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SOMATIC EMBRYOGENESIS FROM EMBRYOGENIC CELL SUSPENSION CULTURES OF CALIFORNIA POPPY, *ESCHSCHOLZIA CALIFORNICA* CHAM.

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SUMMARY

A somatic embryogenesis protocol was developed for *Eschscholzia californica* Cham. (California poppy) using embryogenic cell suspensions and optimized media conditions. Rapidly-growing, finely-dispersed embryogenic cell suspension cultures were established from embryogenic callus and maintained in B5 liquid media supplemented with 0.5 mg l^{-1} (2.26 μ*M*) 2,4-dichlorophenoxyacetic acid. Culture conditions were optimized by investigating the effect of basal media composition, gyratory shaker speed, various carbon sources, different cytokinins, and $AgNO_3$ on the efficiency of somatic embryogenesis. After 40 d in culture, the somatic embryos that formed were counted and their overall growth expressed as packed cell volume. The selected media consisted of either Gamborg (B5) or Murashige and Skoog (MS) salts and vitamins supplemented with 40 g l^{-1} (117 m*M*) sucrose, 0.05 mg l^{-1} (0.22 μ*M*) 6-benzylaminopurine, and 10 mg l^{-1} (58.8 μ*M*) $AgNO_3$. Somatic embryo production was substantially reduced at shaker speeds above 40 rpm. Glucose and sucrose were the most effective carbon sources, whereas fructose, galactose, and maltose resulted in a reduced yield and growth of somatic embryos. The development of somatic embryos was promoted by $AgNO_3$ at concentrations below 10 mg l^{-1} (58.8 μ*M*). A semi-solid medium containing 1.5 g l^{-1} Gel-rite produced the highest frequency of somatic embryo conversion, and promoted the efficient growth of plantlets. Using the reported protocol, over 500 viable somatic embryos were produced per 25 ml of embryogenic cell suspension culture.

Key words: cytokinins; carbon source; embryogenesis; gelling agent; media optimization; plant regeneration.

INTRODUCTION

Eschscholzia californica Cham. (California poppy) is a common ornamental species and a traditional medicinal plant of many indigenous peoples in North America. The roots of California poppy accumulate copious amounts of benzophenanthridine alkaloids, which comprise a unique class of pharmacologically active compounds restricted in occurrence to members of the Papaveraceae and Fumariaceae. Benzophenanthridine alkaloids are also synthesized in cell cultures of California poppy after treatment with various elicitors, such as hydrolyzed fungal cell wall extracts or methyl jasmonate (Schumacher et al., 1987; Gundlach et al., 1992). One of the benzophenanthridine alkaloids produced in California poppy is sanguinarine, which is used commercially as an antiplaque agent in oral hygiene products due to its potent antimicrobial activity. Most of the enzymes involved in the formation of benzophenanthridine alkaloids have been isolated, and some of the corresponding genes have been cloned (Kutchan, 1998). However, the molecular mechanisms that control alkaloid biosynthesis in California poppy are still poorly understood. The development of an efficient protocol for the genetic transformation of California poppy will facilitate a more complete application of advanced molecular and biochemical approaches to study the regulation of benzophenanthridine alkaloid biosynthesis. A California poppy transformation protocol would also create new opportunities for the development of metabolic engineering strategies in this plant.

A reliable and efficient method for regenerating intact plants is essential for the establishment of a California poppy genetic transformation protocol. Plant transformation and regeneration via somatic embryogenesis is a desirable strategy because of the high proliferation potential and low risk of chimeric plant development. Recently, we reported a method for somatic embryogenesis and plant regeneration from seed-derived embryogenic callus cultures of California poppy (Park and Facchini, 2000). Our original protocol represented a significant improvement over previous somatic embryogenesis and plant regeneration procedures for California poppy (Kavathekar and Ganapathy, 1973; Kavathekar et al., 1977). In addition to low embryogenesis and regeneration frequencies, most of the viable embryos produced by these earlier methods displayed abnormal developmental morphology and developed into plantlets only after exposure to low temperatures. In this paper, we report the development of a remarkably efficient protocol for somatic embryogenesis in California poppy using embryogenic cell suspension cultures, and optimized media and culture conditions.

