Effect of Different Growth Regulators on *in vitro* Micropropagation of *Bacopa monnieri*

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DECLARATION

The whole work of the experimental work described in this thesis was carried out by me in Tissue Culture Lab, Department of Biological Sciences, Quaid-e-Azam University, Islamabad. The conclusions are my own research after numerous discussions with my supervisor. I have not presented part of this work for any other degree.

Zaheer Ahmed

I certify that the above statement is correct

Dr. M. Fayyaz Chaudhry
Professor/ Supervisor

Dated.....

APPROVAL

This is to certify that this thesis submitted by **Zaheer Ahmed** is accepted in present form by the Department of Biological Sciences, Quaid-e-Azam University, Islamabad, as satisfying the dissertation requirement for the degree of Master of Philosophy in Microbiology, Quaid-e-Azam University, Islamabad, as satisfying the dissertation requirement for the degree of **Master of Philosophy** in **Microbiology**.

Internal Supervisor _____

(Dr. M. Fayyaz Chaudhary)

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(Dr. Allah Nawaz)

Chairperson: _____

Dated: 19-01-2009

To My Mother

ACKNOWLEDGEMENT

Each and every true prayer is just for Allah the Guardian of the world, who bless the people with his imaginative abilities and all prays for his very last prophet Muhammad (PBUH) enlightened our conscience with the spirit of faith in one Allah, converging all his kindness and mercy upon us.

I have this opportunity to express my gratitude and sincere appreciation to Prof. Dr. M. Fayyaz Chaudhry for his supervision, guidance and encouragement during this work. I'll remain grateful to him for his liberty he graciously granted me to encroach his precious time.
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It is a matter of great pleasure to me to express my sincere feelings to the nice company of my friends. Thanks are also due to my colleagues and lab fellows for their pleasant companionship, which rejoice my days and night.

I was lucky to bestow with enlightened prayers offered in my favor by my beloved mother and family members. I pray I can do best for them in life.

Zaheer Ahmed

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2,4-D	2,4-Dichlorophenoxy acetic acid
BA	6-benzylamino purine
GA_3	Gibberellic acid
IAA	Indole acetic acid
IBA	Indole-3- butyric acid
Kin	Kinetin
NAA	α-nephthalene acetic acid
Zt	Zeatin
PGR	Plant growth regulator
TDZ	Thidiazuron
UV	Ultraviolet

Legends

+	Positive response
++	Good
+++	Very Good
++++	Excellent

CHAPTER 1
INTRODUCTION

The medicinal plants find application in pharmaceutical, cosmetic, agricultural and food industry. The use of the medicinal herbs for curing disease has been documented in history of all civilizations. Man in the pre-historic era was probably not aware about the health hazards associated with irrational therapy. With the onset of research in medicine, it was concluded that plants contain active principles, which are responsible, for curative action of the herbs.

Before onset of synthetic era, man was completely dependent on medicinal herbs for prevention and treatment of diseases. With introduction of scientific procedures the researchers, were able to understand about toxic principles present in the green flora. The scientists isolated active constituents of the medicinal herbs and after testing some were found to be therapeutically active. Aconitine, Atisine, Lobeline, Nicotine, Strychnine, Digoxin, Atropine, Morphine is some common examples (Ayuvedaherbs 2005).

The agro climatic circumstances of the region offer a perfect habitat for natural growth of variety of plants and herbs. Plants were the earliest medicines, and even as current humans have developed sophisticated pharmaceutical chemicals to treat illness, medicinal plants remain an important tool for treating illness in most cultures. Human beings have been utilizing plants for basic preventive and curative health care. According to a survey carried out by WHO, 80% population of developing countries still rely on traditional medicines, mostly plant-based drugs (Anonymous, 1998). A rich heritage of knowledge on preventive and curative medicines was even available in Atharva Veda, Charkha, Sushruta etc.

Moreover 25000 effective plant based formulation available under indigenous medicine. Over one and a half million practitioners of Indian system of medicine use medicinal plants in preventive and curative applications. Since the dawn of history man

has been in search of ways to find cure and relief from mental and physical Illness. Although synthetic organic compounds have contributed in pharmaceutical applications, satisfactory therapy is available only for about one third of all human ailments known at present and several diseases like cancer, AIDS, autoimmune disease continue to evade reasonable solution. The main reason behind the revival of interest in plant based ayurvedic drugs in recent years in the developed nations and developing countries is the side effects and high rising prices of the Allopathic medicines. Herbal remedies have attained popularity among the common people, Due to increasing awareness of personal health maintenance through natural products. The developed nations are also looking for eco-friendly means for treatment of various diseases through plant source.

This has recognized that approximately 9500 plant species have got a vital role in pharmaceutical industries, which are available in sub continent. About 25% of new medicines either consist of plant or plant based derivative. As anticipated by the Exim Bank, the international market of medicinal plants related trade is to tune of US $ 60 billion per year having a growth rate of 7% per annum and annual exports of these plants is valued at Rs.1200 million (Jose *et al.*, 2001).

According to one account, in 1992, at least 74 species of medicinal plants were being commercially traded in the global market; the number has now been significantly increased. In addition to these major species, hundreds of others are bought and sold in lesser quantities across national boundaries, sometimes illegally.

A comparison of the volumes of traded materials with those of the previous decade also provides dramatic evidence of the market's growth. Also in 1990, more than 2000 companies in Europe alone were marketing herbal medicinals, with 30% having a turnover in excess of $20 million- expenditure in the United States on "unconventional, alternative, or unorthodox" therapies reached $13.7 billion dollars during the same year. The so-called "nutraceuticals" sector--consisting of herbal medicines, which are dubbed food or dietary supplements in order to pass FDA criteria more easily--is now estimated to be valued at US$ 27 billion (Anon, 1995).

then stabilizes again. These swings reflect the stages when the plants are over harvested are therefore in short supply and command a high price. At this time natural populations are under extreme stress and some are threatened with possible extinction. National Medicinal Plant Board (NMPB), Govt. of India, has currently short-listed some of the important plant species, which requires immediate attention. Some of them are: Amla, Aswagandha, Brahmi, Giloe, Guggal, Kalmegha, Kuth, Senna, Shatavari.

Technology, Information, Forecasting and Assessment Council (TIFAC, DST, GOI) has also recommended 45 medicinal plant species and specifically recommended 7 plants for immediate attention during 2001-05. They are as follows:

1. *Aloe vera* (Ghrita Kumari), 2. *Bacopa monnieri* (Brahmi), 3. *Centella asiatica* (Mandukparni, Gotukola), 4. *Rawolfia serpenrtina* (Sarpagandha), 5. *Catharanthus roseus* (Periwinkle), 6.*Taxus bacata/T.wallichiana* (Himalayan Yew) and 7. *Artemisia annua.*

Finally, there are significant challenges associated with ensuring the safety, quality and efficacy of plant based medicinal content, contamination with abiotic and biotic factors, adulteration with misidentified plant species, efficiency of manufacturing processes and product handling. The major constraint in the development of medicinal plants is being experienced by the farmers are non availability of quality planting materials of improved varieties, lack of development and extension support in the cultivation and processing, and unorganized marketing(Neetu, 2005).

1.1 History of *Bacopa monnieri*

In the folklore of herbal medicine, certain herbs have been used traditionally as brain or nerve tonics. One of the most popular of these used in neurotonics is, *Bacopa monnieri* also called Brahmi, a name derived from Brahma, the creator god of the Hindu pantheon of deities. It is legendary for its diversity of usage. In the Ayurvedic *Materia medica, Bacopa monnieri* has been recognized for its brain enhancement characteristics.

It is said that the use of *Bacopa monnieri* for memory enhancement goes back 3000 years or more in India, when it was cited for its medicinal properties, especially the memory enhancing capacity, in the Vedic texts Athar-Ved Samhita (3:1) of 800 B.C. and in Ayurveda. Back before written language, ideas and cultural values were transmitted by epic hymns or poems that were committed to memory and transmitted orally from one generation of Brahmins (the highest class of priests) to the next *Bacopa monnieri* is reputed to have played a role in increasing the ability to memorize the great epic poems, possibly helping new generations.

1.2 Salient Features

Synonyms for *Bacopa monnieri*

1. *Bacopa monnieri* (L.) Pennell.
2. Lysimachia monnieri L. Cent.
3. Gratiola monnieri (L.) L.
4. Monnieri cuneifolia michaux.
5. Herpestis monnieri (L.) Kunth

Figure 1: Plant of *Bacopa monnieri.*

Scientific Classification

Kingdom:	Plantae
Division:	Magnoliophyta
Class:	Magnoliopsida
Order:	Lamiales
Family:	Scrophulariaceae
Genus:	*Bacopa*

Species: *Bacopa monnieri*

Habitat and Botanical description

Bacopa monnieri has originated in sub-continent. A genus of spreading herbs, commonly growing in damp and marshy places throughout sub-continent, ascending up to an altitude of 1,320 m, a small creeping, succulent, herb rooting at nodes, stem soft, obtuse- angular, branches ascending, leaves are sessile, opposite decussate, succulent, obovate or oblanceolate in shape, short petiolate, 0.6-2.5 cm in size, flowers solitary axillary, blue or white in color with purple veins, campanulate, pentamerous , capsules ovoid (Mathur and Kumar, 1998). Flowers and fruits appear in summer. Whole plant forms the medicinally useful part.

