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Full Length Research Paper

An efficient somatic embryogenesis based plant regeneration from the hypocotyl of *Catharanthus roseus*

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An efficient and rapid somatic embryogenesis based plant regeneration from hypocotyls of three *Catharanthus roseus* cultivars, Pacifica cherry red (PCR), Heatwave mix color (HWMC), and Mediterranean Rose Red (MRR) was standardized. Hypocotyl-derived primary calluses (HPC) were formed on callus induction medium (MSCP1) after 10 days of culture. Embryogenic calluses (EC) with somatic embryos were induced from HPC on proliferation medium (MSCP2) within 2 weeks of culture. Later, EC were selected and maintained on plantlet formation medium (MSCP3) with periodic subculturing at an interval of 2 weeks. Somatic embryos were germinated well into plantlets on MSCP3 within 6 weeks of culture. As sources of nitrogen, casein hydrolysate (CH; acid free) and L-proline were necessary as standard addenda in the plant regeneration protocol of *C. roseus*. The promotive effects of CH and L-proline not only decreased the culturing time but also increased the number of somatic embryos and their derived plantlets. The percentage of EC and regenerated plant initiated from hypocotyls were higher than those previously reported.

Key words: *Catharanthus roseus*, multi-cultivar, hypocotyls, embryogenic callus, plant regeneration.

INTRODUCTION

Catharanthus roseus is a perennial semi shrub plant belonging to the family Apocynaceae. As an ornamental and medicinal plant, *C. roseus* is cultivated in tropical and subtropical regions of the world. The *Catharanthus* (or Vinca) alkaloids comprise a group of about 130 terpenoid indole alkaloids (TIAs). Many TIAs are true lead compounds for drug development, including two commercially important cytotoxic dimeric alkaloids, vinblastine and vincristine. These are utilized in a number of different cancer chemotherapies (Van der Heijden et al., 2004). Due to the pharmaceutical importance and the low yield in vinblastine and the related alkaloid vincristine, *C. roseus* became one of the best-studied medicinal plants. At present, only precursors and semi-synthetic derivatives of vinblastine and vincristine are obtained by chemosynthesis, cell and hairy roots cultures (Kurz et al., 1981; Bhadra et al., 1993; Hong et al., 2006). Accordingly, it is feasible to establish a genetic engineering method on *C. roseus*, aiming at higher production levels of these low-yield TIAs in the plant (Bhadra et al., 1993; Van der Fits and Memelink, 2000; Hong et al., 2006).

A few studies have been done on tissue culture regeneration protocol that is prerequisite for the generation of genetically transformed *C. roseus* plants. In *C. roseus*, plant regeneration has been reported from callus initiated from a range of explant material; for example, mature

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Abbreviations: PCR, Pacifica cherry red; **HWMC,** heatwave mix color; **MRR,** mediterranean rose red; **HPC,** hypocotyl-derived primary calluses; **EC,** embryogenic calluses; **MSCP,** basal medium; **MSCP1,** callus induction medium; **MSCP2,** proliferation medium; **MSCP3,** plantlet formation medium; **TIAs,** indole alkaloids, **BA,** benzylaminopurine; **NAA,** naphthalene acetic acid; **ZT,** zeatin; **IAA,** indole acetic acid; **RM,** rotary microtome; **DNMRT,** Duncan's new multiple range test; **2,4-D,** 2,4-dichlorophenoxyacetic acid.

zygotic embryo, hypocotyl, cotyledon, leaf petiole, stem node, shoot tip (Dhandapani et al., 2008), and immature zygotic embryo (Kim et al., 2004). However, in most of these reports, the formation of shoots and plantlets in *C. roseus* is rather infrequent and transient and requires long duration. In addition, all of the reports about somatic embryogenesis of *C. roseus* published so far have either unstable regeneration from any type explant or lack the corroboration of morphology and histology, which make genetic transformation studies on *C. roseus* difficult. Somatic embryogenesis resulting in regeneration of whole plant is an important step in the plant transformation method. Successful and stable transformation requires that a single cell gives rise to a plant. Ideal transformation scheme is done via somatic embryogenesis, because from callus, each transformed cell has the potential to produce a plant. Junaid et al. (2007a) reported that a somatic embryogenesis system has been established in *C. roseus* from hypocotyl and cotyledon, respectively. In the research of Dhandapani et al. (2008), hypocotyl and cotyledon of *C. roseus* cultivar 'little bright eye' were regenerated via organogenesis on benzylaminopurine (BA) and naphthalene acetic acid (NAA) containing medium. Two factors, the type of plant growth regulators (combination and concentration), and the genotype specificity may have resulted to this. The genotype-dependent plant regeneration from *C. roseus* cultivar 'little bright eye' leaf petiole and hypocotyl was reported to be important for plant regeneration (Choi et al., 2003; Lee et al., 2003). The phenomenon of genotype-dependent plant regeneration also exists in other plant species (Green et al., 1974; Firoozabady and DeBoer, 1993). Therefore, the extensive application of these protocols on other *C. roseus* cultivars will be limited by genotype specificity.

In this study, three *C. roseus* cultivars randomly selected from different series of variety were tested, aiming at the extensive application of regeneration protocol which can be utilized to genetically transform different *C. roseus* cultivars to create variations.

Two form of nitrogen sources and different plant growth regulators were supplemented into culture medium in order to significantly promote embryogenic callus formation from hypocotyl explants and, thus, highly efficient plant regeneration in a short duration. In this paper, the role of plant growth regulators and culture conditions in embryogenic callus induction, proliferation and regeneration into plantlets were discussed. And the use of casein hydrolysate (CH; acid free) and L-proline to induce an efficient somatic embryogenesis and plant regeneration from *C. roseus* multi-cultivar hypocotyls are reported for the first time.

