MICROPROPAGATION OF SOME IMPORTANT MEDICINAL PLANTS OF KERALA

THESIS
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By

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IN GOD I TRUST

DEDICATED TO MY BELOVED PARENTS

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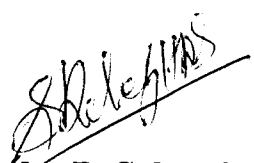
CERTIFICATE

This is to certify that the thesis entitled "**Micropropagation of some important medicinal plants of Kerala** "submitted to the University of Calicut by Delse P. Sebastian for the Degree of **Doctor of Philosophy in Botany**, embodies the results of bonafide original research work carried out by him under my supervision and guidance and the thesis has not previously formed the basis for the award of any degree, diploma, associateship, fellowship or recognition.

Aleyamma Thomas

DECLARATION

I do hereby by declare that that the work presented in this thesis entitled **"Micropropagation of some important medicinal plants of Kerala."** has been originally carried out by me under the guidance of Dr.Aleyamma Thomas, Professor (Retd.), Dept. of Botany University of Calicut; and that the same has not been submitted earlier for any degree or diploma of any other University in India or abroad.



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CONTENTS

Abbreviations

µl	:	Microlitre
2,4-D	:	2,4,Dichlorophenoxyacetic acid
2iP	:	N^6(2-isopentyl)-adenine
B_5	:	Gamborg et al. (1968)
BA	:	6-Benzyl-adenine ($C_{12}H_{11}N_{15}$)
BSA	:	Bovine serum albumine
$CaCl_2$	:	Calcium chloride
g	:	Gram
H_2SO_4	:	Sulphuric acid
HCl	:	Hydrochloric acid
$HgCl_2$	:	Mercuric chloride
IAA	:	Indole-3-acetic acid ($C_{10}H_9\ NO_2$)
IBA	:	Indole-3-butric acid ($C_{12}H_{13}NO_2$)
Kn	:	Kinetin (6-furfurylaminopurine)
mg	:	Milligram
min.	:	Minutes
ml	:	Milli litre
mM	:	Milli molar
mm	:	Millimeter
MS	:	Murashige and Skoog's (1962)
NAA	:	α- naphthalene acetic acid
NaOH	:	Sodium Hydroxide
nm	:	Nanometre
PAGE	:	Poly Acrylamide Gel Electrophoresis
pH	:	Pruissense de hydrogen
PVP	:	Poly Vinyl Pyrolidone
Rf	:	Ratio of fronds
SDS	:	Sodium Dodecyl Suplhate
TLC	:	Thin layer chromatography

INTRODUCTION

INTRODUCTION

Medicinal plants are being used by about 80% of the world population for primary health care (Kamboj 2000). The practice of using medicinal plants for the treatment of various diseases started since the dawn of civilization (Das *et al.,* 1999). In fact ancient man was totally dependant on medicinal plants for the treatment of various ailments (Hussain 1991).

Drugs obtained from plants consist of entire plant or their parts leaves, roots, fruits, seeds, rind etc. Dried plants or plant parts and phytochemicals have been widely used for the preparation of phytomedicines in ayurvedic, allopathic, unani, siddha, homoeopathic and folk medicines. The disease curing properties of plants are associated with their chemical constituents.

Ayurveda, an integral part of Indian culture from vedic ages (1500-800 B.C) mainly uses plant based drugs for the treatment of diseases. Following the discovery of modern medicines in the 18^{th} century, herbal medicines including ayurvedic ones suffered a set back. However, presently there has been an increasing interest for the plant based drugs because of the ready acceptance to local populance, relative inexpensiveness and minimal side effects. The fascination of our holistic system of medicine especially ayurveda, which relies on the use of more than 7000 medicinal plants attained popularity not only in India but also abroad.

Screening and indexing of many plants, which have been used in traditional Indian and Chinese medicines since time immemorial, resulted in novel therapeutics, useful for the treatment of various ailments of man such as rheumatism, kapha, pitha, blood pressure, cancer etc. (Anonymous 1948 and 1959).

India is one of the world`s richest sources of medicinal plants because of its rich geographical diversity, varied climatic and ecological features. About 60% of Indian population depends mainly on Ayurvedic medicines for the treatment of common diseases (Nair *et. al.*, 1992). Presently, the traditional herbal medicines, both nationally and internationally are receiving considerable attention from pharmaceutical industries. The scientific study of traditional medicines, derivatives of drugs, through bioprospection and systematic conservation, domestication and cultivation of the medicinal plants thus assumes great importance in today's context when more people need safe and effective medicine at affordable rate.

It has been estimated that about 30% of pharmaceuticals are derived from green plants and this percentage has risen considerably in recent years. The market for whole plant preparations, often sold as complimentary or safer alternative medicines has also been increasing (Saxena 2002). In most industrialized countries the use of medicinal plants has increased dramatically in the last decade (Rajendra and D`souza 1999). Medicinal plants therefore form

an important part of international commerce. Present global market of medicinal plants or their products is that of Rs.360000 crore annually. Of these Indian share is pegged at Rs.2800 crore and is growing by 7% annually (Abraham 2002). Trade in medicinal plants between developed and developing countries is expected to touch $500 billion by the turn of the century (Banerjee 1998).

As a result of ever-increasing demand for medicinal herbs, the supply of the medicinal plants has dwindled. According to World Health Organization, these starting materials for medicinal preparations represent some 21000 plant species of which 70 to 90 percent are obtained through commercial collection from wild habitat (WHO, 1978 and 1987). Even now, most of the plants used in medicines are collected from their wild habitats and only some species used in larger quantities are cultivated systematically.

Many medicinal plants, which were ignored in the past years, have been over exploited in recent years. The plant collectors increase in number, but the number of plants still found in the wild is progressively declining (Rajendra and D'Souza 1999). Collected plant drugs, especially those wild crafted and traded under the vernacular name, are very prone to mislabeling, making an analytical determination of identity important. Another problem associated with plant collection from wild is the frequent contamination of medicinal plant materials with foreign matter, such as sand, grass and non-drug part of the collected

plants (Harnischfeger 2000). Due to unscrupulous and unscientific collection some of the most valuable medicinal plant species of natural resources are facing extinction. Studies have shown that about 10% of the plants fall into the category of endangered species (Raizada 1993). This may virtually result in termination of the branch of medicine using natural products for the treatment of ailments and related industries (Kulkarni 1995). To over come all these problems it has become imperative to develop methods to conserve and propagate medicinal plants on large scale.

Conservation of medicinal plants and the capability to utilize them in a sustained manner are essential for the well being and continued survival of man. Moreover germplasm conservation is important for the breeding programme as the diversity in the crop harbour genes for various traits.

The conservation can be done by *in situ* method or *ex situ* method. In *in situ* method, plant or the stock of the plant community is protected in its natural habitat as national park or as biosphere reserves. Whereas, in *ex situ* conservation samples of genetic diversity (species) are protected away from their field habitats. *Ex situ* conservation is done through the establishment of gene banks which include genetic resource centers, zoos, botanical gardens, tissue culture collections etc.. Tissue culture techniques are invaluable to compete other conservation strategies particularly for vegetatively propagated and threatened medicinal plants. The major advantageous of *in vitro*

techniques are rapid multiplication and storage of relatively large number of propagules in small space, away from natural vagaries (Sharma 2001). Development of micropropagation protocol is a pre - requisite for *in vitro* conservation (Tyagi and Prakash 2001).

Plant tissue culture techniques are now being used globally for the multiplication and conservation of medicinally important plant species and monitoring their secondary metabolites (Rajasekharan *et al.*,2001; Prakash *et al.*,1999). These techniques ensure the availability of plants throughout the year, production of uniform clones, production of plants with changed genotype, conservation of genetic resources etc.

Micropropagation through direct organogenesis ensures genetic stability in the *in vitro* propagated progeny and it has been successfully practiced for the clonal multiplication of large number of medicinal plants. Micropropagation through indirect organogenesis offers a method for the selection of useful and economically important variants.

Somatic embryogenesis also offers a method for *in vitro* propagation and it enables the production of large number of plants from explants. The method can also be used for the genetic manipulation and conservation of important medicinal plants.

Synthetic seed technology is an exciting and rapidly growing area of research in the field of plant cell and tissue culture. The technology is designed to combine advantages of clonal propagation with those of seed propagation and storage (Datta *et al.,* 1999; Ara *et al.,* 2000). Earlier, synthetic seeds were referred only to encapsulated somatic embryos. However, in the recent past other micropropagules like shoot buds, shoot tips, organogenic or embryogenic calli etc. have also been employed in the production of synthetic seeds.

Medicinal plants are valued for their secondary metabolites such as alkaloids, steroids, flavanoids, terpenes, glycosides etc. The use of plant tissue culture technique for the large scale production of secondary metabolites is advantageous, as there are problems in the extraction of metabolites from field grown plants (due to dependency of the secondary metabolite metabolism to season and environmental constraints during the cultivation). Moreover, in many cases the production of secondary metabolites from cell cultures is higher in comparison to small amount extracted from *in vitro* grown plants (Chand *et al.*, 1999). Plant tissue culture can also be utilized for studying the physiology and biochemistry associated with growth and differentiation in plants.

Considering all these facts into account two important medicinal plants ***Heliotropium keralensis*** and ***Naregamia alata*** were selected for the present investigations.

IMPORTANCE OF THE PLANT MATERIALS

Heliotropium keralensis Sivar & Manilal

Heliotropium keralensis is an endemic seasonal medicinal plant of Kerala. (Sasidharan and Sivarajan 1996; Sivarajan and Balachandran 1994). *Heliotropium keralensis* is a constituent of ayurvedic drugs like vidaryadigana and arkadigana of Vagbhata. The roots of the plant are errhine and enter into the composition of preparations like Vidaryassavam, Vidaryadighastam, Vidaryadileham etc, (Sivarajan and Balachandran,1994). The plant is reported to be bitter and it pacifies all the three morbidities, cleanses wounds and is useful in the treatment of worms, skin diseases, scorpion and snake poisoning, asthma, cough, anaemia, insanity and epilepsy (Ulubelde *et al.,* 1991; Sivarajan and Balachandran 1994). Pyrolozidine alkaloids from *Helioptropium* species have significant antitumour activity (Said and Saeed 1993).

Naregamia alata W & A

Naregamia alata W &A is a small branching undershrub belonging to the family Meliaceae. Due to over exploitation for medicinal uses the plant has become rare in nature (Daniel *et al.*, 1999).

The plant is acrid sweet, cooling, aromatic, alexeteric, vulnerary, emetic, cholagogue, expectorant, depurative and antipyretic (Warrier *et al.*, 1995). The plant contains an alkaloid naregamin, an oxidizable fixed oil, wax, sugar, resin etc. (Khory and Katrak 1999). The roots of *N. alata* are good emetic and cholagogue and is sweet and cooling (Kirthikar and Basu, 1995; Warrier *et al.*, 1995; Chopra *et al.,* 1956).

