

Optimización de la conservación *in vitro* de germoplasma de *Dioscorea spp* por crecimiento mínimo

Optimization of *in vitro* conservation of *Dioscorea spp* germplasm by minimal growth

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Resumen

El ñame Criollo (*Dioscorea alata*) y el ñame Espino (*Dioscorea rotundata*) se constituyen como las dos especies mayormente cultivadas en el departamento de Sucre, Colombia. Por esta razón en la Universidad de Sucre se han implementado técnicas para lograr su conservación mediante la propagación *in vitro*, sin embargo esta metodología conserva las accesiones por un periodo no mayor a los 4 meses, provocando continuos subcultivos, aumento de costos y mano de obra. Por ello la presente investigación tuvo como objetivo establecer medios de cultivo óptimos para la conservación *in vitro* por crecimiento mínimo de diferentes accesiones (*D. alata* y *D. rotundata*) pertenecientes al banco de germoplasma de la Universidad de Sucre, durante un periodo de 8 meses. Esto mediante la modificación del medio de cultivo base MS; con los siguientes osmólitos: sacarosa, manitol y sorbitol. Se evaluaron 8 tratamientos (T) en los siguientes porcentajes T₁ (control) (3:0:0), T₂ (0:1,5:0), T₃ (0:0:2), T₄ (0:1,5:2), T₅ (0:0:1) y T₆ (0:0:3), T₇ (0:1,5:1) y T₈ (0:1,5:3). Cada 30 días se evaluó: supervivencia (%), hojas expandidas (%), longitud del tallo y raíz, número de nudos y raíces, oxidación (%), senescencia foliar (%) y callo (%). Los resultados mostraron que las especies *D. alata* y *D. rotundata*, se conservan de forma óptima, en la combinación T₄ (0:1,5:2), donde se evidencia un alto porcentaje de supervivencia, un mínimo porcentaje de senescencia foliar y un desarrollo restringido en el resto de variables. Garantizando así la disponibilidad y el desarrollo normal de las accesiones en un periodo superior a 4 meses.

Palabras clave: ñame, manitol, sorbitol, sacarosa.

Abstract

Dioscorea alata cv. "Criollo" and *Dioscorea rotundata* cv. "Espino" are constituted as the two cultivars mostly cultivated in the department of Sucre, Colombia. For this reason the University of Sucre has been implementing tissue culture techniques for their multiplication throughout *in vitro* propagation. However, this methodology support accessions for a period about 4 months, causing continuous subcultures, increased costs and labor. Therefore the objective of the present investigation was to establish an optimal culture media for *in vitro* minimal growth conservation of different accessions (*D. alata* and *D. rotundata*) from the University of Sucre yam's genebank, for a period of 8 months by modifying the basic MS culture medium. The following osmolytes were used: sucrose, mannitol and sorbitol in different percentages (S:M:S). Eight treatments (T) in the following percentages were evaluated: T₁ (control) (3:0:0), T₂ (0:1,5:0), T₃ (0:0:2), T₄ (0:1,5:2), T₅ (0:0:1) y T₆ (0:0:3), T₇ (0:1,5:1) y T₈ (0:1,5:3). Every 30 days data was recorded for: survival (%), expanded leaves (%), length of stem and roots, number of leaves and roots, phenolization (%), leaf senescence (%) and callus presence (%). The best results for *D. alata* and *D. rotundata*, were observed in treatment T₄ (0:1,5:2), where a high percentage of survival evidence, a minimum percentage of leaf senescence and a limited response for the other variables. Therefore, these results indicate the potential of this media, to increase 100% *in vitro* growth conservation over the conventional media, and ensuring the viability and normal development of the accessions for more than four months period.

Key words: yam, mannitol, sorbitol, sucrose.

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Introduction

Dioscorea is one of the six genera of the *Dioscoreaceae* family, which is comprised of more than 650 species distributed in tropical areas with high rainfall (Thurston, 1998). The *Dioscorea* sp. yam is a very important tuber for the small-scale producers of the Caribbean Region in Colombia. Many of this region's farming families depend on this crop (Reina, 2012), as well as the markets that are part of the free trade agreements (FTA). Therefore, developing research aimed at understanding its features and finding solutions to the problems presented by the crop is of vital importance.

