

EFFECT OF PLANT GROWTH REGULATORS ON MICROPROPAGATION OF *Catharanthus roseus*

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ABSTRACT:

Research in plant biotechnology is playing a crucial role in the production and conservation of plant-based resources globally. The aim of the work is to establish favourable culture medium condition for regeneration and better growth of *Catharanthus roseus* explant. Plant Micropropagation has also been used as a tool for the propagation of genetically manipulated superior clones. Our attempt was made to develop micropropagation as suitable condition for cloning of *Catharanthus roseus*. After optimizing the culture media of explant culture, the repeated subculturing was performed at regular intervals for 5 weeks. The roots were developed within 10 days. IBA concentration and the period of pulse treatment had significant effects on the average number of roots produced per shoot. Rooted plants were successfully acclimatized at room temperature in soil contained in pots. All plants flowered and set seeds in the greenhouse after 3 months. It is an attempt to highlight some of the important landmarks of tissue culture of medicinal plants and it is also as important recent development in vitro technology.

KEYWORDS: *Micropropagation, Plant growth regulator, Root induction, Biotechnology, Invitro.*

ABBREVIATIONS:

BAP (6-Benzyl Aminopurine), MS (Murashige and Skoog), 2,4-D (2,4-dichlorophenoxy acetic acid), IBA (Indole 3-Butyric Acid), Kin. (Kinetin), UV (Ultraviolet light), PGR (Plant Growth Regulator), IAA (Indole-3-Acetic Acid), NAA (1-Naphthalene Acetic Acid).

[I] INTRODUCTION

The world's botanical resources are being depleted at very fast rates [1,9,13]. Since the later half of the 20th century, there has been exponential population growth rates in these countries, leading to increased demand for plant resources and destruction of their habitats to pave way for agricultural and settlement land among other development activities [9,16,32]. Estimates by the International Union for

Conservation of Nature (IUCN) and the World Wildlife Fund (WWF) indicate that up to 60,000 higher plants species could become extinct or nearly extinct by the year 2050 if the current trends of utilization continue [9]. In addition to their contribution to the integrity of the environment, plants are also invaluable sources of therapeutic agents among other uses. Traditional medicine, which is the major mode

of health care in the rural parts of developing countries, is mostly plant based [14,16].

Cultivation of medicinal plants on the other hand would alleviate pressure from wild sources and also provide a source of livelihood for the unemployed commercial medicinal plant gatherers as well [6,16,30]. *Catharanthus roseus* is a medicinal plant which contains a virtual cornucopia of useful alkaloids, used in diabetes, blood pressure, asthma, constipation and menstrual problem [7,17]. More recently, extracts from *C. roseus* have been shown to be effective in the treatment of various kinds of cancer diseases such as Leukaemia, skin cancer, lymph cancer, breast cancer and Hodgkin's disease [2,5,7,10]. Catharanthus alkaloids are considered as boon to medical science by the nature.

Catharanthus roseus has opposite glossy leaves, of 2-3 inch long; the flowers are white to dark pink with a darker red centre, with a basal tube of 2.5-3 cm long and a corolla of about 2-5 cm diameter with five petal-like lobes [5]. The fruit is a pair of follicles of 2-4 cm long and 3 mm broad. Fruits of *Catharanthus roseus* have many small black and cylindrical seeds [5,6]. It is a rich source of alkaloids. More than 130 different compounds have been so far reported in which vincristine is highly important. Traditionally, *C. roseus* is propagated through seed which leads to genetic segregation and decline in uniform yield of the alkaloid [7,33].

Multiplication of genetically identical copies of a cultivar by asexual reproduction is called clonal propagation and a plant derived from a single individual by asexual reproduction constitutes a clone. In vitro clonal propagation is called Micropropagation [6,14]. The plant tissue culture media generally comprises of inorganic salts, organic salts, vitamins, amino acids, plant growth regulators, carbohydrates and galling agent i.e., agar-agar etc. A special characteristic of plant cells and meristems in which they retain a latent capacity to produce a whole plant is called Totipotency [12]. The survival of explants depends on their rate of microbial contamination and their browning, which pertains not only to them which used for culture initiation but also to

the physiological stage of mother plants and to the season when they were collected [5,33].

The resulting product can have a high degree of phenotypic uniformity since the crop can be artificially manipulated in laboratory to yield large plant population of same growth stage. Micropropagation method can be applied for preservation, conservation and multiplication of threatened/rare plant [8,13]. The main advantages are attributed to potential of combining rapid, large scale propagation of new genotypes, the use of small amount of original germplasm and generation of pathogen free propagules [6,18]. The aim of this experiment is to establish the culture method for rapid mass micropropagation of *C. roseus*. Effects of various growth regulators on explants development were evaluated.

