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ORIGINAL ARTICLE

Cultural conditions affect somatic embryogenesis in *Catharanthus roseus* L. (G.) Don

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Abstract We established an efficient plant regeneration system for *Catharanthus roseus* L. (G.) Don through somatic embryogenesis. Embryogenic callus was induced from hypocotyl of seed germinated in vitro. Somatic embryogenesis in *Catharanthus* has been categorized into three distinct stages: (1) initiation and proliferation of embryo; (2) maturation, and; (3) germination or plantlet conversion. Beside plant growth regulators, various stages of embryogenesis were screened for their response to a wide variety of factors (pH, gelrite, light, sugar alcohols, polyethyleneglycol and amino acids), which affect embryogenesis. All of the tested factors had a small to marked influence on embryogeny and eventual conversion to plantlets. The plantlets were acclimatized successfully in a greenhouse. To our knowledge, this is the first report describing a detailed study of various cultural factors which regulate embryogenesis in *C. roseus*. The results discussed in this paper may be used in mass propagation to produce medicinal raw material, and the embryo precursor cells could be used in genetic modification programmes that aim to improve the alkaloid yield as well.

Keywords *Catharanthus roseus* · Cultural conditions · Plant growth regulators · Somatic embryogenesis

Abbreviations

BA	N^6-benzyladenine
2,4-D	2,4-Dichlorophenoxyacetic acid
NAA	α-Naphthalene acetic acid
GA_3	Gibberellic acid
PEG	Polyethylene glycol
MS	Murashige and Skoog's medium
SE	Somatic embryo
DMRT	Duncan's multiple range test

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Introduction

Somatic embryos (SEs) produced from cultured plant tissues have been reported in a number of plant genera (Bajaj 1995; Thorpe 1995; Brown et al. 1995; Mujib and Samaj 2006) but have been relatively new in *Catharanthus roseus* (Junaid et al. 2004; 2006, 2007a, 2007b). The formation of SEs is, thus, a unique mode of in vitro regeneration and offers many advantages, including the potential for unlimited production of clones with elite traits. The culture of plant tissues via SE on a large scale makes it possible to produce synthetically coated seeds (Redenbaugh 1993; Gray et al. 1995). Secondly, the use of embryogenic cultures has proven to be especially valuable in providing a source of regenerable protoplasts (Chang and Wong 1994; Jimenez 1996).

There are a number of plant growth regulators (PGRs) that are used to induce SEs during culture (Ramage and Williams 2004), and the right balance or the ratio of these PGRs is often the primary empirical basis for the optimization of in vitro SE development (Ochatt et al. 2000; Pan et al. 2003). Aside from PGRs, the most commonly used carbohydrate for plant tissue or cell culture is sucrose, but this is not always the best carbohydrate to achieve regeneration (Krikorian and Berquam 1969; Cheong and Pooler 2004; Junaid et al. 2006). In order to

be beneficial, certain changes in carbohydrate sources are often necessary (Schuller and Reuther 1993; Scott and Lyne 1994; Cabasson et al. 1995; Reidiboym-Talleux et al. 1998; Nuutila et al. 2002; Junaid et al. 2006). There are several other factors [pH, sugar alcohols, activated charcoal, light, polyethylene glycol (PEG), amino acids, solidifying agents, etc.] which also influence embryogenesis (Smith and Krikorian 1990; Koh and Loh 2000; Van Winkle et al. 2003; Cheong and Pooler 2004; Pullman et al. 2005). In the present investigation we evaluated the roles of various factors in different stages of embryogenesis in *C. roseus.*

Materials and methods

Plant material

Almost mature fruits (follicles) of two-year-old *Catharanthus roseus* L. (G.) Don were collected from naturally grown plants in the Jamia Hamdard (Hamdard University, New Delhi) herbal garden at the beginning of August 2003 (average temp 36 ± 2 °C, humidity 70%)

Sterilization

The fruits were washed in running water for 15 min and surface-sterilized in 70% ethanol for 10 min and rinsed three times with sterilized double-distilled water. Seeds were isolated inside a laminar hood from sterilized pods and treated with 0.5% mercuric chloride for 2 min followed by H_2O_2 (1%). After every treatment, the seeds were rinsed four times in sterilized double-distilled water.

In vitro germination of seeds

Surface-disinfected seeds (15–25 in number) were placed in GA-7 Magenta vessels (Sigma, St. Louis, MO, USA) containing 50 ml MS (Murashige and Skoog 1962) medium without organic compounds and PGR. Germinating seedlings were grown until they reached 2 cm in length. They were removed from the culture vessels, and the hypocotyls were excised.

