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CALLUS INDUCTION AND CELL SUSPENSION CULTURE PRODUCTION OF *CATHARANTHUS ROSEUS* FOR BIOTRANSFORMATION STUDIES OF (–)-CARYOPHYLLENE OXIDE

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Abstract

For biotransformational studies, Callus of *Catharanthus roseus* was produced from young shoot tip on Murashige & Skoog medium supplemented with 1.5 mg/litre 2, 4-D and 0.5 mg/litre Kin, solidified by 8 g/litre agar at 25±1°C under complete darkness. The cultures were maintained on the same medium at 25±1 °C in the dark by subculturing after every four weeks. Suspension culture of the plant was produced from induced callus on the same medium except Kin and agar. Cell suspension culture of *Catharanthus roseus* (Madagascar periwinkle) was employed for the first time in order to study the biotransformational capability of (–)-caryophyllene oxide (**1**), and four metabolites, 15-hydroxycaryophyllene oxide (**2**), 4*β*, 5*α*-dihydroxycaryophyll-8(13)-ene (**3**), 2*β*-hydroxycaryophyllene oxide (**4**), and 2-hydroxy-4,5-epoxycaryophyllan-13-ol (**5**) were obtained. Metabolites **4** and **5** were found to be new compounds.

Introduction

Catharanthus roseus is an important medicinal plant, known worldwide for its anticancerous property (Junaid *et al*., 2006). Cell suspension cultures of *C. roseus* have been commonly employed for the structural modifications of various natural and synthetic products (Hamada *et al.*, 1997; Balsevich, 1985; Kergomard *et al.*, 1998; Hamada *et al.*, 2001) such as oxidation, hydroxylation, reduction, isomerization, esterification and glycosylation (Min *et al.*, 2002; Giri *et al.*, 2001). At present, researchers aim to produce substances with antitumor, antiviral, hypoglycaemic, antiinflammatory, antiparasite, antimicrobial, tranquilizer and immunomodulating activities through tissue culture technology (Vanisree *et al*., 2004). Living organism systems such as microbes (fungi, bacteria, etc.), plant cells and organs, insects and animals (including cells in-vitro), are the multi-enzymes systems, therefore, it is possible that a great many products could be yielded from one natural product bioconverted by these systems as an exogenous substrate (Dai *et al.*, 2005). Exploration of the biosynthetic capabilities of various cell cultures has been carried out by a group of plant scientists and microbiologists in several countries during the last decade. Likewise, *Catharanthus roseus* (L.) G. Don, which is an important alkaloid-yielding medicinal plant belonging to family Apocynaceae (Seth & Mathur, 2005), was used to establish callus and its cell suspension culture to examine the conversion eligibility of the (–)-Caryophyllene oxide (**1**), which is a sesquiterpene, a constituent of many essential oils of traditionally used folk medicinal plants and spices (Heymann *et al.*, 1994; Tressel *et al.*, 1978). This paper covering biological aspects of our research paper published earlier, which circumferences the chemical aspects of the experiments (Choudhary, *et al.*, 2004).

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Materials and Methods

Plant material: The plant material apparently healthy and free from any external damage of pest and insect attack was collected from the premises of the H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi (Fig. 1A). Explants; shoot tips were prepared for the initiation removing unnecessary parts and placed under running tap water for 1 hour to wash dust and other unwanted materials. Apical meristems of the explants were cut approximately 1cm in length (Fig. 1B). Different experiments were conducted employing several sterilizing agents using various concentrations for varying time period including commercial bleach (Sodium Hypochlorite, containing 5% active chlorine) prior dipped into jar containing 100% ethanol for surface sterilization and washed with distilled autoclaved water three times each for 5 min.

Callus induction: The callus cultures of the plant were derived from young shoot tips cultivated in 300 ml capacity jars, each having 25 ml of Murashige and Skoog medium (Murashige & Skoog, 1962), supplemented with sucrose (30 g/litre), various concentrations of 2, 4-D (0-2 mg/litre), IBA (0-5 mg/litre), Kin (0-2 mg/litre), BA (0-2 mg/litre) and agar (8 g/litre) for solidification. The pH of the medium was adjusted to 5.8 with 1N HCl or 1N NaOH before autoclaving for 15 min at 121°C at 15 psi. Explants were incubated under complete darkness at 25 ± 1°C. The percentage of the explants producing callus was investigated for each treatment and the number of explants taken were five.

