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Full Length Research Paper

Medium-term conservation and regrowth of the tropical multipurpose species *Genipa americana*

Ana da Silva Lédo[1]*, Camila Santos Almeida[2], Ana Veruska Cruz da Silva[1], Josué Francisco da Silva Junior[1] and Aparecida Gomes de Araujo[1]

[1]Embrapa Coastal Tablelands, Avenida Beira Mar 3250, Aracaju, Sergipe, Brazil.
[2]Federal University of Sergipe, São Cristovão, Sergipe, Brazil.

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The *Genipa americana* L., a fruit-producing species found in several countries and commonly found in the Brazilian Northeast, is very important economically, both for its wood and as a source of food and dye. This study evaluate the effects of abscisic acid (ABA) and the concentrations of salt and sugar in MS medium on reducing *Genipa americana* L. growth for *in vitro* conservation, as well as the effects of benzylaminopurine (BAP) on the regrowth phase. Seedlings from accession CZA, after 90 days of *in vitro* cultivation, were transferred to Murashige and Skoog (MS) medium with 30 g L^{-1} sucrose and various concentrations of ABA (0, 0.5, 1.0, 2.0 and 4.0 mg L^{-1}). In a second assay, various concentrations of MS medium salts and sucrose were tested. During the regrowth phase, nodal segments were placed in MS regeneration medium with 30 g L^{-1} sucrose and BAP (0 or 1 mg L^{-1}). All the ABA concentrations affected the number of leaves, but significant effects on the length of aerial portion were not observed. The MS and ½ MS media with 30 g L^{-1} sucrose slowed plant growth and are recommended for conservation protocols for slow growth. Adding 1 mg L^{-1} BAP to the culture medium caused a greater morphogenic response, and the apical, medial and basal nodes retained morphogenic potential during regrowth.

Key words: *Genipa americana* L., germplasm, abscisic acid, sucrose.

INTRODUCTION

Genipa americana (Rubiaceae) is the well-known native fruit tree in South America and West Indies. Fruits and tree bark are used medicinally as tonic, febrifuge, for venereal diseases, pharyngitis, cough, cold and others. Fruits are used to prepare beverages (Yee et al., 2010). Recently studies reported the utilization of *G. americana* as an anthelminthic in sheep (Nogueira et al., 2014).

The Brazilian Agricultural Research Agency has been increasing *G. americana* L. germplasm collection, characterization and conservation efforts through the genetic resource platform, which was started in 2009 with the creation of an active Germplasm Bank containing 195 genotypes. Each conservation method has its advantages and disadvantages, and complimentary strategies are required to maximize the conservation of genetic diversity, which varies between species (Martin and Pradeep, 2003).

G. americana L. is a perennial species, and the conservation of the genetic resources of this tree is mainly based on field collection due to its physiology. The

*Corresponding author. E-mail: ana.ledo@embrapa.br
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species does not tolerate extreme desiccation; therefore, the behavior of *G. americana* L. during storage is less than optimal (Salomao, 2004; Magistrali et al., 2013).

In the last few years, *in vitro* culture techniques for Germplasm Bank conservation have been widely developed and applied to more than 1,000 species, many of which come from tropical regions. *In vitro* culture is thought to be a very promising method for endangered species for which the seeds cannot be conserved and are valuable for conserving plant genetic material (Harding et al., 1997).

Growth reduction is generally achieved by modifying the environmental conditions and/or the culture medium. The most widely applied technique is temperature reduction, which can be combined with a decrease in light intensity or culture in the dark. Tropical species are often cold-sensitive and have to be stored at higher temperatures, which depend on the cold sensitivity of the species. Modifications of the culture medium can include dilution of mineral elements, reduction of sugar concentration, changes in the nature and/or concentration of growth regulators and addition of osmotically active compounds (Engelmann, 2011).

