at the end of the experiment (10 months), in the presence

of higher concentrations of sucrose, sorbitol or mannitol, as observed in M8 and M12, with 0.0% and 10.0% survival, respectively. Osmotic agents acted as growth retardants by causing osmotic stress to the material under conservation (EL-BAHR et al., 2016).

These carbohydrates reduced the water potential and the availability of water to the explants (HUANG et al., 2014). Osmotic agents like mannitol and sorbitol reduced the absorption of minerals by the cells by means of different osmotic pressures, thus retarding plant growth (TAVAZZA et al., 2015; MUÑOZ et al., 2019). In this context, mannitol, sucrose and sorbitol have been reported to prolong the useful lifetime of *in vitro* cultured tissues (SHARAF et al., 2012). We observed that the amburana plants conserved for 300 days in culture media containing 262.92 mM of sucrose (M4) showed a higher survival percentage (Figure 2A); although, it did not differ from the medium containing 87.64 mM of sucrose and 43.8 mM of mannitol (M5). Conversely, plants grown in culture media containing 262.92 mM of sucrose supplemented with 131.46 mM of mannitol (M8) or sorbitol (M12) presented the lowest survival rates or even death of all plants (Figure 2B).

Other studies have demonstrated that the presence of sorbitol and/or mannitol combined with sucrose in the conservation medium increased the survival percentage of various plant species (TAVAZZA et al., 2015; TAKUR et al., 2015; EL-BAHR et al., 2016; NASIRUDDIN AND ISLAM, 2018).

Regarding the number of senescent leaves, it increased in plantlets grown with the smallest concentration of sucrose (87.6 mM), as well as when