In the Sucre Department, the most cultivated species are the "white yam" (*Dioscorea rotundata*) and "purple yam" (*Dioscorea alata*) with 75% of the total amount cultivated (Reina, 2012). In this area of the country, yam is produced from vegetable form through division of the tubers into portions, causing the transfer of diseases from one cycle to another and from one locality to another, in some cases (Rodríguez, et al. 2008). Therefore, the initiative arose to implement new methodologies for the conservation of this species, such as *in vitro* culture. Through this methodology, the Universidad de Sucre established a germoplasm bank in which the previously mentioned species are protected.

The conservation of this crop in a germoplasm bank constitutes a solution to the problems presented by field cultivation. There are reports about the micropropagation of some edible yams, such as *D. rotundata* cv. "white yam" (Acosta and Beltrán, 2001); *D. alata* cv "Pico de Botella" (Rodríguez and Beltrán, 2001); and somatic embryogenesis in *D. alata* cv "Diamante 22" (Espitia and Quintero, 1999) (cited in Salazar, 2002). However, the *in vitro* cultivation of *Dioscorea* spp. presents disadvantages, such as short periods between subcultures (3 to 4 months), which as a consequence, entails increases in costs and the use of labor, affecting the genetic stability of the accessions and compromising their asepsis (Acedo and Arradoza, 2006).

Therefore, it was necessary to apply a new conservation method that would allow the subculture periods to be extended and thus prevent the loss of material and increases in costs and labor. This method is called conservation by minimum growth, which aims to directly restrict the growth and development of a plant through controlled osmotic stress generated by some components of the culture medium, such as sugars and alcohol sugars in combination with controlled environmental parameters (Neiva and Jiménez, 2010). There are conservation reports on *Dioscorea* spp., such as "*Dioscorea alata* L, "Caraqueño" clone" (Borges et al. 2009). In this study, they managed to conserve and regenerate *in vitro* plants from uninodal segments of the *D. alata* L "Caraqueño"

clone in 9 to 12 months with different concentrations of mannitol, sucrose and BAP. Studies have also been carried out on other species such as "*Dioscorea alata*, *D. rotundata*, *D. bulbifera* and *D. trifida*" (Carmona, et al. 2013), which managed to conserve four *in vitro* yam species using a combination of sucrose, mannitol and sorbitol for a period of more than six months. These methods have been successful, however, in this last research, the species responded differently to the different treatments, so each species had a medium in which it was maintained in the adequate conservation conditions during the research.

For the reasons expressed above, the objective of this work was to establish optimum culture media for the *in vitro* conservation by minimum growth of different accessions belonging to the yam germoplasm bank of the Universidad de Sucre. This was for a period of 8 months and based on the modification of the culture medium with different levels of mannitol and sorbitol, individually and combined.

Materials and Methods

Geographical Location

This research was conducted in the Laboratory of Biotechnology and Cultivation of Plant Tissue of the Universidad de Sucre, Puerta Roja Campus. This university is located in the city of Sincelejo with the geographical position in Colombia of 9° 18' north latitude and 75° 23' west longitude of the prime meridian (Ortega et al. 2011).

General Procedures and Techniques

Obtaining Plant Material

The micropropagation media were prepared for the establishment of mother plants. These consisted of M&S salts (Murashige and Skoog, 1962), sucrose 30 g/L, activated carbon 2 g/L, ANA 0.5 mg/L, BAP 4 mg/L, thiamine 1 mg/L, myoinositol 0.1 g/L, and agar 8 g/L for *Dioscorea alata*. For *Dioscorea rotundata*, media were prepared with M&S salts (Murashige and Skoog, 1962), sucrose 30 g/L, activated carbon 2 g/L, ANA 0.1 mg/L, BAP 0.3 mg/L, thiamine 1 mg/L, myoinositol 0.1 g/L, and agar 3.25 g/L.