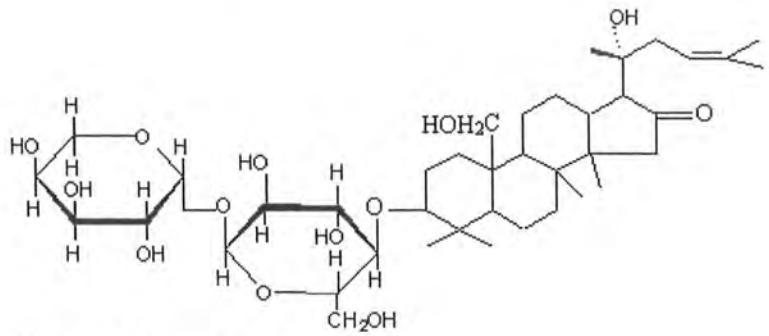
1.3 Propagation and Agronomy

Bacopa monnieri spreads by producing new plants on above ground runners. The new plants can be separated from the parent plant once they have taken root. The natural regeneration of this herb is hampered by death of seedlings at 2-leaved stage and specific habitat (marshy areas) requirements. *Bacopa monnieri* seems to be poor competitor and so it can colonize open spaces only (Tiwari *et al.,* 2000). A field trial involving five Indian accessions of *Bacopa monnieri* was conducted during 1997 to 1998, at Lucknow, Uttar Pradesh, India, to standardize the cultivation procedure for the domestication of this medicinal herb. The accessions monitored for growth and *Bacoside-A* yields over 18 months, could be maintained as perennials, but growth properties were sensitive to the growing season. Loss of shoot biomass occurred in winter (December-February) and the growth rate was higher in the monsoon season (July-September) than in summer (March-June). *Bacoside-A* content of herb was high from September through March and in June. Suitable harvest times for high yields of *bacoside-A* were June and September through November. An accession from Guwahati in Assam state of India yielded more *bacoside-A* than all other accessions (Mathur *et al.*, 2002).

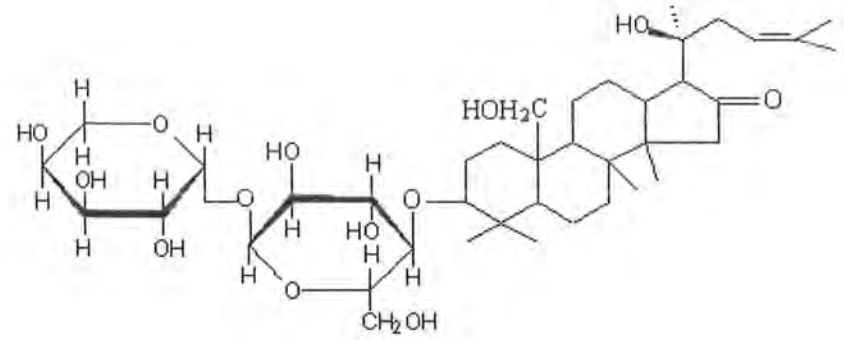
Figure.2: Established *Bacopa monnieri* Plants at Plantation Area

1.4 Chemical Constituents

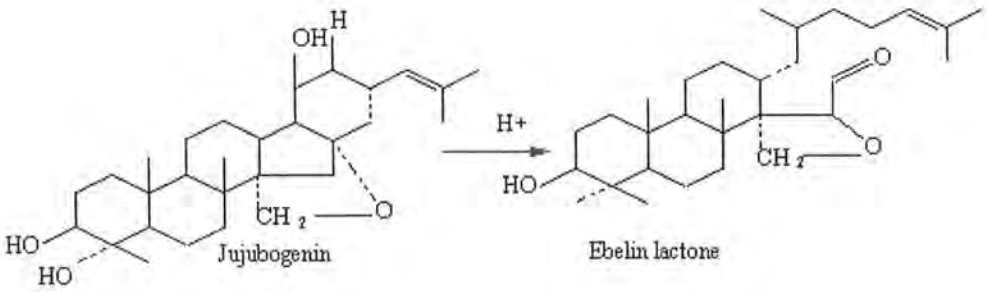
Plant is important due to some medically active compounds. The chemical structures of some of these compounds are as follow: (Sabinsa Corporation, 2001)



BACOSIDE A (Levorotatory)



BACOSIDE B (Dextrorotatory)



(Triterpenoid saponin) (Bacogenin A4)

1.5 What is Plant Tissue Culture?

Plant tissue culture is the aseptic (free from microorganism) culture of any plant part *in vitro*. Tissue culture is utilized in the field of Biotechnology. Whereas, micro-propagation is the rapid vegetative propagation of plants via tissue culture techniques. Micro-propagation permits the manipulation of physical and chemical conditions in the production of large numbers of high quality plant material within a short period of time.

- The production of exact copies of plants that produce particularly good flowers, fruits, or have other desirable traits.
- To quickly produce mature plants.

- The production of multiples of plants in the absence of seeds or necessary pollinators to produce seeds.
- The regeneration of whole plants from plant cells that have been genetically modified.
- The production of plants in sterile containers that allows them to be moved with greatly reduced chances of transmitting diseases, pests, and pathogens.
- The production of plants from seeds that otherwise have very low chances of germinating and growing, i.e., orchids and nepenthes.
- To clean particular plant of viral and other infections and to quickly multiply these plants as 'cleaned stock' for horticulture and agriculture.
- The elimination of diseases and the production of disease free plantlets.
- The rapid production of large numbers of genetically identical plantlets.
- Introduction of new varieties and or genotypes
- Preservation of germplasm
- Production of haploid plants which can be used for plant breeding

Conditions for Tissue Culture

Modern plant tissue culture is performed under aseptic conditions. Living plant materials from the environment are naturally contaminated with microorganisms, so surface sterilization of starting materials (explants) in chemical solutions (usually alcohol or bleach) is required. Mercuric chloride is commonly used as a plant sterilant today. Explants are then placed on the surface of a solid culture medium, but are sometimes placed directly into a liquid medium, particularly when cell suspension cultures are desired. Solid and liquid media are generally composed of inorganic salts plus a few organic nutrients, vitamins and plant hormones. Solid media are prepared from liquid media with the addition of a gelling agent, usually purified agar.

The composition of the medium, particularly the plant hormones and the nitrogen source (nitrate versus ammonium salts or amino acids) have profound effects on the morphology of the tissues that grow from the initial explant. For example, an excess of auxin will often result in a proliferation of roots, while an excess of cytokinins may yield

shoots. A balance of both auxin and cytokinins will often produce an unorganised growth of cells, or callus, but the morphology of the outgrowth will depend on the plant species as well as the medium composition. As cultures grow, pieces are typically sliced off and transferred to new media (subculture) for growth or to alter the morphology of the culture. The skill and experience of the tissue culturist are important in judging which pieces to culture and which to discard.

As shoots emerge from a culture, they may be sliced off and rooted with auxin to produce plantlets which, when mature, can be transferred to potting soil for further growth in the greenhouse as normal plants.

Most method of regeneration requires that a whole plant is regenerated from isolated plant cells or tissues which have been genetically transformed. This regeneration is conducted *in vitro* so that the entire environment and growth medium can be manipulated to ensure a high frequency of regeneration. In addition to a high frequency of regeneration, the regenerable cells must be accessible to gene transfer. The primary aim is therefore to produce, as easily and as possible, a large number of regenerable cells that are accessible to gene transfer. The subsequent regeneration step is often the most difficult step in plant transformation. However it is important that a high frequency of regeneration does not necessarily correlate with high transformation efficiency.

Plants due to their sessile nature and long life span have developed a greater ability to endure extreme conditions and predation than have animals. Many of the processes involved in plant growth and development adapt their metabolism, growth and development to best suit their environment. Particularly important aspects of this adaptation, as far as plant tissue culture and regeneration are concerned, are the abilities to initiate cell division from almost any tissue of the plant and to regenerate lost organs or undergo different development pathways in response to particular stimuli. When plant cells and tissue are cultured *in vitro* they generally exhibit a very high degree of plasticity, which allows one type of tissue or organ to be initiated from another type. In this way, whole plant can be subsequently regenerated.

This regeneration of whole organism depends upon the concept that all plant cells express the total genetic potential of the parent plant, this is called totipotency. Plant cells culture and regeneration do, in fact, provide the most compelling evidence for totipotency.

The Culture Environment

When cultured *in vitro*, all the needs, both chemical and physical, of the plant cell have to be met by the culture vessel, the growth medium and the external environment. The growth medium has to supply all the essential mineral ions required for growth and development. In many cases, it must also supply additional organic supplement such as amino acids and vitamins. Many plant cell cultures, as they are not photosynthetic, also require the addition of a fixed carbon source in the form of a sugar (most often sucrose). One other vital component that must also be supplied is water, the principal biological solvent. Physical factors, such as temperature, PH, the gaseous environment, light and osmotic pressure, also have to be maintained with in the acceptable limits.

Plant Cell Culture Media.

Culture media used for the *in vitro* cultivation of plant cells are composed of three basic components:

1. Essential elements, or mineral ions, supplied as a complex mixture of salts;
2. An organic supplement supplying vitamins and/or amino acids; and
3. A source of fixed carbons; usually supplied as the sugar.

For practical purposes, the essential elements are further divided into the following categories

i. Macronutrients
ii. Micronutrients
iii. An Iron source

Complete plant cell culture medium is usually made by combining several different components.

Media Components.

Some of the useful components of the stock solutions are;

Macronutrients

As it is implied by the name, the stock solution supplies elements that essentially required in large amounts for plant growth and development. Nitrogen, Phosphorus, Potassium, Magnesium, Calcium and Sulphur are usually regarded as macronutrients. These elements usually comprise at least 0.1% of the dry weight of plants. Nitrogen is most commonly supplied as a mixture of nitrate ions (KNO_3) and ammonium ions (NH_4NO_3). Theoretically, there is an advantage in supplying nitrogen in the form of ammonium ions, as nitrogen must be in reduced form to be incorporated into macronutrients. Nitrate ions therefore need to be reduced before incorporation. However, at high concentration, ammonium ions can be toxic to plant cell cultures and uptake of ammonium ions from the medium can cause acidification of the medium. In order to use ammonium ions as the sole nitrogen source, the medium needs to be buffered. High concentration of ammonium ions can also cause culture problem by increasing the frequency of vitrification. Using a mixture of nitrate and ammonium ions has the advantage of weekly buffering the medium has the advantage of weakly buffering the medium as the uptake of nitrate ions OH^{-1} ions to be excreted.

Phosphorus is usually supplied as the phosphate ion of the ammonium, sodium or potassium salts. High concentration of phosphate can lead to the precipitate of medium elements as insoluble phosphate.

Micronutrient

These elements are required in trace amounts for plant growth and development. Manganese, iodine, copper, cobalt, boron, molybdenum, iron and zinc usually comprise

These components are the basic chemical necessities for plant cell culture media. However other additions are made in order to manipulate the pattern of growth and development of plant cell culture.

Plant Growth Regulators

Plant growth regulators are the critical media components in determining the development pathway of the plant cells. The plant growth regulators used most commonly are plant hormones or their synthetic analogues.

