Biotechnological Studies On *Catharanthus roseus L.*

(AN ANTI CANCEROUS DRUG YIELDING PLANT)

A Thesis

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By

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C E R T I F I C A T E

I feel great pleasure in certifying that the thesis entitled " **Biotechnological Studies On *Catharanthus roseus L.* (AN ANTI CANCEROUS DRUG YIELDING PLANT)"**by Monika Sain under my guidance.She has completed the following requirements as per Ph.D regulations of the University.

(a)Course work as per the university rules.

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ABSTRACT

Experimental plant *Catharanthus roseus* which also commonly known as Madagascar periwinkle,is a tropical and subtropical plant belong to the family Apocynaceae.

Catharanthus roseus is medicinally important plant which is being widely used for production of anticancerous drugs by pharmaceutical companies due to the presence of vinblastine and vincristine alkaloids.

Due to over exploitation of this plant for pharmaceutical purpose, demand of raw materials of this plant is constantly rising. To meet the growing demand, a large scale multiplication of this plant has become need of present time.

Keeping this in view, in present study we used leaf, nodal portion and root tips as explants .Morphogenic effect of various concentrations of cytokinins and auxins added singly or in combination supplemented with MS medium was studied during experimental work.

Somatic embryogenesis was observed on combination of 2, 4-D (1.0mg/l) + Kinetin (1.0mg/l) supplemented with MS medium from leaf explant. By encapsulation of these somatic embryos with gelling agent (Sodium alginate and Calcium chloride), synthetic seeds were formed. Effect of natural additives (Banana homogenate and Coconut water) on 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).

Best rooting response was obtained on half strength MS medium containing IBA (5.0mg/l). Effect of different energy sources (glucose, fructose and sucrose) and tryptophan amino acid subjected with three different dark cycles (12hrs, 16hrs and 20hrs) on rooting was also studied.

Qualitative analysis to detect the presence of various bioactive chemicals in leaf of *Catharanthus roseus* was also done. *In-vitro* grown plantlets were successfully acclimatized and then transferred to field condition.Our efforts are continued to transfer more and healthy *in vitro* raised plantlets to field condition.

Candidate's Declaration

I, hereby, certify that the work, which is being presented in the thesis, entitled **Biotechnological Studies On *Catharanthus roseus L.*(AN ANTICANCEROUS DRUG YIELDING PLANT)** is a partial fulfillment of the requirement for the award of the Degree of Doctor of Philosophy, carried under the supervision of Dr. Vandana Sharma and submitted to the Department of Botany , Government College, Kota, University of Kota, Kota represents my ideas in my own words and where others ideas or words have been included. I have adequately cited and referenced the original sources. The work presented in this thesis has not been submitted elsewhere for the award of any other degree or diploma from any Institutions. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that any violation of the above will cause for disciplinary action by the University and can also evoke penal action from the sources which have thus not been properly cited or from whom proper permission has not been taken when needed.

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ABBREVIATIONS

%	-percent
^{0}c	-degree centigrade
µg	-microgram
µM	-micromolar
cm	-centimeter
g	-gram
hrs	-hours
IBA	-Indole-3-butyric acid
IAA	-Indole acetic acid
BAP	-6-benzyl amino purine
NAA	- α-Naphtalene acetic acid
2,4-D	-2,4-Dichlorophenoxyacetic acid
Kinetin	-6-(2- furfury1-7-aminopurine)
CW	-Coconut water
M	-molar
mg	-miligram
Min	-minute
ml	-mililitre
mm	-milimeter
mM	-milimolar
MS	-Murashige and skoog medium
EDTA	-Ethylene Diamene TetraaceticAcid
GA_3	-Gibberellic acid

Introduction

Medicinal Plant – An overview

Medicinal plants have been proved an important therapeutic aid for alleviating the ailments of human kind. The search for longevity and remedies of pain and discomfort drove early man to look into immediate natural surroundings to make the use of plants, animal products and minerals etc.

Medicinal plants contain many of natural products with varying level of bioactivities. Now-a-days, there is a renewal interest in traditional medicine and herbal drugs. Because green medicines are considered as safe and more dependable than synthetic drugs. Many synthetic drugs have adverse side effects **(Nair and Chandra, 2007)**.

An **''Herb''** is a small herbaceous plant and plant parts (stem, leaves, flowers, seeds, fruits, bark, roots, rhizomes and wood etc) valued for its medicinal, aromatic or savory qualities (**Fig A i).**

Medicinal plant produce and contain a variety of chemical substances that act upon the body and used as pharmaceuticals, agrochemical, flavour and fragrance, ingredients, food additives and pesticides (**Fig. A ii)** Now-a-days, many drugs which are commonly used are of herbal origin. Indeed, about 25 percent of the prescribed drugs contain at least one active ingredient derived from plant material.

Medicinal plants are potential resource for uplifting economy and generating employment for the unemployed youth. In India, it is estimated that the collection and processing of medicinal plants contribute to at least 35 million workdays of employment per annum **(Karki, 2012).** Medicinal plant cultivation may prove success model for their development.

Medicinal plants and herbs are a valuable part from which different medicinal system like Ayurveda, Siddha and Unani have been developed. Since time immemorial, medicinal plants have been proved a boon to human kind. Medicinal plants are viewed as a possible bridge between sustainable economic development, affordable health care and conservation of valued biodiversity. So, there is an urgent need to make policies and strategies to harness optimum potential of medicinal plant for multi dimensional socio-economic progress.

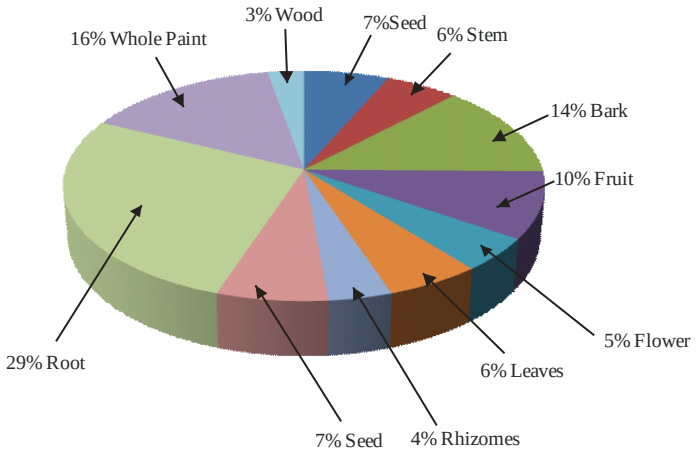

Fig. A(i) Percentage of different parts of medicinal plants used for pharmaceutical purpose

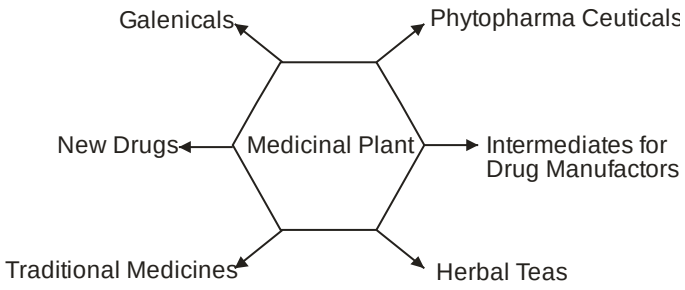

Fig. A (ii) Different uses of medicinal Plant.

History and the present status:-

Early humans developed their dependence on nature for a healthy life.Human kind has been dependent on plant resources for food, clothing, shelter and medicine to cure myriads of ailments since long time. In ancient cultures, tribal people collected information systematically on herbs and developed well defined herbal pharmacopoeias and brought in practice.

Herbs have been prized for their aromatic, flavouring and medicinal property for centuries. There are many references of herbs as wonder drugs in the ancient Indian literature. These herb have magical properties and are used to cure many incurable diseases from tip to top and increase longevity even bring dead back to life.

Traditional Health care system is the only accessible form for majority of population both logistically and economically. The utilization of medicinal plant is not the saga of today but it was mentioned much earlier in Rigveda between 4500 B.C. to 1600 B.C.. Also sixty thousand years ago, physical evidence of the use of herbal remedies some sixty thousand years ago has been found in a burial site of Neanderthal man uncovered in 1960 in a cave in northern Iraq. **(Kunle, 2012)**

'**Ayurveda'** literally means 'knowledge' (**Veda**) of life (**Ayur**) had its beginning in Atharvaveda (Circa 1500 – 100 BC). The theory of Rasa, Vipaka, Virya and Prabhava formed the basis of Ayurveda Pharmacology, which made no clear distinction between diet and drug, as both were vital component of treatment **(Valiathan 1998)** . **Charak,Sushruta** and **Vagbhata** described 700 Herbal drugs with their properties and clinical effects.

During the 18^{th} and 19^{th} centuries, the introduction of allopathic medicine in the form of base chemical and pharmaceutical has developed method for bringing quick relief

which won popularity. In the 1980's due to side effects of allopathic medicine, demand for green medicine is gradually increased.

Recognition of the rising use of herbal medicines led to establishement of the office of alternative medicine by the National Institute of Health USA in 1992. Worldwide herbal medicine received a boost, when the WHO encouraged development to fulfill the needs which were unmet by modern system **(Window and kroll, 1998).** Today, 60% in most of the countries and 70-80% of Chinese, Indian, African and Germany people use herbal medicine.

In India, approximately 1800 plant species are used in Auyurveda, 400 plant species are used in Siddha andapproximately 500 plant species for Homeopathic system of medicines. Thus, approximately 8000 plant species used in traditional system of medicine in India.

Market Survey

Medicinal plants and their phytochemicals are important factors for developing new and urgently needed products for human health care. Considering the revival of interest in natural products, many pharmaceutical companies have started producing a number of herbal preparations. The medicinal plant based industry is growing at the rate of 7 – 15% annually.

The Indian herbal drug market is about $ one billion and the export of herbal crude extracts is about $ 80 million. The top 5 best selling herbal medicine in developed countries are Ginseng (***Panax ginseng***), Turmeric (***Curcuma longa***), Ginkgo(***Ginkgo biloba***) and Garlic (***Allium sativum***).

In recent years, the growing demand for herbal products has led to a quantum jump in volume of plant material traded within and outside the country.India is one of the major raw material producing country.Medicinal plants being exported from India are –***Zingiber officinale*** **(Rhizome),** ***Rauwolfia serpentiana*** **(Root),** ***Swertia chirayita*** **(whole plant),*****Cassia angustifolia*** **(leaf & pod),** ***Acorus calamus*** **(Rhizome),*****Adhatoda vasica*** **(Whole plant),*****Aconitum species*** **(Root),** ***Picrorhiza kurroa*** **(Root)** and ***Colchicum lutem*** **(Rhizom& Seed).**

Secondary metabolites of herbal plant are the source of refined compounds used in pharmaceutical industries. Terpenoids contributed major part of world wide trade $ 7.2 billion, alkaloids $ 3.6 billion and other plant derived compound were about $ 4 billion.

Earth wealth:-

The Earth is home to a rich and diverse array of living organisms. Their genetic diversity and relationship with one another and with the physical environment ultimately constitutes biodiversity.

India is unique in the richness of its plant wealth. Approximately eight percent of world's biodiversity has the potential of becoming major global player in screening and developing plant based formulations, medicines and products.

India's surface land has been grouped into ten district zones and these are further divided into 25 biotic provinces and 426 biomes. The forest areas of these biogeographic zones are classified into 16 major forest types and more than 200 subtypes.

India is one of the twelve megabiodiversity center having 15000 – 18000 flowering plants, 23000 fungi, 2500 algae, 1600 lichen, 1800 bryophytes and 50 million micro organism. About 1500 plants with medicinal uses are mentioned in ancient texts and around 800 plants being used in drug production by industries where only less than 20 species of plants are under commercial cultivation.

According to macro analysis of the distribution of medicinal plants 70% of india's medicinal plants are found in tropical and deciduous forests and 30% are found in temperate and high altitude forests.

Over 70% of the plant collections involve distructive harvesting because of the use of plant parts and the whole plant in case of herbs. It will posses threat to the genetic stocks and to the diversity of medicinal plants, if biodiversity is not sustainably used.

According to the IUCN report for the year 2000, India ranked fifth in the case of threatened plant species and birds. Recently by using IUCN designed CAMP methodology, assessment of the threat status of medicinal plants revealed that about 112 species in southern india, 74 species in northern and central india and 42 species in high altitude of Himalyas are threatened in the wild habitat.

The growing demand of medicinal plant and there products is putting a heavy strain on the existing resources, resulting in a number of species to be either threatened or endangered category.

Medicinal Plants of India

Considering the resurgence of interest in natural products for curing several chronic ailments, the demand of plant based drug is increasing. This scenario needs in depth

and extensive search process of high values medicinal plants used for preparation of drugs in various system of medicine.

The Indian system of medicine (Ayurveda, Unani, Siddha and Homeophathy) largely use plant-based products. In India, many companies like Ajanta Pharmaceuticals, Dabur limited and the Himalayas Drug Company have launched several herbal remedies in the market using the plants like ***Withania somnifera*** **(Ashwagandha),** ***Bacopa monnieri*** **(Brahmi),** ***Terminalia arjuna*** **(Arjuna),*Commiphora wightii* (Guggal),** ***Curcuma longa,*** **(Turmaric) and Triphala (*Emblica officienalis*, *Terminalia chebula* and *Terminalia belerica*.)**

Today there are at least 120 distinct chemical substances derived from plants which are considered important herbal drugs. Some of the most important plant genera which yield frequently prescribed medicines are ***Dioscorea deltoidea, Papaver somniferum, Atropa belladonna, Rauwolfia serpentina, Cinchona officinalis, Albizzia lebek, Picrorhiza kurroa*** **etc.**

Dioscorea species are being frequently use as a tonic in chinese traditional medicine. The active ingredients present in these tubers are **"diogenin"** which is an important medicinal steroids like prednisene, dexamethasone. **(T.sukamot etal:1936)."Capsaicin"** obtained from Capsicum species is also used in pharmaceutical preparation as a digestive stimulant and for rheumatic disorder **(B.S.Soohch etal, 1997).**

Production of **'Morphine'** and **"Codeine"** (Analgesics) from morphologically undifferentiated culture of ***Papaver somniferum*** has been reported two decades back **(T.Furuya et al. 1972; W.H.J. Tam et al.1980).**

Berberine (anti bacterial drug) extracted from **Berberis.** It's productivity was increased in cell cultures by optimizing the nutrients and the level of phytohormones in the growth medium **(F.sato&Y.Yamada, 1984).**

"Taxol", Complex diterpene alkaloid **(ananticancerdrug)** was isolated from ***Taxus brevifolia***(view tree)**(M C wani et al 1994 and venisree and coworkers, 2004).**
There are same major plant drugs of which no synthetic substitute is currently available.

DRUG	PLANT	USE
Reserpine	*Rauwofia serpentina*	Tranquilizer
Gingosensosides	*Panax ginseng*	Antidiabetic
Pilocarpine	*Pilocarpus jaborandi*	Antiglucoma
Curcuminoids	*Curcuma longa*	Anti inflammatory
Digitoxin	*Digitalis sp.*	Cardiotonic
Silybenin	*Silybium marianum*	Antioxidant
Morphine	*Papaver somniferum*	Tranquilizer
Allicin	*Allium sativum*	Anti fungal
Atropine	*Atropa belladonna*	Spasmolytic, cold
Nimbidin	*Azdirachata indica*	Antiulcer
Catechin	*Acacia catechu*	Anti ulcer
Artemisin	*Artemesia annua*	Anti malarial
Gossybol	*Gossypium sp.*	Anti spermatogenic
Glycyrrhizin	*Glycyrrhiza glabra*	Antiulcer

Conservational Strategies :

Medicinal plants are the most important source of life-saving drugs for the majority of the world's population. Due to medicinal importance and other multiple uses, the

demand for medicinally important plant is constantly rising. However, the supply of these value-aided plants has become erratic and inadequate. Population explosion, increased anthropogenic activities and destructive harvesting combined with habitat loss in the form of deforestation has aggravated the problems of availability of the plants.

Over harvesting has become out of control. If the pattern of over usage goes the same way, medicinal plants wealth will surely be in danger. According to world bank report titled as: **''Medicinal Plants: rescuing a global heritage,"** a large number of medicinal plants are being utilized and over harvested, so they will become extinct and endangered.

Therefore the need of conservation of medicinal plants is crucial. To cope up with alarming situation, many efforts have been made by in-situ and ex-situ conservation methods, but biotechnological strategies would open up new vistas in the field of conservation.

Plant Tissue Culture

In recent years, plant tissue culture represent 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. 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.

Tissue culture 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 **(R. Rao & R. Shankar, 2002).**

Large scale plant tissue culture is found to be an alternative approaches to traditional method of plantation as it offers a control supply of biochemicals independent of plant availability. Micropropagation has become an important tool to obtain genetically pure elites rather than having genetically different populations.

Micropropagation is now a well established technique commercialized globally for the rapid production of a number of commercially important plants. More over, the plant multiplication can continue throughout the year irrespective of season and the stocks of germplasm can be maintained for many years **(C.P. Malik, 2007).**

Advantages of Micro-Propagation

- Alternatives to conventional methods of vegetative propagation to enhance the rate of multiplication.
- Large number of plants can be raised from a small (even microscopic) piece of plant tissue within a short span of time.
- It provides reliable and economical method of maintaining pathogen free plants.
- Stocks of germplasm can be maintained for many years.

Three basic methods are used to propagate plants *in-vitro*:- (i) Enhanced axillary shoot proliferation (ii) Denovo formation of adventitious shoots (iii) Somatic or non zygotic embryogenesis.

In-vitro techniques have been proved beneficial for collecting, multiplication and storage of plant's germplasm **(L. Engelmall, 1991).**

The major advantages of a cell culture techniques over the conventional cultivation of whole plants are (1) Useful compounds can be produced under controlled conditions independent of climatic change or soil conditions; (2) Cultured cells would be free of microbes and insects; (3) The cells of any plants, tropical or alpine could easily be multiplied to yield their specific metabolites; (4) Automated control of cell growth and rational regulation of metabolite processes would reduce the cost and improve productivity; (5) Organic substances are extractable from callus cultures.

Synthetic Seed

Synthetic seed technology has revealed new vistas in plant biotechnology. This technology provides methods for preparation of seed analogues called synthetic seed or artificial seeds from axillary buds, somatic embryos, apical tips, protocorm like bodies and embryogenic calli.

For the first time embryogenic cell treated as artificial seed for obtaining plants directly have been reported for several crops of agricultural interest **(S. Kitto and J. Janick 1985 and A.V. Patel, 2000)**. This technology offers tremendous potential in micropropagation and germplasm conservation.

Advantages of synthetic seed technology:-

* Easy handling while in storage
* Easy to transport
* Potential for long storage without losing viability
* Production of large number of identical embryos.
* Determination of role of endosperm in embryo development and germination.
* Study of somaclonal variation and seed coat formation.

Secondary Metabolites

Plant tissue culture hold great promise for controlled production of myriad of useful secondary metabolites. Secondary metabolites such as a alkaloids, terpenoids, flavanoids and glycosides mediate the relation between plants and their environment.

More than 100 alkaloids have been isolated from various plants of apocynaceae family. Biologically active compounds from plant sources have a dramatic impact as medicine. **Quenine** present in **Cinchona** for malaria and **Vincristine** present in ***Catharanthus roseus*** for leukemia (Blood Cancer) are few examples.

In order to obtain high yields suitable for commercial exploitation efforts have been focused on isolating the biosynthetic activities of cultured cells achieved by optimizing the culture conditions, selecting high producing strains and employing precusor feeding, elicitation, transformation method and immobilization techniques **(Diosmose and Misawa, 1993).**

At present researchers aim to produce substance with anti-cancerous, antiviral, hypoglycemic, anti-inflammatory, anti-parasitic, anti-microbial, tranqulizer and immuno-modulating activities through tissue culture technology.

Cancer has become a big question for scientific community as no existing treatments could solve the problems related to this dreadful disease. Cancer (malignant tumour) is an abnormal growth and proliferation of cells. It is a frightfull disease because the patient suffers pain, disfigurement and loss of many physiological processes. It may be uncontrolled and incurable and may occur at any time at any age in any part of the body. It continues to represent the largest cause of mortality in the world and claims over 6 millions.

Research is in well progress since half century but failed to give a right solution to fight against it, However a herbal anti cancerous drug give good hope to the people for cancer by the development in science and technology.

There are many plants with anticancerous property, e.g., ***Azadirachta indica*** **(Nimbidin),** ***Camellia sinensis*** **(Cataechins),** ***Podophyllum emodi*** **(Podophyllin),** ***Camptotheca acuminata*** **(Camptothecine),** ***Plumbago zeylanica*** **(Plumbagin),** ***Withania somnifera*** **(Asparaginase),** ***Catharanthus roseus*** **(Vincristine and Vinblastine),** ***Taxus*** **spp. (Taxol) etc.**

Seeing the need of time, we focussed our experimental studies on standardization of better protocol for large scale multiplication and conservation of an anticancerous drug yielding plant – ***Catharanthus roseus*** **(L) G.Don. (Apocynaceae).**

About the Experimental Plant

Catharanthus roseus (L) G. Don

(Madagascar rosy Periwinkle)

Taxonomical classification :-

Kingdom – Plantae
Phylum – Magnoliophyta
Class – Magnoliopsida
Order – Gentianales
Family – Apocynaceae
Genus – Catharanthus
Species – roseus

Fig. 1 (iii) Picture of *Catharanthus roseus L*.

Binomial Name — *Catharanthus roseus* (L.) .Don.

Common Name — Vinca rosea, Periwinkle, Sadabahar, Sadaphul, Nayantra, Nityakalyani, Old maid.

History

Linnaeus published *Catharanthus roseus* as Vinca rosea in his **"Systema Naturae"** in 1759. In 1928 **Reichenbach** used the name **Lochenra** in his **"Conspectus Regni Vegetabiles"** but without giving a description or reference. In 1835, G.Don. published Catharanthus in **"General system of Gardening and Botany"**. Lochnera is a synonym of **"Catharanthus."**

Morphology

***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. The leaves are oval to oblong, elliptic, acute, rounded apex, 2.5-9.0 cm long and 1-3.5 cm broad glossy green hairless with a pale midrib and a short petiole about 1-1.8 cm. long and

they are arranged in the opposite pairs. The flowers are white to pinkish purple with a dark red eye in the centre with a basal tube about 2.5-3 cm long and a corolla about 2-5 cm diameters with five petal like lobes in terminal or axillary cymose clusters. The fruit is a pair of follicles about 2-4 cm. long and 3 mm. broad. The seeds are oblong, minute and black.

Habital and Distribution

Catharanthus roseus is a perennial, evergreen herb and is best grown as an annual bedding plant in well drained sandy loam in full sun to part shade. Needs regular moisture but avoid overhead watering.

Numerous cultivars have been selected for variation in flower colour (white, mauve, peach, scarlet and reddish orange) and also for tolerance of cooler growing conditions in temperate regions, notable cultivars include **"Albus"** (white flowers), **"Grape cooler"** (rose pink, cool tolerant), the **"Ocellatus group"** (various colours) and **"Pepperment cooler"** (white and red centre, cool tolerant). The synonyms of the plant name include ***Vinca rosea, Ammocalis rosea*** and ***Lochnera rosea*****.** Rose purple *flowers* are being cultivated because of its higher alkaloid contents. Recently, two white flowered varieties with high alkaloid content named **"Nirmal"** and **"Dhawal"** have been released by the CIMAP, Lucknow.

Catharanthus roseus is a native of the Indian Ocean Island of Madagascar. It comprises eight species, seven endemic to Madagascar **(*C. coriaceus, C.lanceus, C. longifolius, C. ovalis, C. scitulus, C.trichophyllus*)** and one, ***C. pusillus*** from India. In the wild, it is found to be an endangered plant and the main cause of their decline is the habitat destruction by the slash and burn agriculture. Abundantly natural glow in many region, particularly in arid coastal location. It is grown commercially for its medicinal uses in Australia, Africa, India and Southern Europe. Cultivated as an ornamental plant almost throughout the tropical and subtropical regions world wide **(Yuan et al, 2011).**

The climatic condition and the soil properties of some European countries are however unfavourable for the cultivation of *Catharanthus roseus* It may be grown only as an annual plant in green house and in plastic tunnels but in such cases the contents of "dimeric indole alkaloids" was observed to be very low**(Pietrosiuk et al, 2007).** In Poland, hydroponics technique is also used for *Catharanthus roseus* cultivation **(Lata et al, 2007)**

Medicinal Uses

Catharanthus roseus has more than 130 know alkaloids known as **"Vinca"** alkaloids, which are used in cancer, diabetes, high blood pressure, asthma, constipation and menstrual problems. 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 extracts 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.

Other uses

Besides medicinal uses, in Europe, it was thought to be an magical plant also that could ward off evil spirits. It is also used as an ornamental in many countries. Exposed *Catharanthus roseus* bioaccumulates heavy metal like cadmium(cd) etc, so it is used in phytoremediation.

Pharmacology

Catharanthus roseus is a chemical factory producing more than 130 different terpenoid indole alkaloids (TIAs), some of which exhibit strong and important pharmacological activities. Some major alkaloids isolated from plant and cell tissue culture of *Catharanthus roseus* have been mentioned here –

Alkaloids Isolated from *Catharanthus roseus*

Alkaloids	**Source of isolation**
N,N-dimethyl- tryptamine	SC
Antirhine	P, SC
Akuammicine	P,*L*, R, C, SC, S
Lochrovicine	*L*
Cavincine	P,*L*, R, C, HR
Lochnerine	SC
Rosicine	*L*
Catharanthine	P,*L*,F, S*l*, C, SC, S
Perivine	P,*L*, F, R, C, SC
Vinervine	SC
Alstonine	R, C
Cathenamine	P
akuammiline, O-deacetyle	*L*,C
Lochnericine	P,*L*, SC
Minovincine	P
Preackuammicine	SL
Rosamine	*L*
Perividine	P
Yohimbine	P, R, SL, C, SC, Hr
Vincoline	P, L

Vindolinine	P,L,SC,S
Lochrovidine	P
Vincarodine	P,L
Vinosidine	R
Cathovaline	*L*
vindolidine[3]	P, F
Vindoline	P,*L*,F,SL,S
Bannucine	P,*L*
Vinsedine	S
Leurosinine	P
Vinsedicine	S
Vinblastine	P,*L*, F, SL, C
Cathericine	P,*L*
Catherine	P,*L*, S
Carosine	P,*L*,F
Vinamidine	P,*L*
Vincristine	P,*L*
Leurosinone	*L*
Cathindine	*L*, R, SC
Rovindine	P,*L*

P = Plant extract, L = Leaf, F = Flower, SL = Seedling, S = Seed, C = Callus culture, SC = Suspension Culture, S = Shoot, Hr = Hairy Root, R = Root

Besides alkaloids, other secondary metabolites have been isolated from *Catharanthus roseus*, including monoterpenoid glucosides **(loganin, secologanin, sweroside, deoxyloganin, dehydrologanin)**, steroids **(catasteron, brassinolids)** phenols, flavinoids and anthocyanins**(rosindin).**

Some of the *Catharanthus roseus* alkaloids are marketed as pharmaceuticals –

Vinblastine : (Velban, VLB-Vincaleukoblastine) : was introduced in 1960 and used as anticancerous drug. It is composed of Catharanthine and Vindoline, present in the leaves of the plant.

Vincristine (Oncovin, LC = Leurocristine) : is an oxidized form of vinblastine and it was introduced in 1963, also used as anticancerous drug. It is very scarce in the plant but it can be transformed in the laboratory from vinblastine and scientifically known as Leurocristine and it is marketed by **Elli-lilly & Company** with name as **ONCOVIN.**

Anhydro vinblastine: have also been claimed for use as an antineoplastic agent in the treatment of cervical and lung cancer.

Ajmalicine (Hydroserpan, Lamuran): was introduced in 1957 for the treatment of hypertension.

Vindesine (Eldisine): It is a derivative of VLB and used for treatment of Leukemia's mylomas and diabetes.

Vinorelbine (Navelbine): is used against lung-carcinomas.

Vinzolidine: shows a greater therapeutic effect in tumours than either VCR or VLB, but severe toxicity of oral dosing schedules had led to early closure of several studies.

Vinflunine : showed a superior anti tumour activity invivo in preclinical tumor model compared to the other *Catharanthus* alkaloids.

Vinxaltine : *Invitro*, it is more active against myloma cells compared with VCR & VLB.