The pH of the culture medium was adjusted to 5.8 and distributed in flasks of 182 cm³, at a ratio of 20 ml per flask. Finally, it was sterilized for 20 minutes at 15 psi and 121 °C. The media were observed for seven days before their use to rule out preliminary contamination.

Three segments were planted per flask with nodes and leaves of the donor plants from the germoplasm bank of the Plant Biotechnology Laboratory of the Universidad de Sucre. The activities to prepare

equipment, sterilize materials and culture media were implemented under aseptic conditions in a horizontal laminar flow cabinet (Astrocel®) in controlled temperature and lighting conditions.

Planting of Explants and Cultivation Conditions

Single-node (without leaves and roots), approximately 1 cm-long segments were used, which were obtained from the micropropagated mother plants at three months from their establishment. The segments were planted, according to the polarity of the plant at the ratio of one explant per flask under a horizontal laminar flow cabinet in aseptic conditions. Finally, they were placed in an incubation room at a temperature of 28 ± 5 °C, relative humidity of 65% and a photoperiod of 12 hours of light with a light intensity of 50 µmol m⁻² s⁻¹.

In Vitro Conservation

Culture Media

These consisted of M&S salts (Murashige and Skoog, 1962), activated carbon 1 g/L, ANA 0.5 mg/L, BAP 4 mg/L, thiamine 1 mg/L, myoinositol 0.1 g/L, and agar 8 g/L for *Dioscorea alata*. For *Dioscorea rotundata*, media were prepared with M&S salts (Murashige and Skoog, 1962), activated carbon 1 g/L, ANA 0.1 mg/L, BAP 0.3 mg/L, thiamine 1.0 mg/L, myoinositol 0.1 g/L, and agar 8 g/L. The medium was modified with different concentrations of sucrose (%), mannitol (%) and sorbitol (%), individually and combined. They consisted of the following treatments: T1 (3:0:0); T2 (0:1, 5:0); T3 (0:0:2); T4 (0:1, 5:2); T5 (0:0:1); T6 (0:0:3); T7 (0:1, 5:1); and T8 (0:1, 5:3). The pH of the culture medium was adjusted to 5.8, then the agar was dissolved for ten minutes on a heating plate and distributed in flasks of 182 cm³ at a ratio of 20 ml per flask. Finally, it was sterilized in an autoclave (Sterilof®) for 20 minutes at 15 psi and a temperature of 121 °C. The media were maintained for seven days (maximum) before being used to rule out any contamination of them. The media of the different treatments were planted in aseptic conditions in a horizontal laminar flow cabinet (Astrocel®).

Evaluation of Conservation

The following variables were measured every 30 days over a period of eight months.

- Length of stem (measured in centimeters with a millimeter ruler from the base of the explant to the last node).
- Length of longest root (cm).
- Number of nodes per explant.
- Number of roots.

- Percentage of expanded green leaves (number of expanded leaves / total number of leaves).
- Percentage of dead leaves (number of dead leaves / total number of leaves).
- Callus percentage (number of *in vitro* plants that generated calluses / total number of plants).
- Oxidation percentage (number of *in vitro* plants with darkening in the tissue / total number of plants). It was assessed by visual observation.
- Survival percentage (number of live *in vitro* plants / total number of plants), Estrada et al. (2009).

Design and Statistical Analysis

This research was conducted under a completely randomized design (CRD) comprised of eight treatments with three repetitions and eight replicas. Normality tests (Shapiro-Wilk) and homogeneity of variance (Bartlett) tests were applied to the data obtained, after which, the normally and homogeneously distributed variables were subjected to an analysis of variance (ANOVA), followed by Tukey's test of multiple comparison of means (α : 0.05), while the non-parametric Kruskal-Wallis test was applied to the other variables. All of the statistical analyses were processed in the R program for Windows (De Mendiburu, 2012).