MATERIALS AND METHODS

Plant materials and sterilization

Three *C. roseus* cultivars, Pacifica cherry red (PCR), Heatwave mix color (HWMC) and Mediterranean rose red (MRR), belonging to different series of variety were used in the present investigation and seeds were purchased from PanAmerican Seed Company, USA. Seeds were surface sterilized in consecutive washings of 75% (v/v) ethanol (2 min) and 20% commercial bleach [5.25% (v/v) NaOCl] (shaken for 10 min at 110 rpm min^{-1}), rinsed three times with sterile distilled water, and then were germinated on Murashige and Skoog (1962; MS) basal medium. Hypocotyl explants about 5 mm were excised from 4-day-old germinated seedlings.

Culture media and culture conditions

The basal medium (MSCP medium) consisted of MS salts (Murashige and Skoog, 1962) supplemented with 150 mg L^{-1} CH, 250 mg L^{-1} L-proline, 30 g L^{-1} sucrose, and 3 g L^{-1} gelrite. The pH of the medium was adjusted to 5.8 before autoclaving at 121°C for 22 min. All of the cultures were incubated at 25 ± 2°C under cool-white fluorescent tubes providing irradiance of 40 µmol m^{-2} S^{-1} during a 16-h photoperiod.

Induction and proliferation of embryogenic callus from hypocotyls

For primary callus initiation, MSCP medium was supplemented with a range of concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D), α-naphthalene acetic acid (NAA) and zeatin (ZT) (*Sigma Aldrich*, St. Louis, USA). These plant growth regulators were used alone or in combinations. Percentage of hypocotyl-derived primary calluses (HPC) and lengthways diameter of callus were determined after 10 days culture. Subsequently, non-brownish and prolifically growing HPC were selected and transferred onto MSCP medium supplemented with 0.5 - 6.0 mg L^{-1} 6-benzylaminopurine (BA) combination with NAA or 3-indole acetic acid (IAA) at a range concentration of 0.1 - 0.6 mg L^{-1} in order to induce and proliferate embryogenic calluses (EC). Percentage of EC and growth rate of callus [both of non-embryogenic calluses (non-EC) and EC] were scored after 2 weeks culture.

Selection of embryogenic callus

EC were selected based on the morphologic characteristics described earlier; *C. roseus* callus morphology differed with growth regulator combinations (Kim et al., 2004; Junaid et al., 2007b; Dhandapani et al., 2008). Embryogenic *C. roseus* callus can be described as mid-friable yellowish and granular callus, compact, greenish-yellow, granular with smaller cells and very dense cytoplasm callus. These types of EC were selected and used for plant conversion studies.

Plantlet formation

For plantlet formation, EC were selected and transferred onto plantlet formation medium consisting of MSCP medium supplemented with different concentrations of BA and IAA. EC were maintained with periodic subculturing at an interval of 2 weeks. After 6 weeks of culture, the percentage of regenerated plantlets from EC was determined, and the lengthways diameter of EC was also measured. Plantlets were subjected to acclimation, transplanted to potting soil and maintained in a growth chamber (25 ± 2°C, approximately 70 µmol m^{-2} s^{-1} from cool-white fluorescent tubes with a 16-h photoperiod).

Histological examination

Samples were collected at critical periods of in *vitro* culture and

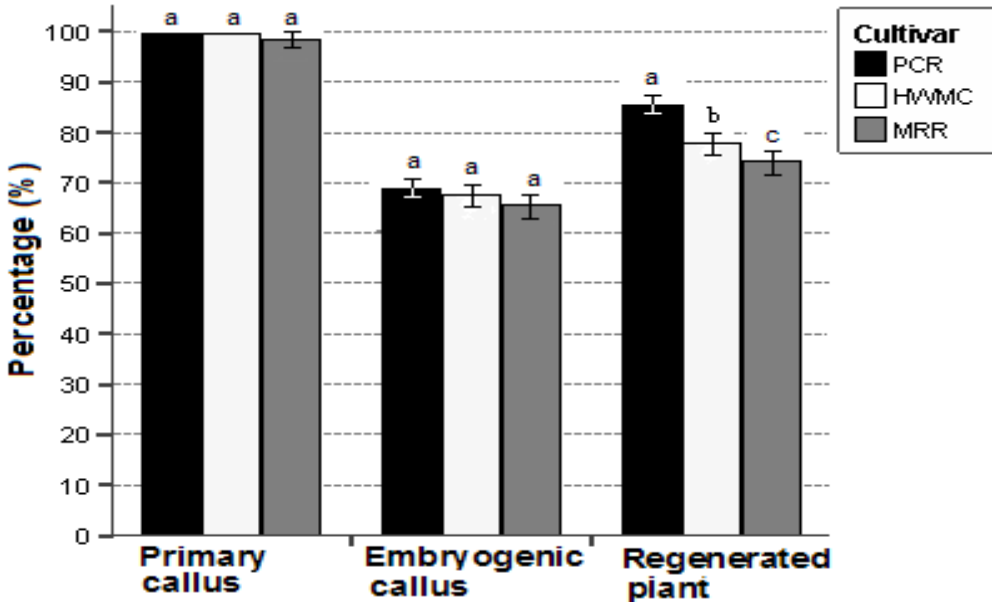

Figure 1. Comparison of somatic embryogenesis based plant regeneration of three *C. roseus* cultivars in different tissue culture stage. Data presented as means for each treatment and repeated thrice. Means followed by same letters within a bar cluster are not significantly different according to Duncan's new multiple range test (DNMRT) at P ≤ 0.05 level. Error bars represent the standard deviations.

fixed in FAA solution (formalin: glacial acetic acid: ethyl alcohol, 0.5: 0.5: 9, v/v/v) for 48 h at 4°C. Thereafter, the samples were stained with 1% haematoxylin for 5 days and dehydrated through a graded series of ethanol, then embedded in paraffin (55 - 60°C). Sections of 10 µm thickness were cut with a rotary microtome (RM 2135, Leica, Wetzlar, Germany), mounted onto glass slides, and then washed twice with xylene for 15 min separately to get rid of paraffin. The histological characteristics of samples were observed under a light microscope.