Slow-growth *in vitro* conservation protocols have been created for several species, including *Plumbago indica* (Charoensub and Phansiri, 2004), *Guarianthe skinneri* (Coello et al., 2009), *Piper* sp. (Silva and Scherwinski-Pereira, 2011), *Gladiolus imbricatus* (RAKOSI-TIAN et al., 2012), *Musa balbisiana* (Kanchanapoom and Promsorn, 2012), *Lilium* sp. (Yun-Peng et al., 2012), *Vitis* sp. (Silva et al., 2012), *Glycyrrhiza glabra* (Srivastava et al., 2013), *Epidendrum chlorocorymbus* (Lopez-Puc, 2013) and *Cocos nucifera* (Lédo et al., 2014).

Numerous parameters influence the efficiency of *in vitro* slow growth storage protocols including the type of explants, their physiological state when entering storage, the type of culture vessel, its volume and the volume as well as the type of closure of the culture vessel (Engelmann, 1991). Although there is no standard procedure for all the genotypes of all the species, it will be possible to develop adequate slow-growth methods that require little manipulation for other species. Recent studies have demonstrated the organogenic capacity of *G. americana* L. plants for *in vitro* propagation (Yee et al., 2010), but strategies for the *in vitro* conservation by minimal growth were not found in the literature. The aim of this study was to evaluate the effects of abscisic acid (ABA) and the concentrations of salt and sugar in Murashige and Skoog (MS) medium on reducing *G. americana* L. growth for *in vitro* conservation, as well as the effects of benzylaminopurine (BAP) on the regrowth phase.

MATERIALS AND METHODS

Plant material and incubation conditions

Seeds taken from the mature fruit of *G. americana* L., occurring in a natural population in Cruz das Almas, BA, Brazil (12°39'10.11''S; 39°07'19.02''W)- CZA accession, were washed with running tap water for 30 min and maintained for 24 h at room temperature. The seeds were then disinfected in a laminar flow cabinet by immersion in 70% (v/v) ethanol for 60 s, followed by 20 min in 2.5% sodium hypochlorite (NaOCl) containing two drops of Tween 20. After that, the seeds were rinsed three times with sterile distilled water to remove minor amounts of disinfection liquid.

Five seeds were inoculated into 250 ml flasks containing 30 ml of MS culture medium (Murashige and Skoog, 1962) with 30 g L^{-1} of sucrose and 4.5 g L^{-1} of Phytagel™ (Sigma-Aldrich Co, Saint Louis, MO, USA) to grow healthy plants for a period of 90 days, which were then used in all the experiments.

The experiments were conducted in the Plant Tissue Culture Laboratory at Embrapa Tabuleiros Costeiros, Aracaju, Brazil. The pH of the culture media was adjusted to 5.8 ± 0.1, and the media were converted to gels with 4.5 g L^{-1} of Phytagel™, followed by autoclaving for 20 min at a temperature of 121 ± 1°C and a pressure of 1.05 atm. The cultures were maintained in a temperature-controlled growth room at 25 ± 2°C, with an average relative humidity of approximately 70% and a light/dark cycle with 12 h of light and a luminosity of 60 µmol $m^{-2}s^{-1}$.

In vitro conservation of *G. americana* L.

To study the effects of ABA and the salt and sugar concentrations of the MS culture on the *in vitro* growth, the *in vitro*-germinated plants were transferred to conservation media (CM) containing, for the assay 1, MS medium salts (Murashige and Skoog, 1962) supplemented with 30 g L^{-1} sucrose and one of five concentrations of abscisic acid (ABA) (0, 0.5, 1.0, 2.0 or 4.0 mg L^{-1}). For the assay 2, different MS salt and sucrose concentrations were used as follows: T1- MS medium + 30 g L^{-1} sucrose, T2- ½ MS + 15 g L^{-1} sucrose, T3- ½ MS + 30 g L^{-1} sucrose, T4- ¼ MS + 15 g L^{-1} sucrose, T5- ¼ MS + 30 g L^{-1} sucrose. All of the media were gelled with 4.5 g L^{-1} Phytagel™.

In vitro regrowth of *G. americana* L.

Nodal segments (apical, medial and basal) from adventitious shoots taken from the *G. americana* L. plants conserved *in vitro* for 150 days under the conditions described above were transferred to growth media. The explants were inoculated into 250 ml flasks, sealed with plastic lids, containing 30 ml of MS culture medium (MS) with 4.5 g L^{-1} Phytagel™ and supplemented with 0 to 1 mg L^{-1} BAP and 30 g L^{-1} sucrose.

