Investigation note

# Sucrose and organic nitrogen sources have an influence in *Agave angustifolia* somatic embryogenesis

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## Abstract

The somatic embryogenesis protocol in *Agave angustifolia* involves the induction of embryogenic calluses, the development and maturation of embryos and their conversion or germination to form a complete plant. In this sense, a judicious selection of the nutrients present in the culture medium is required. In this work, the effect of sucrose concentrations and sources of organic nitrogen on the somatic embryogenesis of *A. angustifolia* was studied. Our results showed that the induction of embryogenic callus and the production of somatic embryos can be controlled positively by changes in the sucrose concentration and are affected by the addition of amino acid sources. The frequency of conversion to seedlings ranged from 90 to 95% with a 100% survival in *ex vitro* conditions.

**Keywords:** *Agave angustifolia*, casein hydrolysate, glutamine, somatic embryogenesis, sucrose.

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*A. angustifolia* is one of most important raw materials for production of high quality mescal, this economic value results in tremendous national and international demand making the specie an important target for *in vitro* mass propagation and genetic improvement.

Somatic embryogenesis is a desirable mode of plant regeneration. However, for particular genotypes, *in vitro* culture conditions and the stages of somatic embryogenesis (acquisition of embryogenic competence, induction and maturation of somatic embryos and conversion to seedlings) must be experimentally optimised especially the compounds of the culture medium.

In this sense, carbohydrates play vital role in plants life, they are substrate of respiration, perform function in synthetic pathway of many compounds and build blocks of macromolecules that may control several developmental processes in the cells (Smeekens, 2000). Sucrose is the most common carbohydrate source used in plant tissue culture and present dominantly in the phloem affected the formation of somatic embryos in culture medium (Nakagawa *et al*., 2001).

On the other hand, in the somatic embryogenesis the growth of cells has high energy demands and synthesize large amounts of proteins and nucleic acids. It is an alternative energy source for rapidly dividing cells and cells that use glucose inefficiently. Cells require nitrogen atoms to build molecules such as nucleotides, amino acids, amino-sugars and vitamins. When glucose levels are low and energy demands are high, cells can metabolize amino acids from organic nitrogen sources for energy. In this sense, glutamine is one of the most readily available amino acids for use as an energy source and it is a major source of energy for many rapidly dividing cell types in vitro because it plays an important role in nitrogen assimilation as it is an intermediate in the transfer of ammonia into amino acids. Likewise, casein hydrolysates can be a source of calcium, phosphate, several microelements, vitamins and most importantly, a mixture of up to 18 amino acids.

Sucrose (6%) only has been used as a carbon source for the induction of somatic embryos in *A. angustifolia* (Arzate-Fernández and Mejía-Franco, 2011), but the effect of different sugar concentrations or the influence of sources of amino acids (glutamine and casein hydrolysate) on somatic embryogenesis of *Agave* has not yet been investigated. In the present work, the effect of different sucrose concentrations and its interaction with organic nitrogen on somatic embryogenesis of *A. angustifolia* has been studied.

To carry out our research, aseptic mature zygotic embryos were dissected from *Agave angustifolia* Haw seeds and used as initial explants for callus induction. This were placed in callus induction medium consisting of quarter-strength MS salt basal medium (Murashige and Skoog, 1992) with 3 mg 2,4-D $L^{-1}$ and 1 mg BA $L^{-1}$ supplemented with L2 vitamins (Phillips and Collins, 1979), in this stage five levels of sucrose (40, 50, 60, 70 and 80 g $L^{-1}$) and organic nitrogen (500 mg L-glutamine or Casein hydrolysate) on the induction of somatic embryogenesis from callus of *Agave angustifolia* Haw. were evaluated. Medium pH was adjusted to 5.6-5.8 before adding the gelling agent (8 g agar $L^{-1}$) (Sigma-Aldrich®) and autoclaving at 121 °C for 20 min. Thus, ten random treatments were assayed. Each treatment consisted of 12 replicates of ten explants each. The cultures were maintained in darkness at 25 ±2 °C for 60 days.

