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Somatic embryogenesis and plant regeneration in *Catharanthus roseus*

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Abstract

Embryogenic callus in *Catharanthus roseus* was initiated from hypocotyl on Murashige and Skoog's (MS) medium supplemented with 1.0 - 2.0 mg dm^{-3} of 2,4-dichlorophenoxyacetic acid (2,4-D) or chlorophenoxyacetic acid (CPA). Calli from other sources were non-embryogenic. Numerous somatic embryos were induced from primary callus on MS medium suplemented with naphthalene acetic acid (NAA) within two weeks of culture. Embryo proliferation was much faster on medium supplemented with 6-benzylaminopurine (BAP). After transfer to medium with gibberellic acid (GA_3, 1.0 mg dm^{-3}) mature green embryos were developed and germinated well into plantlets on MS liquid medium supplemented with 0.5 mg dm^{-3} BAP. Later, embryos with cotyledonary leaves were subjected to different auxins treatments for the development of roots. Before transfer *ex vitro*, plantlets were cultivated on half strength MS medium containing 3 % sucrose and 0.5 mg dm^{-3} BAP for additional 2 weeks. Additionally, the effect of liquid medium has been evaluated at different morphogenetic stages.

Additional key words: auxins, cytokinins, gibberellic acid, *in vitro* culture.

Introduction

Catharanthus roseus belongs to the family *Apocynaceae* and possess several pharmaceutically active compounds including alkaloids. Vincristine and vinblistine are the most important alkaloids which are used against a variety of cancers. Nevertheless, the yield of these two compounds is relatively low. Cell and molecular biological studies have currently been employed to improve alkaloid yield and several key factors that have major control over the biosynthesis of alkaloids have been optimized (Moreno *et al*. 1995, Mujib *et al*. 2002). Various explants, *i.e.*, stem, root and callus were used, most of them responded well in culture, and showed various morphogenetic responses, but very little is known about somatic embryogenesis in *Catharanthus roseus*. Somatic embryogenesis has been currently used in several transgenic research programmes (Rommens *et al*. 2004, Walter 2004). However, the process of embryo formation and conversion is complex and affected by several external factors (Vooková and Kormuťák 2006). Among them, the role of plant growth regulators, especially of 2,4-D in determining the early stages of embryogenesis has been regularly emphasized (Davletova *et al*. 2001, Pasternak *et al*. 2002, Wang *et al*. 2006). Cytokinins are usually less effective but not uncommon (Mujib *et al*. 1998, Iantcheva *et al*. 1999). In the present paper, a suitable method of somatic embryo induction, progression and conversion to plantlets in *Catharanthus roseus* have been described.

Materials and methods

Plant material and sterilization: Semi mature fruits (follicle) of *Catharanthus roseus* were collected from plants grown in Jamia Hamdard herbal garden in the beginning of August 2003. Seeds were isolated from sterilized (70 % ethanol) fruits and treated with 0.5 % mercuric chloride for 2 min followed by 5 % H_2O_2, finally rinsed four times with sterilized double distilled water.

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Abbreviations: *ANOVA* - analysis of variance; BAP - 6-benzylaminopurine; CPA - chlorophenoxyacetic acid; 2,4-D - 2,4-dichlorophenoxyacetic acid; GA_3 - gibberellic acid; IAA - indole-acetic acid; IBA - indole-butyric acid; KIN - kinetin; MS - Murashige and Skoog (1962) medium; NAA - naphthalene acetic acid; 2,4,5-T - 2,4,5-trihydroxy-butyrophenone.
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***In vitro* germination of seeds and culture condition:** Seeds were placed in *Magenta* vessels containing 50 cm^3 of Murashige and Skoog (1962; MS) medium without growth regulators. Germinated seedlings were grown until they had overall 2 cm length. Seedlings were removed from the culture vessels and hypocotyls were excised from each seedling. For embryogenic callus initiation MS medium was supplemented with different growth regulators including 2,4-dichlorophenoxyacetic acid (2,4-D), chlorophenoxyacetic acid (CPA), 2,4,5-tri-hydroxy-butyrophenone (2,4,5-T), naphthalene acetic acid (NAA) and indole-acetic acid (IAA) (*Sigma Aldrich*, St. Louis, USA). These auxins were used alone or in combinations with different concentrations of cytokinins. Embryogenic callus was transferred to liquid and solid medium and used for germination and conversion studies. Embryogenic cultures were maintained for more than 18 months with regular subculture (4 weeks interval). All cultures were incubated under a 16-h photoperiod (cool white fluorescent lamp *F40 T12/CW/EG*) a photon flux density of 100 μmol m^{-2} s^{-1} and temperature of 25 ± 2 °C.

