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OF *Agave salmiana* Otto ex Salm-Dyck subsp.
salmiana USING ZYGOTIC EMBRYO OBTAINED
BY *in-casa* POLLINATION AS EXPLANTS**

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Plant regeneration from indirect somatic embryogenesis of *Agave salmiana* Otto ex Salm-Dyck subsp. *salmiana* using zygotic embryo obtained by *in-casa* pollination as explants

Regeneración de plantas por embriogénesis somática indirecta de *Agave salmiana* Otto ex Salm-Dyck subsp. *salmiana* usando embriones cigóticos obtenidos por polinización *in-casa* como explantes

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ABSTRACT: The use of somatic embryogenesis can be an alternative to produce a large number of somatic seedlings in less time at low cost; however, the viability and success of this method depend on several factors such as the type of explant used, the hormonal balance, among others. In this sense, the present research work evaluated the use of immature zygotic embryos obtained by *in-casa* pollination as explants of *Agave salmiana* Otto ex Salm-Dyck subsp. *salmiana* obtained from different municipalities of the state of Hidalgo, Mexico. After 30-40 days of having pollinated inflorescences or panicles, a total of 89 fruits were obtained, of which 1608 zygotic embryos were rescued *in vitro*, and from these somatic embryos were generated in a nutrient medium (MS) by Murashige and Skoog, supplemented with vitamins L2, 9 μM of the auxin 2,4-dichlorophenoxyacetic (2,4-D) and 1.3 μM of the cytokinin 6-6 benzylaminopurine (BAP). Calli were used in two experiments in the presence of 2,4-D auxins and α -naphthaleneacetic acid (NAA) in combination with the cytokinin BAP. After 40 days of incubation, it was observed that a large number of embryos and embryogenic calli were generated using 4.5-9.0 μM 2,4-D in combination with 0.4-1.3 μM BAP. *In-casa* pollination allows the formation of viable zygotic embryos, and these can be used as explants in somatic embryogenesis, this being an alternative to help the genetic conservation of *Agave* plants in Mexico.

Key words: *In-casa* pollination, embryo rescue, zygotic embryos, auxins

RESUMEN: El uso de embriogénesis somática puede ser una alternativa para producir en menos tiempo un gran número de plántulas somáticas a bajo costo; sin embargo, la viabilidad y el éxito de este método dependen de varios factores como el tipo de explante utilizado, el balance hormonal entre otros. En este sentido, el presente trabajo de investigación evaluó el uso de embriones cigóticos inmaduros obtenidos por polinización-cruzada *in-casa* como explantes de *Agave salmiana* Otto ex Salm-Dyck subsp. *salmiana* obtenidos de diferentes municipios del estado de Hidalgo, México.

Después de 30-40 días de haber polinizado inflorescencias o panículas se obtuvieron un total de 89 frutos, de los cuales se rescataron *in vitro* 1608 embriones cigóticos, y de estos se generaron embriones somáticos en un medio nutritivo (MS) de Murashige y Skoog, suplementado con vitaminas L2, 9 μM de la auxina 2,4-diclorofenoxiacético (2,4-D) y 1,3 μM de la citoquinina 6-6 bencilaminopurina (BAP). Los callos se utilizaron en dos experimentos con presencia de auxinas 2,4-D y el ácido naftalenoacético (NAA) en combinación con la citoquinina BAP. Después de 40 días de incubación, se observó que se generó una gran cantidad de embriones y callos embriogénicos utilizando 4,5-9,0 μM de 2,4-D en combinación con 0,4-1,3 μM de BAP. Después de 60 días de incubación, el porcentaje de germinación de embriones somáticos fue superior al 70% en todos los tratamientos, con una tasa de supervivencia superior al 95% en 21 días. La polinización cruzada *in-casa* permite la formación de embriones cigóticos viables, y estos pueden ser utilizados como explantes en la embriogénesis somática, siendo esto, una alternativa para ayudar a la conservación genética de plantas de *Agave* en México.

