In Vitro Initiation and Early Maturation of Embryogenic Tissue of *Quillaja Saponaria*

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Abstract

A. Vega and D. Prehn. In vitro initiation and early maturation of embryogenic tissue of *Quillaja saponaria*. Quillay (*Quillaja saponaria*) is an endemic tree that grows between IV and IX Region of Chile. The factors that affect the generation of somatic embryos were determined with the objective to study their clonal propagation. Hypocotyls of seeds and apexes of selected adult trees were used for the in vitro culture. The embryogenic induction was performed in 0.5xMS medium supplemented with 13.5 μM 2,4-D and 4.4 μM BAP, during 8 weeks. Later, the embryogenic masses accumulated reserves in medium with 15 mg·L-1 of ABA during 4 weeks. The addition of 4.4 μM BAP to the medium promoted the maturation of pro-embryos to embryos in globular state (Phase Maturation I). In addition, we determined that supplements of casein hidrolysate, magnesium sulphate and glutamine favored the development of the somatic embryos. However, the decrease in the concentration in the gelling agent and the variation in the concentration of oxidized and reduced sources of nitrogen did not significantly affect the maturation. These effects allow developing somatic embryos until globular state, from young and adult tissue of *Q. saponaria*.

Key words: *Quillaja saponaria*, saponins, somatic embryogenesis.

Cien. Inv. Agr. 32(3). 156-166. 2005

INTRODUCTION

Quillaja (*Quillaja saponaria* Molina) is an endemic tree that grows between the 4th and 9th Regions of Chile (Lara, 1997). Saponins are extracted from its wood and bark. These molecules, of great economic importance, are used with pharmaceutical, industrial and agronomic purposes (Hoffmann, 1995; Kensil *et al*. 1991). Besides, they play an important role in the human and animal immunological systems (Osbourn, 1996).

The high demand for bark from *Q. saponaria*, the main source of saponins, and the change in the use of soils have decreased the existence of adult Quillaja trees, especially in the central area of Chile. This has motivated the development of a silvicultural and a forest management project for this species, supported by a propagation program (Prehn *et al*., 2003).

The objective of this propagation program was to develop reproduction protocols for

Received on 09 November 2004; Accepted on 13 August 2005
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quillaja trees in vegetative ways, with a high content of good quality and low toxicity saponins. To achieve this goal, micropropagation techniques were developed from young and adult tissues. One way of micropropagation was based on somatic embryogenesis (formation of an embryo from a cell), without gametes fusion (Tisserat *et al.*, 1979). This method includes an induction stage, which requires the presence of auxins, and an embryo maturation and germination stage in mediums with low levels or auxin free (George, 1993).

Embryogenesis has become the most used method, because of its high coefficient of short term multiplication, the possibility to capsule the embryo for artificial seeds production and the cryopreservation of embryogenic tissue. This method is also used for the possibility to achieve juvenility and for its lower somaclonal variation (Lizt and Jarret, 1991).

Implementing this technique allows the development of many future alternatives for quillaja; for example, improving genetic advances and decreasing the period between generations in an improvement program, multiplication of selected clones, germplasm storage, and rescue of embryos (Haines, 1994; Klimaszewska *et al*. 2005).

This work had the purpose to study the effect of hormone components, the sources of nitrogen and poliethylenglycol in the initiation, maintenance and early maturation of quillaja embryogenic tissue. Besides, the somatic embryos were characterized in the first states of in vitro culture.

MATERIALS AND METHODS

Cultivation medium

The basal medium used in this research was 0.5xMS with half the concentration of basal salts and vitamins, as described by Murashige and Skoog (1962), supplemented per liter with: 50 mg ascorbic acid, 500 mg of hidrolized casein, 185 mg of MgSO4x7H2O, 500 mg of glutamine, 30 mg of alanine, 80 mg of arginine, 250 mg of asparragine, 30 g of sucrose, 6 g Gelrite (Sigma, U.S.A.). PH was adjusted to 5.7 before autoclaving.