MATERIALS AND METHODS

Establishment of embryogenic cell suspension cultures. Embryogenic callus was initiated from seed-derived primary callus of *Eschscholzia californica* Cham. cv. Aurantiaca maintained on agar-solidified MS

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(Murashige and Skoog, 1962) media containing 1.0 mg l^{-1} (5.37 μM) 1-naphthaleneacetic acid (NAA) and 0.5 mg l^{-1} (2.22 μM) 6-benzylaminopurine (BA) as described by Park and Facchini (2000). For the establishment of rapidly growing and finely dispersed cell suspension cultures, 0.5 g (fresh weight) of embryogenic callus was transferred to 125-ml Erlenmeyer flasks containing 25 ml of B5 (Gamborg et al., 1968) liquid media supplemented with 30 g l^{-1} (88 mM) sucrose and 0.5 mg l^{-1} (2.26 μM) 2,4-dichlorophenoxyacetic acid (2,4-D). The medium was adjusted to pH 5.8 and sterilized by autoclaving at 1.1 kg cm^{-2} (121°C) for 20 min. Embryogenic cell suspensions were sub-cultured every 2 wk and maintained at 25°C on a gyratory shaker (100 rpm) under standard cool white fluorescent tubes (Sylvania Gros-Lux Wide Spectrum, Mississauga, ON, Canada) with a flux rate of 35 μmol s^{-1} m^{-2} and a 16-h photoperiod. After 30 d in liquid culture, embryogenic cells were passed through a 308 μm sieve before embryogenesis experiments were initiated. The resulting finely dispersed embryogenic cell suspensions consisted of both single cells and cell aggregates in approximately equal proportions. Stock cultures retained their embryogenic potential for approximately 3 mo., during which time finely dispersed experimental cultures were consistently and efficiently established.

Media optimization experiments. Media optimization experiments were performed by transferring 0.5 ml aliquots from an embryogenic stock culture to 100 × 15 mm Petri plates containing 20 ml of liquid media supplemented with various additives as described below. The Petri plates were sealed with Parafilm (American National Can, Menasha, WI, USA) and maintained on a gyratory shaker, with a shaking speed of 40 rpm, in the dark at 25°C. After 40 d in culture, a 1.5 ml aliquot of the embryo suspension from each treatment was removed using a modified pipette tip (i.e. 5 mm was cut from the end of a standard 1 ml pipette tip to widen the aperture and prevent clogging). Somatic embryos smaller than 475 μm were removed from each 1.5 ml sample by sieving, and the remaining embryos were counted. The overall growth of somatic embryos was then determined by measuring the packed cell volume (PCV). For each treatment, five Petri plates were sampled in triplicate and the data combined, unless stated otherwise.

To optimize basal salt and vitamin concentrations for somatic embryo development, the embryogenic cell suspension cultures were rinsed three times with 2,4-D-free B5 media and resuspended in either half-, 1×-, or 2×-strength hormone-free B5 or MS liquid media, containing 30 g l^{-1} sucrose. Aliquots of the embryogenic cell suspension were also transferred to hormone-free B5 liquid media containing 30 g l^{-1} of either fructose, galactose, glucose, maltose or sucrose to evaluate the effect of different carbon sources on somatic embryo production. The culture medium was further optimized by determining the effect of sucrose concentration between 10 and 50 g l^{-1} on somatic embryo development and overall growth. The effects of the cytokinins BA or kinetin at concentrations up to 1.0 mg l^{-1} (4.4 μM for BA; 4.6 μM for kinetin), and $AgNO_3$ at concentrations up to 20 mg l^{-1} (117.6 μM), were also investigated.

