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Research article

Micropropagación de *Agave angustifolia* Haw., con fines de aprovechamiento y conservación

Micropropagation of *Agave angustifolia* Haw., for use and conservation purposes

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Abstract

Agave angustifolia is a species utilized as raw material for mescal production in the South of the *Estado de México*, which is why the demand for the establishment of plantations of this succulent has increased. Plant tissue culture offers an alternative for mass plant propagation; thus, the objective of the present research was to develop a protocol for the *in vitro* propagation of the species. Germination, shoot multiplication and elongation, and root formation took place under *in vitro* conditions, and the plants were acclimatized in the nursery. The germination percentage was higher than 80 %, and there were differences between disinfection treatments and culture media. The use of 100 % MS medium+0.003 mg L^{-1} of BAP+0.002 mg L^{-1} of IBA+0.1 mg L^{-1} of calcium pantethonate produced a higher number of shoots per plant at 90 and 120 days. With 75 % MS culture medium, 100 % rooting was obtained, with an average of 6.5 roots per plant and 45 mm root length. In the nursery acclimatization stage, there was 100 % survival in the three substrates evaluated and differences were observed in height, diameter and dry matter. In conclusion, a complete protocol was generated for the micropropagation of *Agave angustifolia*, from *in vitro* germination to acclimatization in nursery.

Keywords: Forest biotechnology, tissue culture, xerophytic scrubland, mescal production, *agave* propagation, *in vitro* propagation.

Resumen

Agave angustifolia es una especie que se utiliza como materia prima para producción de mezcal en el sur del Estado de México, por lo cual se ha incrementado la demanda de planta para el establecimiento de plantaciones. El cultivo de tejidos vegetales ofrece una alternativa para la propagación masiva de plantas; por ello, el objetivo de la presente investigación fue desarrollar un protocolo para la propagación *in vitro* de la especie. Bajo condiciones *in vitro* se realizó la germinación, multiplicación y elongación de brotes, y formación de raíces, mientras que en vivero se hizo

la aclimatación de plantas. El porcentaje de germinación fue superior a 80 % y se registraron diferencias entre los tratamientos de desinfección y medios de cultivo. El uso del medio MS al 100 %+0.003 mg L^{-1} de BAP+0.002 mg L^{-1} de AIB+0.1 mg L^{-1} de pantetonato de calcio produjo mayor número de brotes por planta a los 90 y 120 días. Con el medio de cultivo MS al 75 % se obtuvo 100 % de enraizamiento, con un promedio 6.5 raíces por planta y 45 mm de longitud de la raíz. En la etapa de aclimatación en vivero se presentó 100 % de sobrevivencia en los tres sustratos evaluados y se observaron diferencias en altura, diámetro y materia seca. En conclusión, se generó un protocolo completo para la micropropagación de *Agave angustifolia*, desde la germinación *in vitro* hasta su aclimatación en vivero.

Palabras clave: Biotecnología forestal, cultivo de tejidos, matorral xerófilo, producción de mezcal, propagación de agave, propagación *in vitro*.

Introduction

Agaves are endemic to the Americas and have their center of origin in Mexico (Mandujano et al., 2018), where they are mainly found in arid and semi-arid areas (Pérez et al., 2012). The richness of endemic species of the *Agave* L. genus in Mexico is mainly due to the country's heterogeneous habitats, which differ in climate, geology, soils, topography, and altitude, among other environmental factors (León et al., 2013). Some of the taxa are used as raw material for the production of alcoholic beverages and natural fibers, which causes the decline of the natural populations of several taxa (NOM-059-SEMARNAT-2010, 2010). The list of priority species for conservation includes six species of agaves, among them is *Agave angustifolia* Haw. (Secretaría de Medio Ambiente y Recursos Naturales [Semarnat], 2014).

Agave angustifolia (*maguey delgado*, *maguey de monte* or *espadín*) grows wild and extensively in the Western *Sierra Madre*, in the states of *Aguascalientes*, *Chihuahua*, *Durango*, *Jalisco*, *Nayarit*, *Sinaloa*, *Sonora* and *Zacatecas* (Fragoso-Gadea et al., 2021). *Aguamiel* (*maguey* juice) is produced from the sap concentrated in its apical meristematic zones and is utilized to make *pulque*. Its leaves are utilized to cook lamb meat in barbecue, and the flowers are employed in various dishes (Ávila-Lara et al.,

2021). It is also used as a remedy for sprains or broken bones in people or animals (Barrientos et al., 2019).