Classes of Plant Growth Regulators

There are five main classes of plant growth regulators used in plant growth culture, namely:

1. Auxins
2. Cytokinins
3. Gibberellins
4. Abscisic acid
5. Ethylene

Auxins

Auxins promote both cell division and cell growth. The most important naturally occurring auxin is IAA (indole-3-acetic acid), but its use in plant cell culture media is limited because it is unstable to both heat and light. 2.4-Dichlorophenoxyecetic acid (2.4-D) is the most commonly used auxins and is extremely effective in most circumstances. Other auxins are available and some ways more effective or potent then 2.4-D in some instances.

Cytokinins

Cytokinins promote cell divisions. Naturally occurring cytokinins are a large group of structurally related compounds. Of the naturally occurring Cytokinins, two have some use in plant tissue culture media. These are zeatin and 2-isopentyl adenine. Their

use is not wide spread as they are expensive and relatively unstable. The synthetic analogue, kinetin and BAP are therefore used more frequently.

Gibberellins.

There are numerous, naturally occurring, structurally related compounds termed 'gibberellins'. They are involved in regulating cell elongation, and are agronomically important in determining plant height and fruit set. Only a few of the gibberellins are used in plant tissue culture media, GA, being the most common.

Abscisic Acid.

Abscisic acid (ABA) inhibits cell division. It is most commonly used in plant tissue culture to promote distinct development pathways such as somatic embryogenesis .

Ethylene

Ethylene is a gaseous, naturally occurring, plant growth regulator most commonly associated with controlling fruit ripening in climacteric fruits, and its use in plant tissue culture is not widespread.

1.6 Plant Growth Regulators and Tissue Culture.

Concept about plant growth regulators and their use in plant tissue culture media have been developed from initial observation made in 1950s. There is, however, some considerable difficulty in predicting the effects of plant growth regulators because of great difference in culture response between species and even plants of the same cultivar grown under different conditions.

However some principles do hold true and have become the paradigm on which most plants tissue culture regimes are based.

Auxins and cytokinins are the most widely used plant growth regulators in plant tissue culture and are usually together, the ratio of the auxin to the cytokinins determining the type of culture established or regenerated. High auxins to cytokinins ratio generally

favors root formation, whereas high cytokinins to auxins ratio favor shoot formation. An intermediate ratio favors callus production.

Culture Types.

Cultures are generally initiated from sterile pieces of a whole plant. These pieces are termed explants and may consists of pieces of organs, such as leaves or roots or may be specific cell types, such as pollen or endosperms. Many features of explants are known to affect the efficiency of culture initiation. Generally younger more rapidly growing tissue is more effective.

Several different culture type most commonly used in plant transformation are:

Callus

Explants, when cultured on the appropriate medium, usually with both an auxin and cytokinins, can give rise to an unorganized, growing and dividing mass of cells. In culture, this proliferation can be maintained more or less indefinitely, provided that the callus is sub cultured on to fresh medium periodically. During callus formation there is some degree of differentiation both in morphology and metabolism. One major consequence of this differentiation is that the most plant culture loses the ability to photosynthesis. This has important consequence for the culture of callus tissue, as the metabolic profile will probably not match that of the donor plant. This necessitates the addition of another component such as vitamins and most importantly a carbon source to the culture medium, in addition to the usual mineral nutrients.

Callus cultures are extremely important in plant biotechnology. Manipulation of the auxin to Cytokinins ratio in the medium can lead to the development of shoots, roots or somatic embryos from which whole plant can subsequently be produced. Callus cultures can also be used to initiate cell suspensions, which are used in a variety of ways in plant studies.

Cell Suspension Cultures.

Callus cultures broadly speaking fall into one of two categories compact or friable. In compact callus the cells are densely aggregated whereas in friable callus are only loosely associated with each other and the callus becomes soft and breaks apart easily. Friable callus provides the inoculum to form cell suspension cultures. Explants from some plants species or particular cell types tends not to form friable callus, making cell suspension initiation a difficult task. The friability of callus can sometimes be improved by manipulating the medium components or by repeated sub culturing. The friability of the callus can also sometimes be improved by culturing it on semi solid medium.

Protoplast.

Protoplasts are plant cells with the cell wall removed. Protoplasts are most commonly isolated from either leaf mesophyll cells or cell suspensions although. Two general approaches to removing the cell wall can be taken mechanical or enzymatic isolation.

Mechanical isolation although possible often result in low yields, poor quality and poor performance in culture due to substance released from damaged cells. Enzymatic solution is usually carried out in a simple salt solution with a high osmoticum plus the cell wall degrading enzymes. It is usual to use a mix of both cellulase and pectinase enzymes which must be of high quality and purity.

Protoplasts are fragile and easily damaged and therefore must be cultured carefully. Liquid medium is not agitated and high osmotic potential is maintained at least in the initial stages. The liquid medium must be shallow enough to allow aeration in the absence of agitation. Protoplast can be plated out on to the solid medium and callus produced. Whole plant can be regenerated by organogenesis from this callus. Protoplasts are ideal targets for transformation by a variety of means.

Root Culture

Root cultures can be established *in vitro* from explants of the root tip of either primary or lateral roots and can be cultured on fairly simple media. The growth of the roots *in vitro* is potentially unlimited, as roots are indeterminate organs. Although the establishment of root cultures was one of the first achievements of modern plant tissue culture, they are not widely used in plant transformation culture.

Shoot Tip & Meristem Culture.

The tips of the shoot can be cultured *in vitro* producing clumps of shoots from either axillary or adventitious buds. This method can be used for clonal propagation. Shoot meristem cultures are potential alternatives to the more commonly used method for cereal regeneration as they are less genotype dependent and more efficient.

Embryo Culture.

Embryos can be used as explants to generate callus or somatic embryos. Both immature and mature embryos can be used as explants. Immature embryo derived embryogenic callus is the most important method of monocot plant regeneration.

Plant Regeneration.

Whole plants can be regenerated from such cultures. Two methods of plant regenerations are widely used in plant transformation studies i.e. somatic embryogenesis and organogenesis.

Somatic Embryogenesis.

In somatic embryogenesis embryo like structure can develop into the whole plant in a way analogue to zygotic embryos, are formed from somatic tissues. These somatic

embryos can be produced either directly or indirectly. In direct somatic embryogenesis the embryo is formed directly from a cell or small group of cells without the production of an intervening callus. Though common from some tissue usually reproductive tissue such as the nucellus, (styles or pollen), direct somatic embryogenesis is generally rare in comparison with the indirect embryogenesis.

In direct somatic embryogenesis callus is produced from the explant. Embryos can then be produced from the callus tissue or from a cell suspension produced from that callus.

Organogenesis.

Somatic embryogenesis relies on plant regeneration through a process analogue to zygotic embryo generation. Organogenesis relies on the production of organs, either directly from explant or from callus culture. There are three methods of plant regeneration from organogenesis.

The first two methods depend upon adventitious organs arising from a callus culture or directly from an explant. Alternatively, axillary bud formation and growth can also be used to regenerate whole plants from some types of tissue culture.

Organogenesis relies on the inherent plasticity of the plant tissue and is regulated by altering the components of the medium. In particular it is the auxin to Cytokinin ratio of the medium that determines which development pathway the regenerating tissue will take.

It is usual to induce shoot formation by increasing the Cytokinins to auxin ratio of the culture medium. These shoots can then be rooted relatively simple.

1.7 Aims and Objectives

Bacopa monnieri is being indiscriminately exploited because of its medicinal importance, immediate method for its replenishment and cultivation, preferably through tissue culture, becomes an ultimate need. The technique reported in this thesis enable rapid micropropagation of species and possibly other medicinal plants too. The major objective of this study was to optimize medium for callus induction, and direct micropropagation.

Chapter 2

Review of Literature

Plants are an important source of medicines and play a key role in world health (Kala *et al.*, 2005). In almost all regions and cultures of the world, from ancient times till today, plants have been used as medicines (Kala *et al.*, 2006). Today's medicinal plants are important to the global economy, as approximately 80% of traditional medicine preparations involve the use of plants or plant extracts (Viera and Skorupa, 1993; Dhyani and Kala, 2005). The increasing demand for herbal medicines in recent years due to their fewer side effects in comparison to synthetic drugs and antibiotics has highlighted the need for conservation and propagation of medicinal plants. An efficient and most suited alternative solution to the problems faced by the phytopharmaceutical industry is development of in vitro systems for the production of medicinal plants and their extracts. The in vitro-propagated medicinal plants furnish a ready source of uniform, sterile and compatible plant material for biochemical characterization and identification of active constituents (Wakhlu and Bajwa, 1986; Banerjee and Shrivastava, 2006). Recently in a report by the National Medicinal Plant Board (NMPB), Government of India and Technology Information Forecasting and Assessment Council (TIFAC) has recommended immediate attention to few medicinal plants, among which *Bacopa monnieri* prominently features, which makes this plant in the category of highly endangered plants in India (http://www.nmpb.nic.in/prioritisedmedicinalplants.htm).

The urgent need of many identical uniform individual of a selected genotype has led to the development of *in vitro* culture technique as a source of vegetative propagation, in contrast to conventional method, which takes long time for multiplication. (Nin *et al.*, 1996)

Micropropagation of medicinal plants is mainly carried with following objectives: -

- In some plants, seed production is difficult and many a time's seeds do not show proper germination and seedling growth.
- Micropropagation is mainly used in individual plants having elite characters and protects them against segregation or mutation.
- In many medicinal plants, planting material is becoming endangered so necessary to develop micropropagation protocols to preserve germplasm and for distribution during cultivation in new areas.
- Production of medicinal plant seedlings can be carried continuously without seasonal variation and environmental factors. Asexual multiplication using tissue culture techniques can be achieved by three approaches:
    - Enhancing axillary bud break
    - Production of adventitious buds
    - Somatic embryogenesis

The first two approaches lead to plantlets formation via organogenesis through production of uni-polar shoots, which must then be further multiplied, followed by rooting in a multistage process. In contrast, somatic embryogenesis leads to the formation of a bipolar embryo through steps that are often similar to zygotic embryogenesis. Shoot multiplication is widely used for the colonal propagation using the above approaches; it has been possible to produce plantlets of over 70 angiosperms and 30 gymnosperms. Tissue culture techniques have been applied for the propagation of approximately 20% of 7000 known Ayurvedic plant species (Rajendra and D'Souza, 1999).