Data collection and statistical analysis

The length of the aerial portion (LAP), number of leaves (NL), number of leaves with abscissions (NLA) and culture viability (CV) were evaluated in the *in vitro*-conserved plants at 60, 90, 120 and 150 days. The plant viability (plant response under conditions of limited growth) was quantified on a scale with the following categories: 5- the leaves and buds are completely green, 4- the leaves are starting to dry and die, 3- 30-50% of the leaves and buds are dry and dead, 2- more than 50% of the leaves and buds are dry and dead and 1- the leaves and buds are completely dead. The percentage of the explants with morphogenic responses was record after 50 days in the regrowth medium. The plants were considered to have a morphogenic response if they showed signs of direct or indirect organogenesis and/or callogenesis.

The *in vitro* conservation assays had a completely randomized experimental design with five treatments and four replicates, and each treatment consisted of 20 flasks with one plant/flask. The

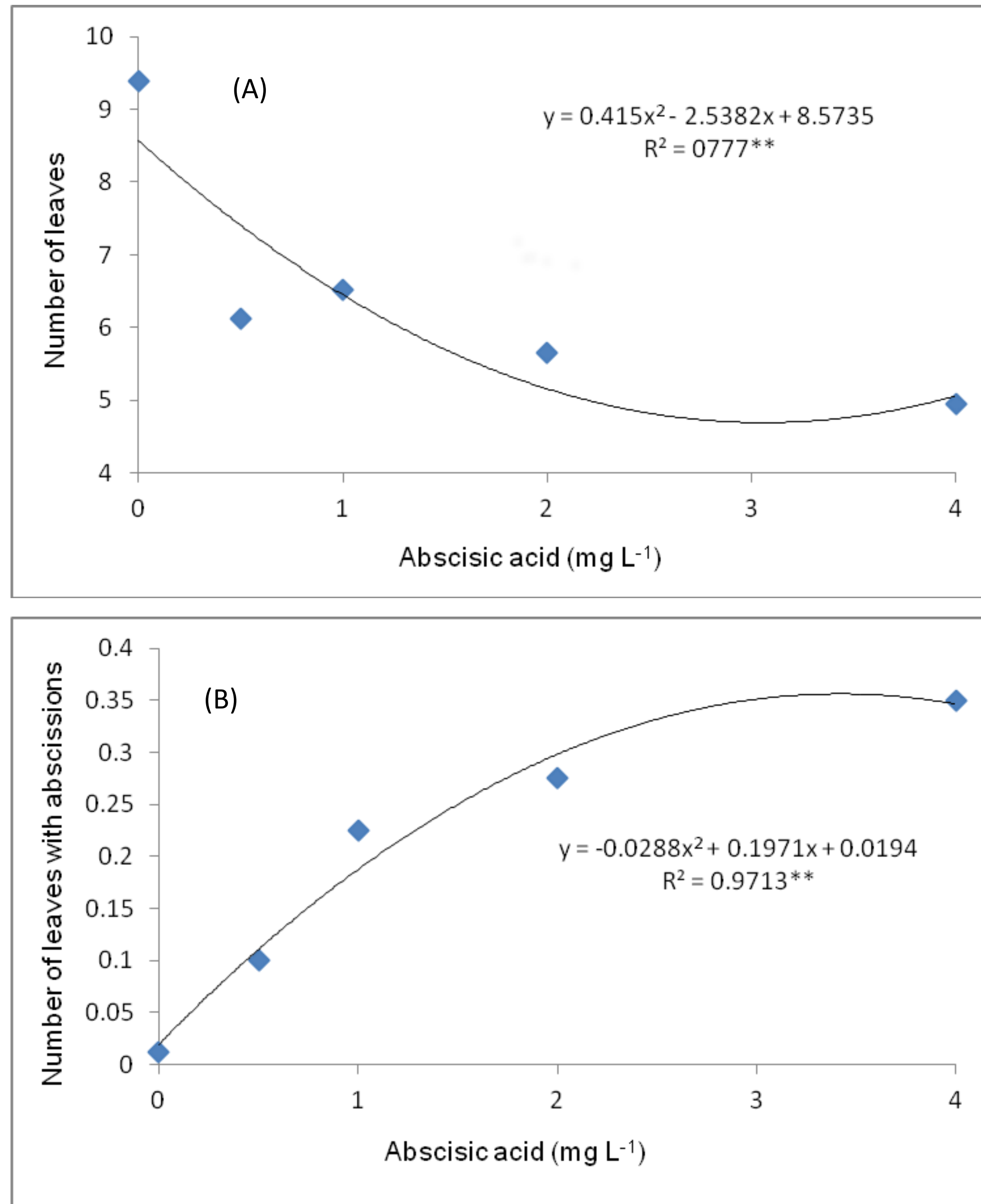

Figure 1. (A) Number of leaves (NL); (B) Number of leaves with abscissions (NLA) on *G. americana* L. plants as a function of the ABA concentration after 150 days of *in vitro* culture.

regrowth assays were completely randomized, while accounting for the factors in the *in vitro* conservation step (five treatments) combined with three types of explants and two concentrations of BAP (factorial 5 x 3 x 2) for a total of 30 treatments with three replicates each. An experimental parcel contained three flasks (one explant/flask). The data of the *in vitro* conservation assays containing ABA were used for the analysis of variance with the F-test and to calculate the fitted equations using polynomial regression. The data of the *in vitro* conservation assay containing various amounts of MS salts and sucrose were submitted to an analysis of variance and compared with the Tukey test at 1 and 5% probability. The data from the regrowth assays were compared using the Tukey test at 5% probability.

RESULTS AND DISCUSSION