Today, one of the strategies with the greatest potential for recovering, multiplying and preserving plant species is *in vitro* culture (Suárez, 2020). For example, in the study by Domínguez et al. (2008) with *Agave cupreata* Trel. & A. Berger, *A. difformis* A. Berger, *A. karwinskii* Zucc., *A. obscura* Schiede and *A. potatorum* Zucc., meristematic tissues extracted from *in vitro* germinated seedlings were used. Multiple shoots were obtained from basal explants in Murashige and Skoog (1962), added with 30 g L^{-1} sucrose, 8 g L^{-1} agar and various cytokinin treatments [6-benzylaminopurine (BA), 6-γ,γ-dimethylallylaminopurine (2iP), cinetin (Cin), thidiazuron (TDZ) and meta-topoline or N6-(metahydroxybenzyl) adenine (MT)].

Literature includes records on the successful use of this technique for the propagation of such agaves as *Agave tequilana* F. A. C. Weber (Ángeles-Espino et al., 2012; Portillo et al., 2007; Ramírez-Malagón et al., 2008), *A. inaequidens* K. Koch (Aureoles-Rodríguez et al., 2008), *A. salmiana* Otto *ex* Salm-Dyck (Ramírez-Malagón et al., 2008), *A. fourcroydes* Lem. (Garriga et al., 2010), *A. grijalvensis* B. Ullrich (Santíz et al., 2012), *A. americana* L. var. *oaxacensis* Gentry (Cruz et al., 2017; Miguel et al., 2013), *A. angustifolia* (Monja-Mio et al., 2015; Ríos-Ramírez et al., 2017) and *A. potatorum* (Luna-Luna et al., 2017).

In the Southern region of the *Estado de México*, mescal production is a deep-rooted tradition in many communities and an important source of income for many families. The use and demand for *A. angustifolia* in this region is generating an increase in the demand for plants to establish commercial plantations. To address this situation, the *Protectora de Bosques del Estado de México*, *Probosque* (Agency for the Protection of Forests of *Estado de México*) is developing biotechnological techniques such as *in vitro* plant tissue culture in order to obtain plants with quality characteristics for establishment in the field. Therefore, the objective of this study was to evaluate the

effectiveness of *in vitro* propagation to produce *A. angustifolia* plants from seeds and their subsequent acclimatization in nursery.

Materials and Methods

Plant material

The project was carried out from June 2021 to December 2023 at *Probosque*'s Forest Biotechnology Laboratory, located in *Metepec*, *Estado de México*. In March 2021, *A. angustifolia* seeds were collected at *La Joya del Campo*, *Lagunita* locality, *Ocuilán* municipality (395569 E, 2130744 N, UTM zone 14 s). Vegetation is xerophilous scrubland; 21.2 °C average annual temperature and 1 085.2 mm average annual rainfall (*Instituto Nacional de Estadística y Geografía* [Inegi], 2019). In the milling process, seeds were manually extracted and cleaned to eliminate impurities. A representative sample of the lot was considered for quality analysis according to International Seed Testing Association (ISTA, 2025) procedures. Seeds showed 46 % viability, 40.25 % germination, 9.36 % moisture, 98.50 % purity, with 92 299 seeds per kilogram, 0.0069 g per seed, and a length of 0.8 to 0.9 mm. The germplasm was placed in high-density polyethylene bags, Captan (*Captán* 50 WP®, Israel) and Thiabendazole (Tecto® 60, Mexico) were added in a 1:1 ratio, enough to impregnate the seeds; the bags were subsequently sealed, placed in hermetically sealed plastic containers and stored in a cold room at 3-5 °C with 90 % relative humidity.