2.1 Explant Source

All parts of the plant have been used as the source for micropropagation. Nodal segments of healthy explants have been however used in most cases. Saba *et al, 1999*, reported use of nodal segments in *Ammi majuss* L., nodal explants of *B. monnieri* were propagated *invitro* using liquid shake cultures (Tiwari *et al.*, 2000).

Tiwari *et al.*, 2000, reported use of nodal segments for clonal propagation of *Centella asiatica*; shoot tip, nodal and internodal segments were reported in *Phyllanthus amarus* (Ghanti *et al.*, 2004). The morphogenetic potential of node, internode and leaf explants of Brahmi, *Bacopa monnieri* (L.) [Wettst] was investigated to develop reliable protocols for shoot regeneration and somatic embryogenesis (Tiwari *et al.*, 1998). Regeneration from callus cultures of *Centella* using stem and leaf explant from green house grown mother plant (Patra *et al.*, 1998).

2.2 Sterilization treatment

Surface sterilization is most important step before inoculation of explant. Different steps have been employed for treatment of explant. *Shrivastava and Rajani, 1999*, have described sterilization treatment of *Bacopa Monnieri*, which includes use of 0.1% Mercuric chloride (w/v) for 2 minutes followed by rinsing thoroughly with sterile distilled water. Different sterilization treatment was followed by Mathur *and* Kumar, 1998, in which leaves and stem explants were shaken for 10 minutes in Tween 20 (Ranbaxy) and Savlon (J & J Company) in water for 10 minutes, rinsed in running water for 30 minutes, treated with 0.1% Mercuric chloride for 3-4 minutes and washed several times with sterile water.

2.3 Multiple Shoot Induction

Murashige and Skoog medium (1962) supplemented with different combinations of growth regulators has been used for growth of herbaceous species. Multiple shoots were obtained from shoot tips (1-2 cm) derived from field-grown plants of *Bacopa monnieri* in Murashige and Skoog medium supplemented with 0.5 mg / litre (Singh *et al.*, 1999; Tiwari *et al.*, 2000), proposed an efficient and rapid method using liquid shake cultures for *in vitro* propagation of *Bacopa monnieri*, a medicinally important herb. This was achieved by culturing nodal explants on liquid MS medium. Compared to single axillary shoot proliferation on a growth regulator free agarified medium, the respective liquid medium induced 4 or 5 shoots per nodal explant 4 weeks after culture. Addition of

6-benzyladenine (0.01-0.1 mg/l) resulted in the increase in morphogenetic response (number of shoots, mean shoot length and number of roots per node explant) in both the types of culture media. Tiwari *et al.*, 2001, reported use of range of cytokinins for multiple shoot induction for *Bacopa monnieri*, with node, internode and leaf explants. Of the four cytokinins (6-benzyladenine, thidiazuron, kinetin and 2-isopentenyladenine) tested thidiazuron (6.8μM) and 6-benzyladenine (8.9μM) proved superior to other treatments. Optimum adventitious shoot buds induction occurred at 6.8μM thidiazuron (TDZ) where an average of 93 shoot buds were produced in leaf explants after 7 weeks of incubation. Multiple shoots were obtained on MS medium supplemented with auxins or/and cytokinins with or without coconut milk. Maximum number of plants was obtained on medium containing Kin/2-ip (0.1 mg/l) and Kin (1 mg/l) in shoot tip and nodal cultures respectively (Tejavathi *et al.*, 2001). Banerjee *et al.*, 1999 reported in Centella that initial sprouting required the presence of BAP (2mg/l) and IBA (0.1 mg/l), however for induction of multiple shoots higher concentration of BAP (3 mg/l) and lower concentration of NAA (0.05mg/l) is required.

2.4 Rooting and Hardening

In medicinal plants rooting of micro shoots have been obtained in MS medium with IAA, IBA, NAA used singly or in combinations or when transferred to hormone free medium. Root induction in *B. monnieri* was observed in MS medium supplemented with 0.5 mg BAP [benzyladenine]/litre within 6 days of culture. In the case of *P. foetida* and *C. asiatica* root induction was observed within 12 and 21 days of culture when single shoots of both the plant species were cultured on MS media supplemented with 0.25 mg BAP + 0.5 mg IBA and 0.5 mg BAP + 1.5 mg NAA/litre, respectively (Singh *et al.*, 1999).

Root induction has also been reported in *Phyllanthus amarus (Ghanti et al., 2004)* using MS medium supplemented with a concentration of 0.5 mg/l of IBA. Rooted shoots were hardened on basal liquid medium and subsequently in sterile oil+vermiculite (1:1). In case of *Withania somnifera (L.)* Dunal, shoots were rooted best (87%) on MS medium

containing 2 mg/M Indole-3-butyric acid (IBA). The plantlets were transferred to the field after acclimatization and showed 60% survival. (Rani *et al.*, 2003).

In *Centella* shoots regenerated from the stem and leaf callus were rooted within 11 days in 1/2 strength MS basal salts supplemented with 0.5 mg/l indole-3-acetic acid and 2% (w/v) sucrose. About 85% of rooted plantlets were acclimatized and transferred to the greenhouse (Patra *et al.*, 1998). Tiwari *et al.*, 2000, tried rooting on different media in *Bacopa monnieri*, i.e. MS media with or without hormones and found that rooting was highest (90%) on full-strength MS medium containing 2.46 mM IBA.

2.5 Callus Culture

Organogenesis has been reported in number of medicinal plants through callus culture. Callus was induced from leaves of *Rauwolfia serpentina [Rauvolfia serpentina]*, on WCR supplemented with coconut milk (CM), extra nitrogen source solution, biotin (10 mg/litre), BAP (2 mg/litre), NAA (0.8 mg/litre) and GA_3 (0.0001 mg/litre). Callus, subcultured on WRC supplemented with CM, BAP (0.1 mg/litre) and NAA (0.1 mg/litre), grew well and differentiated the roots. Differentiated roots dedifferentiated into white callus, which subsequently redifferentiated into roots. This cycle of differentiation was observed repeatedly on further subculture (Mahmood Riffat, 1994). Calli derived from nodal explants of *Bacopa monnieri* cultured on MS medium containing 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), when subcultured on MS medium containing 0.1 or 0.5 mg/l BA or 0.2 mg/l 2,4-D + 0.1 or 0.5 mg/l kinetin, developed somatic embryos. The somatic embryos germinated either on the same media or on MS basal medium and the resulting plantlets were successfully transplanted to soil. (Tiwari *et al.*, 1998). Rani *et al.*, 2003 reported that callus induction in *Withania somnifera (L.)* Dunal was observed from hypocotyl, root, and cotyledonary leaf segments, grown on Murashige and Skoog (MS 1962) medium supplemented with various concentrations and combinations of 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (Kn). Maximum callusing (100%) was obtained from root and cotyledonary leaf segments grown on MS medium supplemented with a combination of 2 mg/lM) 2,4-D and 0.2 mg/lM) Kn. When hypocotyl segments

were used as explants, callus induction was noticed in 91% of cultures, which showed shoot regeneration on MS medium supplemented with 2 mg /l, 2,4-D and 0.2 mg /l Kn.

2.6. Medicinal Importance of *Bacopa monnieri.*

Brahmi is considered as the main rejuvenating herb for nerve and brain cells and, therefore, has played a very important role in ayurvedic therapies for the treatment of cognitive disorders of aging (Sekar, 1996; Anon, 2004; Russo and Borrelli, 2005; Ernst, 2006). It also possesses anti-inflammatory, analgesic, antipyretic, epilepsy, insanity, anticancer and antioxidant activities (Satyavati *et al.*, 1976; Jain *et al.*, 1994; Tripathi *et al.*, 1996; Bafna and Balaraman, 2005; Sinha and Saxena, 2006). It is also used in treatment of asthma, hoarseness, water retention and blood cleaning.

In India the plant is used for all sorts of skin problems- eczema, psoriasis, abscess, ulcerations- it is said to stimulate the growth of skin, hair and nails. Indian Pennywort is also used for chronic rheumatism often as an ointment. In Pakistan, the herbal drug, Brahmi-buti, is used to treat skin diseases, leprosy, epilepsy, eczema, asthma, hoarseness of the voice, and diseases of the nervous system (Shakoor *et al.*, 1994). According to scientists at the Central Drug Research Institute in Lucknow, India, certain "memory chemicals" in *Bacopa,* called bacosides A and B, help repair damaged neurons by enhancing proteins involved in the regeneration of neural-cell synapses (Rastogi *et al.*, 1994). These are the relay stations of the brain that facilitate the transmission of neural impulses. Thus *Bacopa* can be viewed as a neural nourisher, restoring depleted synaptic activity and leading to enhanced memory function. In scientific studies, it has been shown to exert a remarkable and unique effect on neurotransmitters. *Bacopa monnieri* may even be able to revitalize intelligence. Among its many other applications, *Bacopa monnieri* has reportedly been effective in reducing anxiety levels, thereby allowing for further improvement of brain functioning and elevated mental performance. It is also believed to help stabilize the brain waves of epileptics. *Bacopa* is recognized as a treatment for asthma, bronchitis, and hoarseness. In other parts of the body, it has been used successfully as a remedy for rheumatism, for diarrhea, and as a diuretic (increasing

urinary flow). *Bacopa* also has important antioxidant properties and acts as a metal chelator, removing excess damaging metals from the blood, thus limiting the propagation of free radicals. Perhaps most intriguing of all, in human studies Bacopa appears to increase a child's exploratory behavior, improve visual motor performance, and exercise a positive effect on recall and reaction time.

2.7 Phytochemistry

Plant contains two saponins, BacosideA and B. In addition to the Bacosides, *Bacopa monnieri* contains a wide variety of medically active substances, including stigmasterol, sapogenins, and flavonoids. Other compounds include triterpenoid Saponins. *Bacopa monnieri* also contains D-Mannitol, Betulic acid, Beta-Sitosterol, Octacosane, Nicotine, and Amino Acids such as Apha-Alanine, Aspartic acid, Glutamic Acid, and Serine. Alkaloids such as Brahmines, Herpestine and a mixture of three alkaloids were reported from the leaves of this plant. The carbohydrate mostly of BacosideA was shown to be arabinosyl glucose with the arabinose unit as the terminal sugar. BacosideB was found to be dextorotatory where as BacosideA was laevo rotatory. The haemolytic action of Bacoside B is twice that of Bacoside A (Report on Herbal industry, pp : 37 –41). This is because of the differences in the configuration of the carbohydrate parts. Bacoside Yields bacogenins A1, A2, A3 and A4 upon hydrolysis (Chatterji *et al.*, 1965). The other chemical constituents of the plant includes BacosideA1, Hersaponin, Betulinic Acid, Stigmasterol, b-Sitosterol and Stigmastenol (Chatterji *et al.*,1963 ; Jain and Kulshreshtha, 1993).