Due to presence of above mentioned bioactive compounds,*Catharanthus roseus* posses following pharmaceutical activities.

(1) **Anti neoplastic activity :** This activity of bisindole alkaloids of *Catharanthus* is attributed to their abiliy to disrupt microtubules causing dissolution of mitotic spindles and metaphase arrest in dividing cells. Different percentage of the methanolic crude extracts of *Catharanthus* was found to show the significant anti cancer activity against numerous cell types in the *invitro* condition **(Ueda J.V. et al, 2002).**

(2) **Anti diabetic activity :** The ethanolic extracts of the leaves and flower of *Catharanthus* showed a dose dependent lowering of blood sugar in comparable to the standard drug. The hypoglycemic effect has appeared due to the result of the increase glucose utilization in the liver **(R.R. Chattopadhyay et al, 1931).**

(3) **Anti bacterial activity :** The crude extracts from different parts of the plants was tested for anti bacterial activity. The extracts from leaves showed significantly higher efficacy.

(4) **Anti oxidant activity :** The ethanolic extract of the Catharanthus varieties has exhibited the satisfactory scavenging effect in the entire bioassay in a concentration depended manner but *Catharanthus roseus* was found to possess more antioxidant activity than that of C.alba **(Cheruth A. aled et al, 2009).**

(5) **Anti helminthic activity :** Helminthes infections are the chronic illness, affecting human beings and cattle. *Catharanthus roseus* was found to be used from the traditional period as an antihelminthic agent.

(6) **Anti hypotensive activity :** The extracts of leaves of the plant has significant effect in hypotension.

(7) Anti-diarrheal activity — The anti-diarrheal effect of ethanolic extract of *Catharanthus roseus* showed the dose dependent inhibition of the castor oil induced diarrohea. **(A. Kya kulaga. et al, 2011).**

(8) **Anti viral activity —** The anti viral effect of **"Yohimbine"** alkaloid of *Catharanthus roseus* on herpes simplex virus with a cytopathogenic effect showed by **Ozedik.**

Catharanthus roseus is the unique natural source of the well known high value anti cancerous drug alkaloids **vinblastine (VB)** and **vincristine (VC)**. The process of biosynthesis starts with the amino acids tryptophan. Tryptophan amino acid is a key component of bio synthesis of vinblastine via mevalonote path way.

Vinblastine(Fig.C-i) and **Vincristine(Fig.C-ii)** are synthesized from the coupling of the monomeric alkaloids **catharanthine** (Fig. C-iii) and **vindoline** (Fig.C-iv). The coupling process in plant is catalyzed by the enzyme anhydrovinblastine synthase. These dimeric alkaloids are used as anti tumor agents and produced in trace amounts (0.0005% dry weight). The natural high abundance of Vindoline and Catharanthine in *Catharanthus roseus* plant led to the establishment of a semi synthetic process for coupling the monomers either chemically **(Kutney et al, 1976)** or enzymatically using horse radish peroxidase. **(Goodbody et al, 1986).**

Vinblastine may occur in lactifer and idioblast of *Catharanthus roseus*, it accumulates in parenchyma throughout the plant of the two monomers, the Catharanthine has the highest concentration in youngest, fully expanded leaves and the vindoline concentration was highest in leaves 5 and 7 from the apex **(Balsevish and Bishop, 1989).**

Catharanthus roseus is an amazing chemical factory, producing more than 130 alkaloids, some of which exhibit strong pharmacological activities. The most striking biological activity is antitumour effect of alkaloidsVinblastine and Vincristine together with a number of semi synthetic derivatives, known as **"Vinca"** alkaloids.

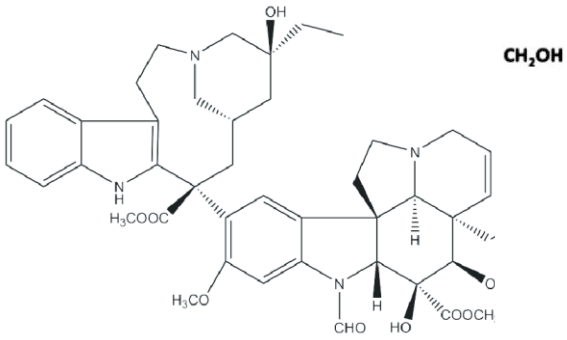

Figure (i) : Vinblastine

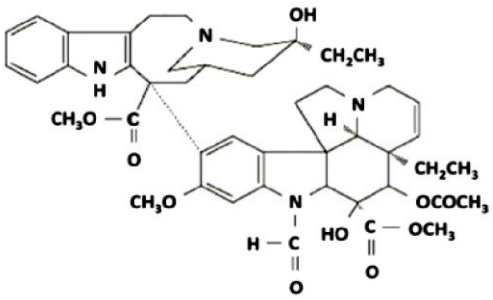

Figure (ii) : Vincristine

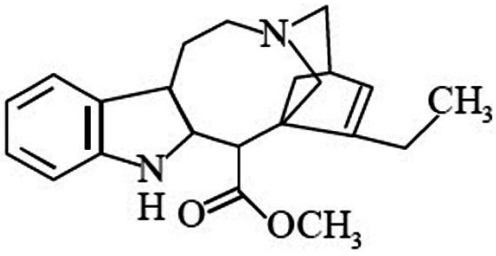

Figure (ii) : Catharanthine

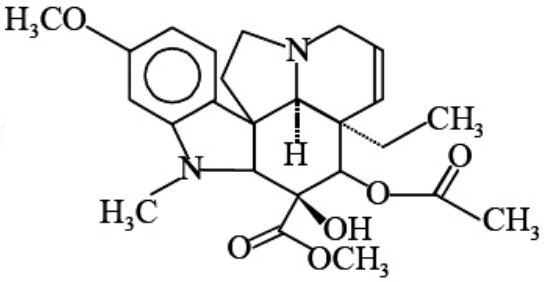

Figure (iv) : Vindoline

Fig. C (i-iv)-Structure of *Catharanthus roseus* alkaloids

Objectives

Today, there are many diseases spreading all over the world causing many deaths, Cancer is a nearly invincible disease that has played havoc in humankind for last few centuries. Due to atmospheric pollution, cancer is increasing in alarming rate. Here is a high level of treatment failures and unpleasant side effects associated with oral anti-cancerous drugs. So, there is an urgent need and desire for alternative treatment.Use of herbal products is becoming popular in the treatment and management of cancer.

Catharanthus roseus is an anticancerous drug yielding plant. It has been known to have alkaloid vincristine and vinblastine which can break malignant cell and cure cancer. The demand of TIAs (terpinoid indole alkaloids) for pharmaceutical purpose is very much and their prices are also high.The content of anti cancerous alkaloids in the raw material derived from naturally occurring whole plant is very low.

Because of over exploitation and habitat destruction of *Catharanthus roseus*, we have to look for the the alternative to keep pace with the growing demand. Considering the medicinal value of the plant in the present study, efforts will be made to develop an efficient protocol for large scale multiplication and plant regeneration in *Catharanthus roseus* an anti cancerous drug yielding plant.

- Selection of most responsive explants for micropropagation as experimental plant.
- To study the effect of different growth regulator on micropropagation of experimental plant.
- To establish the culture condition necessary for callus induction and high frequency regeneration through organogenesis and somatic embryogenesis.
- Acclimatization and hardening of *invitro* regenerated plantlet
- Qualitative analysis to detect the presence of various biochemicals in leaf of *Catharanthus roseus*.

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Review

OF

Literature

Medicinal plants are vital source of compounds for the pharmaceutical industry and traditional medicine. 80% of the population living in developing countries use traditional medicine derived from plants for their primary health care **(De Silva, 1997)**. The success of health depends on the availability of suitable drugs on a sustainable basis.

Cancer is one of the most dreaded diseases of the present time. According to the American cancer societies, deaths arising from cancer constitute 2-3% of the annual deaths recorded world wide is due to cancer. Cancer rates are increasing every year due to atmospheric pollution.

Many traditional healers and herbalists have been treating cancer patients using active compound (alkaloid) of various medicinal plant species since last many years.

Alkaloids are one of the largest classes of secondary metabolites. These compounds contain heterocylic nitrogen that makes them particularly pharmacologically active.**(De Luca and St. Pierre, 2000)**. A large number of alkaloids have been used in medicine and they are the basic components of modern drugs **(Morgan and Shank, 2000).** The anti cancerous alkaloids have been found in families like Apocynaceae, Rubiaceae of Gentiales order **(Verporte et al, 1998).**

Catharanthus roseus **(family-apocynaceae)** is an important medicinal plant of tropical and subtropical India which is used to treat various type of cancer. Its medicinal usage has been already reported in India, British pharmacopoeias and also in indigenous system of medicine. *Catharanthus roseus* is considered to be medicinally potent to combat with various diseases besides cancer. The medicinal value of *Catharanthus roseus* is due to alkaloids like **vindesine, vincaleucoblastine, leurocristine (LC), Catharanthine, Tetrahydroalstonine** present in various parts of plant.

Indiscriminate, ruthless collection of medicinal plants for medicinal purposes is causing rapid depletion of flora, leading to the extinction of many important species. It is possible to save local flora if proper propagation and conservation measures are taken duly **(Gilani et al, 2009)**. So Now a days, there is an increasing interest in using *in vitro* techniques for rapid and large scale propagation of medicinal plants **(Otroshy and Moradi, 2011)**.

Micropropagation by tissue culture offers an alternative way of plant propagation and has the potential to provide high multiplication rates **(Beck and Dunlop, 2001)**.

Catharanthus produces a great variety of terpenoid indole alkaloids (TIAs), most of them are pharmacological activity **(Van der Heyden et al, 2004)**. Vinblastine (VB) and Vincristine (VC) are the most important alkaloids with anti-cancerous property **(Mukherjee et al, 2001)**. Vinblastine is used against several forms of cancers like Hodgkin's disease while vincristine is used in the treatment of leukemias **(Schmeller and Wink, 1998)**. This plant also produces anti hypertensive agents such as ajmalicine and serpentine, which are used to overcome disorders arrhythmias **(Shank et al, 1998)**. These agents improve blood circulation in brain **(Moreno et al, 1995)**. Some of the TIAs are used in the treatment of anxiety (serpentine), arterial hypertension (ajmalicine) **(Kruczynsky and Hill, 2001)**.

The cultivation of plants (for shoot and root) has been practised for rapid biomass production in *Catharanthus roseus* and for *in vitro* biosynthesis of secondary metabolites **(Bietrosiuk et al, 2007)**. The first observations related to the formation of roots from the callus tissue were reported by **Dhruva et al (1977)**.

Ramavatar et al (1978) described the formation of shoots of *Catharanthus roseus* from the callus. Plant regeneration from haploid and diploid callus using different combinations of the plant growth regulators (PGRs) like Kinetin and IAA, was carried out by **Abou-Mandour et al (1979).**

Krueger et al (1982) established leaf organ culture in *Catharanthus roseus* were induced to aspectically on the Murashige and Skoogs revised Tobacco medium (MSRT) supplemented with BAP. The process for conducting plant organ cultures of *Catharanthus roseus* capable of producing significant amounts of indole alkaloids including Vinblastine,Vincrestine , Vindoline, Catharanthine and ajmalicine was patented earlier **(Miura and Hirata, 1986)**. **Endo et al (1987)** induced root and shoot cultures from the seedlings of *Catharanthus roseus.*

Plant regeneration from existing meristem was successfully achieved for quick biomass production in pharmaceutical industries as it helps in obtaining regeneration of a stable invariable genotype **(Pietrosiuk et al, 2007)**. **Furmanowa et al (1994)** successfully regenerated *Catharanthus roseus* of plantlets, when the shoot tips(excised from 7 day old seedlings)were incubated in solid Nitsch and Nitsch (NN) medium **(Nitsch and Nitsch1969)** supplemented with kinetin, BAP, IBA and IAA in various combination.

Although somatic embryogenesis (SE) has been reported in a wide variety of plant genera of angiosperms and gymnosperms **(Thorpe, 1995; Thorpe and Stasoll, 2001; Mujib and Samaj, 2006)**.The report of *in vitro* embryogenesis was rather new in *Catharanthus roseus***(Junaid et al, 2006)**.

High frequency plant regeneration through somatic embryogenesis has been described in *Catharanthus roseus* **(Kim et al, 2004)** from immature zygotic embryo.

There are several factors which control embryogenesis in cultures for example plant growth regulators,carbohydrate, pH, light, amino acids etc.(**Junaid et al,2008**).It has been noted that somatic embryogenesis can also be induced by various stresses in addition to the use of specific hormonal treatments**(Ikeda-Iwai et al, 2003)**. This has

been clearly observed in plants like Carrot, Arabidopsis and several other genera **(Kamada et al, 1993; Touraev et al, 1997; Ikeda Iwai et al, 2003).**

A report made by **Ten Hoopen et al** (2002) described that the temperature has an important influence on growth and ajmalicine production in suspension cultures *Catharanthus roseus*. The optimal temperature for biomass growth and subsequent secondary metabolite production was recorded at 27.5°C.

Bhadra et al (1993) evaluated alkaloids production in selected hairy root cultures of *Catharanthus roseus*because the use of hairy roots has many advantages like their genetic and biochemical stability compare to other cultures **(Khan et al, 2009)**.

Vinblastine and Vincristine are extremely valuable anti neoplastic medicines **(Magdi and Verpoorte, 2002)**. The amounts of these alkaloids in plant are extremely low, So several laboratories worldwide are involved enhance production of these valuable alkaloids through cell and tissue culture **(Ataei-Azimi et al, 2008)**.

The addition of PGR in media affects both culture growth and secondary metabolite production. Cytokinins regulate many aspects of plant growth and differentiation **(Sakai et al, 2001)**. Addition of zeatin to an auxin-free medium resulted in an increase in alkaloid accumulation in *Catharanthus roseus* cell cultures **(Taha et al, 2009)**.

Previous research about the effects of PGRs on Terpenoid Indole Alkaloids in *Catharanthus roseus* was mostly focused on the production of ajmalicins, serpentine, tabersonine, vindoline and catharanthine **(Amini et al, 2009; Mustafa et al, 2009; Peebles et al, 2009)**. There are other reports which also described the effect of PGRs on production of vinblastine**(Pan et al, 2010)**.

Vindoline has been reported in shoot and leaf organ culture of *Catharanthus roseus* **(Constable et al, 1982; Krueger et al, 1982)**.A report of **Hirata et al** (**1987**) confirms that multiple shoot culture induced from *Catharanthus roseus* seedling produced vindoline and catharanthine.

Misawa et al (1988) established an economically feasible process for production of catharanthine by plant cell fermentation or enzymatic coupling. The significant influence of various compounds like vanadyl sulphate, abscisic acid and sodium chloride on catharanthin production have been described by **Smith et al in (1987)** Alkaloids production in *Catharanthus roseus* cell culture and biosynthetic capacity of culture from original explants and regenerated shoots was studied by group of workers**(Gaudet-la, Parisic P, Kurtz Waw and Kuntney JP, 1982)**.

Influence of exogenous hormones on the growth and secondary metabolite production in transformed root cultures were studied **(Giulietti A and Aird E.L.H., 1994)**. In hairy root culture of *Catharanthus roseus*, metabolic rate limitations through precursor feeding **(Morgan and Shanks, 2000)** and effect of elicitor dosage on biosynthesis of indole alkaloids have been reported **(Rijhwani and Shanks, 1998).**

Moreno et al (1996) studied the effect of elicitation on different metabolic pathways involved in secondary metabolism in *Catharanthus roseus*.**Zhao et al (2001 a and b**) tested various fungal elicitors derived from twelve fungi and their effect on improving indole alkaloid production in *Catharanthus roseus* cell suspension culture.

Smith et al (1987 b) reported that the increase in sucrose concentration from 4-10% stimulated alkaloid content in cultured cells of *Catharanthus roseus*.**Dicosmo and Towers (1984)** noted that the addition of 200 mM sorbitol resulted in 63% increase in catharanthine content.

Morgan and shanks (2000) noted that the tissue differentiation plays a significant role for alkaloid yield and in the type of alkaloids produced. For examples, the synthesis of vindoline is restricted to the leaves of the plant,while ajmalicine and serpentine are the major alkaloids found in roots of the plant as well as cell suspension cultures **(Liet al, 2011)**.

Near ultraviolet light (NUV) with the peak at 370 nm stimulated the production of leurosine (one of the major dimeric indole alkaloids) in multiples shoot cultures of *Catharanthus roseus***(Pietrosuik et al. 2007)**. The results suggested that NUV light might specifically stimulate the synthesis of leurosine and Vinblastine from vindoline and cathranthine.

A number of PGRs are used in culture for various *in-vitro* purpose including embryogenesis. In most cases the right balance or the ratio of the compound are the primary basis for optimization of embryogenesis at different stages of embryo development **(Junaid et al , 2008)**.

Hirata et al (**1994**) studied the importance of PGRs in growth and morphological differentiation of tissues in *Catharanthus roseus.* **Yuan and Hu** (**1994**) investigated the influence of different combination of auxins, cytokinin and effect of light intensity on the formation of multiple shoots of *Catharanthus roseus* in *in-vitro* cultures.

Satdive et al. (2003) studied the effect of different concentration of IAA and BAP on the production of ajmalicine in culture flasks using multiple shoots of *Catharanthus roseus.* The roots obtained on NN medium with addition of BAP were white, thin, long and very branched, whereas roots produced on medium with kinetin were yellow, thick and had shorter branches **(Pietrosuk, (1997)**.

An auxin-cytokinin combination was noted to promote regeneration from callus in several studies (**Borgato et al.2007**). The accumulation of PGRS, long callus phases and the use of 2, 4 – D reduced the formation of somatic embryos and caused genetic and epigenetic variations in cultured tissues (**George etal. 2008a).**

In-vitro biomass and alkaloid production in *Catharanthus roseus* are directly influenced by the pH values of the medium. The pH value of 5.5 was found to be optimum for serpentine production **(Dollar etal, 1976)**. It has been reported that alkaloids produced by suspension culture were stored in vacuole and simultaneously storage capacity changed as the changes of pH in the medium and vacuole take place. **(Neumann D. et al 1983)**.

Bioreduction of silver nanoparticles using aqueous leaves extracts of *Catharanthus roseus* was studied by **K. S. Mukunthan et al. (2011)**.

Cell suspension cultures of *Catharanthus roseus* have been commonly used 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, isomerisation, esterification and glycosylation **(Min et al, 2002; Giri et al. 2001)**.

High *in-vitro* production of anti – cancerous indole alkaloids from *Catharanthus roseus* tissue culture was studied by **Azra Ataei-Azimi et al** (**2008**).

S. Rahmatzadeh published a report on regeneration of different explants on different tryptophan free media compositions. In this report, nodal segments have a potential to induce multiple shoots when cultured on MS medium containing BAP(0.5 mg/l)+ NAA (1.0 mg/*l*). However, the rooting media composition was determined as half MS medium supplemented with IBA (0.1 mg/*l*). After addition of tryptophan into this

optimal medium, the maximum shooting and rooting percentage were obtained with 250 mg/l and 350 mg/l tryptophan,**(S. Rahmatzadeh et al. 2014).**

Nural Sariyab studied the effect of phytohormone on micropropagation and shoot induction in *Catharanthus roseus.* Highest percentage of shoot proliferation was observed after 7-14 days of inoculation on MS medium supplemented with 1.0 mg/l BAP + 1.0 mg/l kinetin **(Nurul Sariyal Binti Md. Rajib;2006)**.

On the other side, a report was made by **Farkhanda Manzoor and Farah Aslam** in which in vitro regenereation of *Catharanthus roseus* through shoot tip and nodal portion explants and callus was obtained from leaf, node and fruit explants. Multiple shoots were obtained on all concentration of BAP and NAA, but BAP (1.0 mg/l) showed the best response from both explants. Similarly the best callus response was observed on MS medium supplemented with 2, 4-D (1.0 mg/l) + kin (1.0 mg/l) in all explants **(Farkhanda Manzoor and Farah Aslam, 2013)**.

Nergis kaya and Cuney taki made a report on effect of plant growth regulators on *in-vitro* biomass changing in *Catharanthus roseus*. The leaf explants were cultured on MS medium which supplemented with 5:2, 8:2 10:2 mg/l NAA:BAP under *in vitro* condition. Most effective treatment for biomass production were found as combination of 5:2 mg/l NAA; BAP **(Nergis kaya and Cuney taki, 2013).**

The effect of plant growth regulators on callus induction using different explants to regenerated shoots from different explants as well as from calli in cultures of *Catharanthus roseus* was studied by **Kanta Rani et al (2011).**Based on the results of this study, earliest and maximum callus induction response was observed on 10^{th} day of inoculation from hypocotyl explants under dark conditions on MS medium supplemented with BAP (1.0 mg/l +NAA (1.0 mg/l). For shoot proliferation basal

medium supplemented with BAP (1.5 mg/l) + NAA (1.0 mg/l) was the best or half strength MS medium supplemented with IBA (2.5 mg/l)gave best rooting response. Besides, maximum shoot regeneration response was observed from hypocotyl calli both under light and dark condition on media supplemented with BAP (1.5 mg/l) + NAA (1.0 mg/l) **(Kanta Rani, Pushpa kharb and Renu singh, 2011)**.

Gunjan garg made a report on *in vitro* screening of *Catharanthus roseus* (pink flowered) and (white flowered) were subjected to salinity stresses using different NaCl levels 10 to 100 nm in a shoot proliferating and callus induction on MS medium. The cultivar Rosea was the more tolerant cultivar for callus induction.The leaves of rosea cultivar incubated on MS medium supplemented with 2, 4D (4.06 µm) and Kn (2.3 µm) responded favourable and produce good friable, high biomass callus **(Gunjan Garg, 2010)**.

Akademiai kiado studied *in vitro* micropropagation of *Catharanthus roseus* using axillary bud and shoot tip explants. The highest number of shoots was observed after 45 days of culture in the MS medium supplemented with BAP (4.0 mg/l) + NAA (4.0 mg/l). High frequency of rooting was obtained in half strength MS + IBA (4.0 mg/l) **(Aademiai, Kiado, 2011)**.

F. Matloob and **Co workers** studied the effect of coconut water on micropropagation and shoot induction in *Catharanthus roseus.* Good number of shoot formation was observed on high concentration of Coconut water that is 12% (**F. Matllob** and **Coworkers, 2017).**

Phytochemical analysis and microbial activity of *Catharanthus roseus* was conducted using agar disc diffusion method by **Divya Paikara** and **Co workers.** The qualitative

analysis of phytochemical screening revealed the presence of tannins, alkaloids, flavanoids, terpenoids in *Catharanths roseus* **(Divya Paikara** and **Coworkers, 2017).**

After reviewing the previous work done on micropropagation and alkaloid production of *Catharanthus roseus*, we have realized that large scale propagation of genetically pure and high alkaloid yielding varieties is essential to meet the growing demand of raw materials of *Catharanthus roseus* .

Materials And Methods

In the present study, ***Catharanthus roseus***(an anticancerous drug yielding plant) belonging to family apocynanceae was choosen as experimental plant.*Catharanthus roseus* (L) G.Don is a perennial, everygreen herb that was originally native to the island of Madagascar. It has been widely cultivated of year and growing wild in most warm regions of the world. The leaves are shiny, dark green, oblong-elliptic, acute, rounded apex; flowers are fragrant, white to pinkish purple in terminal or axillary cymose clusters; follicles are hairymany seeded; seeds are oblong, minute and black. It blooms throughout the year and is propagated by seeds or cuttings. The bloom of natural wild plants are pale pink with a purple eye in the centre.

Catharanthus roseus is grown in many gardens as an ornamental plant. It contains more than 130 alkaloids distributed in all parts of the plant in varied proportions. The total alkaloid content in roots amounts to 2-3% or reaches upto 9% in fibrous roots, whereas leaves contain one percent of alkaloid, stem, fruit, seed, pericarp contain 0.48%, 0.40%, 0.18% and 1.14% respectively. The plant produces anti cancer dimeric alkaloids such as vinblastine and vincristine in leaves and accumulates anti-hypersensitive alkaloids such as ajmalicine and serpentine in roots.

Pharmaceutically valuable secondary metabolites have been extracted from the leaves of *Catharanthus roseus* plants. Among these alkaloids, vincristine and vinblastine are used for chemotherapy against many types of cancers. Several other alkaloids, ajmalicine, serpentine and reserpine extracted from this plant are also used for the treatments of blood pressure and urinary disorders. Due to pharmaceutical purpose and anti-neoplastic agents, the demand of *Catharanthus roseus* is increasing and making the plant endangered. So, proper agrotechnique and alternative technologies are required to fulfill the increasing demand.

Biotechnological tools special micropropagation and callus culture are used for large scale plant regeneration with increased bioactive compounds.

Micropropagation of *Catharanthus roseus* has been carried out using various explants viz nodal portion, leaves, root tips. In the present studies, attempt were made to see the effect of different growth regulators used alone or in combination on various explants and callus. *Catharanthus roseus* is also known for its secondary metabolites (alkaloids) which are used as anti cancerous drug. The experiment was also carried out for detection of the bioactive chemicals present in leaf of *Catharanthus roseus*.In order to achieve the goals of the presently research work, following materials and method have been used.

Material

Various parts of *Catharanthus roseus* used as explants for experimental study and were obtained from the garden. Explants used for experiments were leaf, root tip and nodal portion.

Method

For tissue culture experiments following requirements are necessary.

Laboratary

To perform the tissue culture experiments, larger space area is a primary need for preparation of culture media.We need adequate working space to keep all required glasswares, tray with prepared medium. For aseptic inoculation, we require larger space to keep laminar air flow chamber. An incubation chamber should be near to laboratory where racks are arranged to keep the inoculated culture flasks in controlled condition.

Instrument

pH meter **,** autoclave, hot air oven, BOD incubator, Hot plate, Magnetic stirrer, Thermometer, Air conditioner, Room heater are required during culture media preparation, inoculation and incubation.

Glass Wares

The glass wares used for culture experiments are borosil test tube, conical flask and beaker (100 ml, 250 ml, 500 ml and 1000 ml). In addition other used glass wares are graduated measuring cylinder, petridishes, a range of graduated pipettes etc. Before use, glasswares were throughly brushed with alkaline detergent teepol and then washed in running tap water. All glass wares were inverted in a clean tray to drain water and shifted to hot air oven for drying, cotton plugs were made of non absorbent cotton and wrapped with paper or aluminium foil.

Table 1 : (A) Compostion of Murashige and Skoog's medium (1962)

Constituents	Concentration in the medium (mg/l)
Macro inorganic salts	
$(NH_4)\ NO_3$	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
$FeSO_4.4H_2O$	27.8
NO_2 EDTA	37.3
Minor Inorganic Salts	
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
H_3BO_3	6.2
KI	8.3
$Na_2MO_4.2H_2O$	0.25
$CuSO_4.5H_2O$	0.025
$CoCl_2.6H_2O$	0.025
Organic Constituents	
Myoinositol	100
Glycine	2.0
Nicotinic acid	0.5
Pyridoxine HCl	0.5

Thiamine HCl	0.1
Sucrose	30000
Agar-agar	8000

Table 1 : (B) Preparation of stock solution of inorganic and organic compound

(a) Stock solution 1 (Major inorganic 20x)

Chemical compound	Chemical Formula	Amount (mg/l)	Stock (100 ml)
Ammonium nitrate	NH_4NO_3	1650	3.3 gm
Potassium nitrate	KNO_3	1900	3.8 gm
Calcium chloride Dihydride	$CaCl_2.2H_2O$	440	0.88 gm
Magnesium sulphate (Hepta)	$MgSo_4.7H_2O$	370	0.70 gm
Potessium dihydrogen Orthophosphate	KH_2PO_4	170	0.34 gm

(b) Stock solution 2 (Minor inorganic 100x)

Chemical compound	Chemical Formula	Amount (mg/l)	Stock 100 ml
Borric acid	H_3BO_3	6.2	0.062 gm
Potassium iodide	KI	8.3	0.0083 gm
Sodium molybdenum	Na_2MoO_4	0.25	0.0025 gm
Manganese sulphate	$MnSo_4.4H_2O$	22.3	0.223 gm
Zinc sulphate	$ZnSo_4.7H_2O$	8.6	0.086 gm

(c) Stock solution 3 Iron (200x)

Chemical compound	Chemical Formula	Amount (mg/l)	Stock 100 ml
Di-Sodium EDTA	Na_2EDTA	37.3	0.747 gm
Ferrous sulphate	$FeSo_4.7H_2O$	27.8	0.557 gm

(d) Stock solution 4 Vitamin and Organic Compound (100x)

Chemical compound	Amount (mg/l)	Stock 100 ml
Thiamine HCl	0.1	100 ml

Pyridoxine HCl	0.5	0.01 gm
Nicotinic acid	0.5	0.01 gm
Glycine	2.0	0.4 gm
Myo-inositol	100	1.0 gm

(e) Stock solution 5 Micro minor inorganic (100x)

Chemical compound	**Chemical Formula**	**Amount (mg/l)**	**Stock 100 ml**
Cobalt chloride Hexa hydride	$CoCl_2.6H_2O$	0.025	0.0025 gm
Coppler sulphate Penta	$CuSo_4.5H_2O$	0.025	0.0025 gm

Sucrose	3 gm/100ml
Agar-agar	0.8 gm/100ml

Preparation of stock solution of Growth regulators

Stock solution of Auxin : Auxinis taken in a dry test tube and then added few drops of absolute alcohol and shaken well to dissolve it completely. After that double distilled water is added to make final volume.