Germination

The laboratory experiment was started in June 2021. 720 seeds were used for *in vitro* germination. They were washed by manual shaking using a glass rod in a solution with Roma® detergent (1 g L^{-1}) for three minutes and rinsed with running water. Then, the seeds were disinfected with a solution of [aluminum tris-(O-ethyl phosphonate)] Jabali® (Mexico) fungicide (2 g L^{-1}), using a model SP88857100 Thermo Scientific® magnetic stirrer, shaken at 250 rpm for 2 hours. For the germination process, a completely randomized factorial experiment was set up. Factor A was the three-level disinfection process: (D1) 3 % H_2O_2 for 160 min+*Roma*® detergent (1 g L^{-1}) for 3 min+3 % H_2O_2 for 10 min+70 % ethanol for 0.5 min+*Chloralex*® (0.6 % NaClO) for 10 min; (D2) 3 % H_2O_2 for 480 min+*Roma*® detergent (1 g L^{-1}) for 3 min+3 % H_2O_2 for 10 min+70 % ethanol for 0.5 min+*Chloralex*® (0.6 % NaClO) for 15 min; and (D3) 3 % H_2O_2 for 960 min+Roma® detergent (1 g L^{-1}) for 3 min+3 % H_2O_2 for 10 min+Chloralex® (0.6 % NaClO) for 10 min.

Factor B was MS (Murashige & Skoog, 1962) and SH (Schenk & Hildebrandt, 1972) at five levels: (T1) 100 % MS, (T2) 100 % SH, (T3) 50 % MS+0.2 g L^{-1} glutamine, (T4) 50 % SH, and (T5) 50 % MS. 30 g L^{-1} of sucrose and 7 g L^{-1} of bacteriological agar as gelling agent were added to the culture media, and the mixture was adjusted to a pH of 5.7 and then placed (20 mL) in glass vials, which were autoclaved (model Fe-399 *Felisa*®) at 121 °C for 15 minutes.

The 720 seeds were used to set up 15 treatments that resulted from combining the three levels of Factor A (disinfection process) with the five levels of Factor B (culture

media); the experimental unit was a flask with 6 seeds, and 8 replicates were used per treatment, for a total of 120 flasks.

Germination took place in an incubation room at 22 °C, with a relative humidity of 23 %, and a photoperiod of 16 hours of light and 8 hours of darkness. The percentage of germinated seeds per flask was evaluated as a response variable. Data were subjected to a normality test, homogeneous variances, analysis of variance, and Tukey's mean comparison test; a level of $p<0.05$ was considered as a reference (Quinn & Keough, 2002). The analyses were performed using the SAS® 9.4 statistical package (Statistical Analysis System Institute [SAS Institute], 2013).

Multiplication of shoots

Seedlings obtained at the germination stage were used for the multiplication of axillary shoots. For this purpose, individuals with similar characteristics (90 days after germination and with an average height of 5 cm) were chosen, and a cut was made at the root collar to induce the formation of shoots. Five treatments were installed: (T1) 100 % MS; (T2) 100 % MS+0.3 mg L^{-1} of BAP+0.2 mg L^{-1} of IBA; (T3) 100 % MS+0.3 mg L^{-1} of BAP+0.2 mg L^{-1} of IBA+0.1 mg L^{-1} of calcium pantethonate (Andrade et al., 2013); (T4) 100 % MS+0.8 mg L^{-1} of BAP+0.2 mg L^{-1} of IBA, and (T5) 100 % MS+0.8 mg L^{-1} of BAP+0.2 mg L^{-1} of IBA+0.1 mg L^{-1} of calcium pantethonate. The base medium utilized was the MS formulation (Murashige & Skoog, 1962), added with 1 mg L^{-1} of thiamine, 100 mg L^{-1} of Myo-inositol, and 30 g L^{-1} of sucrose. The growth regulators used were: N6-Benzylaminopurine (BAP), indole butyric acid (IBA), and calcium pantethonate. The latter compound has been successfully used in the micropropagation of other plants like *Solanum* L. (Andrade

et al., 2013). The pH of the mixture was adjusted to 5.7, and 7 g L^{-1} of bacteriological agar were added (Enríquez et al., 2005); 30 mL were placed in glass vials and autoclaved (model Fe-399 *Felisa*®) at 121 °C for 15 minutes.

At this stage, a completely randomized experimental design was used, with 10 replications per treatment, and the experimental unit was a jar with three seedlings. The response variables were the number and size of the shoot (mm), measured with a 12 cm vernier caliper (model 5" Anelsam®). The data were subjected to a statistical analysis applying normality test and homogeneous variances tests, an analysis of variance, and a mean comparison test using Tukey's method with a p-value<0.05 (Quinn & Keough, 2002). The analyses were performed with the SAS® 9.4 statistical package (SAS Institute, 2013).