Palabras clave: Polinización-cruzada en casa, rescate de embriones, embriones cigóticos, auxinas.

INTRODUCTION

The genus *Agave* is endemic to America and is the largest and most diverse of the Asparagaceae family with 210 species of angiosperm and monocotyledonous plants; of these, 159 are native to Mexico (García-Mendoza A, 2002). An example is *Agave salmiana* that is distributed mainly in the Mexican states of Tlaxcala, Puebla, Mexico, and Hidalgo (MolinaVega *et al.*, 2021). This species is used in the elaboration of traditional foods, fibers production, pulque and mezcal, saponins, fructans and in obtaining biofuels (Álvarez-Chávez *et al.*, 2021). It is a semelparous species and takes 10 to 15 years to reproduce sexually. In comparison, asexual reproduction takes about five years with the formation of offshoots, being this form of propagation the most used for commercial purposes. However, this has led to the incidence of pests and diseases due to the decrease in genetic variability (Puente-Garza *et al.*, 2015). Plant tissue culture can be a biotechnological tool of great help to preserve the genetic diversity of this species and take advantage of its diverse uses. *In vitro* propagation of *Agave* by the proliferation of axillary buds has been reported in different species between them *A. salmiana* subspecies *crassispina* (Nava-Cruza *et al.*, 2015).

Somatic embryogenesis (SE) is an alternative to the mass culture of plants, which is characterized as a pathway for the regeneration of whole plants from a bipolar structure derived from somatic cells. This process is carried out through the induction of plant cells to dedifferentiate and enter a new biological development program and the subsequent expression and germination of the resulting embryos (Gutierrez-Mora *et al.*, 2012), by using growth regulators such as auxins and cytokinins. Auxins being an essential factor for the initial cell polarization and asymmetrical first division (Asghar *et al.*, 2023).

Some of the factors that have been studied to carry out the embryogenesis process in *Agave* are the type of explant used, the combination and hormonal concentration, osmotic conditions, concentration of amino acids and salts, etc. In the SE of *Agave spp.*, different explants have been used: leaf segments, in *A. victoria-reginae* (Rodríguez-Garay *et al.*, 1996), *A. salmiana* (FloresBenítez *et al.*, 2007), *A. vera-cruz* (Tejavathi *et al.*, 2007) and *A. tequilana* Weber, blue cultivar (Portillo *et al.*, 2007; Rodríguez-Sahagún *et al.*, 2011); stem tissues in *A. victoria-reginae* Moore (Martínez-Palacios *et al.*, 2003) and *A. fourcroydes* Lem (Monja-Mío & Robert, 2016); tissue of bulbil apices in *A. sisalana* (Nikam *et al.*, 2003) zygotic embryos in *A. angustifolia* Haw (Arzate-Fernández & Mejía-Franco, 2011; Reyes-Díaz *et al.*, 2017) The combination of hormones is another of the parameters that has been manipulated to induce ES in different species of *Agave*, being 2,4-D auxin in combination with other hormones the common one of all. Such as the study carried out on *Agave marmorata* in which it was combined with BAP, using mature seeds as explants (Alvarez-Aragón *et al.*, 2020).

Therefore, the use of somatic embryogenesis using zygotic embryos as explants of *Agave salmiana* Otto ex Salm-Dyck subsp. *salmiana*, a product of *in-casa* cross-pollination, could be a useful alternative in the production of plants, conservation of genetic diversity, and for the genetic improvement of this species.