Optimization of somatic embryo conversion and plant regeneration. Mature somatic embryos were transferred to Magenta boxes containing 70 ml of hormone-free B5 solid media. The effect of two different gelling agents on the promotion of somatic embryo conversion and plantlet development was investigated. The plant regeneration medium consisted of B5 salts and vitamins, 30 g l^{-1} sucrose, and various concentrations of Phytagar (Gibco, Burlington, ON, Canada) or Gel-rite (Schweizerhall, South Plainfield, NJ, USA). Incubation of somatic embryos and plantlets was routinely performed on 1.5% Gel-rite in Magenta boxes maintained at 25°C under standard cool white fluorescent tubes (Sylvania Gros-Lux Wide Spectrum) with a flux rate of 35 μmol s^{-1} m^{-2} and a 16-h photoperiod. After 5 wk, plantlets were cultured on hormone-free, half-strength B5 media for 1 mo. before the regenerated plants were transferred to pots containing autoclaved vermiculite. Potted plants were covered with polyethylene bags to maintain high humidity, and kept in a growth chamber at 25°C for 1 wk before transfer to the greenhouse.

Analysis of data. All data collected were submitted to an analysis of variance.

Results and Discussion

A somatic embryogenesis protocol was developed for California poppy using embryogenic cell suspension cultures and optimized media conditions. Cell suspension cultures were established from embryogenic callus and maintained in B5 liquid media supplemented with 0.5 mg l^{-1} (2.26 μM) 2,4-D. Culture conditions for the development and growth of somatic embryos were optimized by testing the effect of basal media composition, various carbon sources, different cytokinins, gyratory shaker speed, and $AgNO_3$. The frequency of induction and the overall growth of somatic embryos were not affected by different strengths of either B5 or MS salts and vitamins, and no significant difference was found between the two media formulations (data not shown). For convenience, B5 at 1×-strength was selected as the basal medium.

The yield of somatic embryos was highest when either glucose or sucrose was used as the carbon source, but significantly decreased with the use of fructose, galactose, or maltose (Table 1). No significant difference was found in somatic embryo development or growth when either glucose or sucrose was used as the carbon source. Although sucrose is the most common form of exogenous carbon provided to plant cell and tissue cultures (Merkle et al., 1995), other sugars have been found to promote somatic embryogenesis in certain species. For example, the use of maltose as the carbon source was shown to improve the yield and morphology of somatic embryos of *Medicago sativa* (Strickland et al., 1987) and *Prunus avium* (Reidiboym-Talleux et al., 1999).

The supply of carbohydrate during somatic embryogenesis is regarded as a critical factor controlling the quantity and quality of somatic embryo formation (Merkle et al., 1995). The production of California poppy somatic embryos was highest when the media contained 20–50 g l^{-1} sucrose, although the data were not found to

TABLE 1

EFFECT OF VARIOUS CARBON SOURCES ON THE PRODUCTION OF SOMATIC EMBRYOS FROM EMBRYOGENIC SUSPENSION CULTURES OF CALIFORNIA POPPY AFTER 40 d IN CULTURE

Carbon source[a]	Globular[b]	Heart[b]	Torpedo[b]	Cotyledon[b]	PCV[c] (ml)
Fructose	18.4 ± 4.5	6.8 ± 2.4	0.4 ± 0.5	0.0	0.40 ± 0.08
Galactose	15.0 ± 2.5	6.4 ± 1.1	1.0 ± 0.5	0.6 ± 0.5	0.52 ± 0.06
Glucose	33.2 ± 4.5	13.6 ± 3.0	2.0 ± 0.7	1.4 ± 0.5	0.83 ± 0.08
Maltose	13.4 ± 3.2	6.0 ± 1.6	0.8 ± 0.4	0.4 ± 0.5	0.50 ± 0.08
Sucrose	33.6 ± 5.5	13.8 ± 3.4	2.6 ± 0.9	1.6 ± 0.5	0.87 ± 0.12

[a] At a concentration of 30 g l^{-1}.
[b] Mean number ± SD of somatic embryos per 1.5 ml of embryogenic cell suspension culture from Petri plates containing 20 ml liquid media.
[c] Mean packed cell volume ± SD per Petri plate containing 20 ml liquid media.

