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EFFECT OF PROMOTING COMPOUNDS OF INDIRECT SOMATIC EMBRYOGENESIS IN THREE *Agave* SPECIES

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ABSTRACT

The *Agave* genus is one of the largest and most widely used in Mexico, mainly for the production of alcoholic beverages. The species in this genus are characterized by their long-life cycles and limited sexual reproduction, which endangers them and demands the search for an alternative that allows mass multiplication and preservation of these species. Somatic embryogenesis (ES) is emerging as a solution to solve this problem, although it is a complex process that depends on a large number of factors and the development of species-specific protocols. Osmotic stress is one of these factors, a condition that can promote the formation and maturation of somatic embryos through the application of compounds such as polyethylene glycol (PEG) or abscisic acid (ABA). So far, no studies have been reported on the effect of osmotic stress on somatic embryogenesis in *Agave* species. In this work we evaluated its effect on the expression and maturation of somatic embryos in *Agave angustifolia*, *A. cupreata* and *A. salmiana*. Its formation was determined using two concentrations of ABA (3 and 9 mg L^{-1}) and two concentrations of PEG (50 and 70 g L^{-1}) in callus obtained from embryonic zygotic axes. Treatments with 9 mg L^{-1} ABA and 50 g L^{-1} PEG favored the formation of somatic embryos in the three species evaluated, in addition to the treatment with 70 g L^{-1} PEG, with which *A. angustifolia* was obtained. Somatic embryo formation was asynchronous, especially for *A. cupreata*. Seedling regeneration from somatic embryos was achieved in *A. angustifolia*, *A. cupreata* and *A. salmiana*.

Keywords: Maguey, osmotic stress, ABA, PEG, ripening.

INTRODUCTION

The *Agave* genus is one of the most widely used in Mexico, mainly for the production of alcoholic beverages, a sector that has grown significantly in recent years, drastically increasing demand for agaves. This has endangered several species due to their long-life cycles (7–15 years); in addition, their sexual reproduction is limited by the same production of alcoholic beverages, making asexual reproduction the main way of propagation (Esparza-Ibarra *et al*., 2015; Garcia-Mendoza, 2002). Such is the case for mezcal species such as *A. angustifolia*, most widely used for mezcal production; *A. cupreata*, which cannot reproduce asexually (Avendaño-Arrazate *et al*., 2015); and *A. salmiana*, a species from which pulque is obtained and has been recently used to obtain

saponins with medicinal potential (Leal-Diaz *et al*., 2016). Therefore, it is strongly advisable to develop propagation protocols that allow for efficient large-scale plant production using biotechnological techniques such as somatic embryogenesis (ES) (Monja-Mio and Robert, 2013).

Indirect somatic embryogenesis is the process by which somatic cells acquire the capability to develop a complete somatic embryo. This occurs through prior disorganization and dedifferentiation triggered by a wide variety of typically stressful stimuli, such as growing conditions, nutrient levels, or the application of plant growth regulators (RCV) (Fehér, 2015; Loyola-Vargas, and Ochoa-Alejo, 2016). This generally consists of two phases: callus induction to disorganize and dedifferentiate cells, and the expression or maturation of somatic embryos, the latter being one of the most problematic since important morphological changes occur for the correct development of somatic embryos, which are generally at different developmental stages (Márquez-Martín *et al.,* 2011; Vale *et al*., 2014).

Osmotic stress has been proposed as an important factor during somatic embryo maturation and has the potential to trigger rapid biochemical changes in the activity of specific proteins and genes during maturation (Leal *et al*., 1995; Kong *et al*., 1998). This restriction in water absorption, low turgor pressure, and reduction in intracellular osmotic potential have the ability to stimulate the maturation of somatic embryos by promoting the accumulation of essential lipids and proteins, simulating the natural desiccation that occurs during seed formation (Merkle *et al*., 1995; Svobodobá *et al*., 1999); in addition, it favors the development of apical and radical meristems (Valencia-Lozano *et al*., 2021).

