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Embryogenesis in *Catharanthus roseus*: Roles of Some External Factors in Proliferation, Maturation and Germination of Embryos

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Abstract *Catharanthus roseus* is an important medicinal plant that contains two well-known anticancerous alkaloids, vincristine and vinblastine. Cell culture technology has been employed for a long time to improve the alkaloid yield. In this chapter, various processes of somatic embryogenesis such as embryo induction, proliferation, maturation and germination are described. In this embryogenic system, embryos showed irregularities in structure and registered poor conversion frequency. Several carbon sources were added in order to improve the embryo quality before germination: 3% fructose or 3–6% maltose were found to be effective during maturation. Plantlet conversion was high on 3–6% maltose and 3% fructose. In addition, suspension culture, indirect embryogenesis and loss of embryogenic potential with time are discussed in brief. The authors felt that the low yields of vincristine and vinblastine may be improved if the single cell embryo origin concept is utilized in a genetic modification program.

1 Introduction

Catharanthus roseus is a fleshy perennial, growing up to 32-in. (80-cm) high. It has glossy, dark green, oval leaves and flowers all summer long. *C. roseus* is native to the Indian Ocean island of Madagascar. This herb is commoner in many tropical and subtropical regions worldwide, including the southern USA.

Extracts of entire dried plant contain many alkaloids of medicinal use. The principal alkaloid is vinblastine or vincaleukoblastine (vinblastine sulfate), sold as Velban. The alkaloid has a growth inhibition effect in certain human tumors. Vinblastine is used experimentally for treatment of neoplasms and is recommended for generalized Hodgkin's disease and resistant choricarcinoma. Another pharmacologically important alkaloid is vincristine sulfate or vincristine. Vincristine is used against leukemia in children. Vinblastine and vincristine in combination has resulted in 80% remission in Hodgkin's disease, 99% in acute lymphocitic leukemia, 80% in Wilm's tumor, 70% in gestational choricarcinoma and 50% in Burkett's lymphoma. There are over 100 other alkaloids in addition to vinblastine and vincristine. Synthetic

vincristine also used to treat leukemia is, however, only 20% effective as compared with the natural product derived from *C. roseus*.

Since 1950, cell culture techniques have been used to improve alkaloid content in *Catharanthus.* The process has been divided into two phases:

1. Establishment of culture
2. Extraction of alkaloids

For establishing culture, various plant parts, i.e., explants (shoot, root, callus, organs, suspension, etc.), have been used. Several key factors that have major control over the biosynthesis of alkaloids have been optimized and were reviewed (Moreno et al. 1995; Mujib et al. 2003). However, the study of somatic embryogenesis has not yet been reported and its importance to enhance yield not assessed. It is a remarkable process by which plant cells are transformed into embryos in culture. Although, the process has been reported in a wide range of plants, plantlet recovery is not always satisfactory. This is partly due to the absence of an optimized system which induces rapid embryo formation and proliferation. The induced somatic embryos also show a range of abnormalities in structure, secondary/adventive embryo formation on primary structures and a higher degree of heterogeneity (Akula et al. 2000; Cho et al. 1998; Ilah et al. 2002). The quality of somatic embryo, in turn, determines the success of maturation and in vitro germination. The low rate of embryo germination and subsequent poor conversion is one of the major challenges in embryogenic research. Somatic embryos with normal morphology also behave differently in different cultural conditions (Soh et al. 2000). A variety of studies have recently been conducted to enhance proliferation rate and plant recovery (Saito et al. 1991; Etlenne et al. 1997; Afreen et al. 2002; Lee et al. 2001). The present chapter describes the role of plant growth regulators in *Catharanthus* and the involvement of external factors like carbohydrate and pH is assessed at different stages of development.

2
Establishment of Somatic Embryo in *Catharanthus*

The process of somatic embryogenesis is a complex multistep process which is divided into the following stages: (1) establishment and maintenance of embryogenic tissues from explant, (2) proliferation of embryos and (3) embryo maturation and germination.