Vegetal material and culture conditions

Quillaja seeds collected between march and april were used from two different trees, San Carlos de Apoquindo, (Santiago Metropolitan Region Chile) "Clones 11 and 12", and sprout apexes from an adult tree. (Antuco, Andes, 8th Region in Chile).

Seeds were disinfected with a disinfection protocol elaborated by Prehn *et al.* (2003) consisting on: 1. A 25 minute-wash in an aqueous mancozeb suspension (Dithane 80 WP, 5 g·L^{-1}) 2. A triple wash in sterilized distilled water for 3 minutes each. 3. An immersion in sodium hypoclorite at 2% for 6 minutes. 4. A triple wash in distilled water for 3 minutes each. 5. Immersion in 60 v. of hydrogen peroxide shaken constantly for 10 minutes, and 6. A triple wash in sterilized distilled water for 10 minutes each. Seeds were further stratified in darkness at 4ºc in Petri dishes containing solid full strength MS medium (Murashige and Skoog, 1962) for 60 hours. Dishes were then transferred to a chamber (20+ 6ºC, 6000 lux) to start germination. Finally, around 1 cm-hypocotiledonar sections were cultivated *in vitro*, that were obtained from the germinated seeds once they reached an extended cotyledon state.

Sprout apexes harvested from the seasonal growth of an adult tree were transferred to the laboratory before 48 hours in a vinclozolin aqueous suspension at 0.025% (Ridomil 50 WP, Basf). They were further disinfected (Prehn *et al.*, 2003) and

established *in vitro* on MS medium supplemented with 1.61 µM kinetin, 1.9 $mg \cdot L^{-1}$ of naftalen acetic acid (ANA), 30 $g \cdot L^{-1}$ of sucrose and 8 $g \cdot L^{-1}$ of agar. Both explants (hypocotyledon sections and apexes) generated calli which were cultivated in a growth chamber (20±6ºC, 6000 lux).

The induction of embryogenic callus on the explants was made in a 0.5xMS medium supplemented with 13.5 µM of 2.4-D (auxin) and 4.4 µM of BAP (citoquinine); 20 $g \cdot L^{-1}$ of sucrose, 2.5 $g \cdot L^{-1}$ of Gelrite; 100 $mg \cdot L^{-1}$ of casein hydrolysate and 250 $mg \cdot L^{-1}$ of glutamine.

Morphological analysis of the somatic embryo in an initiation and early maturation stage

The purpose of this morphological analysis was to correlate the visual morphology of the embryogenic callus with the state of the somatic embryo development. For this, the morphological analysis of the embryos was made in three types of calli; embryogenic, white cotton-like and granular, of Clone 11, present in the initiation, multiplication and early maturation stages, respectively. This analysis was performed with a double differential dyeing protocol adapted from a protocol developed for conifers by Gupta and Durzan (1986). This consisted in: callus dispersion in distilled water, fixation of 10 µL of the callus sample on a slide, dyeing with 10 µL of acetocarmin at 2% for 15 s, wash off excess of dye with distilled water, dyeing with 10µL of Evans Blue at 5%, wash off excess of dye with distilled water and direct observation under an optical microscope.

Effect of abscisic acid (ABA) and polyethylenglycol (PEG) on the development and maintenance of embryogenic calli

For this purpose, embryogenic calli with a granulose and translucent appearance (Dale *et al.*, 1985) were cultivated on the following culture media: 1. 0.5xMS basal medium, 2. Basal medium supplemented with 15 $mg \cdot L^{-1}$ of ABA. 3. Basal medium supplemented with 75 $mg \cdot L^{-1}$ of PEG. 4. Basal medium supplemented with 15 $mg \cdot L^{-1}$ of ABA and 75 $mg \cdot L^{-1}$ of PEG. 13.5 µM of 2.4-D and 4.4 µM of BAP were supplemented in all the treatments. Unless specified, in every of the following tests the frequency of the embryogenic calli, as well as the expected morphology were assessed, according to the development stage: white cotton-like callus in the multiplication stage and granulose callus in the maturation stage. The evaluation was made after 30 days of treatment.