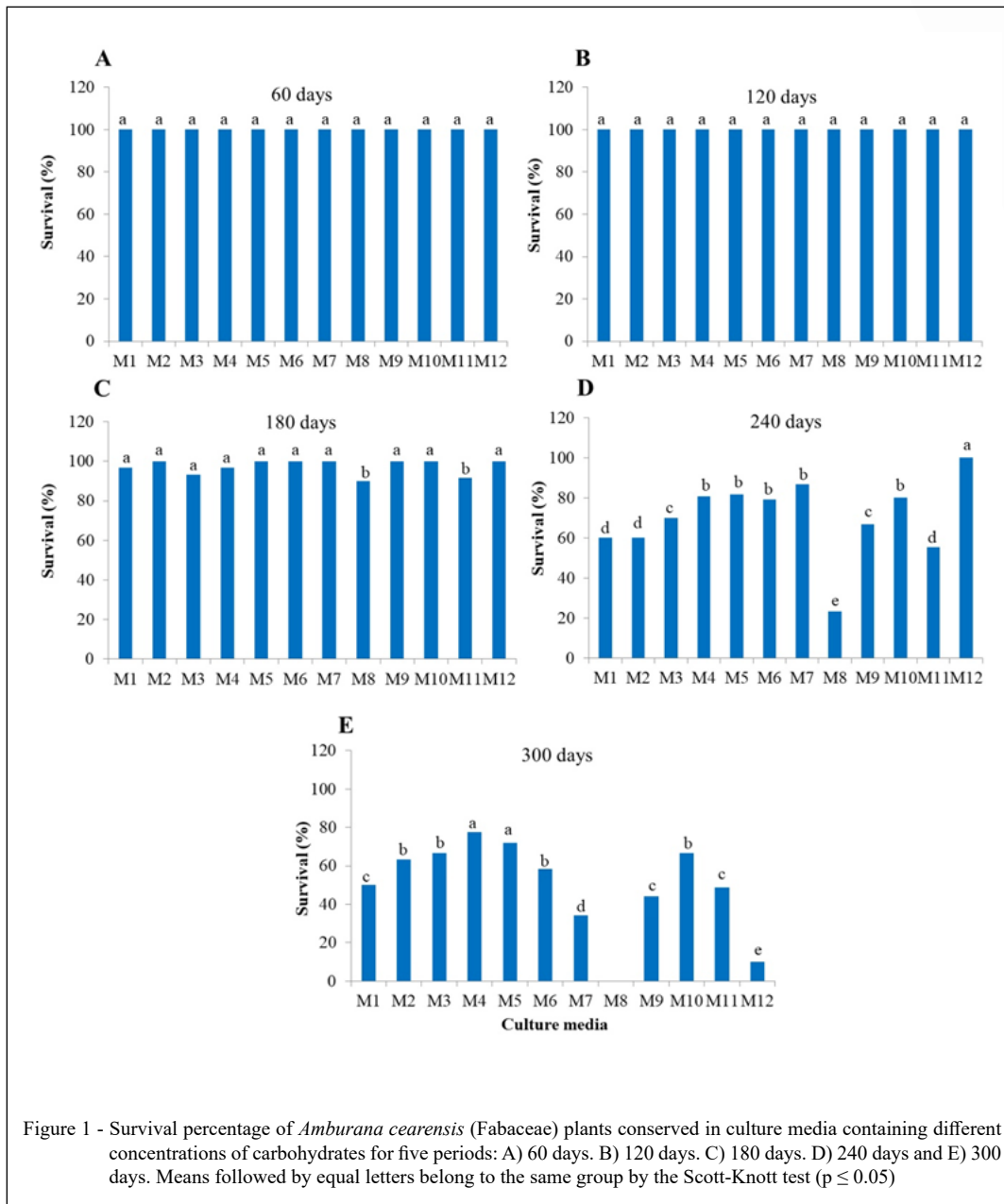


Figure 1 - Survival percentage of *Amburana cearensis* (Fabaceae) plants conserved in culture media containing different concentrations of carbohydrates for five periods: A) 60 days. B) 120 days. C) 180 days. D) 240 days and E) 300 days. Means followed by equal letters belong to the same group by the Scott-Knott test ( $p \leq 0.05$ )

combined with 43.8 mM of sorbitol (M9) (Figure 3A; Figure 2C). This can be explained by the fact that alcohol sugars at low concentrations do not act as reducers of the water potential, thus decelerating the plant growth. However, the lowest number of senescent leaves was observed in the plants grown in media supplemented only with sucrose at the highest concentration (262.92 mM), or combined with mannitol and sorbitol, except for M5 and M9, containing 43.8 mM of these osmotic agents. (Figure

3A). Plants in treatment M12 did not have any leaves with signs of senescence, despite the low survival rate obtained in this culture medium after 300 days.

Some authors have stated that *in vitro* senescence can be attributed to exhaustion of the nutritive capacity of the culture medium, accumulation of inhibitory products resulting from cell metabolism and/or toxicity of the medium after long periods because of evaporation of water from the tubes (GRATTAPAGLIA & MACHADO, 1998).