Induction of embryogenic callus: To examine the effect of 2,4-D, CPA, NAA, 2,4,5-T and other auxins on embryogenic callus formation, hypocotyls of 5 to 7-d-old germinated seeds were placed on MS medium supplemented with a range of auxin concentrations (0.25, 0.5, 1.0, 1.5 and 2.0 mg dm^{-3}). The percentage of hypocotyls which produced calli and somatic embryos were determined after 4 weeks onwards (for more detail see Junaid *et al.* 2004).

Somatic embryo initiation and proliferation: Friable callus (40 - 50 mg) was cultured either on solid or in liquid proliferation medium supplemented with different concentrations of BAP and NAA. The pH of both types of MS media was adjusted to 5.6 before autoclaving at 121 °C for 20 min. The number of somatic embryos (SE) per culture, embryogenesis percentage (percentage of culture showing embryogenesis) and number of somatic embryos (SE) at different developmental stages have been recorded.

Somatic embryo maturation: White opaque cotyledonary somatic embryos (5 - 6 mm length) isolated from the embryogenic culture were used and cultured on different solid and liquid maturation media supplemented with different concentrations (0.0, 0.25, 0.5, 0.75, 1.0 and 1.5 mg dm^{-3}) of gibberellic acid. *Whatman* filter paper of 2 cm radius was used on which somatic embryos were placed in 250 cm^3 Erlenmeyer conical flask with liquid medium (25 somatic embryos per flask). The experiment was repeated twice. Somatic embryo maturation percentage and growth (size and length of somatic embryo) of somatic embryos were measured.

Somatic embryo germination and plantlet formation: For germination and subsequent plantlet formation, matured somatic embryos (SE) were cultured on solid and liquid MS media supplemented with various concentrations of BAP and KIN. Separate application of IBA, IAA and NAA to media were used in order to achieve maximum plant recovery. Five replicates were tested for each concentration (25 SE per conical flask). The response percentage, germination or conversion performance of somatic embryos were scored in terms of root, shoot and total plantlet conversion.

Statistical analysis: Impact of different growth regulators in various concentrations on somatic embryo maturation and germination in both solid and liquid medium was made using *ANOVA* followed by a least significant difference (LSD) test at $P = 0.05$ to compare means.

Results

Embryogenic callus induction: Primary callus of *C. roseus* was initiated using hypocotyl pieces as explants. When hypocotyls were placed on MS medium supplemented with 1.0 - 2.0 mg dm^{-3} either of 2,4-D, NAA or CPA, callus initiation occurred. The callus was separated from the mother tissue and upon transfer to fresh nutrient medium, it grew as a white-yellow friable tissue. It could be maintained on regular subculturing at 3 - 4 weeks intervals.

Initiation and proliferation of somatic embryos: Two weeks old primary callus of hypocotyl origin was cultured on various combinations of plant growth regulators to induce somatic embryogenesis. Addition of 1.0 mg dm^{-3} of NAA was effective in inducing embryos. The other three auxins 2,4-D, CPA and 2,4,5-T induced embryogenesis with lower frequencies (data not presented). Various concentrations of BAP were also added to improve the efficiency of embryogenesis and we observed that during proliferation, the embryos appeared in masses over the entire primary callus derived from hypocotyl. The highest number of somatic embryos (99.25 ± 2.27 per 40 - 50 mg embryogenic callus) was obtained on medium supplemented with 1.5 mg dm^{-3} BAP + 1.0 mg dm^{-3} NAA where mean globular, heart, torpedo and early cotyledonary embryos were 61.5 ± 1.18, 22.5 ± 1.2, 9.0 ± 0.80 and 6.25 ± 1.7, respectively (Fig. 2).