Induction of embryogenic callus

The initial procedure of establishing embryogenic callus was done as described earlier (Junaid et al. 2004). Somatic embryogenesis in *Catharanthus* has been categorized into three distinct stages as reported previously (Junaid et al. 2006): (1) initiation and proliferation of embryo; (2) maturation, and; (3) germination or plantlet conversion.

SE initiation and proliferation

In short, the friable embryogenesis callus (40–50 mg) was cultured on medium, along with 3% sucrose and optimized concentrations of 6.72 μM BA and 5.37 μM NAA (Junaid et al. 2006). The embryogenesis percentage (%) and the number of SEs at different developmental stages were recorded.

SE maturation

Advanced cotyledonary SEs (5–6 mm length) were isolated from proliferated embryogenic culture and placed on maturation medium along with 3% maltose and optimized 2.60 μM GA_3 (Junaid et al. 2006). Data were scored in terms of maturation percentage and growth [(size and length (mm)] of SEs.

SE germination and plantlet formation

For embryo germination green mature SEs were cultured on MS, which was added with 6% maltose and optimized levels of 2.24 μM BA and 7.38 μM IBA (Junaid et al. 2007b). The response percentage and the germination or conversion performances of SEs were noted.

Effect of various factors on somatic embryogenesis

pH

The effect of pH has been examined during the different developmental stages of embryogenesis. The MS was separately kept at various pHs (3.0–7.0) that were adjusted before sterilization; the required pH values were maintained and adjusted with 0.1(N) NaOH or 0.1 (N) HCl.

Gelrite (solidifying agent)

The addition of gelrite (either alone or with agar agar) was used similarly at different embryogenesis stages. Except in the gelrite experiments, all media were added along with agar agar (0.8 gm/L).

Light

The effects of various light periods (8, 12, 16 and 20 h) were evaluated during proliferation, maturation and germination times. Unless otherwise mentioned, all other cultures were kept under a 16 h photoperiod.

Sugar alcohols

In *Catharanthus*, embryos at different developmental stages (proliferation, maturation and germination) showed

specific requirements for carbohydrates. High proliferation, maturation and germination were found at 3% sucrose, 3% maltose and 6% maltose added medium, respectively, so we used these optimized levels of carbohydrates in the present set of experiments (Junaid et al. 2006). Two sugar alcohols (sorbitol and mannitol) were also added to the media at 1.5–3.0 mg/L.

Polyethylene glycol (PEG: 4000)

Various concentrations (1.87, 3.75 and 7.5 g/L) of PEG were added, and its effects were monitored at different stages of embryogenesis.

Amino acids

Four amino acids (L-asparagine, L-glutamine, L-cystine and L-proline) at 100, 300, 500 mg/L were individually added in order to evaluate their impact on embryogenesis. A separate medium with a normal MS glycine concentration (2 mg/L) was used as control.

Acclimatization

Converted plantlets were removed, transplanted into microplastic pots containing sterile soil rite, thoroughly covered with perforated polythene bags, and grown for one month at 25 ± 2 °C. Plantlets were then transferred to pots containing 1:1 soil rite and sand for another 2–3 weeks at room temperature, and finally planted in soil under natural conditions.

Culture condition

Except in the pH experiment, all prepared media were adjusted to pH 5.8 before sterilization. Unless mentioned otherwise, the medium was always added with 3% sucrose and was gelled with 0.8% agar agar. The media were sterilized at 121 °C for 15 min. Cultures were incubated at 25 ± 2 °C under a 16-h photoperiod with cool white fluorescent illumination (100 μmol/m^2/s PFD).

Statistical analysis

The influence of different factors on SE proliferation, maturation and germination were analyzed by one-way analysis of variance (ANOVAs). Values are means of five replicates from two experiments, and the presented mean values were separated using Duncan's multiple range test (DMRT) at $P \leq 0.05$.

Results

Embryogenic callus induction

Hypocotyls of seeds germinated in vitro were cultured on MS along with an optimized concentration of 2,4-D (6.78 μM), on which embryogenic callus was induced. Upon regular subculturing on fresh nutrient medium, it grew further as a white–yellow friable mass; however, the embryogenic potentiality decreased with time. The callus induced from other explant sources was nonembryogenic.

SE proliferation

Hypocotyl-induced embryogenic callus was placed on NAA (5.37 μM) in MS medium, which produced embryos in masses. For further proliferation, SEs were subcultured on earlier optimized concentrations of BA (2.24 μM) + NAA (5.37 μM). They produced a heterogeneous mass with all forms of embryos that were cream in color and showed fast in vitro growth.