Roots, leaves and stems of *N. alata* have been used in the treatment of acute dysentery, asthma, bronchitis, biliousness, ulcer, etc. (Dey and Bahadur, 1973, Chopra *et al.*, 1956; Kirthikar and Basu, 1995)

The whole plant of *N. alata* has been used in the treatment of rheumatism, itch, wounds, ulcers, vitiated conditions of pitha, and vatha, halitosis, cough, pruritus, dysentery, catarrh, anaemia, chronic fever, malarial fever etc. (Chopra *et al.,* 1956; Warrier *et. al.*1995).

OBJECTIVES OF THE PRESENT STUDY

Considering the ever increasing demand from Indian pharmaceutical industry and rapidly depleting natural resources, these two important medicinal plants. ***Heliotropium keralensis*** Sivar & Manilal and ***Naregamia alata*** W&A were selected for the *in vitro* culture studies with the following objectives.

- Direct shoot regeneration from different explants
- Indirect shoot regeneration from different explants,through callus culture
- Induction of somatic embryogenesis
- Encapsulation of somatic embryos and shoot buds and germination studies.
- Induction of roots on *in vitro* shoots
- Successful establishment of micropropagated plants in the field conditions
- Comparative qualitative analysis of secondary metabolites in the root tissues of *in vitro* and field grown plants by TLC.
- Biochemical studies in primary and regenerating calli.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Medicinal plants have been used in Ayurvedic, Unani, Homoeopathic and Allopathic medicines to cure various diseases. Due to large scale and unrestricted exploitation of the natural resources to meet the ever increasing demand by the pharmaceutical industry coupled with limited cultivation and insufficient attempts of replenishment, the wild stock of medicinally important plant species have been markedly depleted. Hence it has become imperative to develop suitable techniques for rapid and large scale production of important medicinal plants to meet the production needs of plant based drugs and conservation of the plants. Plant Biotechnology, in particularly, plant tissue culture has emerged as a novel technology for mass multiplication and germplasm conservation of rare, endangered aromatic and medicinal plants (Sudha and Seeni 1994). It offers many unique advantages over conventional propagation methods such as rapid multiplication of valuable genotypes, expeditious release of improved varieties, production of disease free plants, non-seasonal production, germplasm conservation and novel delivery system of propagules through production of synthetic seeds.

Advances during last five decades in the field have important bearing in realms of industrial technology, production of natural compounds of therapeutic value and in biosynthetic studies of secondary plant constituents. Bajaj *et al.,*

(1988) and George (1996) made wide reviews on tissue culture studies of medicinal plants. In fact, literature on plant tissue culture is too abundant and exhaustive to comprehend in this chapter. Yet an attempt is made to review various aspects of *in vitro* studies, regarding micropropagation, callus culture, somatic embryogenesis, conservation through synseeds, secondary metabolite analysis and biochemical studies performed on medicinal plants with special reference to Boraginaceae and Meliaceae.

CALLUS CULTURE AND MICROPROPAGATION

First significant attempt to culture isolated plant cells on artificial nutrient medium was done by Haberlandt (1902). Later, long term calli cultures were established from carrot cambium by Gautheret (1939) and Nobecourt (1939). In subsequent years different culture media for plant tissue culture were formulated by the work of Gautheret (1940), Hildebrandt (1946), Nitsch (1951), Reinert and White (1956), Murashige and Skoog (1962), White (1963). Gamborg *et al.,* (1968), Schenck and Hildebrandt (1972) etc.

Investigations by Minocha (1980), Bornmann (1983), Villalobos *et al.,* (1984) on culture conditions; White and Gilbey (1966), Kolevska-Pletikapic (1978), Mulder-Krieger *et al.,* (1982) on nutrient requirements; Oka and Ohyama (1975), Minocha (1987), Nadel *et al.,* (1991) on hormonal

requirements; MacRae and Vanstaden (1990), Pochet *et al.,* (1991) on gelling agents etc. revealed some other important aspects of culture.

Propagation of plants using tissue culture technology is called micropropagation. Micropropagation techniques have been employed early especially for the propagation of economically important ornamental and foliage plants, while micropropagation of medicinal plants is also getting increased interest in the present era (Bajaj 1986, Bajaj *et al.,* 1998). The problems associated with conventional seed propagation and vegetative methods can be overcome to a great extent by micropropagation. During the last few years there has been considerable emphasis in propagating medicinal plants through *in vitro* culture techniques (Prakash *et al.,*1999; Komalavally and Rao 2000; Tang 2000; Prabhakar *et al.*, 2001; Tyagi and Prakash 2001; Choi *et al.,* 2002). Presently about 20% of the medicinal plants are propagated through this technique (Rajendra and D'Souza 1999)

Micropropagation studies on some important medicinal plants through organogenesis and embryogenesis are listed in the table 1 and 2.

ORGANOGENESIS

Organogenesis may be either direct or indirect. In direct organogenesis, competence is already present at the culture onset while in indirect

Table 1. Micropropagation studies on some medicinal plant during last five years

Name of the plants	Explant	Type of response	Reference
Acorus calamus	Rhizome buds	Axillary bud multiplication	Harkrishnan & Hariharan 1999
Allium sativam	Stem	Indirect organogenesis	Barandiaran *et al.* 1999
A.sativam	Stem disc	Direct organogenesis	Ayabe & Sumi 1998
A. galanga	Rhizome buds	Axillary bud multiplication	Borthakur *et al.*, 1999
Aegle marmeelos	Node	Axillary bud multiplication	Ajitkumar & Seeni 1998
Alpinia calcarata	Rhizome	Indirect organogenesis	Martin *et al.*, 2002
Anthemis nobilis	Leaf	Direct organogenesis	Echeverrigaray *et al.*, 2000a
Anthemis nobilis	Shoot tip	Multiple shoot	Echeverrigaray *et al.*, 2000b
Aralia cordata	Immature inflorescence	Somatic embryogenesis	Lee et al., 2002
Asparagus officinalis	Microspores	Indirect organogeneis	Peng & Wolyn 1999
Azadiracta indica	Leaf	Direct organogenesis	Eeswara *et al.*, 1998
Bacopa monniera	Node,internode, leaf	Multiple shoot	Tiwari *et al.*, 2001

Becopa monnieri	Stem, leaf, Flower bud	Indirect organogeneis	Tejavathi & Shailaja 1999
Boerhavia diffusa	Node	Axillary bud multiplication	Phukan *et al.*, 1999
Butea monosperma	Cotyledonary node	Shoot multiplication	Kulkarni &D'souza 2000
Calliandra tweedii	Internode, petiole	Somatic embryogenesis	Kumar *et al.*, 2002
Centella asiatica	Leaf	Direct organogenesis	Banerjee *et al.*, 1999
Centella asiatica	Node	Multiple shoot	Tiwari *et al.*, 2000
Cephaelis ipecacuanha	Leaf	Somatic embryogenesis	Rout *et al.*, 2001
Cichorium intybus	Leaf	Direct organogenesis	Pieron *et al.*, 1998
Coleus forskohlii	Leaf,stem,&flower bud	Indirect organogenesis	Suryanarayanan & Pai 1998
Coleus forskohlii	Leaf	Indirect organogenesis	Reddy *et al.*, 2001
Coleus forskohlii	Flower, stem, shoot tip	Indirect organogeneis	Suryanarayan & Pai 1998
Corydalis ambigua	Tuber	Somatic embryogenesis	Hiraoka *et al.*, 2001
Cuminum cyminum	Hypocotyl, internode	Multiple shoot	Tawfik & Noga 2001
Cunila galioides	Axillary bud	Direct organogenesis	Fracaro & Echeverrigaray 2001
Curcuma aromatica	Rhizome buds	Indirect organogenesis	Anand & Hariharan 1999
Dendrobium moschatum	Stem disc	Direct organogenesis	Kanjilal *et al.*, 1999

Echinacea purpurea	Leaf	Indirect organogeneis	Koroch *et al.*, 2002
Echinaceae purpurea	Hypocotyl	Indirect organogeneis	Coker & Camper 2000
Eleutherococcus senticosus	Embryo	Somatic embryogeneiss	Choi *et al.*, 2002
Enicostemma axillare	Leaf	Direct organogenesis	Sudhersan 1998
Eschscholzia californica	Seed	Somatic embryogenesis	Park &Facchini 1999
Gloriosa superba	Shoot tip	Shoot multiplication	Sivakumar &Krishnamoorthy, 2000
Gymnema sylvestre	Seedling node	Multiple shoot	Komala valli & Rao 2000
Gymnema sylvestre	Hypocotyl, cotyledon, leaf	Somatic embryos	Kumar *et al.*, 2002
Houttuynia cordata	Node	Axillary bud multiplication	Handique & Bora 1999
Hybanthus enneaspermus	Seed	Indirect organogenesis	Prakash *et al.*, 1999
Hyoscyamus niger	Shoot tip	Multiple shoot	Prabhakar *et al.*, 2001
Iphigenia indica	Corm , corm bud	Direct organogenesis	Mukhopadhyay *et al.*, 2002
Kaempferia galanga	Rhizome bud	Indirect organogenesis	Vincent *et al.*, 1998
Kaempferia galanga	Rhizome	Multiple shoot	Shirin *et al.*, 2000
Lavandula viridis	node	Multiple shoots	Dias *et al.*, 2002

Lavandula x intermedia	Leaf	Direct organogenesis	Dronne *et al.*, 1999 b
Lilium nepalense	Twin scale	Multiple shoot	Wawrosch *et al.*, 2001
Lippia junelliana	Shoot tip, node	Multiple shoot	Juliani (Jr.) *et al.*, 1999
Liriope platyphylla	Embryo	Somatic embryogenesis	Kim *et al.*, 2000
Litsea cubeba	Shoot tip, node	Multiple shoot	Mao *et al.*, 2000
Mentha spp.	Leaf	Direct organogenesis	Faure *et al.*, 1998
Mentha x piperata	Protoplast	Indirect organogenesis	Jullien *et al.*, 1998
Moricandia nitens	Protoplast	Indirect organogenesis	Tian & Meng 1999
Panax ginseng	Cotyledons	Somatic embryogenesis	Tang 2000
Phyllanthus amarus	Shoot tip	Multiple shoot	Bhattacharyya & Bhattacharya, 2001
Pinus wallichiana	Seedling	Shoot multiplication	Bastola *et al.*, 2000
Piper longum	Leaf	Indirect organogenesis	Philip *et al.*, 2000
Pisonia alba	Node	Axillary bud multiplication	Jagadishchandra *et al.*, 1999
Pittosporum napaulense	Node	Multiple shoot	Dhar *et al.*, 2000
Plumbago spp.	Leaf	Direct organogenesis	Das & Rout 2002

