New Approaches for Micropropagation and Cryopreservation of Agave Peacockii, An Endangered Species

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Abstract

More than 50% of Agave species are endemic to Mexico. Among them, *Agave peacockii* is listed within the list of threatened species that require special protection. In this work, we aimed at developing new supplementary strategies to achieve micropropagation and perform cryopreservation of *in vitro*-grown shoot-tips of *A. peacockii*. For multiplication, the addition of two cytokinins, 6-benzylaminopurine (26.6 µM) and kinetin (27.84 µM) to MS semisolid medium significantly favoured the morphogenetic response and produced the highest shoot generation (87.00±17.18) after 60 d of culture. This interaction was more effective than using the same growth regulators separately. Propagated and rooted plantlets were successfully acclimated with 100% survival and a normal morphological development during greenhouse performance. For cryopreservation, an optimized protocol following droplet-vitrification approach allowed obtaining 98% and 96% regrowth before and after cryopreservation, respectively. Shoot-tips were excised of *in vitro*-propagated plants, subjected to preculture on MS semisolid medium with 0.3 M sucrose for 1d, loaded in solution with 0.4 M sucrose and 1.6 M glycerol for 20 min, exposed to vitrification solution PVS2 for 15 min, and then, immersed in liquid nitrogen in droplets of PVS2 placed on aluminium foil strips. The vegetative growth of cryo-derived plants and of the *in vitro* propagated plants was compared under greenhouse culture conditions. No significant differences were detected in most assessed characteristics after 120 d of acclimatization. The results presented here constitute new viable biotechnological approaches for the *in vitro* propagation and long-term conservation of endangered *Agave* germplasm.

Key Message

Agave peacockii shoot micropropagation was induced combining 6-benzylaminopurine and 6-furfuryl-aminopurine. A droplet-vitrification protocol was optimized to cryopreserve shoot-tips. Greenhouse performance of *in vitro* and cryo-derived plants was similar.

Introduction

The *Agave* genus plays an essential role in the ecology, culture, and commercial production of Mexico. The characteristics of this taxon are outstanding due to the anthropogenic use that has been recorded for more than 10,000 years (Zizumbo-Villarreal et al. 2013). Since then, agave plants have been employed to obtain different products with various purposes, such as food, traditional and commercial distilled and fermented beverages, as well as for other uses like forage, extraction of sapogenins, biofuels and utilization of waste (Pérez-Zavala et al. 2020).

Thinking of *Agave* spp. is thinking of Mexico, because 75% out of all species belonging to the *Asparagaceae* family are found in Mexico and of them, 55% are considered endemic (García-Mendoza et al. 2019a). Among the endemic species, *A. peacockii* was referred by Gentry (1982) as a "rare" species, emphasizing the needing to include it under special protection. Currently, this species of agave occurs in the Tehuacán-Cuicatlán Biosphere Reserve of Mexico and is listed in NOM-059-SEMARNAT-2010 as "Special Protection" (Diario Oficial de la Federación, 2019) and in the Red List of Threatened Species™ (García-Mendoza et al. 2019b), due to its relatively little distribution and extent of occurrence (14,670 km2). In general, *A. peacockii* faces a problem in common with various endemic species in Mexico, which are limited in the wild due to the degradation of their natural populations, besides their non-sustainable use.

Significant progress has been made in using biotechnological tools such as the *in vitro* techniques for collecting, multiplying, and conserving endangered and rare plant species (Cruz-Cruz et al. 2013). Tissue culture methods have led to high propagation rates through shoot organogenesis or somatic embryogenesis as morphogenic pathways (Sarasan et al. 2006). In the case of *Agave* spp., efficient micropropagation protocols have been developed using different growth regulators in the culture medium like 6-benzylaminopurine (BAP) (Domínguez et al. 2008), 6-furfuryl-aminopurine (kinetin-KIN) (Aureoles-Rodríguez et al. 2008), combined or not with low concentrations of auxins such as indole-3-acetic acid (IAA) (Ríos-Ramírez et al. 2017; Aguilar and Rodríguez, 2018), or acid 2,4-dichlorophenoxyacetic (2,4-D) (Santacruz-Ruvalcaba et al. 1999; Álvarez-Aragón et al. 2020). Nevertheless, mass production of shoots is a heterogeneous response that depends on each *Agave* species, the type of explant, and the growth regulators used (Lecona-Guzmán and Reyes-Zambrano, 2017).

In vitro approaches have also been studied for medium- (slow growth) and long-term (cryopreservation) storage of *Agave* germplasm. Using mannitol or sorbitol at 50 g L^{-1} slow growth rate of shoots was induced, which allowed extending the lapses between subcultures up to 10 months without affecting plant regeneration capacity of various threatened agave species, including *A. peacockii* (Pérez-Molphe-Balch et al. 2012). On the other hand, cryopreservation of *A. sobria* apical meristems using the droplet-vitrification technique (Tin and Folgado, 2019) and *A. tequilana* somatic embryos cv. 'Chato' using the V-cryoplate method (Delgado-Aceves et al. 2021) was successfully achieved. They provided new complementary strategies based on the total arrest of metabolic activity and cell division, eliminating the need to perform any subculture.