Experimental design and statistical analysis

The experiment was a randomized block design in the stage of induction and proliferation of EC, consisting of a factorial arrangement of plant growth regulators, three *C. roseus* cultivars, and two types of medium. In the stage of plantlet formation, the concentrations of BA and IAA in the medium were optimized with quadratic orthogonal rotation regression design method.

Each treatment consisted of at least 30 explants and the experiment was repeated thrice. The data of effects of plant growth regulators, CH and L-proline on different stages of embryogenesis and other parameters were analyzed by the statistical analysis system (SAS) software. Values are means of three replicates from three experiments, and the presented mean values were separated using Duncan's new multiple range Test (DNMRT) at P ≤ 0.05 level.

RESULTS AND DISCUSSION

Callus induction from hypocotyls

Hypocotyls of three *C. roseus* cultivars were tested for their best primary callus induction responses to a series of concentrations and combinations of four different types of plant growth regulators. Hypocotyls about 5 mm expanded and began to form calluses after 5 days culture. After 10 days, most of the hypocotyls produced off-white, mid-friable calluses, while the hypocotyls inoculated on MSCP medium with BA and NAA produced yellowish mid-friable, globular calluses (Figure 2A). Although the calluses responses to BA plus NAA showed slightly embryogenic characteristics, the percentage of HPC was low and drastically decreased with increasing concentrations. Brownish calluses were noted at concentrations more than 1.0 mg L^{-1} BA and 0.1 mg L^{-1} NAA. Otherwise, the puniness of promotive effect by 2,4-D alone is contrary to the reports of other investigators. Junaid et al. (2007a) indicated that a maximum of 85% callusing initiated from hypocotyls was recorded with 1.0 mg L^{-1} 2,4-D. In this study, the effect on callusing was improved by 2,4-D plus ZT, and with NAA plus ZT (Table 1).

Compared with the effect of all other combinations of plant growth regulators on callus induction, hypocotyls of all three *C. roseus* cultivars produced the highest percentage of calluses on MSCP medium supplemented with 1.0 mg L^{-1} 2,4-D, 1.0 mg L^{-1} NAA and 0.1 mg L^{-1} ZT (MSCP1 medium).

Somatic embryo initiation and proliferation from hypocotyl callus

In this study, two different approaches were employed to increase the formation of embryogenic calluses. Both

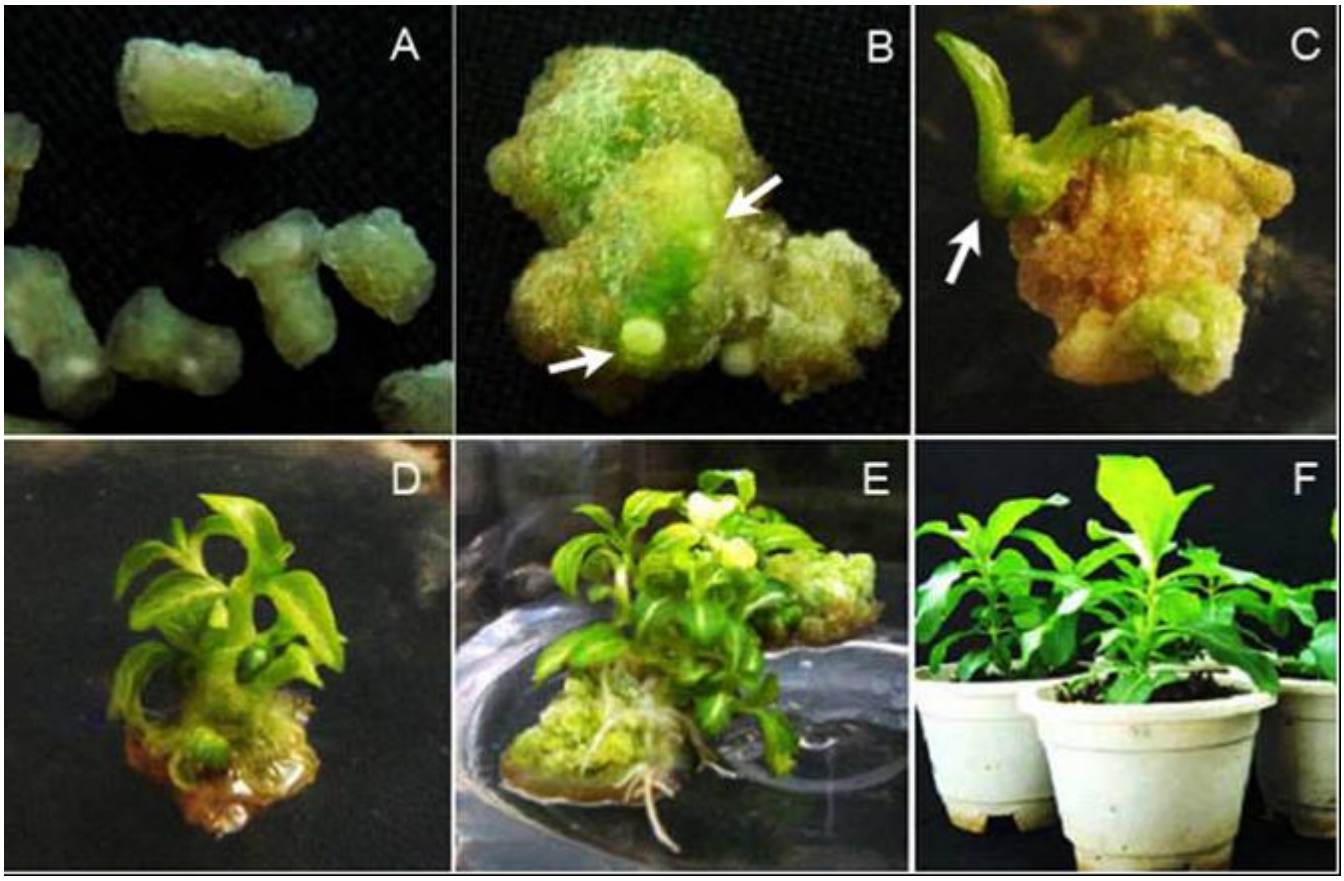

Figure 2. Somatic embryogenesis and plantlet regeneration from hypocotyls of *C. roseus*. (A) Primary calluses induction from hypocotyls on MSCP1 culture medium; (B) embryogenic callus with several globular embryo-like structures (arrows) developing at the surface of the callus on MSCP2 culture medium; (C) somatic embryo germination into leaf-like structures (arrow) on MSCP3 culture medium; (D) non-full-grown plantlets without roots obtained from converted somatic embryos; (E) full-grown plantlets obtained from converted somatic embryos; (F) acclimatized plantlets.