In this regard, compounds such as polyethylene glycol (PEG) and abscisic acid (ABA) have been reported in other ES protocols (Acanda *et al*., 2020; Cruz *et al*., 2022). PEG is a large molecule that is unable to penetrate the cell wall, causing a decrease in the osmotic potential (Klimaszewska *et al*., 2000); whereas ABA is a molecule related to abiotic stress in general, with emphasis on its role in water stress tolerance and osmotic in face of stress synthesis (Parwes *et al*., 2022). The latter has been linked mainly to the ABI-3 gene and the embryogenic cell protein (ECP) genes, both of which belong to the late embryogenesis abundant (LEA) gene family, essential for the process of somatic embryogenesis (Kikuchi *et al*., 2006).

So far, there are no reports of the evaluation of the effect of osmotic stress on ES in the genus *Agave*, so the purpose of the present work was to evaluate the effect of PEG and ABA on the expression and maturation of somatic embryos in *Agave angustifolia*, *A. cupreata*, and *A. salmiana*.

MATERIALS AND METHODS

Plant material

For disinfection and *in vitro* establishment, the method proposed by Martínez-Martínez et al. (2021) was followed. Mature seeds of *Agave angustifolia*, *A. cupreata*, and *A. salmiana* were washed in tap water with 2 mL of liquid detergent and two

drops of Tween® 20 for 15 min. Subsequently, inside a laminar flow hood, seeds were immersed in 70 % ethanol for 1 min, then in 1 % sodium hypochlorite solution for 15 min, rinsed three times with sterile distilled water, and then immersed in 16 µL L^{-1} gentamicin for stratification for 48 h at 4 °C.

Embryogenic callus induction

Ten embryonic zygotic axes were placed in ten 100 x 15 mm plastic Petri dishes with 20 mL of culture medium per box. The culture medium for callus induction (MCICE) proposed by Alvarez-Aragón *et al*. (2020) was used, which was composed of MS salts (Murashige and Skoog, 1962) at 25 % concentration. It was supplemented with L2 vitamins (Phillips and Collins, 1979), 60 g L^{-1} sucrose, 5 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D, Sigma-Aldrich Chemical Company, MO, USA), 3 mg L^{-1} 6-benzylaminopurine (BA, Sigma-Aldrich Chemical Company, MO, USA) and 8 g L^{-1} agar (Sigma-Aldrich Chemical Company, MO, USA).

Cultures were incubated in complete darkness for 60 d at a temperature of 25 ± 2 °C. The percentage of embryogenesis was obtained as follows: [Number of responsive explants] * [100] / 10 = % embryogenic callus.

Somatic embryo maturation

Approximately 0.1 g of embryogenic callus was placed in culture medium for embryo expression and maturation (MCEMES) as proposed by Alvarez-Aragón *et al*. (2020), composed of MS salts at 50 % of their original concentration, 30 g L^{-1} sucrose, and 8 g L^{-1} agar. Two concentrations of ABA (3 and 9 mg L^{-1}) and two of PEG 6000 (50 and 70 g L^{-1}) were evaluated, in addition to the control treatment supplemented with 0.1 mg L^{-1} of 2,4-D.

The number of somatic embryos was determined at 30 d. The cultures were incubated at a temperature of 25 ± 2 °C in complete darkness during 60 d for *A. angustifolia* and *A. salmiana*, and 45 d for *A. cupreata*.

Somatic embryo germination and seedling growth

The obtained somatic embryos in the scutellar phase were transferred to germination culture medium (MCG) composed of MS salts at 50% of their original concentration, 30 g L^{-1} sucrose and 0.5 g L^{-1} activated charcoal (Sigma-Aldrich Chemical Company, MO, USA), free of RCV.