Maturation, germination and plantlet conversion: Individually isolated white-opaque cotyledonary somatic embryos (5 - 6 mm in length) placed on MS medium supplemented with various concentrations of GA_3. After 2 weeks of culture somatic embryos turned green (Fig. 1*A*,*B*) and elongated, many of them also developed visible root axis without cotyledons. GA_3 in concen-

Fig. 1. *In vitro* plant regeneration in *Catharanthus roseus via* somatic embryogenesis. All pictures have been taken at magnification 10×. *A* and *B* - somatic embryos growing on MS maturation medium, solid and liquid media supplemented with 1.0 mg dm^{-3} GA_3; *C* and *D* - germinated plantlets in solid and liquid media originating from somatic embryos; *E* - *Catharanthus* plant regenerated from somatic embryo.

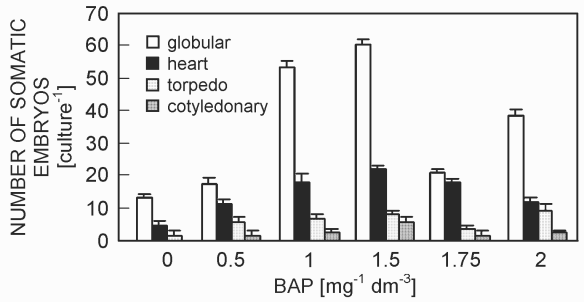

Fig. 2. Different developmental stages of somatic embryos developed on media suplemented with NAA (1.0 mg dm^{-3}) and BAP (as indicated). Data were scored after 7 weeks of culture.

tration 0.5 - 1.5 mg dm^{-3} was observed to be more promotive for this process (Fig. 3).

The growth of embryo (length in mm) was higher (up to 7 of weeks of culture) in solid medium compared to liquid medium (Table 1). In these GA_3 containing maturation media, somatic embryos became green, coiled frequently but were devoided of cotyledonary leaves and roots. Later, for germination (plant conversion) these matured embryos were individually placed on media supplemented with various concentrations of cytokinins (BAP and KIN).

In this study, three different types of responses were mainly observed: *1*) plants with shoot and root *2*) only shoot (without root), and *3*) only roots (devoid of shoots). Of the two cytokinins tested, BAP was found to be more active as compared to KIN in plant conversion (Table 2) programme from matured embryo. Plant conversion, shoot development frequency and subsequent growth, all were high in BAP containing media (0.5 mg dm^{-3} BAP was the most effective concentration). Plant conversion/ germination rate was, however, improved further when liquid medium was used. Many of the converted plantlets (both liquid and solid media) did not have proper root axes or with aborted root ends, therefore, other media combinations involving plant growth regulators that act as root inducers were tested. Shoot, root and plantlet conversion was high on media containing IBA and IAA (Table 3).

Table 1. Length [mm] of matured somatic embryos in solid and liquid MS medium supplemented with different concentrations of GA_3 (25 somatic embryos per culture and data were scored after 7 weeks of culture). Means ± SE of 5 replicates from two experiments. Within each column, values are followed by the same superscript letter are not significantly different at $P = 0.05$ level according to LSD test.

GA_3[mg dm^{-3}]	Solid	Liquid
0.00	5.32 ± 0.90^{e}	3.10 ± 0.70^{d}
0.25	8.45 ± 0.18^{c}	6.47 ± 0.45^{c}
0.50	9.00 ± 0.18^{b}	7.57 ± 0.23^{b}
0.75	9.20 ± 0.18^{b}	8.00 ± 0.52^{a}
1.0	10.00 ± 0.27^{a}	8.85 ± 0.12^{a}
1.5	8.72 ± 0.26^{c}	7.48 ± 0.15^{b}
2.0	7.50 ± 0.37^{d}	6.80 ± 0.24^{c}

Table 2. Shoot (SL) and root (RL) length [mm] of germinated somatic embryo on liquid or solid MS media supplemented with IBA, NAA or IAA [mg dm^{-3}]. Data were scored after 10 weeks of culture. Means ± SE of 5 replicates from two experiments. Within each column, values are followed by the same superscript letter are not significantly different at $P = 0.05$ level according to LSD test.

