New approaches for micropropagation and cryopreservation of *Agave peacockii*, an endangered species

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Abstract
More than 50% out of 129 of Agave species are endemic to Mexico. Among them, *Agave peacockii* is among 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 (87.00 ± 17.18) shoot generation number after 60 days of culture. This interaction was more effective than using the same growth regulators separately. Propagated and rooted plantlets were acclimatized with 100% survival and normal morphological development. For cryopreservation, an optimized protocol following droplet-vitrification allowed obtaining 98% and 96% regrowth before and after cryopreservation, respectively. Shoot-tips (1 mm in length × 1 mm wide) 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 Plant Vitrification Solution 2 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 conditions. No significant differences were detected in most assessed characteristics after 120 days of culture. 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 (BAP) and 6-furfuryl-aminopurine (kinetin). A droplet-vitrification protocol was optimized to cryopreserve shoot-tips. Greenhouse performance of in vitro and cryo-derived plants was similar.

Keywords Agavoideae · In vitro propagation · Droplet-vitrification · Vegetative growth

Abbreviations

| Abbreviation | Description                                      |
|--------------|--------------------------------------------------|
| 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)                |
| PGRs         | Plant growth regulators                          |
| PVS          | Plant vitrification solution                     |
| 2,4-D        | 2,4-Dichlorophenoxyacetic acid                   |
| IBA          | Indole-3-butyric acid                            |

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 and biofuels (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 need 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 decline 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 (Sarasa et al. 2006). In the case of Agave spp., efficient micropropagation protocols have been developed using different plant growth regulators (PGRs) in the culture medium such as 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) (Rios-Ramírez et al. 2017; Aguilar and Rodríguez, 2018), or 2,4-dichlorophenoxyacetic acid (2,4-D) (Santacruz-Ruvalcaba et al. 1999; Alvarez-Aragón et al. 2020). Nevertheless, mass propagation of shoots depends on each Agave species, the type of explant, and the growth regulators used (Lecona-Guzmán et al. 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). Additionally, 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. 2020) was successfully achieved. They provided new complementary strategies based on the total arrest of metabolic activity and cell division (Cruz-Cruz et al. 2013).

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, defoliated until the last fused leaves were left and the roots were cut. 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 a solution 50% (v/v) of commercial bleach (5.25% w/w) 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 L⁻¹ of BAP for shoot stimulation.

For micropropagation, in vitro generated shoots with 2 cm in length and three developing leaves were selected and cultured on MS semisolid basal medium with different combinations of PGRs: 0.00, 4.43, 13.13 or 26.6 μM BAP and/or 0.00, 4.6, 13.92 or 27.84 μM KIN. Culture medium devoid of PGRs 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 mediasterilized 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 days of culture. Six replicates were used per each culture medium studied.

**Rooting and acclimatization**

Five replications of shoots (4–5 cm long) cultured on MS semisolid medium for 30 days were inoculated for root induction. Rooting was carried out using MS semisolid medium modified by reducing NH₄NO₃ 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 PGRs was used as control. The number and length of roots were recorded after 60 days of culture.

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/50–75% relative humidity) and the irrigation was performed by capillarity using 20% liquid basal MS medium devoid of sucrose for 120 days. During acclimatization, survival (%) was evaluated by counting the number of living plants after 60 days 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 days of greenhouse culture in 10 randomly selected plants. The foliar area was calculated according to Montgomery (1911) using the following formula:

\[
\text{Foliar area} = \left(\text{leaf length} \times \text{maximum leaf width}\right)(0.75)
\]

The fresh and dry weights were determined using an analytical Sartorius® balance with an accuracy of 0.1 mg. Drying was performed for 72 h, wrapping the roots in aluminum foils and using an oven at 60 °C. Length was measured with a graduated ruler with 1.0 mm precision.

**Histological analysis**

Root thickness during in vitro rooting was visually compared and analyzed by histological observations. For histology, 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 of treatments with IBA and/or sucrose to analyze different root thickness.

**Cryopreservation of shoot-tips by droplet-vitrification**

In vitro plants regenerated during shoot multiplication experiments were grown on MS semisolid medium containing 0.8% (w/v) agar and maintained 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 3 h (until completing the dissection process) or remained on the same preculture medium for 1 day. Sucrose-preculture for 1 day 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 days.

Cryopreservation experiments were carried out using the droplet-vitrification method. The successive steps of this procedure comprise osmoprotection by treating the shoot-tips with a loading solution (sucrose-glycerol), followed by the osmotic dehydration with the Plant Vitrification Solution 2 (PVS2) composed by 30% v/v glycerol, 15% v/v dimethyl sulfoxide, 15% v/v ethylene glycol, and 13.7% w/v sucrose (Sakai et al. 1990). Subsequently, shoot-tips are inserted into drops of PVS2 placed on aluminum foil strips, which are directly immersed in liquid nitrogen (LN). For cryopreserving A. peacockii shoot-tips, a modified droplet-vitrification protocol to that described by Tin and Folgado (2019) for A. sobria shoot-tips was applied.