Effect of cultural factors on SE proliferation

pH

Somatic embryo proliferation was tested under the influence of various pH levels (in the range of 3.0–7.0). The highest embryogenesis (%) and embryo proliferation (Table 1) were observed in culture maintained at pH 6.0 (Fig. 1a). Only early-stage embryos were present in high numbers at low pH (3.0–3.5), with no advanced-stage embryos observed. Similarly, torpedo and cotyledonary embryos were also missing in culture maintained at higher pH (7.0).

Gelrite

Gelrite showed a negative influence on embryo proliferation, as it decreased the embryo differentiation potentiality of somatic cells (Table 2) when used with agar agar. In two combinations of gelrite and agar [(2.0 + 6.0) and (2.5 + 5.5)], the torpedo and cotyledonary embryos were very rare.

Light

The embryogenic calli were cultured on proliferating medium and were exposed to various light periods (8–20 h). Of the various light conditions, 8–16 h light proved to be very effective for faster SE proliferation. Longer photoperiods reduced embryo differentiation capabilities;

Table 1 Effect of pH on SE proliferation, maturation and plantlet conversion

pH	SE proliferation (%)	SE maturation (%)	Plantlet conversion (%)
3.0	0.0^{i}	56.7^{g}	0.0^{e}
3.5	0.0^{i}	57.8^{g}	0.0^{e}
4.0	29.1^{h}	60.0^{f}	0.0^{e}
4.5	33.3^{g}	62.3^{e}	11.2^{d}
5.0	54.1^{e}	65.5^{d}	13.1^{c}
5.5	68.3^{d}	75.6^{c}	20.8^{b}
5.8	74.3^{c}	80.1^{b}	22.6^{ab}
6.0	85.3^{a}	98.1^{a}	23.1^{a}
6.5	76.8^{b}	42.2^{h}	10.3^{d}
7.0	51.3^{f}	26.7^{i}	12.6^{cd}

Values are means of five replicates from two experiments. Data were scored after seven weeks of culture

Means with common letters within each column are not significantly different at $P \leq 0.05$, according to DMRT

For proliferation, maturation and germination, SEs were cultured on the respective medium

developed embryos even showed morphological irregularities with brown to yellowish patches on their surface.

Sugar alcohols

Murashige and Skoog's medium was added with optimized sugar (sucrose 3%) and sugar alcohols (sorbitol and mannitol) in the range of 1.5–3.0 g/L. Both of the sugar alcohols used showed varied responses. The embryogenesis percentage and the number of SEs decreased significantly in culture with sucrose (1.5%) + mannitol (1.5 g/L) compared to the control (sucrose 3%). The same medium proved to be better than medium that had sucrose (1.5%) + sorbitol (1.5 g/L), where advanced (cotyledonary) forms of SEs were absent (Table 3). The separate addition of sorbitol and mannitol (3%) in place of sucrose (3%) was less effective; mannitol (3%) had some positive effect compared to sorbitol (3%).

PEG

Of the different concentrations of PEG used, only 1.87 g/L showed some influence on embryo proliferation (Table 4), while the other concentrations that were noted to be toxic exhibited poor embryo differentiation compared to the control.

Amino acids

The embryogenic calli were transferred to media with four amino acids [L-asparagine, L-cystine, L-glutamine and L-proline (100, 300 and 500 mg/L)] added. The highest embryogenesis percentages and the maximum SE numbers were obtained in L-glutamine followed by L-asparagine, especially up to a level of 300 mg/L (Fig. 1b; Table 5). Cultures with 500 mg/L of amino acids exhibited inhibitory effects; embryogenesis and embryo number decreased significantly. The cotyledonary form of the embryos was missing in cultures supplemented with L-cystine and L-proline. Some of the SEs isolated from cultures with L-cystine and L-proline were irregular and smaller than usual in size, with yellow to black patches on their surfaces.

SE maturation

Isolated white-opaque cotyledonary SEs were cultured on maturation medium containing 2.60 μM GA_3. SEs turned green and became elongated, while fully matured embryos were dark green in appearance.

Effect of factors on SE maturation

pH

Cotyledonary somatic embryos were placed on the same embryo maturation medium at pH values of 3.0 to 7.0. pH 5.8–6.0 proved to be highly effective, since high embryo maturation percentages with normal embryos were observed (Table 1), while a low pH (3.0) yielded poor to moderate embryo maturation and had 85% abnormal embryos. At a high pH level the embryos were more aberrant structurally and later perished.