2.1
Induction and Maintenance of Embryogenic Tissue

Initiation of callus tissues in *Catharanthus* was induced from various tissues like stem, leaf and root; however, induction of embryogenic callus was

only achieved from hypocotyl tissue derived from in vitro germinated seeds. Two media, namely, Murashige and Skoog (MS) and White, were used, and both proved to be effective in establishing culture. The production of somatic embryos is controlled by various external factors such as carbon sources, nitrogen source, dissolved oxygen and pH. Since Skoog and Miller (1957) the role of auxins in tissue culture especially in somatic embryogenesis has been well established (Dudits et al. 1991; Davletova et al. 2001). A number of auxins, both natural (indole-3-acetic acid, indole-3-butyric acid) and synthetic (such as naphthalene acetic acid, NAA, 2,4-dichlorophenoxyacetic acid, 2,4-D, chlorophenoxyacetic acid, CPA, and 2,4,5-trichlorophenoxyacetic acid) have been regularly added to the culture media for somatic embryogenesis. However, auxin involvement in triggering embryogenesis has been only noted at the early stage of embryogenesis; later on auxins inhibit embryo growth. The removal or addition of lower concentrations of auxins was thus necessary. Auxins like 2,4-D and CPA are also required for the formation of the callus on which the embryo originates from "induced embryogenic cells" (Sharp et al. 1980). The rapid uptake of auxins results in depletion of the medium and in liquid medium they disappear early and eventually increase the plant growth regulator (PGR) level within the tissues. In *Catharanthus*, all the auxins (2,4-D, CPA and NAA) had a profound influence on callusing: the effective concentration only varies and generally lies within 0.5–2.0 mg/L. The hypocotyl callus was friable, light yellow, fast growing and the callus mass transformed into embryogenic tissue. The other explant sources (stem, leaf, etc.) induced calli which are non-embryogenic in nature, being characterized by their compact and nodular appearance and relatively slow growth. The embryogenic calli of hypocotyl origin were routinely maintained on medium supplemented with the same or a lower concentration of auxin alone or in combination with cytokinin (6-benzylaminopurine, BAP). Periodic transfer of tissues (3-week intervals) onto fresh nutrient media kept the callus mass growing and prevented necrosis. Subculture at extended intervals, however, reduced embryogenic ability; this temporary regenerative loss was resumed on restoration of normal cultural conditions.

2.1.1 Indirect Embryogenesis

Somatic embryogenesis has been reported in numerous plant genera where two distinct modes have been recognized. In some cultures, embryogenesis occurs directly without any callus phase, whereas in indirect embryogenesis the embryo develops from already induced meristematic callus clusters. In *Catharanthus* vigorous embryogenesis was established following a callus phase from hypocotyls. Many of the cultures also developed adventitive/secondary embryos. In such cases the growth of primary structures was significantly checked. Several embryos on solid medium were ag-

gregated, laterally coalesced and showed ill-developed roots and other abnormalities.

2.1.2 Suspension Culture

Suspension culture was established by transferring 2–3-week-old embryogenic callus on MS liquid medium containing auxin alone or with cytokinin. Continuous agitation on a gyratory shaker at 120 rpm yielded rapid proliferation of embryogenic callus and released free cells with cell aggregates. The embryogenic cells were small and round, contained abundant starch and divided rapidly to form cell aggregates (two to five cells) of quadrilateral to hexagonal appearance. Some of the single cells were elongated and vacuolated; these cells showed limited cell divisions with transverse end-to-end attachment. After 1 week, a proembryo-like structure developed from cell aggregates and later transformed into globular and heart-shaped embryos. However, elongated and vacuolated cells did not participate in embryogenic processes.

The globular or heart-shaped embryos did not progress to maturity in liquid medium. Use of solid medium at this stage and onwards is important for embryo maturity. This strongly suggests a need of stability (which the semisolid agar provides) to establish a shoot–root axis/polarity at advanced stages of embryo development. However, a second round of callusing and embryogenesis was also simultaneously noted in solid media. A similar arrest of growth of somatic embryos in liquid medium was earlier noted in other plant systems (Soh et al. 2000).

2.2 Proliferation of Embryos

Four to five week old embryogenic calli differentiated into embryos in NAA (1.0 mg/L) added medium; other auxin sources were less effective for production of embryos. A heterogeneous mixture of somatic embryos (globular, heart and cotyledonary) was visible under a simple microscope. Embryos were induced generally in masses along with proliferating clumps of embryogenic callus. Addition of BAP in NAA-supplemented (1.0 mg/L) medium improved the embryo proliferation process (Fig. 1a, Table 1).