Chapter 3
MATERIALS AND METHODS

The present research work was carried out in Plant Tissue Culture Laboratory, Faculty of Biological Sciences, Quaid-e-Azam University, Islamabad. A brief account of the materials and methods used and the procedure adopted is given below.

3.1 Tissue Culture studies of *Bacopa monnieri*

Plant Material:

The explants were collected from vicinity of Quaid-e-Azam University, Islamabad

Chemicals:

The entire chemicals used in the experiment work were of the highest grade of purity. Chemicals were purchased from sigma chemicals Company USA and E.Merck of Germany.

Culture Procedure.

The glass ware used in the preparation of media was made of borosilicate. All the glassware was washed using commercial detergent. Cleaned glassware was dried at 150^0 C for 30 mints in an oven and wrapped with aluminum foil.

Basal Media:

MS (Murashige and Skooge, 1962) medium was used as basal medium in present investigation. The composition is given in Table 3.2

3.2 Composition of Murashige and Skoog (1962) Medium

S.No	Constituent	Formula	Conc. In stock solution g/l	Volume of stock/l of medium (ml)
Macronutrients				
1	Potassium Nitrate	KNO_3	38	50
2	Ammonium Nitrate	NH_4NO_3	33	
3	Calcium Chloride	$CaCl_2.2H_22O$	8.8	
4	Magnesium Sulphate	$MgSo_4.7H_2O$	7.4	
5	Potassium Phosphate	KH_2PO_4	3.4	
Micronutrients				
6	Manganese Sulphate	$MnSO_4.H_2O$	4.4	5
7	Zinc Sulphate	$ZnSO_4.H_2O$	1.72	
8	Boric Acid	H_3BO_3	1.24	
9	Potassium Iodide	KI	1.67	
10	Sodium Molybdate	$Na_2MoO_4.2H_2O$	0.05	
11	Copper Sulphate	$CuSO_4.2H_2O$	0.01	
12	Cobalt Chloride	$CoCl_2.6H_2O$	0.005	
Iron Sources				
13	Sodium EDTA	$Na_2EDTA.2H_2O$	7.46	5
14	Ferrous Sulphate	$FeSO_4.7H_2O$	5.56	
Organic Supplements (Vitamins)				
15	Myo-inositol		20	5
16	Glycine		0.4	
17	Nicotinic Acid		0.1	
18	Pyridoxine-HCL		0.1	
19	Thiamine HCL		0.1	
Carbon Sources				
20	30 g/l of Sucrose was used in MS medium			

Growth Regulators.

Different plant growth regulators were used for organogenesis and callogenesis. Stock solution of growth regulators (2,4 D, BA, IBA, IAA, NAA) were prepared. Five different strengths were used i.e. 0.5mg/l, 1.0mg/l, 1.5mg/l, 2.0mg/l, 2.5mg/l.

Preparation of 1 liter MS Basal Medium

One liter medium was prepared according to following protocols

1. Thirty gms of sucrose was dissolved in 600 ml of distilled water contained in a 2 liter flask.
2. Required amount of macronutrients (50g/l), micronutrients (5g/l), vitamins (5g/l), and iron source (5g/l) from there stock solutions were added.
3. Volume was raised up to one liter with distilled water.
4. The medium was dispensed into test tubes and plant growth regulators were added as per required for the desired concentration.
5. pH was adjusted to 5.5 - 5.8 by 1 N NaOH or 1N HCl
6. Agar (0.8%) was added and medium was boiled with continuous stirring for thorough mixing of agar into medium
7. Dispensing in flasks.
8. Culture vessels were plugged with non absorbent cotton wool wrapped with aluminum foil and autoclaved at 121°C for 20 minutes at 15 lb/in^2

Inoculation Area and Manipulation Tools

Transfer room was cleaned with a commercial detergent on monthly basis and sprayed with 95% ethyl alcohol before start of work. Transfer room was treated with UV rays for 40 minutes before use.

Surgical instruments, Petri dishes, flasks containing distilled water were sterilized in an autoclave at 121°C and 15 lb/in^2 pressure for 20 minutes. Surgical instruments and Petri dishes were autoclaved in an air tight steel box.

Culturing of Tissue

Aseptic transfer of tissue was carried out in a laminar flow transfer cabinet fitted with a HEPA filter. Before using on the Laminar Flow transfer Cabinet working surface were swabbed down with 95% ethyl alcohol or spirit

Surgical instruments, Petri dishes, distilled water and culture vessels were brought into the cabinet and sprayed with ethanol. Then the working area and instruments were exposed to UV light for 20 minutes to ensure sterility. Transfer work was started about half hour after the UV light was switched off. Surgical instruments were dipped in 95% ethyl alcohol. After each manipulation, the instruments were again dipped in ethyl alcohol, re-flamed and reused.

Explants Preparation.

Small fresh plants were collected from the land. These plants then thoroughly washed. The explants used for this purpose were cut into 3-4 mm pieces.

Culture Environment.

The culture flasks were incubated under conditions of well controlled temperature, light intensity and photoperiod. Temperature of culture room for the present study was maintained at 25 ± 2°C with 16/8 hours light dark cycle and light intensity was maintained at 2000 lux throughout the experiments.

Chapter 4
RESULTS

4.1 Callogenesis

Observation on the callusing response of stems and leaf explant incubated with varying concentration of different growth regulators individually and in combination are summarized in the table no. 4.1 and 4.2. In general the explants showed tendency to regenerate shoots rather then to form callus with in the 2-3 weeks of initiation of incubation. Same are the results with combinations as very little callus formation was observed mostly with lower concentrations.

Effect of 2, 4 – D

Different concentrations of 2, 4-D were used from all two explants i.e. leaf and stem on MS medium. Five different concentrations 0.5 mg/l., 1.0 mg/l, 1.5 mg/l., 2.0 mg/l., 2.5 mg/l were used.

It was observed that at lower concentrations of 2, 4-D (0.5 mg/l, 1.0 mg/l, 1.5 mg/l and 2.5 mg/l) induced some callus formation. The callus formed was yellowish green and soft which turned brown after two weeks. Internodes and leaf explants showed almost no callus formation as only one callus was formed at 0.5 mg/l. callus formed was soft and yellowish in color

Effect of IAA.

Five different concentrations 0.5 mg/l., 1.0 mg/l, 1.5 mg/l., 2.0 mg/l., 2.5 mg/l were used to check the callus induced from leaf and stem explants. Very low callus response was observed. The callus derived from stem explants at concentrations of 0.5 mg/l, 1.0 mg/l, and 2.0 mg/l. The callus at these concentrations was light green compact and hard. Callus changed color after 2-3 weeks very low Callogenic response was observed in leaves explants as only two calluses were formed each at 0.5 and 1.0 mg/l.

Effect of NAA

Five different concentrations of NAA from 0.5 mg/l to 2.5 mg/l were also used to check the Callogenic response. Leaves did not show any callogenic response at any of the concentration of NAA used, only one single callus formed at 0.5 mg/l with stem explants. Callus formed was green and fresh.

Effect of IBA

Callogenic response stem explants with IBA were comparatively a bit better. Callus induction was observed at 0.5 mg/l, 1.0 mg/l, and 2.5 mg/l. The resultant callus was green, friable and soft. No Callogenic formation was observed in leaf explants

Effect of BA

Five different concentrations of BA 0.5 mg/l to 2.5 mg/l were used to check the callogenic response. At 0.5 mg/l.1.0mg/l and 2.0mg/l callogenic responses observed in stem explants. But the callus formed was light green and not fresh. Two calluse formed with leave explants at 0.5 mg/l and with 1.5mg/l

Effect of Kinetins

Callogenic response at any concentration of kinetin was poor. Some lower strength i.e. 0.5 mg/l and 1.0 mg/l only showed some callus formation was observed with stem and leaf explants. Five different concentrations 0.5mg/l, 1.0 mg/l, 1.5 mg/l, 2.0 mg/l, 2.5 mg/l were used.

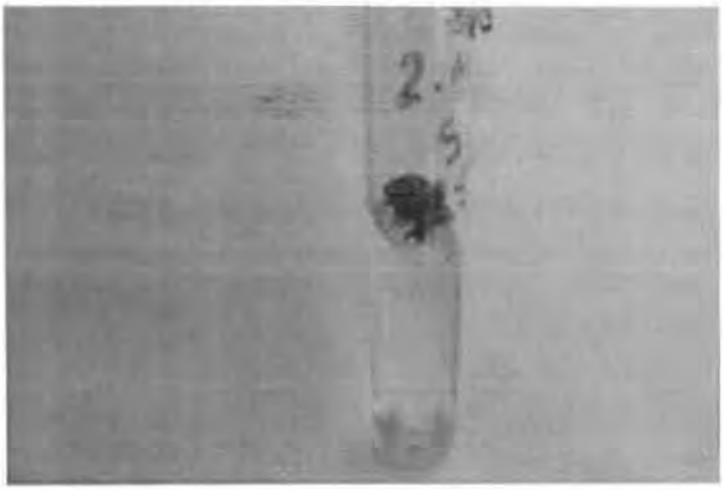
Effect of IBA/BA

Good callogenic response was observed with this combination callus formation was observed with stem explants at 0.5mg/l, 1.0mg/l, 1.5mg/l. Callus formed was fresh and green. One callus each also formed with leave explants at 0.5mg/l and 1.5mg/l. Three different concentrations 0.5 mg/l, 1.0 mg/l, 1.5 mg/l were used.