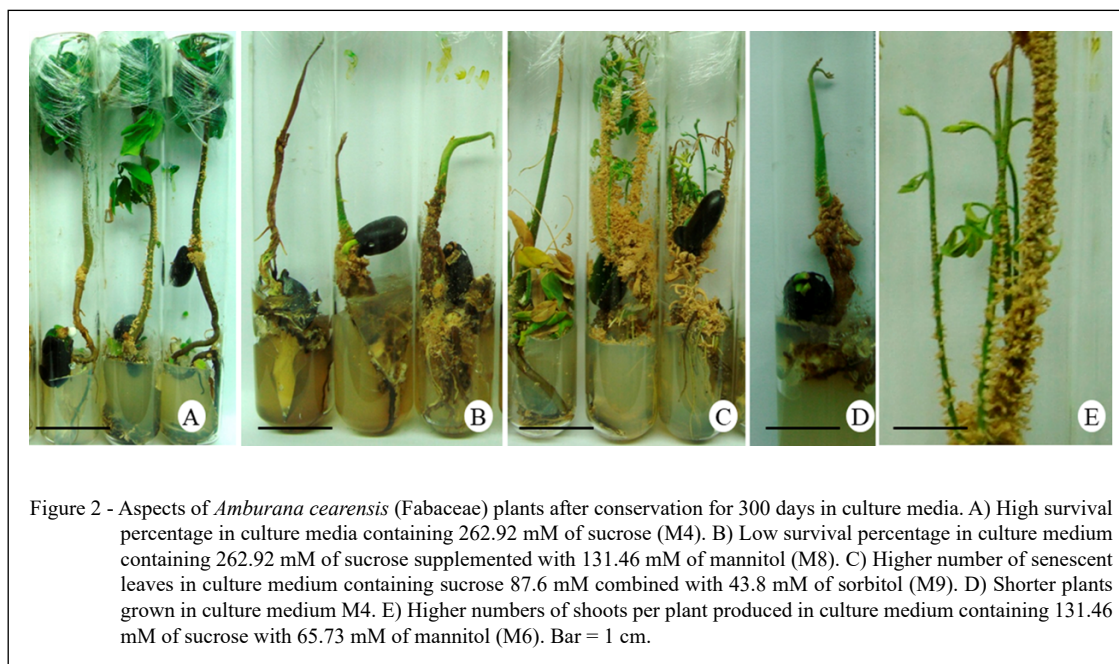


Figure 2 - Aspects of *Amburana cearensis* (Fabaceae) plants after conservation for 300 days in culture media. A) High survival percentage in culture media containing 262.92 mM of sucrose (M4). B) Low survival percentage in culture medium containing 262.92 mM of sucrose supplemented with 131.46 mM of mannitol (M8). C) Higher number of senescent leaves in culture medium containing sucrose 87.6 mM combined with 43.8 mM of sorbitol (M9). D) Shorter plants grown in culture medium M4. E) Higher numbers of shoots per plant produced in culture medium containing 131.46 mM of sucrose with 65.73 mM of mannitol (M6). Bar = 1 cm.

In particular, the accumulation of substances from metabolism is caused by the fact the test tubes are closed, limiting the gas exchanges with the external environment and preventing the release of toxic compounds such as ethylene. This, in turn, is strongly indicated by the presence of fallen leaves from the cultured plants. Therefore, the accumulation of this plant regulator can affect the morphogenesis of the cultures, accelerating the senescence and inducing foliar abscission (GRATTAPAGLIA & MACHADO, 1998; NEPOMUCENO et al., 2009).

For aerial part length, the culture medium supplemented with sucrose at concentration of 175.28 mM (M3) promoted the highest average, of 94 mm (Figure 3B). However, when this carbohydrate was used at the highest concentration (262.92 mM) in the medium (M4), the smallest length was observed (Figure 2D). This result can be explained by the fact that sucrose at high concentrations acts as an osmotic agent, reducing the water potential of the medium and thus hindering the absorption of water and nutrients (JO et al., 2009; CUI et al., 2010; SUMARYONO et al., 2012).

The presence of sucrose, sorbitol or mannitol in the culture medium had a negative effect on the aerial part development. This reduction was most pronounced in the presence of higher concentrations of sucrose (262.92 mM) and sorbitol (131.46 mM) (21 mm); although, this treatment belongs to the same group as M6 (131.46 mM of sucrose + 65.73 mM of

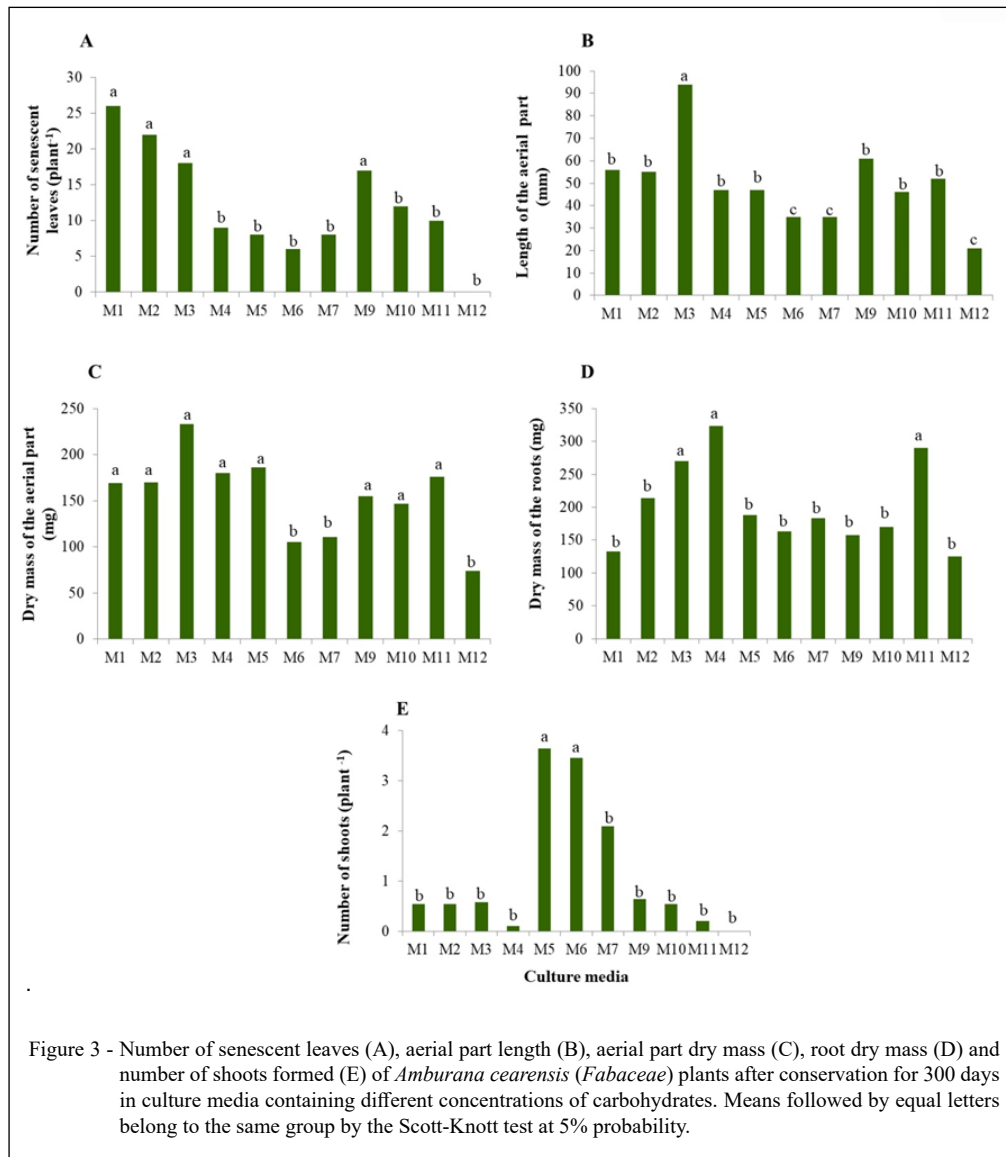
mannitol) and M7 (175.28 mM of sucrose + 87.64 mM of mannitol) (Figure 3B).