TABLE 2

EFFECT OF SUCROSE CONCENTRATION ON THE PRODUCTION OF SOMATIC EMBRYOS FROM EMBRYOGENIC SUSPENSION CULTURES OF CALIFORNIA POPPY AFTER 40 d IN CULTURE

Sucrose concentration (g l^{-1})	Globular[a]	Heart[a]	Torpedo[a]	Cotyledon[a]	PCV[b] (ml)
10	20.2 ± 2.9	8.6 ± 1.1	1.4 ± 0.5	0.8 ± 0.4	0.68 ± 0.06
20	33.0 ± 4.5	14.6 ± 3.6	2.4 ± 0.5	1.6 ± 0.5	0.83 ± 0.08
30	33.6 ± 5.5	13.8 ± 3.4	2.6 ± 0.9	1.6 ± 0.5	0.87 ± 0.12
40	35.6 ± 4.9	16.6 ± 6.5	3.2 ± 1.3	2.6 ± 0.5	1.18 ± 0.15
50	34.8 ± 3.3	15.8 ± 2.3	2.8 ± 0.8	2.2 ± 0.4	1.14 ± 0.04

[a] Mean number ± SD of somatic embryos per 1.5 ml of embryogenic cell suspension culture from Petri plates containing 20 ml liquid media.
[b] Mean packed cell volume ± SD per Petri plate containing 20 ml liquid media.

TABLE 3

EFFECT OF CYTOKININ CONCENTRATION ON THE PRODUCTION OF SOMATIC EMBRYOS FROM EMBRYOGENIC SUSPENSION CULTURES OF CALIFORNIA POPPY AFTER 40 d IN CULTURE

Cytokinin	Concentration, mg l^{-1} (μM)	Globular[a]	Heart[a]	Torpedo[a]	Cotyledon[a]	PCV[b] (ml)
Control	-	33.6 ± 5.5	13.8 ± 3.4	2.6 ± 0.9	1.6 ± 0.5	0.87 ± 0.12
BA	0.01 (0.04)	35.4 ± 4.7	16.8 ± 4.1	3.2 ± 0.8	2.6 ± 0.5	1.28 ± 0.12
	0.05 (0.22)	36.8 ± 6.1	17.6 ± 2.3	3.6 ± 0.5	2.8 ± 0.8	1.33 ± 0.10
	0.1 (0.44)	33.2 ± 2.9	16.0 ± 1.9	2.8 ± 0.8	2.4 ± 0.5	1.15 ± 0.13
	0.5 (2.2)	33.4 ± 3.8	11.6 ± 1.1	1.8 ± 0.4	1.2 ± 0.4	0.85 ± 0.08
	1.0 (4.4)	30.2 ± 5.0	10.0 ± 1.0	1.4 ± 0.5	0.8 ± 0.4	0.79 ± 0.07
Kinetin	0.01 (0.05)	34.2 ± 3.4	15.8 ± 2.8	2.4 ± 0.5	2.0 ± 0.7	0.96 ± 0.14
	0.05 (0.23)	35.4 ± 3.4	15.2 ± 1.9	2.8 ± 0.9	2.4 ± 0.5	1.10 ± 0.13
	0.1 (0.46)	37.8 ± 5.0	17.2 ± 3.1	3.8 ± 0.4	2.8 ± 0.4	1.39 ± 0.21
	0.5 (2.3)	35.2 ± 3.8	16.8 ± 3.0	2.6 ± 0.5	2.4 ± 0.5	1.20 ± 0.14
	1.0 (4.6)	35.0 ± 4.9	15.6 ± 1.5	2.6 ± 0.5	2.0 ± 0.7	1.12 ± 0.16

[a] Mean number ± SD of somatic embryos per 1.5 ml of embryogenic cell suspension culture from Petri plates containing 20 ml liquid media.
[b] Mean packed cell volume ± SD per Petri plate containing 20 ml liquid media.

be significantly different in this range of sucrose concentrations (Table 2). The overall growth of somatic embryos was significantly higher at sucrose concentrations of 40 and 50 g l^{-1} compared to the overall growth of embryos with 20 and 30 g l^{-1} sucrose. Since the overall growth was measured as PCV, and because the differences in the yield of somatic embryos were marginal, higher sucrose concentrations resulted mainly in increased embryo growth. Based on these data, sucrose at a concentration of 40 g l^{-1} was selected as the carbon source for further studies.