Nevertheless, endangered plant species may require new methodological modifications to previously reported protocols. These adjustments may be necessary to improve the adaptation and effectiveness of procedures for *in vitro* culture and long-term germplasm conservation. In addition, they will depend on growth requirements and the response of tolerance to cryopreservation.

This work aimed at developing functional and practical protocols for shoot micropropagation and cryopreservation of shoot-tips of *A. peacockii*, optimizing both procedures and comparing their effects on several characteristics during the vegetative growth of plants derived from both, *in vitro* propagated shoots and from cryopreserved shoot-tips during greenhouse culture.

Materials And Methods

In vitro establishment and propagation

The biological material used in the experiments was donated by the Ethnobotanical Garden of Oaxaca, Mexico. The specimens provided were 2-year-old 20-30 cm long rhizomatous shoots of *A. peacockii*.

For *in vitro* establishment, the rhizomatous shoots were cleaned, rinsed with running water, and defoliated until the last fused leaves were left, cutting the root. Subsequently, they were treated with 46% (v / v) systemic fungicide mefenoxam (RidomilGold®) under continuous stirring for 30 min, and then, rinsed three times with distilled water. Afterwards, rhizomatous shoots were immersed in 50:50 (v / v) chlorine solution and stirred for 10 min, followed by washing three times with sterile distilled water. All oxidized tissues were carefully removed from shoots before being cultured on semisolid basal MS medium (Murashige and Skoog, 1962), supplemented with vitamins L2 (Phillips and Collins 1979) and 5 $mg{\cdot}L^{-1}$ of 6-benzylaminopurine (BAP) for shoot stimulation.

For micropropagation, *in vitro* generated shoots with 2 cm in length and three developing leaves were selected and cultivated on MS semisolid basal medium with different combinations of plant growth regulators: 0.00, 4.43, 13.13 or 26.6 µM 6-benzylaminopurine (BAP) and/or 0.00, 4.6, 13.92 or 27.84 µM kinetin (KIN). Culture medium devoid of regulators was used as control. All media contained 3% (w / v) sucrose and 0.8% (w / v) agar. The pH was adjusted to 5.8 ± 0.02 and media sterilized in an autoclave at 121 ° C for 15 min at a pressure of 1.3 kg cm^{-2}.

Cultures were kept at 25 ± 2 ° C exposed to light intensity of about 27 µmol $m^{-2}s^{-1}$ under a 16/8 h photoperiod (light/dark). The total obtained number of shoots per explant was recorded after 60 d of culture. Six replicates were used per each culture medium studied.

Rooting and acclimatization

Five replications per culture medium of multiplied shoots 4-5 cm long were inoculated for root induction. Rooting was carried out using MS semisolid medium modified by reducing NH_4NO_3 concentration to 5 mM (Castro-Concha et al. 1990) and adding 4.92, 14.76, or 29.25 µM indole-3-butyric acid (IBA) without or with 3 % or 6 % (w / v) sucrose. All culture media were solidified with 0.8% (w/v) agar. Medium devoid of sucrose and regulators were used as control. The number and length of roots were recorded after 60 d of culture. Root thickness during *in vitro* rooting development was also evaluated.

Plantlets with well-developed roots were removed from glass jars, gently washed by taking care not to damage the root system, and then, transferred to 60 cavity seed tray containing sterile peat moss and perlite (6:4). *Ex vitro* cultures were placed under full sun in greenhouse conditions (25 ± 2°C /75% relative humidity) and the irrigation was performed by capillarity using 20% liquid basal MS medium for 120 d. During acclimatization, survival (%) was evaluated by counting the number of living plants after 60 d of culture. The following variables: number of formed roots, root length (cm), root dry weight (mg), number of leaves and total leaf area were evaluated after 120 d of acclimatization in 10 randomly selected plants. The foliar area was calculated according to Montgomery (1911) using the following formula:

Foliar area = (leaf length x maximum leaf width) (0.75)

Analytical Sartorius® balance model BL 2105 (Gottingen, DE Germany) was used to determine the fresh and dry weights. Drying was performed at 60 ° C for 72 h wrapping the roots in aluminum foils and using an oven NOVATECH® model HS35-AIA (Jalisco, Mexico). To measure the length, a graduated ruler of 60 cm with a precision of 1.0 mm was used.

Cryopreservation of apical meristems by droplet-vitrification

In vitro plants regenerated during shoot multiplication experiments were grown on MS semisolid basal medium and kept at the same incubation conditions previously described. Apical shoot-tips (meristematic dome with one leaf primordia, 1 mm in length × 1 mm wide) were aseptically dissected from two-month-old *in vitro* cultures used as donor-plantlets (ten explants by repetition and three replicates). After excision, shoot tips were transferred to preculture MS semisolid medium supplemented with 0.3 M sucrose for approximately 3h (until completing the dissection process) or remained on the same preculture medium for 1d before being subjected to successive steps involved in the droplet-vitrification procedure. Sucrose-preculture for 1d was also applied to batches of shoot-tips dissected from donor-plantlets previously preconditioned on MS semisolid medium with 0.3 M sucrose for 15 d.