Effects of cytokinins and auxins on the maturation of somatic embryos

White cotton-like calli were used, with a whitish and filamentous appearance, having pro-embryos (George, 1993; Hartmann *et al.*, 1997). Samples from these calli, taken from the clones 11, 12, and A14 were germinated in a basal medium supplemented with three hormone treatments: 1. Basal medium supplemented with 4.4 µM of BAP, 2. Basal medium supplemented with 4.5 µM of 2.4-D and 4.4 µM of BAP and 3. Basal medium alone. The effect of each treatment was morphologically evaluated after two months of growth.

Effect of the nitrogen source on the maturation of somatic embryos

White cotton-like calli of the clones 11, 12, and A14 were established on basal medium (0.5xMS), modified with oxidized and reduced nitrogen (NO_3:NH_4+, respectively) in the following proportions: 66:34, 80:20, 60:40 and 64:34. The sources of nitrogen were KNO_3 and NH_4NO_3. For the maturation of the somatic embryos, 4.4 µM of BAP were added to all the treatments.

Effect of the decrease in the gelling agent on the maturation of somatic embryos
For this purpose granulose calli of the clones 11, 12 and A14 were used. These were identified due to a granulose superficial appearance and easy to separate texture (friable). These calli are reported to have embryos in a globular state (George, 1993 and Hartmann *et al.*, 1997). They were grown on basal medium (0.5xMS) modified with 4 and of 6 $mg \cdot L^{-1}$ of Gelrite. 4.4 µM of BAP were added for the maturation of somatic embryos in all the treatments. The effect of every treatment was morphologically evaluated after 1 month of growth.

Design and statistical analysis
All treatments were randomly distributed with four or five repetitions, except in the experiments on the effect of abscisic acid and polietylenglycol on the development and maintenance of the embryogenic callus, where Clones 11 and 12 had two repetitions. The experiment of the effects of cytokinins and auxins on the maturation of somatic embryos had eight repetitions for Clones 11 and 12, and twelve for Clone A14. Each repetition always consisted in a Petri dish of 90 mm-diameter containing four or five embryogenic calli. The results were expressed in every experiment as frequencies of the most embryologically advanced callus, white cotton-like callus in the first test of maintenance, and embryo in globular state in the maturation tests. Prior to the variance test (Statgraphics 2.0) the results were transformed angularly. The averages were separated statistically according to the Duncan multiple comparison (p=0.05).

RESULTS AND DISCUSSION

Morphological analysis of the somatic embryo
The double dyeing procedure allowed to determine the embryogenic possibilities of the cells, and distinguish the different stages of development of the somatic embryo, as expected (Praymod *et al.*, 1987), the acetocarmin dyed with intense red the nucleus and the glycoproteins; while Evans Blue allowed determining cell viability, dying the small nucleus of the suspenders with a blue color. Cells without embryogenic potential displayed a minor color intensity.

Long blue colored cells were observed in the embryogenic calli, with reddish granules on one extreme. This showed the embryogenic potential of the cell to develop the suspensor in the basal cells and the embryo in the terminal cell. These results demonstrate the embryogenic callus having induced cells with embryogenic potential, similar to what Praymond *et al.*, 1987, reported for *Pinus taeda*.

The white cotton-like callus displayed long vacuolated blue cells, corresponding to the suspensor, that were distributed lineally. In one point of these cells, a round cell with reddish granules was observed, representing the origin of the somatic embryo. The granules were bigger than those of the embryogenic callus; this could prove a higher reserve accumulation (glycoproteins) and a more advanced maturation stage according to what Praymond *et al.* described, (1987). This is a characteristic structure of a pre-embryogenic embryo (George, 1993). The granulose callus presented a great amount of reddish sphere cells, arranged in clusters. These would, as reported for other species, correspond to embryos in globular state (Praymond *et al.*, 1987). There were no differences in the callus of all the clones studied.