The addition of either BA or kinetin increased the total number of embryos produced, their degree of maturation, and their overall growth in a manner similar to that observed with callus-derived somatic embryos of California poppy produced from the same embryogenic cell line (Park and Facchini, 2000). Concentrations of BA between 0.01 and 0.1 mg l^{-1} (0.04 and 0.44 μM), and kinetin between 0.1 and 0.5 mg l^{-1} (0.46 and 2.3 μM), caused a significant increase in the formation and overall growth of somatic embryos, compared to the absence of, or treatments with higher levels of, these cytokinins (Table 3). Optimal conditions were selected as 0.05 mg l^{-1} (0.22 μM) BA or 0.1 mg l^{-1} (0.46 μM) kinetin, which represent mean values within the range of the most effective cytokinin concentrations. For further experiments, all media formulations routinely included 0.05 mg l^{-1} (0.22 μM) BA. Previously, we showed that the addition of 0.05 mg l^{-1} (0.22 μM) BA or 0.1 mg l^{-1} (0.46 μM) kinetin to agar-solidified culture media increased the number and conversion frequency of California poppy somatic embryos, and the growth of the resulting plantlets (Park and Facchini, 2000).

The addition of $AgNO_3$ exhibited a positive effect on California poppy somatic embryo production and overall growth at concentrations between 5 and 20 mg l^{-1} (29.4 and 117.6 μM) (Table 4). The addition of 10 mg l^{-1} (58.8 μM) $AgNO_3$, which was selected as the mean of the optimal concentration range between 5 and 20 mg l^{-1} (29.4 and 117.6 μM), resulted in statistically significant increases of 25 and 60% in the yield and growth, respectively, of somatic embryos produced after 40 d in culture, relative to controls (Table 4). $AgNO_3$, a putative ethylene inhibitor, has been used to enhance the production of somatic embryos from carrot embryogenic cell suspension cultures (Roustan et al., 1990), and from wheat and tobacco embryogenic callus cultures (Purnhauser et al., 1987).

A gyratory shaker speed of 40 rpm was found to be optimal for the induction and growth of California poppy somatic embryos in B5 liquid media containing 40 g l^{-1} sucrose, 0.05 mg l^{-1} (0.22 μM) BA, and 10 mg l^{-1} (58.8 μM) $AgNO_3$. A shaking speed of 80 or 120 rpm reduced the number of somatic embryos produced by approximately 25 and 40%, respectively (data not shown). These data are in agreement with those reported for the production of somatic embryos from embryogenic cell cultures of an *Agrobacterium rhizogenes*-transformed cell line of California poppy grown in an 11-l helical-ribbon-impeller bioreactor containing B5 liquid media and 30 g l^{-1} sucrose (Archambault et al., 1994). An impeller speed of 60 rpm resulted in more efficient somatic embryo

TABLE 4

EFFECT OF $AgNO_3$ ON THE PRODUCTION OF SOMATIC EMBRYOS FROM EMBRYOGENIC SUSPENSION CULTURES OF CALIFORNIA POPPY AFTER 40 d IN CULTURE

$AgNO_3$ concentration, mg l^{-1} (μM)	Globular[a]	Heart[a]	Torpedo[a]	Cotyledon[a]	PCV[b] (ml)
0	33.6 ± 5.5	13.8 ± 3.4	2.6 ± 0.9	1.6 ± 0.5	0.87 ± 0.12
1 (5.8)	34.0 ± 3.9	13.0 ± 2.2	2.8 ± 0.8	1.8 ± 0.4	0.93 ± 0.12
3 (17.4)	36.4 ± 4.0	14.4 ± 3.0	2.8 ± 0.4	2.0 ± 0.7	1.01 ± 0.08
5 (29.4)	37.0 ± 4.3	15.6 ± 4.0	3.2 ± 0.8	2.4 ± 0.5	1.16 ± 0.10
10 (58.8)	38.6 ± 6.1	18.2 ± 3.4	3.8 ± 0.8	3.0 ± 0.7	1.37 ± 0.14
20 (117.6)	37.2 ± 3.2	17.6 ± 2.1	3.0 ± 0.7	2.6 ± 0.5	1.24 ± 0.07