[II] MATERIALS

BAP- 6-Benzyl aminopurine, MS- Murashige and Skoog, IBA- Indole 3-butyric acid, Kin.- Kinetin, Hgcl₂ - Mercuric Chloride etc.

The fresh and healthy leaves or stem of *Catharanthus roseus* were collected from the garden Department of Botany, Kota University, India (are shown in figure 1 & 2).



Fig-1. Flowers of *Catharanthus Roseus* plant



Fig-2. Leaf and node explant of *Catharanthus Roseus*

[III] METHODS

Various leaves, nodal segments and root were selected as explants (as shown in table- 1). The explants of 3-4 cm. in length with 2-3 nodes were initially washed with tap water and surface sterilized with 0.1% HgCl₂ [6, 20]. Then these were washed with distilled water which was autoclaved and inoculated aseptically on culture medium. Then pH of the medium was adjusted to 5.8. The medium was then sterilized in an autoclave for 15 min at 121°C. Cultures were incubated at 25- 28°C under 16 hours of photoperiod with cool white fluorescent illumination (100 m mol m⁻² s⁻¹ PFD). The inoculation of the explant was the carried out in a laminar air flow cabinet under sterile conditions. After establishment of explant aseptically on culture media, these were subculture on fresh media. The invitro generated shoots were cut into segments each with one or two node and subcultured at an interval of 5 weeks for further multiplication.

The growth substances in required amount were added and 0.8-1.0% agar-agar (bacteriological grade) was used for solidifying medium [3,4,28]. Complete sterilisation was done by plugging the flasks & tubes with cotton plugs, wrapped with aluminium foil. A higher proportion of cytokinins usually stimulates continue multiplication of auxiliary and adventitious shoots [27]. Auxiliary bud is usually present in the axils of each leaf and every bud has the potential to develop in to a shoot. To obtain full plants the shoot must be transferred to a rooting medium which have differential hormone and salt composition [11,14]. This stage is designed to arrest multiplication and to induce the establishment of fully developed plantlet, shoot elongation and root formation.

Table-1. Different type of explants and its length

S.No.	Type of explant	No. of samples	Shoot length (Mean) in cm.
(i)	Nodal segment from unlopped plant	10	2.7±0.2
(ii)	Soft and green nodal shoot segment	10	2.6±0.2
(iii)	Woody green shoot segment from lopped plant	10	2.7±0.2

Light, temperature, and relative humidity are the important parameters in culture incubation [26,29]. Photosynthetic activity is not very important during initial phases of invitro culture but at later stages the culture materials are induced a certain degree to become autotrophic. So light is essential for morphogenetic processes like shoot and root initiation [4,9,21]. Quality and intensity of light as well as the photoperiod are very critical for the success of certain culture experiments. Blue light promotes shoot formation, while red light induces rooting in many species [3,12].

The optimal hormone combination and concentration for multiple shoot initiation and proliferation was determined. Once these criteria were fulfilled, multiple shoot multiplication was initiated using whole in vitro axillary shoots as explants [24,30]. The shoots were serially subcultured every 3-4 weeks for a period of 16 weeks before rooting experiments were initiated. Shoot tip necrosis was a major problem but it was overcome by prompt subculture every 3-4 weeks and substitution of Gelrite with agar (0.8%) as a gelling agent in the shoot multiplication phase [11,23].

The rooted plants (9±1.0 cm in length) could be taken out gently from the vessels and washed to remove adhered agar and traces of the medium to avoid contamination. A final wash was given with distilled water for 10 min. Steps are taken for successful transfer of plantlet from aseptic environment to Green House environment [25,32]. They were grown for the first few days in the green house under low light, high temperature and high humidity. After few weeks light intensity is raised, ambient temperature and humidity are regulated for the plant to natural growth condition [15,19]. However, they act in interaction with auxins even though the auxin effect is indirect [4,22,31]. Plants transferred to the field have established themselves in the soil and are found to be growing well.