The pH of the medium, a key cultural condition, influences in vitro responses. Thus, a range of pH values (4.0–7.0) were tested to see their effect on embryogenesis. Table 4 shows the influence of the initial pH on the production of somatic embryos. The maximum embryo productivity was recorded in media with pH 5.5–6.0, adjusted before autoclaving. Wetherell and Dougall (1976) earlier observed the same pH range for somatic embryo production in carrot. However, the set pH generally changes in all the media after auto-

Table 1 Somatic embryogenesis in proliferation media. Murashige and Skoog (*MS*) medium contained naphthalene acetic acid (1.0 mg/L) with various 6-benzylaminopurine (*BAP*) concentrations (sources: 40–50 mg embryogenic callus, data scored after the seventh week of culture)

BAP concentration (mg/l)	Embryogenesis (%)	No. of somatic embryos/culture	Different stages of somatic embryos			
			Globular	Heart	Torpedo	Cotyledonary
0.5	43.75 ± 4.20^{d}	38.75 ± 2.27^{d}	18.25 ± 1.7^{c}	12.0 ± 1.6^{c}	6.25 ± 1.70^{b}	2.25 ± 1.2^{b}
1.0	61.75 ± 3.03^{ab}	82.5 ± 3.69^{b}	54.0 ± 1.87^{a}	18.5 ± 2.6^{b}	7.00 ± 1.75^{b}	3.00 ± 0.9^{b}
1.5	73.00 ± 3.67^{a}	99.25 ± 2.27^{a}	61.5 ± 1.18^{a}	22.5 ± 1.2^{a}	9.00 ± 0.80^{a}	6.25 ± 1.7^{a}
1.75	49.00 ± 5.52^{c}	46.25 ± 2.58^{d}	21.5 ± 1.29^{c}	18.5 ± 1.3^{b}	4.00 ± 0.81^{c}	2.25 ± 1.2^{a}
2.0	41.25 ± 4.60^{d}	64.75 ± 3.69^{c}	39.25 ± 1.7^{b}	12.2 ± 1.7^{c}	10.2 ± 1.70^{a}	3.00 ± 0.8^{a}
ANOVA						
F	9.031	11.310	2.346	0.759	1.102	2.011
P^{+}	0.000***	0.000***	0.88 ns	0.44*	0.550 ns	0.492*
LSD 5%	5.102	3.909	2.536	2.176	1.182	1.075

Values are means ± standard errors of at least three replicates. Within each column, values followed by the *same superscript letter* are not significantly different at the $P = 0.05$ level according to the least significant difference (*LSD*) test.
F test significant at $^{***}P < 0.001$, $^{*}P < 0.05$