Plumbago zeylanica	Node	Axillary bud multiplication	Rout *et al.*, 1999 a
Plumbago zeylanica	Stem	Indirect organogenesis	Rout *et al.*, 1999 b
Pothomorphe umbellate	Leaf	Direct organogenesis	Pereira *et al.*, 2000
Santolina canescens	Node	Multiple shoots	Casado *et al.*, 2002
Sapindus mukorossi	Seedling	Axillary bud multiplication	Philomina & Rao 1999
Scabiosa columbaria	Anther & ovule	Indirect organogenesis	Romeijn & Van lammereu 1999.
Sereona repens	Immature embryo	Somatic embryogenesis	Gallo- Meagher & Green 2002
Sesamum indicum	Node	Axillary bud multiplication	Gangopadhyay *et al.*, 1998
Simmondsia chinensis	Node	Multiple shoots	Tyagi & Prakash 2001
Simmondsia chinensis	Leaf	Somatic embryogenesis	Hamama *et al.*, 2001
Solanum nigrum	Leaf	Direct organogenesis	Shahzad *et al.*, 1999
Swainsona salsula	Cotyledon	Direct organogenesis	Yang *et al.*, 2001
Tridax procumbens	Node	Axillary bud multiplication	Sahoo & Chand 1998a
Triphytophyllum peltatum	Node(from in vitro plants)	Multiple shoots	Beingmann & Rischer 2001
Uraria picta	Node	Axillary bud multiplication	Anand *et al.*, 1998

Vetiveria zizanoides	Mesocotyl	Indirect organogenesis	George & Subramanian 1999
Viburnum tinus	Node	Multiple shoot	Nobre et al. 2000
Vitex negundo	Node	Axillary bud multiplication	Sahoo & Chand 1998b
Vitex negundo	Node	Multiple shoots	Sahoo & Chand 1998
Vitex negundo	Internode	Indirect organogenesis	Thiruvengadan & Jayabalan, 2001
Withania somnifera	Meristem	Indirect organogenesis	Teli *et al.*, 1999
Withania somnifera	Stem	Indirect organogenesis	Rani & Growen 1999

Table 2. Some important reports of Micropropagation in Boraginaceae and Meliaceae

Name of the Plants	Explants	Type of response	Reference
Azadiracta indica	Hypocotyl	Indirect organogenesis	Zympman *et al.*, 1996
Azadiracta indica	Germinating seed	Indirect organogenesis	Rier & Obasis, 1996
Azadiracta indica	Anther	Indirect organogenesis	Gautam *et al.*, 1993
Azadiracta indica	Leaf disc	Direct organogenesis	Ramesh & Padhya, 1990
Azadiracta indica	Hypocotyl,cotyledon	Somatic embryogenesis	Wen *et al.*, 1997
Melia azedarach	Node	Direct organogenesis	Dingra *et al.*, 1991
Munronia pumila	Hypocotyl, leaf	Indirect organogenesis	Hirimburegama *et al.*, 1994
Naregamia alata	Mature leaves	Direct & Indirect organogenesis	John *et al.*, 1997
Naregamia alata	Shoot tip	Direct organogenesis	Daniel *et al.*, 1999.
Symphytum officinale	Root, petiole, peduncle, stem, leaf	Indirect organogenesis	Huizing *et al.*, 1983
Symphytum spp.	Bud, root, stem	Direct organogenesis	Harrise *et al.*, 1989

organogenesis competence is achieved during the period of *in vitro* culture (Thorpe *et al.*, 1991; De Klerk *et al.*, 1997).

Direct shoot organogenesis from primary tissue is more desirable than indirect organogenesis (Larkin and Scowcroft 1981; George 1993; Jasrai *et al.,* 1999; Shirin *et al.,* 2000; Sivakumar and Krishnamurthy 2000) and it has been reported in many medicinal plants from various explants especially from shoot tips or nodal explants (Table 1). Explants such as leaf, root etc. have also been used for direct shoot regeneration of many medicinal plants. (Harris *et al.*, 1989; Wawrosch *et al.*,1999; Pereira *et al.*,2000 ; Salvi *et al.*, 2001; Das & Rout 2002). The reports indicate that MS is the most widely used medium and cytokinins are prime important in promoting direct shoot initiation or multiplication from various explants. Among the cytokinins, BA is most widely used. (Harris *et al.*, 1989; Juliani (Jr) *et al.,* 1999; Fracaro and Echeverrigaray 2001; Salvi *et al.,* 2001; Tiwari *et al.,* 2001; Dias *et al.,* 2002). Combinations of cytokinins were also reported to be effective for the induction or multiplication of shoots (Komalavalli and Rao 1997,2000; Thirunavoukkarasu and Debata 1998; Kathiravan and Ignachimuthu 1999; Jagadishchandra *et al.,* 1999; Sivakumar and Krishnamurthy, 2000). Synergistic effect of auxin - cytokinin interaction was also reported in direct shoot regeneration and multiplication of many medicinal plants. (Sahoo and Chand 1998b; Dhar *et al.*,2000;

Echeverrigaray *et al.,* 2000a; Shirin *et al.*, 2000; Tiwari *et al.,* 2000; Salvi *et al.*,2001; Casado *et al.*, 2002).

Indirect organogenesis (i.e. via. callus phase) results in somaclonal variations, hence the method is less desirable for large scale clonal multiplication (Thorpe *et al.,* 1991). However, the variations may be useful for crop improvement (George 1993; Suryanarayanan and Pai 1998). One of the key variables in the chemical regulation of *in vitro* organogenesis is the ratio of auxin- cytokinin present in the medium (Skoog and Miller 1957). Indirect organogenesis and subsequent micropropagation have been reported in many medicinal plants (Table 1).

SOMATIC EMBRYOGENESIS.

Efficient plant regeneration from cell and tissue cultures, preferably through somatic embryogenesis, is one of the constituents of biotechnology and has become a pre- requisite for any *in vitro* manipulation (Eapen and George 1989). Since the first report of somatic embryogenesis in carrot cultures (Reinert 1958; Steward *et al.*, 1958;), considerable data have been accumulated in this aspect and extensively reviewed by various authors (Ammirato 1983; Williams and Maheswari 1986; George1993,1996). Somatic embryogenesis may be either direct or indirect.

Direct somatic embryogenesis has been reported in many medicinal plants like *Calliandra tweedi* (Kumar *et al.*, 2002), *Camellia japonica* (Pedroso and Pais 1995) *Trachyspermum ammi* (Seghal and Abbas 1994), Ginseng (Choi *et al.*,1998a,1999) etc. In direct somatic embryogenesis pre- embryogenic determined cells (PEDCs) develop into somatic embryos directly (Konar and Nataraja 1965) and greater genetic and cytological fidelity is associated with this process (Binsfield *et al.*, 1999; Choi *et al.*, 1999; Iantcheva *et al.*, 1999).

Indirect embryogenesis requires dedifferentiation and acquisition of embryonic state (Sharp *et al.*, 1982) and it has been reported in many medicinal plants like *Panax ginseng* (Tang 2000), *Cuminum cyminum* (Tawfik and Noga 2002), *Thevetia peruviana* (Kumar 1992), *Gymnema sylvestre* (Kumar *et al.* 2002), *Simmondsia chinensis* (Hamama *et al.*, 2001) *Eschscholzia californica* (Park & Facchini 1999), *Eleutherococcus senticosus* (Choi *et al.*, 2002) etc. MS is the most widely used medium for somatic embryogenesis (Tsay and Huang 1998; Choi *et al.*, 1998a). Growth regulator/s in the media influence the embryonic response of cultured cells. Among the different auxins, 2,4-D has been widely used for somatic embryogenic calli (Patil 1998; Whakulum and Sharma 1998; Kitamiya *et al.*, 2000; Kim *et al.*, 2000; Choi *et al.*, 2002). However, other auxins also have been reported to be effective for somatic embryogenesis of many medicinal plants (Hiraoka *et al.*, 2001; Monteiro *et al.*, 2002). Synergistic effect of auxin- cytokinin interaction has also

been reported for the induction of somatic embryogenesis in many medicinal plants (Hamama *et al.,* 2001; Gallo-Meagher and Green 2002; Kumar *et al.*, 2002; Tawfik and Noga 2002). In most of the reports indirect somatic embryogenesis involves, induction of callus from a suitable explant in a medium rich with auxin with or without cytokinin and differentiation of somatic embryos upon transfer of these calli into a medium containing relatively low auxin or hormone free medium.

Maturation and germination of somatic embryos occur on transfer of embryos to the medium without growth regulators (Rout *et al.*, 1995,2001; Kumar 1992) or with low levels of growth regulators (Sehgal and Abbas, 1994; Sinha *et al.*, 2000; Kumar *et al.,* 2002)

SYNSEEDS.

Synseeds are artificially encapsulated somatic embryos, shoot buds, cell aggregates or any other tissue that can be used for sowing as a seed and that possess the ability to convert into a plant under *in vitro* or *ex vitro* conditions, which retain the potential also after storage (Ara *et al.,* 2000). Artificial seeds were first developed and reported by Redenbaugh *et al.*, (1984) in celery. Since then there have been many reports on the development and utilization of synthetic seeds for many plants like *Asparagus officinalis* (Uragami *et al.*, 1990; Mamiya and Sakamoto 2001), *A. cooperi* (Ghosh and Sen 1994), *Daucus carota*

(Wake *et al.,* 1995), *Carica papaya* (Castillo *et al.*, 1998), *Citrus reticulata* (Antonietta *et al.,* 1999), Olive (Micheli *et al.*, 1998), *Humulus lupulus* (Martinez *et al.,* 1999), *Mentha spicata* (Hirai and Sakai 1999), etc.

SECONDARY METABOLITES.

Certain chemical substances present in the plants are not directly concerned with their primary metabolic process, these substances are usually termed as secondary metabolites or secondary products. The disease curing property of the medicinal plants are found to be due to the presence of the secondary metabolites. Since the middle of the century there had been several reports representing the presence or accumulation of secondary metabolites in plant cell cultures or micropropagated plants. Some important reports during the last five years are listed in the table 3.

BIOCHEMICAL STUDIES.

Biochemical analysis of primary calli and regenerating calli helps in better understanding of metabolism leading to organogenesis,. However, there are only few reports showing the biochemical analysis of metabolites and enzyme activity in callus cultures. The following are some important reports in this regard.