Table 4.1: Callogenic Response from stem Explants at Different Concentrations of Growth Regulators

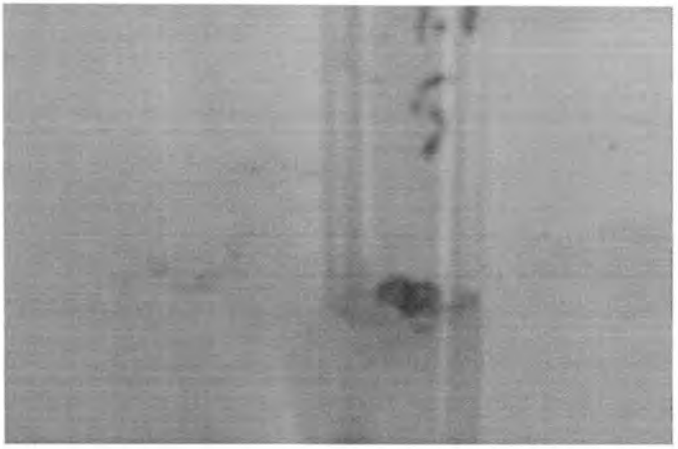
Culture	Growth Regulator	Concentration mg/l	Callogenic Response	Percentage	Appearance
1	2,4 D	0.5	+	20%	Yellowish color
2	2,4 D	1.0		0%	
3	2,4 D	1.5		0%	
4	2,4 D	2.0		0%	
5	2,4 D	2.5		0%	
1	IAA	0.5	+	20%	Light green color
2	IAA	1.0	+	20%	Light green color
3	IAA	1.5		0%	
4	IAA	2.0		0%	
5	IAA	2.5		0%	
1	NAA	0.5		0%	
2	NAA	1.0		0%	
3	NAA	1.5		0%	
4	NAA	2.0		0%	
5	NAA	2.5		0%	
1	IBA	0.5		0%	
2	IBA	1.0		0%	
3	IBA	1.5	+	20%	Green, soft
4	IBA	2.0		0%	
5	IBA	2.5		0%	
1	BA	0.5	+	20%	Light green in color
2	BA	1.0	++	40%	light green in color
3	BA	1.5			

4	BA	2.0	+	20%	yellowish in color
5	BA	2.5			
1	Kin	0.5	++	40%	
2	Kin	1.0	+	20%	
3	Kin	1.5			
4	Kin	2.0			
5	Kin	2.5			
1	IBA/BA	0.5	++	40%	fresh and green
2	IBA/BA	1.0	++	40%	fresh and green
3	IBA/BA	1.5	+	20%	fresh and green
1	BA/NAA	0.5	+	20%	dull green
2	BA/NAA	1.0			
3	BA/NAA	1.5			
1	BA/2,4-D	0.5			
2	BA/2,4-D	1.0			
3	BA/2,4-D	1.5			
1	Kin/IBA	0.5			
2	Kin/IBA	1.0	++	40%	fresh green colored
3	Kin/IBA	1.5			

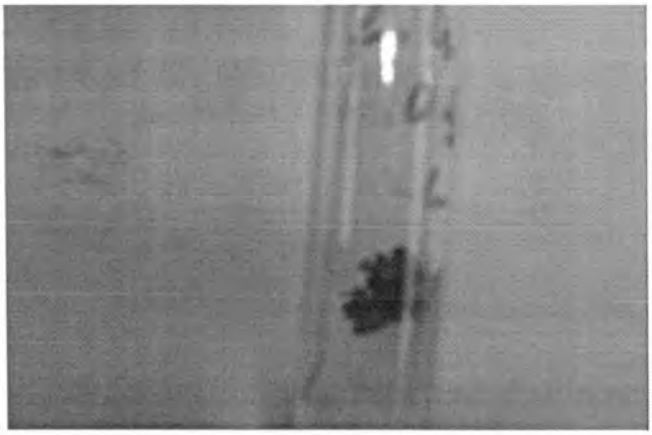


Origin: ***In vitro*** **callogenesis from stem explant**
Nutrition status: **MS Medium**
Hormonal concentration: **0.5 mg/l**
Hormone: **2, 4-D**
Observation Time: **30 Days**

Origin: ***In vitro*** **callogenesis from leaf explant**
Nutrition status: **MS Medium**
Hormonal concentration: **2.0 mg/l**
Hormone: **IAA**
Observation Time: **30 Days**



Origin:	***In vitro* callogenesis from stem explant**
Nutrition status:	**MS Medium**
Hormonal concentration:	**0.5 mg/l**
Hormone:	**NAA**
Observation Time:	**30 Days**



Origin:	***In vitro* callogenesis from leaf explant**
Nutrition status:	**MS Medium**
Hormonal concentration:	**1.0 mg/l**
Hormone:	**IBA**
Observation Time:	**30 Days**

Figure 3.1: Callogenic response of different explant of Bacopa monnieri at different hormonal concentration

4.2 Organogenesis.

Observation on the callusing response of stems and leaf explant incubated with varying concentration of different growth regulators individually and in combination are summarized in the table. 4.3 and 4.4 In general the explants showed tendency to regenerate shoots rather then to form callus with in the 2-3 weeks of initiation of incubation.

Multiple shoot buds without an intervening callus phase, were induced at the cut ends of stems and leaf explants. The emergence of shoot buds was observed visually almost with in two weeks. After the period of 4-5 weeks a thick mat of shoot buds spread over 90-100% of the explant surface. Each plant was then transformed into the dense mass of profusely regenerating shoot buds which made it almost impossible to count the number of shoot buds per unit area

Effect of 2 – 4 – D

Different concentrations of 2, 4-D were used from all two explants i.e. leaf and stem on MS medium. Five different concentrations ranging 0.5 mg/l, 1.0 mg/l, 1.5 mg/l, 2.0 mg/l, and 2.5 mg/l were used.

Almost at all concentrations mostly direct propagation was observed. Internodes and leaf explants showed direct shoot bud generation without any callus formation. Explants initiated growth within 2-3 weeks of inoculation. Shoots were fresh and green with green small leaves

Effect of IAA.

Different concentrations of IAA were used from all two explants i.e. leaf and stem on MS medium. Five different concentrations ranging 0.5 mg/l, 1.0 mg/l, 1.5 mg/l, 2.0 mg/l, and 2.5 mg/l were used.

.Direct propagation without an intervening callus phase was observed at the cut ends of stem and leaves explants. Growth was observed mostly after 2-3 weeks of inoculation. Shoots and leaves were fresh and green.

Effect of NAA

Different concentrations of NAA were used from all two explants i.e. leaf and stem on MS medium. Five different concentrations ranging 0.5 mg/l, 1.0 mg/l, 1.5 mg/l, 2.0 mg/l, and 2.5 mg/l were used.

Direct propagation was observed at all concentrations of leaves and stem explants. Explants initiated growth within 2-3 weeks of culture. Multiple shoots with fresh green leaves formed.

Effect of IBA

Different concentrations of IBA were used from all two explants i.e. leaf and stem on MS medium. Five different concentrations ranging 0.5 mg/l, 1.0 mg/l, 1.5 mg/l, 2.0 mg/l, and 2.5 mg/l were used

Fresh green healthy shoot buds were observed at almost all concentrations both with stems and leaves. Shoot induction was observed after 2-3 weeks. Shoots formed were fresh green with multiple leaves.

Effect of BA

Different concentrations of BA were used from all two explants i.e. leaf and stem on MS medium. Five different concentrations ranging 0.5 mg/l, 1.0 mg/l, 1.5 mg/l, 2.0 mg/l, and 2.5 mg/l were used

Mostly direct propagation was observed with multiple shoot formation was observed after 2-3 weeks of inoculation. Shoots formed were fresh and green and showed good rapid growth.

Effect of Kinetin

Different concentrations of IBA were used from all two explants i.e. leaf and stem on MS medium. Five different concentrations ranging 0.5 mg/l, 1.0 mg/l, 1.5 mg/l, 2.0 mg/l, and 2.5 mg/l were used

Almost all leaf and most of the stems explants showed direct propagation at all concentrations. Direct propagation emerged after 2-3 weeks which showed good growth with fresh green multiple shootings. Five different concentration 0.5mg/l, 1.0mg/l, 1.5mg/l, 2.0mg/l, 2.5mg/l were used.

Effect of BA/IBA

Different concentrations of BA/IBA combination were used for explants of leaf and stem on MS medium. Three different concentrations ranging 0.5/0.05 mg/l, 0.5/1.0 mg/l, and 0.5/0.25 mg/l were used.

Direct propagation was observed in almost all concentrations with multiple green shoot formation. Direct propagation initiated after 3 weeks, with multiple fresh green growth.

Effect of BA/NAA

Different concentrations of BA/NAA combination were used for explants of leaf and stem. Three different concentrations ranging 0.5/0.05 mg/l, 0.5/1.0 mg/l, and 0.5/0.25 mg/l were used.

Good response on direct propagation was observed in all different concentrations used. Direct propagation was observed after 3-4 weeks. Resulting shoots were fresh green and showed good growth.

Effect of BA/2-4-D

Different concentrations of BA/2-4-D combination were used for explants of leaf and stem. Three different concentrations ranging 0.5/0.05 mg/l, 0.5/1.0 mg/l, and 0.5/0.25 mg/l were used.

Amongst all three concentration that were used with BA/2, 4-D combination. Direct shoot propagation with multiple fresh green rapidly growing shoots were observed. Shoot start appearing after 2-3 weeks.

Effect of Kin/IBA

Different concentrations of BA/2-4-D combination were used for explants of leaf and stem. Three different concentrations ranging 0.5/0.05 mg/l, 0.5/1.0 mg/l, and 0.5/0.25 mg/l were used.

Some low response for direct propagation was observed with stem explants, but good percentage showed direct propagation with leaf explants. Growth observed after 3 weeks with fresh green plants seen multiple shoots.