Table 1. Effect of 2,4-D, NAA and ZT on callus induction from the hypocotyl explants of three *C. roseus* cultivars, PCR, HWMC and MRR (data were scored after 10 days of culture).

Growth regulators	Concentrations ($mg\ L^{-1}$)	Percentage of callus (%)		
		PCR	HWMC	MRR
2,4-D	0.5	37.78 ± 1.11 b	35.56 ± 1.11 b	34.44 ± 1.11 c
	1.0	61.11 ± 1.11 a	56.67 ± 1.92 a	57.78 ± 1.11 a
	2.0	41.11 ± 1.11 b	38.89 ± 1.11 b	44.44 ± 1.11 b
	3.0	25.56 ± 1.11 c	23.33 ± 0.00 c	24.44 ± 1.11 d
2,4-D + ZT	0.5 + 0.1	53.33 ± 0.00 c	51.11 ± 1.11 c	48.89 ± 1.11 b
	1.0 + 0.1	84.44 ± 1.11 a	82.22 ± 1.11 a	81.11 ± 2.22 a
	2.0 + 0.2	76.67 ± 1.11 b	76.67 ± 0.00 b	77.78 ± 1.11 a
	3.0 + 0.3	32.22 ± 1.11 d	31.11 ± 1.11 d	28.89 ± 1.11 c
2,4-D + NAA + ZT	0.5 + 0.5 + 0.1	88.89 ± 1.11 b	87.78 ± 1.11 c	88.89 ± 1.11 b
	1.0 + 1.0 + 0.1	100.00 ± 0.00 a	100.00 ± 0.00 a	98.89 ± 1.11 a
	2.0 + 2.0 + 0.2	92.22 ± 1.11 b	91.11 ± 1.11 b	91.11 ± 1.11 b
	3.0 + 3.0 + 0.3	38.89 ± 1.11 c	37.78 ±1.11 d	38.89 ± 1.11 c

Data presented as means ± SE. Means followed by same letters within a column are not significantly different according to Duncan's New Multiple Range Test (DNMRT) at $P \leq 0.05$ level.

Table 2. Effect of BA, NAA and IAA on embryogenic callus induction and proliferation of three *C. roseus* cultivars, PCR, HWMC and MRR (data were scored after 2 weeks of culture).

Growth regulators	Concentration (mg L^{-1})	Growth rate of callus (%)			Percentage of EC (%)		
		PCR	HWMC	MRR	PCR	HWMC	MRR
BA+NAA	0.5 + 0.1	80.41 ± 0.12 a	80.35 ± 0.22 a	80.22 ± 0.29 a	25.56 ± 1.11 e	24.44 ± 1.11 e	23.33 ± 0.00 e
	1.0 + 0.1	50.69 ± 0.60 b	50.11 ± 0.20 b	49.82 ± 0.25 b	31.11 ± 1.11 d	32.22 ± 1.11 d	28.89 ± 1.11 d
	2.0 + 0.2	37.06 ± 0.50 c	36.52 ± 0.41 c	36.01 ± 0.96 c	43.33 ± 0.00 c	42.22 ± 1.11 c	44.44 ± 1.11 c
	3.0 + 0.3	35.08 ± 0.40 d	34.52 ± 0.60 d	34.41 ± 0.76 d	54.44 ± 1.11 b	54.44 ± 1.11 b	51.11 ± 1.11 b
	5.0 + 0.5	31.05 ± 0.11 e	31.26 ± 0.79 e	31.38 ± 0.64 e	68.89 ± 1.11 a	67.78 ± 1.11 a	65.56 ± 1.11 a
	6.0 + 0.6	22.40 ± 0.48 f	21.99 ± 0.77 f	22.37 ± 0.64 f	17.78 ± 1.11 f	15.56 ± 1.11 f	16.67 ± 1.92 f
BA+NAA	0.5 + 0.1	60.32 ± 0.22 b	60.01 ± 0.10 b	60.10 ± 0.22 b	36.67 ± 0.00 b	37.78 ± 1.11 c	37.78 ± 1.11 b
	1.0 + 0.1	44.14 ± 0.16 c	44.05 ± 0.11 c	44.29 ± 0.27 c	52.22 ± 1.11 a	47.78 ± 1.11 b	50.00 ± 1.92 a
	2.0 + 0.2	40.25 ± 0.12 d	40.45 ± 0.42 d	40.36 ± 0.54 d	54.44 ± 1.11 a	52.22 ± 1.11 a	52.22 ± 1.11 a
	3.0 + 0.3	65.25 ± 0.11 a	65.12 ± 0.20 a	65.29 ± 0.27 a	30.00 ± 0.00 c	30.00 ± 0.00 d	28.89 ± 1.11 c
	5.0 + 0.5	14.07 ± 0.23 e	14.14 ± 0.16 e	14.18 ± 0.31 e	12.22 ± 1.11 d	11.11 ± 1.11 e	11.11 ± 1.11 d
	6.0 + 0.6	5.71 ± 0.34 f	5.48 ± 0.34 f	5.63 ± 0.55 f	1.11 ± 1.11 e	1.11 ± 1.11 f	1.11 ± 1.11 e
2,4-D + NAA + ZT	1.0+1.0+ 0.1	52.00 ± 0.10	51.18 ± 0.31	51.46 ± 0.46	22.22 ± 2.22	22.22 ± 1.11	25.56 ± 1.11