Table 3. Some important reports showing the presence of secondary metabolites in culture or micropropagated plants during the last five years

Plant Name	Secondary metabolites	Reference
Datura metel	Tropane alkaloids	Cusido *et al.*1999
Datura stramonium	Littorine & Hyoscyamine	Zabetakis *et al.*,1999
Dianthus caryophyllus	Dianthin	Messeguer *et al.*,1999
Dionaea muscipula	Naphthoquinones	Hook, 2001
Drosera spp.	Naphthoquinones	Hook, 2001
Galphima glauca	Galphimine-b	Osuna *et al.*, 1999
Glycyrrhiza glabra	Flavanoids	Li *et al.*, 1998
Morinda elliptica	Anthraquinones	Abdullah *et al.*, 1998.
Pothomorphe umbellata	4-nerolidyl catechol	Pereira *et al.*,2000
Rauwolfia sellowi	Indole alkaloids	Rech *et al.*,1998
Rosmarinus officinalis	Carnosic acid	Caruso *et al.*, 2000
Tabernaemontana elegans	Terpenoid indole alkaloids	Lucumi *et al.*, 2002
Taxus cuspidate	Taxane	Son *et al.*,2000
Taxus spp.	Paditaxel	Su *et al.*, 2002

Kavikishor (1987) reported biochemical changes during growth and organogenesis in callus culture of tobacco. Kavikishor and Mehta (1988) analyzed the changes in enzyme activities during growth and organogenesis in dark grown tobacco callus cultures. Kavikishor and Mehta (1989) reported carbohydrate oxidation and accumulation of metabolites during organogenesis in callus cultures of tobacco. Vincent *et al.*, (1992) reported changes in enzyme activities in organ forming and non- organ forming callus cultures of *Kaempferia galanga*. Yadav *et al.,* (1995) reported changes in protein and carbohydrate metabolism and callus regeneration in tobacco. Biochemical characteristics of differentiating callus cultures of *Feronia limonia* was analysed by Purohit *et al.,* 1996. *Alarmelu et al.,* (1997) reported changes in enzymes activities and phenol content in *in vitro* callus cultures of *Panicum maximum*. Patra *et al.,* (1999) reported metabolic changes during callus regeneration in *Centella asiatica*. Changes in enzyme and differentiation in calli of *Vigna radiata* were reported by Sakhuja and Chawla (1999).

MATERIALS AND METHODS

MATERIALS AND METHODS

Micropropagation methods such as organogenesis (direct and indirect), somatic embryogenesis and synthetic seed production have great importance in the propagation and conservation of medicinal plants of pharmaceutical relevance. Taking this into account, two important medicinal plants viz. ***Heliotropium keralensis*** **Sivar & Manilal** and ***Naregamia alata*** **W&A** were selected for the present experimental studies. This chapter briefly describes the materials and methods adopted in the present studies under various headlines.

GLASSWARE

Culturing was carried out in conical flasks (100-250ml), culture tubes of different sizes (25x200 mm, 25x150 mm and 18x180 mm) of borosil or corning and in jam bottles (500 ml). The culture vessels were cleaned with liquid detergent (Extran) followed by thorough washing in tap water. The cleaned vessels were rinsed with double distilled water and dried in a hot air oven at 100^0 C. The conical flasks and culture tubes were plugged with non-absorbent cotton wrapped in cotton gauze. Polypropylene caps were also used for capping culture tubes. The jam bottles were capped with aluminum or polypropylene closures.

CHEMICALS

All the chemicals used in this present experiments were of analytical

grade from British Drug House (BDH), India ; E. Merck (India) Ltd; Hi-Media, India; Qualigens ,India and Sigma Chemical Company, U.S.A .

PREPARATION OF MEDIA

Three different media viz. MS (Murashige and Skoog 1962) medium, B_5 (Gamborg *et. al.,* 1968) medium and White's (White 1963) medium were used in the present experiments for culture establishment (Table- 4). The basal media were manipulated with different auxins and cytokinins (Table-5) in different concentrations and combinations. Half strength MS medium supplemented with auxins were also tested for *in vitro* rooting experiments.

For making media, fresh stock solutions were prepared once in a month. Separate stock solutions were prepared for both micro and macro nutrients, vitamins, amino acids and chelating agents of various media and hormones according to the standard methods described by George (1993). All stock solutions were stored in the refrigerator at 4^0 C. Required volumes of stock solutions were pipetted out and carbohydrate source were added before making up the final volume of the media with double distilled water.

The pH of the media were adjusted to 5.7 (for MS and B_5) or 5.5 (for White's) using 1N HCl and 1N NaOH. For the preparation of semi- solid media 0.8% agar was added as the gelling agent and the media were heated to boiling for proper mixing.

Table 4- Compositions of culture media (mg/l)

Ingredients	MS	B_5	White's
$(NH_4)2SO_4$	-	134	-
$(NH_4)NO_3$	1650	-	-
KNO_3	1900	2500	80
$Ca(NO_3)_2$	-	-	300
$CaCl_2$ $2H_2O$	440	150	-
Mg SO_4 $7H_2O$	370	250	720
Na_2SO_4	-	-	200
KH_2PO_4	170	-	-
NaH_2PO_4 H2O		150	16.5
KCl		-	65.0
$FeSO_4$ 7H2O	27.2	27.8	-
Na_2EDTA	37.3	37.3	-
$Fe_2(SO_4)$		-	2.5
$MnSO_4$ $4H_2O$	22.3	-	7.-
Mn SO_4 H2O	-	10.0	-
$ZnSO_4$ 7H2O	8.9	2.0	3.0
H_3BO_3	6.2	3.0	1.5
KI	0.83	0.75	0.75
Na_2MoO_4 $2H_2O$	0.25	0.25	-
$CuSO_4$ $5H_2O$	0.025	0.025	-
$CoCl_2$ $6H_2O$	0.025	0.025	-
Myo- Inositol	100	100	-
Nicotinic acid	0.5	1.0	0.5
Pyridoxine HCl	0.5	1.0	0.1
Thiamine HCl	0.1	10.0	0.1
Glycine	2.0	-	3.0
Ca D-panthothenic acid			1.0
Sucrose	30,000	20,000	20,000

Table-5. List of Plant growth regulators used in the study.

Compound	Chemical Formula	Molecular weight
Auxins		
IAA	Indole-3-acetic acid ($C_{10}H_9NO_2$)	175
IBA	Indole-3-butric acid ($C_{12}H_{13}NO_2$)	203.20
NAA	α- naphthalene acetic acid ($C_{12}H_{10}O_2$)	186.20
2,4,D	2,4,Dichlorophenoxyacetic acid ($C_8H_6Cl_2O_3$)	221
Cytokinins		
BA	6-Benzyl-adenine ($C_{12}H_{11}N_{15}$)	225
KIN	Kinetin (6-furfurylaminopurine)($C_{10}H_9N_5O_5$)	215.20
2iP	N^6(2-isopentyl)-adenine	203.25

Later, the media were dispensed into appropriate culture vessels (culture tubes or conical flask or jam bottles). The culture vessels were closed with closures as described earlier and were autoclaved for 20 minutes at 1.06 Kg/cm^2 at 121^{0}C.

EXPLANTS AND SURFACE STERILIZATION

Healthy explants such as shoot tips, nodes, internodes, young leaves and roots of *H.keralensis* were collected during the months of December to May (since it was a seasonal plant) from plants growing in Calicut University Campus and the explants of *N. alata* (shoot tips, nodes, internodes, young leaves, flower petals and roots) were collected from one year old plants growing in Calicut University Botanical Garden.

The explants were washed thoroughly under running tap water, followed by treatment with 5% extran (v/v) for 5 minutes and subsequently washed five to seven times with sterile double distilled water. The explants were then surface disinfected with 0.1% mercuric chloride solution (w/v) for 4-20 minutes or 70% ethanol for 3 minutes or 0.5% sodium hypochlorite solution (15-30 minutes) under Laminar air flow cabinet. After decanting the sterilant, the explants were washed three to four times with double distilled water. After the surface sterilization the explants were cultured on different nutrient media under aseptic conditions.

CULTURE CONDITIONS

The inoculations were done under the laminar air flow cabinet taken due care to maintain aseptic conditions. The instruments such as scalpels, forceps, blade holders, blades etc. were flamed in rectified sprit and cooled before use. All the cultures were maintained at 25 ± 1°C under 16 h photoperiod provided by white fluorescent tubes (2000 lux). Suspension cultures were aerated using gentle shaking in a shaker (Certomat)

CONTROL OF PHENOLIC EXUDATION OF *H. KERALENSIS.*

To control the phenolic exudation in the cultures of *H.keralensis,* activated charcoal (0.06-1%) or PVP (1g/l) or ascorbic acid (100mg/l) were used in culture media. Periodic subculturing (at weekly intervals) to fresh media with same compositions were also tested to overcome the problem.

DIRECT SHOOT REGENERATION

Different explants (shoot tips, nodes and roots) of *H.keralensis* and *N.alata* (shoot tips nodes and leaves) were cultured on different media augmented with various concentrations and combinations of growth regulators (cytokinins and auxins) for direct shoot induction of the plants (Table 5). To study the effect of sucrose on direct shoot regeneration, varying concentrations of sucrose (1-5%) were incorporated in MS media supplemented with most effective growth regulator combinations observed from earlier experiments.

INDIRECT SHOOT REGENERATION

Shoot regeneration potential via. callus phase of different explants of *H.keralensis* and *N. alata* were studied by culturing on MS medium fortified with different combinations of cytokinins and auxins. (Table 5)

SOMATIC EMBRYOGENESIS

Heliotropium keralensis

Different explants (leaf, internode and node) of *H. keralensis* were cultured on MS medium supplemented with varying concentrations and combinations of growth regulators for the induction of embryogenic calli. The embryogenic calli were later transferred to MS basal liquid or semi solid media for the induction and maturation of somatic embryos.

Naregamia alata

Leaf explants were cultured on MS medium supplemented with lower concentration (0.1mg/l) of auxins for direct somatic embryogenesis in *N.alata*. For the induction of indirect somatic embryogenesis i.e. via callus phase, embryogenic calli obtained from various explants when cultured on MS medium supplemented with 2,4- D (0.1-3.0 mg/l) were transferred to MS basal semi solid or liquid media.

Mature embryos were cultured on MS semisolid medium with or without growth regulators for the germination of somatic embryos.

ENCAPSULATION OF SOMATIC EMBRYOS/SHOOT BUDS.

Mature somatic embryos and micropropagated shoot buds (unipolar) of both species were used in encapsulation studies. For encapsulation, various levels (1-5%) of sodium alginate solutions containing ingredients of MS medium (without $CaCl_2$) were prepared. The pH was adjusted to 5.7. Solutions of $CaCl_2$ $2H_2O$ of different strengths (25,50,70 and100mM) were prepared in double distilled water. The solutions were sterilized in an autoclave.

The embryos/shoot buds selected for encapsulation were blot dried and transferred to sodium alginate solution under aseptic conditions. Using a sterilized pipette having 0.5-1.0 cm diameter at the tip, the alginate solution with the propagules were drawn and dropped in to $CaCl_2$ solution. Each drop was adjusted to contain a single embryo or a shoot bud. They were allowed to remain in the $CaCl_2$ solution for 20-25 minutes. After the incubation period the $CaCl_2$ solution was decanted off and the beads with embryo/shoot tip (synseed) were washed three times with sterilized MS basal medium. The synseeds (artificial seeds) were either cultured on MS medium for germination or conserved by storing at 4^0 C. The viability and the efficacy of the synseeds were tested by culturing on MS basal semi- solid medium after three and six months of storage.