Gelrite

We noted that the maturation decreased with increasing gelrite concentration (Table 2). This also changed embryo morphology; a high level of gelrite turned the embryos green/yellow within a week or two.

Light

A few advanced SEs were kept under various light regimes for about seven weeks. More dark and extra light exposure proved to be less important, where embryos were mostly small and remained white or light green. A longer duration of the light period (12 and 16 h) improved the embryo maturation percentage, with the highest (81.1%) occurring at 12 h.

Sugar alcohols

Optimized maltose (3%) was used along with two sugar alcohols (sorbitol and mannitol) to evaluate their effect on

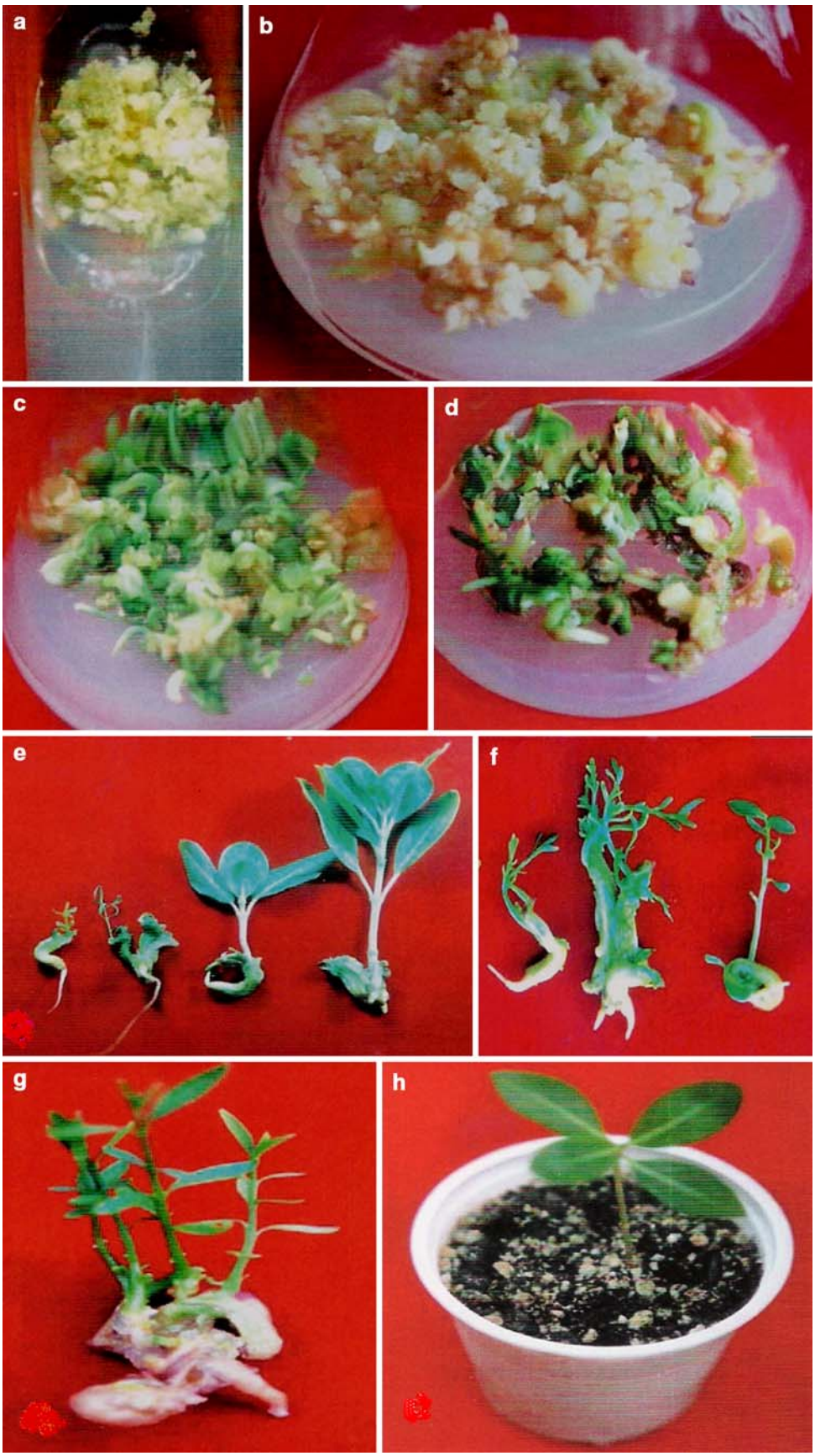

Fig. 1 Influences of various cultural factors on *Catharanthus* somatic embryogeny. **a** SE proliferation at pH 5.8–6.0. **b** Embryos grown on medium with L-glutamine (300 mg/L). **c** Embryos were grown at 3g/L added sorbitol. **d** SE maturation at 3.75 g/L added polyethyleneglycol. **e** SE germination and plantlet conversion at pH 6.0. **f** SE germination and plantlet conversion at 16 h photoperiod. **g** Germinated embryos developed on MS medium with sugar alcohol (maltose 3% + mannitol 1.5 g/L) added. **h** Plantlet growing in ex vitro conditions