Stock Solution of Cytokinin : Cytokinin (BAP orkinetin) is taken in a dry test tube and added with few drops of 1 N NaOH and shaken well to dissolve it. Then distill water is added to make final volume. Store them in a refrigerator at 4°–5°C.

Culture Media Preparation

Murashige and Skoog's (1962) medium was used as basal medium throughout the tissue culture experiment **(Table 1-A).** Stock solution were stored at 4°C in refrigerator and were used in desired proportions. Stored stock solutions were used upto 15 days of preparation.

Steps involved in the preparation of 1 litre of MS medium, are as follows :

(a) Take 500 ml double distilled water in a measuring cylinder of 1 litre capacity.

(b) Add stock solution 1, 2, 3, 4 and 5 **(Table 1B - a, b, c, d)** of quantity 50 ml, 20 ml, 10 ml and 10 ml respectively to the above distilled water in a cylinder.

(c) Add sucrose (30 gms) and Myo inositol (100 mg) to the above solution.

(d) Make the final volume to 1 litre by adding double distilled water and mix properly.

(e) Adust the pH to 5.8 using pH meter.

(f) Heat the medium and add 8 gm of agar in 1 litre, shake it vigorously to make homogenous medium.

(g) Definite aliquots of one litre medium were distributed in the culture vessel. Generally 20-25 ml in test tube, 40 ml in 100 ml flask and 100 ml in 250 ml flask was distributed. Test tubes and flasks were plugged with sterile cotton plugs (made of non absorbent cotton) and covered with paper and auto claved at 121°C (15 p.s.i) for 15 to 20 minutes. Test tubes were placed on racks in tilted position to make slants to have large surface area for the explants.

Surface sterilization of explants :

Like media, plant tissues are also disinfected before using for culture experiments. Explants like leaf segments, nodal portion and root tip were taken from plants growing in **in vivo** conditions or natural habitat. These were placed in different bottles and covered with net and washed for 30 minutes under running tap water to remove all the adhering dust particles and microbes from the surface. The explants were then washed with liquid detergent (teepol) for another 15 minutes and then thoroughly washed with water to remove detergent.

After that explants were treated with anti biotic and bavistin solution for another 20-30 minutes separately to remove bacterial and fungus infection. After proper washing with distilled water to remove anti biotic and fungicide, explants were transferred into tween-20 solution (2-3 drops in 100 ml distilled water) under the sterile conditions (in LAF chamber). The explants were treated with 0.1% $HgCl_2$ solution for 1-2 minutes depending upon the explants. The explants were then thoroughly washed (4-5 washings) with sterilized distilled water to remove traces of $HgCl_2$.

Inoculation of explants

All the experimental manipulations were carried out in aseptic conditions in laminar air flow fitted with a bactericidal ultra violet tube (15W, peak emission 2537A°). The bench or working area of laminar air flow was thoroughly cleaned with cotton dipped in alcohol. The surfaces of all the vessels and other accessories such as instruments (spatula, forceps, needles and scalpel etc.), spirit lamp were also cleaned with alcohol. Alcohol was then sprayed in the chamber with the help of an atomizer. The chamber was then sterilized with ultraviolet tubelight which is switched on before an hour of inoculation.

All culture experiments were performed after 45 minutes of switching off the ultra violet tubelight.

Culture conditions :

All the cultures were incubated in an air-conditioned room at temperature of 25 ± 2°C and relative humidity 70-80%. The source of illumination was fluorescent tubes (40W) and incandescent bulb (25 W). The intensity of illumination was maintained 3500 lux. An illumination of 16 hour light and 8 hours dark was provided to the cultures.

Calculation

$$\textbf{Standard error} = \frac{\text{Standared deviation}}{\sqrt{n}}$$

n = total number of explants cultured

Explant Culture

Surface sterilized explants were subjected to growth hormone used singly and in combination. Observations were recorded weekly.

Callus Culture and Morphogenesis:

Callus induced from different explants was subjected to various growth regulators to see the effect on callus growth and morphogenesis.

Induction of roots in *in-vitro* regenerated shoots:- For root induction, *in-vitro* raised shoots were separated aseptically and subjected to various root induction medium. Observations were recorded every week.

Hardening of *in-vitro* developed plantlets :

In-vitro raised plantlets with strong root system are transferred in small plastic containers having mixture of sand, soil and peat moss, These plastic containers are covered with another piece of plastic container or plastic bags. So that humidity can be maintained. For air circulation, small hole is made. To acclimatize the plant to natural, a small opening is made in inverted plastic cover and gradually widened this opening with time. At regular intervals, pots are irrigated with inorganic nutrient solution.

Plantlets are handled with full care, till 6 weeks. After 6 weeks, plantlets are transferred in earthen pot and gradually brought to natural conditions.

Synthetic seed/ Artificial seed formation :

Encapsulation of somatic embryos, embryogenic calli, apical meristem, micropropagules, protocorms by gelling agent is called synthetic seed formation and it is preferred for germplasm conservation, long term storage, easy handling and transportation across the border.

Major steps to be followed during the present experiment of synthetic seed formation :

1. **Induction and selection of plant tissue to be encapsulated**
   (a) Establishment of callus culture – sterilized explants were cultured on MS medium supplemented with 2, 4-D and combination of 2, 4-D with different growth regulators.
   (b) Induction of embryogenic callus and somatic embryogenesis.
   (c) Confirmation of somatic embryo formation and selection of somatic embryo of suitable stage for encapsulation.

2. **Preparation of gelling solutions for encapsulation**
   (a) **Sodium alginate solution** : Sodium alginate solution of 2.50 gm sodium alginate powder and separately added in 100 ml distilled water to prepare sodium alginate solution of different percentage. Prepared solutions were sterilized for 20 minutes.
   (b) **Calcium chloride solution (1.5%)** – 1.5 gm calcium chloride powder is dissolved into 100 ml distilled water to get 1.5% calcium chloride solution.

3. **Encapsulation of somatic embryos**

(a) **In laminar air flow bench**, somatic embryos/embryogenic calli are teased and separated and picked up with sterilized forceps and dropped into sodium alginate solution. After 4-5 minute, these somatic embryos are transferred into $CaCl_2$ solution and there they are stirred for 10-15 minutes. As a result of the ion exchange between Na^+ and Ca^{++} ions, calcium alginate beads are formed.

(b) **Storage of artificial seeds** : Encapsulated beads are transferred to autoclaved glass vessel for storage at 4°C in refrigerator.

Qualitative test for detection the presence of various bioactive chemicals present in plant material.

Step I :- Preparation of leaf powder : leaves are collected, washed and shade dried then they are ground with mortar and pestle for powder formation. Fine powder is collected and used further for analysis.

Step II :- Preparation of extract : Extracts of leaf powder is prepared in 95% ethanol

StepIII :- Following tests are performed for qualitative analysis of bioactive chemicals.

Test for Carbohydrates

Small amount of extracts are dissolved in little quantity of distilled water and filtered separately. The filtrates were used to test presence of carbohydrates.

Molisch's Test : The extract is treated with Molisch reagent and concentrated sulphuric acid is added from the sides of the test tube to form a layer.

Benedict's Test : To the filtrate added 2 ml benedict's reagent and boiled in water bath.

Test for Starch :

Dissolve 0.015 gm of iodine and 0.075 gm of potassium iodide in 5 ml of distilled water and 2-3 ml of extract is added to perform test.

Test for protein :

Ninhydrin Test: We took the 3 ml of extract and added 3 drops of ninhydrin solution. This tubes shifted in boiling water bath for 10 minutes.

Test for Alkaloids :

The extract was first basified with ammonia and extracted with chloroform. The chloroform extract was acidified with dilute hydrochloric acid. The formed acid layer was used for testing the alkaloids.

Wagner test: The acid layer was treated with few drops of Wagner's reagent. Formation of reddish brown precipitate indicates the presence of alkaloids.

Mayer's test: The acid layer was treated with few drops of mayer's reagent.

Test for Saponin:

Foam test: Small amount of extract is shaken with little quantity of water to perform this test.

Test for flavnoids:-

Alkaline test:- In ethanolic extract of leaves powder of *Catharanthus roseus,* few drops of sodium hydroxide and desolve HCl was added.

Test for Anthraquinone:-

Borntrager Test:- Dilute H_2So_4 was added in ethanolic extract of leaves powder of *Catharanthus roseus* or boiled. Then it was filtered and cooled. To the cold filtrate, 3ml of Benzene was added and mixed. The Benzene layer was seprated and ammonia (2ml) was added.

Observations

And

Results

For the present study**,** different explants (leaf segment, nodal portionand root tip) of experimental plant *Catharanthus roseus* were taken from in vivo grown 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 A,B,C,D,E and F).**

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 eg.leaf segment, nodal portion and root 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.

Effect of BAP: In this set of experiment, from **leaf segment** explants shoot bud formed in BAP supplemented medium but there was no callusing observed even after 6 weeks. Good number of shoots formed on medium containing 3.0 mg/l BAP cultures. But these shoots did not grow further **(Plate-2, Figure A, Table 2).**

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 concentration, 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 (leaf 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 **leaf explants** with shoot bud or organized structure **(Plate 2, Fig B, Table 2).** From **nodal explants**, shoot emergence without callusing was noticed on all tried concentration of kinetin (1.0 mg/l – 3.0 mg/l).

Effect of NAA: On MS medium supplemented with 0.5 mg/l – 3.0 mg/l NAA, only scanty callus induction was noticed from **leaf explant** with no shoot buds. Formed callus was whitish and slow growing **(Plate 2, Fig C, Table 2)**

In case of **nodal explants**, few shoots could be noticed on all concentration 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.

Effect of IBA: On all tried concentration of IBA that is 0.5 mg/l-3.0 mg/l, neither callusing nor shoot bud formation was noticed from **leaf explants** and **nodal explants**.

Effect of IAA: All tried concentration of IAA could not initiate callusing and shoot formation even after 2 months of inoculation from leaf explants. Very few shoots were noticed from nodal explants without callus formation **(Table-2).**

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 was 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.

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 favourable for callusing but found conducive for shoot bud formation from nodal explants.

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

Table 2: Effect of growth hormones on different explants of *Catharanthus roseus* L.

Hormone concentration (mg/l)	Explants response after 21 days		
	Leaf segment	Nodal portion	Root tip
0.5 BAP	C^{-}, S^{+}	C^{-}, S^{++}	NR
1.0 BAP	C^{-}, S^{+}	C^{-}, S^{+++}	NR
3.0 BAP	C^{-}, S^{++}	C^{-}, S^{+++}	NR
0.5 Kinetin	C^{+}, S^{+}	C^{-}, S^{+}	NR
1.0 Kinetin	C^{++}, S^{+}	C^{-}, S^{++}	NR
3.0 Kinetin	C^{++}, S^{+}	C^{-}, S^{+}	NR
0.5 NAA	C^{+}, S^{-}	C^{-}, S^{+}	NR
1.0 NAA	C^{+}, S^{-}	C^{-}, S^{+}	NR
3.0 NAA	C^{+}, S^{-}	C^{-}, S^{+}	NR
0.5 IBA	C^{-}, S^{-}	C^{-}, S^{-}	NR
1.0 IBA	C^{-}, S^{-}	C^{-}, S^{-}	NR
3.0 IBA	C^{-}, S^{-}	C^{-}, S^{-}	NR
0.5 IAA	C^{-}, S^{-}	C^{-}, S^{+}	NR
1.0 IAA	C^{-}, S^{-}	C^{-}, S^{+}	NR
3.0 IAA	C^{-}, S^{-}	C^{-}, S^{+}	NR
0.5 2, 4-D	C^{++}, S^{-}	C^{-}, S^{+}	NR
1.0 2, 4-D	C^{+++}, S^{-}	C^{-}, S^{+}	NR
3.0 2, 4-D	C^{+++}, S^{-}	C^{-}, S^{+}	NR

C^{-} = callus absent, C^{+} = Poor callus, C^{++} = Callus formation in moderate amount, C^{+++} = Very good callus, S^{-} = Shoot induction absent, S^{+} = Shoot induction in low number, S^{++} = Shoot induction in moderate number, S^{+++} = Profuse shoot formation

NR= not response

PLATE:-1

Various steps of surface sterilization of explants of *Catharanthus roseus L.*

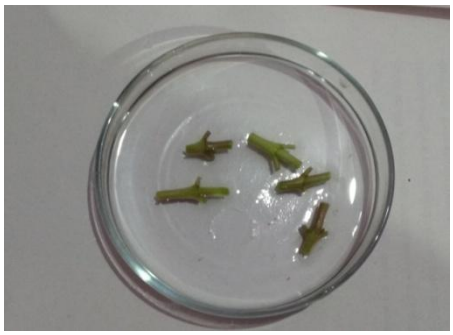

Fig. A. Explants in tap water solution

Fig B. Explants in antibacterial

Fig C. Explants in antifungal solution of Bavestin solution

Fig D. Explants in Twin – 20

Fig E. Explants treatment in $HgCl_2$ and Ethanol

Fig F. Explants transferred into culture medium

PLATE:-2

Effect of growth hormones on leaf explants of Catharatnthus roseus L.

Fig A. Shoot formation on MS + BAP (3.0 mg/l)

Fig B. Callus and shoot induction on MS + kinetin (1.0 mg/l)

Fig C. Scanty callus induction on MS +NAA (1.0mg/l)

Fig D. Callus induction on MS + 2, 4-D (3.0mg/l)

Effect of different combination of 2, 4-D wth Kinetiin and BAP on Callus proliferation raised from leaf explants of *Catharanthus roseus L*.

Callus was raised from leaf explants of *Catharanthus roseus* on MS medium supplemented with 2, 4-D (3.0 mg/l). This concentration of 2, 4-D proved favourable for callus induction. Callusing started after 5-6 weeks of inoculation.

Callus induced from leaf explants was further subcultured on MS medium supplemented with different concentratons of 2, 4-D in combination with kinetin and BAP. Observations were recorded every week.

When 2, 4-D was applied combination with Kinetin, Callus induction was induced at all conceentration **(Table: 3, Plate: 3 FigA, B and C).** 70%-80% Culture response was recorded on combination of 1.0 mg/l. 2, 4-D + 1.0 mg/l kinetin. On these combinations, profuse shoot formation was observed after 1 month of inocubation. **(Plate: 3, Fig B).**

Initially, callus turned light green with dark green patches and small shoot bud initials also appeared on upper surface of callus. When the 2, 4-D concentration was low in the combination with kinetin that is 0.5 mg/l, no organized structures were formed and callus growth was also slow **(Plate: 3 A).**

BAP in combination with 2, 4-D also promoted callus proliferation in all concentration, but on this combination, little callus proliferation was obsreved than combination of 2, 4-D with kinetin. In this Combination, few number of shoot initial was observed. Percentage of culture responded was low. Callus was slow growing **(Table: 3, Plate: 4, fig (A and B).**

On comparing observations, we concluded that 2, 4-D in combination with Kinetin showed more stimulatory effect on callus proliferation than combination of 2, 4-D

with BAP. Medium supplemented with 2, 4-D (1.0 mg/l) with Kinetin(1.0 mg/l) was proved favourable for callus proliferation. In this combination, callus was healthy, light and dark green with profuse shoot formation.

Table: 3 Effect of combination of 2-4-D with Kinetin and BAP on Callus proliferation raised from leaf explants of *Catharanthus roseus* L.

Growth Hormone (mg/l) 2, 4-D + Kn	**Callus response (%)**	**Description of Callus after 4 Weeks**		
		Proliferation Scoring	**Color**	**Morphological Changes**
0.5 + 0.5	35	C^{+}, E^{-}	Brown	Dried
0.5 + 1.0	40	C^{+}, E^{-}	Whitish	Small growth, no organised structure
1.0 + 0.5	75	C^{+++}, E^{++}	Light and darkGreen	Profuse Shoot bud Initials
1.0 + 1.0	80	C^{++++}, E^{++++}	Light and darkGreen	Profuse Shoot formation Occured.
1.5 + 0.5	70	C^{+++}, E^{++}	Light and dark Green	Few number of Shoot bud Initial was observed.
1.5 + 1.0	65	C^{++}, E^{++}	Light and dark green	Shoot initiation
2, 4-D + BAP				
0.5 + 0.5	30	C^{+}, E^{-}	Brown	Dried
0.5 + 1.0	40	C^{+}, E^{-}	Light Green	Only callus, noorganogenesis
1.0 + 0.5	60	C^{++}, E^{-}	White withGreen Patch	Only callus, noorganogenesis
1.0 + 1.0	65	C^{++}, E^{++}	Light and darkGreen	Few number of Shoot bud Initial
1.5 + 0.5	55	C^{+}, E^{+}	off white	Shoot initiation
1.5 + 1.0	50	C^{+}, E^{+}	off white	Shoot initiation

PLATE:-3

Effect of 2, 4-D in combination with kinetin on callus proliferation raised from leaf explants of *Catharanthus roseus L.*

Fig A. Callus proliferation on MS medium MS supplemented with 2, 4-D (0.5 mg/l) + kinetin (1.0 mg/l)

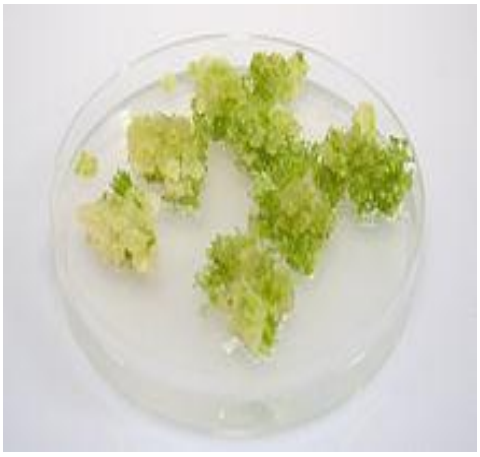

Fig B.Callus proliferation on supplemented with 2, 4-D (1.0mg/l) + kinetin (1.0mg/l)

Fig C. Callus proliferation on MS medium supplemented with 2, 4-D (1.5mg/l) + kinetin (1.0mg/l)

PLATE:-4

Effect of 2, 4-D combination with BAP on callus proliferation raised from leaf explants of *Catharanthus roseus L.*

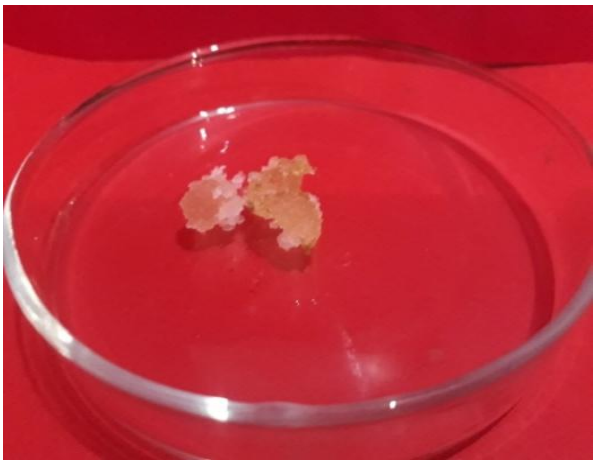

Fig A. Callus proliferation on MS medium supplemented with 2,4-D(0.5 mg/l) +BAP(1.0mg/l)

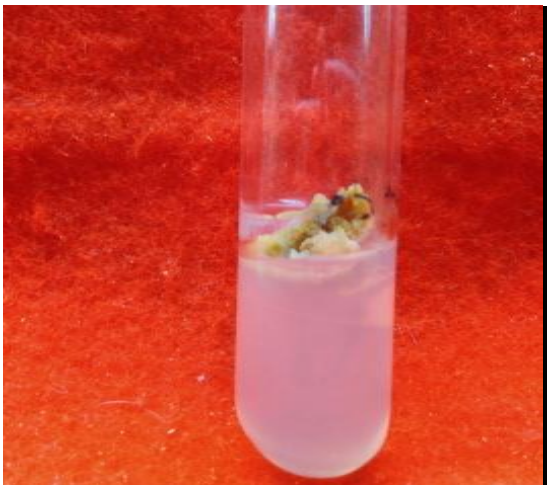

Fig B. Callus proliferatin on MS medium supplemented with 2, 4-D (1.5mg/l) + BAP (1.0mg/l)

Effect of different natural additives on callus proliferation raised from leaf explants of *Catharanthus roseus* L.

This experiment was performed to see the effect of different natural addtives (Banana Homogenate and Coconut Water) on callus proliferation raised from leaf explants of *Catharanthus roseus*.

Callus induced from leaf segment explants of *Catharanthus roseus* on MS medium supplemented with 2, 4-D (3.0mg/l) was further subcultured on MS medium supplemented with various natural additives. Observations were recorded every week.

In our studies, we set an experiment to see the effect of banana homogenate on callus proliferation. Here, homogenate means banana macerated in grinder. MS medium supplemented with 3.0mg/l 2, 4-D without banana homogenate showed better Callus proliferation. When we added 10% Banana homogenate to MS medium with 2, 4-D (3.0 mg/l), 40% cultures showed callus proliferation but increasing concentration of banana homogenate inhibited callus proliferation.Further, so we concluded that adding banana homogenate is not much favourable for callus proliferation **(Table: 4, Plate: 5, Fig A, B).**

Coconut water used at 10% strength with 2, 4-D (3.0 mg/l) was found more conducive for callus proliferation. We noticed that increase in strength of coconut water to 20% triggered shoot formation also. Callus formed in these cultures was showed both shades of green light and dark **(Table: 4, Plate: 5, Fig C).**

Further increasing the concentration of coconut water to 30% was found to be inhibitory for shoot generation. But a peculiar observation was noticed that the callus was more compact and dark green at 30% of coconut water with 2, 4-D in few cultures but did not show shoot regeneration (**Table: 4, Plate: 5, Fig D**).

Overall, our results showed that MS medium enriched with coconut water (10% and 20%) was the best for callus proliferation and growth in comparison to banana homogenate.

Table: 4 Effect of different natural additives on callus proliferation raised from leaf explants of *Catharanthus roseus* L.

Natural Additives + MS	**Callus response (%)**	**Description of Callus after 4 Week**		
		Proliferation Scoring	**Color**	**Morphological Changes**
Control (MS)	50%	C^{+}, E^{-}	Light Green	Few Shoot bud initials
Banana Homogenate				
5%	20%	C^{+}, E^{-}	Brown	Dried
10%	40%	C^{+}, E^{+}	Off White	Few Shootbud initials
20%	25%	C^{+}, E^{-}	Brown	Dried
30%	20%	C^{+}, E^{-}	Brown	Dried
Coconut Water				
5%	50%	C^{++}, E^{+}	Light Green	Few Shoot Bud Initials
10%	70%	C^{+++}, E^{++}	Light anddark green	Profuse Shootformation occurred
20%	60%	C^{++}, E^{++}	Light anddark green	Profuse Shoot formation occurred
30%	55%	C^{++}, E^{+}	Light green	Profuse Shootbud initials

PLATE:-5

Effect of different natural additives (Banana Homogenate and Coconut water) on callus proliferation raised from leaf explants of *Catharanthus roseus L.*

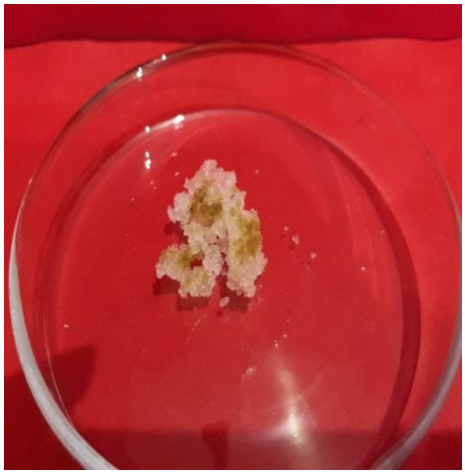

Fig A. Callus proliferation on MS medium MS+ 2,4-D with 10% Banana Homogenate

Fig B. Callus proliferation on MS medium +2, 4-D with 30% Banana Homogenate

Fig C. Callus proliferation on MS medium +2, 4-D with 10% Coconut Water

Fig D. Callus Proliferation on MS medium +2, 4-D with 30% Coconut Water

Nodal explants –

Surface sterilized nodal explants of experimental plant *Catharanthus roseus* L. were inoculated on MS medium supplemented with cytokinins and auxins added singly and in combination. Observations for morphogenic response were recorded every week.

Morphogenic Response of nodal explants of *Catharanthus roseus* L. on cytokinins (BAP and Kinetin) added singly–

Effect of BAP –

Nodal explants were inoculated on MS medium supplemented with different concentrations of BAP which were 0.5 mg/ℓ, 1.0 mg/ℓ, 2.0 mg/ℓ, 3.0 mg/ℓ, 4.0 mg/ℓ and 5.0 mg/l. After 10 days of inoculation, green, healthy and delicate shoots started emerging from nodal explants. After 3 weeks, among five different concentration of BAP tried the good number of shoots were observed on medium supplemented with 1.0 mg/ℓ BAP and percentage culture response was 80-85% but shoots ofgood length were observed on medium containing 1.0 mg/ℓ BAP . As shown from **Table: 5, Graph-1,** when BAP concentration was increased from 1.0 mg/ℓ to 5.0 mg/ℓ, number of shoots were decreasedin mostly cultures **(Table: 5, Plate: 6, Fig A, B and C**).

Effect of Kinetin

Nodal explants were inoculated on MS medium supplemented with kinetin (0.5 mg/ℓ, 1.0 mg/ℓ 2.0 mg/ℓ, 3.0 mg/ℓ 4.0 mg/ℓ and 5.0 mg/ℓ) added singly. After 10 days of inoculation, we observed that dark green, healthy, small and delicate shoots started emerging and number of shoots was less seen than observed in BAP supplemented cultures. A good length of shoots (approximate 3.0cm) and highest culture response (70 – 75%) was observed on 3.0 mg/ℓ kinetin supplemented cultures. It was noticed that when kinetin concentration was increased to 4.0 mg/ℓ, it resulted in reducing in the number of shoots**(Table: 5, Plate: 6, Fig D, E and F).**Further addition of kinetin that is 5.0 mg/ℓ caused the reduction in number of shoots **(Table-5) (Graph-1).**

When nodal portions of *Catharanthus roseus* were inoculated on MS medium supplement with different concentration of BAP and kinetin, after 3 weeks we observed that shoot formation was observed in all concentration of both cytokinin but number of shoots and shoot length was higher in BAP supplemented cultures, which indicates that BAP was favourable for shoot proliferation and elongation.

From this experiment, we noticed and concluded that kinetin do not have more conducive effect on callusing and shoot initiation in nodal explants.

Table: 5 -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)	Hormone concentration (mg/ℓ)	Culture Response (%)	No. of Shoots (mean ± SE)	Shoot length (in cm) (mean ±SE)
BAP	Kinetin			
0.5	-	60-65	3.15 ± 0.28	1.21 ± 0.24
1.0	-	80-85	7.12 ± 0.45	1.80 ± 0.28
2.0	-	70-75	3.40 ± 0.81	2.54 ± 0.65
3.0	-	55-60	2.40 ± 0.24	1.36 ± 0.74
4.0	-	50-55	2.30 ± 0.31	1.19 ± 0.21
5.0	-	30-35	1.73 ± 0.87	1.10 ± 0.15
-	0.5	55-60	2.57 ± 0.21	1.11 ± 0.71
-	1.0	60-65	4.67 ± 1.22	1.70 ± 0.56
-	2.0	60-65	4.80 ± 0.24	1.52 ± 0.94
-	3.0	70-75	5.38 ± 0.39	1.76 ± 0.29
-	4.0	50-55	2.50 ± 0.20	1.68 ± 0.11
-	5.0	40-45	2.31 ± 0.30	1.27 ± 0.85

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.