Table 4.3: Direct Propagation Response from stem Explants at Different Concentrations of Growth Regulators

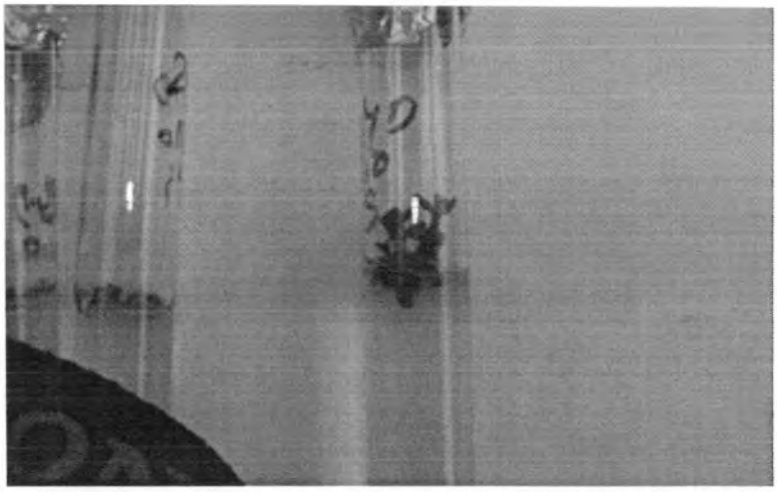
Culture	Growth Regulator	Concentration mg/l	Direct Propagation	Percentage response	Appearance
1	2,4 D	0.5	+++	60%	Fresh green
2	2,4 D	1.0	++	40%	Fresh green
3	2,4 D	1.5	++	40%	Fresh green
4	2,4 D	2.0	+++	60%	Fresh green
5	2,4 D	2.5	++++	80%	Fresh green
1	IAA	0.5	++	20%	Fresh green
2	IAA	1.0	+++	60%	Fresh green
3	IAA	1.5	+++	60%	Fresh green
4	IAA	2.0	++++	80%	Fresh green
5	IAA	2.5	++++	80%	Fresh green
1	NAA	0.5	++	40%	Fresh green
2	NAA	1.0	++	40%	Fresh green
3	NAA	1.5	+++-	60%	Fresh green
4	NAA	2.0	++++	80%	Fresh green
5	NAA	2.5	+++	60%	Fresh green
1	IBA	0.5	++	40%	Fresh green
2	IBA	1.0	+++	60%	Fresh green
3	IBA	1.5	++++	80%	Fresh green
4	IBA	2.0	+++	60%	Fresh green
5	IBA	2.5	++++	80%	Fresh green
1	BA	0.5	+++	60%	Fresh green
2	BA	1.0	++	40%	Fresh green
3	BA	1.5	++	40%	Fresh green

4	BA	2.0	+++	60%	Fresh green
5	BA	2.5	+++	60%	Fresh green
1	Kin	0.5	++	40%	Fresh green
2	Kin	1.0	+	20%	Fresh green
3	Kin	1.5	+++	60%	Fresh green
4	Kin	2.0	++	40%	Fresh green
5	Kin	2.5	++++	80%	Fresh green
1	IBA/BA	0.5/0.05	++	40%	Fresh green
2	IBA/BA	0.5/1.0	+	20%	Fresh green
3	IBA/BA	0.5/0.25	+++	60%	Fresh green
1	BA/NAA	0.5/0.05	+++	60%	Fresh green
2	BA/NAA	0.5/1.0	++	40%	Fresh green
3	BA/NAA	0.5/0.25	++++	80%	Fresh green
1	BA/2,4-D	0.5/0.05	+++	60%	Fresh green
2	BA/2,4-D	0.5/1.0	++	40%	Fresh green
3	BA/2,4-D	0.5/0.25	++++	80%	Fresh green
1	Kin/IBA	0.5/0.05	+	20%	Fresh green
2	Kin/IBA	0.5/1.0	+	20%	Fresh green
3	Kin/IBA	0.5/0.25	+++	60%	Fresh green

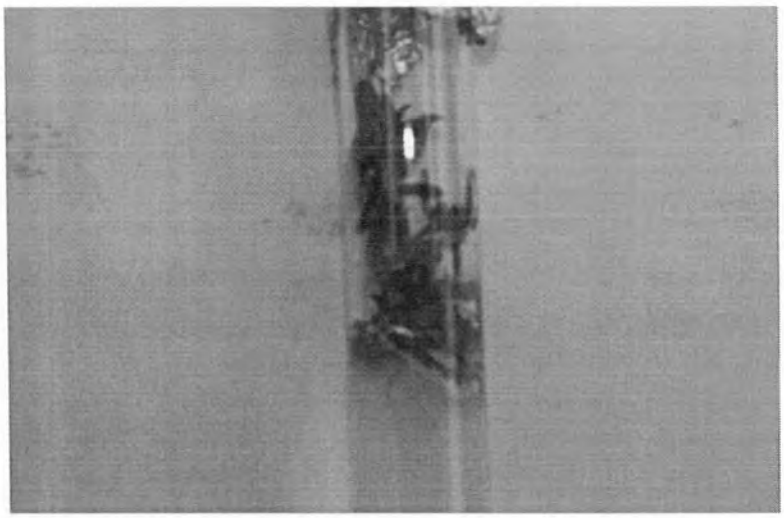
Table 4.4: Direct Propagation Response from leave Explants at Different Concentrations of Growth Regulators

Culture	Growth Regulator	Concentration mg/l	Direct Propagation	Percentage	Appearance
1	2,4 D	0.5	++++	80%	Fresh green
2	2,4 D	1.0	+++	60%	Fresh green
3	2,4 D	1.5	+++	60%	Fresh green
4	2,4 D	2.0	++++	80%	Fresh green
5	2,4 D	2.5	++++	80%	Fresh green
1	IAA	0.5	++	40%	Fresh green
3	IAA	1.5	+++	60%	Fresh green
4	IAA	2.0	++++	80%	Fresh green
5	IAA	2.5	++++	80%	Fresh green
1	NAA	0.5	+++	60%	Fresh green
2	NAA	1.0	+++	60%	Fresh green
3	NAA	1.5	+++	60%	Fresh green
4	NAA	2.0	++++	80%	Fresh green
5	NAA	2.5	+++	60%	Fresh green
1	IBA	0.5	+++	60%	Fresh green
2	IBA	1.0	+++	60%	Fresh green
3	IBA	1.5	++	40%	Fresh green
4	IBA	2.0	+++	60%	Fresh green
5	IBA	2.5	++++	80%	Fresh green

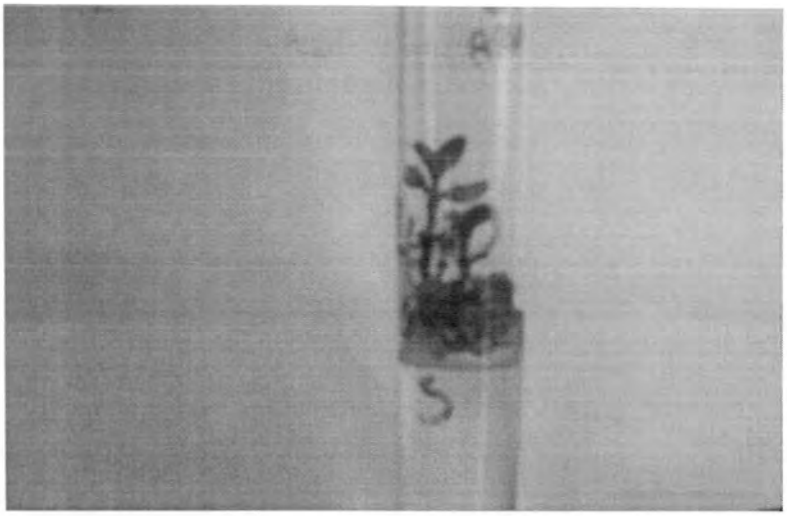
1	BA	0.5	+++	60%	Fresh green
2	BA	1.0	++	40%	Fresh green
3	BA	1.5	++	40%	Fresh green
4	BA	2.0	+++	60%	Fresh green
5	BA	2.5	+++	60%	Fresh green
1	Kin	0.5	+++	60%	Fresh green
2	Kin	1.0	+++	60%	Fresh green
3	Kin	1.5	++	405	Fresh green
4	Kin	2.0	+++	60%	Fresh green
5	Kin	2.5	++++	80%	Fresh green
1	BA/IBA	0.5/0.05	++	40%	Fresh green
2	BA/IBA	0.5/1.0	++++	80%	Fresh green
3	BA/IBA	0.5/0.25	+++	60%	Fresh green
1	BA/NAA	0.5/0.05	+++	60%	Fresh green
2	BA/NAA	0.5/1.0	++	40%	Fresh green
3	BA/NAA	0.5/0.25	++++	80%	Fresh green
1	BA/2,4-D	0.5/0.05	+++	60%	Fresh green
2	BA/2,4-D	0.5/1.0	++	40%	Fresh green
3	BA/2,4-D	0.5/0.25	++++	80%	Fresh green
1	Kin/IBA	0.5/0.05	++	40%	Fresh green
3	Kin/IBA	0.5/0.25	+++	60%s	Fresh green
2	Kin/IBA	0.5/1.0	+++	60%	Fresh green



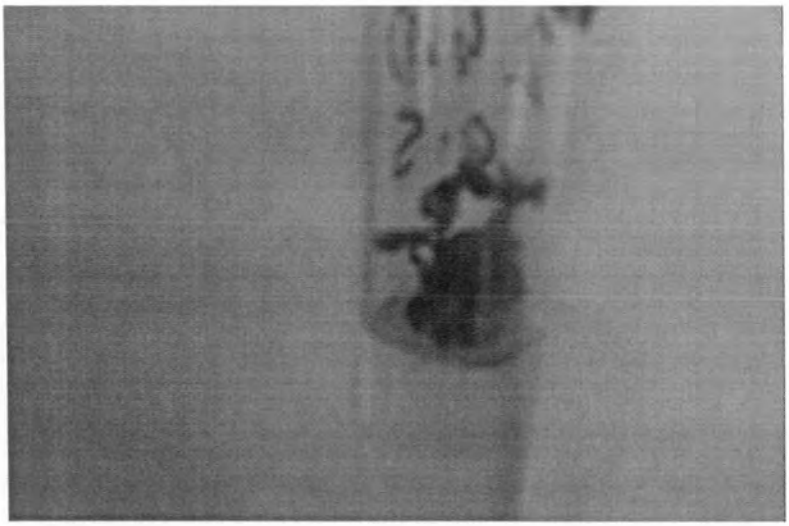
Origin: In vitro shooting from stem explant
Nutrition status: MS Medium
Hormonal concentration: 2.0 mg/l
Hormone: 2-4-D
Observation Time: 30 Days