maturation (Table 6). Maltose with mannitol improved the SE growth (Fig. 1c) and embryogenesis percentage compared to control (maltose 3%). The culture with maltose and sorbitol added together was found to be ineffective and the SEs developed were irregular and were light green. The use of sorbitol (3%) in place of maltose retarded embryo growth (data not shown), which later turned brown and died completely within one week of culture.

Table 2 The influence of gelrite and agar agar on SE proliferation, maturation and plantlet conversion

Combinations (gelrite + agar, g/L)	SE proliferation (%)	SE maturation (%)	Plantlet conversion (%)
0.0 + 8.0 (control)	73.0[a]	80.3[a]	54.2[b]
0.5 + 7.5	70.3[b]	72.1[b]	61.0[a]
1.0 + 7.0	59.7[c]	66.2[c]	59.1[a]
1.5 + 6.5	43.2[d]	51.0[d]	0.0[d]
2.0 + 6.0	31.1[e]	40.5[e]	0.0[d]
2.5 + 5.5	20.0[f]	21.4[f]	0.0[d]
3.0 + 5.0	11.8[h]	10.7[g]	0.0[d]
3.0 + 0.0	16.2[gh]	23.5[f]	13.4[c]

Values are means of five replicates from two experiments. Data were scored after seven weeks of culture

Means with common letters within each column are not significantly different at $P \leq 0.05$, according to DMRT

For proliferation, maturation and germination, SEs were cultured on the respective medium

PEG

The effects of various concentrations of PEG were tested, and a significant increase in percentage maturation and growth (length) was observed (Fig. 1d) in medium with maltose (3%) + PEG (3.70 g/L) added (Table 7). PEG was inhibitory when used alone at a level of 7.50 g/L, as only 45% embryo maturation was observed.

Amino acids

Table 5 shows the influence of amino acids on SE maturation. L-Glutamine (up to 300 mg/L) and L-asparagine at 300 mg/L were highly effective compared to the control and other amino acids. However, a significant reduction in maturation rate and embryo growth (length) was observed in cultures with 500 mg/L of L-glutamine and L-aspara-

Table 3 Effect of sugars and sugar alcohols on somatic embryogenesis; embryogenic callus (40–50 mg) was cultivated on MS along with optimized concentrations of BA (6.72 μM) + NAA (5.37 μM)

Treatment	Callus showing embryogenesis (%)	Number and types of somatic embryos			
		Globular	Heart	Torpedo	Cotyledonary
Sucrose 3% (control)	73.0[a]	61.5[a]	22.5[a]	9.0[a]	6.3[a]
Sucrose 1.5% + sorbitol 1.5 g/L	14.1[c]	12.4[b]	5.8[b]	2.5[b]	0.0[c]
Sucrose 1.5% + mannitol 1.5 g/L	65.4[b]	58.3[a]	20.5[a]	8.5[a]	3.2[b]
Mannitol 3 g/L	8.8[d]	4.3[c]	1.7[c]	0.0[c]	0.0[c]

Values are means of five replicates from two experiments. Data were scored after seven weeks of culture

Means with common letters within each column are not significantly different at $P \leq 0.05$, according to DMRT

Table 4 Influence of PEG on embryogenesis; embryogenic callus (40–50 mg) was cultivated on MS with optimized concentrations of BA (6.72 μM) + NAA (5.37 μM) and 3% sucrose

Treatment	Callus showing embryogenesis (%)	Number and type of somatic embryo			
		Globular	Heart	Torpedo	Cotyledonary
Sucrose (%) + PEG (g/L)					
3 + 0.0 (control)	73.0[a]	61.5[a]	22.5[a]	9.0[a]	6.3[a]
3 + 1.87	70.9[b]	55.7[b]	21.9[a]	8.1[a]	4.6[b]
3 + 3.75	48.7[c]	43.1[c]	10.5[b]	4.7[b]	0.0[c]
3 + 7.50	18.4[d]	8.4[d]	4.2[c]	2.3[c]	0.0[c]