The cultures were incubated for 60 d at a temperature of 25 ± 2 °C and a 16 h photoperiod. The germination percentage was obtained as follows: [Number of germinated embryos] * [100] / total embryos in MCG = % germination.

Seedling acclimatization

Seedlings regenerated by ES, regardless of maturation treatment, were rinsed with tap water to remove all residue from the culture medium and transferred to trays with 2.2 x 3.6 cm phenolic foam cylinders (peatFOAM ®) and placed in a floating root

system (Martínez-Martínez *et al*., 2021). They were kept for 30 d in a polyethylene micro tunnel with a minimum of 70 % humidity at 27 ± 5 °C. The survival percentage was evaluated, which was obtained as follows: [Number of surviving plants] * [100] / total seedlings = % survival.

Statistical analysis

In the present study, the percentage of embryogenic callus, the average number of somatic embryos for each treatment, and the percentage of germination were evaluated. A completely randomized design was used, with three replicates per species. The data obtained were subjected to an analysis of variance using Stathgraphics software version 5.0. Where significant differences were found, a mean comparison test (DMS method) was performed with a significance level of 95 %.

RESULTS AND DISCUSSION

Induction of embryogenic callus

Explant dedifferentiation began after the first 12 d of culture initiation (ddic) in MCICE (Figure 1A) with no distinction in the area of the embryonic zygotic axes or their orientation in the culture medium. It differed from previous reports (Hartweck *et al*., 1988; Kysely and Jacobsen, 1990), in which disorganization and dedifferentiation occurred only at one explant site. Non-embryogenic callus with a compact, whitish and smooth appearance (Figure 1B) and embryogenic callus characterized by being friable with a beige coloration (Figure 1C) were obtained at 60 ddic, similar to what has been previously reported in indirect ES of *Agave* species (Portillo *et al*., 2012; Martínez-Martínez *et al*., 2021).

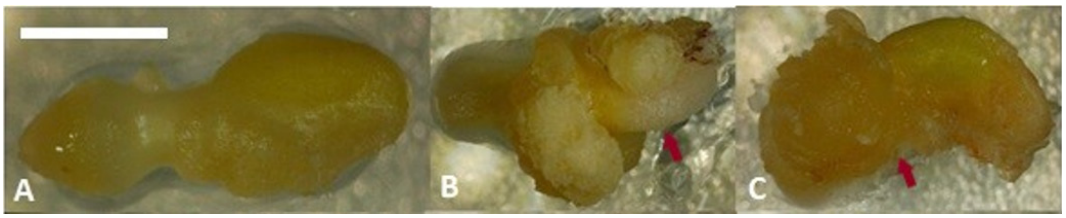

Figure 1. Callus formation in *Agave angustifolia*. A: explant with disorganization 12 days after initiation of culture; B: non-embryogenic callus (arrow); C: embryogenic callus (arrow). Bar: 5 mm.

The presence of both embryogenic and non-embryogenic tissue in the same culture is frequently reported in other models (Fehér *et al*., 2003). It is known that non-embryogenic cells can secrete molecules into the culture medium (Hecht *et al*., 2001), which, when sensed by other competent cells, promote the formation of somatic embryos (Pennell *et al*., 1992; Santa-Catarina *et al*., 2004). In this sense, the presence of non-embryogenic callus in the cultures may be necessary to obtain somatic embryos

of the three *Agave* species, where the expression of somatic embryos was observed in a section of the callus while presenting a morphology considered non-embryogenic in others (Figure 2).