Data presented as means ± SE. Means followed by same letters within a column are not significantly different according to Duncan's New Multiple Range Test (DNMRT) at $P \leq 0.05$ level.

approaches were aimed at choosing the best combination and concentration of plant growth regulators, thereby inducing somatic embryo and subsequent plant regeneration. 10-day-old HPC were cultured on MSCP medium with different concentrations of BA combined with NAA, or IAA. The growth rate of callus (both of non-EC and EC) and percentage of EC were determined after 2 weeks culture. It was observed that during proliferation, the somatic embryos emerged from hypocotyl callus surface. In addition, EC grew more slowly than non-EC. MSCP medium with 5.0 mg L^{-1} BA and 0.5 mg L^{-1} NAA (MSCP2 medium) was the most effective medium in inducing and proliferating somatic embryos. Although somatic embryos on MSCP medium with BA and IAA were faster elongated than those on MSCP medium with BA and NAA, the quantity of somatic embryos was less. As control, the frequency of EC from HPC continuously subcultured on MSCP1 medium was probably underestimated because of overgrowth of non-EC. Accordingly, BA plus NAA can increase the quantity of somatic embryos, but the effects of BA plus IAA on somatic embryo germination and subsequent plantlet formation should also be regarded.

Somatic embryo germination and plantlet formation

As mentioned above, BA plus IAA induced a promotive effect on germination and plantlet formation of somatic embryos. Thereby, the concentrations of BA and IAA in plantlet formation medium were optimized with quadratic orthogonal rotation regression design aiming at a higher efficiency of plant regeneration (Table 3).

After 2 weeks of culture on MSCP2 medium, mid-friable, yellowish, granular and compact, greenish-yellow, granular calluses were selected from off-white non-EC by color and texture. EC with somatic embryos were maintained on plantlet formation medium containing different concentrations of BA and IAA (Table 3) with periodic subculturing at an interval of 2 weeks.

After 6 weeks of culture, growth coefficient (EC diameter of 6 weeks culture, inoculated EC diameter), differentiation coefficient (regenerated plantlets number, inoculated EC number) and differentiation degree (growth coefficient × differentiation coefficient) were determined. The model of BA, IAA and differentiation degree was constructed and analyzed by SAS software; the

Table 3. The results of quadratic orthogonal rotation regression design of BA and IAA.

Factor levels		Concentrations (mg L^{-1})		Results		
6-BA	IAA	6-BA	IAA	Growth coefficient	Differentiation coefficient	Differentiation degree
-1	-1	0.87	0.23	1.78	1.13	2.01
-1	1	0.87	0.87	1.41	1.23	1.73
1	-1	2.63	0.23	2.43	1.67	4.06
1	1	2.63	0.87	2.52	2.03	5.12
-1.414	0	0.50	0.55	0.96	0.93	0.89
1.414	0	3.00	0.55	2.47	1.80	4.45
0	-1.414	1.75	0.10	1.99	1.20	2.39
0	1.414	1.75	1.00	2.14	1.40	3.00
0	0	1.75	0.55	2.30	1.47	3.38
0	0	1.75	0.55	2.31	1.83	4.23
0	0	1.75	0.55	2.29	1.60	3.66
0	0	1.75	0.55	2.32	1.63	3.78
0	0	1.75	0.55	2.35	1.57	3.69
0	0	1.75	0.55	2.42	1.50	3.63
0	0	1.75	0.55	2.35	1.53	3.60
0	0	1.75	0.55	2.37	1.63	3.86

Concentrations of BA and IAA were arranged by parameter table of two factors. The treatment of BA plus IAA at factor level 0 was repeated eight times in order to estimate SE. Data were scored after 6 weeks of culture.

regression equation was $Y = 3.7290 + 1.3067x_1 + 0.2050 x_2 + 0.3337x_1x_2 - 0.3926 x_1^2 - 0.3813 x_2^2$. The results of lack of fit test ($F_1 = 3.4391 < F_{0.05}$（3，7= 4.3468) and significance test ($F_2 = 32.1316 > F_{0.01}$ 5，10= 5.6363) proved that the regression equation was reliable. The association between BA, IAA and plantlet formation can thus, be well reflected.

Data was further analyzed by SAS software for optimal concentrations of BA and IAA. Nineteen (19) combination schemes (differentiation degree ≥ 4.5) were gained among the 121 combination schemes when the step length was 0.2828 between the values of factor levers. According to the frequency distribution and 95% confidence interval, the optimal concentrations were 1.75 mg L^{-1} BA and 0.55 mg L^{-1} IAA (Table 4).

The percentages of regenerated plantlet of three *C. roseus* cultivars were recorded after 6 weeks cultured on the optimized plantlet formation medium (MSCP medium supplemented with 1.75 mg L^{-1} BA and 0.55 mg L^{-1} IAA, MSCP3 medium) (Figure 1). In this study, two different types of responses were observed: 1) A maximum of full-grown plantlets with well developed shoots and roots,
2) a few of non-full-grown plantlets had only roots, or shoots which had to be transferred onto half MS medium (that is, without any plant growth regulator) in order to induce rooting efficiently. When the plantlets with well-developed roots and shoots were finally transferred to outdoor, about 100% survival occurred. This regeneration protocol is well suitable for multi-cultivar *C. roseus* regeneration via somatic embryogenesis. The maximum percentages of HPC, EC and regenerated plant of all three cultivars were gained in this study (Figure 1). Similar responses of primary callus and EC formation were observed in three cultivars, which relational data was analyzed by DNMRT at $P \leq 0.05$ levels. Although three cultivars, PCR, HWMC and MRR, were significantly different in plant regeneration, the maximum percentage (74.44% to 85.56%) was achieved (Table 2).