Values are means of five replicates from two experiments. Data were scored after seven weeks of culture

Means with common letters within each column are not significantly different at $P \leq 0.05$, according to DMRT

Table 5 Effects of different amino acids on SE proliferation, maturation and plantlet conversion

Amino acid (mg/L)	SE proliferation (%)	SE maturation (%)	Plantlet conversion (%)
MS (control)	73.2^{e}	87.6^{c}	59.1^{b}
L-Asparagine 100	71.5^{e}	77.7^{d}	65.4^{b}
L-Cystine 100	65.9^{f}	56.3^{f}	39.2^{d}
L-Glutamine 100	80.4^{c}	91.1^{c}	68.1^{a}
L-Proline 100	48.4^{g}	49.2^{g}	42.6^{c}
L-Asparagine 300	90.3^{b}	93.2^{b}	10.4^{e}
L-Cystine 300	74.2^{d}	61.1^{e}	8.3^{f}
L-Glutamine 300	99.7^{a}	97.2^{a}	11.9^{e}
L-Proline 300	81.6^{c}	50.3^{g}	6.5^{fg}
L-Asparagine 500	42.9^{h}	56.9^{f}	0.0^{h}
L-Cystine 500	9.6^{i}	32.2^{i}	0.0^{h}
L-Glutamine 500	48.2^{g}	41.2^{h}	5.04^{g}
L-Proline 500	6.3^{j}	20.5^{j}	0.0^{h}

Values are means of five replicates from two experiments. Data were scored after seven weeks of culture

Means with common letters within each column are not significantly different at $P \leq 0.05$, according to DMRT

For proliferation, maturation and germination, SEs were cultured on the respective medium

Table 6 SEs in maturation medium (MS + 2.60 μM GA_3) with different sugars and sugar alcohols added (25 SEs/culture)

Treatment	Length after 5 weeks (mm)	Length after 7 weeks (mm)
Maltose 3% (control)	9.7^{a}	11.5^{a}
Maltose 1.5% + sorbitol 1.5 g/L	7.3^{b}	9.1^{b}
Maltose 2.0% + sorbitol 1.5 g/L	6.8^{b}	8.4^{c}
Maltose 1.5% + mannitol 1.5 g/L	7.1^{b}	8.9^{b}
Maltose 2.0 + mannitol 1.5 g/L	9.9^{a}	11.8^{a}
Mannitol 3 g/L	4.7^{c}	6.9^{d}

Values are means of five replicates from two experiments. Data were scored after seven weeks of culture

Means with common letters within each column are not significantly different at $P \leq 0.05$, according to DMRT

gine added. At higher concentrations, embryos became brownish to black and finally perished.

SE germination and plantlet conversion

An earlier study indicated that plant conversion was high in medium supplemented with BA (2.24 μM) + IBA (7.38 μM), so we used this combination as the germination medium and tested the roles of other factors in embryo germination.

Effect of cultural factors on SE germination

pH

A pH range of 5.8–6.0 had a significant effect on plantlet conversion (Fig. 1e; Table 1). The low and high pH levels (3.5 and 7.0) were only useful up to the maturation stage, having less influence on plantlet formation.

Gelrite

The presence of gelrite in the medium decreased plantlet conversion (Table 2); higher concentrations only accelerated early root formation.

Light

Various light periods were employed to investigate the influence of photoperiod on embryo germination. Of the light conditions tested, a significant increase in SE germination was noted at a 16-h light period; however, it decreased as the dark period increases, and as the photoperiod was increased too. Isolated embryos incubated in the dark for an extended period (16 h) produced light green or yellow patches on their surface. The number of shoots arising from individual embryos decreased; rooting was completely inhibited at a 16-h photoperiod. Complete plantlets were observed when embryos were exposed to moderate photoperiods (8–16 h) (Fig. 1f).

Sugar alcohols

In this experiment maltose (6%) was used alone or in combination with two sugar alcohols (sorbitol and mannitol). The SE conversion percentage was high (70%) in medium containing maltose (3%) + mannitol (1.5 g/L). Better root and shoot development were noted on medium with maltose (4%) + mannitol (1.5 g/L) (Table 8). The addition of 3 g/L sorbitol and mannitol together also proved less effective for plant conversion and shoot development (Fig. 1 g); only roots were induced that were thin and transparent with reduced growth. Individual applications of sorbitol and mannitol were ineffective during embryo germination (data not shown).