For cryopreservation experiments, a modified droplet-vitrification protocol to that described by Tin and Folgado (2019) for *A. sobria* shoot-tips was applied. After preculture, shoot-tips were treated with a loading solution containing MS basal medium with 0.4 M sucrose and 1.6 M glycerol for 20 min in the dark, and then exposed to the vitrification solution PVS2 (30% v/v glycerol, 15% v/v dimethyl sulfoxide, 15% v/v ethylene glycol, and 13.7% w/v sucrose) (Sakai et al. 1990), previously stored in the refrigerator and used to induce osmotic dehydration before liquid nitrogen immersion. A preliminary assessment to verify the tolerance of shoot-tips to dehydration with PVS2 was performed. Shoot-tips precultured with 0.3 M sucrose for 1d were loaded for 20 min, and then, exposed to pre-chilled PVS2 solution for different exposure times: 15, 30, 45, 60, 75, 90, and 120 min. Survival (%) was evaluated for each PVS2 exposure time 15 d after reculture by counting the shoot-tips that remained yellow-green color and started displaying a growth response. Reculture for 15 d took place first, using a MS semisolid medium with 0.3 M sucrose for 2 d, and then, MS semisolid medium with 0.44 µM BA.

Based on the optimization of exposure time to PVS2, shoot-tips precultured with sucrose for 3h and those precultured for 1 d derived or not of previously preconditioned plantlets, were loaded for 20 min and dehydrated with cold PVS2 solution for 15 min. About 5 min before finishing the dehydration treatment, PVS2 droplets were placed over the shoot-tips before being transferred to sterile aluminum foil strips (0.5 × 2.0 cm), and then, foils were placed in 2 mL cryovials prefilled with liquid nitrogen (LN). The cryovials were stored in LN for 30 min. For rewarming, the aluminum strips with samples were removed from the cryovials and rapidly immersed in an unloading solution (MS basal medium with 1.2 M sucrose, pH 5.8) for 20 min in the dark. After cryopreservation, shoot-tips were transferred to Petri dishes (5 cm diameter) containing MS semisolid medium with 0.3 M sucrose for 2 d, followed by the reculture on semisolid MS basal medium with 0.44 µM BAP until 30 d. All samples were always kept in the dark during the first week for recovery. Regrowth of shoot-tips was expressed as the percentage of explants that exhibited elongation of meristematic dome and developed leaves after 15 d of post-cryopreservation recovery. The new shoots were rooted onto MS semisolid medium with reduced NH_4NO_3 content and devoid of hormones for 60 d. Acclimatization of plantlets (4 cm long) with well-developed roots was performed under the same conditions described above. Survival (%) of acclimated cultures was also evaluated after 60 d. The same variables: number, length, and dry weight of roots, number of leaves, and total leaf area of plants derived from cryopreserved shoot-tips were recorded as previously described and compared with those of the propagated non-cryopreserved plants after 120 d of acclimatization.

Histological analysis

Fresh roots were embedded in polyethylene glycol (PEG) 1450 M mass in a 1:4 proportion (PEG: deionized water), according to Burger and Richter (1991). A rotatory microtome was used to obtain 10 µm sections from the samples in PEG; then, they were stained with a double treatment using safranin 0.5% (1:1 w/v) and 0.5% astral blue (1:1 w/v). A light microscope was used to analyze the tissues. Six replicates were taken per treatment showed in rooting experiment.

Statistical analysis

Experiments were performed using a completely randomized design. Quantitative data are presented as the mean ± Standard Error (SE), and the differences among means were determined by one-way analysis of variance (ANOVA) following Fisher's Least Significant Difference (LSD) test ($p \geq 0.05$). All statistical analyses were carried out using the Minitab® statistical software 17.2.1.

Results And Discussion

In vitro establishment and propagation

In vitro establishment and propagation of *A. peacockii* were successfully achieved using rhizomatous shoots as starting explant. The significantly ($p \geq 0.05$) higher number (87.00±17.18) of shoot generation was obtained by the interaction of the highest concentrations of both hormones BAP (26.6 µM) and KIN (27.84 µM) added to MS basal culture medium. On the other hand, root formation in the new proliferated shoots was only detected when the media without growth regulator or supplemented with KIN were used (Table 1).