At this stage, the percentage of callus induction and callus weight were recorded. At this stage, the percent of formation and weight of callus were registered. Sixty days after culture initiation (DACI), the calluses of the explants that responded to the treatments for callus induction were transferred to embryo expression medium: half-strength MS salts, 0.5 mg 2,4-D $L^{-1}$, and 30 g sucrose $L^{-1}$, gelled with 3 g Gelrite® $L^{-1}$. The calluses were incubated under the same environmental conditions as in the preceding step for another 60 days. At this stage, the number of somatic embryos (SE) was registered. For plant regeneration, all developed SE were transferred to flasks with germination medium (Arzate-Fernández and Mejía-Franco, 2011). In order to improve plant development and to enhance root proliferation, regenerated plantlets (4-5 cm in length) from SE 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 three day intervals. Afterward, all regenerated plantlets were transferred to greenhouse conditions. To evaluate the effect of each treatment, using the data on percentage and weight of induced calluses (60 DACI) and number of somatic embryos (120 DACI), an analysis of variance (F test) and Tukey's multiple range test ($p< 0.05$) were performed in Statgraphics PLUS® software.

As a result, embryogenic calli were soft and yellowish (Figure 1A), starting initiation at the apical end of the explant, this response was observed in all treatments. Somatic embryos were induced when they transferred to expression media from embryogenic callus cultured in different concentrations of sucrose (40, 50, 60, 70 and 80 g $L^{-1}$) and organic nitrogen (500 mg of L-glutamine or Casein hydrolysate). After 1 week, globular-shaped embryos were further developed into heart and torpedo-shaped embryos.

Cotyledonary embryos were observed on the most (90-95%) of the calli 60 DACI after subculture (Figure 1B). Various stages of somatic embryogenesis were observed simultaneously on the same callus in culture media added with nitrogen sources (Figure 1C) indicating that somatic embryogenesis in *A. angustifolia* is an asynchronous phenomenon in these conditions causing the number reduction of somatic embryos in a cotyledonary state at 120 DACI (Table 1). Table 1 also shows the number of embryos on embryogenic callus when different sucrose concentrations were used. Somatic embryogenesis was significantly increased with increasing sucrose concentration, but the development of normal embryos was low at the higher concentration of 80 g $L^{-1}$.

Induced somatic embryos were transferred onto expression medium where they developed into entire plantlets within 2 weeks (Figure 1D). Average germination rate of somatic embryos was about 90-95%. One hundred percent of rooted plantlets were successfully transferred in to mixture of compost, perlite and soil when they developed into normal plants in the greenhouse with an average of 95% survival. Phenotypic variability was not observed in plants in this experiment.

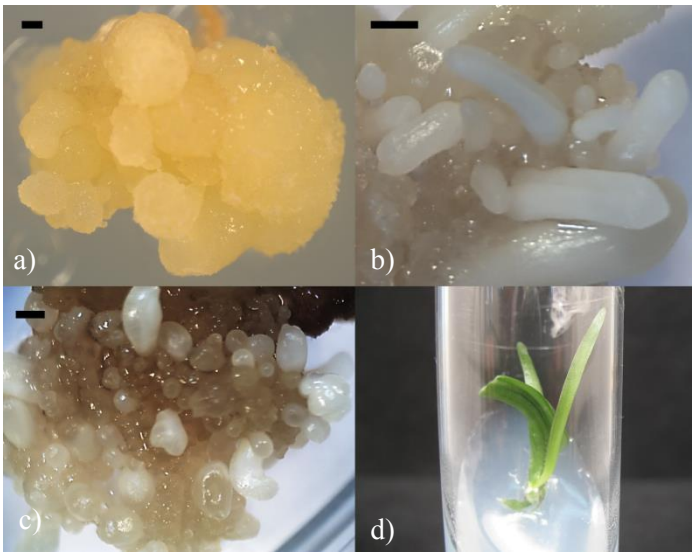


**Figure 1. Somatic embryogenesis in *Agave angustifolia*. a) cmbryogenic callus; b) cotyledonary embryos, c) variability of somatic embryos on the same callus in culture media added with nitrogen sources; y d) plantlet** (Bar: 1 mm).