MATERIALS AND METHODS

Plant material. In the April-June 2017 period, panicles and inflorescences of *Agave salmiana* Otto ex Salm-Dyck subsp. *salmiana* in the stage of sexual reproduction were collected in the municipalities El Arenal (N: 20° 10' 39.1" W: 98° 53' 56.1" 2217 m.s.n.m, N: 20° 10' 36.8" W: 98° 53' 54.5" 2239 m.s.n.m., N: 20° 10' 37.1" W: 98° 53' 51.5" 2246 m.s.n.m, N: 20° 10' 38.4" W: 98° 53' 53.1" 2267 m.s.n.m), Cardonal (N: 48° 78' 94.0" W: 22° 77' 97.3" 1961 m.s.n.m, N: 20° 36' 03.8" W: 99° 06' 40.0" 1953 m.s.n.m, N: 20° 36' 03.8" W: 99° 06' 40.0" 1953 m.s.n.m.), San Agustín Tlaxiaca (N: 51° 40' 99" W: 22° 18' 749" 2624 m.s.n.m, N: 50° 96' 66" W: 22° 25' 077" 2301 m.s.n.m) and Emiliano Zapata (N: 19° 39' 20.2" W: 98° 33' 10.4" 2502 m.s.n.m). The taxonomic classification was made according to dichotomous keys (Little & Gentry, 1983). The plant material was kept in the laboratory in containers with clean water (flower vases) at an average room temperature of 21-23 °C.

Pollen viability tests. Mature flowers were emasculated, and anthers were collected and placed on a sheet of bond paper at room temperature, for three days, to carry out the pollen harvest and its subsequent viability test. The viability of pollen grains was observed through the formation and growth of the pollen tube. The pollen grains were placed in Petri dishes (100 mm x 15 mm) with 25 mL of culture medium containing 102.69 g L⁻¹ sucrose, 0.02 g L⁻¹ boric acid, 0.287 g L⁻¹ calcium nitrate and 5 g L⁻¹ agar, subsequently incubated for 30 minutes at 25 °C (López Díaz & Rodríguez Garay, 2008).

***In-casa* cross-pollination.** After checking the viability of pollen and when the flower was receptive with the presence of a pollen drop in the stigma, pollination was carried out as described by Rodríguez-Garay *et al.*, (2018).

Callus formation. After 30 to 40 days post-pollination, the fruits were collected from the panicles and disinfected with 96% v/v alcohol and flamed within a laminar flow hood, then opened with a sterile scalpel and extracted the immature viable seeds (black color). The immature zygotic embryos were extracted with the help of a stereoscopic microscope, fine forceps, and a scalpel under aseptic conditions (Rodríguez-Garay *et al.*, 2018). These immature zygotic embryos were used as explants. Embryos were placed in Petri dishes (100 mm x 15 mm) containing 25 mL of MS medium; supplemented with 30 g L⁻¹ sucrose, L₂ vitamins (Phillips & Collins, 1979), 8 g L⁻¹ agar, 9.0 µM 2,4-D (Sigma-Aldrich D8407) and 1.3 µM of BAP (Sigma-Aldrich B3408) (Portillo *et al.*, 2007). The pH was adjusted to 5.8 prior sterilization in an autoclave. Five explants (immature zygotic embryos) were seeded in each Petri dish and incubated at 27 °C ± 2 °C for 40 days under total darkness.

Experimental method

To increase the number of genotypes that could form somatic embryos, the explants that formed calluses were distributed in two experiments that differed by the use of plant growth regulators and their concentration.

Experiment 1: A 3 × 3 bifactorial experiment was designed: The first factor was 2,4-D in concentrations of 4.5, 9.0, and 13.5 µM, in combination with the second factor BAP in concentrations of 0.4, 1.3 and 2.2 µM (Portillo *et al.*, 2007).

Experiment 2: A 3×3 bifactorial experiment was designed, using the auxin α naphthaleneacetic acid (NAA) (Sigma-Aldrich N0640) in concentrations of 1.34, 2.7 and 4.06 μM , and the cytokinin BAP in concentrations of 2.5, 5.0 and 7.05 μM (Flores-Benítez *et al.*, 2007).

Induction medium

For each of the experiments, MS medium (Murashige & Skoog, 1962), supplemented with 30 g L^{-1} sucrose, L_2 vitamins (Phillips & Collins, 1979) and 8 g l^{-1} agar was used, with plant growth regulator, respectively.

