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Media culture factors affecting somatic embryogenesis in *Agave angustifolia* Haw

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ABSTRACT

A complete protocol of somatic embryogenesis involves induction of embryogenic callus, embryo development, embryo maturation, and their conversion or germination to form complete plants; in this sense judicious selection of nutrient medium, growth regulators, and physical culture environment is required. In this work, culture medium factors that influence somatic embryogenesis in *Agave angustifolia* were investigated. In an induction medium (IM) we evaluated the effect of three sucrose concentrations, three plant growth regulator (PGR) combinations, two groups of vitamins and two sources of amino acids. We observed that somatic embryos (SE) in medium containing 6% sucrose concentration grew vigorously, while those induced in medium with 8% sucrose were abnormally shaped and did not develop fully. In contrast, a higher sucrose concentration (10%) inhibited development of explants. Embryogenic callus cultured in IM containing 13.59 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 4.44 μM 6-benzyladenine (BA) only produced SE, while those explants assayed in IM with 11.34 μM abscisic acid (ABA) did not improve embryogenesis response. Phillips-Collins (L2) vitamins induced a higher number of SE (34 ± 0.4) than Murashige–Skoog (MS) vitamins (1.7 ± 0.7). High levels of amino acids (500 mg l^{-1} L-glutamine or casein hydrolysate) were not effective in promoting embryogenesis. Conversion frequency to plantlets ranged from 95 to 100% with 100% survival under *ex vitro* conditions.

1. Introduction

The *Agave* species have a great industrial potential for the production of food, cellulose, fibres, sugars, pharmaceutical compounds, syrups, sapogenins, and ornamental plants (Portillo et al., 2007), as an energy crop to produce biofuel and as raw material to produce alcoholic beverages such as tequila or mescal. This economic value results in a tremendous national and international demand of the *Agave* species, making the genus an important target for *in vitro* mass propagation and genetic improvement.

For the genus *Agave*, somatic embryogenesis protocols have been reported *for A. victoria-reginae, A. sisalana, A. tequilana, A. Veracruz* and *A. angustifolia* (Rodríguez-Garay et al., 1996; Martínez-Palacios et al., 2003; Nikam et al., 2003; Portillo et al., 2007; Tejavathi et al., 2007; Arzate-Fernández and Mejía-Franco, 2011). *A. angustifolia* is one of most important raw materials for production of high quality mescal.

Somatic embryogenesis forms the basis of cellular totipotency in higher plants. Under *in vitro* conditions, one or a few somatic cells of the explant should be competent to receive a signal (endogenous or exogenous) for the developmental restructuring towards the embryogenic pathway. It triggers the pathway of embryogenic development (commitment) leading to somatic embryo formation. In this fate, plant cells attempt to establish a new programme through changes in pH gradients of all cell compartments, arresting differentiated functions, reactivating the cell cycle and re-organising gene expression as well as metabolism (Feher et al., 2003). However, for a particular genotype, the *in vitro* forms of somatic embryogenesis, the optimum conditions (potential, competence, induction, and commitment) have to be experimentally optimised (Feher et al., 2003).

Thus, the establishment of improved micropropagation procedures

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; BA, 6-benzyladenine; ABA, abscisic acid; MS, Murashige–Skoog; PGR, plant growth regulator