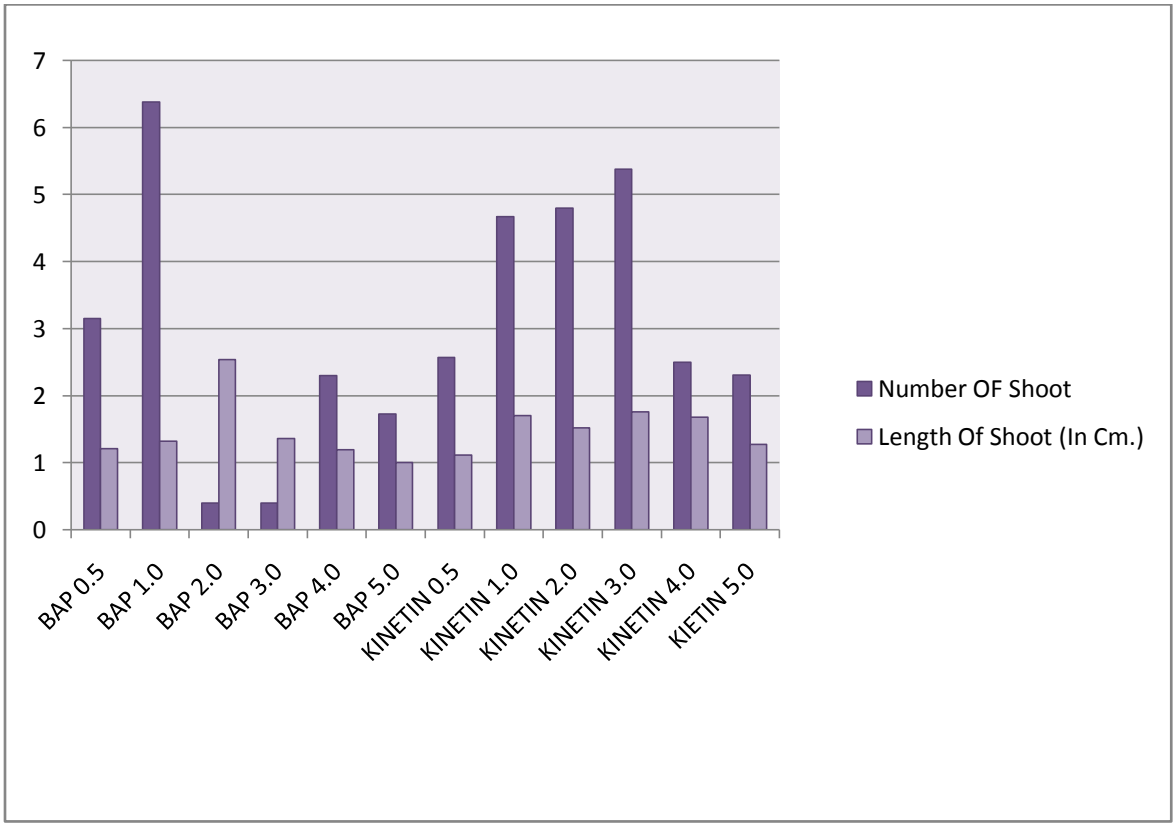

PLATE:-6

Morphogenic response of nodal explants of *Catharanthus roseus L.* on MS medium supplemented with various concentrations of cytokinins (BAP and kinetin) added singly

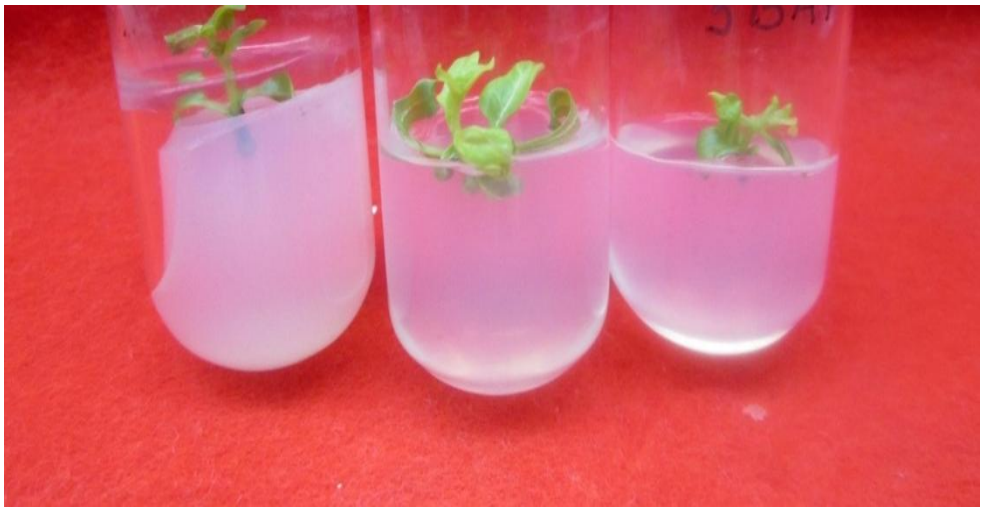

Fig. (A-C). Showing morphogenic response of varius concentration of BAP (1.0mg/l, 3.0mg/l and 5.0mg/l)

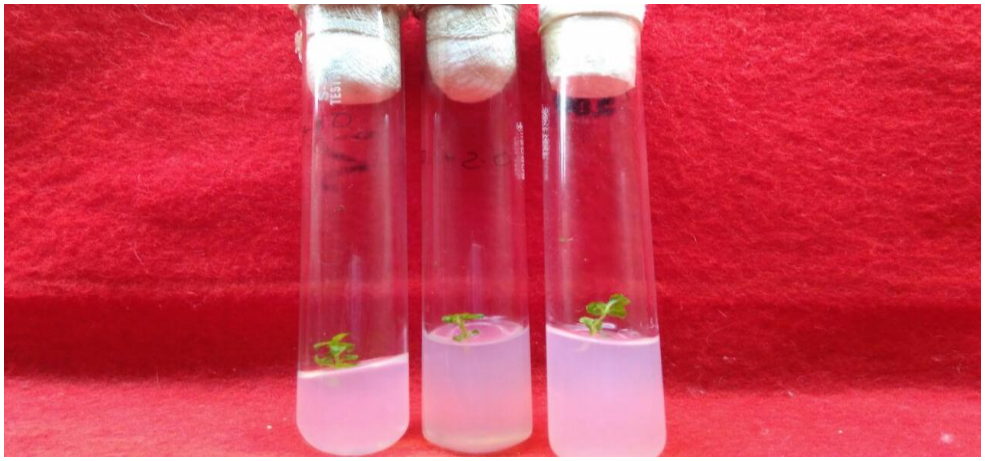

Fig. (D-F). Showing morphogenic response of varius concentration of kinetin (1.0mg/l, 3.0mg/l and 5.0 mg/l)

Morphogenic response of nodal explants of *Catharanthus roseus* on MS medium supplemented with various concentrations of auxins (NAA, 2,4–D, IBA and IAA) added singly.

Effect of various concentrations of different auxins added singly on nodal explants of *Catharanthus roseus* was observed in this set of experiment. Observations were recorded every week.

Effect of NAA

Nodal explants of *Catharanthus roseus* were inoculated on MS medium supplemented with different concentrations of NAA (0.5 mg/ℓ – 4.0 mg/ℓ). On 0.5 mg/ℓ of NAA supplemented cultures, culture response was poor (30-35%). In very few cultures, shoot initiation was observed after 25 days of incubation but shoots did not grow and turned brown. A good number of shoots was observed on medium supplemented with 4.0 mg/ℓNAA. This response was observed in 70% cultures. These shoots were green healthy and average length of shoots was approximate 3 cm but after 4 weeks, these shoots turned yellow and did not growfurther.

With this experiment, we can say that NAA has conducive effect on shoot formation from nodal explants of *Catharanthus roseus (***Table-6, Plate-7 fig A, Graph-02).**

Effect of 2, 4–D

Nodal explants were inoculated on 2, 4–D supplemented MS medium. On MS medium supplemented with 3.0 mg/l 2,4-D, 50-55% cultures response was recorded with few numbers of shoots but shoot did not multiply further and dried later **(Table-6, Plate-7 Fig B, Graph-2).** On MS medium supplemented 4.0 mg/l 2, 4-D nodal portion showed slight swelling after 2 weeks of inoculation in some cultures. After 4 weeks, this swelling turned into callus which seemed healthy and green at few sites

Effect of IBA

Various concentration (0.5 mg/ℓ – 4.0 mg/ℓ) of IBA were failed to induce any change in all tried cultures of nodal explants of *Catharanthus roseus*. There was no response even after 4 – 5 weeks of incubation and nodal explants turned brown in 100% cultures.

Effect of IAA

Nodal portion of *Catharanthus roseus* were inoculated on MS medium containing various concentrations of IAA (0.5 mg/ℓ– 4.0 mg/ℓ). On very low concentration of IAA that is 0.5 mg/ℓ and high concentration of IAA that is 4.0 mg/ℓ, we observed poor culture response. Better response of cultures was recorded on 2.0 mg/ℓ of IAA containing medium, where shoots were also emerged but shoots did not grow for longer period **(Table-6, Plate-7 fig C, Graph-02).**

On comparing the effect of different auxins on nodal portion of *Catharanthus roseus* with the above experiment, we analyzed that NAA supplemented cultures showed best response in terms of shoot initiation and elongation in comparison of otherauxin (2, 4D, IBA and IAA) containing cultures **(Table-6).**In this experiment2,4-D supplemented cultures showed conducive effect on callus formation.

Table: 6 - Morphogenic response of nodal explants of *Catharanthus roseus* on MS medium supplemented with various concentrations of auxins (NAA, 2, 4 – D, IBA and IAA) added singly.

Hormone cosncentrations (mg/ℓ)	Hormone concentrations (mg/ℓ)	Culture Response (%)	No. of Shoots (mean ± SE)	Shoot length (in cm) (mean ±SE)
NAA	2, 4 –D			
0.5	-	30-35	0.85 ± 0.10	0.50 ± 0.21
1.0	-	50-55	2.23 ± 0.12	0.77 ± 0.39
2.0	-	55-60	2.40 ± 0.34	1.51 ± 0.28
3.0	-	60-65	3.65 ± 0.34	1.81 ± 0.30
4.0	-	65-70	3.80 ± 0.24	2.11 ± 0.94
-	0.5	15-20	Only initiation	-
-	1.0	35-40	1.63 ± 0.74	0.12 ± .026
-	2.0	40-45	1.73 ± 0.92	0.52 ± 0.21
-	3.0	50-55	2.50 ± 0.75	1.01 ± 0.81
-	4.0	20-25	1.77 ± 0.23	0.16 ± 0.28
IBA	IAA			
0.5		-	-	-
1.0		-	-	-
2.0		-	-	-
3.0		-	-	-
4.0		-	-	-
	0.5	15-20	0.50 ± 0.28	0.28 ± 0.31
	1.0	45-50	1.62 ± 0.40	0.62 ± 0.28
	2.0	60-65	2.28 ± 0.71	1.53 ± 0.26
	3.0	50-55	0.53 ± 0.33	0.86 ± 0.26
	4.0	35-40	0.28 ± 0.18	0.15 ± 0.18

Graph: 2 Morphogenic response of nodal explants of Catharanthus roseus L. on MS medium supplemented with various concentrations of auxins (NAA, 2, 4 – D, IBA and IAA) added singly.

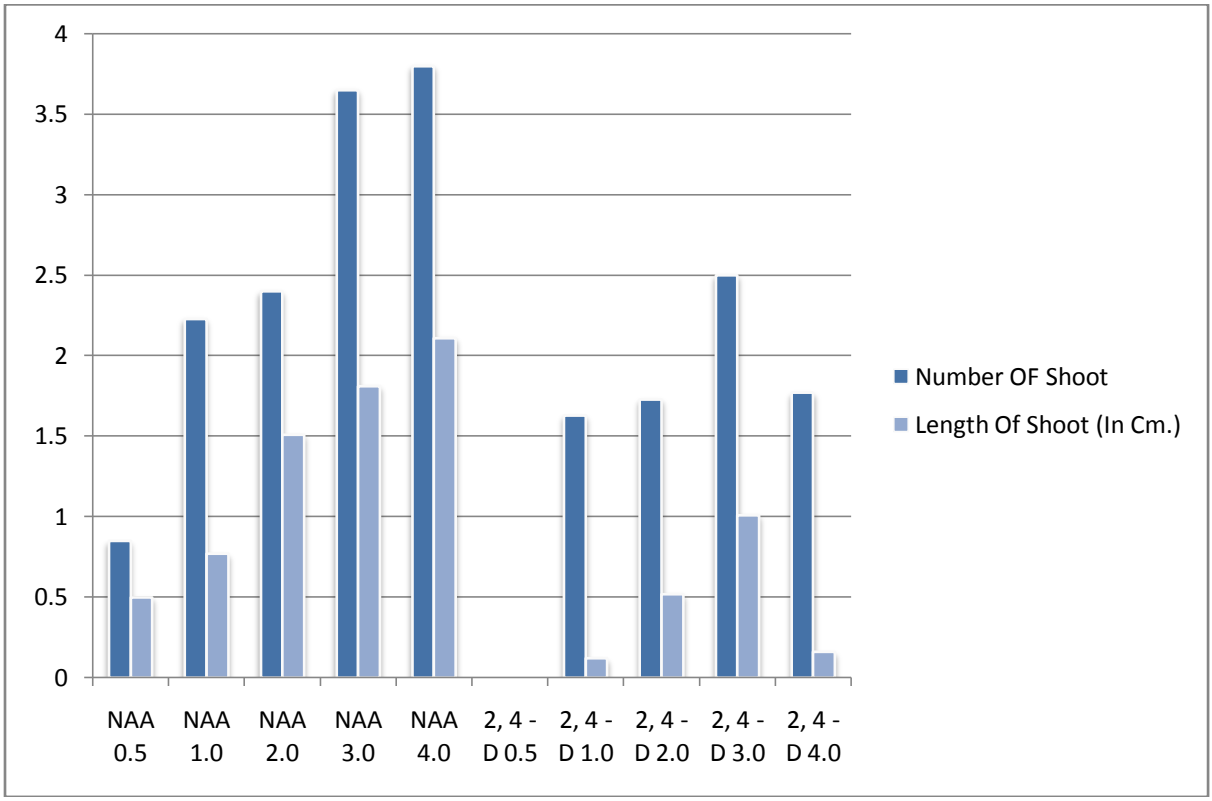

PLATE:-7

Morphogenic response of nodal explants of *Catharanthus roseus* on MS medium supplemented with various concentration of auxins (NAA, 2, 4-D, IBA and IAA) added singly

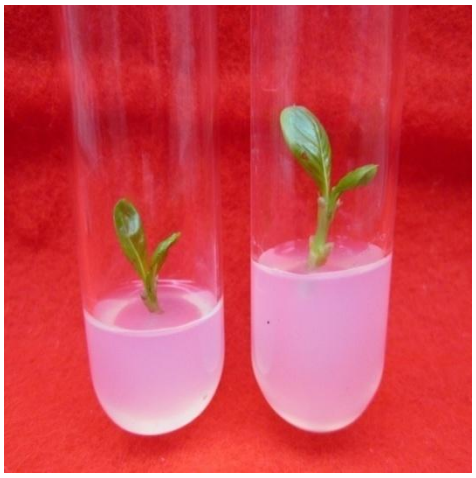

Fig A. Showing morphogenic response on MS + NAA (4.0mg/l)

Fig. B Showing morphogenic response on MS + 2, 4-D (3.0mg/l)

Fig C. Showing morphogenic response on MS + IAA (2.0mg/l)

Morphogenic response of nodal explants of *Catharanthus roseus* L. on combination of cytokinins (BAP + Kinetin), incorporated into MS medium

To see the interactive effect of cytokinins (BAP + kinetin), nodal explants were cultured on MS medium supplemented with various concentration of BAP (0.5 mg/ℓ – 1.5 mg/ℓ) in combination of various concentrations of kinetin (0.5 mg/ℓ – 3.0 mg/ℓ).

In **first set** of experiment, concentration of BAP was kept constant that is 0.5 mg/ℓand kinetin strength was increased from 0.5 mg/ℓ to 3.0 mg/ℓ; shoots emergence was observed after a week in 70-75% cultures. After 3 weeks, we observed that low concentration of BAP (0.5 mg/ℓ) with low concentration of kinetin (0.5 mg/ℓ) evoked better response in terms of shoot numbers and shoot length. Medium supplemented with 0.5 mg/ℓ BAP in combination of 2.0 mg/ℓkinetin was found favourable for producing good number of shoots. Recorded average length of shoots was approximately 4cm. Culture response (80-85%) was observed. But further addition of higher strength of kinetin which was 3.0 mg/ℓ caused the reduction in number of shoots and shoot length **(Table-7, Plate-8, Fig A, Graph-3).**

In **second set** of experiment, concentration of BAP was kept constant that is 1.0 mg/ℓand kinetin concentration was increased from 0.5 mg/ℓ to 3.0 mg/ℓ. Maximum numbers of cultures showed shoot initiation in combination of 1.0 mg/ℓ BAP with 1.0 mg/ℓkinetin 70% cultures showed shoot initiation. Number of shoots observed on this combination was also good. However, further addition of kinetin concentration 2.0 mg/ℓto 3.0 mg/ℓhad inhibitory effect on shoot initiation **(Table-7, Plate-8, Fig B, Graph-3).**

In **third set** of experiment, concentration of BAP was kept constant that is 1.5 mg/ℓand kinetin concentration was increased gradually from 0.5 mg/l-3.0 mg/l. On

combination of 1.5 mg/ℓBAP with 0.5 mg/ℓ kinetin, 65% cultures showed shoot formation and length of shoots approximately 2cm. However, further addition of kinetin concentration that is 2.0 mg/l and 3.0 mg/l did not initiate shoot formation and elongation. During experiment, we concluded that low concentration of BAP (0.5 mg/ℓ) in combination with 2.0 mg/l of kinetin proved favourable combination in this set of experiment for multiple shoot formation from nodal explant of *Catharanthus roseus* L**. (Table-7, Plate-8, Fig C, Graph-3).**

Shoots emerged from nodal explants were healthy and dark green in colour as shown in **Plate-8**. There was no callus formation observed in all combination of BAP with kinetin.

Table 7:-Morphogenic response of nodal explants of *Catharanthus roseus* L. on combination of cytokinins (BAP + Kinetin), incorporated into MS medium

Hormone concentrations (mg/ℓ)	Hormone concentrations (mg/ℓ)	Culture Response (%)	No. of Shoots (mean ± SE)	Shoot length (in cm) (mean ±SE)
BAP	KINETIN			
0.5	0.5	60-65	2.14 ± 0.51	1.41 ± 0.28
0.5	1.0	60-65	5.10 ± 0.28	2.71 ± 0.36
0.5	2.0	80-85	6.23 ± 0.51	3.42 ± 0.39
0.5	3.0	30-35	4.10 ± 0.41	2.41 ± 0.38
1.0	0.5	65-68	2.10 ± 0.26	1.26 ± 0.21
1.0	1.0	70-75	5.23 ± 0.63	3.89 ± 0.24
1.0	2.0	60-65	4.38 ± 0.51	2.85 ± 0.26
1.0	3.0	65-70	3.76 ± 0.28	2.41 ± 0.38
1.5	0.5	65-70	2.57 ± 0.40	2.18 ± 0.41
1.5	1.0	60-65	2.28 ± 0.28	1.38 ± 0.29
1.5	2.0	60-65	2.16 ± 0.26	0.84 ± 0.28
1.5	3.0	50-60	1.58 ± 0.28	0.68 ± 0.24

Graph-3 Morphogenic response of nodal explants of *Catharanthus roseus* L. on combination of cytokinins (BAP + Kinetin), incorporated into MS medium

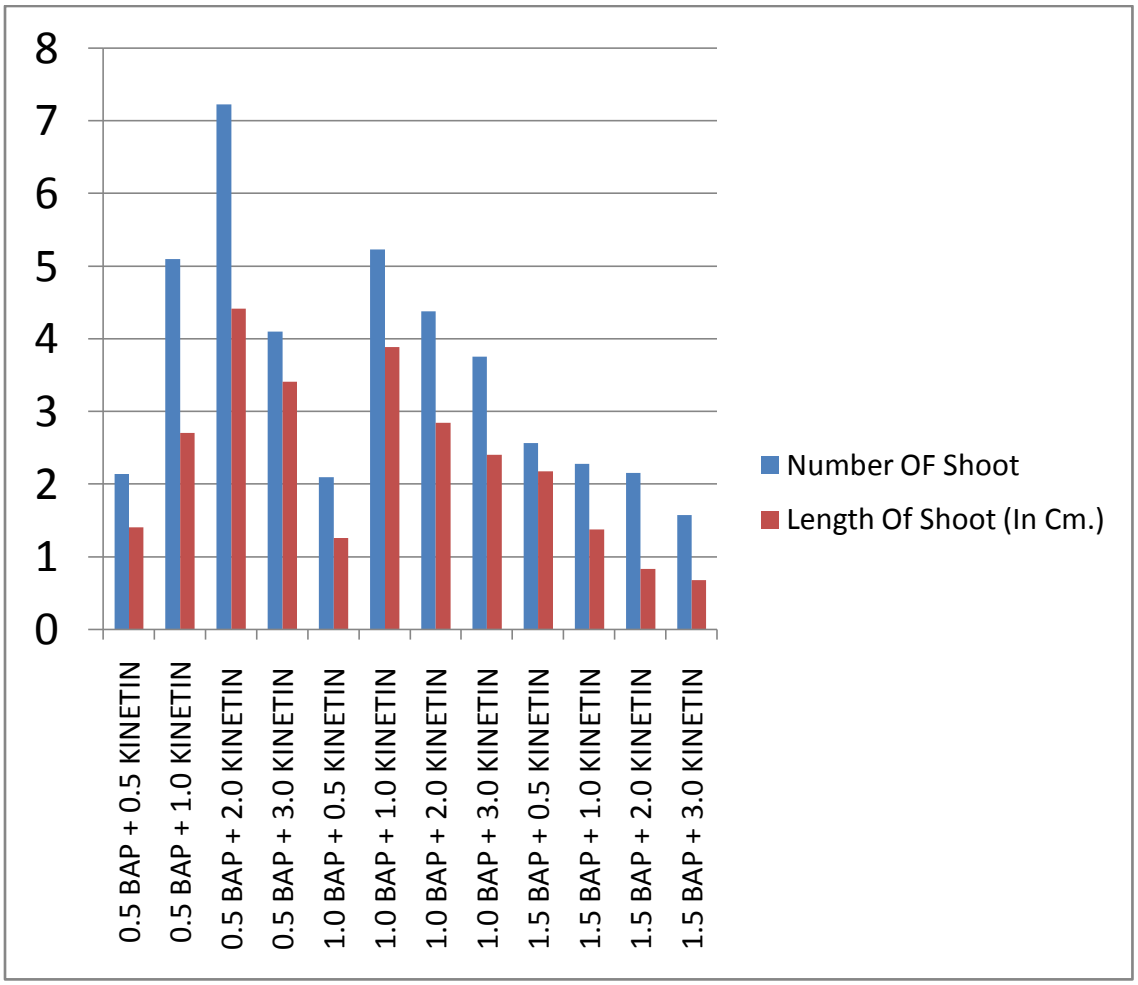

PLATE:-8

Morphogenic response of nodal explants of *Catharanthus roseus L.* on combination of cytokinin (BAP + kinetin), incorporated into MS medium

Fig.A. Showing morphogenic response on Combination of MS + BAP (0.5mg/l) + kinetin (2.0mg/l)

Fig.B.Showing morphogenic response on combination of MS + BAP (1.0mg/l) + kinetin (1.0mg/l)

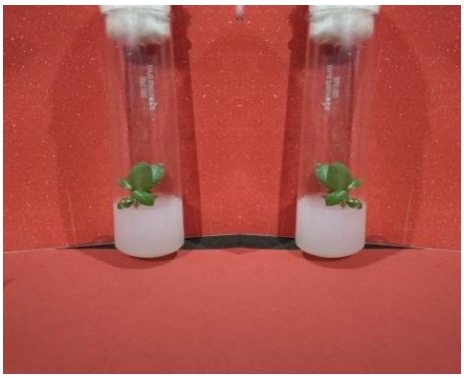

Fig.C. Showing morphogenic response on combination of MS + BAP (1.5mg/l) + kinetin (0.5mg/l)

Morphogenic response of nodal explants of *Catharanthus roseus* L. on combination of cytokinin (BAP) and auxin (NAA), incorporated into MS medium.

In the present experiment, when concentration of BAP (0.5 mg/ℓ) was kept constant in medium and concentration of NAA was increased from 0.5 mg/ℓ – 3.0 mg/ℓ. Shoots emergence was recorded after one week of inoculation.Culture response was 70 – 80%. 0n the combination of 0.5 mg/ℓBAP with 0.5 mg/ℓNAA, shoot emergence was observed in 70% cultures Addition of 1.0 mg/ℓ NAA, increased the number of shoots and shoot length. Shoot length increased upto 4cm. However, further addition of higher concentration of NAA which was 2.0 mg/ℓ and 3.0 mg/l NAA had inhibitory effect on shoot formation and elongation **(Table-8, Plate-9, FigA, Graph-4).**

In the second set of experiment, on the concentration of 1.0 mg/ℓ BAP in combination with 1.0 mg/ℓ NAA, good number of shoots was observed in 70-80% cultures. On addition of higher concentration of NAA (2.0mg/l and 3.0mg/l) reduced shoot emergence **(Table-8, Plate-9, Fig B, Graph-4).**

When concentration of BAP (1.5 mg/ℓ) was kept constant and concentration of NAA was raised from 0.5 mg/ℓ to 3.0 mg/ℓ, after 20-25 days of incubation, we observed that less number of shoots was recordedin comparison to other combination of BAP and NAA that is 1.0 mg/l BAP and 1.0 mg/l NAA **(Table-8, Graph-4).**

So, we concluded that high concentration of BAP (1.5 mg/ℓ) was not conducive for shoot proliferation but relatively low concentration of BAP that is 1.0 mg/ℓ and 0.5 mg/ℓ induced stimulatory effect on multiple shoot formation and shoot elongation as shown in table-8.

Having a glance on the recorded result of experiments and observation,we conculded that lower concentration of BAP that is 0.5 mg/ℓ in combination of NAA (1.0 mg/l) was found favourable for multiple shoot formation. But combination of low concentration of BAP (1.00 mg/l) combination with moderate concentration of NAA (2.0 mg/ℓ) proved better for shoot elongation **(Table-8, Plate-9, Fig A and B, Graph-4).**

There was no callus formation observed in above combination.

Table 8:-Morphogenic response of nodal explants of *Catharanthus roseus* L. on combination of cytokinin (BAP) and auxin (NAA), incorporated into MS medium.

Hormone concentrations (mg/ℓ)	Hormone concentrations (mg/ℓ)	Culture Response (%)	No. of Shoots (mean ± SE)	Shoot length (in cm) (mean ±SE)
BAP	NAA			
0.5	0.5	65-70	6.50 ± 0.27	3.87 ± 0.39
0.5	1.0	70-75	7.30 ± 0.64	5.97 ± 0.17
0.5	2.0	75-80	5.02 ± 0.76	3.06 ± 0.22
0.5	3.0	70-75	3.78 ± 0.57	2.17 ± 0.47
1.0	0.5	75-80	4.08 ± 0.28	2.27 ± 0.36
1.0	1.0	80-85	6.24 ± 0.24	5.29 ± 0.18
1.0	2.0	80-85	5.86± 0.22	5.21 ± 0.27
1.0	3.0	70-75	5.24 ± 0.36	2.37 ± 0.48
1.5	0.5	65-70	3.08 ± 0.28	1.25 ± 0.29
1.5	1.0	55-60	4.02 ± 0.92	1.42 ± 0.56
1.5	2.0	55-60	2.70 ± 0.82	0.98 ± 0.27
1.5	3.0	50-55	2.60 ± 0.70	0.52 ± 0.21

Graph: 4 Morphogenic response of nodal explants of *Catharanthus roseus* L. on combination of cytokinin (BAP) and auxin (NAA), incorporated into MS medium.