Each experiment resulted in nine treatments, with eight and seven replications respectively for both experiments. Each experimental replication consisted of four calluses per Petri dish (nodular calluses, reliable and beige in color). They were incubated at $27^\circ\text{C} \pm 2^\circ\text{C}$ for 40 days under total darkness. After this period, the number of calluses that could express somatic embryogenesis was quantified, as well as the number of somatic embryos formed in each callus before its maturation and germination.

Statistical analysis. A unifactorial analysis of variance (ANOVA) with a level of significance of 0.05 was performed, using the PAleontological STatistics program version 3.20 (Hammer & Harper, Norway).

The response surface methodology was used with the help of Design-expert software version 7.1.5 (Stat-Ease, Minneapolis), adjusting the data to a Miscellaneous 3^2 design with 4 points in the center to estimate the optimal conditions within the experimental area, (Table 1).

Table 1. Experimental range of the levels of the two independent variables in terms of real values in the experiments of induction of somatic embryogenesis in *Agave salmiana* Otto ex Salm-Dyck subsp. *salmiana*.

Variables	Symbol	Coded variable levels		
		-1	0	+1
6-bencilaminopurine (BAP)	X1	0.4 μM	1.3 μM	2.2 μM
2,4-diclorofenoxiacetic acid (2,4-D)	X2	4.5 μM	9.0 μM	13.5 μM

Maturation and germination of somatic embryos. The somatic embryos generated in both experiments were transferred to Petri dishes (100 x 15 mm) containing 25 mL of MS medium with the addition of 30 g L^{-1} sucrose, 500 mg L^{-1} glutamine, 250 mg L^{-1} casein hydrolysate and supplemented with L_2 vitamins, and 6 g L^{-1} phytigel (Sigma P8169) (Santacruz-Ruvalcaba *et al.*, 1998). For this purpose, eight embryos per Petri dish were incubated at $27^\circ\text{C} \pm 2^\circ\text{C}$ with a photoperiod of 16 h light for 60 days, to quantify the percentage of germination.

Propagation. Two germinated plants without roots were placed in each bottle containing 100 mL of MS medium supplemented with 30 g L^{-1} sucrose, 44.3 μM BAP, 0.18 μM 2,4-D, and 8 g L^{-1} agar (Santacruz-Ruvalcaba *et al.*, 1999). The pH was adjusted to 5.8 prior sterilization in an autoclave. The cultures were incubated at $27^\circ\text{C} \pm 2^\circ\text{C}$ for 60 days with a photoperiod of 16 h light to generate the formation of multiple shoots.

For rooting, the same light and temperature conditions were used; however, the culture medium did not contain plant growth regulators and with a salts reduction of 50% of the MS medium. Finally, the rooted plants were transplanted to vessels made of expanded polystyrene (EPS) with an equivalent mixture of peat moss, vermiculite, and soil (1: 1: 1). The survival rate of plants was quantified 21 days after the initiation of incubation.

RESULTS

Pollen viability tests in most of the collected parent plants indicated that 90% of the collected pollen could form a pollen tube. In Figure 1, the general process of the formation of zygotic embryos is depicted. 89 fruits were formed after 30 to 40 days as a product of the *in-casa* pollination. 25 out of 89 fruits were selected randomly, of which 1608 immature zygotic embryos were rescued. Seven of the rescued embryos had two cotyledons. 744 (46.3%) out of 1608 immature zygotic embryos could form calli, and 30 (1.8%) of them expressed somatic embryogenesis after 40 days of incubation in the induction medium; however, it was observed that only explant formed somatic embryos within 20 days.