The effect of cytokinins alone or in combination with auxins has been previously tested for micropropagation of different *Agave* species as *A. cantala, A. fourcroydes* and *A. sisalana* (Binh et al. 1990); *A. parrasana* (Santacruz-Ruvalcaba et al. 1999); *A. inaequidens* (Aureoles-Rodríguez et al. 2008); *A. cupreata, A. difformis, A. karwinskii, A. obscura and A. potatorum* (Dominguez et al. 2008); *A. marmorata* (Aguilar and Rodríguez, 2018). However, until now, the use of only two cytokinins has never been reported for the same purpose in *Agave*. It is well known that each species responds differently to growth regulators used as supplements (Domínguez et al. 2008). Based on the auxin-cytokinin interactions, Garriga et al. (2010) found that combining Thidiazuron with BAP and IBA in the MS basal medium allowed improving shoot multiplication percentage in *A. fourcroydes*. Another efficient micropropagation protocol was achieved in *A. americana* via indirect organogenesis using 2,4-D and BAP (Lara-Hidalgo et al. 2017). According to Pérez-Molphe-Balch et al. (2012), *A. peacockii* has shown a higher shoot production rate (up to 17.1 shoots per explant) than other species in the medium containing 0.5 µM BAP. However, in the present study, we have remarkably increased the results by combining BAP and KIN to generate multiple shoots proliferation (87.00±12.93). During our experiments, the combined effect of these two cytokinins significantly improved the morphogenetic response compared to those obtained by only using BAP or KIN in the culture medium. Figure 1 (a to d) illustrates different stages of the micropropagation process of *A. peacockii* shoots.

Rooting and acclimatization

The rooting medium with 3% sucrose and supplemented with the highest concentration (29.25 µM) of IBA, allowed obtaining the significantly ($p \geq 0.05$) higher (8.6±1.01) number of formed roots (Table 2). *In vitro* developed roots were classified as thick, a common characteristic detected in the roots formed on any IBA-supplemented media. By contrast, longer (up to 20 cm in length) and thinner roots were obtained only using sucrose as an additive (Figure 2).

The observed differences in thickness during the radicular development led to further investigations to determine roots' anatomy and cellular structure. The histological analysis showed that thin roots did not present a well-organization regarding epidermis, cortex, endodermis, pericycle and secondary root formation, unlike what was observed in the thick roots' structural organization after the same culture time.

Nevertheless, regardless of the anatomical characteristics of roots, all rooted plantlets were successfully acclimated with 100% survival and normal morphological development. After 120 d of acclimatization, a more remarkable evolution of both the aerial part and root system was observed in those plants previously rooted in culture media with IBA and sucrose than in plants rooted in sucrose-free media (Table 3). It is well known that sugars usually serve as carbon source and energy during *in vitro* culture, but they can also change the water potential of medium (Lipavská and Vreugdenhil,1996). This might explain the experimented stimulus in root elongation by the effect of this component, which, combined with IBA, accelerated the early vegetative growth during the greenhouse performance of plants.

Cryopreservation of apical meristems by Droplet-Vitrification

The preliminary assessment of the critical osmoprotective step associated to dehydration with the vitrification solution PVS2, demonstrated a decrease in regrowth of agave shoot-tips from 100 % at 15 min down to 60% after 90 min of exposure at 25 °C (Figure 3). The detrimental effect was progressively detected by the increase of the exposure time longer than 30 min. Therefore, treatment for 15 min was selected as the best condition to dehydrate *A. peacockii* shoot-tips prior to cryopreservation.

Once the best dehydration time was determined with PVS2, the following cryoprotective experiments focused on optimizing the preculture duration of shoot-tips on a semisolid medium with 0.3 M sucrose. Before liquid nitrogen immersion, results revealed no significant differences in regrowth whatever the preculture time (3 h or 1 d) used or whether the mother plantlets from which shoot-tips were isolated had been pretreated with sucrose for 15 d before dissection. However, 1d of preculture resulted in the most effective treatment in both stages, before (-LN) and after (+LN) cryopreservation, because it allowed obtaining significantly higher regrowth (98% and 96%, respectively) without the 15 d-preconditioning of the donor-plantlets (Figure 4). Therefore, following the droplet-vitrification procedure, the best protocol for cryopreservation of *A. peacockii* shoot tips involved: 1d-preculture on semisolid medium with 0.3 M sucrose, treatment for 20 min in loading solution containing MS medium with 0.4 M sucrose and 1.6 M glycerol, exposure to cold vitrification solution PVS2 for 15 min and then, ultra-rapid cooling and warming. Recovery and regrowth of shoot-tips after cryopreservation using the optimized protocol is presented in Figure 5.

The cryogenic protocol defined for *A. peacockii* shoot tips included modifications that simplified and shortened the process's duration compared to the first report on cryopreservation of *A. sobria* shoot-tips (Tin and Folgado, 2019). The highest (87%) post-cryopreservation regrowth of *A. sobria* shoot-tips was achieved by pretreating donor-plantlets for 15 d in medium enriched with 0.3 M sucrose. By contrast, the highest (96%) post-cryopreservation regrowth of *A. peacockii* shoot tips was achieved using 1d-preculture of shoot-tips on semisolid medium with 0.3 M sucrose and without requiring the pretreatment of donor plants. Therefore, 1d-preculture of shoot-tips on semisolid medium resulted more effective than extended pretreatment of donor plants at the same concentration of sucrose and replaced the use of liquid medium with 20 mg L^{-1} of ascorbic acid during dissection as Tin and Folgado (2019) previously reported.