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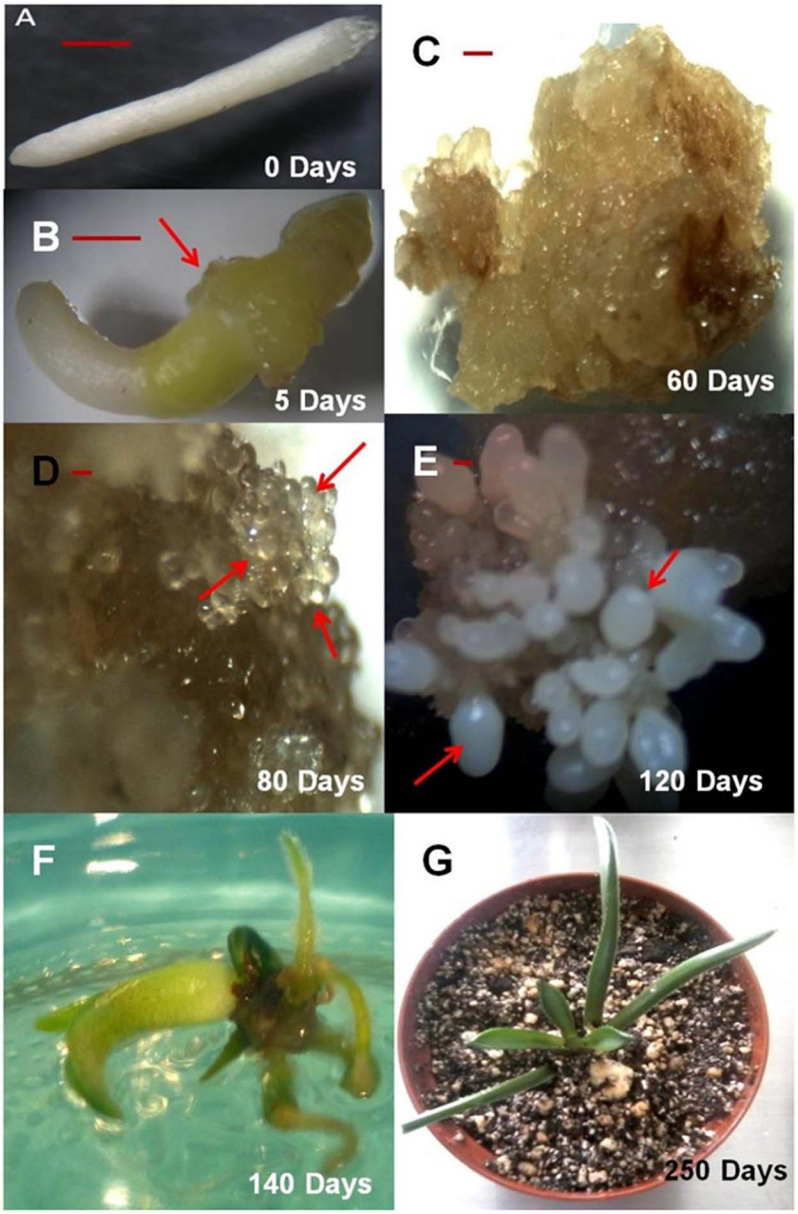

Fig. 1. Somatic embryogenesis in *Agave angustifolia* Haw. obtained with vitamins L2, 14 μM 2,4-D, 5 μM BA and 60 g l^{-1} of sucrose. (A): explant, (B): callus initiation, (C): creamy-white friable callus, (D): globular masses, (E): mature somatic embryos, (F): rooted plantlet, (G): plantlet exposed to natural conditions. Bar: 1 mm.

by testing media culture factors is desirable and could help to increase the efficiency of protocols. In this work, we present data on the influence of some culture medium components (highlighting carbon concentration, plant growth regulators and nitrogenous substances) on growth and development of embryogenic callus to optimize *in vitro* regeneration potential in *A. angustifolia*. The results showed that these factors affected induction frequency of embryogenic calluses, size of embryogenic calluses then differentiation potential of calluses for somatic embryo formation.

2. Materials and methods

2.1. Explant source and surface sterilization

The experimental materials were *Agave angustifolia* Haw seeds, collected from wild plants in Zumpahuacan, State of Mexico. The seeds were washed in running water with detergent for 10 min, surface-sterilized in 96% ethanol for 1 min, followed by immersion in 5% calcium hypochlorite solution (CaClO) for 15 min, and rinsing three times with sterile distilled water (SDW). Later, they were soaked in SDW for 96 h. Under laminar flow beach, aseptic mature zygotic embryos (Fig. 1A) were dissected from seeds and used as initial explants for callus induction.