Effects of ABA, MS salts and sucrose concentrations on the *in vitro* slow-growth of *G. americana* L.

ABA significantly affected the NL and the NLA on the *G. americana* L. plantlets. The NL exhibited a quadratic behavior with a gradual reduction as a function of increasing ABA concentration (Figure 1A), as seen, increasing concentrations of ABA led to increased NLAs (Figure 1B). Although ABA affected the NL and NLA, a

Table 1. Number of leaves (NL), length of the aerial portion (LAP), number of leaves with abscissions (NLA) and culture viability (CV) in different concentrations of MS salts and sucrose.

Treatments	NL	LAP (cm)	NLA	CV
MS + 30 g L^{-1} sacarose	8.79±1.82^{c}	4.69±0.62^{b}	0.08±0.14^{a}	4.10±0.63^{c}
½ MS + 15 g L^{-1} sacarose	9.46±1.57^{bc}	5.37±0.40^{a}	0.08±0.18^{a}	4.73±0.35^{ab}
½ MS + 30 g L^{-1} sacarose	10.13±2.08^{ab}	4.73±0.77^{b}	0.05±0.10^{a}	4.44±.52^{bc}
¼ MS + 15 g L^{-1} sacarose	11.00±1.76^{a}	5.47±0.22^{a}	0.15±0.56^{a}	4.63±0.28^{ab}
¼ MS + 30 g L^{-1} sacarose	11.20±1.61^{a}	5.12±0.26^{ab}	0.00^{a}	4.94±0.11^{a}
Probability (P > F)	0.00001	0.00001	0.5655	0.00001
VC (%)	13.56	10.26	17.16	8.97

Averages in the same column followed by the same letter are not significantly different at a 5% probability level according to the Tukey test. VC= Variation Coefficient.

Table 2. Number of leaves (NL), length of the aerial portion (LAP), number of leaves with abscissions (NLA) and culture viability (CV) as a function of time cultured in different concentrations of MS salts and sucrose.

Time (days)	NL	LAP (cm)	NLA	CV
60	8.72±0.82^{b}	4.916±0.45^{a}	0.04±0.09^{a}	4.78±0.35^{a}
90	9.32±1.51^{b}	4.984±0.59^{a}	0.03±0.08^{a}	4.64±0.47^{a}
120	10.78±1.84^{a}	5.127±0.60^{a}	0.04±0.11^{a}	4.51±0.51^{ab}
150	11.64±2.01^{a}	5.273±0.64^{a}	0.17±0.05^{a}	4.33±0.55^{b}
Probability (P > F)	0.00001	0.0816	0.2453	0.0018
VC (%)	13.56	10.26	17.16	8.97

Averages in the same column followed by the same letter are not significantly different at a 5% probability level according to the Tukey test. VC= Variation Coefficient.

significant effect on the LAP (cm) and plant viability were not observed.