ROOTING OF *IN VITRO* SHOOTS

For root induction in *H. keralensis* and *N. alata,* the shoots (> 3cm) were excised from primary cultures and cultured on semi solid MS medium supplemented with 1BA(0.1-3mg/l), or NAA (0.1-3mg/l) on IAA (0.1-3mg/l) individually.

ACCLIMATIZATION AND TRANSFER OF PLANTLETS TO FIELD

The plantlets, regenerated through various *in vitro* techniques, with healthy root and shoot systems were taken out from culture medium and washed gently with distilled water for removing all traces of medium from the roots. The washed plantlets were then transferred to small plastic cups containing sterile sand. The pots were then covered with polythene bags or small bottles to maintain high humidity and kept in plant growth chamber. The plantlets were moistened with water. The polythene bags/bottles were removed after fifteen to twenty days. The plantlets were later transferred to larger pots containing sterile sand and soil (1:1 ratio) and kept under shade in the net house for another two weeks before transferring to field.

SECONDARY METABOLITE ANALYSIS

The comparative analysis of the alkaloids present in the roots of *in vitro* and field grown plants of both *H.keralensis* and *N.alata* were performed by thin layer chromatography (TLC).

Roots from field grown and *in vitro* plants of *H.keralensis* and *N.alata* were collected separately and washed thoroughly in running tap water. Blot dried roots of one gram from each sample were extracted with ethanol. The ethanol extracts were then transferred to china dishes and the dishes were kept on boiling water bath for evaporating to dryness. The precipitates were dissolved in 1 N sulphuric acid (H_2SO_4) and filtered using whatmans No.1 filter paper. The filtrates were neutralized with ammonium hydroxide solution (1N). After making ammoniacal the filtrates were extracted with diethyl ether in separating funnel. Anhydrous sodium sulphate was also added during the extraction in the separating funnel. The ether fraction from the separating funnels were transferred to beakers and concentrated to 3 ml, by heating and later transferred to airtight borosilicate glass tubes having closures and were kept in refrigerator.

Plates were made with silica gel according to Stahl (1969) by mixing 10g silica gel (G60) in 20 ml. of double distilled water and was spread on a clean TLC plate (20x20cm) with a spreader at a thickness of 0.25mm. The plates were dried in air and activated at 110^{0}C in hot air oven and allowed to cool. The samples prepared from field-grown and from *in vitro* plants were applied adjacently on the TLC plate each in 5µl and 10µl quantities. A micropipette and spotting guide were used for the spotting of samples. After loading the samples, the plates were allowed to air dry and kept in a developing tank pre saturated with chloroform: acetone : ammonium hydroxide in the ratio of 30:70:2. After allowing the solvent

front to ascend about 15cm from the sample spot, the plates were taken out and allowed to evaporate the solvent. For visualizing the alkaloids, the plates were sprayed with 5% concentrated H_2SO_4 (in ethanol) and allowed to dry. Later the plates were heated at 110 ^{0}C for 15 minutes in a hot air oven for colour development. The Rf values of charred spots were measured by using the spotting guide.

$$Rf = \frac{\text{Distance travelled by the alkaloid}}{\text{Distance travelled by the solvent front}}$$

BIOCHEMICAL STUDIES

To compare primary callus (induced on MS medium supplemented with 2mg/l IAA) and regenerating callus (on MS medium supplemented with 0.5 mg/l IAA + 2mg/l BA) biochemical studies were conducted. Total sugars, total phenolics, total proteins, difference in the activities of peroxidase and polypeptide profiles were studied for the same. The studies were attempted in order to correlate the possible role in organogenesis.

Estimation of Sugars

Sugars present in the calli were estimated by Anthrone method (Yemm and Willis, 1954). One gram dried callus was extracted in 1 ml of ethanol, 0.2 ml of this extract was mixed with 1.8 ml of distilled water and 4 ml of freshly prepared, chilled anthrone reagent. The reaction mixture was kept in boiling water bath for

10 min. and green colour developed was recorded at 620 nm. The amount of sugar present in the sample was calculated from the standard graph prepared by plotting concentrations of glucose on x-axis.

Estimation of Phenolics

Estimation of phenolics in the calli was done according to Folin method (Swain and Hillis, 1959). One gm of callus was extracted in 5ml ethanol. To 0.5 ml of this extract 4.5 ml of distilled water and freshly prepared 0.5 ml of folin reagent were added. The assay mixture was incubated for 3 min. and 2 ml of 20% sodium carbonate solution was added. The reaction mixture was kept over a boiling water bath for 1 min. Tubes were taken out and the absorbance was read at 650 nm against a reagent blank at room temperature. To calculate the total phenolic content of the samples, standard curve of caffein was made and from this the amount was calculated as mg/g fresh weight of samples.

Estimation of Protein

Protein estimation of the calli was done by phenol method (Lowry et. al., 1951). One gm of callus was extracted in 5 ml of phosphate buffer. The homogenate was centrifuged and supernatant was used for protein estimation. To 0.1ml of protein extract 0.9ml of distilled water and 5ml of Lowry's reagent were added with constant stirring and incubated for 20 min. Then 0.5 ml of folin-ciocalteau reagent was added to each sample and incubated in dark for 30 min. The optical density of blue colour developed was read at 660 nm. The amount of

protein (in milligrams) of the samples was calculated from the standard graph of bovine serum albumin.

Peroxidase assay

Peroxidase assay was measured by the method of Racusen and Foote (1965). Calli (0.5 mg) were homogenized with 0.1 ml of Tris –HCl buffer (pH-8) and pinch of abrasive (sterile sand) to ensure maximum disruption of cell walls using a prechilled mortar and pestle at 4 ^{0}C (Sadasivan and Manickam, 1996). The macerate was centrifuged at 12500 rpm for 20 min. at 4 ^{0}C. The clear supernatant was taken and stored in small aliquots at 4 ^{0}C. The extract was used as source for the enzyme.

To 0.2 ml of enzyme extract 3.5 ml of phosphate buffer (pH6.5) and 0.1 ml freshly prepared O- dianisine were added in a clean dry cuvette. The assay mixture was incubated at 28-30^{0}C and the cuvette was placed in a spectrophotometer at 430 nm. To the assay mixture 0.2 ml of hydrogen peroxide (0.2 M) was added and mixed well. A stopwatch was started and the absorbance was read at 30 min. intervals. The increase in absorbance was plotted against time. From the linear phase change in absorbance per minute was read. The enzyme activity was expressed in terms of increased absorbance per unit mg protein. A water blank was also used in the assay.

Polypeptide profiles

Polypeptide profiles of the samples were determined by SDS-PAGE.

i. Preparation of running and stacking gels.

Glass plates used for casting the gels were of 19 cm x 17.5 cm (4mm thickness)(Genei, Bangalore.) The plates were cleaned by soaking them in chromic acid overnight, rinsed with water, then with ethanol. The plates were then placed on clean tissue papers and were allowed to air dry.

Teflon spacers (1mm thickness) were used between the plates to obtain gels of uniform thickness. The plates were then placed in the casting unit.

The resolving gel mixture (Table 6) was then poured into the space between the glass plates, leaving sufficient space at the top for stacking gel. Little amount of iso-propanol was added at the top to obtain even surface for the resolving gel. After polymerization the iso-propanol was decanted and stacking gel mixture (Table 7) was poured at the top of the resolving gel and a comb was inserted at the top.

After polymerization of the stacking gel comb was carefully removed to expose the sample wells which were rinsed with reservoir buffer (Table 8) using a syringe fitted with a needle. Gel along with glass plates was later placed in the electrophoretic chamber.

Table 6. Resolving Gel mixture

Constituents	Volume
Distilled water	24 ml
Tris 1.5 M (pH 8.8)	15 ml
30% Acrylamide solution (29.2% Acrylamide and 0.8 % Bis-acrylamide in distilled water)	20 ml
10% SDS	0.6 ml
10%APS	0.3 ml
TEMED	0.02 ml

Table 7. Stacking Gel Mixture

Constituents	Volume
Distilled water	18 ml
Tris 1.5 M (pH 8.8)	7.8 ml
30% Acrylamide solution	4 ml
10% SDS	0.3 ml
10%APS	0.3 ml
TEMED	15 µl

Table 8. Reservoir buffer (pH 8.3)

Constituents	Quantity
Glycine	14.4g
Trizmabase	3g
10% SDS	10ml
Distilled water	800ml

Table 9.Sample buffer

Constituents	Quantity
Trizmabase	0.075 g
SDS	0.4 g
2-mercapto ethanol	1 ml
Distilled water	7 ml
Bromophenol Blue	0.02 g
Glycerol	2 ml

ii. Sample preparation and running

One gram callus was homogenized at 4^{0}C with 2 ml of 0.1 M Tris-HCl buffer (pH-8) containing 0.5 M sucrose, 0.1% ascorbic acid, 0.056M 2-mercaptoethanol. This was centrifuged at 12500 rpm for 20 min. at 4^{0}C. The supernatant was used as the source of protein. The supernatant was mixed with sample buffer (Table 9) in 2:1 ratio and 2µl of bromophenol blue (Glycerol and Bromophenol blue 1:1 ratio) was added to it. These samples were heated in a boiling water bath for 8 min. to ensure denaturation of the protein. Molecular weight markers (mixture of 6 standard proteins of known molecular weight) or BSA (Bovine serum albumin) dissolved in sample buffer along with bromophenol blue was also heated in boiling water bath for 8 min.

Wells were loaded with 15µl of samples and 5µl of molecular marker or BSA was also loaded in a parallel track of each gel. Care was taken to avoid mixing of proteins of different wells. The reservoir buffer was put in the upper and lower chambers of the gel apparatus very slowly before loading the samples.

Electrophoretic runs were made for 3-4 hrs. at 20 mA. As soon as the runs were over i.e., the tracking dye front had migrated very close to the bottom of the gel the run was stopped.

Table 10. Staining solution

Constituents	
Coomassie Blue	1.25 g
Methanol	250 ml
Glacial acetic acid	45 ml
Made up to 500 ml with distilled water	

Table 11.Destaining solution

Constituents	
Methanol	100 ml
Glacial acetic acid	70 ml
Made up to 1000ml with distilled water	

iii. Staining and de-staining

Gels were first kept in a fixative (50% methanol : 10% acetic acid in water) solution for 30 min. Then the gels were placed in staining solution (Table 10)

After staining, the gels were rinsed with double distilled water twice and destained with the destaining solution (Table 11) with slow and constant shaking in a rocker (Genei Bangalore). The destaining solution was changed frequently until back ground of the gel became clear.