Morpho-histological aspects

After 10 days of culture on MSCP1 medium, swelling and growth of the hypocotyls were observed, resulting in the development of primary callus which presented a yellowish, amorphous, and mid-friable texture (Figure 2A). The primary callus quickly proliferated and considerably increased in size on MSCP2 medium, and greenish-yellow embryogenic callus with several globular embryo-like structures were formed within 2 weeks culture (Figure 2B). The embryogenic callus were selected and maintained on MSCP3 medium, where growths of somatic embryos as well as signs of polarization were observed. Figure 2C shows the somatic embryo germination into leaf-like structures which further developed into non-full-grown plantlets without roots (Figure 2D) or full-grown plantlets (Figure 2E). The regenerated plantlets transferred to soil were moved to greenhouses (Figure 2F) where they grew into full-sized *C. roseus* plants.

Histological analyses of primary callus structure showed

Table 4. Frequency distribution of BA plus IAA combinations with differentiation degree ≥ 4.5.

Factor levels	6 - BA		IAA	
	Degrees	Frequencies	Degrees	Frequencies
-0.2828	0	0	2	0.11
0	0	0	3	0.16
0.2828	0	0	3	0.16
0.5656	0	0	3	0.16
0.8484	5	0.26	3	0.16
1.1312	7	0.37	3	0.16
1.14140	7	0.37	2	0.11
SE	0.0525		0.1242	
95% Confidence interval	1.07 ~ 1.25		0.35~ 0.79	
Concentrations interval	1.66 ~ 1.84mg L^{-1}		0.33 ~ 0.77L^{-1}	
Interim factor level	1.1610		0.5656	
Interim	1.75mgL^{-1}		0.55mgL^{-1}	

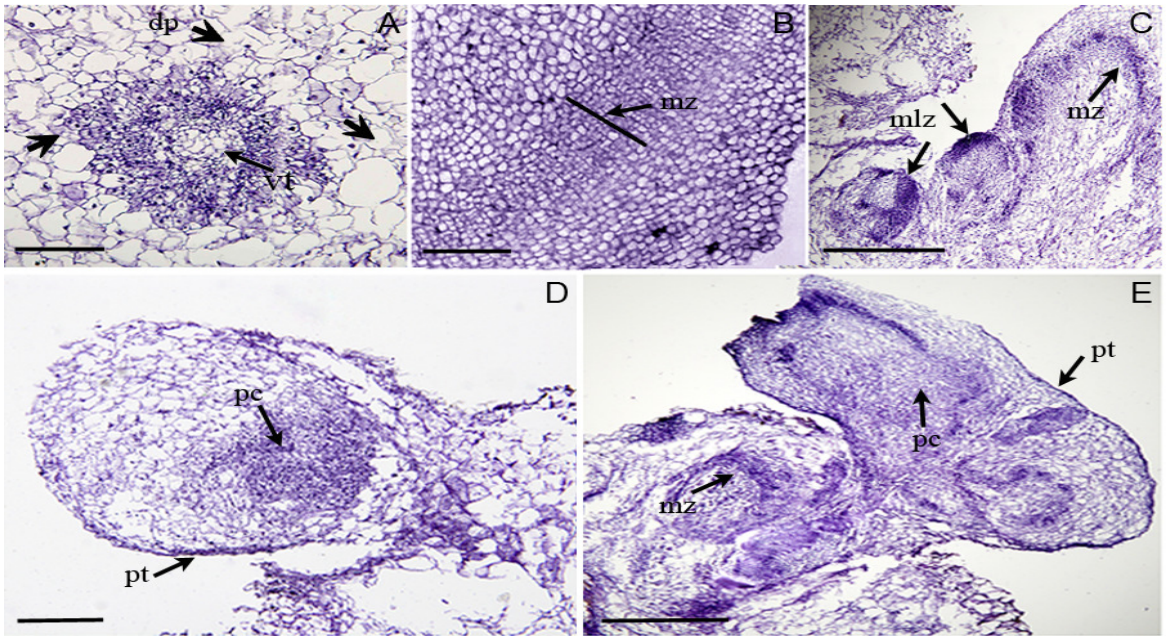

Figure 3. Histological analyses of *C. roseus* somatic embryogenesis. (A) First cell division events (arrows) observed in cells adjacent to the vascular tissue and the degenerating parenchyma (arrow); (B) the meristematic zone (arrow) of primary callus; (C) somatic embryo meristem-like zone (mlz, arrow) developing at the callus surface; (D) globular somatic embryo showing polarization signals, a well-defined protoderm and a well-developed procambium in proliferation culture conditions; (G) somatic embryo without vascular connection in germination culture conditions with the shoot-like structures. mlz, Meristem-like zone. Scale bars: A, B, D = 200 µm; C, E = 500 µm.

that the first cellular division events were observed to occur simultaneously in several cells adjacent to the vascular tissue, while the remaining parenchymatic cells degenerated (Figure 3A) after inoculation on MSCP1 medium about 1 week, these actively dividing cells progressed to burst through the epidermis of the explants, resulting in primary callus. The radial growth of the primary callus was ensured by the presence of a meristematic zone composed of small meristematic isodiametric cells with a centred nucleus and abundant, dense cytoplasm (Figure 3B). In proliferation culture conditions, development of globular embryo structures arising from different regions of the embryogenic callus mass was observed. Figure 3C shows a section of an