Based on these morphological descriptions, the flow of the somatic embryogenesis in quillaja, from induction to early maturation

was: a) embryogenic callus, b) white cotton-like, and c) granulose calli.

Effect of abscisic acid (ABA) and polietylenglycol (PEG) on the development of the embryogenic callus

According to the existing information, a stage of accumulation of reserves is required in the pro-embryogenic cells, in order to develop mature somatic embryos. Experimental evidence in different species, suggest that this stage can be iniciated with PEG (Pulmann *et al.*, 2003) and / or ABA incorporated to the *in vitro* culture medium (Linossier *et al.*, 1997; Stasolla *et al.*, 2002; Stasolla and Yeung, 2003). The results of this work demonstrated the existence of an embryogenic callus response to the addition of PEG, ABA, PEG plus ABA, and the control treatment (Table 1).

Table 1. Effect of polyethyleneglicol (PEG) and abscisic acid (ABA) on embriogenic masses in 30 days.

Treatments	Frecuency of embryogenic masses[1]			
	Clon			Mean
	A14	11	12	
Effect on growth and development				
ABA+PEG	0.16a[2]	0.00a[2]	0.00a[2]	0.05a[2]
PEG	0.00a	0.50a	0.10a	0.20a
ABA	0.52b	0.50b	0.51b	0.51b
Control	0.40ab	0.30ab	0.00ab	0.23ab
Effect on maintenances				
ABA+PEG	0.00a[2]	0.00a	0.00a	0.00a
PEG	0.04a	0.20a	0.00a	0.08a
ABA	0.48c	0.70c	0.30c	0.49c
Control	0.56b	0.30b	0.10b	0.32b

[1] Data are the mean frequencies of 180 calli.

[2] Means in each column followed by the same letters are not significantly different according to Duncan (p = 0.05).

