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In-vitro propagation of *Catharanthus roseus* and their pharmaceutical value

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Abstract- *In-vitro* propagation is a method using culturing explant in appropriate culture nutrient medium under sterile condition. Experimental plant *Catharanthus roseus* commonly known as Madagascar belong to the family Apocynaceae. It tropical and has been spread throughout the tropical and subtropical by human activities through its primary traditional use were for people with diabetes, and anticancer effect. It is medicinally important plant widely used for the production of anticancerous drugs by pharmaceuticals companies due to the presence of vinblastine alkaloid. Due to the over exploitation of this plant for pharmaceuticals use as raw materials. We use axillary bud; shoot tip, node and internode explants of *Catharanthus roseus*. Morphogenic effect of various concentrations of cytokinins and auxins added singly or in combination supplemented with MS medium was studied during experimental work. All the cultures were incubated at 25±26°C under 16 hour's photoperiod with light and 8 hours in dark. Somatic embryogenesis was observed on combination of 2, 4-D (1.0mg/l) + Kinetin (1.0mg/l) supplemented with MS medium from shoot apex. Effect of natural additives (compost of sand + gold sand + soil homogenate and shootex) on axillary bud and callus formation was also studied. In our study, combination of BAP (0.5mg/l) + NAA (1.0mg/l) supplemented with MS medium proved to be optimal for the production of good number of shoots from nodal explant. Different size and different positions (Basal, Middle and Distal) of nodal explants along the stem length of *Catharanthus roseus* were cultured on MS medium supplemented with BAP (3.0mg/l). Present study provides the mass production and conserved of *Catharanthus roseus* in short times, for pharmaceutical raw materials.

Key words: *In-vitro* propagation, Explant, *Catharanthus roseus*, Alkaloids, MS medium, Cytokinins, Auxins, Morphogenic.

INTRODUCTION

In-vitro propagation is a method using culturing explant in appropriate culture nutrient medium under sterile condition. Micro propagation is also used to promote germplasm storage for maintenance of disease-free stock in controlled environmental conditions[1], and long term via cryopreservation[2]. *In-vitro* propagation is a method using culturing explant in appropriate culture nutrient medium under sterile condition. Plant parts will be used in culture technique and referred to as micro-propagation. Plant tissue culture forms the back bone of Plant Biotechnology; this includes micro propagation, induction of some clone's somatic hybridization, cryopreservation and regeneration of transgenic plants Plant cell and tissue culture has already contributed significantly to crop improve and has great potential for the future. It has also a great potential for the propagation of important crops like, *Cassava* spp, *Phaseolus* spp, *Solanum* spp.[3] The micro-propagation of elite or selected plants showed good results which benefit the agriculture, horticulture and forestry.[4] *In-vitro*

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Propagation of ornamental and medicinal plants is widely used to produce active compounds for herbal and pharmaceutical industries.[5] *In-vitro Propagation* will enable rapid multiplication and sustainable use of medicinal plants for future generations. *Catharanthus roseus* (Apocynaceae) is an important medicinal plant (2n). Linnaeus, 1759 published *Catharanthus roseus* as *Vinca rosea* in his – Systema Naturae. Reichenbach, 1928 used the name Lochenra in his -Conspectus Regni Vegetabiles but without giving a description or references. G. Don, 1835 published *Catharanthus* in -General system of Gardening and Botany. *Lochnera* is a synonym of -*Catharanthus*. In this study, tissue culture propagation of *Vinca roseus* using explants from stems and leaves was investigated. Single and combined treatments of different phytohormones were added to Murashige and Skoog (MS) media.[6] Catharanthus is an important herbal medicinal plant, in Ayurveda its numerous diseases including diabetes, malaria and Hodgkin's lymphoma, heigh blood pressure, Vinca alkaloid use for cancer.[7] Antiviral, hypoglycemic, anti-inflammatory, antimicrobial, antiparasitic antimicrobial, tranquilizer and immune modulating activities through tissue culture technology.[8] In recent years, plant tissue culture represents a potential renewable source to obtain genetically pure elite population under *in-vitro* conditions.

The concept of *in vitro* plant tissue culture was first developed by a German scientist Gottlieb Haberlandt (Father of tissue culture) in 1902.