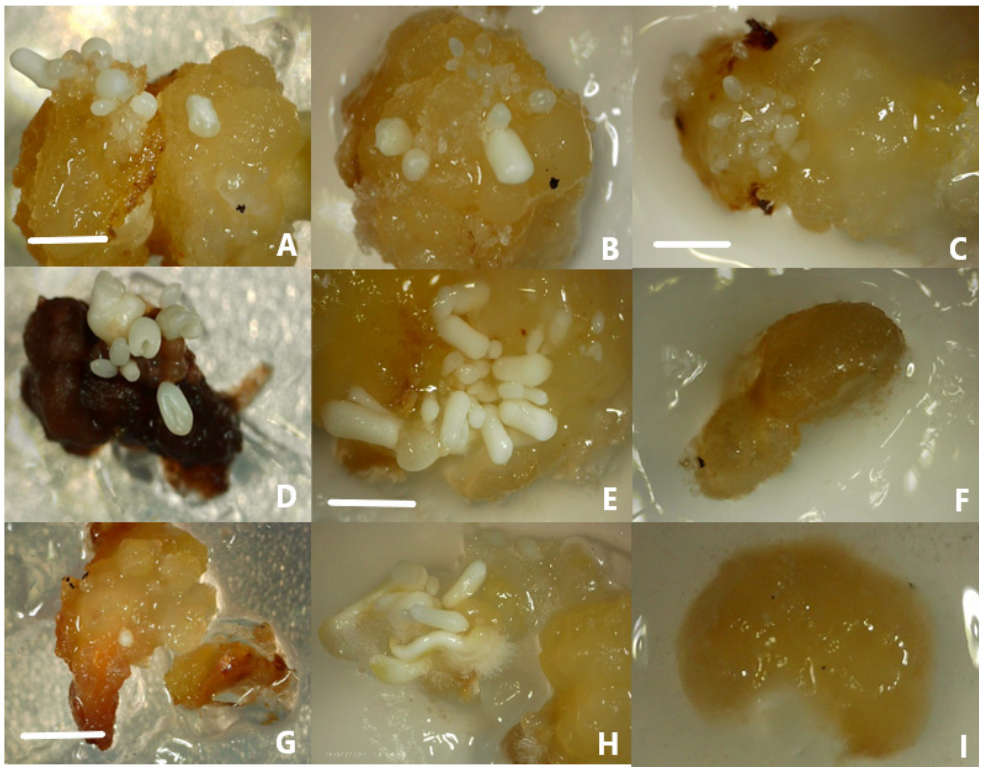

Figure 2. Expression and maturation of somatic embryos in *Agave angustifolia* (A, B, C), *A. salmiana* (D, E, F), and *A. cupreata* (G, H, I) after 30 d in MCEMES. From left to right: (A, D, G) treatment with 9 mg L^{-1} ABA; (B, E, H) 50 g L^{-1} PEG; (C, F, I) 70 g L^{-1} PEG. Bar: 5 mm.

Evaluation of osmotic stress on somatic embryo expression and maturation

The first somatic embryos were observed from day 18 (78 ddic) in the control for *A. cupreata*, in the treatment with 9 mg L^{-1} ABA for *A. salmiana*, as well as the 50 g L^{-1} PEG treatment for *A. angustifolia* and *A. salmiana.* Somatic embryo formation was observed in all three species at 90 ddic in treatments with 9 mg L^{-1} ABA (Figure 2A, 2D, 2G) and 50 g L^{-1} PEG (Figure 2B, 2E, 2H). In addition, somatic embryo formation was observed in *A. angustifolia* treated with 70 g L^{-1} PEG (Figure 2C). These findings support previous reports for other species such as *Picea abies* L. (Svobodobá *et al*., 1999), *Daucus carota* (Kikuchi *et al*., 2006), *Cicer arietinum* L. (Mishra *et al*., 2012), *Cunninghamia lanceolata* (Zhou *et al*., 2017), *Passiflora edulis* (Cruz *et al*., 2022), and *Ocotea catharinensis* Mez. (Santa-Catarina *et al*., 2004), where osmotic stress positively affected somatic embryo formation and maturation.