Although ABA participates in the regulation of many physiological processes of whole plants (Tuteja 2007), it is regarded as an inhibitor of plant growth and therefore usually used as a growth retardant in plant tissue culture (Engelmann, 1991; Rai et al., 2011). Various authors have pointed out that while exogenous ABA alone suppresses shoot regeneration, however the absence of effect on the length of the aerial portion it is possible is linked to genetic characteristics of this species.

There was no significant increase in the variables analyzed as a function of *in vitro* culture time. ABA slowed *G. americana* L. plant growth (number of leaves) at the studied concentrations and may be a promising compound for *in vitro* conservation. Studies with higher concentrations of ABA should be conducted to evaluate the effect on deceleration of growth in length of the aerial part. Regarding the MS salts and sucrose concentrations, the analysis of variance revealed significant effects on the following variables: the NL, LAP and plant viability. The *in vitro* culture time did not significantly affect the LAP or NLA.

We observed that treatments with ¼ of the MS medium salts, independent of the sucrose concentration, led to the formation of larger leaves in the plants and had greater results than the other treatments. The MS and ½ MS media with 30 g L^{-1} sucrose reduced the growth of the aerial portion (Table 1) while maintaining good plant viability.

Decreasing the salt concentrations in the culture media led to an increase in plant development, as indicated by the LAP, contrary to what was expected and what has been observed in several species when used as an *in vitro* conservation strategy (Table 2). According to Malaurie and Borges (2001), reducing the concentration of mineral salts and sucrose in the culture medium allows for the maintenance of plants at a minimal growth rate. The opposite effects observed in this study can probably be explained by endogenous factors.

Similarly, the culture media with ¼ MS salts and 15 or 30 g L^{-1} sucrose show promise for creating *in vitro* germination protocols for *G. americana* L. that decrease production costs by requiring lower concentrations of MS salts than what is required for rapid plant growth (Table 2). In addition, decreasing the salts improved plant viability, and the ¼ MS culture medium with 30 g L^{-1} sucrose had higher values than the other treatments.

The *in vitro* culture time significantly affected the NL and viability. At 150 days, there were significantly more leaves than at the other time points (Table 2). However, the *G. americana* L. plants had better viability at 60 and

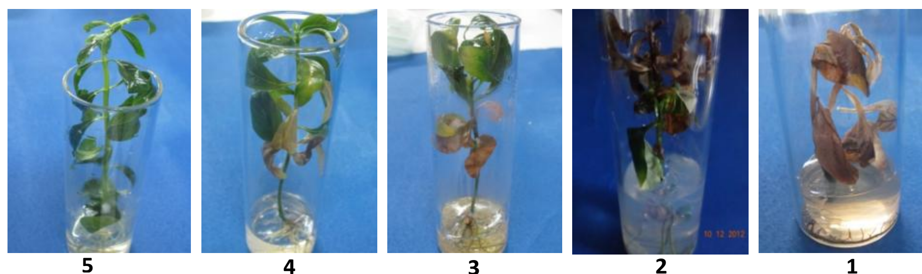

Figure 2. Rating scale for analyzing *G. americana* L. plant viability under slow-growth conditions, 5- the leaves and buds are completely green, 4- the leaves are starting to dry and die, 3- 30-50% of the leaves and buds are dry and dead, 2- more than 50% of the leaves and buds are dry and dead and 1- the leaves and buds are completely dead.

Table 3. Percentage of the morphogenic response of various types of explants (regrowth medium) in the presence of ABA (conservation medium) after 50 days of culture *in vitro*.

Types of explants	Morphogenic response (%)
Basal	78.34±24.84^{b}
Medial	90.00±19.03^{ab}
Apical	95.00±12.20^{a}
Probability (P > F)	0.0200
VC(%)	19.11

Averages in the same column followed by the same letter are not significantly different at a 5% probability level according to the Tukey test. VC= Variation Coefficient.