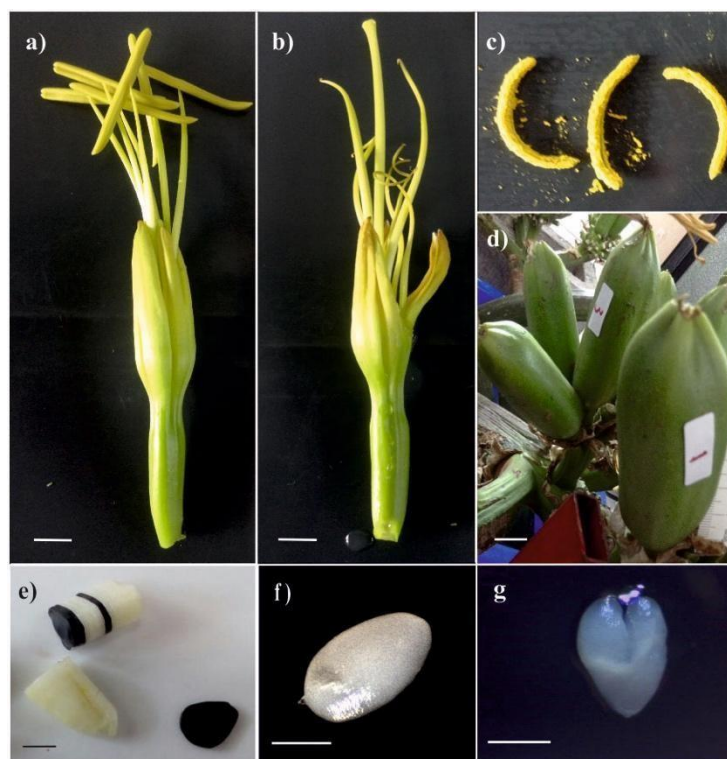


Fig. 1. Formation of zygotic embryos by *in-casa* cross-pollination of *Agave salmiana* Otto ex Salm-Dyck subsp. *salmiana*. **a)** Flowers ready to be emasculated (Bar = 1cm). **b)** Mature flower ready to be pollinated (Bar = 1cm). **c)** Mature and open anthers for pollen harvest. **d)** Fruit ripening (Bar = 1cm). **e)** Viable (black color) and non-viable (white color) seeds (Bar = 5mm). **f)** Monocotyledon zygotic embryo (Bar = 1mm). **g)** Dicotyledon zygotic embryo (Bar = 0.5mm).

The analysis of variance (ANOVA) for the results of experiment 1 showed that there was a statistically significant difference between the treatments, after quantifying the number of calli that expressed somatic embryogenesis ($p = 0.040$) (Table 2). The response surface methodology predicted the number of calli expressed by somatic embryogenesis as a function of BAP and 2,4-D, and the results were adjusted to a second-order equation:

$$y = 1.098 - 0.148 x_1 + 0.218 x_2 - 0.007 x_1 x_2 - 0.058 x_1^2 - 0.014 x_2^2$$

Table 2. Number of calli that expressed somatic embryogenesis of *Agave salmiana* Otto ex Salm-Dyck subsp. *salmiana* in nine treatments with different concentrations of growth regulators: 2,4-D / BAP.

BAP / 2,4-D	4.5 μM	9.0 μM	13.5 μM
0.4 μM	1.750 \pm 0.250 ^a	1.875 \pm 0.479 ^{ab}	1.125 \pm 0.227 ^c
1.3 μM	1.250 \pm 0.250 ^c	1.500 \pm 0.189 ^{ac}	1.125 \pm 0.227 ^c
2.2 μM	1.250 \pm 0.313 ^c	1.000 \pm 0.267 ^{bc}	0.500 \pm 0.189 ^c

*Number of calluses per Petri dish (Maximum value 4) \pm standard error

*Values with the same letter do not have a statistically significant difference ($p < 0.05$) using the LSD test.

The model predicted that the optimal concentration of plant growth regulators within the experimental area was 0.44 μM BAP and 7.39 μM 2,4-D with a response of 1.812 calli expressing SE. According to the model, the factor with the highest level of significance was auxin 2,4-D ($p = 0.003$). Figure 2 shows the response surface as a function of different concentrations of BAP and 2,4-D. It was found that there was no significant statistical difference between treatments ($p = 0.456$) when quantifying the number of somatic embryos formed.