[IV] STATISTICAL ANALYSIS

In this study leaf and nodal segment were used for the regeneration of *Catharanthus roseus* plant. Experiments were carried out to establish favourable culture medium conditions for

explant regeneration and growth. Aseptic culture establishment and bud break depends upon amount of contaminations and physiological status of the collected explants during various seasons of the year. For the shoot induction from nodal explants various concentration of cytokinins were tried. The Auxins like 2, 4-D, IBA and the cytokinins like BAP, Kinetin were used in the medium [9,11,31]. It was also observed that the glassware also affect the rate of multiplication, maximum number of multiplication was observed in culture bottles as compare to conical flask.

4.1. Effect of 2, 4-D on leaf explant

The MS medium was used by me have various levels of 2, 4-D (0.1 to 6.0 ml/L) concentration. Leaf explant was inoculated in this culture medium. Observation was recorded in term of number of days, taken in the development of callus. In first experiment I used very low concentration of 2, 4-D i.e. 0.1 ml/L. This will have no effect on cultured leaf segment.

In the case of high level of 2, 4-D concentration slight swelling was appeared, looking like callusing. In the next sample high level of 2, 4-D (3.0 - 6.0 ml/L) induced variable amount of callus from leaf segment (as shown in table-2 & figure 3). In my experiment the best result was obtained by the use of 2,4-D of a concentration of (4.0 ml/L). The response by the leaf explant is best and its response is seen in 13 days with this concentration. We can successfully use 2,4-D as a callus stimulating agent.

Table-2. Effect of various concentration of 2, 4-D on different explant

S.No.	Concentration of 2,4-D (ml/L) in culture medium	Development of callus by leaf explant (No. of days)	Response by leaf segment
(i)	0.1	No response	NR
(ii)	1.0	20	C-
(iii)	2.0	18	C+
(iv)	3.0	16	C++
(v)	4.0	13	C+++
(vi)	5.0	19	C+
(vii)	6.0	21	C-

(Number of replicates kept during experiment was 10)

Here:- C- = Slight swelling C+ = Poor callus
C++ = Moderate callus C+++ = Very good callus
NR = No response

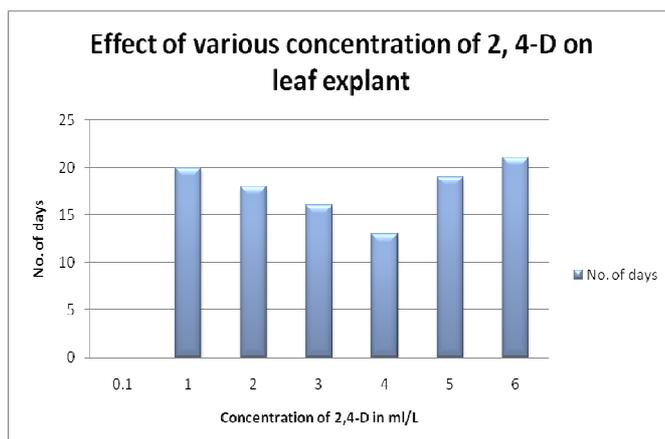


Fig-3. Effect of Cytokinin on Multiple Shoot Induction

4.2. Effect of BAP on nodal segment

The MS medium was used by me had various levels of BAP (1.0 to 8.0 ml/L) concentration. Node segment explant was inoculated in this culture medium. Observation was recorded in term of number of days, taken in the development of bud breaking. In first experiment I used very low concentration of BAP that is 1.0 ml/L, and due to this little composition very small effect on nodal segment is observed. In the case of high level of BAP concentration bud breaking stimulated. In the next sample high level of BAP (2.0 - 8.0 ml/L) induced variable amount of bud breaking from nodal segment (as shown in table-3 and figure 4). In this experiment the best result was obtained by the use of concentration of BAP (6.0 ml/L). The response by the nodal explant is best and its response in 4 days. We can successfully use BAP as a bud stimulating agent.

Table-3. Effect of various concentration of BAP on nodal explant

S.No.	Concentration of BAP (ml/L)	Bud breaking of nodal explant (No. of days)	Response by nodal segment
(i)	1.0	12	C-
(ii)	2.0	10	C+
(iii)	3.0	9	C+
(iv)	4.0	7	C++
(v)	5.0	6	C+++
(vi)	6.0	4	C++++
(vii)	7.0	8	C++
(viii)	8.0	11	C-

(Number of replicates kept during experiment was 10)

Here:- C- = Slight bud breaking C+ = Poor bud breaking
C++ = Moderate bud breaking C+++ = Very good bud breaking
C++++ = Excellent bud breaking