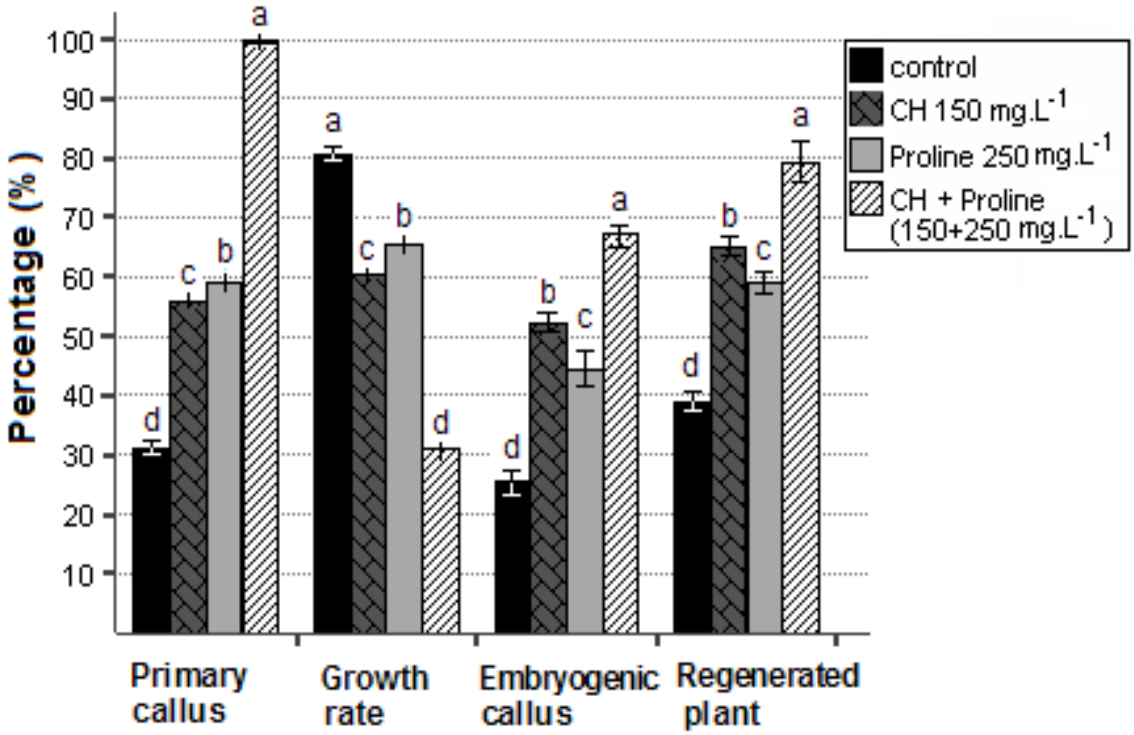

Figure 4. Effect of CH and L-proline on somatic embryogenesis based plant regeneration of three *C. roseus* cultivars in different tissue culture stage. Data presented as means from explants of all three cultivars for each treatment. Means followed by same letters within a bar cluster are not significantly different according to Duncan's new multiple range test (DNMRT) at $P \leq 0.05$ level. Error bars represent the standard deviations.

embryogenic callus with somatic embryos, histological analyses revealed that somatic embryo structures arose from the meristematic zone, and the presence of dome-shaped, meristem-like domains with several layers of typically meristematic cells enclosing a central corpus region (dark areas, mild areas) also exhibited embryogenic competence, resulting in the development of additional globular somatic embryos. Most of the well-formed somatic embryos presented a well-delimited protoderm, complete polarization and the developing procambium which can be clearly distinguished (Figure 3D) as well as the characteristics of somatic embryos at the globular stage. Subsequently, mature somatic embryo germinated into shoot or root structures (Figure 3E) on plantlet regeneration medium and could be converted into plantlets (Figure 2F).

Effect of casein hydrolysate and L-proline on somatic embryogenesis

Sources of nitrogen have often proved to be beneficial in promoting somatic embryogenesis and subsequent plant regeneration. CH at low levels increases somatic embryogenesis in wild carrot (Halperin and Wetherell, 1965). L-proline has been used to promote callus initiation and growth of embryos in Datura (Sanderst and Burkholder, 1948). CH together with L-proline notably influence *Agrobacterium*-mediated transformantion of barley (Bartlett et al., 2008). The present study was an attempt to increase EC formation and thus, plant regeneration in *C. roseus* using medium containing organic nitrogen supplements in the form of CH and L-proline which also act as the sources of carbon and NADPH.

CH (150 mg L^{-1}) and L-proline (250 mg L^{-1}) were tested singly or in combination to study their effects on somatic embryogenesis based plant regeneration of *C. roseus*, and the medium without these was used as control. Data are presented as means from explants of all three cultivars for each treatment (Figure 4). In the stage of primary callus induction, percentage of callusing was recorded after about 10 days culture. The hypocotyls responded quickly to either CH or L-proline, and within 10 days, callusing occurred compared with the control, which need at least two to three weeks for callus induction.

Almost 100% primary callusing was achieved when the medium contained both CH and L-proline, which was approximately 3 times higher than that of the control. Addition of CH or L-proline alone was beneficial to EC formation and differentiation of somatic embryos was further enhanced by the simultaneous addition of CH and L-proline. The percentages of EC and regenerated

plant were approximately 2.5 and 2 times higher, respectively, than that of the control. Growth rate with high proportion of EC was low. The full-grown plant duration was decreased to 3-4 months. Accordingly, CH and L-proline were necessary as standard addenda in the tissue culture regeneration protocol of *C. roseus*.

CONCLUSION

All of the reports about somatic embryogenesis of *C. roseus* published so far have either infrequent, transient regeneration from any type of explants or lack the corroboration of morphology and histology. Somatic embryogenesis resulting in regeneration of whole plant is an important step in the plant transformation method. Successful and stable transformation requires that a single cell gives rise to a plant. Ideal transformation scheme is that done via somatic embryogenesis, because from callus each transformed cell has the potential to produce a plant. In addition, the genotype-dependent somatic embryogenesis and plant regeneration had been confirmed in many plant species; for example, maize (Green et al., 1974; Jain et al., 1996), cotton (Firoozabady and DeBoer, 1993; Sakhanokho et al., 2001; Rao et al., 2006). In *C. roseus*, the genotype-dependent somatic embryogenesis based plant regeneration was also reported to be important (Choi et al., 2003; Lee et al., 2003), which make a large-scale of genetic transformation studies on *C. roseus* difficult.

In this study, three main factors had been considered for broad applicability and high efficiency of *C. roseus* hypocotyls regeneration system. These are the genotype specificity, the combinations and concentrations of plant growth regulators, and the form of extrinsic nitrogen source.