Shoot elongation

A. angustifolia shoots obtained at the multiplication stage after 180 days were selected. Healthy 3 cm long shoots were placed in the culture media corresponding to each treatment: (T1) 75 % MS; (T2) 75 % MS+3 mg L^{-1} of kinetin+3 mg L^{-1} of BAP; (T3) 75 % MS+3 mg L^{-1} of kinetin+10 mg L^{-1} of BAP; (T4) 75 % MS+3 mg L^{-1} of BAP+0.3 mg L^{-1} of NAA; (T5) 100 % MS; (T6) 100 % MS+3 mg L^{-1} kinetin+3 mg L^{-1} BAP; (T7) 100 % MS+3 mg L^{-1} kinetin+10 mg L^{-1} BAP; (T8) 100 % MS+3 mg L^{-1} BAP+0.3 mg L^{-1} NAA. The decision was made to test the combination of cytokinins and auxins, given that the effect of these plant hormones on the elongation of *A. angustifolia* shoots is not clear in the literature.

The experimental design was completely randomized, with ten replications per treatment, and the experimental unit was a jar with three seedlings. The response

variable was shoot height (mm), measured with a 12 cm vernier caliper (model 5" Anelsam®). The data were subjected to a statistical analysis, applying normality and homogeneous variances tests, an analysis of variance, and a mean comparison test using Tukey's method with a *p*-value<0.05 (Quinn & Keough, 2002). The SAS® 9.4 statistical package was used (SAS Institute, 2013).

Root formation

The plants obtained at the elongation stage were subjected to rooting. For this purpose, a MS culture medium was utilized at 75 % concentration without growth regulators. The process of root formation occurred within a period of 8 to 12 weeks in an incubation room, under the same laboratory conditions described above. The length (cm) and root collar diameter (mm) were measured with a 12 cm vernier caliper (model 5" Anelsam®), and the number of roots was counted. The average for 300 plants was estimated.

Acclimatization

The 300 rooted plants were used at the acclimatization stage. The average characteristics of the individuals were: five leaves, 7.8±0.8 cm in height, 1±0.1 cm root collar diameter and 6±0.2 cm root length. Plants were placed in three volume mixtures

of four different substrates (soil, pumice, Multiperl® agrolite, and Berger® peat moss): (T1) Soil (40 %)-pumice (30 %)-peat moss (30 %), (T2) Soil (50 %)-pumice (50 %), and (T3) Soil (40 %)-pumice (30 %)-agrolite (30 %). The soil properties used were as follows: pH (5.6), *EC* (0.04 dS m^{-1}), *OM* (7.8 %), N (0.25 %), P (5.2 mg kg^{-1}), K (0.1 $cmol^{+}$ kg^{-1}), Ca (7.7 $cmol^{+}$ kg^{-1}), Mg (2.1 $cmol^{+}$ kg^{-1}), Na (0.1 $cmol^{+}$ kg^{-1}), Fe (90 $mg{\cdot}kg^{-1}$), Cu (1 $mg{\cdot}kg^{-1}$), Zn (2 $mg{\cdot}kg^{-1}$) and Mn (4 $mg{\cdot}kg^{-1}$).

32×32 cm plastic trays were used, with 25 cavities for 16.5 cm long tubes, a 6 cm upper diameter, a 4.5 cm lower base, and a volume of 285 cm^3 were utilized for planting. After transplanting, each tube was covered with a polypropylene bag to maintain the moisture. The trays were placed in a pre-acclimatization room at a temperature of 24 °C, with a 41 % relative humidity and lighting with LED lamps (16 hours of light and 8 hours of darkness). After eight days, the polypropylene bag was removed to verify the leaf turgor and plant survival; the plants were transferred to 25 cm long nursery bags with a 12.5 cm diameter and established under greenhouse conditions at an average temperature of 20.5 °C and with 52 % relative humidity.