90 days than at 120 and 150 days. Although the plant viability did decrease, the minimum value for this variable was 4.33 (Figure 2), indicating that the overall plants maintained excellent vigor over the culture period. The MS medium and the medium with ½ the concentration of MS salts with 30 g L^{-1} sucrose decreased the growth rate of the *G. americana* L. plants.

Effects of type of explant and BAP on the *in vitro* regrowth of *G. americana* L. from CM containing ABA

The type of explant and the interaction between the CM and BAP had significant effects during the *in vitro* regrowth of the plants. The presence of ABA in the CM or BAP and the other interactions did not significantly affect the morphogenic response. According to Table 3, the apical segment had the highest morphogenic response (93.33%), and the basal segment had the lowest response (78.34%). Overall, all of the explants had good morphogenic responses. Although ABA decreased the growth rate of the plants, the explants maintained their morphogenic potential during recovery. These results are different from those obtained by Silva and Scherwinski-Pereira (2011), who showed that ABA negatively impacted regenerations from *Piper* sp. explants.

The analysis of the morphogenic response as a function of ABA (in the CM) and BAP revealed that there was not a statistical difference between the explants grown in the presence or absence of BAP (Table 4). However, the explants with 0.5 or 2.0 mg L^{-1} of ABA during the conservation phase that were subcultured in the presence of 1.0 mg L^{-1} BAP had a greater morphogenic response than those maintained in the absence of this regulator. The explants maintained in the absence of BAP during the *in vitro* regeneration step had a larger organogenic response (84.44%). However, the explants in the presence of BAP had an 81.12% regeneration rate *in vitro* with an indirect organogenic response (Table 5). For the factors of the type of explant and the CM (ABA), we observed a morphogenic response with direct and indirect organogenesis, but a higher percentage of the explants had direct organogenesis.

Effects of type of explant and BAP on the *in vitro* regrowth of *G. americana* L. from CM with different sucrose and MS salts concentrations

The CM, BAP and the CM x BAP interaction had a significant effect on *in vitro* regrowth *in vitro,* while the explant type and the other interactions did not. As shown in Table 6, the morphogenic response of the explants maintained in MS medium + 30 g L^{-1} sucrose or ½ MS + 15 g L^{-1} sucrose during the conservation phase was lower

Table 4. Percentage of morphogenic response as a function of ABA (conservation medium) and BAP (regrowth medium) after 50 days of culture *in vitro*.

Morphogenic response (%)		
ABA (mg L^{-1})	**BAP (mg L^{-1})**	
	0	**1**
0	94.45±13.61^{aA}	88.89^{aA}
0.5	66.68^{aB}	94.45^{aA}
1.0	94.45^{aA}	83.34^{aA}
2.0	77.78^{aB}	100.00^{aA}
4.0	88.89^{aA}	83.34^{aA}
Probability (P > F)	0.0185	
VC (%)	19.11	

Averages followed by the same lowercase letter in columns and the same uppercase letter in are not significantly different at a 5% probability level according to the Tukey test. VC= Variation Coefficient.

Table 5. Percentage of morphogenic response as a function of the type of explant, the presence or absence of BAP (regrowth medium) and ABA (conservation medium) after 50 days of culture *in vitro.*

Type of explants	**EOS (%)**	**CALLO (%)**	**DO (%)**	**IO (%)**
Basal	21.67	0	38.33	40
Medial	10	0	45	45
Apical	6.67	1.67	55	36.67
BAP (mg L^{-1})	EOS (%)	CALLO (%)	DO (%)	IO (%)
0	15.56	0	84.44	0
1	10	1.1	7.78	81.12
ABA (mg L^{-1})	EOS (%)	CALLO (%)	DO (%)	IO (%)
0	8.33	0	55.56	36.11
0.5	19.44	0	41.67	38.89
1.0	11.11	0	47.22	41.67
2.0	11.11	0	38.89	50
4.0	13.89	2.78	47.22	36.11

EOS – Oxidized, contaminated and unresponsive plants; CALLO – callogenesis; DO – direct organogenesis; IO – indirect organogenesis.