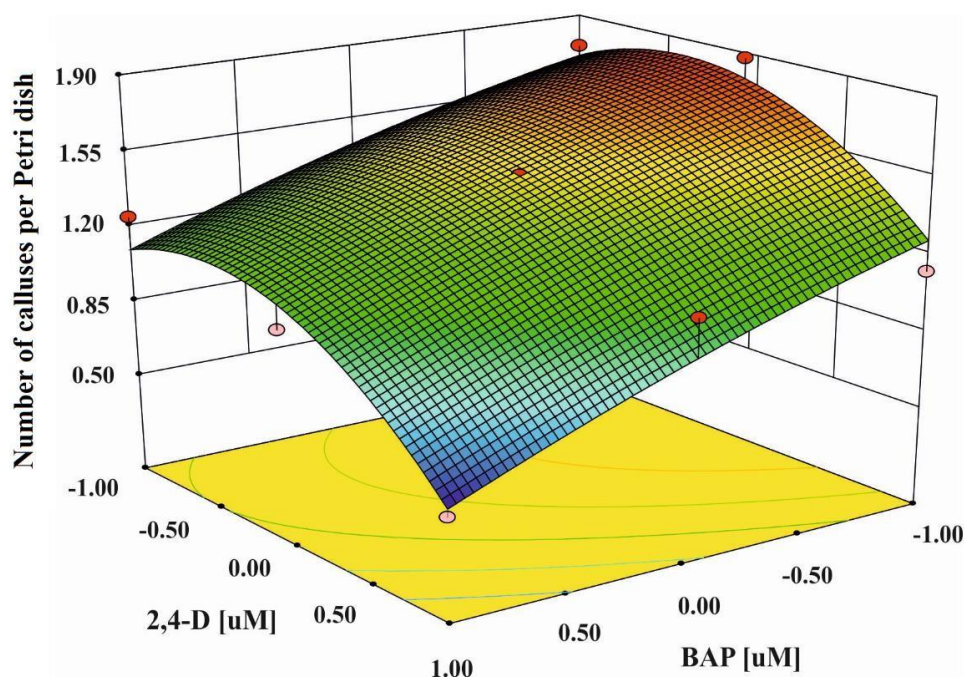


Fig. 2. Contour plot of the surface for the number of embryogenic calluses (Z) using the interaction of the variables (X1) cytokinin BAP and (X2) auxin 2,4-D. Having as answer the number of calluses formed per Petri dish (The coding of the data is shown in Table 1).

In experiment 2, the ANOVA demonstrated that there was no statistically significant difference between treatments when quantifying the number of calli that expressed somatic embryogenesis ($p = 0.315$), as well as in the number of somatic embryos formed ($p = 0.617$). However, when comparing the results of both experiments, a statistically significant difference was observed in the number of genotypes (each zygotic embryo) that expressed somatic embryogenesis ($p = 0.035$), and in the number of somatic embryos formed ($p = 0.000$) (Table 3).

Table 3. Comparison of two experiments with different auxin in combination with BAP for the SE of *Agave salmiana* Otto ex Salm-Dyck subsp. *salmiana*.

Experiment	Number of calluses per Petri dish	Number of somatic embryos per callus
(1) 2,4-D / BAP	1.264 ± 0.137	42.419 ± 5.858
(2) NAA / BAP	0.873 ± 0.099	10.698 ± 1.925

Number of calluses per Petri dish have a Maximum value $4 \pm$ standard error.

The germination percentage of the somatic embryos in experiments 1 and 2 was $97.11 \pm 0.98\%$ and $73.03 \pm 5.43\%$, respectively. There was a 95% survival rate as a result of the acclimatization. Figure 3 shows some images of the process.