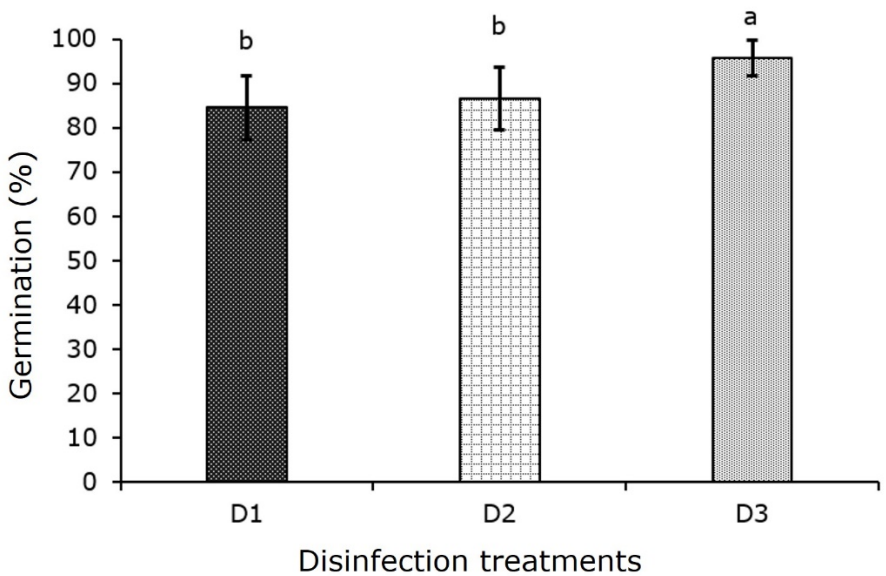
After six months, height (cm), root collar diameter (cm), and root length (cm) were measured with a 12 cm vernier caliper (model 5” Anelsam®). The number of roots and the dry matter content of the plant were also quantified. During this period, the plants were watered every other day, and no fertilizer was applied.

The data were subjected to statistical analysis by means of an analysis of variance and a comparison of means test using Tukey's method with a p-value<0.05 (Quinn & Keough, 2002); for this purpose, the SAS® 9.4 statistical package (SAS Institute, 2013) was utilized.

Results and Discussion

Disinfection treatments

Differences were obtained between disinfection treatments ($p<0.05$), culture media ($p<0.05$), and interaction of disinfection treatments and culture media ($p<0.05$) on germination. In this sense, disinfection treatment D3 registered the highest germination percentage (Figure 1), and the lowest percentage corresponded to treatments D1 and D2; thus, not all disinfection methods proved equally effective in reducing contamination. Method D3 had the longest immersion time in H_2O_2, which explains its effectiveness. In the study by Aguilar-Rito et al. (2024), the highest levels of contamination were observed with the $Ca(ClO_2)$ and NaClO treatments, while the lowest percentage of seed contamination occurred with the treatments with H_2O_2.



Equal letters indicate that there is no significant difference ($p<0.05$). D = Disinfection.

Figure 1. Percentage of germination by disinfection treatment.

Treatments D1 and D2 included the use of ethanol. However, this compound and other small alcohols, being natural surfactants of aqueous/organic interfaces, act on the cell

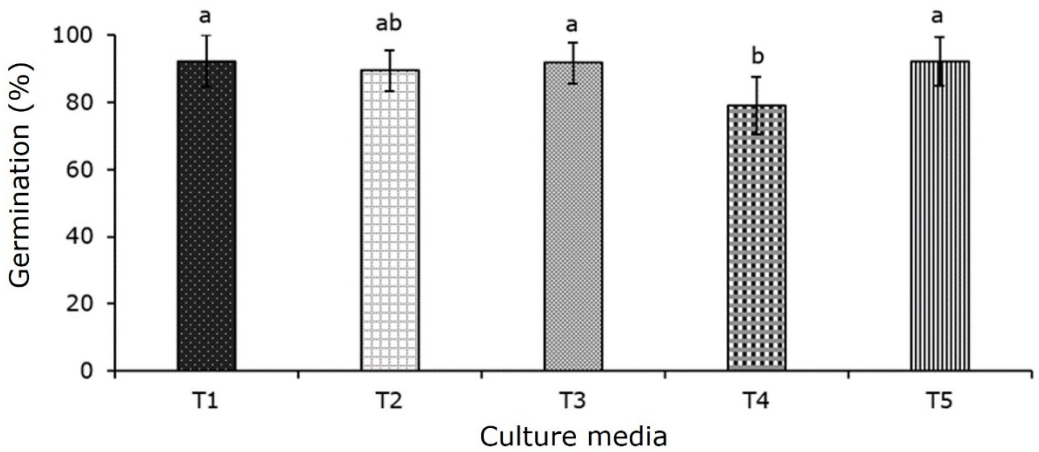
membrane by mechanically stressing it and inhibiting the natural adsorption of terminal groups at these interfaces (Rivera & Lima, 2013), and thereby damage plant tissues.