He isolated single fully differentiated individual plant cell from different plant species and culture them in nutrient medium containing glucose, peptone and knop salt solution.[9] However, those cultures did not grow further. But he concluded that every cell of the plant body is totipotent i.e. capable of giving rise to a new plant under proper nature condition. *In-vitro* propagation has now become a well-established technique for culturing and studying the physiological behavior of isolated plant organs, tissue, cells, protoplasts and even cell organelles under precisely controlled physical and chemical conditions.[10] Large scale plant tissue culture is found to be an alternative approach to traditional method of plantation as it offers a control supply of biochemical independent of plant availability.[11]

Micro-propagation has become an important tool to obtain genetically pure elites rather than having genetically different populations.

Micro-propagation is now a well-established technique commercialized globally for the rapid production of a number of commercially important plants. Moreover, the plant multiplication can continue throughout the year irrespective of season and the stocks of germplasm can be maintained for many years.[12]

Catharanthus roseus (L) G. Don (2n = 16) is an important medicinal plant of family Apocynaceae which contains a virtual cornucopia of useful alkaloids used in different diseases. It is a perennial evergreen herbaceous plant growing to 1 m tall. *Catharanthus roseus* has more than 130 know alkaloids known as Vinca alkaloids, which are used in cancer, menstrual problems, asthma, diabetes, high blood pressure, and constipation.[13] Recently, Vinblastine and Vincristine alkaloids of *Catharanthus roseus* have been shown to be effective in the treatment of various dreaded diseases like childhood leukemia, skin cancer, malignant lymphoma, breast cancer, Hodgkin's disease, Wilm's disease, choriocarcinoma, Kaposi sarcoma, neuroblastoma, mycosis, fungoides and cardio vascular maladies.

It was also used as folk medicine in the ancient period. In India, the juice of leaves is used as application to bee sting/wasp sting. As home-made remedies, it was used to ease prolong congestion, inflammation, sore throats and carribean. An extract from the flowers was used to make a solution to treat eye irritation and injections. It has been used as a poultice to stop bleeding. It is used as an astringent and diuretic.

MATERIAL & METHODS

Plant material and culture conditions:

In this Research, healthy and elite axillary buds, shoot apex, nodal, and intermodal explants (3-4cm long) of *Catharanthus roseus* were obtained from campus of Jai Prakash Vishwavidyalaya Chapra (Saran) Bihar, India. Young branches of cut and use as explants were to sterilization procedures washing in running tap water.[14] They were further washed in 1% detergent for 1-2 Minutes and then washed in tap water for 6-8 times. Finally, 2 washing were done using sterilized distilled water and then explants were taken for culture.

Medium and Culture Conditions:

Murashige and Murashige and Skoog (MS) medium containing 0.8% agar and sucrose 30gm were used during the experiment. pH of the medium was adjusted to 5.8.

Growth supplemented with various concentrations. Plants Growth Regulators used were: 2,4-D, Kinetin, NAA, IBP and BAP in different combinations. The experiments were conducted in a completely randomized design. Thirty-five replicates were used and repeated three times. All the treatments were statistically analyzed by Duncan's Multiple Range Test (DMRTS).

RESULTS & DISCUSSION

For the present study, different explants (Nodal, Internodal portion and Shoot tip) of experimental plant *Catharanthus roseus* were taken from young plants. Explants having the meristamatic tissue were preferred for the culture experiments. Explants were cultured in the medium after following surface sterilization procedure and then incubated in growth chamber.

The sterilization procedure included- washing of explants with running tap water, soaking of explants in an aqueous solution containing antibiotic (0.03 % streptomycin), repeated washing with distilled water, treating explant with antifungal reagent (0.2% Bavistin - BASF India limited) containing solution followed by treatment with 0.01% Mercuric chloride for 1-2 minutes. Explants were than washed thoroughly with autoclaved sterilized water to remove traces of all used disinfectants and washed repeatedly (Plate 1 fig. C) *Catharanthus roseus* (L).

Effect of growth hormones on different explants of *Catharanthus roseus* L.

Explant culture: Experiments were conducted for large scale multiplication of plant in-vitro by using various explants e.g., Internodal segment, nodal portion and shoot tip of *Catharanthus roseus*. Effect of cytokinins (BAP and kinetin) and auxins (NAA, IBA, IAA and 2, 4-D) added singly was observed and recorded.

Table 1- Morphogenic Response of nodal explants of *Catharanthus roseus* L. on MS medium supplemented with various concentrations of cytokinins (BAP and Kinetin) added singly.