2.2. Callus induction

Initial explants were placed in callus induction medium (IM) consisting in quarter-strength MS salt basal medium (Murashige and Skoog, 1962). In this IM, the following external factors were assessed: sucrose (60, 80 and 100 g l^{-1}), plant growth regulators (PGR) (13.59 μM 2, 4-D and 4.44 μM BA with or without ABA and only 11.34 μM ABA), supplemented or not with 500 mg l^{-1} L-glutamine or casein hydrolysate (as amino acids source), with or without MS (Murashige and Skoog, 1962) or L2 (Phillips and Collins 1979) vitamins. These factors were added to evaluate their influences on embryogenic callus induction (Table 1). Thus, 81 treatments were assayed. The pH of the medium was adjusted to 5.6–5.8 before adding the gelling agent (8 g l^{-1} agar) and autoclaving at 121 °C for 20 min. Petri dishes (9 × 1.5 cm) were filled with 25 ml of medium and sealed with Allupack®.

Each treatment consisted of 12 replicates each with ten explants.

Table 1
Factors evaluated and their effect on somatic embryogenesis of induced *Agave angustifolia* Haw calluses.

Factors						Callus induction (%)+		Callus weight of (g)+		Callus type	Somatic embryos per explant++	
Sucrose (g l^{-1})	Plant growth regulators (PGR) (μM)			Vitamins	Source of amino acids (500 mg l^{-1})							
	2,4-D	BA	ABA									
60 (T_0)	13.59	4.44	0	L2	None	30.6 ± 4.0	a	1.2 ± 0.5	b	E	34 ± 0.4	a
80	13.59	4.44	0	L2	None	28.0 ± 4.2	a	1.7 ± 0.3	a	E	17 ± 0.4	b
100	13.59	4.44	0	L2	None	18.0 ± 3.0	b	1.0 ± 0.7	b	NE	0	
60	13.59	4.44	11.34	L2	None	0		0		–	–	–
60	0	0	11.34	L2	None	7.2 ± 1.5	d	0.8 ± 0.2	bc	NE	0	
60	13.59	4.44	0	L2	L- glutamine	14.6 ± 3.0	bc	0.8 ± 0.4	bc	NE	0	
60	13.59	4.44	0	L2	Casein hydrolysate	10.6 ± 2.3	cd	0.7 ± 0.5	c	NE	0	
60	13.59	4.44	0	MS	None	18.6 ± 3.2	b	1.0 ± 0.2	b	E	1.7 ± 0.7	c
60	13.59	4.44	0	None	None	15.3 ± 2.3	b	0.9 ± 0.6	b	NE	0	

The medium consisted of quarter-strength MS salts and 8 g l^{-1} of agar. Mean ± standard error. Embryogenic callus (E). Non-embryogenic callus (NE). Means in a column with same letters are not significantly different by Duncan's multiple range test at $P < 0.05$. +60 days of cultures; ++ 120 days of culture. Data is from 12 replicates of 10 explants per replicate. T_0; control treatment.

The cultures were maintained in darkness at 25 ± 2 °C for 60 days. In this stage, the percentage of callus induction and callus weight were scored.

2.3. Somatic embryogenesis and plant regeneration

Sixty days after culture initiation (DACI), the creamy-white and friable calluses of those that responded to the treatments were transferred to embryo expression medium (EM): half-strength MS salts, 0.5 μM 2, 4-D, 30 g l^{-1} of sucrose, and gelled with 3 g l^{-1} gelrite. The medium was adjusted to pH as in the IM medium. The calluses were incubated under the same environmental conditions as in the preceding step for 60 days more. In this stage, the type of callus (embryogenic or not) and number of somatic embryos (SE) were scored.

For plant regeneration, all developed SE were transferred to flasks with germination medium (GM) (Arzate-Fernández and Mejía-Franco, 2011). All cultures were incubated at 25 ± 2 °C with a 16-h photoperiod under fluorescent light (16 μmol s^{-1} m^{-2}).

2.4. Hardening

In order to improve plant development and to enhance root proliferation, regenerated plantlets (4–5 cm in length) from SE were removed from culture flasks, and after washing their roots under running water, they were transferred to pots containing a mixture of compost, perlite and soil (1:1:1). They were maintained at 25 ± 2 °C with a 16-h photoperiod under fluorescent light (16 μmol s^{-1} m^{-2}) for 20 days and watered using a spray gun at 3-day intervals. Afterward, all regenerated plantlets were transferred to greenhouse conditions.

2.5. Statistical analysis

To evaluate the effect of each treatment, an analysis of variance (F test) and Duncan's multiple range test ($P < 0.05$) were performed using the data on percentage and weight of induced calluses (60 DACI) and number of somatic embryos (120 DACI).