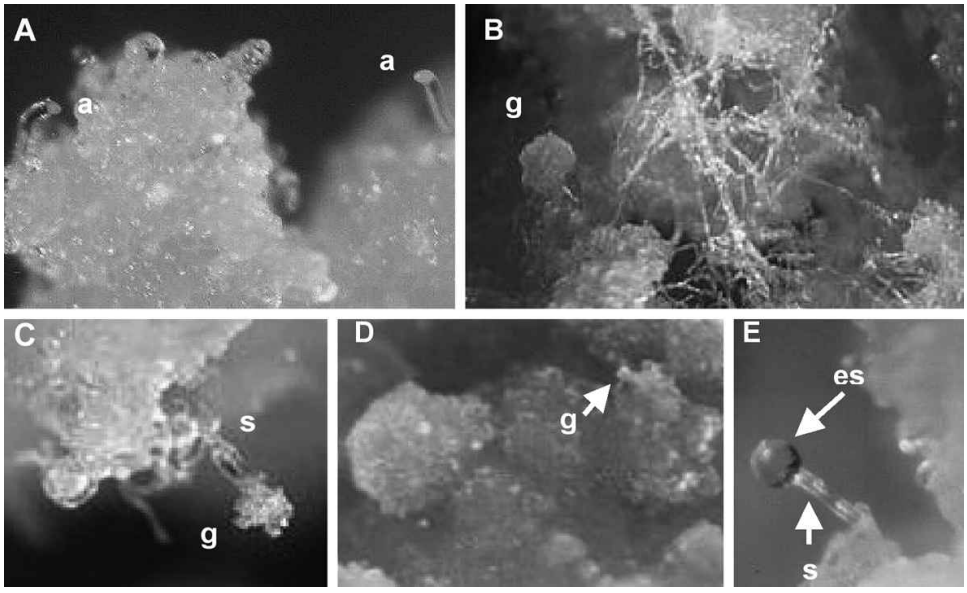

Figure 1. Scheme for maturation of embryiogenic callus of quillay, from accumulation of reserves to globular embryos. (A) Cell with reserve accumulation in apical zone (opaque color) (a), medium with abscisic acid 4X. (B) Spherical cell on the callus 2X. (C) Structure of long cells (s) with spherical cell on the callus surface (g), Clone A14 4X. (D) Globular somatic embryos on maturation callus Clone A14, medium with BAP (4,4 μM) 3X. (E) Somatic embryos (es) with suspensor cell (s) Clone A14 4X.

The analysis of variance showed statistically significant differences (p= 0.05) between the treatments. In this experiment the white cotton-like callus represented the most advanced callus, with embryos in pre-embryogenic state. In the reserve storage stage, the quillaja callus had a better morphological response to the addition of ABA than PEG, independent of the used clones. This could be due to the accumulation of storage proteins, in response of the formation of small vacuoles, and the proliferation of the endoplasmatic reticule, caused by ABA (Maquoi *et al.* 1993) (Figure 1a). Thus, ABA increases the frequency of more developed somatic embryos (Goebel *et al.*, 1993) promoting maturation (Stasolla and Yeung 2003). Nevertheless, Bell *et al.* (1993) point out that high concentrations of ABA inhibit somatic embryogenesis. This could explain the absence of results in growth and morphology of the callus, when adding PEG and ABA simultaneously to the medium (Table 1). PEG decreases the osmotic potential, causing a higher nutrient absorption (Lipavska *et al.*,2000), increasing ABA concentration in the cell. This could explain the growth inhibition and the somatic embryogenesis obtained through this treatment.

Linossier *et al.* (1997) report that PEG addition to the medium favors the conversion of pro-embryogenic mass to a globular shape, being the response species and cultivar dependent. In this case, the effect described above was not observed (Table 1).

Based on these results, the phase of reserve accumulation in the quillaja embryogenic callus requires a subculture on 0.5xMS medium supplemented with 15 $mg \cdot L^{-1}$ of ABA and amino acids for four weeks.

Mantainance of the embryogenic callus
A propagation program based on somatic embryogenesis requires a maintenance medium for the embryogenic callus in order to allow the proliferation of the embryogenic tissue. In our experience, the addition of PEG, ABA, PEG plus ABA and the control treatment, had an effect on the maintenance of the embryogenic callus, in a medium in which the 2.4-D concentration was decreased (from 13.5 μM to 4.5 μM) (Table 1). This was also described by George (1993) and Kosky (1998).

The stereoscopic microscope showed the white cotton-like callus with globose surface cells, on top of long braided cells (Figure 1C). These structures were arranged in groups and were present in a higher quantity than in the previous test, due probably to the 2.4-D decrease, allowing the beginning of the somatic embryo maturation (George, 1993; Gogte and Nadgauda, 2000). The white cotton-like callus in the control medium showed the highest quantity of braided and globule structures in small groups (Figure 1B), indicating that the control medium was appropriate for the embryogenic tissue proliferation. When comparing the development of a zygotic embryo with a somatic embryo, the long structures observed corresponded to the suspensor (Figura 1C-E, s), which braided to form the sphere-like cells (Figure 1B-D, g), and thus originating the embryo (Figure 1C).

The average frequency of white cotton-like callus in the different treatments is shown in Table 1; they were statistically separated into three groups: 1. The media supplemented with PEG presented the lowest embryogenic capacity; 2. The media without PEG and ABA presented a moderate embryonic maturation; and 3. the medium supplemented with ABA presented the most advanced embronic maturation. Nevertheless, the control medium without PEG and ABA (basal medium complemented with 4.5 μM of 2.4-D and

4.4 µM of BAP) would be the best medium for maintenance because the higher growth obtained, with a better embryogenic tissue proliferation and with an appropriate morphology for the transfer to the maturation medium. The decrease in the auxin concentration favored a better development of the embryogenic structures, and increased its growth. These results are coherent to what Gogte and Nadgauda reported (2000).