A preliminary assessment to verify the tolerance of shoot-tips to dehydration with PVS2 was performed. Shoot-tips precultured on MS semisolid medium with 0.3 M sucrose for 1 day 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. Loaded shoot-tips were exposed to pre-chilled (stored in the refrigerator) PVS2 solution for different exposure times: 15, 30, 45, 60, 75, 90, and 120 min. Survival (%) was evaluated for each PVS2 exposure time 15 days after reculture by counting the shoot-tips that remained yellow-green color and started displaying a growth response. Reculture for 15 days took place first, using a MS semisolid medium with 0.3 M sucrose for 2 days, and then, MS semisolid medium with 0.44 μM BAP and 3% (w/v) sucrose.

Based on the optimization of exposure time to PVS2, shoot-tips precultured with sucrose for 3 h and shoot-tips precultured for 1 days, excised from plantlets with or without preconditioning for 15 days were treated with the loading solution (20 min) and dehydration with cold PVS2 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 x 2.0 cm), and then, foils were placed in 2 mL cryovials prefilled with 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 days, followed by the reculture on semisolid MS medium with 0.44 μM BAP and 3% (w/v) sucrose until 30 days. All samples were maintained 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 days of post-cryopreservation recovery. The new shoots were rooted onto MS semisolid medium with reduced NH₄NO₃ (5 mM) and devoid of PGRs for 60 days. Acclimatization of plantlets (4 cm long) with well-developed roots was performed under the same conditions described above. Survival (%) of acclimatized cultures was also evaluated after 60 days. 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 days of acclimatization.

**Statistical analysis**

Experiments were performed using a completely randomized design. Quantitative data are presented as the mean ± Standard Error (SE). Shoot-tip proliferation (levels of BAP and KIN) and rooting (levels of IBA and sucrose) experiments were evaluated by two-way analysis of variance (ANOVA). Results of cryopreservation assays were processed by one-way ANOVA. All differences among means were determined following Fisher’s Least Significant Difference (LSD) test (p ≤ 0.05). 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. A significantly higher number (87.00 ± 17.18) of shoot generation was obtained by the interaction of the highest concentrations of both PGRs: 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 PGRs 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 such 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. diffornis*, *A. karwinskii*, *A. obscura* and *A. potatorum* (Domínguez et al. 2008); *A. marmorata* (Aguilar and Rodríguez, 2018). However, until now, the combination of two cytokinins had never been tested and reported for multiplication 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

**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 days of culture

| Concentration of growth regulators (μM) | Number of shoots Proliferation* | Number of roots in shoot* |
|----------------------------------------|---------------------------------|--------------------------|
| **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
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 shows the parent plant (a) and the effect of different PGRs after 60 days culture (b, c, d) of *A. peacockii*.

**Rooting and acclimatization**

During in vitro rooting, the interaction of IBA and sucrose factors was statistically significant (p ≤ 0.05). The significantly higher number (8.6 ± 1.01) of formed roots was achieved using medium with 3% sucrose supplemented with the highest concentration (29.25 µM) of IBA (Table 2). However, significantly longer roots (20.52 ± 3.02 and 18.8 ± 1.86) were observed in media containing only sucrose (6 and 3%, respectively). Unlike in vitro rooting, after 120 days of greenhouse culture, a more notable development of both the aerial part and the root system was detected in those plants previously rooted in culture media with sucrose and lower concentrations of IBA (Table 3). These results indicated that high IBA concentrations were not required to produce strong (with a significantly higher number of roots), well-rooted (with significantly longer roots) plants during greenhouse culture. The reason why in vitro rooting was better at higher concentrations of IBA (Table 2) but rooting during acclimatization was better in plantlets coming from lower concentrations (Table 3) could be because the high dose of auxin supplied exogenously, when combined with the endogenously synthesized, exceeds the level required for an optimal response and causes partial inhibition under greenhouse conditions.