3. Results and discussion

3.1. Callus induction, somatic embryogenesis and plant regeneration

Once the zygotic embryo swelled, early callus induction was observed at the apical end of the explant, especially 5 DACI (Fig. 1B). A creamy-white, friable callus (Fig. 1C) was observed on medium supplemented with 60 or 80 g l^{-1} of sucrose or with MS or L2 vitamins. In contrast, in the medium containing 100 g l^{-1} sucrose a creamy-brown callus that had stopped growing was observed. Maximum weight of callus (1.7 ± 0.3 g) was observed 60 DACI on the medium supplemented with 80 g l^{-1} of sucrose (Table 1).

Induced calluses on medium containing 2,4-D (13.59 μM) and BA (4.44 μM) plus sucrose 60 g l^{-1} with L2 or MS vitamins or 80 g l^{-1} with L2 vitamins responded to somatic embryogenesis 60 DACI, and after 20 days on expression medium, they produced globular masses at the surface (Fig. 1D), the somatic embryos at the globular stage were characteristically creamy-yellow in colour, this morphological characteristic coincides with that reported by Tejavathi et al. (2007) and Arzate-Fernández and Mejía-Franco (2011) in *A. vera-cruz* and *A. angustifolia*, respectly. After 50–60 days (110–120 DACI) of incubation on embryo EM, the embryogenic calluses developed clusters of mature somatic embryos (Fig. 1E). However, the globular structures obtained in the induction medium with MS vitamins did not differentiate into mature somatic embryos.

Somatic embryos started to germinate when cultured on plant growth regulator-free half-strength MS medium and developed into well-rooted plantlets 140 DACI (Fig. 1F). Even though the induced calluses in medium with 80 g l^{-1} of sucrose were embryogenic, those hyper-hydric somatic embryos that developed from them did not develop into plantlets.

Overall, the most effective medium for induction of embryogenic callus and regeneration of *A. angustifolia* plantlets contained quarter-strength MS salts and L2 vitamins with 13.59 μM 2,4-D and 4.44 μM BA plus 60 g l^{-1} of sucrose (Table 1). The conversion frequency of somatic embryos to plantlets varied from 95 to 100%.

3.2. Effect of evaluated factors

3.2.1. Sucrose

It is well known that carbohydrates control several developmental processes in the cells. Sucrose is the most commonly used carbohydrate in plant tissues culture because it has multiple roles, including prevision of carbon and energy, causing an osmotic effect (Hazarika, 2003). In our study, the effect of sucrose concentrations significantly affected maturation and subsequent germination and conversion of *A. angustifolia* somatic embryos; similar results were reported by Arzate-Fernández and Mejía-Franco (2011).

These findings are similar to those reported by Trembley and Tremblay (1991), Shibli et al. (1992), George (1993), Mehta et al. (2000), Huang and Liu (2002), and Kim and Kim (2002), who considered that the addition of sucrose in the culture medium acts as an osmotic agent that may introduce stress in somatic cells towards the

embryogenic pathway allowing the formation of somatic embryos. However, callus growth and embryo formation decrease in the presence of high sucrose concentration because it negatively affects chlorophyll formation causing a reduction in optimal callus turgor and embryo formation.

3.2.2. Plant growth regulators

All aspects of plant development are regulated by plant hormones which may act individually or in a concerted fashion. In tissue cultures, internal hormone concentrations are, in a very complex way, influenced by the plant growth regulators (PGR) present in the culture medium.

In this sense, abscisic acid (ABA) has been reported to be an important PGR for somatic embryogenesis, especially in the stages of embryo induction and maturation. It synchronizes the cultures and inhibits aberrant development during transition from globular to later stages. Exogenous ABA promotes synthesis of embryo-specific proteins and mRNA in order to enhance somatic embryo formation (Kikuchi et al., 2006). However, throughout the present assay, the addition of ABA (alone or with 2,4-D and BA) was ineffective in promoting callus induction and consequently embryo formation (Table 1).