Effect of cytokinines and auxins on the maturation of somatic embryos

A maturation stage is required for the development of mature somatic embryos, after the phase of proliferation and storage of the induced embryogenic tissue. This stage is featured by a decrease of the auxin concentration, allowing cell polarization (George, 1993; Kosky, 1998). According to the results, there were statistically significant differences (p=0.05) between the treatments on the response of the white cotton-like callus of the different clones, with pre-embryogenic embryos, to the basal medium complemented with 4.4 µM of BAP, the maintenance medium (basal medium complemented with 4.4 µM of BAP and 4.5 µM of 2.4-D) and the basal medium without growth regulators (control basal medium) (Table 2). The granulose callus represented the most advanced callus of this experiment; globular state embryos were observed when the cells were morphologically analyzed. The BAP treatment was statistically different from the medium without hormones and the maintenance medium. Lee and Lee (2003) also reported an increased in the maturation of the somatic embryos of *Dicentra spectabilis* when supplementing the maturation medium with BAP but without 2.4-D. This demonstrates the inductor effect of the cytokinins in the maturation of the quillaja somatic embryos. According to the literature, the maturation of the somatic embryos occurs in hormone free medium. However, the inclusion of cytokinins is beneficial for a great amount of dicotyledoneous species (Chaudhury and QU, 2000). This is due to that cytokinins promote the growth of preformed embryos, being frequently added to the maturation medium (George, 1993).

Table 2. Effect of citoquinine (BAP, bencilaminopurine) and auxines (2,4 D) on the maduration of embryogenic masses after 60 days.

Treatments	Frequency of granulose callus[1]			
	Clones			Promedio
(concentration, µM)	A14	11	12	
Control	0.19a[2]	0.13a	0.16a	0.16a
2,4 D+BAP (13,5+ 4,4)	0.19a	0.22a	0.22a	0.21a
BAP (4,4)	0.88b	0.56b	0.66b	0.70b

[1] Data represent the mean frequencies of 336 calli.
[2] Means in each column followed by the same letters are not significantly different according to Duncan (p = 0.05).

The stage of maturation of quillaja embryo was developed in a medium with 4.4 µM of BAP and without auxins during eight weeks. This medium decreased the amount of long structures in the white cotton-like callus, and at the same time the embryos grew polarized, forming granulose structures on the surface (Figure 1B-D, g). These granulose structures were formed by spheric-like cells, which originated from long structures. These grains matured to globular state (Figure 1E).

Effect of different sources of nitrogen on the maturation of somatic embryos

Somatic embryos in globular state must mature to cotyledonar state. The concentration and the relation between the oxidized and reduced nitrogen may influence the maturation of somatic embryos (George, 1993), as they modify the pH of

the culture medium. The content and proportion of the nitrogen sources, generally used in the indirect embryogenesis on dycotiledoneous species, is based on the 0.5x MS medium (George, 1993).

Nuutila *et al.* (1991) in somatic embryogenesis in *Betula pendula*, found a more effective maturation medium, in a 80:20 proportion of oxidized and reduced nitrogen (NO3: NH4+, respectively). However, a maturation medium with a 60:40 (NO3:NH4+) proportion was more effective for corn somatic embryogenesis (Suprasanna *et al.*, 1991).

Independent of the quillaja clones, the four combinations of oxidized and reduced nitrogen used in this research developed a similar response to the control medium (basal medium 0.5xMS, 66:34 NO3:NH4+) showing no statistically significant differences (p=0.05) between treatments. Therefore, the recommended proportion of oxidized and reduced nitrogen for the maturation of embryogenic calli of quillaja would be 66:34 NO3:NH4+, respectively.