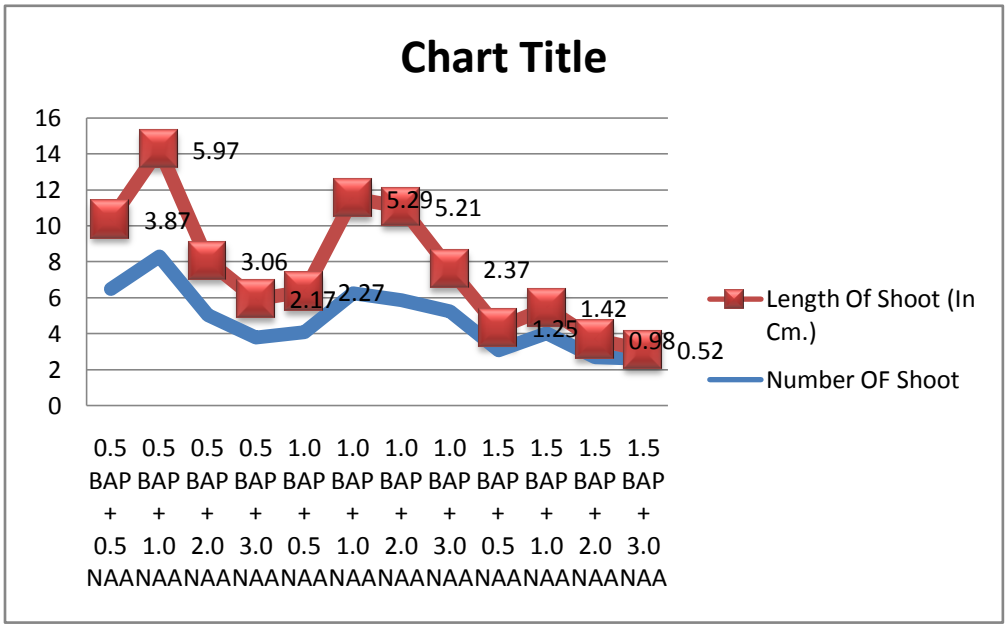

PLATE:-9

Morphogenic response of nodal explants of *Catharanthus roseus L.* on combination of cytokinin (BAP) and auxin (NAA), incorporated into MS medium

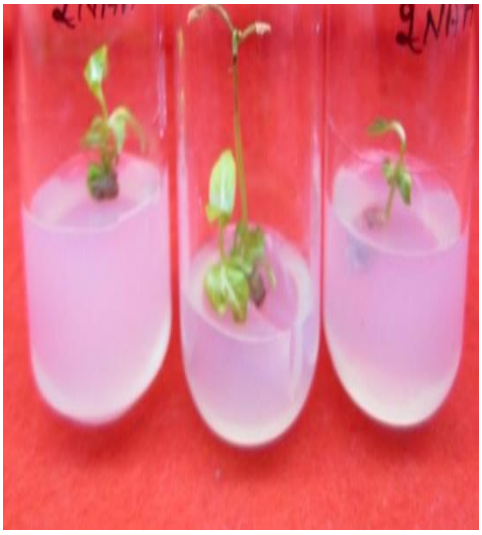

Fig. A. Showing morphogenic response on combination of MS + BAP (0.5mg/l) + NAA (1.0 mg/l)

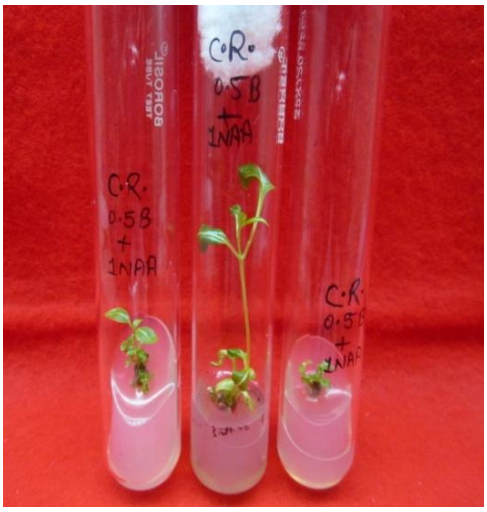

Fig. B. Showing morphogenic response on combination of MS + BAP (1.0 mg/l) + NAA (2.0 mg/l)

Morphogenic response of nodal explants of *Catharanthus roseus* L on combination of cytokinin (BAP) and auxin (2, 4-D), incorporated into MS medium.

In the present experiment, effect of 2,4-D and BAP used in combination was studied. By keeping the concentration of BAP constant that is 0.5 mg/l and concentration of 2, 4-D was raised from 0.5 mg/l to 3.0 mg/l, shoot emergence was recorded after a week. After 3 weeks of incubation, highest number of shoots were noticed having the combination of 0.5 mg/l BAP with 3.0 mg/l 2, 4-D, 70% cultures showed shoot formation. Longer shoots were noticed on combination of 0.5 mg/l BAP with 1.0 mg/l 2, 4-D. The length of shoots was approximately 3cm **(Plate 10, Fig A).**

In second set of experiment, BAP concentration was kept constant at 1.0 mg/l in medium and concentration of 2, 4-D was increased from 0.5 mg/l to 3.0 mg/l. 60% culture response showed shoot proliferation. After 3 weeks, we observed that few shoot were formed but increasing concentration of 2, 4-D helped in shoot proliferation **(Table-9, Plate- 10, Fig B, Graph-5).**

In the subsequent experiment, when BAP concentration was slightly increased from 1.0 mg/l to 1.5 mg/l and kept constant and similarly 2, 4-D concentration was increased from 0.5 mg/l to 3.0 mg/l. After 21 days, shoot emergence was noticed but shoots were not healthy and did not grow further **(Table-9, Plate- 10, Fig C, Graph-5).**

Seeing the observation, we concluded that low concentration of BAP (0.5 mg/l) and high concentration of 2, 4-D (3.0 mg/l) was favourable for shoot proliferation.

Table 9:-Morphogenic response of nodal explants of *Catharanthus roseus* L on combination of cytokinin (BAP) and auxin (2, 4-D), incorporated into MS medium.

Hormone concentrations (mg/ℓ)	Hormone concentrations (mg/ℓ)	Culture Response (%)	No. of Shoots (mean ± SE)	Shoot length (in cm) (mean ±SE)
BAP	2 ,4-D			
0.5	0.5	55-60	1.78 ± 0.76	0.58 ± 0.21
0.5	1.0	55-60	2. 08 ± 1.07	2.88 ± 0.12
0.5	2.0	60-65	2.24 ± 0.28	2.26 ± 0.26
0.5	3.0	60-70	3.81 ± 1.20	1.82 ± 0.31
1.0	0.5	55-60	1.08 ± 0.83	0.76 ± 0.23
1.0	1.0	60-65	1.68 ± 0.21	1.28 ± 0.28
1.0	2.0	60-65	2.09 ± 0.71	2.12 ± 0.31
1.0	3.0	60-65	2. 12 ± 0.42	2.16 ± 0.6
1.5	0.5	55-60	1.38 ± 0.21	1.76 ± 0.81
1.5	1.0	55-60	1.60 ± 0.61	1.41 ± 0.86
1.5	2.0	50-55	1.78 ± 0.58	0.78 ± 0.29
1.5	3.0	50-55	0.86 ± 0.28	0.56 ± 0.86

Graph: 5 Morphogenic response of nodal explants of *Catharanthus roseus* L on combination of cytokinin (BAP) and auxin (2, 4-D), incorporated into MS medium.

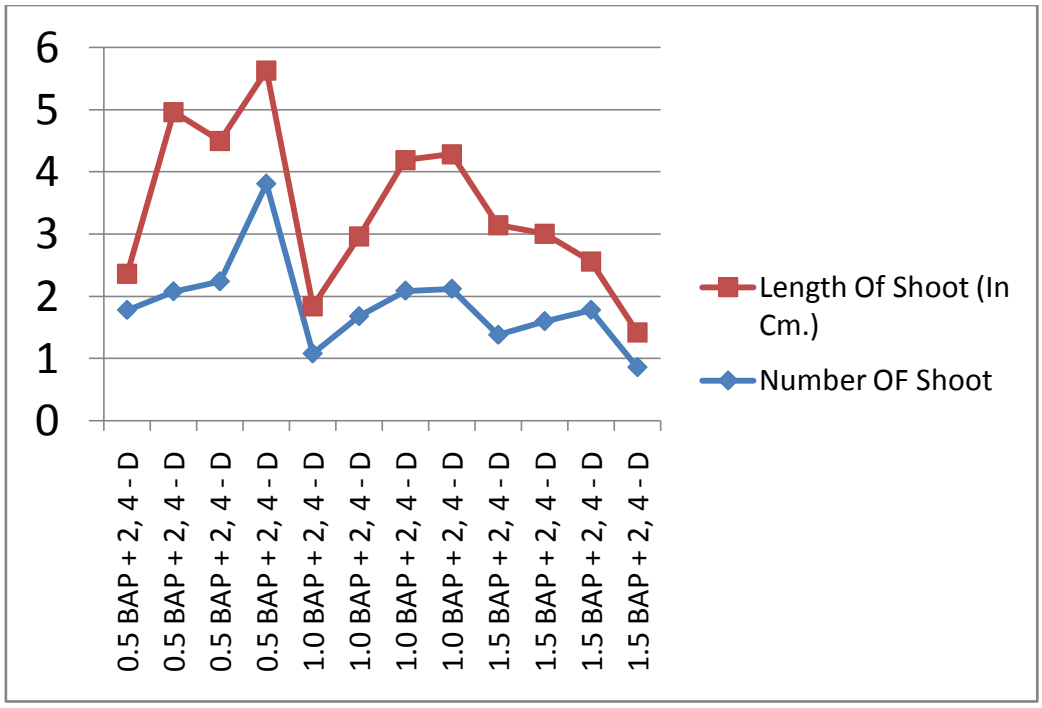

PLATE:-10

Morphogenic response of nodal explants of *Catharanthus roseus L.* on combination of cytokinin (BAP) and auxin (2, 4-D), incorporated into MS medium

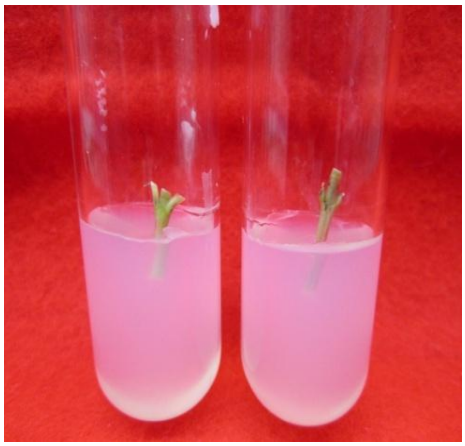

Fig A. Showing morphogenic response on combination of MS+ BAP (0.5mg/l) + 2, 4-D (3.0mg/l)

Fig B. Showing morphogenic response on combination of MS + BAP (1.0mg/l) + 2, 4-D (3.0mg/l)

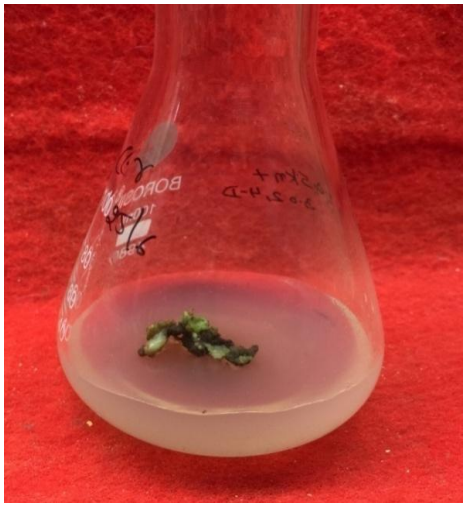

Fig C. Showing morphogenic response on combination of MS + BAP (1.5mg/l) + 2, 4-D (2.0mg/l)

Morphogenic response of nodal explant of *Catharanthus roseus* L. on combination of cytokinin (kinetin) and auxin (NAA), incorporated into MS medium.

In the present experiment, nodal explants of *Catharanthus roseus* L. were inoculated on MS medium supplemented with various concentration of cytokinin (kinetin) in combination of auxin (NAA).

When concentration of kinetin was kept constant (0.5 mg/l) and NAA concentration was raised from 0.5 mg/l to 3.0 mg/l. After 3 weeks, shoots were observed in 60-70% cultures.

A good number of shoots was found on combination of (kinetin 0.5mg/l + NAA(1.0 mg/l) and culture response was 80% but longer shoots were noticed in combination of 0.5 mg/l kinetin and 0.5 mg/l NAA **(Table-10, Plate-11, Fig A, Graph-6).** So, we concluded that low concentration of NAA (0.5 mg/l) with moderate concentration of kinetin (1.0 mg/l) was proved favourable for producing a good number of shoots which elongated later.

In second set of experiment, when concentration of kinetin was kept constant at 1.0 mg/l and NAA concentration was increased from 0.5 mg/l to 3.0 mg/l. After 3 weeks, we observed that culture response was 60%.But number of shoot recorded was low.

However, increasing concentration of NAA upto 2.0 mg/l had stimulating effect on shoot formation **(Table-10, Graph-6**). Further addition of 3.0 mg/l NAA inhibited the shoot initiation.

In the subsequent experiment, when kinetin concentration was increased at 1.5 mg/l and kept constant in medium and NAA concentration was varied from 0.5 mg/l to 3.0 mg/l.

In most of cultures having combination of 1.5 mg/l kinetin + 2.0 mg/l NAA showed good number of shoots and length of shoots recorded was about 2-3 cm **(Table-10, Plate-11, FigB, Graph-6**). We observed that up to 2.0 mg/l NAA in the medium in combination with kinetin (1.5 mg/l) proved best for shoot formation and further growth.

On analysing the result of above combination, we observed that moderate concentration of NAA along with high concentration kinetin proved favourable for multiple shoot formation.

Table 10:-Morphogenic response of nodal explants of *Catharanthus roseus* L. on combination of cytokinin (kinetin) and auxin (NAA), incorporated into MS medium.

Hormone concentrations (mg/l)	Hormone concentrations (mg/l)	Culture Response (%)	No. of Shoots (mean ± SE)	Shoot length (in cm) (mean ±SE)
Kinetin	NAA			
0.5	0.5	50-60	0.67 ± 0.32	1.21 ± 0.82
0.5	1.0	75-80	1.86 ± 0. 62	0.86 ± 0.32
0.5	2.0	65-70	1.50 ± 1.60	0.82 ± 1.02
0.5	3.0	60 - 65	1.28 ± 0.76	0.56 ± 0.26
1.0	0.5	60 - 65	0.86 ± 0.69	0.56 ± 0.28
1.0	1.0	60-65	1.16 ± 0.23	1.89 ± 0.38
1.0	2.0	60-65	1.22 ± 0.61	0.86 ± 0.14
1.0	3.0	55-60	1.02 ± 0.29	0.52 ± 0.37
1.5	0.5	55 – 60	1.21 ± 0.25	1.18 ± 0.26
1.5	1.0	60 – 65	1.72 ± 0.31	1.72 ± 0.46
1.5	2.0	70 – 75	2.62 ± 0.81	1.62 ± 0.23
1.5	3.0	50 – 55	2.32 ± 0.18	1.32 ± 1.32

Graph: 6 Morphogenic response of nodal explants of *Catharanthus roseus* L. on combination of cytokinin (kinetin) and auxin (NAA), incorporated into MS medium.

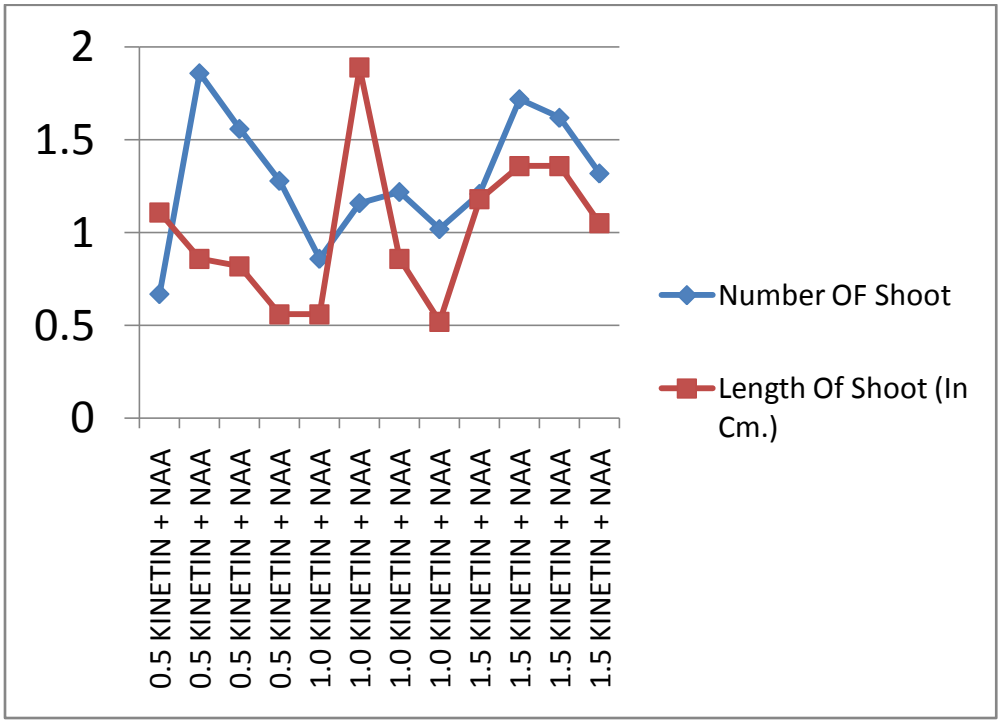

PLATE:-11

Morphogenic response of nodal explants of *Catharanthus roseus L.* on combination of cytokinin (kinetin) and auxin (NAA) incorporated into MS medium

Fig. A. Showing response on combination of MS + kinetin (0.5mg/l) + NAA (1.0mg/l)

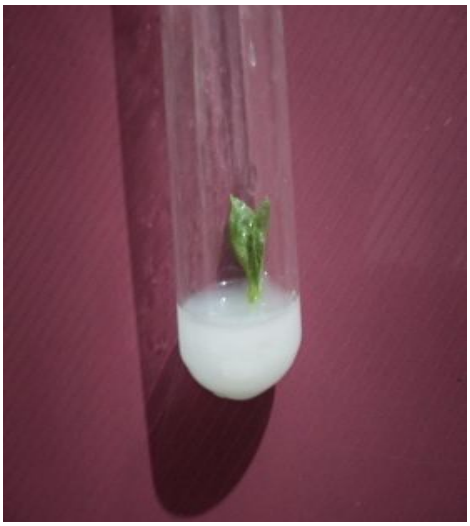

Fig B.Showing response on combination of MS + kinetin (1.5mg/l) + NAA (2.0mg/l)

Morphogenic response of nodal explant of different size and different position (basal, middle and distal) along the stem length of *Catharanthus roseus* cultured on MS medium supplemented with BAP.

In this experiment, nodal piece of different size and different position (basal, middle and distal) along the stem length of *Catharanthus roseus* is taken to observe morphogenic response.These nodal pieces were inoculated in MS medium supplemented with BAP (3.0 mg/l).

After 20-25 days of inoculation, we observed that nodal piece of 1.5 cm induced a good number of shoots among the different position of nodal piece along the stem length (basal, middle and distal) of *Catharanthus roseus.* In these cultures, middle position of nodal piece showed a very good number of shoot formation in comparison to other positions of nodal piece that were distal and basal. Culture response recorded was 60-70%.

On analyzing the observation of this experiment , we concluded that size and relative position of nodal pieces along the stem length of *Catharanthus roseus* had stimulatory effect on shoot formation**(Tale-11, Plate-12, Fig A, B and C).**

Talble-11 Morphogenic response of nodal explants of different size and different position (basal, middle and distal) along the stem length of *Catharanthus roseus* cultured on MS medium supplemented with BAP.

Size of explants (in cm)	Position of the node along the stem length	Culture Response(%)	Number of Shoot formation
1.0 cm	Basal	-	-
	Middle	10 to 20	+
	Distal	5 to 10	+
1.5 cm	Basal	50 to 60	++
	Middle	60 to 70	+++
	Distal	30 to 40	++
2.0 cm	Basal	20 to 30	+
	Middle	40 to 50	++
	Distal	10 to 20	+

- = no response,

+ = very few number of shoot formation

++ = moderate number of shoot formation

+++ = very good number of shoot formation

PLATE:-12

Morphogenic response of nodal explant of different size and different positions (basal, middle and distal) along the stem length of *Catharanthus roseus L.*

Fig A. Response of 1.0cm long nodal explants of middle position

Fig B. Response of 1.5cm long nodal explants of middle position

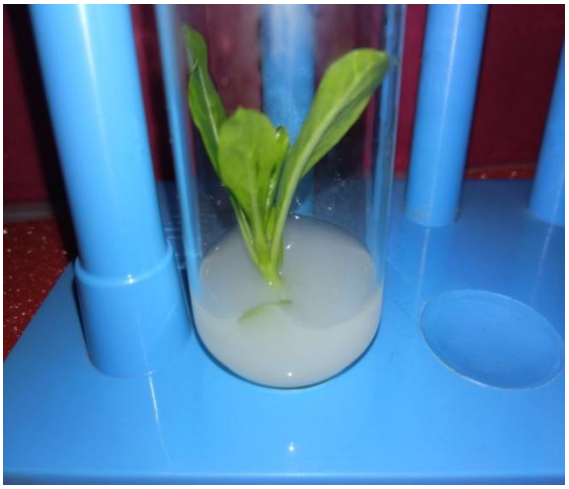

Fig C. Response of 2.0cm long nodal explants of middle position

Root induction in *in-vitro* raised shoots of *Catharanthus roseus* L.

In vitro raised shoots (4-6 cm long) were used for induction of roots. Three different experiments were set for root induction.

(A) In vitro raised shoots were inoculated on MS medium (1/2 strength) supplemented with different concentration of various growth hormone(BAP, NAA, IAA and IBA).

(B) In vitro raised shoots were inoculated on different concentration of IBA with 1/2 MS medium strength.

(C) In vitro raised shoots were inoculated on different energy source (sucrose, fructose and glucose).

A. Rooting response of *in vitro* raised shoots of *Catharanthus roseus* L. on MS medium (1/2 strength) supplemented with different concentrations of growth hormones.

In this experiment, to find out most favourable growth hormone for rooting. *In-vitro* raised shoots were transferred on MS medium (1/2 strength) supplemented with single application of BAP, NAA, IAA and IBA with concentration range of 0.5 mg/l, 1.0 mg/l and 2.0 mg/l.

When the cultures were left for long period (more than 6 weeks), feeble roots emerged in cluster in few culture Roots were white in colour and length was not much. Table-12 showed the result obtained after 2 weeks rooting induction. The time was extended for optimum condition.

Effect of BAP

No root induction could be seen even after six weeks of inoculation. We concluded that BAP has no stimulatory effect on root induction.

Effect of NAA

On NAA supplemented MS medium, after 45 days, very few cultures showed root initiation on MS medium incorporated with 1.0 mg/l and 2.0 mg/NAA added singly . Low concentration of NAA (0.5mg/l) could not induce root initiation **(Table-12, Plate-13, Fig A, Graph-7).**

Effect of IAA

In IAA containing cultures, all concentration of IAA showed positive response for rooting but percentage and quality of roots were not good. Higher concentration of IAA induced good root system .But poor roots were formed on low concentration of IAA(0.5mg/l) . In this concentration, roots did not grow further**(Table-12, Plate-13, Fig B, Graph-7).**

Effect of IBA

In IBA containing cultures, all concentration of IBA showed stimulatory effect on rooting induction. The rooting was increased with increased concentration of IBA in the culture medium. A good percentage (78%) of rooting responce was observed on 2.0 mg/l IBA supplemented cultures. The roots were long thick and creamish in colour **(Table-12, Plate-13, Fig C, Graph-7).**

Overall, the study revealed tha IBA immediately initiated rooting response compare to the treatment of BAP, NAA and IAA. Apparently roots were thread like, contractile, slightly thin and whitish to creamy coloured.

Table 12: Rooting response of *in-vitro* shoots of *Catharanthus roseus* L. inoculated on MS medium (1/2 strength) supplemented with various concentrations of different growth hormones (BAP, NAA, IAA and IBA) added singly

Growth hormone conc. (mg/l)	Culture response (%)	Response after 15 days	Response after 30 days	Response after 45 days
½ MS basal medium	NR	NR	NR	NR
0.5 BAP	NR	NR	NR	NR
1.0 BAP	NR	NR	NR	NR
2.0 BAP	NR	NR	NR	NR
0.5 NAA	NR	NR	NR	NR
1.0 NAA	36%	NR	NR	Root initiation
2.0 NAA	32%	NR	NR	Root initiation
0.5 IAA	48%	NR	NR	Root initiation
1.0 IAA	62%	NR	NR	Small thread like roots appearing
2.0 IAA	65%	NR	Root initiation	Small thread like roots appearing
0.5 IBA	60%	NR	Root initiation	Small thread like roots emerged
1.0 IBA	73%	Root initiation	Small thread like roots emerged	White thread like roots emerged
2.0 IBA	78%	Root initiation	Small thread like roots appeared	Creamish long thread like roots appeared

Graph-6 Rooting response of *in-vitro* shoots of *Catharanthus roseus* L inoculated on MS medium (1/2 strength) supplemented with various concentrations of different growth hormones (BAP, NAA, IAA and IBA) added singly

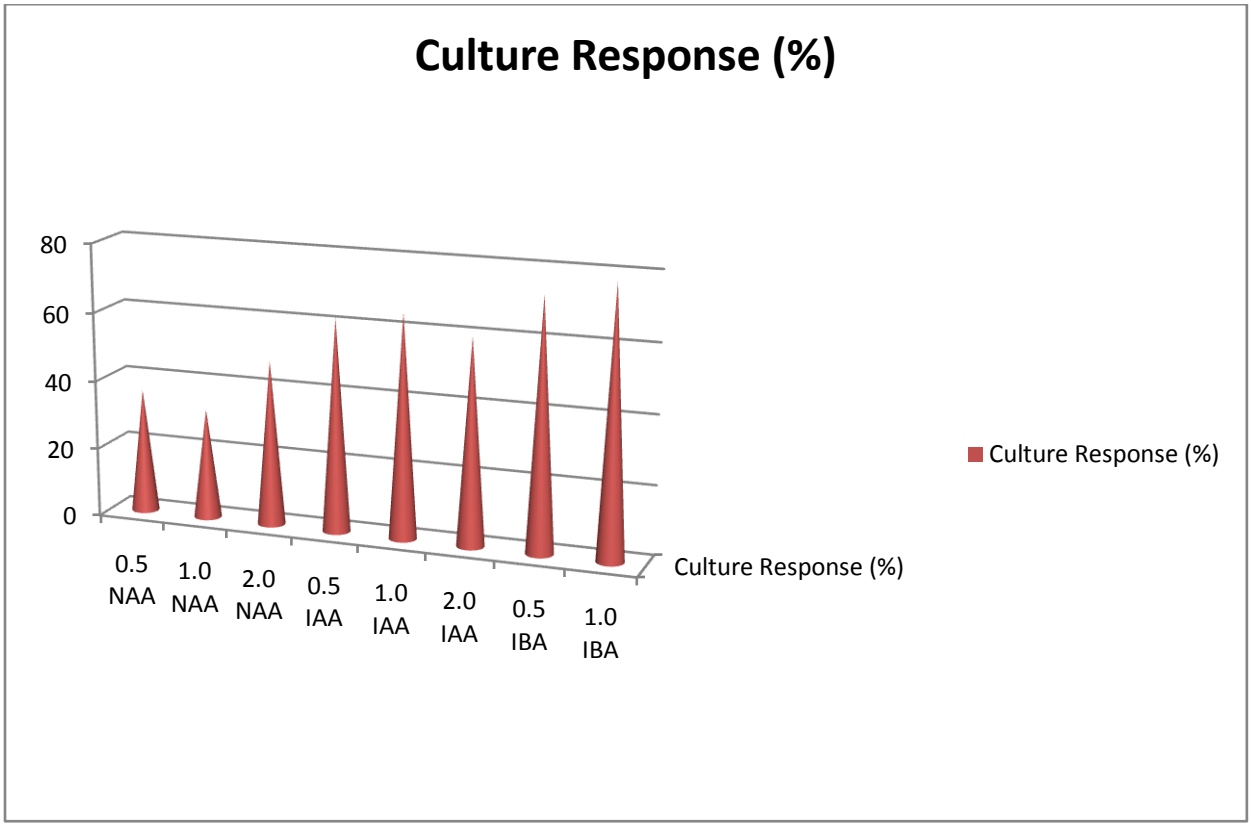

PLATE:-13

Rooting response of *in-vitro* raised shoots of *Catharanthus roseus L.* on half strength MS medium supplemented with different concentrations of growth hormone

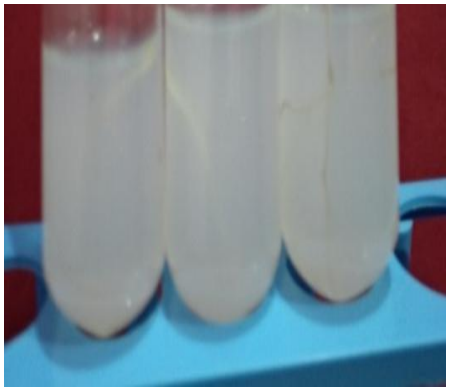

Fig A. Showing response on ½ MS + different concentration of NAA (0.5 mg/l, 1.0 mg/l and 2.0 mg/l)

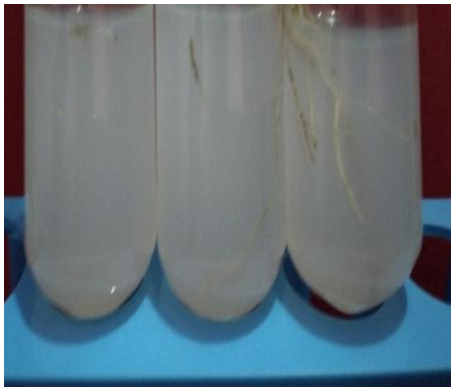

Fig B. Showing response on ½ MS + different concentration of IAA (0.5 mg/l, 1.0 mg/l and 2.0 mg/l)

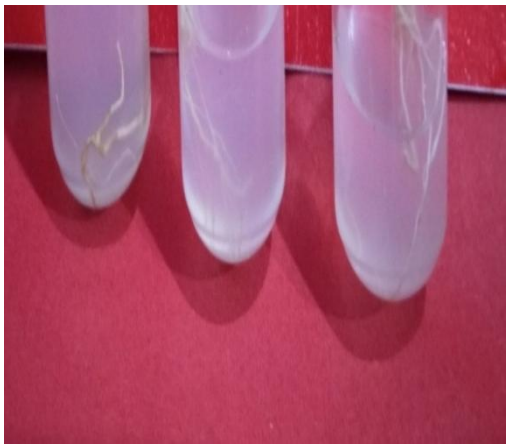

Fig C. Showing response on ½ MS + different concentration of IBA (0.5 mg/l, 1.0 mg/l and 2.0 mg/l)

Root induction response of *in-vitro* raised shoots of *Catharanthus roseus* L. on half strength MS medium supplemented with different concentratioin of IBA.