Suspension culture: Cell suspension cultures were derived from static cultured calli in Erlenmeyer flasks (500 ml), each containing 200 ml of the Murashige & Skoog medium, supplemented with ingredients as mentioned above, except Kin and agar. After 15 days of preculturing on a gyratory platform shaker at 100 rpm and 25 ± 1°C with a 16h photoperiod, a solution of substrate (40 mg in 1 ml of acetone) was added to each flask through a 0.2 µM membrane filter and the flasks were placed on a shaker for 8 days. Actively growing cultured callus were used for initiation of cell suspension cultures by transferring 5, 10, 15, and 20 gms of it in Erlenmeyer flasks

Biotransformation protocol: The time course study was carried out by taking aliquots from culture on daily basis and the content of transformation was analyzed by TLC. A negative control containing only plant cell suspension culture and a positive control containing compound **1** in the medium was also prepared in order to check the presence of plant metabolites in the cell culture and the chemical changes as a result of chemical reaction (if any) due to media components, respectively.

Extraction and isolation procedure: After 10 days of incubation, the cells and the media were separated by filtration. The filtrate was extracted with CH_2Cl_2 (3 × 1.5 litre) and the cells were extracted in an ultrasonic bath with CH_2Cl_2 (3 × 500 ml) at r. t. The combined extract was dried over anhydrous Na_2SO_4, and concentrated under reduced pressure which afforded a brown residue (1.2 g). The transformed metabolites were isolated from this gummy crude by column chromatography (silica gel) with petroleum ether/EtOAC gradient, afforded metabolites **2** (8 mg, 4% yield, with petroleum ether/EtOAc 81 : 19), **3** (12 mg, 6% yield, with pet. ether-EtOAc, 75 : 25), **4** (15 mg, 7.5% yield, with pet. ether-EtOAc, 74 : 26), and **5** (9.2 mg, 4.6% yield, with pet. ether-EtOAc, 64 : 36). Some quantity of substrate **1** was also recovered unchanged (51 mg). All metabolites subjected to spectroscopic techniques for the structure elucidation.

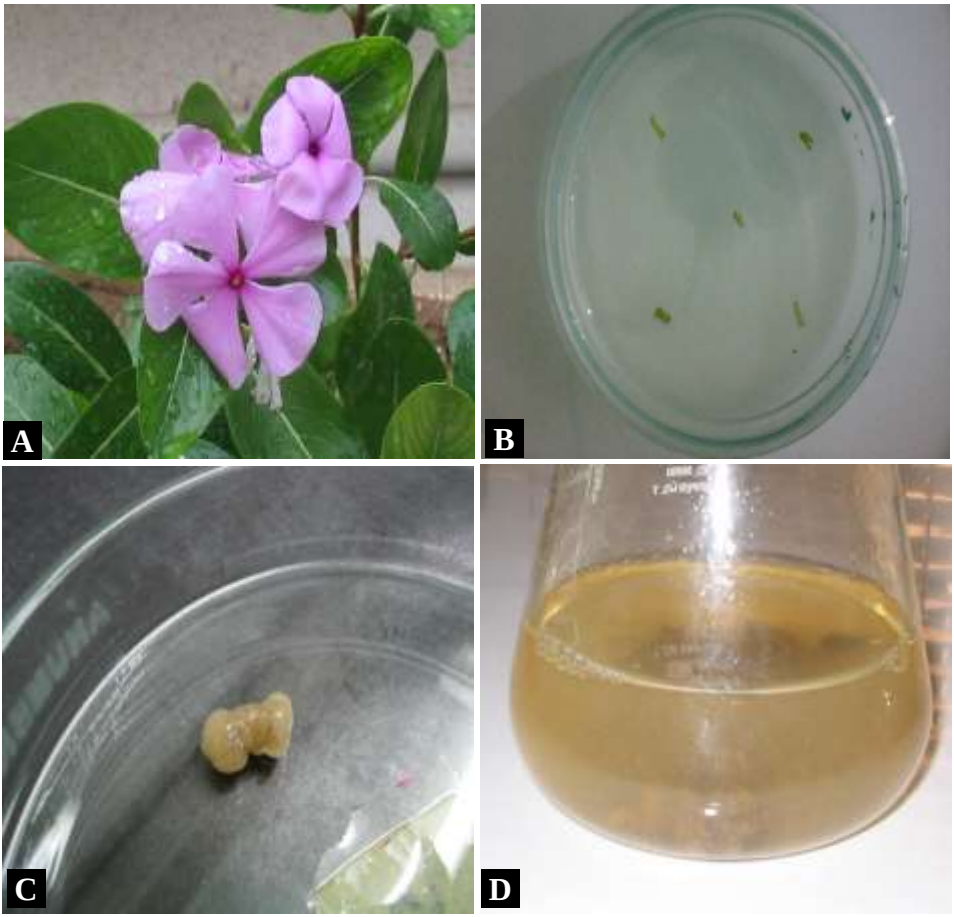

Fig. 1. C*atharanthus roseus* plant (A) explants for callus induction (B) callus produced (C) and cell suspension culture (D).