Preculture for 1d on semisolid medium supplemented with 0.3M sucrose is usually the most applied pretreatment of any cryopreservation procedure for organized tissues like shoot-tips (Gonzalez-Arnao et al., 2014). This conditioning step also allows explants to recover from the dissection stress and, followed by a loading treatment in a sucrose-glycerol solution, has proved to enhance the acquisition of tolerance to dehydration with PVS2 (Sakai and Engelmann, 2007). Therefore, this combination's beneficial effect has led to increased post-cryopreservation survival of shoot-tips subjected to different vitrification-based procedures (Takagi et al. 1997; Matsumoto et al. 2014; Valle Arizaga et al. 2017). Another modification of the first reported protocol (Tin and Folgado 2019) was performing dehydration at room temperature using a pre-chilled solution of PVS2, instead of dehydration at 0°C placing the samples on ice.

We can assume that *Agave* spp., have a high tolerance to cryopreservation, since with the two species (*A. sobria* and *A. peacockii*) tested until now, post-cryopreservation results have exceeded 80% in the first case (Tin and Folgado, 2019), and 90% in our studies. Different protocols following droplet-vitrification approach have been successfully applied to cryopreserve organized structures of several endemic and endangered plant species such as adventitious shoot-tips of *Paraisometrum mileense* (Lin et al. 2014); shoot-tips of *Castilleja levisecta* Greenm (Salama et al. 2018); shoot apices and axillary buds of *Dianthus* taxa (Halmagyi et al. 2020); shoot-tips of *Pogostemon yatabeanus* (Lee at al. 2021).

A process of cryopreservation imposes several stressful conditions which can affect survival and potentially alter genetic stability (Harding, 2004), therefore, optimizing the composition of the post-cryopreservation reculture medium is also essential to prevent induction of somaclonal variation. In this sense, cryopreserved shoot tips were firstly recovered on medium with low (0.44 µM) concentration of BAP for 30d, and then, transferred and maintained in culture using a modified (NH_4NO_3 reduced to 5 mM) MS semisolid medium with 3% sucrose and devoid of growth regulators. After 60 d of culture, the new plantlets spontaneously developed roots and were transferred to greenhouse culture conditions. After 120 d of acclimatization, the features (number, length, dry weight of roots, leaves number, and total leaf area) compared between plants derived from cryopreserved shoot-tips and propagated non-cryopreserved plants are summarized in Table 4.

Evaluation of vegetative growth of greenhouse-grown plants showed no significant differences in four out of five compared characteristics between plants regenerated from micropropagated shoots and the plants obtained after cryopreservation of shoot-tips. Therefore, the general analysis indicates no significant effect of cryopreservation altering the vegetative growth of agave plants. Our results agree with other authors' reports using droplet-vitrification with PVS2 to cryopreserve shoot tips of different plant species. Zhang et al. (2015) found that root formation of *Argyranthemum* during an early stage of greenhouse-performance was less in cryo-derived plants than in plants regenerated after micropropagation; however, this initial difference in rooting performance did not influence the genetic stability and other morphological characteristics during the further development of plants. On the other hand, no significant differences were detected in the vegetative growth of *in vitro* and cryo-derived plants of *Actinidia* spp. under greenhouse culture conditions (Zhang et al. 2020). In this regard, Pawłowska et al. (2019) found that cryopreservation of wild rose shoot-tips did not have any adverse effect on biochemical attributes or pollen characteristics in field-grown plants.

Field or greenhouse performances are critical to validate the success of a micropropagation process and the effectiveness of a cryopreservation protocol. The results presented here provide new verified biotechnological approaches up to greenhouse culture conditions. Their implementation will allow further signs of progress addressed to the propagation and safe conservation of plant germplasm, besides supporting new research on *Agave* spp.

Conclusion

In this study, we reported a new alternative for successful micropropagation of *Agave* using *A. peacockii* as a study case, an endemic and vulnerable species from Oaxaca, Mexico. The significantly higher generation of new shoots was obtained by supplementing MS semisolid medium with BAP and KIN. The interaction of these two cytokinins induced a significant stimulatory effect on the morphogenic response and provided an effective method for large-scale propagation, which can also be helpful for other *Agave* species. The

histological analysis during rooting of micropropagated plants allowed the anatomical characterization of roots. The structural organization level was proportional to the thickness of formed roots and did not affect survival or the morphological development of plants during greenhouse performance.

A modified droplet-vitrification protocol was successfully optimized for cryopreservation to guarantee efficient cryogenic storage of agave shoot-tips. No significant differences were detected in most assessed characteristics when compared the vegetative growth of *in vitro* and cryo-derived plants under greenhouse culture conditions. Therefore, the results presented here represent new potential biotechnological strategies for the *in vitro* propagation and long-term conservation of *Agave* germplasm.