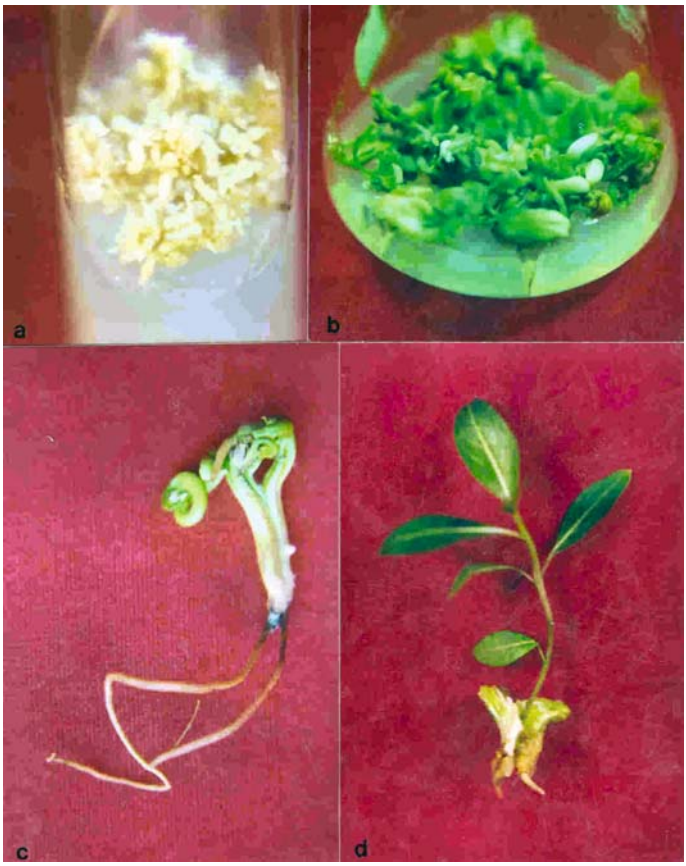

Fig. 1 Somatic embryogenesis and plant regeneration in *Catharanthus roseus* **a** A heterogeneous mixture of somatic embryos in proliferation medium. **b** Large green somatic embryo in maturation medium. **c** Somatic embryo with black necrotic zone at shoot–root axis. **d** Somatic embryo regenerated plantlet

claving and during the culture period (Smith and Krikorian 1990; Owen et al. 1991; Huang et al. 1993; Sakano 1997) and may alter embryo production ability. The mature embryo productivity was also similarly high (Table 5) when the initial pH was adjusted to 5.5–6.0.

2.3 Embryo Maturation, Germination and Role of Carbon Sources

In in vitro culture plant cells or tissues show little autotrophic property, even the apparently green tissues are not fully autotropic and need external carbon for energy. The addition of various carbon sources in the media enhances cell growth, regeneration and also influences somatic embryogenesis (Verma

and Dougall 1977). However, poor embryo quality limits plantlet conversion frequency. Recently, a number of treatments have been adapted to embryos involving the use of abscisic acid, sugar, sugar–alcohol, poly(ethylene glycol), etc. during maturation and germination (Xing et al. 1999; Lipavska and Konradova 2004; Robichaud et al. 2004). Sucrose is generally the carbon source of choice; however, other sugars are used frequently in tissue culture. In this chapter the roles of various carbon sources are evaluated at different stages of embryogenesis.

Individual white-opaque cotyledonary somatic embryos were cultured on MS medium fortified with gibberellic acid (1.0 mg/L) for maturation. Somatic embryos turned green (Fig. 1b), increased in length and occasionally became coiled but did not germinate into plantlets. However, the green embryos germinated well (Fig. 1d) in media supplemented with BAP (0.5 mg/L). The maturation and germination were influenced by carbohydrate sources (Tables 2, 3) as the somatic embryos increased in size in all the sugar sources tested and maintained steady growth up to the seventh week of culture. The 3% level of carbohydrate is more active than the 6% level in which embryo growth was slow and this tendency was noted for all carbon sources, such as maltose, glucose, fructose and even sucrose. Germination, i.e., plantlet conversion, is high in 3–6% maltose and 3% fructose, whereas 3% glucose and 6% sucrose/fructose had little effect on germination. In some of the sugars

Table 2 Somatic embryo in maturation media (MS + 1.0 mg/L gibberellic acid), added with different carbohydrates

Treatment	Initial length of embryos (mm)	Length after 5 weeks (mm)	Length after 7 weeks (mm)
Sucrose 3%	5.70 ± 0.5^{bc}	8.50 ± 0.3^{b}	10.05 ± 0.2^{c}
Sucrose 6%	5.52 ± 0.2^{bc}	7.275 ± 0.2^{a}	9.05 ± 0.3^{dc}
Maltose 3%	6.57 ± 0.3^{a}	9.675 ± 0.2^{a}	11.47 ± 0.3^{a}
Maltose 6%	5.80 ± 0.6^{b}	8.300 ± 0.2^{bc}	9.54 ± 0.3^{cd}
Glucose 3%	6.37 ± 0.2^{a}	8.750 ± 0.2^{b}	10.725 ± 0.5^{b}
Glucose 6%	6.00 ± 0.4^{ab}	7.533 ± 0.2^{d}	8.625 ± 0.4^{c}
Fructose 3%	5.725 ± 0.5^{b}	8.675 ± 0.3^{b}	10.625 ± 0.4^{b}
Fructose 6%	5.175 ± 0.3^{c}	7.42 ± 0.8^{cd}	9.70 ± 0.2^{c}
ANOVA			
F	8.416	12.262	13.12
P^{+}	0.000^{***}	0.000^{***}	0.002 ns
LSD 5%	0.542	0.657	0.559