Results and Discussions

Sterilization of explants is very important step in establishing tissue culture of any plant, all upcoming phases are really dependent on it. In our experiment, the concentration at which highest degree of the explants viability; 95% obtained was 15% of Sodium hypochlorite for 10 min. At lower concentration i.e., at 10%, 50% explants were contaminated and explants were start dieing as we went towards higher concentrations gradually. Plant tissue culture as have been afforded a magnificent history to have valuable products from several and numerous medicinal plants through plant cell suspension cultures with the sole help of plant growth regulators. Out of 40 combinations (M2 to M41, Table 1a and 1b) best combination of the hormones (**M14,** Table 1a) with Murashige & Skoog medium observed for the cell suspension culture preparation for biotransformation studies was 1.5 mg/litre 2, 4-D and 0.5 mg/litre Kin (Fig. 1C). Off white callus with friable texture in all replicates was found to be the best when this callus transferred into the liquid medium it start disintegrating easily making good suspension culture. Experimental control (M1) was also run i.e. without hormones

on the same medium to deduce the hormonal effect. On control neither curling nor swelling observed. Referring other combinations, such as 2, 4-D (0.5 mg/litre–1.5 mg/litre) with Kin (0.25 mg/litre–1.0 mg/litre) except above mentioned combination (Table 1a) produced callus but not as required for the suspension culture, for instance on upper and lower neighboring combinations, cells with the off white color with the 100% frequency proliferated but with compact texture, which can not be suitable for single with micro-aggregated cell suspension culture. On other concentrations that did not show promising color and texture of the callus, at higher concentrations of Kin with the companion dedifferentiated hormone greenish calli with hard texture were notified which is also not fit for suspension culture. Likewise, IBA with the BA showed similar results with respect to calli features, lower level of IBA did not respond as of 2, 4-D because its effect is many times lesser at the same concentration. Contrasting effect can be seen on M38 and M39 but calli texture was matchless. Hard and undefined texture was observed of the Callus produced on auxins only but this is not the case when we see in combination with cytokinins, which suggests that cytokinins have synergetic effect with auxins on proliferating cells particularly in this case.

15 gms of the friable callus was found enough to form suspension in 15 days from inoculation time in the liquid medium (Fig. 1D). Lesser amount of the callus such as 5 and 10 gms were producing cell suspension culture as well but were taking more time than the selected weight but at higher amount of callus, cell death was observed due to the collision of the callus clumps. Addition of Kin causes differentiation of the callus in light condition and agar for solidification of liquid medium respectively. That is why these were not used in the suspension culture. Agitation of the suspension at lower rpm bring about lower level of cell dispersion, on the contrary at higher rpm might have risk of cell collision that is why rpm kept at moderate speed i.e., 100 rpm. The substrate was dissolved in acetone and filtered through sterile filter paper in order to avoid any particle which could hinder process of bioconversion and minimize the chances of getting the suspension contaminated.

Suspension was regularly investigated for the conversion of the substrate into derivatives using TLC up to 8 days. Positive and negative controls were also run along with original experiment to examine the plant metabolites and other contamination which may create false positive results. The whole system was left for 10 days so that substrate could be transformed completely but it was not entirely converted then it was immediately extracted and subjected to column chromatography to isolate and purify the analogues produced. Enzyme system of the *Catharanthus roseus* was found active during whole time course experiments as it converted the substrate, regularly.

The sesquiterpene(-)-caryophyllene oxide (**1**) was subjected to biotransformation with the plant cell suspension culture of *Catharanthus roseus*, resulting in the formation of four metabolites 2-5 (Fig. 2). Hydroxylated metabolite (**2**) was obtained when hydroxylation occurred at C-15 of compound (**1**). When epoxide ring of the compound (**1**) opened in the trans-fashion resulted in the formation of metabolite (**3**). When hydroxylation occurred at C-2 of compound (**1**), it resulted in the formation of metabolite (**4**). Addition of water molecule in compound (**1**) on its double bond and additional hydroxylation occurred at C-2 resulted in the formation of metabolite (**5**). It can be proposed that conversion of compound (**1**) into (**3**) appeared first because of highly strained ring of epoxide in compound (**1**) opened spontaneously by the enzyme. It can also be proposed that conversion of compound (**1**) into (**2**) and (**4**) appeared, directed by the H-1 β, resulting in the hydroxylation occurred at C-15 β, hydroxylation occurred at C-2 in beta-fashion, resulting in the formation of metabolite (**4**). Same directing effect can be observed in the formation of metabolite (**5**), in which H-1 β and H-9 α directed the hydroxylation at C-2 and C-13.

Table 1a. 2, 4-D and Kin effect on the callus induction.