Hormone concentration mg/l Kinetin	Hormone concentration mg/l BAP	Culture Response (%)	No. of Shoots (mean ± SE)	Shoot length (in cm) (mean ± SE)
0.5	-	55-60	1.0± 0.35	1.11. ±0.71
1	-	60-65	1.01± 0.28	1.70±0.54
1.5	-	60-65	2.0±0.37	1.52±0.92
2	-	70-75	1.02±0.22	1.74±0.30
2.5	-	50-55	1.0±0.32	1.64±0.27
3	-	40-45	1.0±0.15	1.68±0.11
-	0.5	60-65	2.57± 0.21	1.21 ±0.24
-	1	80-85	4.67± 1.22	1.78±0.26
-	1.5	70-80	4.78±0.26	2.52±0.63
-	2	55-60	5.36±0.29	1.38±0.76
-	2.5	50-55	2.50±0.20	1.17±0.19
-	3	30-35	2.31±0.30	1.10±0.15
Hormone concentration mg/l	No. of Shoots	Shoot length (cm)	No. of Shoots (SE)	Shoot length (SE)
Kinetin 0.5	1	1.11	0.35	0.71
Kinetin 1.0	1.01	1.7	0.28	0.54
Kinetin 1.5	2	1.52	0.37	0.92
Kinetin 2.0	1.02	1.74	0.22	0.3
Kinetin 2.5	1	1.64	0.32	0.27
Kinetin 3.0	1	1.68	0.15	0.11
BAP 0.5	2.57	1.21	0.21	0.24
BAP 1.0	4.67	1.78	1.22	0.26
BAP 1.5	4.78	2.52	0.26	0.63
BAP 2.0	5.36	1.38	0.29	0.76
BAP 2.5	2.5	1.17	0.2	0.19
BAP 3.0	2.31	1.1	0.3	0.15

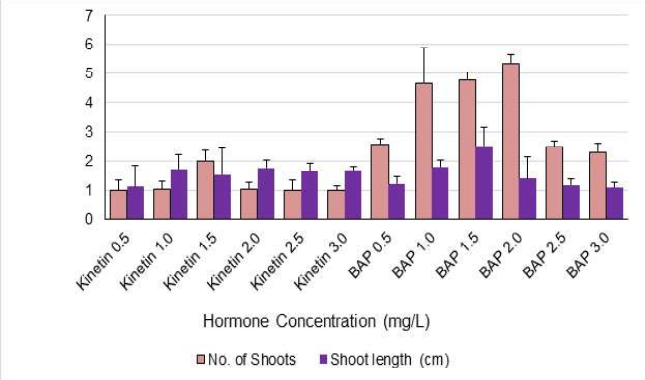
Effect of BAP

In this set of experiment, from shoot explants bud formed in BAP supplemented medium but there was no callusing observed even after 6 weeks. Good number of shoots formed on medium containing 1.0 mg/l BAP cultures. But these shoots did not grow further (Graph 1, and Table-1).

In case of nodal explants, shoot buds or very small shoots were observed on MS medium supplemented with 0.5 mg/l-3.0 mg/l BAP but on these concentrations, callus formation was totally absent. Shoot bud initiation was noticeable after 2 weeks of inoculation. Among all tried concentration of BAP (1.0 mg/l and 3.0 mg/l) proved conducive for shoot proliferation. In this experiment, BAP did not prove stimulatory effect on callusing from both the explants (internodal segment and nodal portion).

Effect of kinetin

On 0.5 mg/l – 3.0 mg/l kinetin containing MS medium cultures, callus induction was noticed from Internodal explants with shoot bud or organized structure (Graph 1, Table 1). From nodal explants, shoot emergence without callusing was noticed on all tried concentration of kinetin (1.0 mg/l – 3.0 mg/l).



Graph 1- Morphogenic Response of nodal explants of *Catharanthus roseus* L. on MS medium supplemented with various concentrations of cytokinins (BAP and Kinetin) added singly.

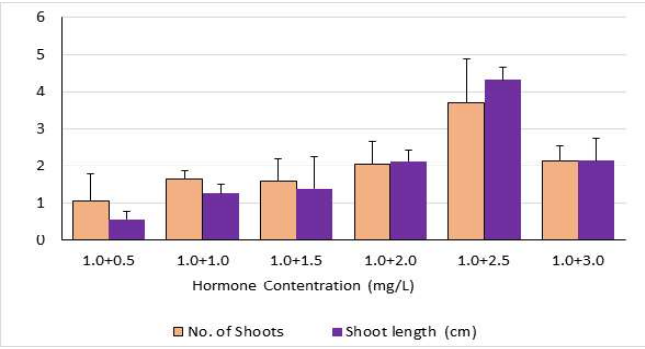
Table 2- Morphogenic Response of nodal explants of *Catharanthus roseus* L. on MS medium supplemented with various concentrations of cytokinins (BAP and 2,4-D) added singly.