Effect of the gelling agent on the maturation of somatic embryos

Quillaja somatic embryos maintained their globular state without signs of maturation. Therefore, the maturation medium I was modified. The concentration of the gelling agent could be critical for the maturation of somatic embryos (Klimaszewska *et al.* 2000) The second maturation stage depends of the osmotic potential, which determines a higher availability of water to allow a better growth of the callus (Prehn *et al.*, 2003). The analysis of variance however, did not demonstrate significant differences (p= 0.05) between the treatments with 4 and 6 $mg \cdot L^{-1}$ of Gelrite. On the other hand, Pullman *et al.* (2005) reported the gelling agents to heavily influence the ABA absorption, suggesting that their concentration could influence the maturation of the somatic embryos in a negative way.

For the endemic species quillaja, so far only the *in vitro* micropropagation has been described. The results of this research demonstrate the possibility to propagate this species *in vitro*, with the benefits associated to somatic embryogenesis, consisting mainly in the increase of the multiplication rate and the possibility of tissue cryopreservation. Nevertheless, future research must complement the regeneration protocol of quillaja *via* somatic embryogenesis to allow the development of the embryo maturation to reach the cotyledonar state and germination; at the same time additional research should address the acclimatization of plants obtained through *in vitro* micropropagation.

CONCLUSIONS

The explants of both seed hypocotyl and apexes of adult quillaja trees are able to make somatic embryogenesis, showing no morphological differences in the stages of initiation, multiplication and beginning of maturation in the flow of somatic embryogenesis. In quillaja, the flow of somatic embryogenesis stated in this investigation and the conditions to obtain it, would be the following: 1. Embryogenic induction phase, in a 0.05xMS medium complemented with 13.5 µM of 2.4-D (auxin) and 4.4 µM of BAP (cytokine). 2. Phase of accumulation of reserves in a 0.5xMS medium complemented with 15 $mg \cdot L^{-1}$ of abscisic acid. 3. Phase of proliferation of embryogenic tissue, in a 0.5xMS medium complemented with 4.5 µM of 2.4-D and 4.4 µM of BAP. 4. Somatic embryo maturation phase, from pre-embryogenic state to globular state, in

presence of 4.4 μM of BAP (cytokinin), but auxin free.

RESUMEN

El quillay es un árbol endémico chileno que crece entre las regiones IV y IX. Con el objetivo de mejorar su propagación clonal se determinaron los factores que afectan la generación de embriones somáticos. Para el cultivo *in vitro* se utilizaron hipocotilos de semillas y ápices de árboles adultos seleccionados de tres genotipos. La inducción embriogénica de los explantes se realizó en medio 0,5 x MS complementado con 13,5 μ*M* de 2,4-D y 4,4 μ*M* de BAP, durante 8 semanas. Posteriormente, los callos embriogénicos acumularon reservas al adicionar al medio 15 $mg \cdot L^{-1}$ de ABA durante 4 semanas. La remoción del 2,4-D y la mantención de la concentración de 4,4 μ*M* de BAP en el medio de cultivo promovieron la maduración de pro-embriones a embriones en estado globular (Fase de Maduración I). Además, se determinó que los suplementos de hidrolizado de caseína, sulfato de magnesio y glutamina beneficiaron el desarrollo de los embriones somáticos. En cambio, la disminución en la concentración en el gelificante y la variación en la concentración de fuentes oxidadas y reducidas de nitrógeno no afectaron significativamente la maduración. Los efectos estudiados permiten desarrollar embriones somáticos hasta estado globular tanto de tejido juvenil como de tejido adulto de *Quillaja saponaria* de tres genotipos.

Palabras clave: Embriogénesis somática, *Quillaja saponaria*, saponinas.

AKNOWLEDGEMENT

The authors thank FONDEF for financing this investigation, FONDEF project D97I2010.

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