The D3 method was developed in the Forest Biotechnology Laboratory at *Probosque*, where it is routinely utilized for seed disinfection; its application resulted in a higher germination percentage compared to treatments D1 and D2 (Figure 1). This procedure contrasts with the technique employed by Aguilar-Rito et al. (2024), who used 3 % H_2O_2 for 24 h+30 % copper sulfate for 10 min+0.1 % $HgCl_2$ for 10 min and obtained 100 % disinfection, although with variable germination rates. Other disinfection techniques involve the use of 96 % ethanol for 1 min+3 % NaClO for 10 min (Santacruz-Ruvalcaba et al., 2022) or 70 % ethanol for 3 min+0.6 % NaClO for 15 min (Correa-Hernández et al., 2022). Finally, plants from seeds show genetic variability compared to those obtained from Mother plants, which remain genetically uniform (Arzate-Fernández et al., 2016). For conservation purposes, micropropagation from seeds is recommended because it maintains the genetic diversity.

Culture media for germination

The highest germination percentage was achieved with T1, T3, and T5 culture media (Figure 2). Conversely, the lowest amount of germinated seeds was recorded with T4; although T2 followed a similar trend. This behavior suggests that the MS medium provides a higher germination rate. The results of the present investigation are superior to those documented by Téllez et al. (2023), who used a MS medium with 50 % concentration of its inorganic components, supplemented with sucrose 30 g L^{-1} and bacto-agar 8.5 g L^{-1}, pH 5.7 and obtained 56 % germination in *A. potatorum* seeds stored for six months at 5 °C. The same authors indicate a germination rate of

31.8 % in seeds germinated in substrate under greenhouse conditions. Aguilar-Rito et al. (2024) used a MS medium supplemented with 30 g L^{-1} sucrose and 0.5 g L^{-1} activated carbon, without plant growth regulators and gelled with 8 g L^{-1} agar, and obtained a germination percentage of 85 % for *A. angustifolia* seeds analyzed in the same harvest year. Therefore, the generated protocol increased the germination percentage in agave seeds compared to conventional germination methods, *i. e.*, 46 % in substrate germination and 89 % on average under *in vitro* conditions.



Equal letters indicate that there is no significant difference (p<0.05). T = Treatment.

Figure 2. Percentage of germination by culture medium.

Multiplication of shoots

After establishment in the culture medium, there were differences between treatments in the average number of shoots obtained per explant (p<0.05). At 90

days, T3 had the highest number of shoots and, with the exception of T1, the rest of the treatments showed an increase in the number of shoots at 120 days; T3 and T4 were superior. Treatment T1 did not develop shoots because it contained neither cytokinins nor auxins (Table 1). Faisal et al. (2018) report that shoot apices of *Ruta graveolens* L. show no morphogenic response in a MS medium without a growth regulator. Auxins are unique hormones that can be subjected to polar transport, create gradients, and regulate cell differentiation; therefore, they are responsible for shoot morphogenesis (Pasternak & Steinmacher, 2024). High levels of cytokinins together with low auxin concentration synergistically affect cell division and *in vitro* plant regeneration (Fatima et al., 2011).

Table 1. Shoot development in *Agave angustifolia* Haw., 90 and 120 days after establishment in different culture media.

Treatment	Number of shoots per explant at 90 days	Number of shoots per explant at 120 days
T1	0±0c	0±0c
T2	1.9±0.9bc	4.7±1.2ab
T3	4.9±1.1a	6.0±1.3a
T4	3.6±0.6ab	5.5±1.1a
T5	1.2±0.6bc	2.9±1.0b

Equal letters indicate that there is no significant difference ($p<0.05$).