Abbreviations

KIN 6-furfuryl-aminopurine (kinetin)

BAP 6-benzylaminopurine

ANOVA Analysis of variance

PEG polyethylene glycol

LN Liquid nitrogen

LSD Least significant difference

L2 Vitamins Phillips and Collins (1979)

MS Medium Murashige and Skoog (1962)

PVS Plant vitrification solution

2, 4-D 2, 4-dichlorophenoxyacetic acid

IBA indole-3-butyric acid

Declarations

Authors' contributions

L-DA and MT-GA wrote and reviewed the manuscript. L-DA, LP and FJ-RP designed and performed the shoot generation experiments. L-DA and LP conducted histological cuts, rooting and acclimatization experiments. L-DA, FJ-RP and RF developed the cryopreservation experiments. LD and MT-GA conducted the statistical analysis. All the authors read and approved the manuscript.

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Tables

Table 1. Effect of different concentrations of BAP and/or KIN on the *in vitro* generation of new shoots and roots from *Agave peacockii* shoots after 60 d of culture.

Concentration of growth regulators (µM)		Number of shoots proliferation (mean ± SE) *		Number of roots in shoot (mean ± SE) *	
BAP	KIN				
0.00	0.00	0.83±1.32	f	2.33±1.86	a
4.43	0.00	33.00±11.52	d	-	
13.13	0.00	56.00±18.38	bc	-	
26.6	0.00	60.66±16.77	b	-	
0.00	4.64	1.66±1.50	f	4.00±1.89	a
4.43	4.64	41.33±8.98	cd	-	
13.13	4.64	28.00±15.04	de	-	
26.6	4.64	58.83±14.63	bc	-	
0.00	13.92	12.83±3.43	ef	2.5±1.64	a
4.43	13.92	52.16±17.87	bc	-	
13.13	13.92	53.50±18.98	bc	-	
26.6	13.92	60.83±13.93	b	-	
0.00	27.84	13.6±9.11	ef	4.10±2.56	a
4.43	27.84	45.00±17.01	bcd	-	
13.13	27.84	45.16±15.19	bcd	-	
26.6	27.84	87.00±12.93	a	-	

∗Values represent means ± standard errors from six replicates.

* Different letters within the columns are significantly different according to the LSD test (p ≥0.05). (-) No roots

Table 2. Effect of different concentrations of IBA and/or sucrose in the rooting medium of *A. peacockii* plantlets after 60 d of culture.

Treatment	Factors		Number of roots		Root length (cm)		Characteristic of roots
	IBA (µM)	Sucrose %	(mean ± SE) *		(mean ± SE) *		
1	0	0	1.2±0.40	g	10.62±2.94	defg	thin
2	4.92	0	1.8±0.40	fg	9.98±2.98	efg	thick
3	14.76	0	2.8±1.16	ef	8.58±1.46	g	thick
4	29.25	0	4.4±1.01	cd	8.94±4.90	fg	thick
5	0	3	2.2±0.74	fg	18.8±1.86	ab	thin
6	4.92	3	6.6±1.49	b	13.32±0.81	cde	thick
7	14.76	3	5.8±0.74	bc	12.72±1.89	cdef	thick
8	29.25	3	8.6±1.01	a	11.46±2.58	defg	thick
9	0	6	4.0±1.09	de	20.52±3.02	a	thin
10	4.92	6	4.4±1.01	cd	15.86±2.80	bc	thick
11	14.76	6	6.6±0.80	b	14.26±3.80	cd	thick
12	29.25	6	6.8±2.22	b	12.98±0.87	cde	thick

Values represent means ± standard errors of five replicates.

* Different letters within the columns are significantly different according to the LSD test ($p \geq 0.05$).

Table 3. Effect of rooting culture medium on the vegetative development of *Agave peacockii* plants after 120 d of acclimation.

Treatment	Factors		Number of roots		Root length (cm)		Root dry weight (mg)		Leaves number		Total leaf area	
	IBA (µM)	Sucrose %	(mean ± SE) *		*		*		*		*	
1	0	0	5.0±1.58	de	6.06±2.57	d	0.0208±0.01	f	5.0±1.00	f	3.40±1.25	f
2	4.92	0	5.0±1.58	de	8.42±3.55	cd	0.0364±0.01	f	5.4±1.34	ef	4.12±1.08	ef
3	14.76	0	5.0±0.70	de	12.52±6.17	bc	0.0586±0.02	ef	6.2±0.44	cde	5.19±1.25	def
4	29.25	0	4.2±2.16	e	6.42±5.76	d	0.0328±0.01	f	5.0±1.58	f	4.67±3.14	def
5	0	3	6.4±1.14	cd	13.8±6.04	bc	0.0714±0.02	def	6.0±0.70	def	6.33±1.83	cde
6	4.92	3	9.8±1.30	a	17.16±2.82	ab	0.1827±0.05	a	7.8±0.44	a	8.73±1.58	abc
7	14.76	3	7.8±0.83	bc	20.2±4.36	a	0.1580±0.04	ab	7.2±0.44	abc	9.16±1.56	ab
8	29.25	3	8.0±1.22	bc	16.12±2.34	ab	0.1514±.05	abc	7.0±0.70	abcd	9.40±2.2	ab
9	0	6	7.2±1.09	bc	17.04±3.14	ab	0.1553±0.07	ab	7.4±0.54	ab	9.27±2.15	ab
10	4.92	6	7.2±0.83	bc	17.12±3.44	ab	0.1213±0.04	bcd	7.8±0.44	a	8.29±2.52	abc
11	14.76	6	8.4±1.94	ab	17.86±2.92	ab	0.1375±0.05	abc	7.6±0.54	ab	9.61±2.55	a
12	29.25	6	6.6±0.89	cd	16.5±5.14	ab	0.0982±0.04	cde	6.6±0.89	bcd	6.96±0.96	bcd