PEG

The addition of PEG has been reported to enhance embryo maturation and germination in a number of plant species. So we used PEG for the same purpose and its effect was evaluated in *Catharanthus*. PEG at a low concentration (3.75 g/L) was stimulatory and resulted in better germination (Table 9), but higher PEG levels decreased the

Table 7 Effect of PEG on embryo maturation; MS with GA_3 (2.60 μM) + 3% maltose (25 SEs/culture)

Treatment Maltose (%) + PEG (g/L)	Length after 5 weeks (mm)	Length after 7 weeks (mm)
3 + 0.0 (control)	9.7[c]	11.5[d]
3 + 1.87	9.8[c]	11.9[c]
3 + 3.75	10.9[a]	13.9[a]
3 + 7.50	10.5[b]	12.7[b]

Values are means of five replicates from two experiments. Data were scored after seven weeks of culture

Means with common letters within each column are not significantly different at $P \leq 0.05$, according to DMRT

Table 8 SE germination in BA (2.25 μM); MS contained sugars and sugar alcohols (25 SEs/culture)

Treatment	Plant conversion (RL + SL)	
	RL (mm)	SL (mm)
Maltose 6% (control)	9.4[a]	12.8[a]
Maltose 3% + sorbitol 1.5 g/L	6.7[b]	10.1[b]
Maltose 4% + sorbitol 1.5 g/L	7.3[b]	10.3[b]
Maltose 3% + sorbitol 3 g/L	0.0[c]	0.0[c]
Maltose 3% + mannitol 1.5 g/L	10.1[a]	13.0[a]
Maltose 4% + mannitol 1.5 g/L	10.2[a]	13.1[a]

Values are means of five replicates from two experiments. Data were scored after ten weeks of culture

Means with common letters within each column are not significantly different at $P \leq 0.05$, according to DMRT

germination rate. All of the plantlets derived at low PEG levels were apparently healthy, while plantlets developed at higher levels of PEG were weak.

Amino acids

L-Glutamine at 100 mg/L followed by L-asparagine markedly improved germination and plantlet conversion (Table 5). At a higher concentration (500 mg/L), L-asparagine, L-cystine and L-proline hindered the normal growth and developmental process. Although the morphology of converted plantlets was barely disturbed, the overall growth was checked at higher concentrations of amino acids.

Ex vitro transplantation

After a week of incubation at room temperature, plantlets with well-developed roots and shoots were finally transferred to the field (Fig. 1h). 100% survival was observed and the plants flowered normally.

Table 9 SE germination (plantlet conversion) in MS medium containing BA (2.24 μM) along with 6% maltose and various PEG concentrations (25 SEs/culture)

Treatment	Plant conversion (SL + RL)	
Maltose (%) + PEG (g/L)	SL (mm)	RL (mm)
6 + 0.0 (control)	12.5[b]	9.4[b]
6 + 1.87	12.9[b]	9.4[b]
6 + 3.75	13.7[a]	9.9[a]
6 + 7.50	11.1[c]	8.6[c]

Values are means of five replicates from two experiments. Data were scored after ten weeks of culture

Means with common letters within each column are not significantly different at $P \leq 0.05$, according to DMRT

Discussion

In nature, embryogenesis is the fusion of male and female gametes, termed "zygotic embryogenesis," but in a culture, plant somatic cells also have the potential to produce embryos, in a process called "somatic embryogenesis." Two types of embryogenic processes have been observed in plant systems: (a) direct embryogenesis (directly on the explant) and (b) indirect embryogenesis (intervening callus phase). In our experiments, embryogenic calli were induced on hypocotyls of seeds germinated in vitro, and then embryos were produced (i.e., the indirect approach).

We have evaluated the roles of several factors in *Catharanthus* embryogeny. In the present investigation, the highest embryogenesis percentages, embryo proliferations, maturations and germinations were obtained when the pH was adjusted to 5.8–6.0. A similar influence of pH was also noted in other groups of plants (Smith and Krikorian 1990; Owen et al. 1991; Huang et al. 1992). Increasing the gelrite concentration resulted in decreased embryo proliferation, with low numbers of torpedo and cotyledonary embryos, and it reduced the maturation and germination of embryos as well. In the Miho 51–4 line of *Chamaecyparis obtusa*, however, the embryo yield increased on medium with 3.0 g/L gelrite (Taniguchi et al. 2004). Similarly, in *Pinus taeda* somatic embryos were induced on medium containing 2.5 g/L gelrite (Pullman et al. 2003). In our study, the application of gelrite proved to be less effective; increasing the level of gelrite in the medium was reported to reduce the bioavailability of magnesium, calcium, zinc, manganese and other inorganic and organic salts (Van Winkle et al. 2003).