In this experiment, *in-vitro* raised shoots were transferred on half strength MS medium with different concentration of IBA respectively 0.5 mg/l, 1.0 mg/l, 2.0mg/l ,3.0 mg/l,4.0mg/l and 5.0 mg/l.

When *in-vitro* raised shooting plantlets were cultured on half strength MS medium with incorporation of increasing concentration of IBA (0.5 mg/l - 5.0 mg/l). After 3 weeks, we observed that all concentration of IBA induced root initiation and higher concentration of IBA had stimulatory effect on rooting .Observation showed low concentration of IBA was not as effective as high concentration of IBA **(Table 13 , Graph-7, Plate; 14 fig A,B and C).**

After 3 weeks, we observed that good roots were produced on all concentration of IBA. The highest rooting response (85%) was recorded on 5.0 mg /l IBA.Apparently roots seem to be originating from basal part of shoots..

On comparing the all observation, we concluded that half strength MS medium containing with higher concentration of IBA (5.0 mg/l) was proved to be best for root induction.

Table 13: Root induction response of *in-vitro* raised shoots of *Catharanthus roseus* L. on half strength MS medium supplemented with different concentratioins of IBA.

Hormone concentration (mg/l)	Rooting Response (%)	No. of roots (mean ± SE)	Root length (in cm) (mean ±SE)
Half strength MS medium			
0.5 IBA	15-20	Only root initiation	-
1.0 IBA	45-50	0.34 ± 0.41	0.21 ± 0.18
2.0 IBA	65-70	0.62 ± 0.36	0.48 ± 0.61
3.0 IBA	70-75	2.40 ± 0.27	0.87 ± 0.21
4.0 IBA	70-75	2.46 ± 0.52	1.26 ± 0.08
5.0 IBA	80-85	3.60 ± 0.41	2.68 ± 0.12

Graph-7 Rooting response of *in-vitro* raised shoots of *Catharanthus roseus L.* on half strength MS medium supplemented with different concentratioins of IBA.

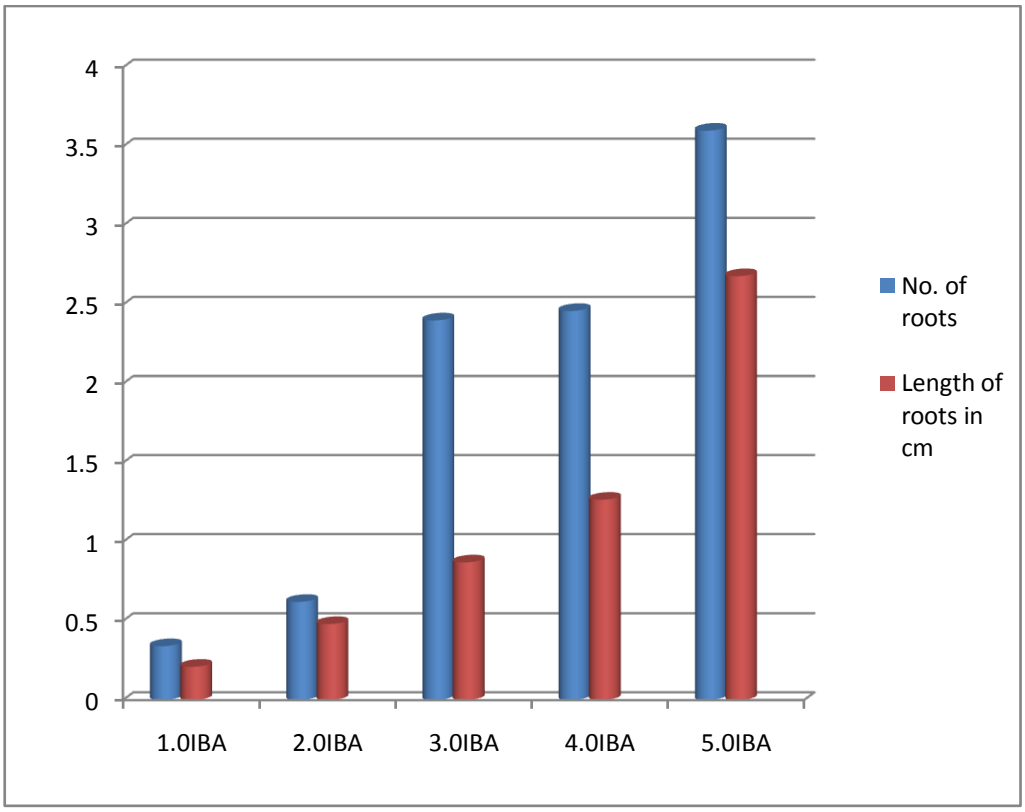

PLATE:-14

Rooting response of *in-vitro* raised shoots of *Catharanthus roseus L.* on half strength MS medium supplemented with different concentrations of IBA

A **B** **C**

Fig A. Showing response on ½ MS + IBA (1.0mg/l)

Fig B. Showing response on ½ MS + IBA (5.0mg/l)

Fig C. Showing response on ½ MS + IBA (3.0mg/l)

Effect of various types of energy sources on root proliferation of *in-vitro* raised shoots of *Catharanthus roseus L.* on half strength MS medium supplemented with IBA.

In this experiment, half strength MS medium with IBA (3.0 mg/l) was taken as the standard medium for rooting and different energy sources like sucrose, glucose and fructose were added singly to the standard medium for root induction and proliferation.

When rooted shoots were subcultured on half strength MS medium supplemented with 3% and 6% sucrose with 3.0 mg/l. IBA containing medium .After 15 days, we observed that both concentration of sucrose triggered early response on root proliferation but very good response was noticed on 6% concentration of sucrose. When these cultures were left for longer period (45 days), healthy roots were appeared shown in **Plate15, fig A and B.**

When glucose was used as energy source instead of sucrose delayed root initiation was noticed after 30 days of inoculation.Little root initiation was observed in second set of experiment **Table ;14, Graph 7.**

In subsequent experiments,when fructose was used as energy source instead of sucrose.we observed that very few cultures showed root proliferation after 45 days.

Overall, our result showed that sucrose at 6% in MS medium supplemented IBA(3.0 mg/l) was found to be the most promising energy source for root proliferation in comparison to other energy sources (glucose, fructose).

Table 14: Effect of various types of energy sources on root proliferation of *in-vitro* raised shoots of *Catharanthus roseus L.* on half strength MS medium supplemented with IBA.

Energy Source + MS+IBA (3.0 mg/l)	Culture response (%)	Proliferation of roots after 15 days	Proliferation of roots after 30 days	Proliferation of roots after 45 days
3% sucrose	70	Little proliferation	Very small thread like roots	Small thread like, whitish roots
6% sucrose	88	Little proliferation	Small thread like, whitish roots	Branched, long thread like, creamy whitish roots
3% glucose	30	----	Little proliferation	Very small thread like thin, whitish roots
6% glucose	50	----	Little proliferation	Very small thread like thin, whitish roots
3% Fructose	10	----	Little proliferation	Very little proliferation
6% Fructose	20	----	Little proliferation	Very little proliferation

PLATE:-15

Rooting response of in-vitro raised shoots of *Catharanthus roseus* L. on half strength of MS medium with various types of energy sources

Fig A Showing response on ½ MS + IBA (3.0mg/l) + sucrose (6%)

Fig B. Showing response on ½ MS + IBA (3.0mg/l) + glucose (6%)

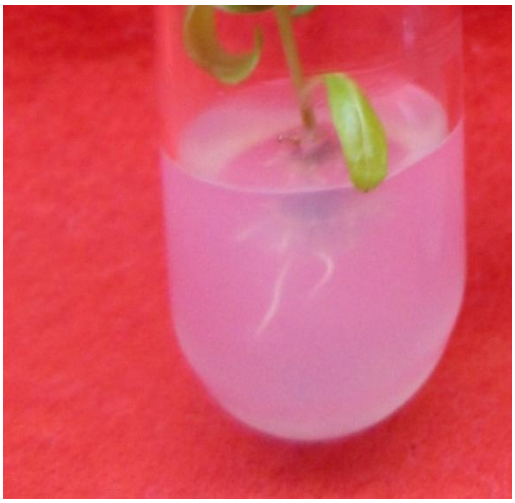

Fig C. Showing response on ½ MS + IBA (3.0 mg/l) + fructose (6%)

Effect of tryptophan concentrations and duration of dark periods on root proliferation by subculture of in vitro raised rooted shoots of *Catharanthus roseus* L.

To see the effect of tryptophan concentrations and durations of dark periods on root proliferation, *in-vitro* raised rooted shoots were subcultured on ½ MS medium supplemented with 3.0 mg/l IBA with two different concentration of tryptophan (150 ml and 250 ml) in 3 different dark cycles of 12 hours, 16 hours and 20 hours.

After 3 weeks of inoculation, we observed that root proliferation on standard rooting media (MS medium with 3.0mg/l IBA) with 250 ml tryptophan was higher than 150 ml tryptophan. In 150 ml tryptophan added standard medium, simultaneous rooting response was lower in 12 hours duration of darkperiod but tended to increase as a stimulates of higher duration of dark period **(Table-15, Plate 16 fig A,B and C)**

The result revealed that higher concentration of tryptophan (250 ml) subjected with long duration of dark period (20 hours) have more conductive effect on root proliferation.

Table 15:- Effect of tryptophan concentrations and duration of dark periods on root proliferation by subculture of *in-vitro* raised rooted shoots of *Catharanthus roseus* L.

Darkperiod (Dark)	Tryptophan Concentration (ml) + ½ MS + IBA (3.0mg/l)	Rooting Response (%)	Number of root formation
12 hr	150	-	-
	250	10 to 20	+
16 hr	150	40 to 50	++
	250	60 to 70	++
20 hr	150	50 to 60	++
	250	70 to 80	+++

+ = normal root formation, ++ = moderate root formation

+++ = vigorous root formation,

++ = moderate root formation

+++ = Vigorous root formation, - = no root formation

PLATE:-16

Effect of tryptophan concentrations and dark periods on root proliferation by subculture of in-vitro raised shoots of *Catharanthus roseus* L.

Fig A. Showing response of 12hrs dark period cycle on 250ml tryptophan + ½ MS + IBA (3.0 mg/l) concentration

Fig B. Showing response of 16hrs dark period cycle on 250ml trptophan+ ½ MS + IBA (3.0 mg/l) concentration

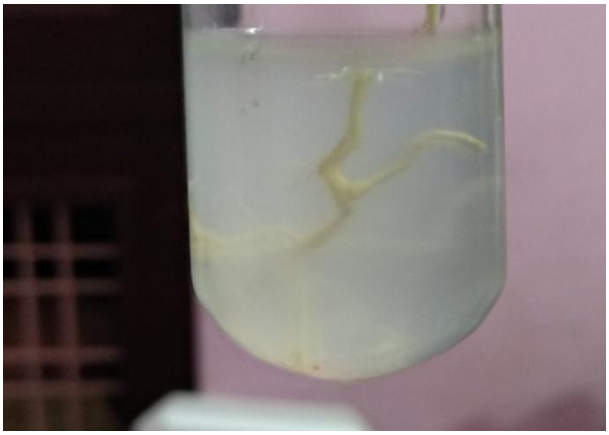

Fig C. Showing response of 20hrs dark period cycle on 250ml tryptophan + ½ MS + IBA (3.0 mg/l) concentration

Acclimatization of *in-vitro* raised plantlets of *Catharanthus roseus L.*

The regenerated plantlets of*Catharanthus roseus* were taken out aspectically from the culture vessel and washed with autoclaved water carefully to remove traces of agar. After washing, *in vitro* rooted plantlets of *Catharanthus roseus* were transferred carefully to culture bottles filled (¼ th) with soilrite composition (Sand; Soil and peat moss composition).

After 3weeks of growth, plantlets were transferred to field condition for further growth **(Plate 17, figA and C).** Though we could transfer regenerated plants to field condition by acclimatizing and following a harding procedure but still more efforts are required for a good number of *in vitro* raised plants to transfer field condition.

PLATE:-17

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

Fig A. Plantlets of *Catharanthus roseus L* in soilrite mixture Vessel covered with plastic container

Fig B. Acclimatized plantlet of *Catharanthus roseus*

Synthetic seed formation in *Catharanthus roseus L.* by encapsulation of somatic embryos :-

Synthetic seed are the commonly described as encapsulated embryos. Synthetic seed propagation technology is currently considered an effective and efficient alternative method of propagation in several commercially important agronomic and horticultural crops.

Artificial seed technology provides an alternative method for formation of seed analogue from micropropagules like auxillary shoot buds, apical shoot tip, embryogenic calli, somatic embryos and protocorm like bodies. It provides a tremendous potential for micro propagation and germplasm conservation.

Catharanthus roseus is a tropical and subtropical plant belonging to the family Apocynaceae. It is an important source of anti cancerous alkaloids Vinblastine and Vincrustine or anti hypertensive alkaloids such as ajmalicine, serpentine and reserpine.

Due to medical importance, it is used in pharmaceutical preparation. To meet this growing demand, large scale production of *Catharanthus roseus* is essential. So, there is a strong need of alternative method of large number of seed availability irrespective to the season.

The aim of present study is to develop a protocol for synthetic seed formation by encapsulating somatic embryos raised from leaf explants of *Catharanthus roseus* using calcium gel matrix.

Embryogenic callus having organized structure similar to somatic embryos was raised from leaf explants of *Catharanthus roseus* on MS medium supplimented with combination of 2,4-D (1.0 mg/l) and kinetin (1.0 mg/l). This embryogenic calli with emryoids was separated and selected for artificial seed formation.

Callus pieces which were showing white globular structure were taken out from culture flasks under asepctic condition and teased to separate somatic embryoids.
Teased and separated somatic embryos were dipped into sodium alginate solution prepared in half strength MS medium with antibiotic and then transferred into $CaCl_2$ solution.

After few minutes, these artificaial seeds were put into serilized petriplates. Calcium alginate capsule were formed by the ion exchange between Na^+ and Ca^{++}. These prepared synthetic seed were preserved in refrigerator at 4^0c **(Plate 18, fig A**).

We are in the process of prepairing more number of synthetic seeds so, that we can confirm the viability of these *in-vitro* prepared artificial seeds. Hence, we can have a standardized protocol of production of large number of synthetic seeds.

PLATE:-18

Synthetic seed formation in *Catharanthus roseus L.* by encapsulation of somatic embryos

Initiation of callus from leaf explants on 2, 4-D medium

↓

Induction of somatic embryogenesis on combination of 2, 4-D (1.0 mg/l) + kinetin(1.0 mg/l) with MS medium

↓

Prolific somatic embryogenesis

↓

Fully developed somatic embryos separated and dipped in ½ MS medium and sodium algenate (2.5%) and than treated with $CaCl_2$ solution (1.5%)

↓

Ca ions induce rapid cross linking of Na alginate to produce gel bead

↓

Each containing encapsulated embryos

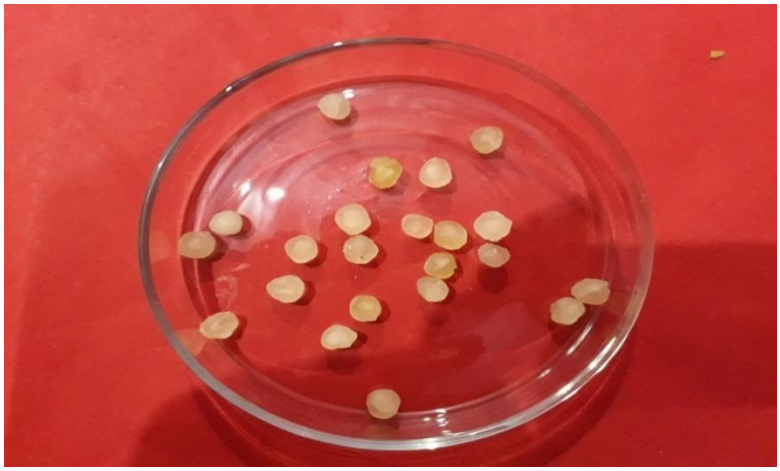

Fig A. Synthetic seeds in petriplate

Qualitative test for detection of various bioactive chemicals present in leaf of *Catharanthus roseus* L.

Chatharanthus roseus L. is an important medicinal plant belongs to the family Apocynaceae and posseses several pharmaceutically active compounds. It contains more than 100 alkaloids, distributed in all parts of the plant in varied proportions. The total alkaloid content in roots amount to 2 – 3%. Leaves contain one percent of alkaloids, stem, fruit, seed and pericarp contain 0.48%, 0.40%**,** 0.18%, and 1.14% respectively **(B. Karthikeyan et. al, 2010).**

Catharanthus roseus L. is an erect herbaceous perennial plant which is a chief source of patented cancer and hypertensive drugs. It is also known for usage in the treatment of diabetes, stomach pain, high blood pressure, nose bleeding, sore throat and urinary disorder **(R. Verpoorte et al, 1993).**

Seeing the extensive pharmacological action of the present bioactive chemicals, qualitative analysis of the leaves was performed in the present experiment.

Plant material –

The leaves of *Catharanthus roseus* L. were collected in the month of May and June from field.

Preparation of leaf powder of *Catharanthus roseus* L.

The collected leaves were washed; shade dried and powdered with mechanical pulveriser for size reduction. It was then passed through muslin cloth and the fine powder was collected and used for the experiment and preparation of extract.

Preparation of ethanolic extract:-

For preparation of ethanolic extract of leaf powder of *Catharanthus roseus,* 50gm dried powder of leaf Catharantheus roseus was weighed and transferred into conical flask and further, it was macerated with sufficient amount of 95% ethanol. Filtrate was obtained by filtering through filter paper. Then ethanolic filtrate of leaf powder of *Catharanthus roseus* was stored in 10-15^{0}c.

Following tests were performed to detect presence of phytochemicals in ethanolic extract of leaf powder of *Catharanthus roseus*.

Test for carbohydrate:-

Small amount of ethanolic extract of leaf powder of *Catharanthus roseus* was dissolved in little quantity of distilled water and filtered separately. The filtrate was used for confirming the presence of carbohydrate..

Molisch Test:- The filtrate was treated with Molisch reagent and concentrated sulphuric acid was added from the sides of the test tube to form a layer. Appearance of reddish colour confirmed the presence of carbohydrates in our test(**Plate 19 fig A).**
Another test to confirm the presence of carbohydrate is Benedict Test.

Benedict Test:- The filtrate was treated with 2ml of Benedict reagent and boiled in water bath. Formation of reddish brown precipitate confirmed the presence of carbohydrate in ethanolic extract of leaf powder of *Catharanthus roseus***(Plate19 fig**

Test for starch:-

Iodine Test:- First, we prepared Iodine solution by dissolving Iodine(0.015gm) and potassium Iodide in 5ml of distilled water. We added 3-4 drops of Iodine solution in 2-3 ml of ethanolic extract of leaf powder of *Catharanthus roseus*. We observed blue colour in this test, which confirm the presence of starch of ethanolic extract of leaf powder of *Catharanthus roseus***(Plate 19 fig C).**

Test for Protien:-

Ninhydrin Test:- To perform this test, we added three dropes of ninhydrin solution in 3ml of ethanolic extract of leaf powder of *Catharanthus roseus*. This tube was shifted in water bath. We observed purple colour after 10 minutes. Purple colour formation confirmed the presence protein in our test**(Plate19 fig D).**

Test for Flavanoids:-

Alkaline Test:- In ethanolic extract of leaf powder of *Catharanthus roseus*, we added few drops of sodium hydroxide. In test tube, dark yellow colour was appeared which disappears after adding dilute HCL that indicates the presence of flavanoids in our test **(Plate19 fig E).**

Test for Alakloids:-

Firstly, ethanolic extract was alkaline with ammonia then extracted with chloroform. The chloroform extract was acidified with dilute HCL and formed acid layer. The formed acid layer was used for following test

Wagner Test:- Formed acid layer was treated with few drops of wagner reagent. Reddish brown precipitate is formed in our test, which confirms the presence of alkaloids in ethanolic extract leaf powder of *Catharanthus roseus* roseus **(Plate19 fig F).**

Mayer Test :- Few drops of Mayer reagent added in formed acid layer. We observed the creamy white precipitate in this test which confirms the presence of alkaloids **(Plate19 fig G).**

Test for Anthroquinone:-

Borntrager Test:- In this test, we took small amount of ethanolic extract of leaves powder of *Catharanthus roseus* and 1-2ml of dilute HCL was added and then boiled. Then it was filtered and cooled. Benzene (3ml) was added in cold filtrate. Formed Benzene layer was separated and 3ml of ammonia was added. Dark red colour ammonical layer was observed in our test, which confirms the presence of anthraquinone in ethanolic extract of leaf powder of *Catharanthus roseus***(Plate19, fig H).**

Test for Seponin:-

Foam Test:- We took small amount of ethanolic leaf extract of leaf of *Catharanthus roseus* and shook with little quantity of water. In this test, foam was formed that confirms the presence of saponin in ethanolic extract of leaf of *Catharanthus roseus* **(Plate19, fig I).**

Table 16: Qualitative test for detection of various bioactive chemicals present in leaf of *Catharanthus roseus* L.

Chemical Test	Result	Chemical Test	Result
Test for carbohydrates		Test for Alkaloids	
Molesch's test	+	Wagner's test	+
Benedict's test	+	Mayer's test	+
Test for starch		Test for anthraquinone	
Iodine test	+	Borntrager's test	+
Test for protein		Test for saponin	
Ninhydrin test	+	Foam test	+
Test for flavanoids			
Alkaline reagent test	+		

'+' positive test showing present of bioactive chemical

'–' Negative test showing present of bioactive chemical

PLATE:-19

Result of qualitative test for detection of various bioactive chemicals present in ethanolic extract of leaf of *Catharanthus roseus L.*

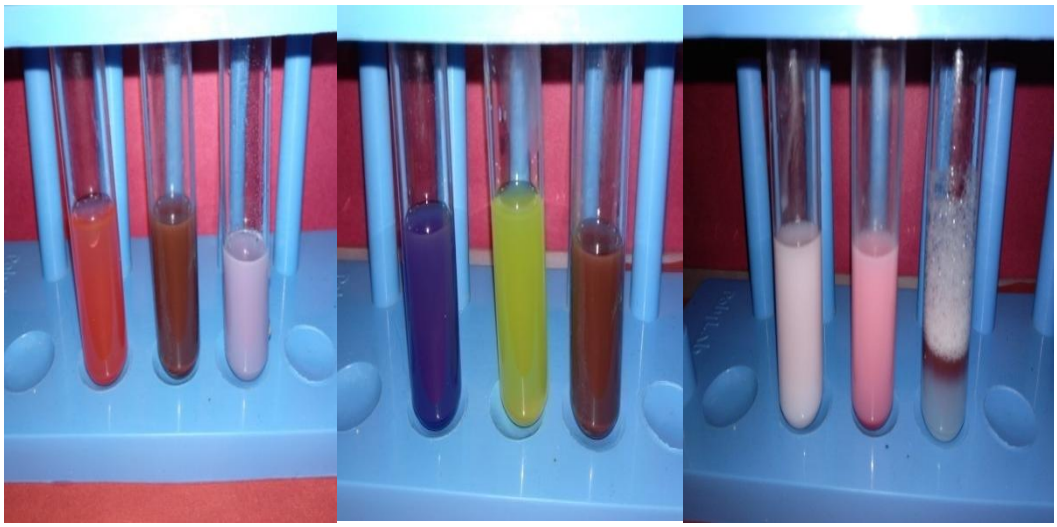

Fig A. Appearance of reddish colour indicates presence of carbohydrate (Molish test)

Fig B. Formation of red brown precipitate indicates presence of carbohydrate (Benedict test)

Fig C. Blue colour indicates presence of starch (Iodine test)

Fig D. Formation of purple colour indicates presence of protein (Ninhydrin test)

Fig E. Appearance of yellow colour indicates the presence of flavanoids (Alkaline test)

Fig F. Formation of raddish brown precipitate indicates the presence of alkaloid (Wagner test)

Fig G. Creamy white precipitate indicates the presence of alkaloid (Mayer test)

Fig H. Appearance of rosy pink colour indicates the presence of anthraquinone (Borntrager's test)

Fig I. Formation of foam indicates of flavnoids (Foam test)

Discussion

Over the centuries, medicinal plant has become an important part of daily life despite the progress in modern medical and pharmaceutical industry.

The growing interest in herbs and their ability to offer economical uses is part of the movement towards greener economics and life styles. This movement is based on the belief of that the plants have a vast potential for their uses as a curative medicine.

Medicinal plant will also maintain their importance in the search for new, valuable sources of drugs and lead compounds. In the view of the steadily rising demands on these valuable drugs, attention should be paid to adopt alternative method for rapid multiplication.

In recent years, culture of plant cell and tissue has gain considerable importance as a promising tool for multiplying and improving medicinally important plants and also to obtain genetically pure elite population under *in-vitro* condition.

Micro propagation through tissue culture provides fast and dependable methods for production of large number of uniform plant let in short time. The plant multiplication can continue throughout the year irrespective of season and the stocks of germplasm can be maintained for longer period.

Apocynaceae family plants are used for medicinal purpose due to the presence of several bioactive compounds in different part of the plant. The growing demand of medicinally important plants has caused a serious reduction in native population due to over harvesting and deforestation.