PGR		Liquid		Solid	
		SL	RL	SL	RL
IBA	0.25	15.00 ± 1.5^{a}	13.70 ± 2.0^{c}	4.80 ± 1.5^{d}	15.80 ± 0.3^{c}
	0.50	13.10 ± 1.6^{b}	16.50 ± 2.3^{b}	8.70 ± 0.7^{a}	20.60 ± 0.9^{b}
	0.75	10.20 ± 0.6^{cd}	18.00 ± 0.6^{a}	5.92 ± 0.8^{c}	23.30 ± 0.8^{a}
NAA	0.25	9.77 ± 0.6^{d}	5.04 ± 2.7^{h}	7.72 ± 0.6^{b}	9.20 ± 0.3^{e}
	0.50	7.27 ± 0.9^{e}	8.00 ± 0.5^{f}	5.27 ± 0.9^{c}	10.50 ± 0.5^{e}
	0.75	5.47 ± 0.4^{f}	9.67 ± 0.5^{e}	3.47 ± 0.4^{e}	13.90 ± 1.7^{d}
IAA	0.25	14.40 ± 1.4^{b}	10.20 ± 0.2^{de}	8.37 ± 0.4^{a}	6.25 ± 1.2^{g}
	0.50	11.30 ± 1.1^{c}	8.27 ± 1.3^{f}	5.77 ± 0.4^{c}	7.70 ± 1.3^{fg}
	0.75	10.20 ± 1.0^{cd}	7.92 ± 0.8^{g}	4.10 ± 0.6^{d}	13.00 ± 1.0^{d}

Table 3. Shoot length [mm] of germinated somatic embryo on liquid or solid MS media supplemented with BAP or KIN [mg dm^{-3}]. Data were scored after 10 weeks of culture. Means ± SE of 5 replicates from two experiments. Within each column, values are followed by the same superscript letter are not significantly different at $P = 0.05$ level according to LSD test.

PGR		Liquid	Solid
BAP	0.0	3.40 ± 0.8^{g}	2.80 ± 1.0^{h}
	0.25	9.00 ± 0.6^{e}	6.10 ± 1.0^{f}
	0.50	18.80 ± 1.9^{a}	12.50 ± 1.9^{a}
	0.75	15.80 ± 1.8^{b}	9.67 ± 0.9^{c}
	1.00	12.30 ± 2.3^{d}	8.67 ± 0.6^{cd}
KIN	0.0	2.90 ± 0.8^{gh}	2.10 ± 0.6^{h}
	0.25	7.27 ± 0.5^{ef}	5.50 ± 1.5^{fg}
	0.50	14.40 ± 1.9^{c}	9.00 ± 1.1^{c}
	0.75	14.00 ± 1.0^{c}	10.30 ± 0.7^{b}
	1.00	11.70 ± 2.3^{d}	7.25 ± 2.5^{de}

Among the tested combination of auxins, IBA applied together with NAA (0.25 + 1.5 and 0.5 + 1.0 mg dm^{-3}) was most effective for root, shoot development and plantlet conversion. These were followed by IAA + IBA combinations (data not presented). Matured cotyledonary green embryos rapidly germinated into plantlets (Fig. 1*D*) with well developed shoot and root at a maximum frequency 53 ± 2.64 in liquid medium compared to 44.64 ± 4.50 in solid medium (Fig. 1*C*). Shoot development frequency was also very high (61 ± 4.0 %) in liquid medium, but when somatic embryos formed roots with or without rudimentary shoot, the effect of solid medium was more prominent. In combinations NAA + IAA, IAA + IBA and IBA + NAA plantlets grew well with no major differences (data not shown), but in liquid medium conversion frequency was much better compared to solid one. Root growth was, however, more vigorous on solid medium.

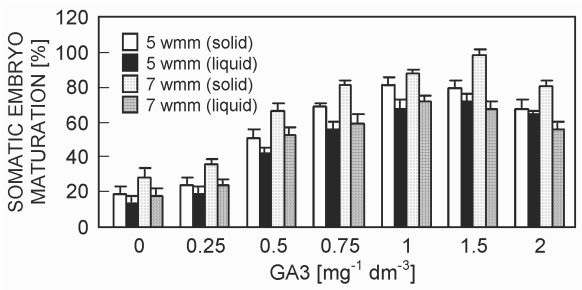

Fig. 3. Maturation of somatic embryos on different concentrations of GA_3. MS solid and liquid media were used. 5 wmm, 7 wmm - somatic embryo maturation percentage after 5 and 7 weeks in maturation medium, respectively.