In cultures, plant cells or tissues show poor autotrophic properties and need external carbon for energy. The addition of exogenous carbon to the medium enhances cell growth; it accelerates regeneration and influences in vitro embryogenesis (Verma and Dougall 1997; Strickland et al.

1987; Blanc et al. 1999). In a previous study, the present authors (Junaid et al. 2006) noted enhanced maturation and germination on medium added with maltose. We also evaluated the effect of sugar alcohol in this *Catharanthus* study. Mannitol effectively enhanced maturation and germination, while sorbitol was noted to be ineffective. The various carbohydrate sources have been previously shown to influence in vitro growth and development, including embryo maturation and somatic embryogenesis in general, in other species (Scott and Lyne 1994; Reidiboym-Talleux et al. 1998; Nuutila et al. 2002; Cheong and Pooler 2004).

The quality of the SE determines the frequency of plantlet conversion. Recently, a number of treatments have been adopted in order to improve embryo quality through the use of abscisic acid, sugars, sugar alcohol, PEG, etc., during maturation and germination (Xing et al. 1999; Lipavská and Konrádová 2004; Robichaud et al. 2004). Embryogenesis in *Catharanthus* was influenced by the culture photoperiod; a 16-h light period proved very effective for faster embryo proliferation, while a 12-h light period improved embryo maturation and germination. In several *Pinus* species, however, the role of light was noted to be less important, and it even inhibited embryogenesis (Druart 1981; De March et al. 1993; Machado et al. 1995). Although light or dark photoperiod-induced somatic embryogenesis has been reported previously in the literature (Fiore et al. 1997; Torne et al. 2001), knowledge of the molecular mechanisms that cause this influence is limited. Analysis of SE induction in wheat has, however, indicated that the light-induced expression of some proteins is involved in this process and phytochrome was noted to be an important key molecule (Nato et al. 2000; Kevei and Nagy 2003).

In the present study, attempts have been made to improve the quality of SEs by using compounds like high molecular mass osmoticum PEG. This only improved proliferation when used alone, while maltose and PEG used together enhanced embryo germination. The response has been reported to be very similar in several other plant genera and species like *Chamaecyparis obtusa* (Taniguchi et al. 2004), *C. pisifera* (Maruyama et al. 2002) and *Abies nardmanniana*) (Norgaard 1997). Increased concentrations of PEG or carbon perhaps alter the osmolarity level of the medium, which is responsible for a lower embryo yield. Appropriate osmolarity of the medium was previously noted to be an important factor in the successful establishment of somatic embryogenesis (Gupta and Grobe 1995). The combined application of ABA and PEG has become a routine method for stimulating SE maturation in some conifer genera (Bozhkov and Von Arnold 1998) and in selected tree species such as *Havea braziliensis* (Linossier et al. 1997). Information on the promotive effects of pH, PEG, etc. on cell differentiation is also noted to be inadequate. These factors are often applied as stressor to create forced environmental signal(s) for acquiring embryogenic competence (Choi et al. 1998; Pasternak et al. 2002; Ikeda-Iwai et al. 2003). These stresses trigger or alter gene expression, produce stress proteins (Rey et al. 2002; Puigderrajols et al. 2002), and or affect cell cycle regulation, which regulate embryogenesis (Gaj 2004).

In treatments where amino acids were added, the highest levels of embryogenesis, proliferation and maturation were observed for moderate concentrations of L-glutamine followed by L-asparagine. For SE germination and plantlet conversion, lower concentrations were, however, very effective. Increased embryogenesis upon the addition of amino acids to the culture medium have also been reported in other plant species; glutamine promoted embryogenesis in peaches and nectarines (Bhansali et al. 1990) and in *Eucalyptus* (Pinto et al. 2002). L-Proline enhanced somatic embryogenesis in the early stages of culture of floribunda rose (Marchant et al. 1996) and *Miscanthus* (Holme et al. 1997). Cheong and Pooler (2004) recently reported that the addition of L-cystine, L-glutamine and L-proline, particularly at higher concentrations, was ineffective for *Prunus incisa* cv February pink embryogenesis.

In conclusion, the present study demonstrates the effects of a wide variety of cultural factors (pH, gelrite, light, sugar alcohols, PEG and amino acids) on different developmental stages of embryogenesis in *C. roseus*. This procedure may offer new possibilities for genetic transformation and faster plant propagation, including transgenics, as an efficient somatic embryogenesis protocol has been established in this species. This study provides valuable information that could be used in the future to improve the yields of vinblastine and vincristine; it could also be used as a medicinal raw material to isolate other alkaloids present in the plant.

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