For present study, we selected *Catharanthus roseus*whichcontains a wide range of medicinally important alkaloids such as Vinblastine and Vincristine. These alkaloids

are used in the treatment of cancer. The other alkaloids such as Ajmalicine and Serpentine are also used as sedative compounds.

Pharmaceutical production of this valuable alkaloid (Vinblastine, Vincristine, Ajmalicine and Serpentine) exploits plants of *Catharanthus roseus* as a raw material. However, tissue culture is an alternative approach for large scale multiplication of this desired plant.

Morphogenic effect of different growth hormones on different explants of *Catharanthus roseus*:-

Leaf segment, nodal portion and root tips of*Catharanthus roseus* were taken as explants to see the morphogenic effect of various auxins (IAA, IBA, NAA, 2, 4-D) and cytokinins(BAP and Kinetin) added singly in MS medium.

In present study, shoot initiation was found from both nodal portion and leaf explant on MS medium supplemented with different concentration of cytokinin (BAP and Kinetin). This observation of our study is similar to the report of **M. Naeem(2017).** In their study, they observed initiation of multiple shoots in cultures having various concentrations of cytokinins (BAP and Kinetin).

Similar to our findings, study of **E. I. Mokaden(2012)**and **A. Swanberg et al(2008)** states that from leaf explants, few number of shoot bud initials were formed as compared to nodal explants on MS medium supplemented with Cytokinin(BAP and Kinetin).

In our study, when nodal portion was treated with cytokinin (BAP and Kinetin), we observed a very low number of shoots produced on medium treated with alone kinetin.In our experimental work BAP was proved to be superior than the Kinetin in regeneration of shoots from nodal portions. This findingis similar to the report made

by **Karim et el in 2003**, who too found that Kinetin did not show encouraging results as far as multiple shoot formation is concerned from nodal portion and leaf explant.

Similar report was reported by **Fadilah(2011)** that when BAP was used alone, cultures showed good number of shoots in comparison to Kinetin.

But the report of **A. Kumar et al(2009)** is contrary to our findings. According to them, Kinetin is solely responsible for profuse shoot bud initials in their study.

Very similar to our results, in study of **D. K. Kar** and **S. Sen (1985),** BAP used singly acts as a good shoot bud inducer at higher concentration. In other words BAP is favourable for multiple shoot induction.

Superiority of BAP in terms of shoot induction to other cytokinins has also been confirmed in other plants like ***Arachis hypogeal*** **(Matre et al, 1987**).

In present study, no callus initiation was observed from both leaf and nodal portion in BAP supplemented medium but the report of **K. Rani et al (2011)** is contrary to our findings, Where BAP singly showed callus induction from both the explants leaf and nodal portion.

As in our experiment, callus induction was observed on MS medium supplimented with kinetin, NAA and 2, 4-D used solely, similar results were also obtained in research report of **K. Rani at al(2011).**

In present experiment, auxins failed to induce shoot bud formation from leaf explants.Contrary to our result, in report of **Swanberg and Dai(2008),** in NAA

supplemented medium some shoot bud initiation from leaf explants was observed but in our result shoot initiation was totally absent from leaf explants in NAA supplemented medium.

Similar to our study, **R. Singh et al (2011)** also reportedi n *Catharanthus roseus* that auxins induced shoot bud formation from nodal explants but IBA did not trigger shoot initiation from nodal explants.

In present experiment, root tips did not show any response on any of the growth hormone which is similar to the report of **M. S. Biradar** and **Co workers(2011).**

But dissimilar to our experiment, in report of **K. Rani et al(2011),** for root tip explant, high level of auxin is best for induction of shoots and for further growth, high cytokinin is required.

Morphogenic effect of combination of auxin and cytokinin on shoot induction and proliferation from nodal explants of *Catharanthus roseus*:-

On combination of cytokinins, good number of shoots were recorded on low concentration of BAP(0.5 mg/l)+ kinetin(2.0mg/l) but shoots were not healthy and did not grow further similar report was observed by **M. Sharon** and **Coworkers(2011).**

Cytokinin in combination with auxin also play important role for shoot formation. In our study, different concentration of BAP in combination with different auxins concentration was used for shoot induction.

MS medium when fortified with auxin and cytokinine in different combination showed variation in the number of shoots formed. In present experiment, when nodal explants were treated with combination of BAP and NAA, average number of shoots was

observed and highest numbers of shoots were observed on MS medium supplemented with 0.5mg/l BAP with 1.0mg/l NAA.

In report of **Kanta Rani et al(2011),** maximum growth of shoots from nodal explant of *Catharanthus roseus* was observed on MS medium supplemented with 1.5mg/l BAP + NAA (1.0mg/l). Similar results were seen in our experiment also. But contrary to our report where we observed combination of BAP with NAA was favourable for shoot multiplication, report of**A. Kumar(2009)** says that BAP in combination with NAA did not respond satisfactory, where as shoot multiplication is concerned.

When 2, 4-D, IAA and IBA were used in place of NAA with BAP, good number of shoot formation was observed on combination of BAP and IBA. These result of our study are similar to the report made by **M. Rajib(2006).**

In present experiment, nodal explants of *Catharathus roseus* were inoculated on MS medium with various concentration of kinetin in combination of NAA and 2, 4-D. We observed a good number of shoots were formed on MS medium supplemented with combination of kinetin and NAA.

In report of **S. R. Mehta** and **S. D. Subramaniam(2005),** multiple shoots were obtained on MS supplemented with NAA (0.46µm)with kinetin (0-27µm), after 3 weeks of inoculation. In their study further increase in the concentration of NAA and kinetin did not havebetter results as we noticed in our study.

Morphogenic response of nodal segments taken of (different size and different positions) along the stem length of *Catharanthus roseus* L. :

In our study, larger size of nodal explants that is 1.5cmlong induced good number of

shoots than the nodal segments taken of small size(1.0cm).Among the different position of nodal explant along the stem length (basal, middle and distal), middle position of nodal segments showed good number of shoot formation in comparison to segment taken from other positions which were distal and basal. Similar findings were observed by **A.Swanberg** and **W. Dai (2008),** where position was deciding factor for shoot formation.

Nhut et al(2007) reported that size and position of nodal explants along stem length probably played critcal role in shoot regeneration because larger tissues had more nutrient reserves, which can promote more shoot differentiation and middle position of nodal explants have normal concentration of auxin than other position(basal and distal). This might be the reason of shoot proliferation from middle position of nodal segment in our study too.

Rooting response of *in-vitro* raised shoots of *Catharanthus roseus* in MS medium supplemented with different concentrations of growth hormone:-

In the present experiment, *in-vitro* raised shoots were transferred on ½ strength MS medium supplemented with different concentration of NAA, IAA and IBA.

On half strength of MS basal medium, root emergence from *in-vitro* raised shoots was totally absent in our experiment but in the report of **A. Kumar(2009),** roots were induced and elongated in half strength MS medium which is dissimilar to our study.
In our study, we concluded that IBA triggered early rooting inducion as compare to NAA and IAA added singly.

When the culture were left for more than 5 weeks in rooting medium, highest rooting response was observed on IBA supplemented medium as compared to other auxin treated medium (IAA, NAA). This finding is similar to report made by **Kanta Rani et**

al(2011) on *Catharanthus roseus*, there also IBA was preffered for root induction.

Contrary to our experiment, report of **Fadilah(2005)** stated that NAA was the best rooting induction medium compare to IBA for *Catharanthus roseus*.

Report of **Nari (2009)** stated that IAA was completely ineffective in root induction which is dissimilar to our study where, IAA stimulates the root initiation.

In our experiment, half strength MS medium with IBA was taken as the standard medium for root proliferation and different energy sources like sucrose, glucose and fructose were added saperatly to the standard MS medium for root proliferation. We found that sucrose is most promising energy source for root proliferation in comparison to other energy sources (glucose and fructose) because sucrose can efficiently move across the cell wall.

Similar to our result, in report of **M.Zahara et al.(2017),** sucrose was found to be the most promising energy source for root proliferion.

During our study, we tried to see the effect of that tryptophan (amino acid) on root proliferation. We noticed that high concentration of tryptophan enhanced root proliferation being the best in our experiment even better than IBA. When we subjected the cultures having tryptophan to dark period and compared the result.

Dark period of 20 hours triggered root proliferation. In other words, longer dark period enhanced root proliferation in cultures compare to shorter dark period that is 12 hours and 16 hours. The reason behind the good root proliferation in dark period may be etiolation occurs which dilutes the component of cell wall. This process increases the intake of the outer growth hormone into the cell resulting in the root proliferation.

On increasing the tryptophan concentration, soluble sugar content in roots is also gradually increased that is due to the role of tryptophan in biosynthesis of chlorophyll pigments.

Our result revealed that increase in tryptophan concentration enhanced root proliferation. Our findings are similar with the report made by **S. Rahmatzadeh et al (2014).**

Keeping the explants in the dark enhanced organogenesis was also evidenced in other species too **(Choi et al, 2001; Daiand Castillo, 2007**).

Similar to our result, **A.Swanberg** and **W.Dai(2008)** reported that long duration of dark period (20/4 hr) have more stimulatory effect on root proliferation compare to shorter (12hr and 16hr) dark period.

In-vitro raised planlets were gradually brought to field condition after hardening procedure and acclimatization. Mostly researchers preferred hardening and acclimatizatoin before transfer in vitro grown plantlets in to field.

In the experiment of **A.K.Jain** and **Coworker 2014,** the plantlets were transferred in pot having vermiculite culture and peatmoss and gradually transferred in field condition.

We could transfer very few healthy plants to field condition.We are trying to transfer for more number of in vitro regenerated plants to field condition. The result of our experiments confirm that *in-vitro* propagation can be proved as an alternative source for large scale shoot multiplication and plant regeneration to fulfill the rising demand of *Catharanthus roseus* which is our experimental plant.

Somatic embryogenesis acts as an alternative to organogenesis for regeneration of

plants. In our study profuse somatic embryogenesis was observed on MS supplemented with combination of 2,4-D (1.0 mg/l) with kinetin (1.0 mg/l) from leaf explants. We made artificialseeds from these somatic embryos by encapsulation with sodium alginate and calcium chloride and subsequently preserved at low temperature.

Similar to our result, an encapsulation of embryos to obtain synthetic seed was established in *Catharanthus roseus*. Leaf derived callus containing somatic embryos were encapsulated in sodium alginate and calcium chloride solution. (**M.Maqsood et al, 2012).** Sodium alginate acts as an artificial endosperm, providing nourishment to the growing embryos, protecting embryos from damage and facilitates embryo's germination.

In other experiment we made qualitative analysis of leaf of *Catharanthus roseus* was performed to find out the presence of various medicinally important bioactive principles present in *Catharanthus roseus*.

In our study, we confirmed the presence of carbohydrates, protein, antraquinone, alkaloids, saponins, flavanoids in leaf powder of *Catharanthus roseus* which was confirmed by various test.

Phytochemical analysis of leaf of *Catharanthus roseus* reveals the presence of alkaloids, terpenoids, saponin, quinine, protein, flavnoids and steroid. Presence of these bio active compounds was also confirmed by thin layer chromatography in *Catharanthus roseus***(K.Kabeshetal, 2015).**

In leaf powder of *Catharanthus roseus,* carbohydrates and protein were absent in report made by **D.Paikar et al, 2017.** But our experiment shows presence of carbohydrates and protein in leaf of *Catharanthus roseus*.

Conclusion

Plants remain an importance source for medicine since ancient time. Among the valuable medicinal plant, *Catharanthus roseus* is an important anti-cancerous drug yielding plant. *Catharanthus roseus* has more than 130 known alkaloids which are used in the treatment various types of cancer and dread diseases.

Demand of pharmaceutical industries and other multiple uses have led to over exploitation of this medicinally potent plant. Tradition cultivation method does not fulfill the growing demand of the market.

Therefore, alternative method is required for large scale production of uniform plant material of anti-cancerous drug yielding plant *Catharanthus roseus*.

The protocol developed through the present study will be useful for large scale multiplication of *Catharanthus roseus*.

The present effort is a successful attempt of developing rapid and reproducible *in-vitro* micropropagation system from nodal explants. Size and position of nodal explants along the stem length play significant role in shoot formation and elongation. Half strength MS medium supplemented with IBA has been proved best medium for rooting response.

In our experiment, effect of different energy sources(glucose,fructose and sucrose) and tryptophan amino acid along with dark period on rooting response was studied and monitered.

We standarised the favourable media for fast growing callus and its proliferation. Further we also made attempt to see the effect of natural additives(banana homogenate and coconut water) on callus proliferation raised from leaf explants of *Catharanthus roseus*.

In the present study, we also synthesis artificial seeds by encapsulation of somatic embryos with gelling agent sodium alginate and calcium chloride. Prolific callus could be obtained on combination of moderate concentration of 2, 4-D and kinetin both.

To conclude, we have devised a protocol of plant regeneration from different explants using various growth regulators. During the study, we reach the coclusion that combination of cytokinin and auxin, size and position of nodal explants, types of energy source (sucrose, glucose and fructose) and tryptophan along with dark period cycle (12hrs,16hrs and 20hrs) was also affected the regeneration capacity of *Catharanthus roseus*.

Qualitative analysis of dried leaf was done to detect the presence of various bioactive chemicals responsible for therapeutic actions of *Catharanthus roseus*. This study also strength our knowledge of herbal drugs with their scientific bases.

The above result confirm that *in vitro* culture techniques are an alternative method for lagre scale multiplication of *Catharanthus roseus.*

The integrated approaches of this culture system will provide the basis for future development of novel, safe, effective and high quality products for consumers and pharmaceutical industries.

Application for society:-

Catharanthus roseus is an anti ancerous drug yielding plant. It produces several commercially valuable metabolites including the anticancer agent vinblalstine, vincristine and hyper intensive drugs ajmalicine and serpentine. Its extracts are used in various dermatological preparations, so it has developed into a true mlti-purpose plant. Our work on micro propagation of *Catharanthus roseus* is a step to meet the growing demand of plant used as a raw metirial for pharmaceutical purpose.

Our devised protocol for large scale multiplication and *in vitro* regeneration of these plants would definitely help as source of tissues for the biochemical characterization of medicinally active compounds and will increase the opportunities for the use of this plant in both traditional and modern health care.

The protocol used in present study is simple, easy to carry out and can provide optimum conduction for callus enhanced yields of bioactive principles in pharmaceutical industry for drug prepration.

Future prospectus:-

1. Optimization of other culture conditions as (light, temperature, aeration, elicitors, biotic and abiotic) are to be used to increase alkaloids production in *in vitro* grown plants.
2. Comparative biochemical analysis of in-vitro and in-vivo plant.
3. Genetic modification can be very useful for the production of desire level of secondary metabolites.
4. Salt tolerance varieties can be screened using physiological parameters.
5. Hairy roots culture can be developed by biotechnology for large scale alkaloid production.

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Summary

Medicinal plants are the most valuable source of life saving drugs since ancient time. They contain a fascinating array of natural products with varying level of bioactive principle. Today, renewal interest in herbal products increased a great demand of plants products within and outside the country. So, many medicinally important plants are being considered "Endangered".

Catharanthus roseus L. is a medicinal plant with a history of anti-cancerous property. Cancer is one of the most dreaded diseases of the present time. Due to over exploitation and extensive use of *Catharanthus roseus,* we have to look for the alternative to keep pace with the growing demand of this medicinally valuable plant. So we, focused our study on *Catharanthus roseus* (L.) an anti cancerous drug yielding plant.

To meet the rising demand, we need the raw plants material in adequate amount, so we require an efficient protocol for large scale multiplication of *Catharanthus roseus*.

Tissue culture technique is an efficient tool for large scale multiplication of this anticancer medicinal plant in short time, limited space and irrespective of season. Through this technique, we can get uniform plant material and genetically superior disease free plants. Germplasm conservation can be achieved with this technique. Such plant can be used for the extraction of bio active compounds for pharmaceutical purpose.

About the experimental plant–*Catharanthus roseus* is an important medicinal plant of apocynaceae family. It is also know as "**Sadabahar" or Madagascar rosy Periwinkle**. It is our experimental plant

Catharanthus roseus is native of the Indian Ocean Island of Madagascar. It is a perennial, evergreen herb and cultivated throughout tropical and subtropical parts of India, including Africa, Australia and South European countries. In the wild, Now it is said to be an endangered plant and the main cause of their decline is the habitat destruction by the slash and burn agriculture.

Catharanthus roseus contains a virtual cornucopia of useful alkaloids known as "Vinca alkaloids", which are used in treatment of various disease like diabetes, Cancer, blood pressure, constipation and menstrual problems. But it is mainly known for its anti cancerous properties. "**Vinblastin"** and **"Vincristine"** alkaloids of *Catharanthus roseus* have been shown to be effective in the treatment of different type of cancer specially childhood leukemia and Hodgkin.

Due to multiple uses, the demand of *Catharanthus roseus* is constantly on the rise However, the supply is rather erratic and inadequate which forced us to take this plant as our experimental plant. To meet the growing demand of this plant for pharmaceutical industry and also for other purposes to mankind, large scale multiplication of this plant has become the need of present time. So, we selected *Catharanthus roseus* as our experimental material.

The experimental study was done in following steps:

Explant culture:-Effect of cytokinins (BAP and Kinetin) and auxins (NAA; IAA, IBA and 2, 4 – D) used singly on different explants of *Catharanthus roseus*.

Leaf segment, **nodal explantand root tips** of *Catharanthus roseus*were taken as explants to see the morphogenic effect of various concentration of cytokinins (BAP and Kinetin) and auxin (NAA, IAA, IBA and 2, 4 – D) added singly in MS medium. In root tip explants, there was no response observed (neither callus or nor shoot formation) on all tried concentration of cytokinins and auxins used singly.

Leaf Segment explants

(a) Effect of cytokinins and auxins –

In this set of experiment from leaf segment explants, shoot bud formed in BAP supplemented medium but there was no callusing observed even after 6 weeks. Scanty callus with few shoot bud was recorded on higher concentration of kinetin (1.0 mg/l and 3.0 mg/l) from leaf segment explants. Scanty callus was induced on all tried concentration of NAA (0.5, 1.0 and 3.0mg/l). Neither callusing nor shoot formation was occurred on all tried concentrations of IBA nor IAA from leaf explants. On 0.5 mg/l – 3.0 mg/l2, 4–D supplemented MS medium, prolific callus formation was noticed from leaf explants. This little callus was whitish and greenish at few sites.

(b) Subculturing of callus

Callus raised from leaf explants of *Catharanthus roseus* on MS medium supplemented with 2, 4-D (3.0 mg/l) was left for few more weeks to grow. When callus grew in good amount, few cultures were maintained to have stock callus and callus of rest cultures were cut into small pieces and placed into freshly prepared medium with combination of different concentration of 2, 4–D with kinetin and BAP. Good response of morphogenesis and organogenesis could be noticed on 2, 4-D (1.0 mg/l) combination with kinetin (1.0 mg/l). After one month, profuse shoot formation was observed and good culture response was observed on this combination.

(c) **Effect of natural additives on callus proliferation**

When callus raised from leaf explants of *Catharanthus roseus* was placed on MS medium supplemented with 2, 4-D (3.0 mg/l) with various natural additives like homogenate of Banana and Coconut water.

The type and concentration of natural additives influenced callus proliferation. Among two additives, Coconut water at 10% concentration was the most effective in promoting the growth of callus. High concentration of coconut water was found to be inhibitory for early regeneration and callus was non embryogenic.

Nodal Explants

(a) Effect of cytokinins and auxins

When nodal explants of *Catharanthus roseus* were inoculated in MS medium supplemented with different concentration of BAP, Kinetin, NAA, IBA, IAA and 2, 4-D added singly. Comparative study showed that number of shoots and length of shoots were higher in BAP supplemented MS medium than kinetin. In NAA supplemented cultures, shoot emerged from nodal portion but they turned brown and they did not grow further. On high concentration of IAA and 2, 4-D, low percentage of culture response was recorded. IBA was not favourable for shoot formation.

(b) Effect of combination of cytokinin and auxin

Among all tried combination, BAP (0.5 mg/l) + NAA (1.0 mg/l) was proved best combination for multiple shoots formation. In this combination, tillering of shoots was observed and length of shoots was approximately 5-6cm. On 0.5 mg/l BAP + 2.0 mg/l kinetin supplemented media also showed shoots formation.

(c) Effect of positions and size of nodal explants

Different sizes of nodal explants (1.0 cm, 1.5 cm and 2.0 cm) and relative positions of node along the stem length of *Catharanthus roseus* (Basal, middle and distal order) were cultured on MS medium supplemented with BAP (3.0mg/l). The best shooting was obtained from 1.5 cm long nodal explants located in the middle order position along the stem length of *Catharanthus roseus*.

(d) Root induction on *in vitro* regenerated shoots

Among all tried concentration of IAA, Cultures supplemented with 2.0 mg/l IAA in MS medium showed positive response but percentage of cultures responded was significantly low. Good response of rooting was observed on 5.0 mg/l IBA supplemented with ½ strength MS medium. Various concentrations of NAA and BAP failed to induce rooting in *in-vitro* regenerated shoots of *Catharanthus roseus*.

(e) Effect of different energy source on root proliferation:

When half strength MS medium with IBA (3.0 mg/l) was selected as the standard medium for rooting and different energy sources like sucrose, glucose and fructose were added singly to the standard medium for root proliferation. After 15 days of inoculation, glucose and fructose did not have conductive effect on root proliferation but sucrose containing cultures showed better response, as sucrose triggered good number of root formation with elongated rapidly later. After 45 days *roots* appeared long thread like and branched.

(f) Effect of dark period and tryptophan concentrations on root proliferation

In this experiment tryptophan amino acid was introduced with ½ MS medium + IBA (3.0mg/l) with two different concentrations of tryptophan (150ml and 250ml). These cultures were subjected to three different cycles of dark period that are 12hrs, 16hrs and 20hrs.

After 3 weeks of inoculation, in 250ml tryptophan added standard medium, simultaneous rooting response was lower in 12hr duration of dark period but tended to increase as consequences of higher duration of dark period up to 20 hrs.

(g) Hardening of *in-vitro* raised plantlets of *Catharanthus roseus*

In-vitro raised plantlets were taken out from culture flasks aseptically and roots were washed with autoclaved water gently and carefully.

These *in-vitro* regenerated plantlets were then shifted to plastic container having a mixture of sand, soil and peat moss and covered with another plastic container to save from harsh environment condition. After hardening procedure these plantlets were transferred to field.

Synthetic seed formation:-

Plant propagation using artificial or synthetic seed developed from somatic and non zygotic embryos have a great importance in plant conservation.

In synthetic seed formation, encapsulation of somatic embryos formed *in-vitro* are encapsulated by gelling agent for long term storage, easy handling and easy transport across the border.

Very good somatic embryogenesis was observed on MS medium supplemented with combination of 2, 4-D (1.0 mg./l) with Kinetin(1.0 mg/l). These fully developed somatic embryos were teased and separated from each other outside the flask under aseptic condition and used for encapsulation by using gelling agent sodium alginate (2.5 %) and calcium chloride (1.5%) to from synthetic seeds.

Qualitative analysis for detection the presence of bioactive chemicals present in ethanolic extract of leaf powder of *Catharanthus roseus*

(a) Preparation of ethanolic extract

Extract of leaf powder was prepared. Firstly, dried leaves were mechanically pulverized by mortor and pestle or converted into powder form. This powder was dissolved in 95% ethanol.

(b) Test for detection of various bioactive chemicals

Qualitative test were performed for detection of bioactive chemicals present in ethanolic extract of leaf powder of *Catharanthus roseus*. After analysis, we could detect carbohydrate, starch, flavanoids, protein, alkaloids, saponin and anthraquinone are present in leaves of *Catharanthus roseus*.

Bibliography

A Giri, Y. Dhingra, C.C.Giri, A singh, O.P. ward and M.L. Narasu (2011). **Biotransformation using plant cells, organ cultures and enzyme systems: Current trends and future prospects.**Biotechnol. Adv.,19;175-199.

A. A. Mao ,(2002).***Oroxylum indicum vent*** **A potential anti cancer medicinal plant**. Ind .Jrn.Tra. Knowledge, 1(1); 17-21.

A. EI-Sayedemd and G. A. Cordell ,(1981).***Catharanthus***; **A new antitumor bi indole alkaloid from *Catharanthusroseus* .J. Nat. Prod., 44; 289-293.**

A. K. Shukla,(2005).**Molecular studies on biosynthesis of shoot alkaloids in *Catharanthus roseus* (L)G. Don.**Ph.D thesis, Department of Lucknow, India.

A. Sharma, S. Bansali and A. Kumar,(2013**). *In-vitro* Callus induction and short regeneration in *Eclipeta alba*(L)**. Hassk, Int. J. of life Sci. and Pharma Res.,3(2);22-40.

A.EI-Sayed and G.A. Cordell, (1981). **Catharanthus alkaloids. XXXIV; Catharanthamine, a new antitumor bisindole alkaloid from *Catharanthus roseus*.** Journal of Natural product , 44(3) ; 289-93.

A.Giri and M.L.Narasu, (2000). **Transgenic hairy roots : recent trends and applications.** Biotechnol. Adv., 18; 1-22.

A.H.Scragg and S.Ashton, (1990). **Growth of *Catharanthus roseus* suspensions for maximum biomass and alkaloid accumulation**. Enzyme and microbial Technology ,12; 292-298.

A.Junaid, A. Mujib, F.Zohra and M.P. Sharma, (2010). **Variations in Vinblastine productionat different stages ofsomatic embryogenesis, embryo and field grown plantlets of *Catharanthus roseus* L. (G) Don, as revealed by HPLC**,*In-vitro* cell. Dev. Biol. Plant, 46:348-353.

A.K. Jain and Dinesh K.Meena, (2014).**In-vivo and In-vitro comparative study of primary metabolites and anti oxidant activity of *Catharanthus roseus***, Asian Journal of plant Sci. and Res., 4(2); 42-46.

A.K. Verma and R.R. Singh, (2010).**Induced dwarf mutant in *Catharanthus roseus* with enhanced anti bacterial activity,** Indian J. of Pharm. Sci., 72(5); 655-657.

A.K. Verma, R.R singh and S.Singh, (2013).**Mutation breeding in *Catharanthus roseus* (L) G.Don : An overview,** Journal of pharmaco. and phytochemistry, 2(1); 334-336.

A.Kruczynski, J.M. Barret, (2002). **Novel aspects of natural and modified Vinca alkaloids.** Current Medicinal chemistry-Anti-Cancer Agents, 2:55-70.

A.kyakulaga ,T.Alinda, V.P. Brenda and O.E. Patrick, (2011).**Invivo anti diarrheal acitivity of the ethanolic leaf extract of *Catharanthus roseus* Linn. (Apocynaceae**) **in wistar rates**. African Journal of pharmacy and pharmacology, 5 (15) ; 1797-1800.

A.Pietrosiuk, M. Furmanowa and B.Lata,(2007). ***Catharanthus roseus:* micropropagation and *in-vitro* techniques.** Springer Sci., 6: 459-473.

A.Roy Maithy, N. Banerjee and S. Mandal, (2005).**Structural and functional parameters of mating and seed germination in mutants of *Catharanthus roseus* (Apocynaceae).**J.Apple. Bio.Sci., 31(2); 145-149.

A.V. Patel, I.Pusch, G.Mix-Wagner and K.D. Vorlop (2000). **A novel encapsulation technique for the production of artificial seeds.** Plant cell Rep.,19; 868-874.

Anonymous, (2010). **Achieving 2010 Biodiversity Target : India's Contribution. Report, Ministry of Environment and Forest, Govt. of India, New Delhi.**

Ashwin B. Keshirsagar and V.D. Deshmukh, (2011). **Regeneration of *Asparagus racemous* L.through tissue culture.** IndianJournals com. Bio.inflote.,8(1); 61-64

AzraAtaei-Azimi, B .D. Hashemloian, H. E.brahimzadeh and A. Majd, (2008).**High in vitro production of anti canceric indole alkaloid from periwinkle (*Catharanthus*) Tissue culture .**African Journal of Biotechnology,7(16);2834-2839.

B. Deus Neumann, J. Stockigt, M. H. Zenk ,(1987). **Radio immune assay for the quantitative determination of catharanthine.** Plant medica, 53;184-188.

B. R. O.Keefe, G. B. Mahady, J. J. Gills and C. W. Beecher ,(1997). **Stable vindeline production in transformed cell cultures of *Catharanthus roseus*.**J.Nat. Prod., 60;261-264.