Secondary somatic embryogenesis: Secondary somatic embryos were developed on the surface of the primary somatic embryos on various media, although it was the best on solid BAP (1.5 mg dm^{-3}) and NAA (1.0 mg dm^{-3}) containing medium (unpublished). A maximum of 3 - 4 somatic embryos were observed on one primary embryo. Except cotyledonary stage all other three morphological types of embryos (globular, heart, torpedo) were visible. The appearance of secondary embryos hindered normal primary embryo growth and conversion, and even secondary callus formation was observed from primary sources.

Preparation and establishment of plantlets for outdoor transfer: Somatic embryo regenerated plants (Fig. 1*E*) with well developed shoots and roots were cultured on ½ MS medium supplemented with 3 % sucrose and 0.5 mg dm^{-3} BAP for further development of new shoots. Within 2 - 3 weeks, the plantlets developed multiple shoots and roots which grew well on liquid medium. After additional 2 weeks they could be transferred to outdoor.

Discussion

Somatic embryogenesis from hypocotyl has been reported in details in *Catharanthus roseus*, an important medicinal plant. In the present study, callus induction was observed from a variety of explant sources but only the hypocotyl callus has proved to be embryogenic; calli from other sources were non-embryogenic. This differential responses may be due to varied level of endogeneous plant growth regulators and other physiological gradients which are present in different explants. Similar behaviour was previously noted in

many other plants (Wernicke and Milkovits 1986, Mujib *et al*. 1996). Using combination of NAA and BAP, all stages of embryos were visible in a non synchronous fashion at varying numbers. In other plant species, 2-4-D influence on embryo induction and participation at initial stages of development has been widely reported (Gray *et al*. 1993, Mujib and Samaj 2006). Here, we have noticed that in *Catharanthus* 2,4-D is less effective compared to NAA.

Generally, two different types of embryogenesis have been observed: direct embryogenesis when embryos develop directly on explant without any intermediate callus formation and indirect embryogenesis where embryos arise on meristematic callus masses. In *Catharanthus*, embryos were produced indirectly on callus but never directly on hypocotyl explant. Furthermore, secondary/adventitive embryos were produced simultaneously on primary embryo structures, preferably on solid medium. Similar induction of secondary embryogenesis was earlier reported in embryogenic studies of many plant systems (Barbulova *et al*. 2002, Iantcheva *et al*. 2001)**.** However, both maturation and conversion of secondary embryos were poor. On the other hand, primary embryos advanced well towards maturity, and short application of GA_3 was found to be very effective in maturation before conversion. The embryo maturation and subsequent conversion represent a complex process. Various substances such as sugars, polyethylene glycol and sugar-alcohol in the medium substantially improve maturation and germination (Xing *et al*. 1999, Lipavská and Konrádová 2004, Robichaud *et al*. 2004). GA_3 induced enhanced embryo maturation and germination was also reported in current studies (Corredoira *et al*. 2003a, Junaid *et al.* 2006). In liquid medium, maximum plant recovery was achieved as shoot, root and total conversion were high on BAP or auxin containing medium. In *Catharanthus,* there has been no previous report on somatic embryogenesis in liquid medium nor any comparison has been ever made. The influence of liquid medium at different stages of embryogenesis was reported earlier for some plant species (Tautorus *et al.* 1994, Afreen *et al*. 2002) Somatic embryogenesis based plant regeneration reported here could be very useful for continuous regeneration of somatic embryos/plantlets for alkaloid production. The embryo precursor cell could be used in *Catharanthus* genetic manipulation studies.

Summary: The process of somatic embryogenesis and plant regeneration has been categorized into following stages: *1*) Embryogenic callus was established from hypocotyl on MS medium supplemented with 2,4-D, CPA within three weeks; *2*) somatic embryos were observed on media supplemented with NAA and BAP, they rapidly proliferated and produced more embryos; *3*) for maturation, GA_3 was very effective, the cotyledonary white opaque embryos turned into green embryos; *4*) **s**omatic embryos germinated and converted into plantlets on MS medium supplemented with BAP; *5*) the liquid medium promoted embryo growth and improved plantlet conversion. The plantlet recovery time from primary callus to well developed plantlets was around 4.5 - 5 months.

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