in the absence of BAP than in the other treatments. These results suggest that the plants maintained in media that slow growth during the conservation phase recover less, necessitating the addition of BAP during the regrowth phase. There was no difference between the morphogenic responses of the explants maintained in the different culture media containing BAP (1 mg L^{-1}), as the media with ½ MS and 30 g L^{-1} sucrose or ¼ MS with 15 or 30 g L^{-1} sucrose resulted in a 100% morphogenic response rate in the explants. The MS medium with 30 g L^{-1} sucrose and ½ MS with 15 or 30 g L^{-1} sucrose had lower morphogenic responses in the absence of BAP than in the presence of BAP (Table 7). The presence of 1.0 mg L^{-1} BAP positively affected regrowth *in vitro*, with a significant increase in the morphogenic response of the explants grown in culture media containing the cytokine.

The inclusion of BAP combined with sucrose and various concentrations of MS salts in the culture media also led to a greater morphogenic response in the explants with callogenesis. In the absence of BAP, more explants showed direct organogenesis (Table 7). Although, the *G. americana* presents open pollination and this aspect may have influenced the higher variation coefficient in the morphogenetic responses, due to greater variation genetics of explants, the type of explant and conservation medium considerably increased regeneration with callus formation and the number of explants with a direct organogenesis response. Further studies should be performed to determine the regrowth medium that promotes the most regeneration in explants maintained under these conditions during the conservation step.

Conclusions

This is the first report on *in vitro* conservation by slow-growth of *G. americana* L., and our study is a

Table 6. Percentage of morphogenic response of the explants from accession CZA as a function of the conservation medium and the presence or absence of BAP (regrowth medium) after 50 days of culture *in vitro*.

Morphogenic response (%)		
Conservation medium	**BAP (mg L^{-1})**	
	0	**1**
MS + 30 g L^{-1} sacarose	16.67^{cB}	75.0^{aA}
½MS + 15 g L^{-1} sacarose	61.11^{bB}	94.45^{aA}
½MS + 30 g L^{-1} sacarose	72.22^{abB}	100.0^{aA}
¼MS + 15 g L^{-1} sacarose	94.45^{aA}	100.0^{aA}
¼MS + 30 g L^{-1} sacarose	88.89^{abA}	100.0^{aA}
Probability (P > F)	0.0212	
VC (%)	24.42	

Averages followed by the same lowercase letter in columns and the same uppercase letter in are not significantly different at a 5% probability level according to the Tukey test. VC= Variation Coefficient.

Table 7. Percentage of morphogenic response as a function of explant type, the presence or absence of BAP (regrowth medium) and the conservation medium after 50 days of culture *in vitro*.

Type of explants	EOS (%)	CALLO (%)	DO (%)	IO (%)
Basal	35	41.67	23.33	0
Medial	28.34	43.33	28.33	0
Apical	26.67	16.67	38.33	18.33
BAP (mg L^{-1})	EOS (%)	CALLO (%)	DO (%)	IO (%)
0	43.33	0	56.67	0
1	16.67	67.78	3.33	12.22
Conservation medium	EOS (%)	CALLO (%)	DO (%)	IO (%)
MS + 30 g L^{-1} sacarose	58.33	27.78	8.33	5.56
½MS + 15 g L^{-1} sacarose	36.11	38.89	22.22	2.78
½MS + 30 g L^{-1} sacarose	36.11	33.33	27.78	2.78
¼MS + 15 g L^{-1} sacarose	13.89	36.11	47.22	2.78
¼MS + 30 g L^{-1} sacarose	5.56	33.33	44.44	16.67

EOS – Oxidized, contaminated and unresponsive plants; CALLO – callogenesis; DO – direct organogenesis; IO – indirect organogenesis.

contribution to the germplasm conservation of this tropical multipurpose species. *G. americana* L. *in vitro* cultures can be conserved for five months in MS and ½ MS medium plus 30 g L^{-1} sucrose without losing regeneration capacity. The inclusion of 1 mg L^{-1} BAP in the regrowth medium leads to a greater morphogenic response in explants taken from plants in slow-growth conditions. The apical, medial and basal nodal segments have morphogenic potential during the regrowth phase. Studies with higher concentrations of ABA should be conducted to evaluate the effect on deceleration of growth in length of the aerial part.

Conflict of Interest

The authors have not declared any conflict of interest.

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