Values are means ± standard errors of five replicates with six embryos in each replicate. Within each column, values followed by the *same superscript letter* are not significantly different at the $P = 0.05$ level according to the LSD test.
F test significant at $^{***}P < 0.001$

Table 3 Somatic embryo germination (plantlet conversion) in BAP (0.5 mg/L). MS medium also contained different sugars and concentrations

Treatments	Plant conversion (RL + SL) RL (mm)	SL (mm)	Only root RL (mm)	Only shoot SL (mm)	Leaf number LN
Sucrose 3%	5.80 ± 0.7^{d}	8.57 ± 0.4^{d}	–	11.80 ± 0.4^{b}	3.5 ± 1.2^{a}
Sucrose 6%	–	–	11.92 ± 0.5^{a}	9.275 ± 0.4^{d}	2.0 ± 1.8^{a}
Maltose 3%	8.17 ± 0.2^{b}	11.6 ± 0.4^{b}	–	11.00 ± 0.1^{bc}	2.5 ± 1.2^{a}
Maltose 6%	9.36 ± 0.4^{a}	12.80 ± 0.1^{a}	9.35 ± 0.5^{b}	12.77 ± 0.7^{a}	3.0 ± 2.1^{a}
Glucose 3%	–	–	–	9.625 ± 0.6^{d}	1.5 ± 1.2^{a}
Glucose 6%	5.30 ± 0.4^{d}	8.55 ± 0.5^{d}	–	11.00 ± 0.4^{c}	2.5 ± 2.3^{a}
Fructose 3%	7.26 ± 0.3^{c}	9.55 ± 0.5^{c}	–	11.75 ± 0.1^{bc}	2.5 ± 1.2^{a}
Fructose 6%	–	–	7.30 ± 2.6^{b}	7.925 ± 0.3^{c}	2.2 ± 2.6^{a}
ANOVA	0.752	0.267	2.646	0.379	0.567
F	0.043*	0.0674 ns	0.048*	0.820 ns	0.345*
P^{+}	0.536	0.554	0.436	0.700	2.455
LSD 5%					

Data were recorded after 10 weeks on germinating medium following 7 weeks in maturation media. Values are means ± standard errors of five replicates with six embryos per replicate per treatment. Values followed by the *same superscript letter* are not significantly different at the $P = 0.05$ level according to the LSD test.

Dashes indicate that there were no converted plantlets and and there was no root development.

F test significant at $^{*}P < 0.05$

Table 4 Effect of pH on somatic embryo proliferation in *Catharanthus roseus*

pH values	No. of somatic embryo/culture	Different stages of somatic embryos Globular	Heart	Torpedo	Cotyledonary
4.0	53.66 ± 2.4	31.34 ± 2.6	16.00 ± 1.7	4.66 ± 0.5	2.33 ± 0.5
4.5	61.00 ± 2.4	32.00 ± 1.6	21.00 ± 1.0	5.00 ± 2.0	3.00 ± 2.6
5.0	61.33 ± 2.6	34.67 ± 2.0	16.00 ± 3.6	6.33 ± 2.0	4.33 ± 2.0
5.5	69.66 ± 3.2	40.33 ± 1.2	16.67 ± 3.7	7.00 ± 2.0	5.66 ± 1.5
5.8	80.33 ± 2.9	49.34 ± 2.3	21.67 ± 4.0	9.33 ± 2.1	7.00 ± 2.4
6.0	99.25 ± 2.2	61.50 ± 1.1	22.50 ± 1.2	9.00 ± 0.8	6.25 ± 1.7
6.5	59.00 ± 7.4	30.66 ± 1.6	20.33 ± 2.0	4.00 ± 2.6	4.00 ± 2.0
7.0	24.00 ± 3.5	15.33 ± 1.2	8.66 ± 3.7	–	

- Values are means ± standard errors of at least 3 replicates.
- Hormones for proliferation (MS+NAA 1.0 mg/L)+BAP (1.5 mg/L)
- Incubation period: 6th weeks of culture.
- Sugar: Maltose 6%
- Inoculam: Embryogenic callus