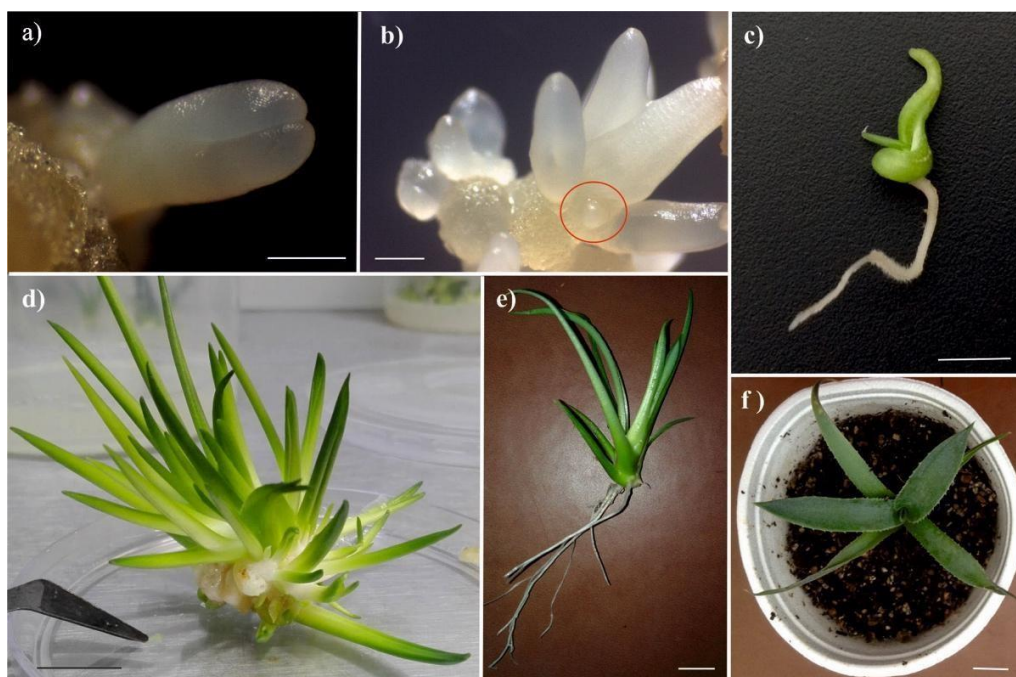


Fig. 3. *In vitro* propagation of *Agave salmiana* Otto ex Salm-Dyck subsp. *salmiana* by somatic embryogenesis. **a)** Dicotyledonous somatic embryo (heart) (Bar = 0.5 mm). **b)** Globular and torpedo structures seen under the stereo microscope on the surface of the embryogenic callus. (Bar = 0.5 mm). **c)** Germination of a somatic embryo (Bar = 5 mm). **d)** Bud formation (Bar = 1 cm). **e)** Rooted plant ready to be transplanted to soil (Bar = 1cm). **f)** Plant regenerated in substrate after 21 days (Bar = 1cm)

DISCUSSION

In-casa cross-pollination could be a useful tool for the conservation of the genetic diversity of agave plants of the same species and variety. The previous procedure can be carried out in the laboratory under controlled conditions for the production of hybrids through sexual reproduction (Rodríguez-Garay *et al.*, 2018), avoiding open pollination of unknown species/varieties. The results obtained in this proposal allowed the rescue of zygotic embryos, which were used as explants in the process of somatic embryogenesis, facilitating the selection of phenotypes and the production of cell lines with genetic diversity of *Agave salmiana* Otto ex Salm-Dyck subsp. *salmiana* for conservation and propagation purposes (Angeles Vázquez *et al.*, 2018).

The genus *Agave* is composed of monocotyledonous plants, which are characterized by having only one cotyledon in their embryonic phase (Rodríguez-Garay, 2016). However, in this work, embryos with two cotyledons were found, similar to those reported for *Agave tequilana* with an incidence of 4% (Ayala-González *et al.*, 2014). The presence of more than one cotyledon in this genus represents an opportunity to study the mechanisms that gave rise to its unique cotyledon since the phylogenetic groups of monocots and dicots are closely related (Ayala-González *et al.*, 2014).