Although the regeneration of *A. angustifolia* via indirect somatic embryogenesis has previously been reported (Arzate-Fernández and Mejía-Franco, 2011). Availability of the sugar in the culture medium has been found to affect somatic embryogenesis in many plant species (Mehta *et al*., 2000; Huang and Liu, 2002; Kim and Kim, 2002). In this study, increasing sucrose concentration enhanced induction of embryogenic callus and somatic embryos (Table 1). Plant cell, tissue or organ culture normally requires the incorporation of a carbon source to the culture medium (George, 1993) and sucrose has been used as the major carbon source in tissue culture.

**Table 1. Effect of five levels of sucrose and its interaction with organic nitrogen on the induction of somatic embryogenesis from callus of *Agave angustifolia* Haw.**

| Sucrose (g $L^{-1}$) | Organic nitrogen* | Formation of callus (%)+ | Weight of callus (g)++ | | Somatic embryos per explant+++ | |
|---|---|---|---|---|---|---|
| 40 | - | 35.83 ±3.57 | 0.62 ±0.04 | e | 8.2 ±0.47 | f |
| | + | 40.83 ±3.12 | 0.52 ±0.02 | f | 5.02 ±0.3 | g |
| 50 | - | 37.5 ±3.28 | 0.91 ±0.02 | c | 19.33 ±0.36 | c |
| | + | 40 ±3.25 | 0.67 ±0 | e | 11.02 ±0.46 | e |
| 60 | - | 33.58 ±1.38 | 1.22 ±0.06 | b | 35.71 ±0.27 | a |
| | + | 39.16 ±2.87 | 0.94 ±0.03 | c | 23.41 ±0.68 | b |
| 70 | - | 38.33 ±3.21 | 1.64 ±0.05 | a | 34.53 ±0.69 | a |
| | + | 32.5 ±2.78 | 0.78 ±0.02 | d | 18.71 ±0.28 | c |
| 80 | - | 28 ±4.2 | 1.7 ±0.3 | a | 17 ±0.4 | c |
| | + | 37.5 ±2.75 | 1.26 ±0.05 | b | 16.22 ±0.22 | d |

Mean ± standard error; *= without (-) or with (+) Casein hydrolyzate (250 mg $L^{-1}$) and L-glutamine (250 mg $L^{-1}$). Means in column with same letters are not significantly different by Tukey's multiple range test at $p< 0.05$. +, ++= 60 days of cultures; +++= 120 days of culture. Data is from 10 treatments with 12 replicates of 10 explants per replicate.

Sucrose can serve as a carbon source during somatic embryogenesis (Kim and Kim, 2002) and also as an osmotic regulator (Biahoua and Bonneau, 1999). However, it is a common knowledge that the role of high sugar concentration in somatic embryogenesis may impact the cell osmolarity (Hazarika, 2003). Therefore, the role of sucrose in the present study could be interpreted as both nutritional and osmotic regulatory functions of this carbohydrate. The result of this study showed that higher concentrations of sucrose improve maturation of somatic embryos (Table 1). Increasing sucrose concentration in the medium may create the osmotic stress, but helps to improve somatic embryogenesis. Therefore, it could be suggested that, osmotic effect of sucrose may cause normal development of somatic embryos. The positive effect of high osmolarity may mimic the osmolarity alterations that occur surrounding the embryo in nature (Merkle *et al*., 1995).

On the other hand, casein hydrolysate and glutamine have been the principal sources of nitrogen utilized in tissue culture and the growth of *A*. *angustifolia* callus tissue was significantly affected by the addition of amino acids specifically glutamine and casein This answer suggested that organic nitrogen was a growth-limiting factor in agave cultures. Also, the type and concentration of amino acid significantly affected the expression of *A*. *angustifolia* somatic embryos, as concentration increases the development decreases (Table 1).

## Conclusions

Induction of embryogenic callus on zygotic embryos explants and the production of somatic embryos from embryogenic calli could be controlled by changes in sucrose concentration and is affected by the addition of sources of amino acids. The results of this study also showed that the high percentages of somatic embryos could successfully be regenerated to form entire normal plants. Establishment of conditions required for the high frequency of regeneration via somatic embryogenesis would facilitate protoplast culture, somatic hybridization, genetic transformation and artificial seed production in *A*. *angustifolia*.

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