[a] Mean number ± SD of somatic embryos per 1.5 ml of embryogenic cell suspension culture from Petri plates containing 20 ml liquid medium.
[b] Mean packed cell volume ± SD per Petri plate containing 20 ml liquid media.

production, and greater somatic embryo conversion and plant regeneration frequencies, than a speed of 100 rpm. Moreover, most somatic embryos that formed at an agitation speed of 100 rpm did not exhibit normal developmental morphology (Archambault et al., 1994). Although the culture conditions were different, comparison of our results to those of Archambault et al. (1994) supports the suggestion that somatic embryogenesis in California poppy is more efficient with reduced agitation of the suspension cultures.

Various stages in the production of somatic embryos from embryogenic cell suspension cultures under optimized media conditions are shown in Fig. 1. A multitude of globular- and heart-stage embryos developed within 2 wk after embryogenic suspensions were transferred to B5 liquid media supplemented with 40 g l^{-1} sucrose, 0.05 mg l^{-1} (0.22 μM) BA, and 10 mg l^{-1} (58.8 μM) $AgNO_3$ (Fig. 1A). Under the same conditions, a large number of torpedo- and cotyledonary-stage embryos formed within 5 wk (Fig. 1B).

Somatic embryo conversion increased significantly as the concentration of gelling agent was reduced, and was generally higher on Gel-rite than on agar (Table 5). A semi-solid medium containing 1.5 g l^{-1} Gel-rite produced the highest rates of somatic embryo conversion (Table 5). No significant difference in the growth of shoots and roots was observed when plantlets were grown on either agar or Gel-rite, except at the highest concentration (10 g l^{-1}) of agar tested, which produced significantly less growth compared to all of the other conditions (Table 5). Due to its ability to promote the highest frequency of somatic embryo conversion and the efficient growth of plantlets, 1.5 g l^{-1} Gel-rite was selected for