Hormone concentration				
BAP+2,4-D (mg/l)	Culture Response (%)	No. of Shoots (mean ± SE)	Shoot length (in cm) (mean ±SE)	
1.0 + 0.5	55-60	1.06 ± 0.74	0.56 ± 0.21	
1.0 + 1.0	60-65	1.66 ± 0.23	1.26 ± 0.26	
1.0 + 1.5	55-60	1.60 ± 0.61	1.41 ± 0.84	
1.0 + 2.0	60-65	2.07 ± 0.61	2.14 ± 0.29	
1.0 + 2.5	60-70	3.71 ± 1.18	4.32 ± 0.34	
1.0 + 3.0	60-65	2.14 ± 0.40	2.16 ± 0.60	
BAP+2,4-D (mg/l)	No. of Shoots	Shoot length (cm)	No. of Shoots (SE)	Shoot length (SE)
1.0 + 0.5	1.06	0.56	0.74	0.21
1.0 + 1.0	1.66	1.26	0.23	0.26
1.0 + 1.5	1.60	1.41	0.61	0.84
1.0 + 2.0	2.07	2.14	0.61	0.29
1.0 + 2.5	3.71	4.32	1.18	0.34
1.0 + 3.0	2.14	2.16	0.40	0.60

Effect of 2,4-D

Prolific callusing was observed on higher concentration of 2,4-D (1.0mg/l and 3.0 mg/l) from shoot segment explants but there were no shoot buds or shoot formation noticed on any of the concentration of 2, 4-D even after 6 weeks. On low concentration of 2, 4-D that is 0.5 mg/l, callus induction was noticed but not better than higher strength of 2, 4-D tried in this set of experiment (Graph 2, Table 2). Formed callus was slow going.

From nodal explants, there was no callus formation was recorded but shoot emergence was noticed in few cultures however, they dried later. So, 2, 4-D did not prove favorable for callusing but found conducive for shoot bud formation from nodal explants.



Graph 2- Morphogenic Response of nodal explants of *Catharanthus roseus* L. on MS medium supplemented with various concentrations of cytokinins (BAP and 2,4-D) added singly.

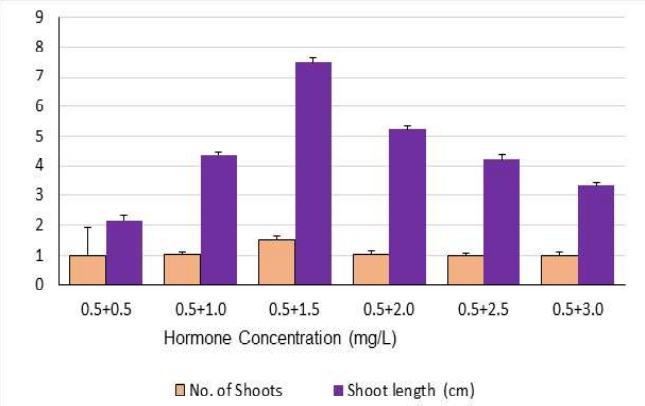
From root tip explant, there was no response observed (neither callus nor shoot formation) on all tried concentration of cytokinins and auxins used singly.

Effect of IBA

All tried concentration of IBA could not initiate callusing and shoot formation even after 2 months of inoculation from nodal explants. Very few shoots were noticed from nodal explants without callus formation (Graph 3, Table 3).

Effect of NAA

On MS medium supplemented with 0.5 mg/l - 3.0 mg/l NAA, only scanty callus induction was noticed from internodal explant with no shoot buds. Formed callus was whitish and slow growing (Graph 4, Table 4)

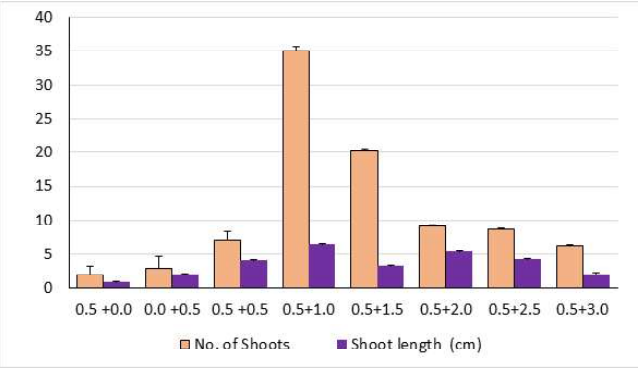


Graph 3- Morphogenic Response of nodal explants of *Catharanthus roseus* L. on MS medium supplemented with various concentrations of cytokinins (BAP and IBA) added singly.