Values represent means ± standard errors of five replicates.

* Different letters within the columns are significantly different according to the LSD test (p ≥0.05).

Table 4. Comparison of root development and of vegetative growth between micropropagated non-cryopreserved (-LN) plants and plants derived of cryopreserved (+LN) *A. peacockii* shoot tips after 120 d of acclimation.

Treatment	Number of roots		Root length (cm)		Root dry weight (mg)		Leaves number		Total leaf area	
-LN	6.4±1.14	a	13.8±6.04	a	0.0714±0.02	a	6.0±0.70	a	6.33±1.83	a
+LN	4.5±0.09	b	12.47±3.47	a	0.0519±0.03	a	5.5±0.90	a	5.60±2.44	a

Rooting was carried out in modified (NH_4NO_3 reduced to 5 mM) semisolid MS medium during 60 d.

*Means with the same letter do not differ significantly according to the LSD test (p ≥0.05).

Figures

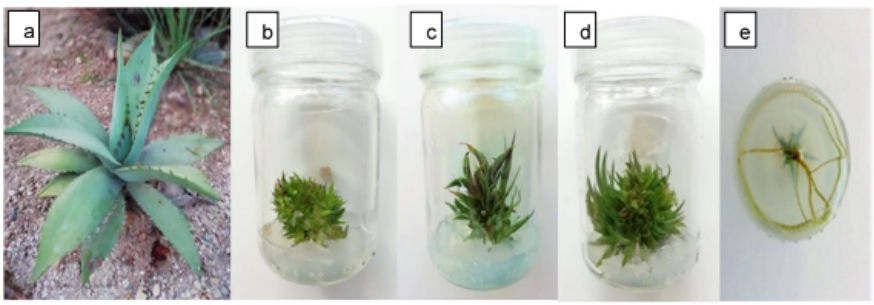

Figure 1

Micropropagation of Agave peacockii. a) Donor plant (two-year-old); b) Developed plantlets after 60 d of culture on MS semisolid medium with 26.6 µM BAP; c) Developed plantlets after 60 d of culture on MS semisolid medium with 27.84µM KIN; d) Developed plantlets after 60 d of culture on MS semisolid medium supplemented with 26.6 µM BAP and 27.84µM KIN and e) Developed roots in clusters of plantlets after 60 d of culture on MS semisolid medium BAP-free.

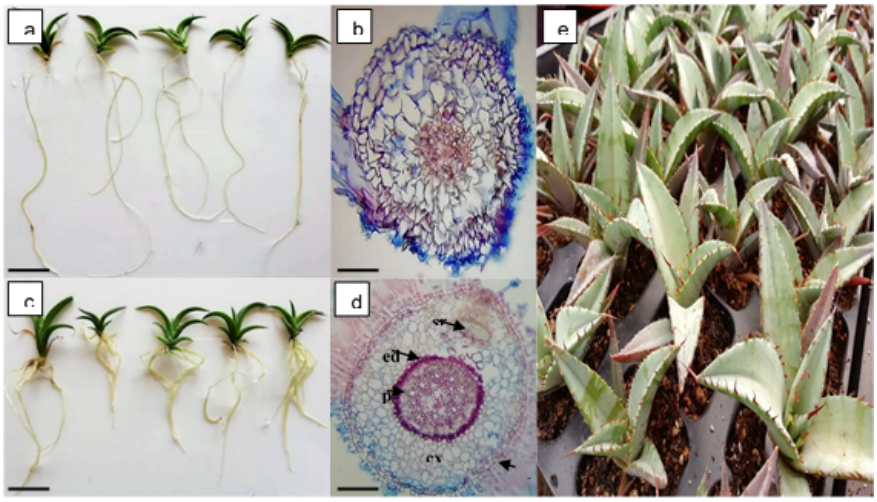

Figure 2

Rooting of Agave peacockii shoots. a) Rooted shoots on MS semisolid medium supplemented with 3% sucrose; b) Example of cross histological section of thin-root (Bar= 50 µm); c) Rooted shoots on MS semisolid medium supplemented with 29.5µM IBA and 3% sucrose (Bar= 2.0 cm); d) Example of cross histological section of thick-root (Bar= 200 µm), sr: secondary root, ed: endodermis, pr: pericycle, cx: cortex, ep: epidermis and e) Three-month-old micropropagated plants transferred to substrate peatmoss- perlite (6:4)