So far, no system has been found to be suitable for somatic embryogenesis and subsequent plant regeneration in multi-cultivar of *C. roseus*. Three *C. roseus* cultivars belonging to different series of variety, PCR, HWMC and MRR, were tested in the present investigation. Almost 100% primary callus induction occurred in all three cultivars on MSCP1 medium after 10 days of culture. Junaid et al. (2007a) indicated that a maximum of 85% callusing initiated from hypocotyls was recorded with 1.0 mg L^{-1} 2,4-D, and that *C. roseus* hypocotyl was regenerated via organogenesis (72%) on BA and NAA containing medium. In this study, somatic embryos were produced indirectly on EC in MSCP medium supplemented with 5.0 mg L^{-1} BA and 0.5 mg L^{-1} NAA. EC rapidly proliferated and produced more embryos. A maximum of 2-4 somatic embryos were observed on one callus after 2 weeks of culture. Through somatic embryogenesis, high percentages of regeneration from all three *C. roseus* cultivars hypocotyls (74.44 to 85.56%) were achieved while EC with somatic embryos were maintained on MSCP3 medium. As sources of nitrogen, the use of CH and L-proline for somatic embryogenesis and subsequent plant regeneration has been reported in a number of studies (Sanderst and Burkholder, 1948; Halperin and Wetherell, 1965; Bartlett et al., 2008). Therefore, it was not surprising as to why promoting effect on somatic embryo formation and plant conversion was observed in the present study. Here, CH and L-proline were found to be notably beneficial in somatic embryogenesis based plant regeneration of *C. roseus*. The corroboration of somatic embryogenesis characteristics was obtained by morphological observation and histological examination (Figures 2 and 3).

In conclusion, the results of this study have clearly demonstrated the establishment of an efficient system for plant regeneration via induction of embryogenic callus in *C. roseus*. Our regeneration system is also relatively rapid as the well developed plantlets can be obtained within 3 - 4 months. Furthermore, the tissue culture and regeneration system described above were almost equally efficient in three diverse cultivars, PCR, HWMC and MRR. The robust regeneration system reported here could be very useful for continuous regeneration of somatic embryos/plantlets for alkaloid production, and may offer an efficient and ideal system for large-scale genetic transformation in *C. roseus*.

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REFERENCES

Bartlett JG, Alves SC, Smedley M, Snape JW, Harwood WA (2008). High-throughput *Agrobacterium*-mediated barley transformation. Plant Methods, 4: 22-27.

Bhadra R, Vani S, Shanks JV (1993). Production of indole alkaloids by selected hairy root lines of *Catharanthus roseus*. Biotechnol. Bioeng. 41: 581-592.

Choi PS, Lee SY, Chung HJ, In DS, Choi DW, Liu JR (2003). Assessing competence for adventitious shoot formation in hypocotyl explant cultures from *Catharanthus roseus* cultivars. J. Plant Biol. 46: 90-94.

Dhandapani M, Kim DH, Hong SB (2008). Efficient plant regeneration via somatic embryogenesis and organogenesis from the explants of *Catharanthus roseus*. *In Vitro* cell dev. Biol. Plant, 44: 18-25.

Firoozabady E, DeBoer DL (1993). Plant regeneration via somatic embryogenesis in many cultivars of cotton (*Gossypium hirsutum* L.). *In Vitro* cell dev. Biol. 29: 166-173.

Green CE, Phillips RL, Kleese RA (1974). Tissue culture of maize (*Zea mays* L): initiation, maintenance, and organic growth factors. Crop Sci. 14: 54-58.

Halperin W, Wetherell DF (1965). Ammonium requirement for embryogenesis *in vitro*. Nature. 205: 519-520.

Hong SB., Peebles C, Shanks JV, San KY, Gibson SI (2006). Terpenoid indole alkaloid production by *W. roseus* hairy roots induced by *Agrobacterium tumefaciens* harboring *rol ABC* genes. Biotechnol. Bioeng. 93: 386-390.

Junaid A, Mujib A, Bhat MA, Sharma MP, Samaj J (2007a). Somatic

embryogenesis and plant regeneration in *Catharanthus roseus*. Biologia. Plantarum. 51: 641-646.
Junaid A, Mujib A, Sharma MP, Tang W (2007b). Growth regulators affect primary and secondary somatic embryogenesis in Madagaskar periwinkle (*W. roseus* (L.) G. Don) at morphological and biochemical levels. Plant Growth Regul. 51: 271-281.
Kim SW, In DS, Choi PS, Liu JR (2004). Plant regeneration from immature zygotic embryo-derived embryogenic calluses and cell suspension cultures of *Catharanthus roseus*. Plant Cell Tiss. Org. Cult. 76: 131-135.
Kurz WGW, Chatson KB, Constabel F, Kutney JP, Choi LS, Kolodziejczyk P, Sleigh SK, Stuart KL, Worth BR (1981). Alkaloid production in *Catharanthus roseus* cell cultures VIII. Planta Med. 42: 22-31.
Lee SY, Choi PS, Chung HJ, In DS, Choi DW, Liu JR (2003). Comparison of adventitious shoot formation in petiole explant cultures of 20 cultivars of *Catharanthus rosesus*. J. Plant Biotechnol. 5: 59-61.
Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15: 473-497.
Rao AQ, Hussain SS, Shahzad MS, Bokhari SY, Raza MH, Rakha A, Majeed A, Shahid AA, Saleem Z, Husnain T, Riazuddin S. (2006). Somatic embryogenesis in wild relatives of cotton (*Gossypium* Spp.). J. Zhejiang Univ. Sci. B. 7: 291-298.
Sakhanokho HF, Zip A, Rajasekaran K, Saha S, Sharma GC (2001). Induction of highly embryogenic calli and plant regeneration in Upland (*Gossypium hirsutum* L.) and Pima (*Gossypium barbadense* L.) Cottons. Crop Sci. 41: 1235-1240.
Sanders ME, Burkholder PR (1948). Influence of amino acids on growth of Datura embryos in culture. Botany, 34: 516-526.
Van der Fits L, Memelink J (2000). ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. Science, 289: 295-297.
Van der Heijden R, Jacobs DI, Snoeijer W, Hallard D, Verpoorte R (2004). The *Catharanthus* alkaloids: pharmacognosy and biotechnology. Curr. Med. Chem. 11: 607-628.