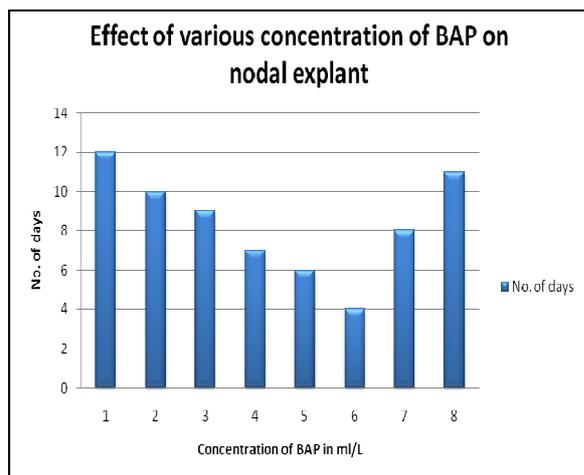


Fig- 4. Effect of BAP on Multiple Shoot Induction

4.3. Effect of Kinetin on nodal segment

The MS medium used in culture medium had various levels of Kinetin (2.0 to 10.0 ml/L) concentration. Nodal explant was inoculated in culture medium. Observation was recorded in term of number of days, taken in the shoot multiplication. In first experiment I used very low concentration of Kinetin that is 2.0 ml/L, and no effect was observed on shoot multiplication.

In the case of high level of Kinetin concentration shoot multiplication initiated. In the next sample high level of Kinetin (4-10 ml/L) induced variable amount of shoot multiplication from nodal segment (as shown in table-4 and figure 5). We can successfully use kinetin as a shoot stimulating agent, in my experiment the best result obtained in the concentration of Kinetin (6.0 ml/L). In the above concentration the response in the shoot multiplication is best and it has been taken 12 days.

Table-4. Effect of various concentration of Kinetin on shoot multiplication

S.No.	Concentration of Kinetin (ml/L)	Shoot multiplication (No. of days)	Response
(i)	2.0	25	C+
(ii)	4.0	18	C++
(iii)	6.0	12	C+++
(iv)	8.0	16	C++
(v)	10.0	20	C+

(Number of replicates kept during experiment was 10)

Here:- C+ = Poor shoot multiplication C++ = Moderate shoot multiplication C+++ = Excellent shoot multiplication

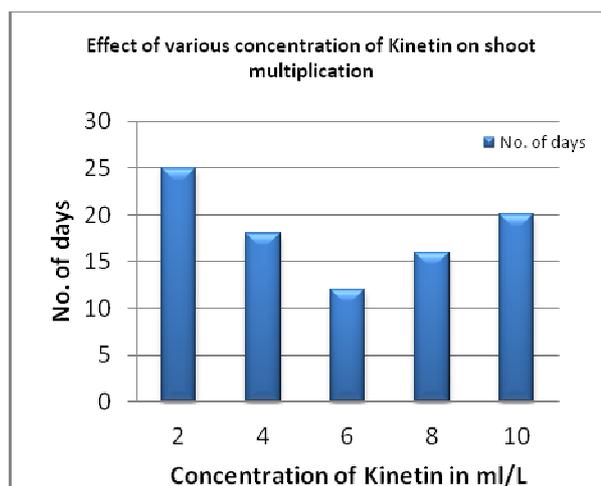


Fig- 5. Effect of Kinetin on Root Induction

4.4. Effect of IBA on nodal segment

The culture medium had various levels of IBA concentration (2.0 to 14.0 ml/L). Nodal explant was inoculated in this culture medium. Observation was recorded in term of number of days, taken in the development of root. In first experiment I used very low concentration of IBA that is 2.0 ml/L. I found no response by the nodal segment. At the high levels of IBA concentration, root slightly appeared, but it took a long time period. In the next sample when high level of IBA (4.0-14.0 ml/L) was used induced variable amount of root from nodal segment was observed (as shown in table-5 and figure 6). We can successfully use IBA as a root stimulating and growing agent. The best root multiplication results were obtained after 10 days in the IBA concentration (10.0 ml/L).