The embryogenic calli obtained in this work showed beige, yellow, and green colorations, with friable and compact textures, many of them nodular. These characteristics have been found in embryogenic calli of *Agave victoria-reginae* (Martínez-Palacios *et al.*, 2003), *Agave sisalana* (Nikam *et al.*, 2003), and *Agave tequilana* (Portillo *et al.*, 2007), showing a specific morphology for embryogenic callus where they are generally friable and nodular of a beige coloration. After 20 days of incubation in the induction medium, somatic embryos were observed in some of the explants. This incubation period is shorter than that reported for other *Agave* species such as *A. victoria-reginae* (Rodríguez-Garay *et al.*, 1996), *A. tequilana* (Rodríguez-Sahagún *et al.*, 2011), *A. fourcroydes* (Monja-Mio & Robert, 2013), *A. sisalana* (Carneiro *et al.*, 2014). This period is attributed to the type of explant used, in which the suspensor plays a significant role since several studies indicate that it is made up of totipotent cells that have the possibility of producing somatic embryos (Liu *et al.*, 2015). In this work, it was observed that in most of the explants (zygotic embryos), the formation of embryogenic callus was initiated in the suspensor. As can be seen in Figure 3. At the end of 40 days of incubation, indirect embryogenesis occurred, which has been described in most *Agave* species (Arzate-Fernández & Mejía-Franco, 2011).

Recent studies have reported the use of zygotic embryos as explants to carry out the process of somatic embryogenesis in the *Agave* genus, rescued from seeds formed by open pollination, such as the one reported for *A. angustifolia*, *A. cupreata* and *A. salmiana*, the which were subjected to osmotic stress induced by the application of compounds such as polyethylene glycol (PEG) and abscisic acid (ABA), reaching percentages of embryogenic callus formation above 50% (Hernández-Solis *et al.*, 2023). Other factors have also been explored, such as the concentration of vitamins in the induction medium, such as Thiamine, using the same type of explants (embryonic axes) in *A. angustifolia*, demonstrating that the higher the concentration of this compound, it is possible to obtain a greater number of embryos somatic (Reyes-Díaz *et al.*, 2020.)

The addition of plant growth regulators in the *in vitro* culture indirectly causes local variations in the auxin concentration of the explant, possibly triggering the *de novo* synthesis and relocation of the endogenous auxin. Also, it has been demonstrated that the auxin analog 2,4-D can act directly as an inducer of genes related to stress (Fehér, 2015). Besides, it has been shown that it causes hypermethylation of DNA, inducing the expression of the MET1 gene that leads to the reprogramming of the genome and the acquisition of embryogenic competence (Leljak-Levanić *et al.*, 2004).

The auxin 2,4-D is the most used analog in SE protocols in different species, including the genus *Agave* (Monja-Mio & Robert, 2016). Finally, the results obtained in this work show that the SE of *Agave salmiana* Otto ex Salm-Dyck subsp. *salmiana* is favored by relatively high concentrations of 2,4-D with the addition of low concentrations of the cytokinin BAP, as established for other *Agave* species (Delgado-Aceves *et al.*, 2021).

CONCLUSIONS

The use of complete inflorescence of *Agave salmiana* Otto ex Salm-Dyck subsp. *salmiana in-casa* cross-pollination allowed obtaining viable zygotic embryos after 30 days post-pollination, which presented embryogenic capacity when using an MS medium supplemented with vitamins L2, 2,4-D and ANA in combination with the cytokinin BAP. The presence of 2,4-D auxin compared to ANA, in a 40-day incubation period in the dark, increased the number of somatic embryos formed and the number of embryogenic calli. The proposal to carry out *in-casa* crosspollination facilitates the conservation of diversity through the controlled pollination of a certain species or variety of *Agave*. Furthermore, it is possible to induce and express the embryogenic capacity of zygotic embryos of *Agave salmiana* Otto ex Salm-Dyck subsp. *salmiana*, this being an alternative to help the genetic conservation of *Agave* plants in Mexico.

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