![Fig. 1 Micropropagation of *Agave peacockii*. a Parent plant (2-year-old); b Developed plantlets after 60 days of culture on MS semisolid medium with 26.6 µM BAP; c Developed plantlets after 60 days of culture on MS semisolid medium with 27.84 µM KIN; d Developed plantlets after 60 days of culture on MS semisolid medium BAP-free](image)

| Treatment | Factors | Number of roots* | Root length* (cm) |
|-----------|---------|------------------|------------------|
|           | IBA (µM) | Sucrose (%)      |                  |
| 1         | 0        | 0                | 1.2 ±0.40        | 10.62 ±2.94    |
| 2         | 2.8      | 0                | 1.8 ±0.40        | 9.98 ±2.98     |
| 3         | 4.4      | 0                | 2.8 ±1.16        | 8.58 ±1.46     |
| 4         | 8.9      | 0                | 4.4 ±1.01        | 6.94 ±4.90     |
| 5         | 2.2      | 3                | 2.2 ±0.74        | 18.8 ±1.86     |
| 6         | 6.6      | 3                | 6.6 ±1.49        | 13.32 ±0.81    |
| 7         | 5.8      | 3                | 5.8 ±0.74        | 12.72 ±1.89    |
| 8         | 8.6      | 3                | 8.6 ±1.01        | 11.46 ±2.58    |
| 9         | 4.0      | 3                | 4.0 ±1.09        | 20.52 ±3.02    |
| 10        | 4.4      | 3                | 4.4 ±1.01        | 15.86 ±2.80    |
| 11        | 6.6      | 6                | 6.6 ±0.80        | 14.26 ±3.80    |
| 12        | 6.8      | 6                | 6.8 ±2.22        | 12.98 ±0.87    |

*Values represent means ± standard errors of five replicates

*Different letters within the columns are significantly different according to the LSD test (p ≤ 0.05)
The addition of IBA was also not significantly effective when media without sucrose were used. 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.

**Histological analysis**

The composition of in vitro rooting media influenced root thickness. Thick roots were always detected when using any IBA-supplemented media. By contrast, longer and thinner roots were always obtained when only using sucrose as an additive (Fig. 2).

The histological analysis during in vitro radicular development allowed identifying differences in roots’ anatomy and cellular structure depending on thickness. Thin roots did not present a well-defined epidermis, cortex, endodermis, pericycle and secondary root formation, unlike thick roots’ structural organization after the same culture time. Nevertheless, regardless of the anatomical characteristics of roots, all rooted plantlets were successfully acclimatized with 100% survival and normal morphological development.

**Cryopreservation of shoot-tips 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 (Fig. 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 day) used or whether the mother plantlets from which shoot-tips were isolated had been pretreated with sucrose for 15 days before dissection. However, 1 day 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 day-preconditioning of the donor-plantlets (Fig. 4). Therefore, the best procedure for cryopreservation of *A. peacockii* shoot-tips involved: 1 day-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 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 Fig. 5.

The cryogenic protocol defined for *A. peacockii* shoot-tips included modifications that simplified and shortened the process's duration compared to the 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

| Treatment | IBA (µM) | Sucrose (%) | Number of roots* | Root length* (cm) | Root dry weight* (mg) | Leaves number* | Total leaf area* |
|-----------|----------|-------------|------------------|-------------------|-----------------------|---------------|-----------------|
| 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 ± 1.34 def |
| 5         | 0        | 3           | 6.4 ± 1.14 cd    | 13.8 ± 6.04 bc    | 0.0714 ± 0.02 def     | 6.0 ± 0.70 def| 6.3 ± 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 ± 0.05 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)
15 days in medium enriched with 0.3 M sucrose. By contrast, the highest (96%) post-cryopreservation regrowth of *A. peacockii* shoot-tips was achieved using 1 day-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 was more effective than extended pretreatment of donor-plantlets at the same sucrose concentration. This preculture had not been tested before in *Agave*, and replaced the use of liquid medium with 20 mg L$^{-1}$ of ascorbic acid like reported Tin and Folgado (2019).

Preculture for 1d on semisolid medium supplemented with 0.3 M sucrose is usually the most applied pretreatment of any cryopreservation procedure for organized tissues like shoot-tips (González-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 et al. 2021).

The 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 MS semisolid medium with low (0.44 μM) concentration of BAP for 30 days, and then, transferred and maintained in culture using a modified (NH₄NO₃ reduced to 5 mM) MS semisolid medium with 3% sucrose and devoid of PGRs. After 60 days of culture, the new plantlets spontaneously developed roots and were transferred to greenhouse culture conditions. 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 after 120 days of greenhouse culture 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.

The rooting and acclimatization conditions tested with cryo-derived plants allowed to evaluate the biological response without the influence of any PGR in the in vitro rooting medium. This demonstrated that plants recovered after cryopreservation could grow and develop in the greenhouse even when had been rooted in suboptimal conditions. Since the greenhouse behaviour of micropropagated and cryo-derived plants did not show significant differences, the
use of optimized rooting conditions (addition of low IBA concentrations to modified MS semisolid medium) in the future, must induce further improvements during greenhouse culture.

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 improvements addressed to the propagation and safe conservation of plant germplasm, besides supporting new research on *Agave* spp.

### Conclusions

In this study, we reported a new approach to 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 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 conditions. Therefore, the results presented here represent new potential biotechnological strategies for the
in vitro propagation and long-term conservation of Agave germplasm.

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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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Declarations

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