Results and Discussion

Experiment 1: Effect of Mannitol and Sorbitol on the in Vitro Conservation of *Dioscorea Alata*

The results obtained in this research confirm that the use of the different osmotic agents affects *in vitro* survival of the *D. alata* species. In this sense, the individual use of mannitol in the culture medium of this species is not advisable, because it causes the death of all the explants. This is possibly due to low availability of nutrients and carbon because of the limited absorption of water by the plant caused by the reduction of the culture medium's water potential, as a result of the addition of said osmoregulator. This phenomenon has been observed by Cárdenas and Villegas (2002), who found that the use of mannitol as an osmoregulator generates more negative osmotic potentials than sorbitol (Table 1).

On the other hand, the use of sorbitol (3%) or a mannitol-sorbitol combination (1.5 - 2%), allows greater rates of survival to be achieved and therefore, it is possible to ensure the availability of this crop for those who need it. The explanation for this phenomenon is associated with sorbitol's capacity to generate less severe osmotic stress than other osmoregulators and its use as a source of energy for the explants (Table 1).

Table 1. Percentages of survival, expanded leaves, dead leaves, calluses and oxidation in *Dioscorea alata* at eight months of *in vitro* conservation.

Treatment	Survival (%)	Expanded Leaves (%)	Dead Leaves (%)	Callus (%)	Oxidation (%)
T ₁	91.07 ab	85.25 ab	5.98 abc	0.00	0.00
T ₂	0.00 c	-	-	-	-
T ₃	90.48 ab	72.06 b*	15.98 a	0.00	0.00
T ₄	100.00 a*	95.83 a	0.35 c*	0.00	0.00
T ₅	75.00 b	85.43 ab	11.65 a	0.00	0.00
T ₆	100.00 a*	88.00 ab	6.91 ab	0.00	0.00
T ₇	62.50 bc	83.47 b*	3.08 bc	0.00	0.00
T ₈	76.19 bc	82.34 b*	4.52 abc	0.00	0.00
TEST	Kruskal	Kruskal	Kruskal	Not applicable	Not applicable

Subtitle: Percentages with different letters by columns significantly differ for $p < 0.05$, where (*) is a desirable attribute for *in vitro* conservation and (-) is an absent value due to death of the explants.

T₁: Sucrose 3%	T₂: Mannitol 1.5%	T₃: Sorbitol 2%
T₄: Mannitol 1.5% + Sorbitol 2%	T₅: Sorbitol 1%	T₆: Sorbitol 3%
T₇: Mannitol 1.5% + Sorbitol 1%	T₈: Mannitol 1.5% + Sorbitol 3%	

When measuring the percentage of the expanded leaves, it was found that this variable responds differently to the treatments used with T₃, T₇ and T₈, obtaining the lowest values. Differences were also observed in leaf size. The leaves generated by culture media with sucrose and sorbitol were larger than those obtained using mannitol. This was probably due to the lack of nutrients for the development of these structures (Table 1).

Regarding leaf senescence, use of the mannitol-sorbitol combination (1.5 - 2%) is the most appropriate, because the appearance of dead leaf tissue is almost zero, possibly due to the limited development of explants growing in these conditions. Additionally, the stimulating effect of sorbitol on necrosis of the leaf tissue must be taken into account, especially when it is used individually. Similar results were obtained by Carmona et al. (2013), who found that use of the mannitol-sorbitol combination prevents leaf senescence in the *Dioscorea alata* *in vitro* plants.

On the other hand, in this research, it was found that the use of different osmoregulators does not promote oxidation, nor the appearance of unorganized cell tissue (callus) in the explant (Table 1).

As shown in Table 2, the length of the stem varies depending on the source of carbon used with the T₇ and

T₈ treatments presenting the least development. Therefore, the effect of the interaction between the mannitol and sample is shown, which causes a considerable reduction in the growth of the explant leading to "dwarf" plants, characterized by small stems and leaves, as well as nodes very close to each other. This phenomenon is the result of the interaction of the plant genotype with an inert sugar such as mannitol together with a moderately metabolizable sugar such as sorbitol. These results do not coincide with those obtained by Borges et al. (2009), who found that use of mannitol does not significantly affect the length of the stem during *in vitro* conservation of the *Dioscorea alata* "Caraqueño" clone species.