It has been reported that auxins and cytokines in the medium promote callus formation. These PGR have a spectrum of biological activities. At the cell level, responses to these PGR involve rapid changes in cell expansion, effects on cell division and meristem activity, as well as differentiation of specific cell types (Seijo, 2000). In this respect, it has been suggested that the induction of somatic embryogenesis is promoted by a physiological stress function of auxins, especially 2,4-dichlorophenoxyacetic acid (2,4-D). Indeed, many authors have postulated that presence of 2,4-D in the medium for induction of somatic embryogenesis is necessary for *A. victoria-reginae, A. vera-cruz, A. sisalana* and *A. tequilana* (Rodríguez-Garay et al., 1996; Martínez-Palacios et al., 2003; Nikam et al., 2003; Portillo et al., 2007; Tejavathi et al., 2007). In a previous report, Arzate-Fernández and Mejía-Franco (2011) suggest that use of 13.59 μM 2,4-D for induction of somatic embryos in *A. angustifolia* is recommendable. Similar findings were observed in our study when that concentration was replicated as the control treatment (T_0) (Table 1).

3.2.3. Amino acids source and vitamins

As pointed out by Kohlenbach (1978), the source of organic nitrogen in the medium plays a crucial role in manipulating embryogenesis. As a nitrogen source, L-glutamine alone or in combination with casein hydrolysate, has been reported to be critical for somatic embryogenesis, which may contribute to the enlargement of the nucleotide pool required for rapid cell proliferation and growth of the embryogenic tissue. Moreover, Tejavathi et al. (2007) suggested the use of L-glutamine in the phase of maturation-conversion of somatic embryos for good results in *A. vera-cruz*. According to our results, a nitrogen source is not essential for embryo induction because no somatic embryos were observed in calluses that developed on medium containing amino acids as a source of organic nitrogen.

On the other hand, it has been mentioned that vitamins are nitrogenous substances synthesized in suboptimal quantities to serve catalytic functions in enzyme systems, especially in plant cells grown in *in vitro* conditions. Thus, culture media are often supplemented with vitamins (standard media formulations and modifications thereof) to enhance growth (Al-Khayri, 2001). In our study, L2 vitamins tended to be superior in terms of a higher number of somatic embryos (34 ± 0.4), whereas frequency of somatic embryos decreased (1.7 ± 0.7) when MS vitamins were used (Table 1). One of the major differences between MS and L2 vitamins is the thiamine concentration (0.1 and 2 mg l^{-1}, respectively). It has been reported that an essential compound in media for plant tissue culture is the vitamin B1 or thiamine, which has been considered an enzymatic cofactor in carbohydrate metabolism, glycolysis, the pentose phosphate pathway and the tricarboxylic acid cycle (Goyer, 2010). In this sense, Bunik and Fernie (2009) reported that thiamine is essential in the Krebs cycle for energy production, nitrogen assimilation and amino acid metabolism. Also, Kruger and Von Schaewen (2003) mentioned that thiamine is a cofactor utilized by the enzyme transketolase in the Calvin cycle where ribulose-1,5-bisphosphate is regenerated from phosphoglycerate formed during CO_2 fixation. In addition, thiamine has been also shown to play an important role as cofactor in response to abiotic (water, osmotic, salt, light, oxidative, and heat) and biotic stress in plants (Goyer, 2010).

3.3. Hardening

Regenerated and well-developed plantlets were transferred to a mixture of compost, perlite and soil. Plantlets transferred to pots and kept for 20 days under controlled environment survived well when exposed to natural conditions (Fig. 1G). The survival rate of these plantlets was 100%. No morphological variant among somatic embryo-callus regenerated plants was observed.

4. Conclusions

Recently, cloning of *Agave* species has been successfully achieved throughout the world. Signalling molecules and stress conditions of the isolated somatic cells under given *in vitro* conditions will force the cells towards the embryogenic pathway. Competent cells can respond to a variety of conditions by the initiation of embryogenic development. It can also be hypothesized that the initiation of somatic embryogenesis using *Agave* zygotic embryos is a general response to a multitude of parallel signals (including growth regulators, stress factors, carbon source, vitamins, and alteration of gene expression).

The present study identified some of the factors that affect *A. angustifolia* somatic embryo production. While the concentration of exogenous auxin is the most critical factor in the embryogenic process, others have major effects on somatic embryogenesis, as is the case of sucrose concentration and vitamin formulation.

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