Table-5. Effect of various concentration of IBA on root development

S.No.	Concentration of IBA (ml/L)	Root development (No. of days)	Response in root development
(i)	2.0	NR	NR
(ii)	4.0	NR	NR
(iii)	6.0	20	C+
(iv)	8.0	17	C++
(v)	10.0	10	C+++
(vi)	12.0	14	C++
(vii)	14.0	18	C+

(Number of replicates kept during experiment was 10)

Here:- C+ = Poor root development C++ = Moderate root development C+++ = Excellent root development NR = No response

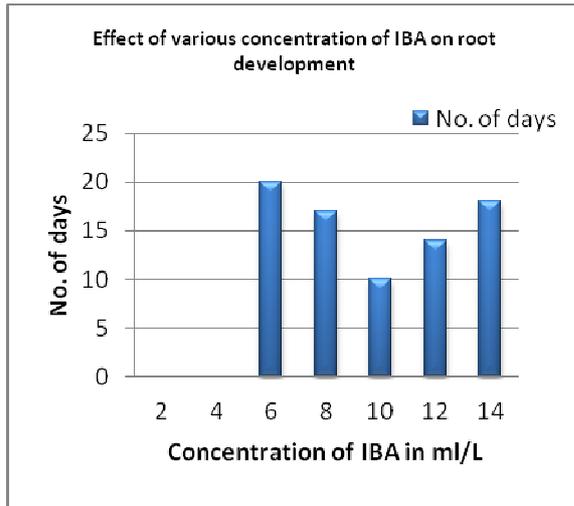


Fig- 6. Effect of IBA on Root Development

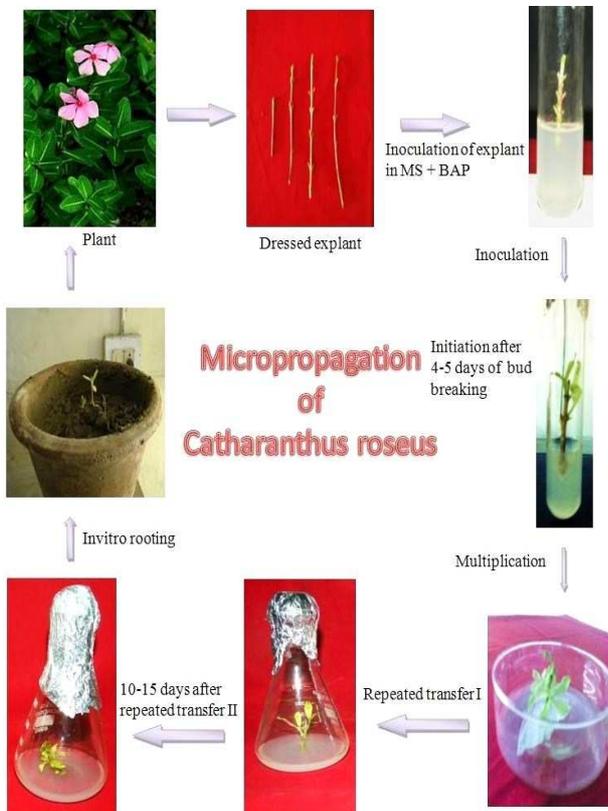


Fig- 7. Concluded whole micropropagation cycle of *Catharanthus Roseus*

[V] CONCLUSIONS

Micropropagation technology is being used for mass multiplication of elite forest species and also for production large quantities of planting material with desired characteristics. Despite the many achievements and potentials of plant biotechnology, its industrial and commercial applications are still limited by a number of factors. The need of hours is to concentrate more

on the research aspects for improvement of these techniques. In conclusion, we developed a reproducible micropropagation system for field grown culms of *C roseus*, which incorporates surface sterilization for 10 min with 0.1% aqueous mercuric chloride. The mainly used plant hormones in culture medium are such as 2, 4-D, IBA, BAP, and Kinetin. In the case of 2,4-D slight swelling apparent in the low concentration and in high level of concentration variable amount of callus obtained from leaf segment with the concentration of 2,4-D (4.0 ml/L) and the leaf explant took 13 days to give good response. We can say that 2,4-D used as a callus stimulating agent.

BAP concentration stimulates bud breaking in the nodal segment. We can successfully use BAP as a bud stimulating agent, the best result obtained in the concentration of BAP (6.0 ml/L). In the above concentration the response by the nodal explant is best and its response is obtained in 4 days. Plant growth hormones such as kinetin used to initiate shoot multiplication. By the use of IBA, we can stimulate root development, but it took a long time. In the high concentration of IBA, variable length of root was induced from nodal segment. The best root development results were obtained after 10 days in the IBA concentration (10.0 ml/L).

Kinetin induced variable amount of shoot multiplication from nodal segment. We can use kinetin as a shoot stimulating agent, in my experiment the best result obtained in the concentration of Kinetin (6.0 ml/L). In the above concentration the response in the shoot multiplication is best and it 12 days. A concluded whole cycle of micropropagation is shown in figure 7.

We hope that this research work is more useful, innovative and also give a new direction for further research and future aspects in this field.

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