Additionally, the number of roots in this species varies depending on the osmoregulator used with the use of low concentrations of sorbitol causing the lower development of this variable, which is advisable for *in vitro* conservation, since it permits the restriction of nutrient absorption by the explant. However, when using sorbitol concentrations over 1%, individually or combined, a larger number of roots is produced.

Therefore, although the use of sorbitol together with mannitol generates a greater number of roots, these are characterized by being small, possibly because the plant needs to increase the amount of structures to absorb nutrients from the medium to supply its metabolic

Table 2. Stem length, number of roots, length of root and number of nodes in *Dioscorea alata* at eight months of *in vitro* conservation.

Treatment	Stem Length (cm)	Number of Roots	Root Length (cm)	Number of Nodes
T ₁	2.46 a	4.94 bc	6.86 a	8.24
T ₃	1.64 a	5.47 abc	3.89 b	7.52
T ₄	0.94 bc	8.63 a	3.73 b	7.78
T ₅	1.12 b	3.60 c*	3.87 b	5.78
T ₆	2.53 a	5.23 abc	5.06 ab	7.63
T ₇	0.78 c*	7.78 ab	1.45 c*	5.78
T ₈	0.75 c*	8.48 ab	1.36 c*	6.54
TEST	Kruskal	Tukey	Tukey	Tukey

Subtitle: Percentages with different letters by columns significantly differ for $p < 0.05$, where (*) is a desirable attribute for *in vitro* conservation.

T₁: Sucrose 3%	T₃: Sorbitol 2%	T₄: Mannitol 1.5% + Sorbitol 2%
T₅: Sorbitol 1%	T₆: Sorbitol 3%	T₇: Mannitol 1.5% + Sorbitol 1%
T₈: Mannitol 1.5% + Sorbitol 3%		

needs. Therefore, the explants in these treatments "prefer" to increase the number of roots even if their size is greatly reduced.

On the other hand, the results obtained show that use of different sources of carbon does not affect the formation of nodes in the *Dioscorea alata* species at eight months of *in vitro* conservation. These results coincide with those obtained by Borges et al. (2009).

Based on the above, it is possible to confirm that the *Dioscorea alata* species responds most adequately to the T₄ treatment. In other words, the combined use of mannitol-sorbitol in the culture medium permits the *in vitro* conservation of this species for eight months,

because this treatment has the highest rate of survival and the lowest percentage of leaf senescence, as well as presenting restricted development in the rest of its variables (Figure 1).

Experiment 2: Effect of Mannitol and Sorbitol on the *in Vitro* Conservation of *Dioscorea Rotundata*

The results obtained in this test show that the rate of survival in the *Dioscorea rotundata* species is affected by the use of different osmoregulators, with the T₁, T₃, T₄ and T₇ treatments presenting the greatest values and therefore, considered appropriate for the *in vitro* conservation of this species. Furthermore, the individual



Figure 1. Aspect of the *Dioscorea alata* plants at eight months of *in vitro* conservation. a) Plants in medium of 3% sucrose (bar = 8.5 cm). b) Plants in mannitol-sorbitol medium (1.5-2%) (bar= 8.5 cm).

use of mannitol in the culture medium is inadequate, because the rate of survival in these conditions is too low. These results do not coincide with those reported by Carmona et al. (2013), who found that the rate of survival in the *Dioscorea rotundata* species is not affected by the use of different osmotic regulators, especially when using mannitol. A possible explanation for this phenomenon may be related to the genotype of the plant material used, since they belong to different cultivars.

Additionally, the results obtained show that the percentage of expanded and dead leaves also varied with respect to the type of carbon source used. Therefore, the use of the mannitol-sorbitol combination generates reduced values of this variable, thus showing a delay in the growth and development of the explants.

Likewise, the evaluation of leave senescence in the plants indicated that the mannitol-sorbitol combinations are adequate for *in vitro* conservation, provided that high concentrations of sorbitol are not used (above 2%), because in both tests, the presence of sorbitol is usually associated with high percentages of leaf senescence. Similar results were obtained by Carmona, et al. (2013).

With regard to the oxidation and callus percentages, similar results were obtained to those presented by *Dioscorea alata*. In other words, there was not a significant effect on the appearance of these characteristics when using different sources of carbon.