Media codes	Growth regulator (mg/litre)		Callus formation frequency %	Description of callus	
	2, 4-D	Kin		Color	Texture
M1	0	0	-	-	-
M2	0.5	0	40	Off white	Compact
M3	0.5	0.25	40	Off white	Compact
M4	0.5	0.5	40	Off white	Compact
M5	0.5	0.75	40	Light green	Compact
M6	0.5	1.0	40	Light green	Compact
M7	1.0	0	40	Off white	Compact
M8	1.0	0.25	40	Off white	Compact
M9	1.0	0.5	80	Off white	Compact
M10	1.0	0.75	80	Greenish off white	Compact
M11	1.0	1.0	100	Greenish off white	Compact
M12	1.5	0	100	Off white	Compact
M13	1.5	0.25	100	Off white	Friable cum compact
M14	**1.5**	**0.5**	**100**	**Off white**	**Friable granular**
M15	1.5	0.75	100	Greenish off white	Compact
M16	1.5	1.0	100	Greenish off white	Compact
M17	2.0	0	100	White	Hard
M18	2.0	0.25	100	White	Hard
M19	2.0	0.5	100	White	Hard
M20	2.0	0.75	100	Greenish White	Hard
M21	2.0	1.0	100	Greenish White	Hard

Table 1b. IBA and BA effect on the callus induction.

Media codes	Growth regulator (mg/litre)		Callus formation frequency %	Description of callus	
	IBA	BA		Color	Texture
M22	1.0	0		Only swelling and curling observed	
M23	1.0	0.5	-	Only swelling and curling observed	
M24	1.0	1.0	-	Only swelling and curling observed	
M25	1.0	1.5	-	Only swelling and curling observed	
M26	1.0	2.0	20*	Greenish	Compact
M27	2.0	0	60	Off white	Compact
M28	2.0	0.5	60	Off white	Compact
M29	2.0	1.0	60	Greenish off white	Compact
M30	2.0	1.5	60	Greenish off white	Compact
M31	2.0	2.0	60	Greenish off white	Compact
M32	3.0	0	80	Off white	Compact
M33	3.0	0.5	80	Off white	Compact
M34	3.0	1.0	80	Off white	Compact
M35	3.0	1.5	80	Greenish off white	Compact
M36	3.0	2.0	80	Greenish off white	Compact
M37	4.0	0	100	Off white	Compact
M38	4.0	0.5	100	Off white	Compact
M39	4.0	1.0	100	Off white	Compact
M40	4.0	1.5	100	Greenish off white	Compact
M41	4.0	2.0	100	Greenish off white	Compact

Key: 20% callus produced means 1/5 explants giving response

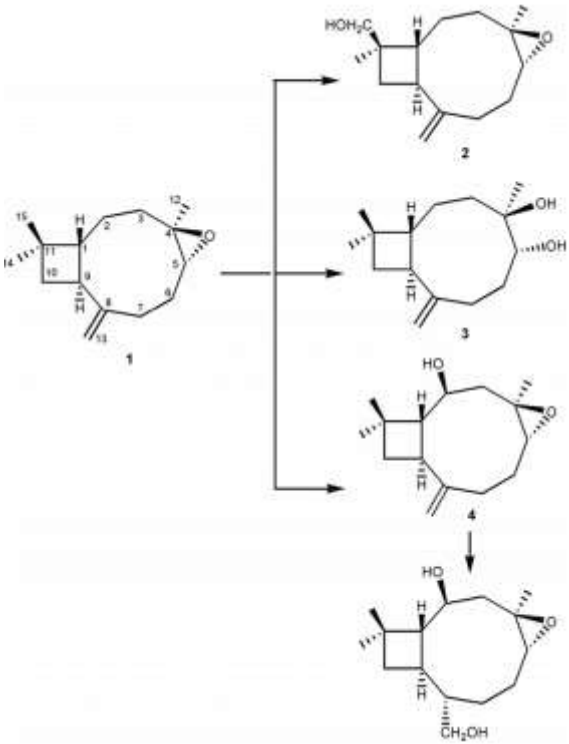

Fig. 2. Biotransformation of compound 1 by cell suspension culture of *Catharanthus roseus*. (Choudhary *et al*., 2004).

Conclusion

Best callus of *Catharanthus roseus* for the biotransformation studies was obtained at 1.5 mg/litre 2, 4-D with Kin at 0.5 mg/litre. When **(–)-**Caryophyllene Oxide were incubated with the Suspension culture of the same plant it produced four metabolites: 15-hydroxycaryophyllene oxide (**2**), 4*β*, 5*α*-dihydroxycaryophyll-8(13)-ene (**3**), 2*β*-hydroxycaryophyllene oxide (**4**), and 2-hydroxy-4,5-epoxycaryophyllan-13-ol (**5**) were obtained. Metabolites **4** and **5** were found to be new.

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