At 90 days, T3 generated the highest number of shoots per plant, and at 120 days the best treatments were T3 and T4. In the present study, due to the unavailability of TDZ, calcium pantethonate was used; this compound has been used in the micropropagation of *Solanum mammosum* L. and *Solanum hirtum* Vahl generating the highest number of leaves and plant length (Andrade et al., 2013). Cytokinins and auxins are commonly used in shoot multiplication. According to Domínguez et al. (2008), the highest shoot production in *Agave cupreata* and *A. karwinskii* is obtained

with 1 and 1.5 mg L^{-1} of BAP, while in *A. difformis* and *A. obscura*, it is achieved with 0.2 mg L^{-1} TDZ.

In *Agave nussaviorum* García-Mend., Miguel-Luna et al. (2023) observed that, at 80 days, 6.2 shoots were formed in the culture medium with 1 mg L^{-1} of BAP and 75 % in MS, 2.0 shoots with 0.5 mg L^{-1} of BAP and 50 % MS, and 4.3 shoots with 0.5 mg L^{-1} of BAP and 75 % MS. Finally, the combination of growth regulators with low doses of auxin (between 0.025 and 0.5 mg L^{-1}) and high doses of cytokinin (between 3 and 10 mg L^{-1}) stimulates the production of shoots from axillary buds in *A. angustifolia* (Ríos-Ramírez et al., 2017), *A. guiengola* Gentry (Chávez-Ortiz et al., 2021), and *A. fourcroydes* (Zhang et al., 2013).

Shoot elongation

The 75 % MS medium without growth regulators (T1) was the most effective for shoot elongation (Table 2). The evidence indicates that only T2 produced the lowest shoot growth.

Table 2. Shoot elongation of *Agave angustifolia* Haw., 60 days after establishment in a culture medium.

Treatment	Shoot length (mm) 60 days
T1	15.4±3.3a
T2	5.9±1.5b
T3	12.4±3.2ab
T4	12.9±1.3ab

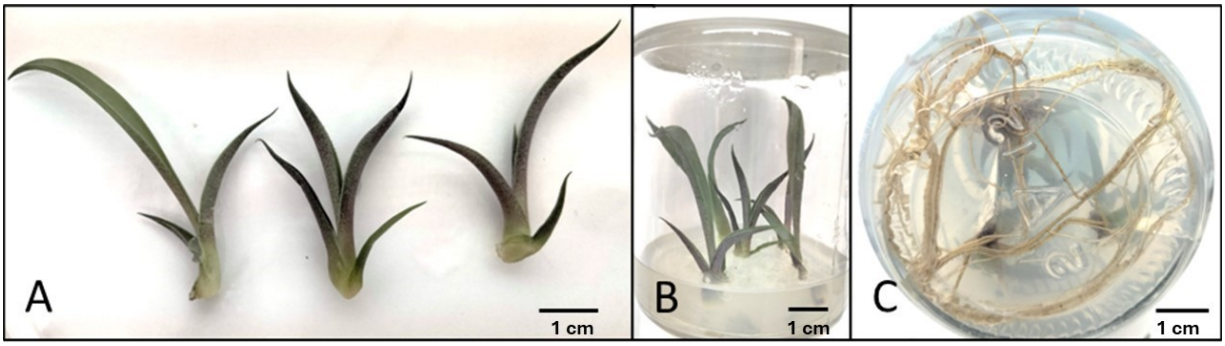
T5	10.5±2.5ab
T6	9.9±1.5ab
T7	9.0±2.3ab
T8	9.7±1.7ab

Equal letters indicate that there is no significant difference ($p<0.05$).

The study conducted by Santacruz-Ruvalcaba et al. (2022) on *in vitro* propagation with *A. maximiliana* Baker indicates that the use of a MS medium with 5 mg L^{-1} indole-3-butyric acid results in longer shoot length. On the other hand, Faisal et al. (2018) observe that, in the case of *Ruta graveolens*, a MS medium enriched with 10 μM benzyladenine generates the shoots with the greatest length. However, further studies are needed to elucidate the effect of the combination of auxins and cytokinins on the growth of *A. angustifolia* shoots, as published research does not evaluate this stage separately.