STATISTICAL ANALYSIS

All experiments in the present study were conducted in a completely randomized design. Mean and standard errors were calculated according to the method of Misra and Misra (1983). The data were from 20 replicates in two experiments.

PHOTOGRAPHS

The photographs in the present study were taken using a Pentax K 1000/ Nikon camera.

OBSERVATION AND RESULTS

Present investigations developed efficient and reproducible protocols for the micropropagation of two important medicinal plants viz., ***Heliotropium keralensis*** and ***Naregamia alata*** through direct shoot induction, callus regeneration, somatic embryogenesis and synseeds from various explants. The observations and results of the experiments are entitled as follows

HELIOTROPIUM KERALENSIS

Efficient and reproductive protocols have been developed for organogenesis and somatic embryogenesis of *H. keralensis* by culturing explants such as shoot tip, node, internode, leaf and root on different media supplemented with various hormonal combinations.

Among the three surface sterilants (sodium hypochlorite, ethanol and mercuric chloride) tested, surface sterilization with 0.1% $HgCl_2$ for 6 and 7 min was most effective for explants of shoot system (shoot tip, node, internode and leaf) and root respectively.

The problem of phenolic exudation was tried to control by using additives such as activated charcoal (0.06-1%), PVP (1 g/l) and ascorbic acid (100 mg/l) or by periodic subculturing to the medium of same composition. Among these weekly subculturing to the medium of same composition

produced best results. Addition of 1% activated charcoal was also an effective method to overcome the problem. But the addition retarded growth in culture, hence it was not used in subsequent experiments.

DIRECT SHOOT REGENERATION

For the induction of direct multiple shoots, different explants like Shoot tip, node, and root were cultured on different media such as MS, B_5 and White's supplemented with different combinations and concentrations of growth regulators.

i) Multiple shoot regeneration from shoot tip

Three different basal media (MS, B_5, White's) were tested for selecting an appropriate culture medium for *H.keralensis*. Shoot tips showed elongation upto 2 cm. within 20 days when cultured on MS basal medium. However, shoot tips cultured on B_5 basal medium required 40 days for elongation up to 2 cm. and shoot tips cultured on White's basal medium did not show any response even after 50 days of culture. On the basis of this observation MS medium was selected for further experiments.

Multiple shoots were formed from the explants when cultured on MS medium supplemented with BA (0.5-4.0 mg/l) or BA (0.5-3.0 mg/l) + Kn (0.5-3.0mg/l) or BA (0.5-3.0mg/l) + Kn (0.5-3.0mg/l) +IAA (0.5 mg/l) (Plate 1 A,B,C) (Table 12) . Of the two cytokinins (BA and Kn) tested, BA was effective

Table 12. Effect of growth regulators on shoot induction from shoot tip explants of *H. Keralensis*

Growth regulator (mg/l)			% of Response	No. of Shoots/Explant	Shoot Length (cm)
BA					
0.1			60	1	5.2 ± 0.25
0.5			60	3.2 ± 0.42	4.5 ± 0.64
1.0			70	6.2 ± 0.25	3.8 ± 0.24
1.5			75	8.47 ± 0.73	3.7 ± 0.43
2.0			85	10.31 ± 0.11	3.14 ± 0.12
3.0			85	13.14 ± 0.33	2.94 ± 0.76
4.0			70	12.7 ± 0.16	2.88 ± 0.14
Kn					
0.5			30	1	3.1
1.0			40	1	2.96
BA + Kn					
0.1	0.1		35	1	4.1 ± 0.45
0.5	0.5		70	4.68 ± 0.39	3.92 ± 0.26
1.0	1.0		80	7.8 ± 0.13	3.88 ± 0.24
2.0	2.0		90	12.1 ± 0.62	3.82 ± 0.38
3.0	3.0		90	14.2 ± 0.57	3.79 ± 0.46
4.0	4.0		85	10.3 ± 0.18	3.7 ± 0.14
BA + Kn + IA					
2.0	2.0	0.5	80	10.1 ± 0.74	5.6 ± 0.76
3.0	3.0	0.5	85	12 ± 0.26	5.3 ± 0.43
BA + Kn + NA					
0.5	0.5	0.5	100%	bc	-
1.0	1.0	0.5	100%	bc	-

bc- Basal callusing
Data from 20 replicates in two experiments(Mean ±SE)
Growth period 50 days

for the induction of multiple shoots. BA at different levels (0.5-4.0 mg/l) on MS medium produced multiple shoots from the explants within 20 days. There was a linear correlation between the increase in BA concentration upto the optimal level and shoot multiplication. For direct shoot regeneration, 3.0 mg/l was the optimum concentration of BA. At lower concentration (0.1 mg/l) of BA shoot tip only elongated with an average length of 5 cm within 50 days (Table 12).

Addition of Kn along with BA on MS medium showed an enhancement in shoot multiplication. A combination of BA (3.0 mg/l) + Kn (3.0 mg/l) on MS medium produced highest number (14) of shoots from the explants (Plate 1B).

Auxins (IAA and NAA) when used alone on MS medium no shoot multiplication was observed from the explant. The combinations of auxins with cytokinins (BA + Kn) on MS medium were less effective when compared to MS medium supplemented with cytokinins (BA + Kn). However the auxin cytokinin combination promoted elongation of shoots. The average shoot length was highest (6.0 cm) on MS medium supplemented with BA (2 mg/l) + Kn (2 mg/l) + IAA (0.5 mg/l) (Table 12).

ii) Multiple shoot regeneration from nodal explants

Multiple shoot regeneration from nodal explants was observed on MS medium supplemented with BA (0.5-4 mg/l) or BA (0.5-4.0 mg/l) + Kn (0.5-4.0 mg/l) or BA (0.5-4.0mg/l) + Kn (0.5-4.0 mg/l)+ IAA (0.5 mg/l) (Plate 2 B,C).

The explants cultured on these media showed their first response by initial enlargement of the existing axillary buds followed by bud break within two weeks. BA at the range 0.5- 4.0 mg/l induced multiple shoots from the explant within 20 days. Number of shoots/explant increased with increase in BA concentration upto 3.0 mg/l. Further increase in concentration of BA decreased shoot multiplication from the explant. However length of shoots decreased with increase in BA concentration. Kn (0.5–3.0 mg/l) when supplemented singly on MS medium no multiple shoot formation was observed. However, single shoot was formed from each axil of the nodal explants at 1.0 mg/l and 2.0 mg/l concentrations of Kn in MS medium (Plate 2 A).

Combination of Kn (0.5 – 3.0 mg/l) and BA (0.5 – 3.0 mg/l) in MS medium showed high rate of shoot multiplication from the nodal explant, about 12 shoots were formed within three weeks. Highest number of shoots were induced on the MS medium containing 3mg/l BA and 3mg/l Kn.

Addition of auxin (IAA) in conjunction with cytokinins (BA + Kn) showed negative effect on shoot multiplication. However, the combination was favourable for shoot elongation (as in the case of shoot tip explants) (Table 13).

Table 13. Effect of growth regulators on shoot induction from nodal explants of *H. keralensis*

Growth regulator (mg/l)			% of Response	No. of Shoots/node	Shoot Length (cm)
BA					
0.5			40	3.1 ± 0.81	5.1 ± 0.76
1.0			55	3.56 ± 0.26	4.72 ± 0.1
1.5			60	5.1 ± 0.41	4.5 ± 0.51
2.0			75	7.6 ± 0.14	4.21 ± 0.32
3.0			75	11.2 ± 0.12	
4.0			70	10.35 ± 0.58	4.2 ± 0.46
Kn					
0.1			--	--	--
0.5			--	--	--
1.0			40	1	3.2 ± 0.21
3.0			5.0	1	3.3 ± 0.18
BA + Kn					
0.5	0.5		70	3.2 ± 0.65	5.2 ± 0.64
1.0	1.0		75	4.28 ± 0.22	4.78 ± 0.21
2.0	2.0		75	10.2 ± 0.33	4.78 ± 0.21
3.0	3.0		90	12.3 ± 0.74	3.58 ± 0.14
4.0	4.0		85	11.8 ± 0.12	3.3 ± 0.76
BA + Kn + IAA					
0.5	0.5	0.5	100	bc + 2.2	6.0 ± 0.18
1.0	1.0	0.5	100	bc + 3.48	5.6 ± 0.36

Data from 20 replicates in two experiments(Mean ±SE)
Growth period 50 days

Table 14. Effect of growth regulators on direct shoot induction from root explants of *H. keralensis*

Growth regulator (mg/l)	% of Response	No. of Shoots/node	Shoot Length (cm)
BA			
1.0	--	--	--
2.0	--	--	--
3.0	30	1.83 ± 0.11	3.2 ± 0.61
4.0	40	2.12 ± 0.21	3.1 ± 0.34
5.0	40	2.04 ± 0.08	2.85 ± 0.14
Kn			
1.0	--	--	--
2.0	--	--	--
3.0	--	--	--
4.0	--	--	--
BA + Kn			
2.0 2.0	--	--	--
3.0 3.0	30	4.6	3.1 ± 0.46
4.0 4.0	55	5.4	2.9 ± 0.25
5.0 5.0	55	5.2	2.86 ± 0.17
BA + Kn + IAA			
1.0 1.0 1.0	Callusing only	-	-
2.0 2.0 1.0	Callusing only	-	-
BA + Kn + NAA			
0.5 0.5 0.5	Callusing only	-	-
1.0 1.0 1.0	Callusing only	-	-
BA + Kn + 2,4-D			
0.5 0.5 0.5	Callusing only	-	-
1.0 1.0 1.0	Callusing only	-	-

Data from 20 replicates in two experiments(Mean ±SE)
Growth period 50 days

iii) Multiple shoot regeneration from root explants

Direct shoot regeneration from root explants of *H. keralensis* was observed on MS medium supplemented with BA (2-5 mg/l) and on MS medium supplemented with BA (2-5 mg/l) and Kn (2-5 mg/l (Plate 3 A,B,C) (Table 14). BA was found as the essential cytokinin for direct shoot regeneration from root explants. The optimal concentration was 4.0 mg/l BA in MS medium for direct shoot regeneration from root explants. Kn did not evoke any response when tested alone with MS medium. However a combination of BA (4.0 mg/l) and Kn (4.0 mg/l) was most effective for direct shoot regeneration from root explants (Table 14).