The results obtained show that reduction in the growth of the stem may be achieved through the use of the mannitol-sorbitol combination or the application of sorbitol in concentrations over 1%. Likewise, it is possible to achieve a considerable reduction in the formation and elongation of roots through the use of said combination. Therefore, these conditions are advisable for the *in vitro* conservation of this species, that is the T₄ and T₇ treatments. Additionally, the results indicate that there is not a significant effect on the formation of nodes on *in vitro* plants when using different osmoregulators in the culture medium.

Finally, taking into account the previously mentioned proposals, it is possible to confirm that the T₇ and T₄ treatments are the most adequate for the *in vitro* conservation of the *Dioscorea rotundata* species, as they allow their growth and development to be significantly reduced in terms of the variables of stem and root length, as well as the number of roots, maintaining high rates of survival and a minimum percentage of leaf senescence (Figure 2).

Table 3. Percentages of survival, expanded leaves, dead leaves, calluses and oxidation in *Dioscorea rotundata* at eight months of *in vitro* conservation.

Treatment	Survival (%)	Expanded Leaves (%)	Dead Leaves (%)	Callus (%)	Oxidation (%)
T ₁	100.00 a*	94.95 ab	1.39 bc*	4.17	0.00
T ₂	33.33 c	83.83 abc	0.00 c	0.00	0.00
T ₃	100.00 a*	93.70 ab	1.53 bc*	0.00	0.00
T ₄	100.00 a*	75.70 bc	1.04 bc*	0.00	0.00
T ₅	86.31 b	8.06 a	0.40 bc*	0.00	0.00
T ₆	91.67 ab	79.50 bc	6.14 a	0.00	0.00
T ₇	100.00 a*	69.53 c*	2.49 ab	0.00	0.00
T ₈	95.83 ab	72.60 c*	0.55 bc*	0.00	0.00
TEST	Kruskal	Kruskal	Kruskal	Kruskal	Not applicable

Subtitle: Percentages with different letters by columns significantly differ for $p < 0.05$, where (*) is a desirable attribute for *in vitro* conservation.

T₁: Sucrose 3%	T₂: Mannitol 1.5%	T₃: Sorbitol 2%
T₄: Mannitol 1.5% + Sorbitol 2%	T₅: Sorbitol 1%	T₆: Sorbitol 3%
T₇: Mannitol 1.5% + Sorbitol 1%	T₈: Mannitol 1.5% + Sorbitol 3%	

Table 4. Stem length, number of roots, root length and number of nodes in *Dioscorea rotundata* at eight months of *in vitro* conservation.

Treatment	Stem Length (cm)	Number of Roots	Root Length (cm)	Number of Nodes
T ₁	2.15 a	5.37 a	6.40 a	5.75
T ₃	1.17 ab*	1.67 bc	1.48 c	4.51
T ₄	1.24 ab*	1.45 cd*	1.39 c	5.49
T ₅	2.17 a	2.37 ab	2.40 ab	5.96
T ₆	1.84 ab*	2.26 ab	2.28 b	6.36
T ₇	1.17 ab*	1.33 cd*	0.70 d*	4.65
T ₈	1.10 ab*	1.92 bc	1.69 c	5.24
TEST	Tukey	Kruskal	Kruskal	Tukey

Subtitle: Percentages with different letters by columns significantly differ for $p < 0.05$, where (*) is a desirable attribute for *in vitro* conservation.

T₁: Sucrose 3%	T₂: Mannitol 1.5%	T₃: Sorbitol 2%
T₄: Mannitol 1.5% + Sorbitol 2%	T₅: Sorbitol 1%	T₆: Sorbitol 3%
T₇: Mannitol 1.5% + Sorbitol 1%	T₈: Mannitol 1.5% + Sorbitol 3%	