B. Tal, J.S. Rokem and I.Gold berg, (1983**). Factors affecting growth and product formation in plant cells grown in continuous culture.** Plant cell Rep. ,2; 219-222.

B.D. Singh, (2003). **Plant tissue cultures. Biotechnology.** KalyaniPubli**.**, 16; 204-224.

B.Ghosh and S.Sen, (1994). **Plant regeneration from alginate encapsulated somatic embryos of *Asparagus* cooperi baker**. Plant cell Rep., 13: 381-385

B.P. Pandey, (1988). **An Introduction to economic botany.** S.Chand and Comp. (Pvt.) Ltd., 8; 166-169.

B.Pierre, F.A. Vizquez- Flota and V.DeLuca, (1990). **Multicellular Compartmentation of *Catharanthus roseus* alkaloid biosynthesis predicts intercellular translocation of a pathway intermediate.** The plant Cell, 11, 887-900.

C. A. Jaleel, R. Gopiand R. Paneerselvam ,(2009).**Alterationism non enzymatic antioxidant component of *Catharanthus roseus* exposed to paclobutrazol, gibberellic acid and pseudomonas fluorescens.**Plant Omics. J., 2;30-40.

C. H. Chenm, N. E. Stenberg and J. G. Ross, (1977). **Colonal propagation of big blue stem by tissue culture.** Crop Sci.,17;847-850.

C.A. Jaleel and R.Panneerselvam, (2007).**Variations in the antioxidative and indole alkaloid status in different parts of two varities of *Catharanthus roseus*: An important folk herb.**Chinease J.of pharmacology and Toxicology, 1(6); 487-494.

C.Kalidas, V.R. Mohan and A. Daniel, 2010. **Effect of auxin and cytokinins on vincrustine production by callus cultures of *Catharanthus roseus***L. (**Apocynaceae**). Trop. Subtrop. Agro. ecosystems ,12; 283-288.

C.P. Kala, (2006). **Medicinal plants of the high altitude cold desert in India; diversity, distribution and traditional uses.** InternationalJ**.** of Biodiversity Science and Management, 2(1); 43-56.

C.P. Malik, (2007**). Applications of biotechnology innovations in pharmaceuticals and nutraceuticsim multitherapetic medicinal and special plants**. Aavishkar Publisher Jaipur, 2; 243-265.

C.W.T Lee-Parsons and A. J. Rogee ,(2006). **Precursor limitation in methyl jasmonate-induced *Catharanthus roseus* cell culture.** Plant cell Resp.,25;607-612.

Cheruth Abdul Jaleel, (2009).**Soil Salinity regimes Alters Antioxidant Enzyme Activities in two varieties of *Catharanthus roseus*.** Botany research International, 2(2) ; 64-68

D. Favretto , A. Piovan and R. Caniato ,(2011).**Monitoring the production yields of vincristine and vinblastine in *Catharanthus roseus* from somatic embryogenesis semiquantitative determination by flow injection electrospray, ionization mass spectrometry.**Rapid communication in mass spectrometry ,15(5) ;364-369.

D. Pahwa, 2009. **Catharanthus alkaloids**. Scribd, 1-9.

D. W. Lane, (1979). **Regeneration of Pear plants from shoot meristem tips. Plants** Sci. Lett., 16; 337-342.

D.C.W. Brown and T.A. Thorpe, (1995). **Crop improvement through tissue culture.** World J. Microbiol. Biotechnol. ,11; 409-415.

D.W.S. Mok and M.C. Mok ,(2001).**Cytokinin metabolism and action.** AnnualReviewofplantphysiologyandmolecularBiology,52; 89-118.

E. M. Linsnaier, F. Skoog, (1965). **Organic growth factor requirements of tobacco tissue cultures.** Physiol plant, 18; 100-127.

E.F. Vachin and F.W. Went, (1949**). Some pH changes in nutrients solutions**. Bot. Gaz., 110; 605-618

F. Constabel, S. Rambold, K. B. Chatson, W. G. W. Kurz and J. P. Kutney ,(1981). **Alkaloid spectra of cell lines derived from one single leaf.**Plant cell report, 1; 3-5.

F. Sasse, K.H. Knobloch and J. Berli, (1982). **Introduction of secondary metabolism in cell suspension cultures of *Catharanthus roseus, Nicotiana tabaccum* and *Peganum larmala*.** 5th Intl. Cong. Tokyo,343-344.

F. Skoog and C. O. Miller, (1957). **Chemical regulation of growth and organ formation in plant tissue cultures *invitro*.** Symp. Soc.Exp.Biol., 11; 118-131.

G. Blasko, G. A. Cordell ,(1997). **Isolation, Structure, Elucidation and biosynthesis of the bisindole alkaloids of *Catharanthus roseus*.** In: Brossi A. Suffness M. eds. the alkaloids Vol.37 San Diego, CA; Academic press, 1-76.

G. M. Gragg and D. J. Newman, (2005).**Plants as source of anticancergents.** J. Ethnopharmacal,100(1-2);72-79.

G.H. Svoboda and D.A. Blake, (1975).**The phytochemistry and pharmacology of *Catharanthus roseus* (L) G.alkaloids**. Marcel Decker, New York , PP. 45-82.

G.M. Gragg and D.J. Newman, (2005). **Plants as a source of anti-cancer agents**. J.Ethnopharmacol,100; 72-79.

G.S. Panwar and S.K. Guru, (2011). **Alkaloid profiling and estimation of reserpine in *R.serpentina* Plant by TLC, HP-TLC and HPLC.** Asian J. of plant Sci. ,393-400.

G.Taylor, (1968). **Introduction of symposium on vincristine.** Cancer Chemother. Rep., 52; 453-460.

H. E.brahimzahed, A. Ataei-Azimi and M. R. Noori-Daloi,(1996).**The distribution of indole alkaloids in different organ of *Catharanthus roseus* G. Don.** J. Sch. Pharm.,6(1,2);11-24.

H. Lipavaska and D. Vreugdentil, (1996). **Uptake of mannitol from media by *in* vitro grown plants.** Plant cell tissue org. cult., 45; 103-107.

H.E. Street, (1966). **The nutrition and metabolism of plant tissue and organ cultures , methods, biology and physiology**.Ed. Wilmer, E.N., 534-629.

H.Hamada, H.Yasumune, Y.Fuchikamic, T.Hirata, I.Sattler,H.J.Williams and A.I.Scott(1997**). Biotransformation of geraniol, nerol(+) and carvone (-) by suspension cultured cells *Catharanthus roseus***, phytochemistry, 44; 615-621.

H.O. Kodja ,D.Liu, J.M. Merillon, F. Andreau, M. Raideav and J.C. Chemieux, (1989). **Stimulation of cytokynins, accumulation of indole alkaloids by suspension culture of *Catharanthus roseus* (L) G.Don.** Z. Natur forsch, 35; 551-556.

H.Rukhama, N. Shagofta, F. Aslam and F.Manzoor, 20013.**Comparison of *in-vitro* response of micropropagation and callogenesis of medicinal plant, Vinca Rosea.** J.Agric. Res., 51(1); 9-17.

H.S. Nyandieka,(1978). **Searches for clinically Expoitable, Biochemical differences between normal and cancer cells, Glyoxalase system in malingnancy and its relation to cellular proliferation.** Clin.Biochem., 11; 150-155.

H.S. Taha, M.K. El-Bahr and M.M.S. El-Nasr, (2008). ***In-vitro* studies on Egyption *Catharanthus roseus* (L) G. Don,: Calli production, direct shootlets regeneration and alkaloid determination**. J.Appl. Sci. Res., 4(8) ; 1017-1022.

I. S. Johnson, J. G. Armstrong, M. Gaman and J. P. burnett ,(1963**). The vinca alkaloids; A new class of oncolytic agents**.Cancer res., 23; 1390-1427.

Ili Asaka, M.Hirotani, Y.Asada and T.Furuya, (1993). **Production of ginsenoside saponins by culturing ginseng (*Panax ginseng*) embryonic tissue in bioreactors. Biotechmol.** Lett. 15; 1259-1264.

J. Ahmadi, R. Mohammadi and Gh.A. Garousi, (2014**). *Invitro* Micropropagation of *Catharanthus roseus* (L) G.Don. via shoot multiplication.** Journal of Molecular and cellular research, 27(1); 14-25.

J.Balsevich,(1985),**Biotransformation of 10-hydroxyeranial and related compounds by a cell suspension culture of *Catharanthus roseus* ; the formation of reduced products.** Plant Med., 51; 128-132.

J. Balsevitch, L.R. Hogge, A.J. Berry. D.E. Games and L.C. Mylchreest ,(1988). **Analysis of indole alkaloids from leaves of *Catharanthus roseus* by means of supercritical fluid chromatography mass spectrophotometary.** J. of natural products, 51(6); 1173-1177.

J. P. Kutney, L. S. Choi, J. Nakano, H .Tsukamoto, C. A. Boulet and M. Mchugh, (1991).**Process of synthesis of vinblastine and vincristine.**U. S. Pat., 5047528.

J. Prajakta Patil and J.S. Ghosh, (2010).**Anti microbial Activity of *Catharanthus roseus*- A detailed study.** British Journal of pharmacology and Toxicology, 1(1) ;40-44.

J. Zhao, W. Zhu and Q.Hu,(2001). **Enhanced catharanthine production in *Catharanthus roseus* cell culture by combined elicitor treatment in shake flasks and bioreactors.** Enzyme Microbiol. Technol. J.,28;673-681.

J.Aslam, A. Mujib, S.A. Nasim and M.P. Sharma, (2009).**Screening of vincrustine yield in exvitro and *in-vitro*somatic embryos derived plantlets of *Catharanthus roseus* L.(G).Don**. Sci. Hort., 119 ; 325-329.

J.Y. Veda, Y.Tezuka and A.H. Banskota, (2002).**Anti proliferative activity of Vietnamese medicinal plants.**Biological pharmaceutical bulletin, 25(6) ; 753-60.

K. A. Hassan, A. T. Brenda, V. Patrick and O. E. Patrick ,(2011).**In vivo antidiarrheal activity of the ethanolic leaf extract of *Catharanthus roseus* Linn (Apocyanaceae) in Winstar rats.** Afr. J. Pharm. Pharmacol., 5(15);1797-1801.

K. Baskaran, K.V.N.S. Srinivas and R.N. Kulkarni, (2013).**Two induced macro-mutants of periwinkle with enhanced contents of leaf and root alkaloids and their inheritance.**Industries crops & products, 43; 701-703.

K. Hirata, A. Yamanaka, N. Kurano, K. Mijamoto and Y. Muira ,(1987).**Production of indole alkaloids in multiple shoot culture of *Catharanthus roseus*(L.) G. Don.**Agric. Biol. Chem., 51;1311-1317.

K. M. Lila ,(2005).**Valuable secondary products from in vitro culture secondary products *invitro*.**CRC Press, LLC.

L. Hamma and M. Baaziz ,(2001). **Somatic embryogenesis and plant regeneration from leaf tissue of Jojoba.** Plant cell tissue org. cult.,65;109-113.

L. K. Pareek ,(2005).**Trends in plant tissue culture and Biotechnology**.Jodhpur India Agrobios.,350.

L.B.Zhang; L.H. Gou and S.V. Zeng,(2000).**Preliminary study on the isolation of endophytic fungus of *Catharanthus roseus* and its fermentation to produce product of therapeutic value.** Chinese traditional Heral Drugs, 11 : 805-807.

L.R. Muhammad, N. Muhmmad, A. Tanveer and S. N.Bagir, (2009).**Antimicrobial activity of different extracts of *Catharanthus roseus*,.**Clin.Exp. Med. J., 3;81-85.

M .Vanisree, C.YueLee, S. Fung Lo, S. Manohar Nalawade, C. Yin Lin and H. ShengTsay,(2004).**Studies on the production of some important secondary metabolites. Bot. Bull.** Acad. Sin.,45;1-22

M. A. Alba Bhutkar and S.B. Bhise ,(2011). **Comparative studies on Antioxidant properties of *Catharanthus roseus*.** International Journal of Pharmaceutical Techniques, 3(3);1551-1556.

M. H. Zenk, (1997). **Plant tissue culture and its biotechnological application. G**Ed. By W. Barz, E. Reinhard and M. H. Springer, Varlag, New York, pp 1-7.

M. M. Gupta, D. V. Singh, A. K. Tripathi, R. Pandey, R. K. Verma, S. Singh, A. K. Shasany and S. P. S. Khanuja, (2005). **Simultaneous determination of vincristine, vinblastine, catharanthine and vindoline in leaves of *Catharanthus roseus* by high performance liquid chromatography**. Journal of chromatographic Sci.,43;450-453.

M. Magnotta, J.Murata, J.Chen, De Luca V,(2006) .**Identificationof a low vindoline accumulating cultivarof *Catharanthus roseus* (L) G.Don. by alkaloid and enzymatic profiling**. Phytochemistry, 67;1758-1764.

M. Misawa, M. Haryashi and S.Takayama, (1985). **Accumulation of antineoplastic agents by plant tissue culture. In; K.H. Neumann (ed.) primary and secondary metabolism of plant cell cultures.**springer-verlag, Berlin, Heidelberg, PP-235.

M. R. Setty, G. A. Harisha, Y. Jayanath and H. G.Ashok Kumar,(2014) . **Production of secondary melabolites from *invitro* cultures of *Rauwolfia serpentina.*Benth.**Int. J. of Svi. Res. Eng. Tech., 2(12);844 -852.

M. Rafiq, M.V. Pahot, S.M. Mangrio, H.A. Nagvi and I.A. Qarshi,(2007). ***In-vitro* nodal propagation and biochemical analysis of field established *Stevia rebaudiana* Bertoni**. Pak. J.Bot; 39(7) 2467-2474.

M. Smith, H. Kobayashi, M. Gawienowski and D.P. Briskin,(2002).**An *invitro* approach to investigate chemical synthesis by three herbal plants.** plant cell tissue org. Cutt, 70; 105-111.

M. V. Gonzale sand A. G. Tolentino ,(2014).**Extraction and Isolation of the alkaloids from *Acacia* Bark: Its Antiseptic potential.**Inter. J. Sci.& Tech. Res.,3(1);119-124.

M.E. Kuchne, P.A. Matson and W.G. Bornmann, (1991).**Selective synthesis of vinblastine, Leurosidine, Vincovaline and 20—epivincovaline.** Journal of organic chemistry, 56;513-582.

M.Faheem, S.Singh. B.S. Tanwer, M.Khan and A Shahzad,(2011). ***In-vitro* regeneration of multiplication shoots in *Catharanthus roseus*- An important medicinal plant.**Adv**.** Appl**.** Sci. Res., 2(1); 208-213.

M.H. Zenk, H.El. Shagi, H. Arens, J. Stockigt, E.W. Weiler and D. Dues, (1977). **Formation of indole alkaloids serpentine and ajmalicine in cell suspension cultures of *Catharanthus roseus***. Plant Tissue Culture and its Biotechnological Applications, pp; 27-44.

M.Jayanthi, N.Sowbala, G.Rajalakshmi, U Kamagavalli, V.Sivarkumar, (2009).**Study of Anti Hyper glycemic effect of *Catharanthus roseus* in Alloxam included of diabetic rats.** International Journal of pharmacy and pharmaceutical Sci., 4;19-28.

M.Maqsood A, Mujib and Z.H. Siddiqui, (2012).**Synthetic Seed Development and conversion to plantlet in *Catharanthus roseus* (L) G.Don**. Biotechnology, 11(1) ; 37-43.

M.Misawa, (1994). **Plant tissue culture : An alternative production of useful metabolite,** Bio. international Inc., Toronto, Canada, PP; 18-19.

M.R. Naryana, B.P. Dimri and M.N.A. Khan ,(1977).**Cultivation of Periwinkle Form Bulletin.** CIMPO, Lucknow, 8; 1-9.

M.S. Swaminathan, (1972). **Mutaional reconstruction of crop Ideotypes in induced mutation and plant improvement.** IAEA, Vienna, 155-180.

M.Vanisree, C.Y.Lee, S.F.Lo, S.M. Nalawade, C.Y. Lin and H.S. Tsay ,(2004). **Studies on the production of some important secondary metabolites from medicinal plants by plant tissue cultures**, Bot.Bull.Acad.Sin.,45;1-22.

M.Venkateswarly, B.N. Susheelamma, P.Kumar and K.Subha, (1988). **Studies on induced mutation of frequency in *Catharanthus roseus* (L.) G.Don. by gamma rays and EMS individually and in combination.** Indian J. Genet., 48; 313-316.

N. Vijayasree, P. Udayasri, K .Y .Aswani, B. B. Ravi, K. Y. Phani and V. M. Vijay, (2010). **Advancement in the production of secondary metabolites**. J. Not. Prod., 3;112-123.

N.R. Farnsworth, G.H. Svoboda and R.N. Blomster, (1968).**Anti viral activity of selected *Catharanthus roseus* alkaloids,** Journal of pharmaceutical Sci. 57:2174-75.

N.R. Fransworth, R.N.Blomster, D.Damratoski, W.A.Meer and L.V Camarato,(1964).**Studies on *Catharanthus* alkaloids,vi. Evaluation means by of thin layer chromatography and cerric ammonium sulphate spray reagent,** J. of Nat. Prod., 27;302-314.

N.Tanaka, M. Takao and T.matsumoto. (2004) **Vincamine production in multiple shoot culture derived from hairy roots of Vinca major.** Plant cell tiss. org. Cult. J., 41; 61-64.

O. P. Saxena, Z. Rathod, S. Singh and M. Christian ,(2012).**Micropropagation of selected medicinally useful plants**. In; Advances in botany- Indian Botanical society communication,pp.219-248.

Olivera J.Fenn, (2003). **Cancer Vaccines : Between the idea and reality Nature reviews immunology.**1(2); 630-640.

P. Christou, T.Capell, A. Kohli, J. A. Gatehouse and A.M.R. Gatehouse,(2010).**Recent development and future prospects in insect pest control in transgenic crops.**Trends Plant Sci.,11;302-308.

P. Mishra and S. Kumar ,(2000). **Emergence of Periwinkle *Catharanthus roseus* as a model system for molecular biology of alkaloids; Phytochemistry, Pharmacology, Plant biology and in vivo and *invitro* cultivation.**Journal of medicinal and Aromatic Plant Sciences,22;306-337.

P. R.H. Moreno, R. Vander Heijden and R. Verpoorte, (1993). **Effect of terpenoid Precursor feeding and elicitation on formation of indole alkaloids in cell suspension cultures of *Catharanthus roseus***, Plant cell Rep. J., 12;702-705.

P.D. Yadav, S. P. Bharadwaj, M. Yedukondolu, C.H. Methushala, A. Ravi Kumar,(2013). **Phytochemical Evaluation of *Nyctanthes arbortristis*, *Neriumoleander* and *Catharanthus roseus*.**Indian J. Bio., 1(3);333-338.

P.F. Wareing and I.D.J. Phillips, (1981). **Growth and differentiation in plants**. Pergamon Press. Oxford, New York, 381-398.

P.Mishra, G.C. Uniyal and S.Sharma, (2001)**. Pattern of diversity for morphological and alkaloid yield related trades among theperiwinkle *Catharanthus roseus*-accessions collected from in and around Indian, Subcontinent**. Genetic Reserarch in crop evolution ,48;273-286.

P.R.H. Moreno, R.Vander Heijden and R.Verpoorte, (1998).**Cell and tissue cultures of *Catharanthus roseus* :** A literature Survey-II, Plant cell Tissue and organ culture, 42;1-25.

R .Bhadra and J. V. Shanks,(1997).**Transient studies of Nutrient uptake, grow and indole alkaloid accumulation in heterotrophic cultures of hairy roots of *Catharanthus roseus*.** Biotechnol Bioeng.,55;527-534.

R. J. Krueger and D. P. Carew ,(1982). **Production of vindoline in root regeneration of *Catharanthus roseus*.** Planta med.,45;56-60.

R. L. Noble, (1990).**The discovery of the vinca alkaloids chemotherapeutic agents against cancer.** Biochemistry and cell biology, 68;1344-1351.

R. O. B. Wijesekera, (1991).**The Medicinal Plant industry.**CRC press, pp.52-7.

R. Sanjay Biradar and B. D. Rachetti, (2013).**Extraction of some secondary metabolites & Thin layer chromatography from different part of *Centella asiatica* .**American J. of life Sci.,1(6);243-247.

R. Vander Heijden, D. I. Jacobs, W. Snoeijer, D. Hallard and R Verpoorate,(2004). **The *Catharanthus* alkaloids**: Pharmacognosy and biotechnology Current medicinal chemistry;11;607-628.

R. Vorpoorte, A. Contin and J. Memelink,(2002), **Biotechnology for the production of plant secondary metabolites.** Phytochemical Review, 1;13-25.

R.B. Singh, (1992). **Current status and future prospects of plant biotechnologies in developing countries in Asia.** In : Sasson A., Costarini, Editors. Plant Biotechnologies for Developing countries. London; 141-162.

R.Garcia- Gonzales, K.Quiroz, B.Carrasco and P.Caligari, (2010**). Plant tissue culture : Current status, opportunities and challenges**. Cien Inv. Agr., 37(3); 5-30.

R.J. Krueger, D.P.Carew, J.H.C. Lui and E.J. Staba, (1982). **Initiation, maintenance and alkaloid content of *Catharanthus roseus* leaf organ cultures**. Planta Medica, 45; 56-57.

R.K. Joshi, B.Kar and S.Nayak, (2011).**Exploiting EST databases for the mining and characterization of short sequence repeat (SSR) markers in *Catharanthus roseus* L,** Bioinformation, 5(9); 378-381.

R.N. Kulkarni and N. Suresh, (1999). **In heritance of characters in Periwinkle : leaf pubescence and corolla colour**. J. Herbs Spices. Med., 6; 85-88.

R.R. Chattopadhyay, S.K. Sarkar and S.Ganguli, (1991).**Hypoglycemic and anti hyperglycemic effect of leaves of Vinea rosea Linn.**Indian Journal of physiology and pharmacology, 35;145-57.

R.Seth and A.K.Mathur,(2005).**Selection of 5-methyl tryptophanresistant callus leries with improved metabolic flux towards terpenoid Indole alkaloid synthesis in *Catharanthus roseus***. Curr. Sci., 89;3-10,

S .Guha and S. C. Maheshwari, (1964). **In vitro production of embryos from others of *Datura*.**Nature (Lond),202-497.

S. C. Ohadoma and H. U. Michael,(2011).**Effects of co-administration of methanol leaf extract of *Catharanthus roseus* on the hypoglycemic activity of metformin and glibenclamide in rats,** Asian Pac. J. Trop. Biomed.,4(6);475-477.

S. Gautam, A mishra and T. Tiwari, (2011). **Cathranthus Alkaloids and their Enhanced Production Using Elicitors : A Review**. International Journal of Pharmacy & Technology, 3(1); 713-724.

S. H. antell, J .A. Mathews and R.A.Mckee ,(1985). **Principles of biotechnology**. Blackwell Sci. Publication, Oxford, UK, pp 1-269.

S. K. Rijhwani and J. V. Shanks,(1998). **Effects of eliator dosage and exposure time on biosynthesis of indole alkaloids by *Catharanthus roseus* by hairy root cultures,** Biotech. Prog., 14;442-446.

S. Prasanthi, T.Kranthi, M.L.S. Bharani, R.Rani, B.Syamala and K. Srinivasulu ,(2010). **Cancer Vaccines : A Mile stone in Cancer Therapy,** 6(12); 259-269.

S. S.Bhojwani and M. K. Rajdan ,(1983).**Plant tissue culture theory and practice**. Elsevier Sci. Publisher, Amsterdam pp.1-502.

S. Stem ,(2000).**Introductory Plant Biology.**8th, Mc. Grow Hill Companies Inc.,pp.238-247.

S. Sukwenkim, I. D. Su, P. Sonchoi and R. J. Liu(2004) ,**Plant regeneration from Immature Zygotic Embryo-Derived Embryogenic calluses and cell suspension cultures of *Catharanthus roseus***. Plant cell tissue organ.Cult., 76(2);131-135.

S.Agarwal, Simi Jacob, N.Chettri, S.Bisoyi, A.Tazeen, A.B. Vedamurthyand V.Krishna, (2011).**Evaluation of *In-vitro* Anti helminthic Activity of *Catharanthus roseus* extract.** Internatinal Journal of pharmaceutical Sci. and Drug Res., 3(3).

S.Fatima, A. Mujib, S.A. Nasim and H. Siddiqui, (2009). **Cryopreservation of embryogenic cell suspension of *Catharanthus roseus*. (G) Don.** Plant cell Tiss. Org. Cult., 98 ; 1-9.

S.G.Rakshmi, S Vijayalakshmi and D. Rajeswari,(2013).**Pharmacological activities of *Catharanthus roseus*:** A Persective Review. Int. J. Pharm. Bio. Sci.,4(2);431-439.

S.K. Chatterjee, (2013**). The cultivation of *Catharanthus roseus* in India : Production of natural compounds by cell culture methods**. Proc. Int. Symp. plant cell culture, Munchen frg., 74-85

S.Morimoto, Y.D. Goto and Y.Shoyama, (1994).**Production of lithospermic acid band rosmarinic acid in callus tissue and regenerated plantlets of *Salvia miltirrahiza*.** J. Nat. Prod., 57; 817-823.

S.N. Singh, P.Vats and S.Puri ,(2001).**Effect of an antibiobetic extract of *Catharanthus roseus* on enzymatic activities in streptomycin induced diabetic rates.** Journal of Ethnopharmacylogy, 76; 269-77.

S.P. Rout, A.K. Choudary, D.M.Das and A Jain, (2009).**Plants in traditional medicinal system- Future source of new drugs.** I.J.P.P.S., 1(1); 2-10.

S.R. Ramachandra and G. A. Ravishanker, (2002). **Plant cell cultures; Chemical factories of secondary metabolites**, Biotechnol Adv.,20;1001-1053.

S.S.Purohit and S.K.Mathur ,(1990).**Fundamental of biotechnology, Agro**.Botanical Publisher(India)Bikaner, India,pp1-398.

Saifullah Khan,(2011).**Callus Induction and cell suspension culture production of *Catharanthus roseus* for Biotransformation Studies of Caryophyllene Oxide**, Pak J. Bot., 43(1); 467-473.

T. Khan, D. Krupadanam and V.Anwar, (2008). **The role of phytohormone on the production of berberine in the calli culture of an endangered medicinal plant, turmeric (*Coscimium fenustratumL*.).**Afr. J. Biotechnol., 7; 3244-3246.

T. Murashige and F. Skoog,(1962).**A revised medium for rapid growth and bio assays with tobacco tissue cultures.**Physiol. plant,15;473-497.

T. Thorpe , (2007). **History of plant tissue culture**. J.Mol. Microbial Biotechnol. ,37; 169-180.

T.Naaranlahti, S.Auriola and S. P.Lapenjoki , (1991). **Growth related dimerization of vindoline and catharanthine in *Catharanthus roseus* and effect of wounding on the process.** Phytochemistry, 30; 1451-1453.

V. De. Luca and B. St. Pierre ,(2000).**The cell and developmental biology of alkaloid biosynthesis.** Trends in plant science, 5;168-173.

V. M. LoyolaVargas, M. Mendez-Zeel, M. Monforte-Gonzalez and M. L. Miranda,(1992). **Serpentine accumulation during greening in normal and tumor tissues of *Catharanthus roseus*,** J. Plant Physiol,140;213-217.

Vander Heijdenetal,(2004**). The Catharanthus Alkaloids Current Medicinal Chemistry**,11(5);601-625.

Vinod U. Borde, Prashant P. Pangrikar, M.S. Wadiwar and sunil Tekala ,(2011). **Extraction and Thin Layer Chromatography of Alkaloids from Bael (*Aegle marmelos*) Leaves**. Journal .of Eco. biotechnology, pp ;1-40.

Y. Sreevalli, R.N. Kulkarni and K. Baskaran, (2002). **Inheritance of flower colour in periwinkle orangered corolla and white eye**. J. Hered, 92; 55-58.

Y.N. Shukla, A. Ravi and S.Kumar, (1997). **Effect of temperature and pH on the extraction of total alkaloids from *Catharanthus roseus* leaves.** J. of medicinal and Aromatic plant Sci., 19; 430-41.

Y.Sidhu, 2010.***In-vitro* micropropagation of medicinal plants by tissue culture.**The Plymouth Students Scientist, 4(1); 432-449.

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