High osmotic pressure acts to suppress the metabolism of plants (CUI et al., 2010), a fact reflected in the lower growth of aerial part of *A. cearensis*. These results are partially concordant with those obtained by TAVAZZA et al. (2015), who observed a reduction of the aerial part length of artichoke plants (*Cynara cardunculus* var. *scolymus* L.) cultivated in the presence of mannitol, while in the medium supplemented with sucrose and sorbitol there were no significant differences.

The aerial part dry mass confirms the smaller accumulation of biomass noted in M6 (105.0 mg), M7 (111.0 mg) and M12 (74.0 mg) treatments. Therefore, the presence of the osmotic agents sorbitol and mannitol, as well as the highest concentration of sucrose tested, acted to suppress the metabolism of *A. cearensis* plants and their growth (Figure 3C).

Regarding root dry mass, there was an increase proportional to the rising concentrations of sucrose, in the media M1, M2, M3 and M4 (Figure 3D). These results corroborated those of other authors, who have reported an increase of root length and root dry mass with rising sucrose concentrations in the culture medium in various species (GEORGE, 2008; JO et al., 2009).

Contradictorily, the smallest root dry mass (125 mg) was obtained in treatment M12,



although this did not differ statistically from the values observed in M1, M2, M5, M6, M7, M9 and M10. Similar results were reported by FARIA et al. (2006), studying the effect of sucrose and sorbitol on *Passiflora gibertii* N.E.Br., where the smallest rooting percentages occurred in culture medium supplemented with sorbitol.

The plants also presented formation of lateral shoots, both at the base of the cotyledon and in the lateral buds, in all the treatments except M12 (262.92 mM of sucrose with 131.46 mM of sorbitol, Figure 3E). The highest average numbers of shoots were produced by the plants in M5 (87.6 mM of sucrose with 43.8 mM of mannitol) and M6

(131.46 mM of sucrose with 65.73 mM of mannitol) treatments, with respective values of 3.64 and 3.45 shoots/plant (Figure 3E; Figure 2D).

## CONCLUSION

The culture medium with 262.92 mM of sucrose (M4) or the combination of 87.64 mM of sucrose with 43.80 mM of mannitol can be used for *in vitro* conservation of *Amburana cearensis*, by favoring the normal development of the preserved plants and resulting in better appearance, with predominance of uniform green leaves during the storage process for 10 months.

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## DECLARATION OF CONFLICT OF INTERESTS

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

## AUTHORS' CONTRIBUTIONS

All authors contributed equally for the conception and writing of the manuscript.

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