All regenerated shoots were free from any basal callus formation at their proximal end. Addition of IAA (0.5 mg/l) was found to be least effective either singly or along with BA and Kn. Instead the combination induced callusing from root explants.

iv) Effect of nutrient media

Mineral salt composition of culture media significantly affected direct shoot regeneration of *H. keralensis*. To select the best medium for direct shoot

Figure 1. Effect of nutrient media on shoot induction from shoot tips of *H. keralensis*

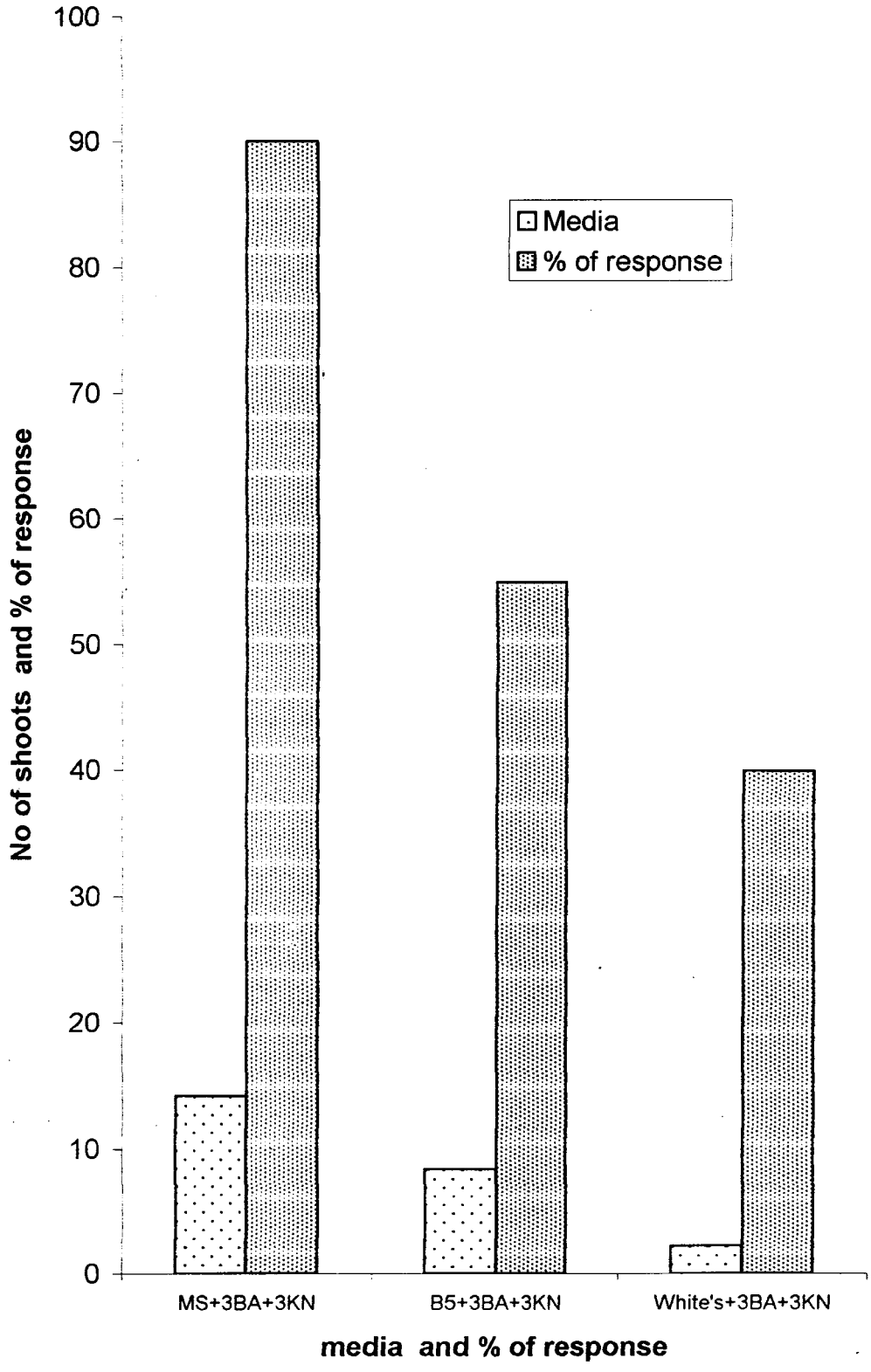
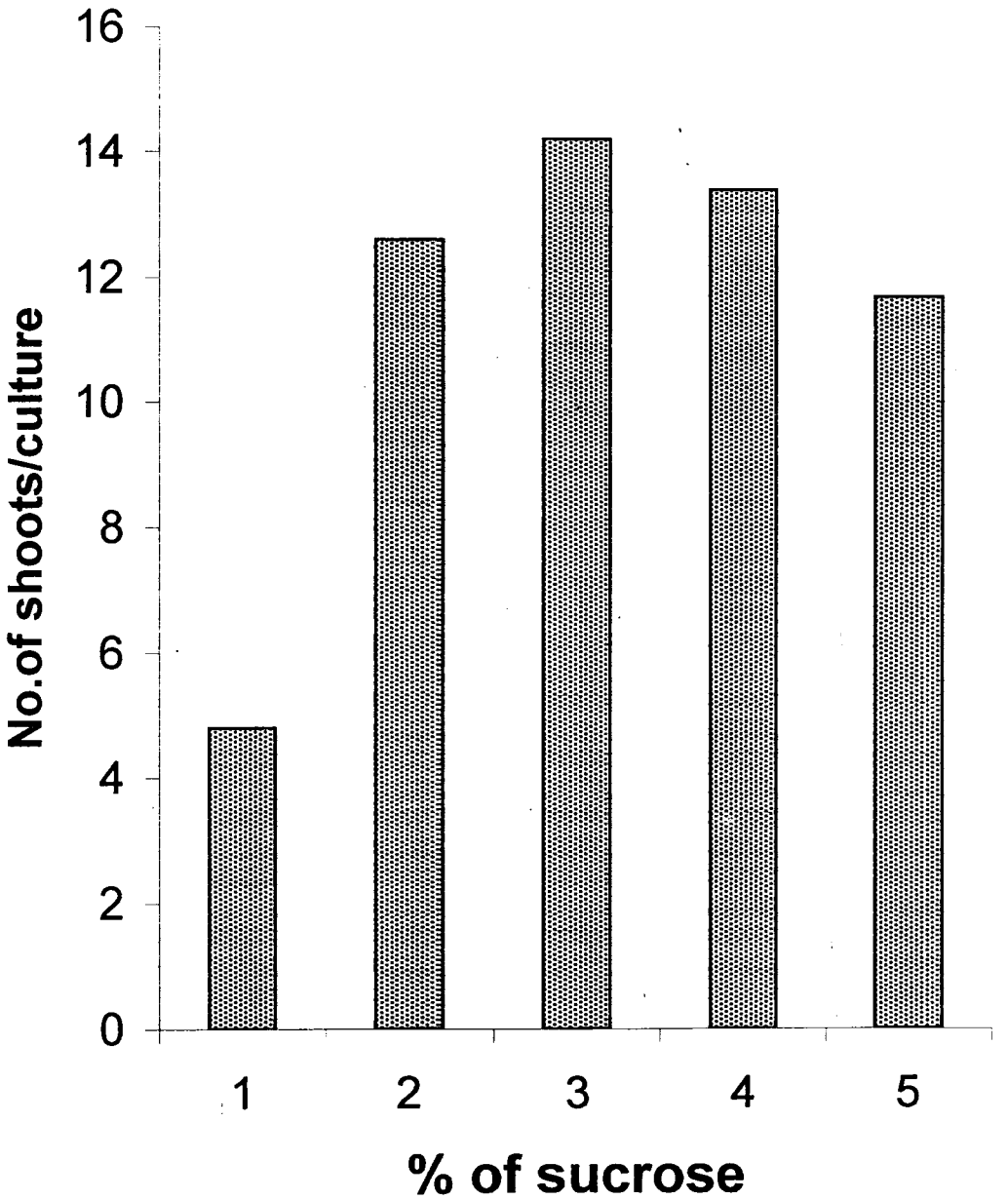


Figure 2. Effect of sucrose on shoot induction from shoot tips of *H. keralensis* (MS medium with 3mg/l Kn + 3mg/l BA)



regeneration, shoot tip explants were cultured on different nutrient media (MS, B_5, White's) supplemented with BA (3mg/l) and Kn (3mg/l). Establishment rate (90%) and multiplication (14) were highest on MS medium (Figure 1).

v) Effect of sucrose concentration

To find out the optimal level of sucrose for the induction of multiple shoots of *H. keralensis*, shoot tip explants were cultured on MS medium with varying levels of sucrose (1-5%) along with 3.0 mg/l BA + 3.0 mg/l Kn. Shoot multiplication was observed with high degree of variation in all the concentrations of sucrose tested and found that 3% was the optimum. Increase or decrease in sucrose concentration resulted in a decrease in shoot multiplication (Figure 2).

vi) Subculturing

To study effect of subculturing on shoot multiplication, shoot tips were excised from *in vitro* shoots and cultured on MS medium with BA (3.0 mg /l) and Kn (3.0 mg /l). No significant change in shoot multiplication was observed during subculture.

INDIRECT SHOOT REGENERATION

The shoot regeneration potential through callus phase from various explants viz. node, internode, leaf and root to various concentrations and combination of growth regulators in MS medium was studied.

i) Callus induction

For callus induction the explants were cultured on MS medium supplemented with BA, Kn, 2iP, IAA, NAA and 2,4-D either alone on in combinations (Table 15).

Effect of BA

Callus formation was observed on MS medium containing BA at the range 2- 4 mg/l from leaf and internodal (Plate 4 A) explants. These calli were green, friable and were meristematic in nature. However, there was a progressive increase in the amount of callus with the increase in BA concentration upto 3 mg/l. Further increase in BA concentration beyond the optimal level (3 mg/l) did not show any progressive change in callus proliferation. Root and node explants did not produce any callusing on MS medium supplemented with BA (Table 15).

Effect of Kn

MS medium with Kn (0.5 – 3.0 mg/l) was found as ineffective in inducing callus from any explant tried (Table 15).

Table 15. Effect of growth regulators on callus induction from various explants of *H. keralensis*

Growth regulators(mg/l)		Explants			
BA		Leaf	Internode	Root	Node
0.5		--	--	--	--
1.0		--	--	--	--
2.0		+	+	--	--
3.0		++	+	--	--
4.0		++	++	--	--
Kn					
0.5		--	--	--	--
1.0		--	--	--	--
2.0		--	--	--	--
3.0		+	--	--	--
IAA					
0.5					+
1.0		+	+	--	++
2.0		++	+	--	++
3.0		++	++	+	+++
2,4-D + 2iP					
0.5	0.5	+	+	+	++
0.5	1.0	++	+	+	++
0.5	2.0	+++	++	++	+++
0.5	3.0	+++	+++	++	++++
0.5	4.0	++	++	++	+++
2,4-D + BA					
0.5	0.5	+	+		+
1.0	1.0	++	++	++	+++

+ =Very slight; ++ =Little; +++ =Moderate ; ++++= Profuse
Data from 20 replicates in two experiments
Growth period 50 days

Effect of IAA

MS medium supplemented with IAA at the range 0.5 – 4.0 mg/l induced callus from leaf, internode and nodal explants within 12 days. Higher concentration of IAA (≥ 3mg/l) was effective for callus induction from root explants. The calli were pale white and friable (Plate 5 B). The calli later turned brown and died within 60 days.