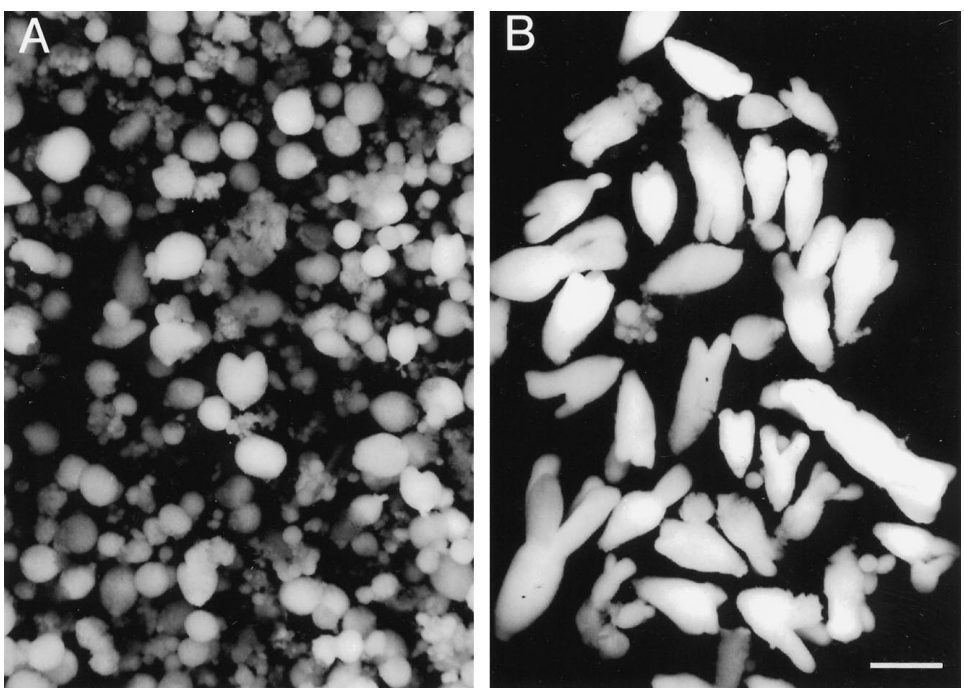

FIG. 1. Somatic embryogenesis from embryogenic suspension cultures of *Eschscholzia californica*. After 2 wk in liquid culture, embryos at the globular and heart stages (A) had formed from the embryogenic cell suspension cultures in liquid media containing B5 salts and vitamins, 40 g l^{-1} (117 mM) sucrose, 0.05 mg l^{-1} (0.22 μM) BA, and 10 mg l^{-1} (58.8 μM) $AgNO_3$. After 5 wk, embryos at the torpedo and cotyledonary stages (B) had developed. *Bar* = 1 mm.

TABLE 5

EFFECT OF GELLING AGENT CONCENTRATION ON THE CONVERSION FREQUENCY OF SOMATIC EMBRYO AND THE GROWTH OF PLANTLETS OF CALIFORNIA POPPY AFTER 5 wk IN CULTURE

Gelling agent	Concentration (g l^{-1})	Conversion[a] (%)	Shoot length[b] (cm)	Root length[b] (cm)
Agar	10.0	55	1.7 ± 0.2	0.7 ± 0.2
	8.0	62	1.9 ± 0.3	0.9 ± 0.2
	6.0	69	2.0 ± 0.3	0.9 ± 0.2
Gel-rite	3.5	68	2.0 ± 0.2	0.9 ± 0.2
	2.5	75	2.2 ± 0.3	1.3 ± 0.2
	1.5	79	2.3 ± 0.3	1.2 ± 0.2

[a] From 100 somatic embryos tested in three independent experiments.
[b] Values represent the mean ± SD of 25 measurements in three independent experiments.

the routine preparation of semi-solid media. The addition of low concentrations of agar (0.3 g l^{-1}) or Gel-rite (0.1 g l^{-1}) to culture media was also reported to improve somatic embryo conversion from immature embryo-derived cell suspension cultures of *Angelica sinensis* (Tsay and Huang, 1998). Approximately 80% of the California poppy somatic embryos which converted to plantlets were successfully transferred to soil and grew into fertile plants. The frequency of plant recovery was the same as that obtained using a protocol for somatic embryogenesis from embryogenic callus cultures of California poppy (Park and Facchini, 2000). The normal morphology of plants produced from liquid culture-derived somatic embryos was also consistent with our previous work (Park and Facchini, 2000). In contrast, other somatic embryogenesis protocols for California poppy resulted in the development of plants with abnormal morphology (Kavathekar and Ganapathy, 1973; Kavathekar et al., 1977). Moreover, our protocol did not require cold treatment to promote somatic embryo conversion (Kavathekar et al., 1977).

The reported protocol for the production of California poppy somatic embryos from embryogenic cell suspension cultures under optimized liquid-culture conditions represents a substantial improvement over our previous method based on the use of embryogenic callus cultures (Park and Facchini, 2000). Using the protocol reported in this paper, over 500 viable somatic embryos were produced per 25 ml of embryogenic cell suspension culture. In contrast, only about 45 viable embryos were produced from approximately the same mass of embryogenic callus cultured on agar-solidified media (Park and Facchini, 2000). The results of these studies can be considered directly comparable because the same embryogenic cell line was used. The regeneration of plants from suspension-cultured embryogenic cells offers an improved opportunity to develop a highly efficient genetic transformation protocol for California poppy. In this context, it should be noted that the protocol can be effectively scaled to larger volumes in 125 ml Erlenmeyer flasks without loss of efficiency (data not shown).

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