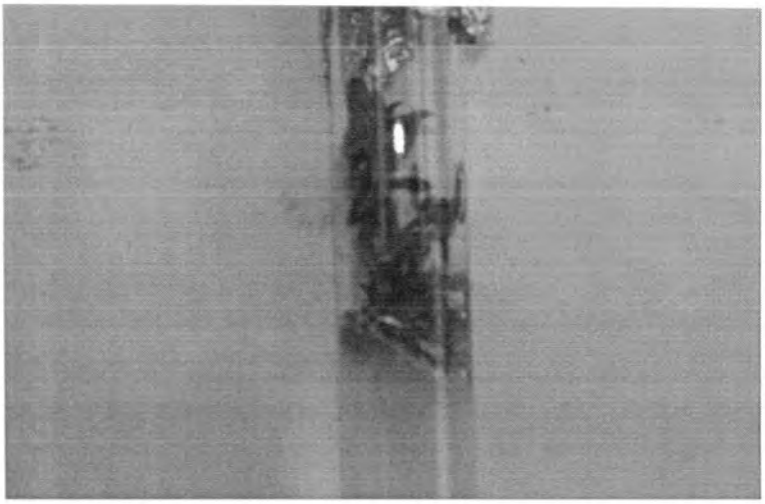
Origin: In vitro shooting from leaf explant
Nutrition status: MS Medium
Hormonal concentration: 1.0 mg/l
Hormone: IAA
Observation Time: 30 Days



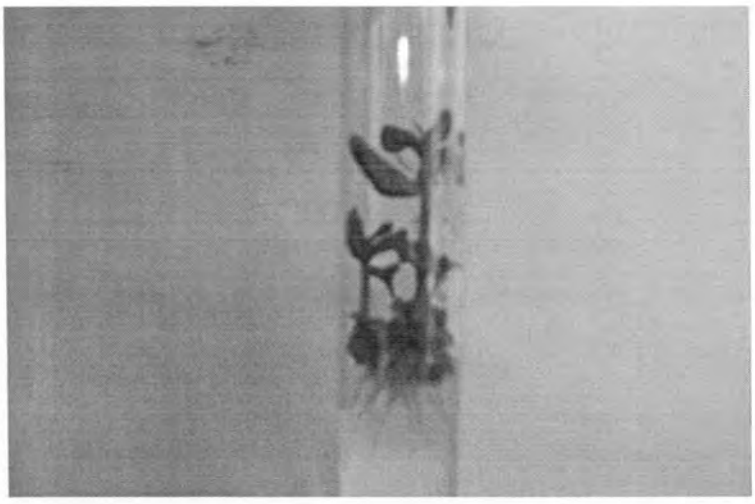
Origin:	**In vitro shooting from stem explant**
Nutrition status:	**MS Medium**
Hormonal concentration:	**2.0 mg/l**
Hormone:	**NAA**
Observation Time:	**30 Days**



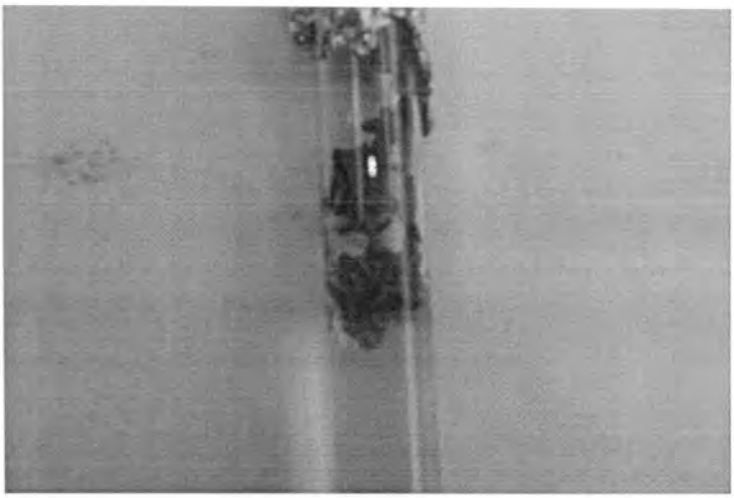
Origin:	**In vitro shooting from leaf explant**
Nutrition status:	**MS Medium**
Hormonal concentration:	**2.0 mg/l**
Hormone:	**IBA**
Observation Time:	**30 Days**



Origin:	**In vitro shooting from stem explant**
Nutrition status:	**MS Medium**
Hormonal concentration:	**1.5 mg/l**
Hormone:	**BA**
Observation Time:	**30 Days**



Origin:	**In vitro shooting from leaf explant**
Nutrition status:	**MS Medium**
Hormonal concentration:	**2.5 mg/l**
Hormone:	**Kinetin**
Observation Time:	**30 Days**



Origin:	**In vitro shooting from leaf explant**
Nutrition status:	**MS Medium**
Hormonal concentration:	**0.5/1.0 mg/l**
Hormone:	**BA/2, 4-D**
Observation Time:	**30 Days**

Figure 3.2: Direct propagation response of different explant of Bacopa monnieri at different hormonal concentration

Chapter 5
DISCUSSION

Tissue culture of *Bacopa monnieri*

Plant propagation using explants is widely used area in plant cell and tissue culture and is being currently used to propagate elite genotype of several crop plants in a number of countries.

Callogenic or direct Micropropagation response was different at different concentration of plant growth regulators either in single or in combination from leaf, stem and cotyledon explants. Green, soft, friable and compact callus was considered best. While fresh green multiple shoot regeneration was considered best.

Multiple shoot buds, without an intervening callus phase, were induced at the cut ends of stem and leaf explants most of the time. The emergence of shoot buds was observed visually on the 2-3 weeks of incubation. After period of 4-5 weeks a thick mat of shoot buds spread over 90-100% of the explant surface. Each explant was transformed into a dense mass of profusely regeneration shoot buds which made it impossible to count the number of shoot buds per unit area. There was an increase in the concentration. Furthermore the shoot induction response of the leaf explants was qualitatively better than that of the stem explants at all concentrations.

At lower dose of 2, 4 D callus formation was observed with stem explants. Callus formed was yellowish green in color. Almost no callus response was observed with higher doses of 2, 4 D. Same is the case with leave explant where direct propagation was observed.

It was observed that 2, 4, D showed good response for direct propagation with multiple shoot formation, but the callogenic response is poor for higher doses. This is in

accordance with Tiwari *et al.*(1998) who has reported callus formation from nodal explants of *Bacopa* cultured on MS medium containing 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D)

Callogenic response at all concentration of NAA and IAA was either absent or very poor from all explants. As leaf explant did not show callogenesis at any concentration of NAA, while stem explants showed some callogenic response at lower concentrations. Callus formed was yellowish green. Shoot growth was observed after few days from those calluses but it died within few weeks.

However good direct shoot induction with fresh green and multiple shoot formation was observed with almost all strengths of IAA and NAA. This growth initiated after about 2-3 weeks. These findings are according to the findings of Shrivastava *et al.* (1998) which states direct shoot induction without any intervening callus.

Different concentrations of BA and Kin did not show good callogenic response. Some callus formation was observed at lower concentration but mostly direct shoot induction was observed. Srivastava *et al.* (2002) observed direct regeneration of shoots and roots occurred in nodal explants in *Bacopa* on MS medium containing BAP. However this oppose Rani *et al.* (2003) who reported that callus induction was observed from hypocotyls, root, and cotyledonary leaf segments, grown on MS medium supplemented Kn.

However, direct propagation was observed with almost all strengths of BA and Kin. This is accordance to Tejavathi *et al.* (2001) also found that maximum numbers of plants were obtained on medium containing Kin in shoot tip and nodal cultures of *Bacopa monnieri* respectively.

Combination of IBA/BA, BA/NAA, BA/ 2,4,D, Kin/IBA all showed very poor callogenic response. Almost all strengths showed direct shoot induction. This confirms the results of Shrivastva and Rajani. 1999, reported that out of two cytokinins used BAP

was found to be more suitable than Kn as BAP resulted in quicker and better response than the latter while addition of NAA (0.2mg/l) proved synergistic Srivastava *et al.*, (2002). Tiwari *et al.*, (2000), also reported that addition of BAP resulted in the increase in number of shoots, mean shoot length and number of roots/explant. Srivastava *et al.*, (2002), observed direct regeneration of shoots and roots occurred in nodal explants in Bacopa on MS medium containing NAA (0.1 mg/l) and BAP (0.5 mg/l).

Conclusion

Herbs are being used since ancient time to maintain health, to treat disease and regain the healthy state of mind and body. They have been used in traditional forms of Indian medicine and have provided solutions to even those health problems that have defied modern science. However, due to over exploitation they are on the verge of extinction.

Bacopa monnieri L. Penn., commonly known as Brahmi, has been used in Indian System of Medicine for centuries for many from snakebite to headache. It is used most often as a brain tonic and a memory enhancer. The demand of *Bacopa* is met from natural population, which leads to put heavy strain on existing natural population and hence slow depletion of this important herb. Tissue culture techniques can be used to attain rapid multiplication of the elite clones and germplasm conservation of *Bacopa monnieri*

Contrary to earlier reports of the use and need of very high concentrations of cytokinins for Brahmi growth, the present work has developed a method of improving in vitro propagation by developing a novel improved protocol highlighting efficient reproducible and reliable techniques for mass multiplication of a medicinally and economically important herb *Bacopa monnieri*. In the present work we have attempted to develop suitable micropropagation protocol and try to improve existing protocol of *Bacopa monnieri*.

Our results show that growth hormones are required for adventitious shoot-bud induction in *B. monnieri* has a high morphogenesis potential, and the explants readily responded to hormones in the culture medium and formed multiple shoot buds. While there are reports of a requirement for high concentrations for shoot regeneration (Yang and Read Bhuyan *et al.*1997). We obtained a high level of shoot-bud regeneration with higher concentrations, which stimulated the induction and continuous proliferation of shoot buds from the explants. Leaves were found to be a superior explant material for shoot regeneration. *B. monnieri* leaf explants continued to expand exponentially and regenerate new shoots. The system showed a potential for a continuous supply of shoots of *B. monnieri* leaf and stem explants showed a very poor callusing capability. Even 2, 4-D, which is often included in tissue culture media for callus initiation and callus culture (Sunderland, 1973) could not induce the formation of proliferating callus in *B. monnieri.*

Chapter 6

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