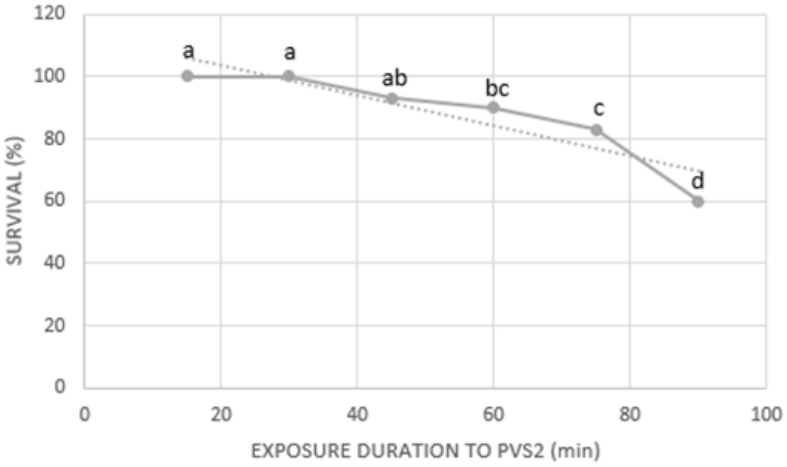

Figure 3

Effect of exposure duration to vitrification solution PVS2 at 25 °C on survival of pretreated Agave peacockii shoot-tips. Surviving shoot-tips maintained a yellow-green color after 1 week of culture. Data are the mean ± SE (n = 3). Means marked with the same letter do not differ significantly according to the LSD test ($p \geq 0.05$). Shoot tips were precultured on MS semisolid medium with 0.3 M sucrose for 1 d and loaded in solution containing basal MS medium supplemented with 0.4 M sucrose and 1.6 M glycerol during 20 min in dark before exposure to PVS2.

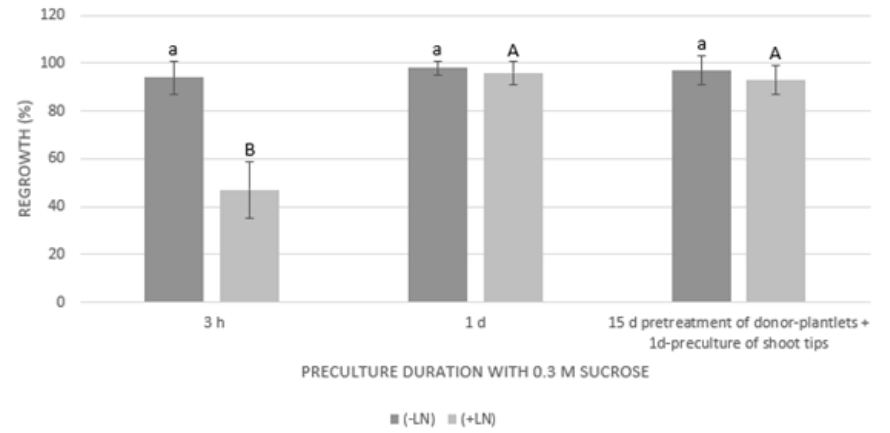

Figure 4

Effect of preculture duration on semisolid medium with 0.3 M sucrose on regrowth (%) of A. peacockii shoot tips before (-LN) and after (+LN) cryopreservation. Results are means of three experiments with 3 replicates and 30 shoot tips in each one. Same letters are not significantly different according to the LSD test ($p \geq 0.05$).

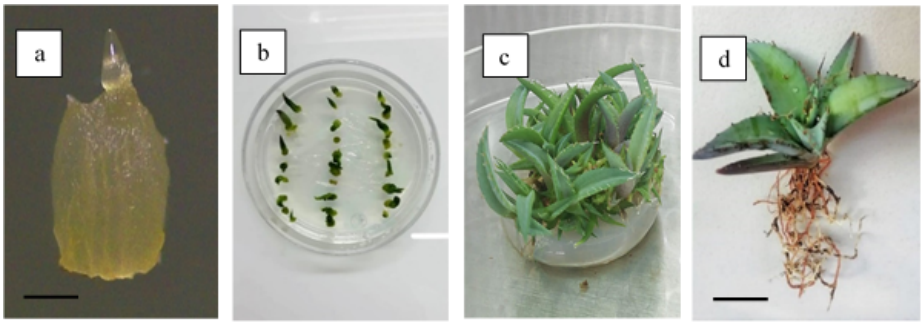

Figure 5

Cryopreservation of Agave peacockii shoot tips by droplet-vitrification a) Apical shoot-tip dissected of two-month-old plantlets, Bar=0.5 mm; b) Growth recovery of cryopreserved shoot-tips after 15 d of reculture; c) Regrown shoots derived of cryopreserved shoot tips after 30 d of reculture; d) Plant regenerated of a cryopreserved shoot-tip after 150 d of culture under greenhouse conditions, Bar=2.0 cm.