Table 3- Morphogenic Response of nodal explants of *Catharanthus roseus* L. on MS medium supplemented with various concentrations of cytokinins (BAP and IBA) added singly.

Hormone concentration BAP+IBA (mg/l)	Culture Response (%)	No. of Shoots (mean ± SE)	Shoot length (in cm) (mean±SE)	
0.5+0.5	20-30	1.0± 09	2.15. ±0.16	
0.5+1.0	50-60	1.01± 0.10	4.37±0.12	
0.5+1.5	60-65	1.5± 0.15	7.48±0.14	
0.5+2.0	70-75	1.02±0.11	5.25±0.13	
0.5+2.5	75-80	1.0±0.08	4.20±0.17	
0.5+3.0	80-85	1.0±0.12	3.30±0.15	
BAP+IBA (mg/l)	No. of Shoots	Shoot length (cm)	No. of Shoots (SE)	Shoot length (SE)
0.5+0.5	1	2.15	0.9	0.16
0.5+1.0	1.01	4.37	0.1	0.12
0.5+1.5	1.5	7.48	0.15	0.14
0.5+2.0	1.02	5.25	0.11	0.13
0.5+2.5	1	4.2	0.08	0.17
0.5+3.0	1	3.3	0.12	0.15

In case of nodal explants, few shoots could be noticed on all concentration Effect of 2,4-D: Prolific callusing was observed on higher concentration of 2,4-D (1.0mg/l and 3.0 mg/l) from leaf segment explants but there were no shoot buds or shoot formation noticed on any of the concentration of 2, 4-D even after 6 weeks. On low concentration of 2, 4-D that is 0.5 mg/l, callus induction was noticed but not better than higher strength of 2, 4-D tried in this set of experiment (Plate 2, figure D). Formed callus was slow going of NAA (1.0 mg/l to 3.0 mg/l) but there was no callus formation in any of the cultures even after 6 weeks.



Graph 4- Morphogenic Response of nodal explants of *Catharanthus roseus* L. on MS medium Supplemented with various concentrations of cytokinins (BAP and NAA) added singly.

Table 4- Morphogenic Response of nodal explants of *Catharanthus roseus* L. on MS medium Supplemented with various concentrations of cytokinins (BAP and NAA) added singly.

Hormone concentration BAP+NAA (mg/l)	Culture Response (%)	No. of Shoots (mean ± SE)	Shoot length (in cm) (mean±SE)	
0.5 +0.0	10-15	2.0±1.2	1.0±0.06	
0.0 +0.5	20-25	3.0±1.75	2.10±0.07	
0.5 +0.5	60-65	7.11± 1.30	4.13 ±0.08	
0.5+1.0	80-85	35.12± 0.65	6.46±0.09	
0.5+1.5	70-75	20.24±0.24	3.32±0.11	
0.5+2.0	55-60	9.20±0.13	5.34±0.10	
0.5+2.5	50-55	8.74±0.15	4.32±0.12	
0.5+3.0	30-35	6.32±0.14	2.10±0.12	
BAP+NAA (mg/l)	No. of Shoots	Shoot length (cm)	No. of Shoots (SE)	Shoot length (SE)
0.5 +0.0	2	1	1.2	0.06
0.0 +0.5	3	2.1	1.75	0.07
0.5 +0.5	7.11	4.13	1.3	0.08
0.5+1.0	35.12	6.46	0.65	0.09
0.5+1.5	20.24	3.32	0.24	0.11
0.5+2.0	9.2	5.34	0.13	0.1
0.5+2.5	8.74	4.32	0.15	0.12
0.5+3.0	6.32	2.1	0.14	0.12

Experimental steps for *In- vitro* propagation of *Catharanthus roseus* with various concentration of plant growth hormones.

A. Healthy plant **B.** Explant **C.** Surface sterilization in soap **D.** Explant transfer

E. 1 + 2. Explant innoculate in medium **F.** Growth shows **G.** Shoot initiation

H. Shoot apex **I.** Shoot proliferation **J.** Shoot Growth **K.** Shoot and ROOT Initiation

M. Shoot growth **N.** Shoot and Root develop.

Fig:- Steps of *In-vitro* propagation of *Catharanthus roseus in* M. S. medium with various P. R. G.

Acclimatization of *In-vitro* raised plantlets of *Catharanthus roseus*.

Fig. A- Transfer in pot **Fig. B- Putting in shad place** **Fig. C- Planting for sell**

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