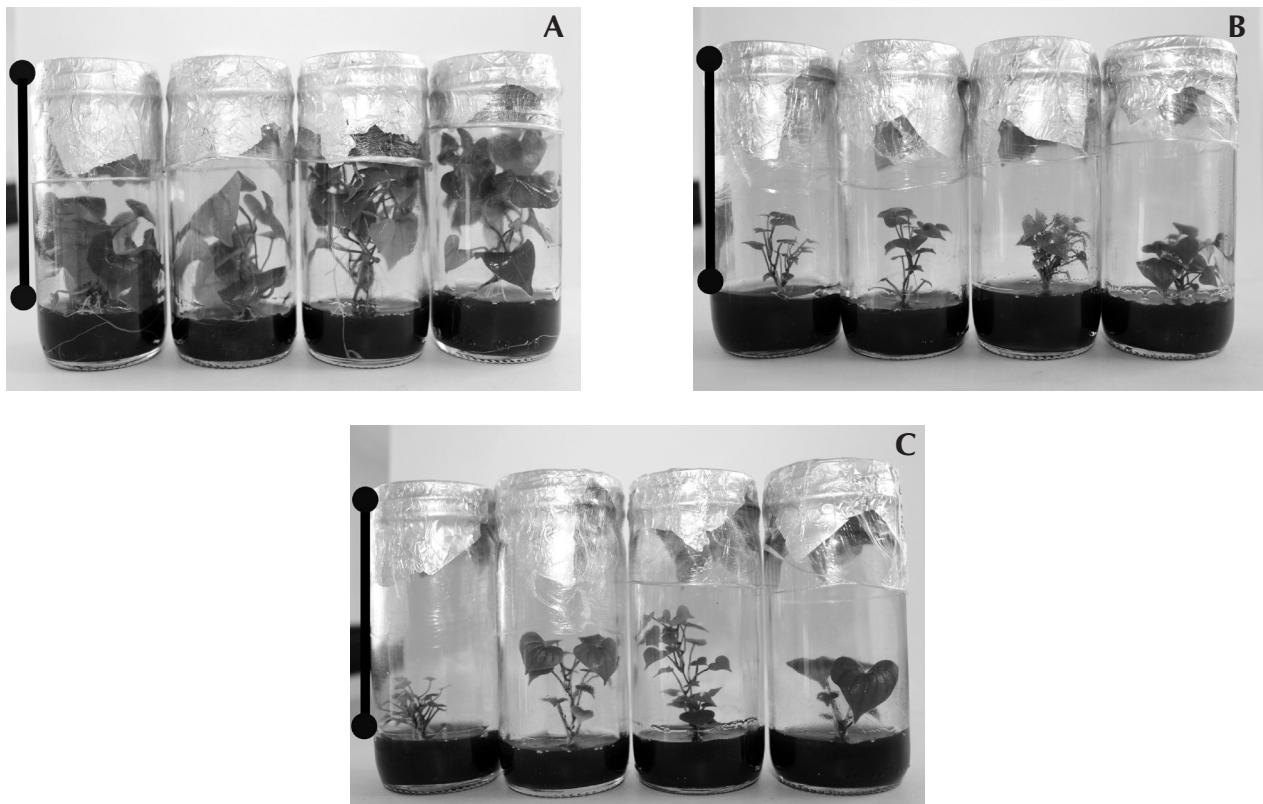


Figure 2. Aspect of the *Dioscorea rotundata* plants at eight months of *in vitro* conservation. a) Plants in medium of 3% sucrose (bar = 8.5 cm). b) Plants in mannitol-sorbitol medium (1.5-2%) (bar= 8.5 cm). c) Plants in mannitol-sorbitol medium (1.5-1%) (bar= 8.5 cm)

Conclusion

Finally, we can conclude that the medium comprised of salts (M&S) + mannitol 15 g/L + sorbitol 20 g/L + activated carbon 2 g/L + thiamine 1 mg/L + myoinositol 0.1 g/L + agar 8 g/L allows the effective conservation of *in vitro* plants from single-node segments of *Dioscorea alata* supplemented with 4 mg/L of BAP and 0.5 mg/L of ANA, and *Dioscorea rotundata* supplemented with 0.3 mg/L of BAP and 0.1 mg/L of ANA, for eight months with high survival percentages and low percentages of leaf senescence, showing restricted development of *in vitro* plants.

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