Statistical analysis revealed no significant differences between *A. angustifolia* and *A. salmiana*, as well as between the best treatments (50 g L^{-1} of PEG and 9 mg L^{-1} of ABA) for obtaining somatic embryos (Table 1). The number of somatic embryos obtained varied: within the same treatment, while in some explants an average of three was observed, others exceeded ten (Table 1). This is possibly due to the fact that the primary explants were embryonic zygotic axes obtained from open-pollinated seed, resulting in each callus having unique genetic characteristics that influenced the response to osmotic stress treatments (Merkle *et al.*, 1995; Namasivayam, 2007). In addition, genotype-specific differences could have played a role, given the variances among the three species in terms both the number of embryos and the percentage of embryogenesis. These differences are common among different species, even if they are of the same genus (Loyola-Vargas and Ochoa-Alejo, 2016).

Table 1. Expression and maturation of somatic embryos in osmotic stress treatments with ABA and PEG in *Agave angustifolia*, *A. salmiana* and *A. cupreata* at 90 days after initiation of culture.

Treatment	Species	Percentage of embryogenic callus	Number of embryos
Control	*A. angustifolia*	0.0 ± 14.64ab	0.0 ± 3.62 b
	A. salmiana	0.0 ± 14.64ab	0.0 ± 3.62 b
	A.cupreata	83.3 ± 14.64ab	11.2 ± 3.62b
ABA 3 mg L^{-1}	*A. angustifolia*	0.0 ± 14.64c	0.0 ± 3.62b
	A. salmiana	0.0 ± 14.64c	0.0 ± 3.62b
	A.cupreata	0.0 ± 14.64c	0.0 ± 3.62b
ABA 9 mg L^{-1}	*A. angustifolia*	33.3 ± 14.64a	5.0 ± 3.62a
	A. salmiana	33.3 ± 14.64a	15.4 ± 3.62a
	A. cupreata	33.3 ± 14.64a	4.8 ± 3.62a
PEG 50 g L^{-1}	*A. angustifolia*	66.6 ± 14.64a	8.3 ± 3.62a
	A. salmiana	16.6 ± 14.64a	14.0 ± 3.62a
	A.cupreata	50.0 ± 14.64a	3.0 ± 3.62a
PEG 70 g L^{-1}	*A. angustifolia*	33.3 ± 14.64bc	11.0 ± 3.62b
	A. salmiana	0.0 ± 14.64bc	0.0 ± 3.62b
	A.cupreata	0.0 ± 14.64bc	0.0 ± 3.62b

[†]Mean values per column with different letter are statistically different ($p \leq 0.05$).

In the three *Agave* species, ABA at a concentration of 9 mg L^{-1} favored somatic embryo development, assisting in genetic reprogramming by acting as the main messenger molecule in the face of abiotic stress (Karami and Saidi, 2010; Parwes *et al*., 2022). This has been reported in relation to several genes essential for ES such as embryogenic cell protein (ECP) genes or the ABI-3 gene in several species (Karami and Saidi, 2010; Kikuchi *et al*., 2006). It also interacts with other RCVs such as polyamines, which are crucial molecules for stress modulation that regulate ABA synthesis when applied

exogenously and which, in turn, are regulated by ABA produced by the same osmotic stress (Parwes *et al*., 2022).

PEG at 50 g L^{-1} favored the development of somatic embryos in all species (Figure 2B, 2E, 2H); however, at 70 g L^{-1}, its formation was only observed in *A. angustifolia* (Figure 2C). This is owed to the fact that their large molecules cause a restriction in water absorption; osmotic changes favor physiological changes in somatic embryos that help them acquire polarity with the development of meristems (Valencia-Lozano *et al*., 2021). PEG-treated cultures have been reported to increase meristematic activity, endogenous ABA content, and polyamines, as well as proteins related to glycolytic and light-responsive processes required for embryo development (Cruz *et al*., 2022), as well as the regulation in the gene expression related to differentiation and development (Vale *et al*., 2014). Both molecules, ABA and PEG, promote the accumulation of essential lipids and proteins in the maturation process of somatic embryos, being comparable to that of embryonic zygotic axes (Elhiti and Stasolla 2022; Merkle *et al*.,1995; Svobodobá *et al*., 1999).