Table 5 Effect of pH on somatic embryo maturation

pH values	Matured embryo/culture	Forms of embryos Normal embryo (%)	Abnormality (%)
4.0	18.00 ± 3.00	10.00	50.00
4.5	18.67 ± 3.51	33.99	21.94
5.0	19.66 ± 3.05	51.00	7.98
5.5	21.65 ± 0.57	60.84	3.95
5.8	22.48 ± 0.34	73.42	3.08
6.0	24.00 ± 2.0	78.34	2.20
6.5	29.00 ± 0.81	84.00	2.34
7.0	6.33 ± 1.53	7.98	10.32

- Values are means ± standard errors of at least 3 replicates.
- Hormones for plant maturation: MS + GA_3 (1.0 mg/L)
- Incubation period: 6 weeks
- Sugar: Maltose 6%
- Inoculum: 30 embryo/culture

tested, a black necrotic zone developed at the shoot–root junction as a mark of an adverse effect (Fig. 1c).

Except for glucose, the sugar level only induced primary roots without any visible shoot and has little importance in a plantlet multiplication program. The involvement of carbohydrate sources on embryo maturation and germination was observed earlier in some systems (Alemanno et al. 1997; Li et al.

1998; Corredoira et al. 2003). But the entire physiology is still very complex to understand fully.

3 Loss of Embryogenic Potential

In *Catharanthus*, embryogenesis is very fast and readily induced from hypocotyl. The potentiality decreases with the age of the culture. The plant growth regulator that was active previously is less effective with the age of the cultures. The lost potentiality was recovered at least partially, where the combination and level of PGR was replaced with a new set of combinations. Early subculturing (2-week interval) has proved to be effective also to some degree. This incidence, however, is common in tissue culture; changes in ploidy of the culture cells and inhibitors released by the aging tissues were previously described as some of the reasons responsible for this embryogenic loss.

4 Conclusion and Some Areas of Interest

C. roseus is a medicinal plant well known for its anticancerous properties. In cell culture techniques several tissues/explants have been used to establish culture; however, the importance of somatic embryogenesis has not been realized fully in an alkaloid improvement program. The present study indicates that embryos were produced in large numbers in solid media; however, in some cases embryogenesis is associated with embryo abnormalities like aggregation of proembryos/embryos, ill-developed roots, secondary callusing and embryogenesis, and root degeneration. Use of bioreactors may minimize such irregularities (Denchev et at. 1992; Hvoslef-Eide et al. 2002) and it also has the ability to improve biomass growth and to increase differentiation and plantlet production. Despite its many promises, the use of a vessel or bioreactor is still not integrated in alkaloid research.

Two different pathways of somatic embryogenesis have been discussed in plant systems, i.e., direct embryogenesis on explant and indirect embryogenesis via a callus phase. In both cases, the origin of the embryo is said to be from a single cell, which is easily amenable to genetic modification. The approaches like *Agrobacterium tumefaciens* mediated genetic alteration, T-DNA insertional mutagenesis, in vitro mutagenesis and selection of induced mutants, and protoplast fusion may generate new cell lines/plants with improved yield.

The process of embryogeny, particularly the aspect of maturation, germination or plantlet conversion, is a complex mechanism of interdisciplinary nature involving embryology, physiology, biochemistry and other subjects. Although

many of the facts have been addressed quite successfully in recent times, there are still questions that remain unanswered. Reduction in structural abnormalities will definitely increase the regenerability of somatic embryos. Besides, proper embryo selection and their transfer to optimized germination medium, selection of germinated rooted plantlets and their transfer to soil for acclimatization are some of the important stages and/or cultural practices that need more attention for success and reproducibility of plantlet production.

Embryonal masses have been preserved for many purposes. In *Catharanthus* the cryopreservation method has recently been established where the pretreatment, cryoprotectants, cooling and thawing processes have been optimized (Mannonen et al. 1990). Storage in liquid nitrogen and mineral oil is also used for the preservation of genetically engineered cells. On receiving appropriate cultural conditions, superior cell lines with high alkaloid producing ability will resume normal growth (Bacchiri 1995), but the information is still not enough in *Catharanthus*.

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