Root formation

The use of a 75 % MS culture medium prompted 100 % rooting, with an average of 6.5 roots per plant and 45 mm root length (Figure 3). These results coincide with those of Bautista-Castellanos et al. (2020) in *A. potatorum*, a species that is difficult to root, when they used a MS medium at 75 and 100 % concentration of salts without auxins; the authors point out that 73 % of the shoots form adventitious roots.



A = Explants for root induction; B = Explants established in MS medium; C = Root formation.

Figure 3. Root formation stage in *Agave angustifolia* Haw.

Aclimatization

At the nursery acclimatization stage, there was 100 % survival in the three treatments (Table 3, Figure 4). These results agree with those of Aguilar and Rodríguez (2018), who in *A. marmorata* Roezl at the acclimatization stage observed a 100 % survival in a mixture of peat moss with agrolite and peat moss with river sand. Other studies with Mexican agaves (*A. cupreata*, *A. difformis*, *A. karwinskii*, *A. obscura* and *A. potatorum*) have estimated survival rates of 53, 73, 60, 100 and 73 %, respectively, in commercial potting soil and sand (1:1) (Domínguez et al., 2008).

Table 3. Characteristics evaluated in *Agave angustifolia* Haw. plants after six months of acclimatization in nursery.

Substrate	Height (cm)	Root collar diameter (cm)	Root length (cm)	No. of roots	Dry matter (%)
T1	13±0.1ab	1.6±0.1a	17.5±0.6 a	11± 1a	15.0±1.4 ab
T2	13.6±0.5a	1.6±0.2a	17.5±0.5 a	11± 2a	17.0±0.7 a
T3	12.3±0.5b	1.3±0.1b	18.0±0.1 a	11± 1a	12.1±1.4 b

T1 = Soil (40 %)-pumice (30 %)-peat moss (30 %); T2 = Soil (50 %)-pumice (50 %); T3 = Soil (40 %)–pumice (30 %)-agrolite (30 %).

A = Soil (40 %)–pumice (30 %)-agrolite (30 %); B = Soil (40 %)-pumice (30 %)-peat moss (30 %); C = Soil (50 %)-pumice (50 %).

Figure 4. Development of *Agave angustifolia* Haw. on different substrates.

In the present study, differences in height, diameter and dry matter content between treatments were detected. The use of a higher percentage of soil in T2 favored a greater growth in height and biomass content, which is explained by a higher concentration of nutrients and moisture retention. In addition to providing nutrients,

organic matter increases soil porosity, moisture holding capacity, and aeration (Osman, 2013). The T3 substrate exhibited the lowest growth in height, root collar diameter, and dry matter content. Stone materials (agrolite and pumice) do not provide nutrients and retain less moisture than peat moss, which explains the lower plant development. For this reason, inorganic materials (pumice, *tezontle* —a reddish, porous volcanic rock—, and agrolite) are more widely used, individually, in hydroponic cultivation (Gayosso-Rodríguez et al., 2016), where the supply of water and nutrients to the plants is precisely controlled. In this experiment, no nutrients were applied to the substrate, which resulted in no differences in root length and number of roots. Particularly, phosphorus supply is related to root growth (Havlin et al., 2016).

Conclusions

A complete protocol was generated for the micropropagation of *Agave angustifolia*, from *in vitro* germination to acclimatization in nursery. The survival rate at the acclimatization stage was 100 %, *i. e.*, the 300 plants obtained at the rooting phase were established in the nursery. The specimens were obtained from seed germination, which gives them greater genetic variability, a key aspect in the conservation of this forest resource. Therefore, *in vitro* production of *A. angustifolia* is a feasible alternative for plant propagation in greater quantities and quality for the establishment of commercial mescal agave plantations in the *Estado de México*. This will help to reduce the pressure that exists due to the overexploitation of the species in its natural habitat.

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Conflict of interest

The authors declare that they have no conflict of interest.

Contribution by author

Armandina de la Cruz-Olvera: planning and execution of laboratory work and drafting of the manuscript; Miriam Serrano-Muñoz: drafting and revision of the manuscript; Adolfo Armando Rayas-Amor: drafting and revision of the manuscript; René García-Martínez: statistical analysis of data, drafting and revision of the manuscript.

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