The presence of both scutellar and globular phase somatic embryos was observed in all treatments, indicating a lag in the development of somatic embryos (Figure 3A). This is a common phenomenon in ES protocols which originates from embryogenic callus cells at different stages (Zegzouti *et al*., 2001; Souza *et al*., 2011). This variability in stages can be synchronized, mainly by filtering and separating embryogenic cells by type and size (Liu *et al*., 2021; Othmani *et al*., 2009; Souza *et al*., 2011), keeping cells at a similar stage while physically isolating embryogenic cells, a necessary condition for the conversion of these to somatic embryos (Lowe *et al*., 1985).

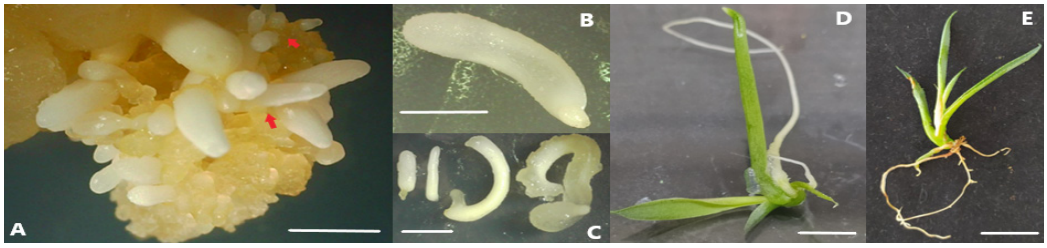

Figure 3. Indirect somatic embryogenesis in *Agave* spp. A: expression of somatic embryos at different stages (arrows) in callus under osmotic stress treatment at 30 d in MCEMES, bar: 5 mm; B: somatic embryo in scutellar phase, bar: 2 mm; C: somatic embryos obtained from osmotic stress treatments, normal scutellar somatic embryos (first two on the left) and elongated and deformed somatic embryos (last two on the right), bar: 5 mm; D: seedling after 60 d in MCG, bar: 10 mm; E: plant after 30 d in *ex vitro* acclimatization, bar: 3 cm.

Somatic embryo germination and seedling growth

After 60 d (*A. angustifolia* and *A. salmiana*) and 45 d (*A. cupreata*) in MCEMES, somatic embryos in the scutellar phase (Figure 3B) were transferred to MCG for germination. Even when *A. cupreata* somatic embryos of remained a shorter period of time in MCEMES, there were somatic embryos that developed faster and started to elongate and deform (Figure 3C), so they were not used in the germination phase.

A hundred percent (100 %) of the somatic embryos in MCG medium germinated and developed completely, with no morphological anomalies were observed, and the seedlings displaying green leaves and white roots. After 60 d at MCG, the seedlings showed two to four leaves measuring 4 to 6 cm in length and a developed root system (Figure 3D). The high percentage of somatic embryo germination in *Agave* spp. is similar to what has been reported previously (Alvarez-Aragón *et al*., 2020; Monja-Mio and Robert, 2013).

Seedling acclimatization

The survival rate of seedlings obtained by ES after 30 d of *ex vitro* acclimatization was 95 % (Figure 3E). This value is similar to previous results for the *Agave* genus, since these species do not face significant adaptation challenges (Portillo *et al*., 2007; Naziri *et al*., 2018). The plants obtained showed more intense leaf coloration, three to four leaves between 7 and 10 cm in length, in addition to a vigorous root system.

CONCLUSIONS

This is the first report of osmotic stress-induced somatic embryogenesis in *Agave angustifolia*, *A. salmiana* and *A. cupreata*. Osmotic stress induced by ABA and PEG favored the maturation of somatic embryos capable of regenerating seedlings in the three *Agave* species. Further research is needed to achieve better synchronization of somatic embryo developmental stages.

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