Effect of 2,4-D

MS medium fortified with 2,4-D (0.5 – 3.0 mg/l) was found effective in inducing callus from leaf, internode and node explants within 10 days. The calli were pale white and friable. The calli later turned brown and died within 50 days(Plate 4B). Root explants also produced calli at higher concentration of 2,4-D (≥ 2 mg/l).

Effect of 2,4-D + BA

Various explants were cultured to study the synergistic effect of growth regulators 2,4-D (0.5 -1.0mg/l) and BA (0.5 – 2.0mg/l) in callus induction of *H. keralensis*. Among the tested concentrations, 1.0mg/l 2,4-D + 2.0mg/l BA were most effective and produced enormous amount of calli from all the explants cultured. These calli were pale green, friable and meristematic (Plate 5 A). The calli regenerated shoots up on culture on shoot induction medium.

Effect of 2,4-D + 2iP

MS medium containing the combinations of 2,4-D + 2iP was found best for callus induction of *H. keralensis*. Callus proliferation was also high in this combination. Callus induction was observed within 10 days from leaf, internode and node explants. Calli developed on root explants only after 15 days. The calli were green, friable (Plate 6 A) and had the potential to regenerate shoots on shoot induction medium. Among the tested concentrations, 0.5 mg/l 2,4-D + 3.0 mg/l 2 iP was found as optimal for callus induction and proliferation from all the explants.

ii) Callus regeneration

An enormous amount of calli were obtained from nodal explants on MS medium supplemented with 2,4-D (0.5 mg/l) + 2 iP (3.0 mg/l). The same callus was selected for regeneration studies to find out optimum growth regulator combination on MS medium for callus regeneration. A combination of BA (3.0 mg/l) + IAA (0.5 mg/l) on MS medium produced highest number of shoots (11) per gram calli (Plate 5 D). Of the two cytokinins (BA and Kn) tested, BA effectively regenerated shoot from calli (Plate 4 C) however, Kn with MS medium was ineffective for the regeneration of shoots from the calli (Table 16).

Table 16. Effect of growth regulators on callus regeneration of *H. keralensis*

Growth regulator(mg/l)	% Response	No. of shoots/Gram of Callus
BA		
0.5	60	3.7 ± 0.76
1.0	75	4.4 ± 0.28
2.0	75	7.6 ± 0.42
3.0	80	9.8 ± 0.17
4.0	80	9.4 ± 0.24
Kn		
0.5	Proliferation only	-
1.0	Proliferation only	-
2.0	Proliferation only	-
3.0	Proliferation only	-
BA + IAA		
0.5 0.5	40	2.7 ± 0.41
1.0 0.5	65	5.2 ± 0.12
2.0 0.5	60	7.8 ± 0.57
3.0 0.5	75	10.8 ± 0.32
4.0 0.5	75	10.6 ± 0.63

Data from 20 replicates in two experiments (Mean ±SE)
Growth period 50 days

Though callus induction was observed with various growth regulators on MS medium, only calli obtained on MS medium containing BA, BA + 2,4-D & 2,4-D + 2 ip were found to be regenerative on subsequent cultures. The regeneration capacity of various calli were tested by subculturing on MS medium supplemented with IAA (0.5 mg/l) and BA (3.0 mg/l) (Plates 4 C,D; 5 D; 6 B). Weekly transfer of callus to medium with the same composition was necessary to retain morphogenic potential of the calli. Brown coloured calli formed from various explants or light green calli which turned brown in the absence of subculturing at weekly intervals never showed regeneration (Plate 5 C). However in the shoot regenerated cultures also the part of the calli which did not regenerate shoots within 30 days turned brown but it did not affect growth of regenerated shoots.

SOMATIC EMBRYOGENESIS

Somatic embryogenesis in *H. keralensis* was established by the subculturing of embryogenic callus on MS hormone free medium.

i) **Induction of embryogenic calli**

Leaf, internode and nodal explants were cultured on MS medium fortified with 2,4-D, IAA, BA and 2iP either alone or in combinations to induce embryogenic calli (Table 17).

Table 17. Effect of growth regulators on embryogenic calli induction from leaf, node and internodal explants of *H. keralensis*

Growth regulators (mg/l)		Embryogenic Potential	% of Response			No. of embryos/g of calli in suspension
IAA			Leaf	Internode	Node	
1.0		NE	85	70	80	-
2.0		NE	90	95	95	-
2,4-D						
1.0		NE	85	90	100	-
2.0		NE	90	80	90	-
IAA + BA						
0.5	1.0	NE	85	70	75	-
0.5	2.0	NE	80	85	90	-
0.5	3.0	NE	90	80	90	-
0.5	4.0	NE	85	90	85	-
IAA + 2iP						
0.5	1.0	E	50	45	40	5.1
0.5	2.0	E	65	50	55	6.2
0.5	3.0	E	60	65	70	11.8
0.5	4.0	E	70	60	70	8.7
2,4-D + 2iP						
0.5	0.5	NE	65	85	70	-
0.5	1.0	NE	80	85	85	-
0.5	2.0	NE	85	90	85	-
2,4-D + BA						
0.5	1.0	NE	65	70	80	-
0.5	2.0	NE	70	75	80	-

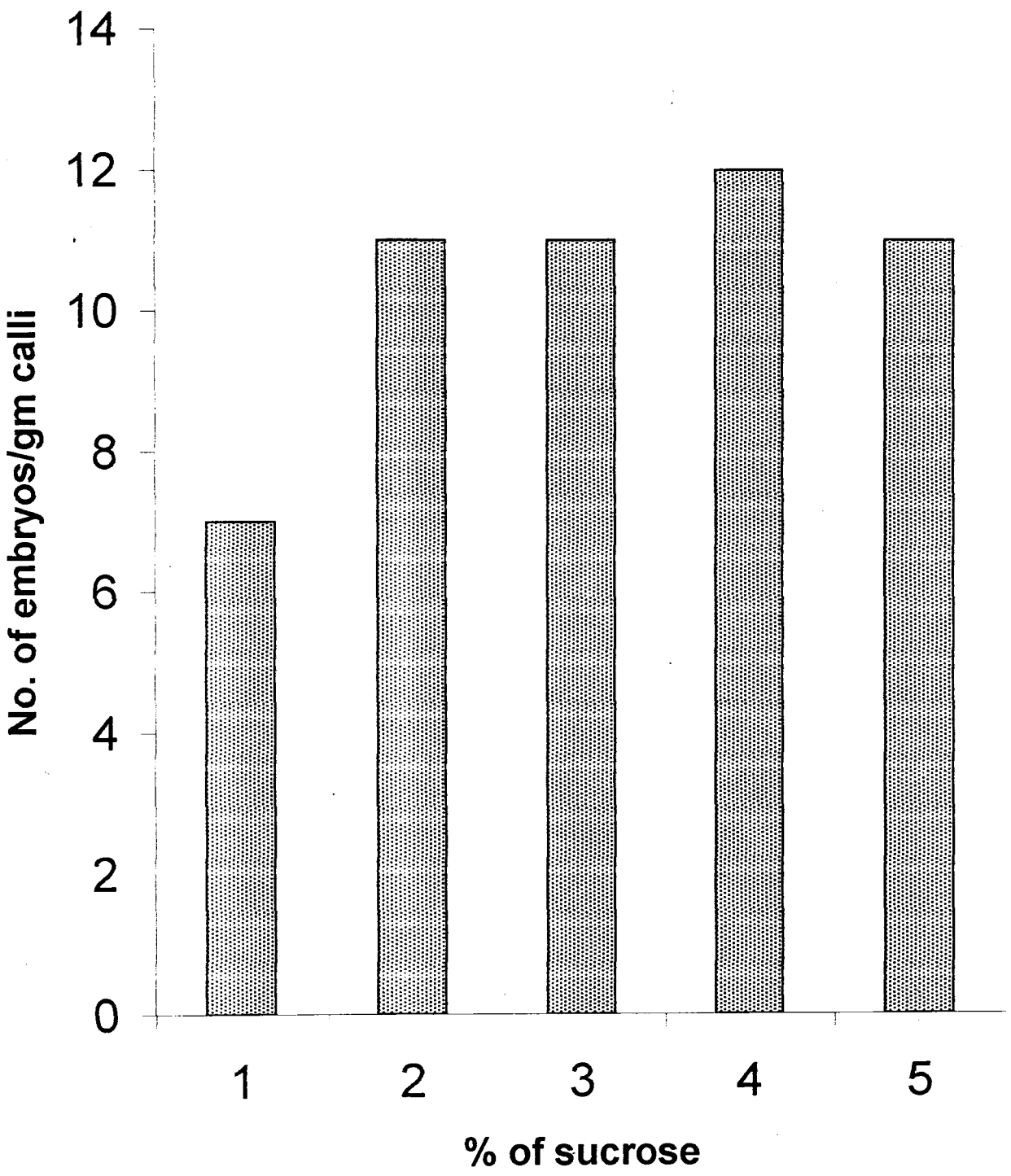
Data from 20 replicates in two experiments
Growth period 90 days
E-Embryogenic calli
NE-Non embryogenic calli

All explants responded well and callus induction was observed within 10 days in all the tested combinations. Nodal explants responded earlier (within 7 days) followed by internode (8 days) and leaf (10 days) explants. However, later experiments revealed that only the calli formed on IAA (0.5 mg/l) and 2 iP (1- 4 mg/l) combinations in MS medium were embryogenic. Other calli were non-embryogenic. The embryogenic calli were white and non-friable (Plate 7 B). But non-embryogenic calli were white and friable (Plate 7 A). Combination of IAA (0.5mg/l) + 2 iP (3.0 mg/l) on MS medium produced calli with highest embryogenic potential from all the explants tested.

ii) Induction and maturation of somatic embryos

Embryogenic calli upon transfer to MS hormone free suspension medium showed proliferation and formation of somatic embryos. Somatic embryos of various developmental stages (Globular, heart, torpedo) (Plate 7D,E) were formed from embryogenic calli within 90 days. Weekly transfer of embryogenic calli to fresh liquid medium of same composition was an essential requisite for the formation of somatic embryos. Failure in subculture resulted in browning and subsequent death of calli. Calli from nodal explants on MS + IAA (0.5mg/l) + 2 iP (1-4 mg/l) combinations produced globular embryos even in semi-solid MS hormone free medium (Plate 7C). However, the embryos turned brown soon after their formation, but browning did not affect the germination capacity of somatic embryos.

Figure 3.Effect of sucrose on somatic embryogenesis of *H. keralensis*



In general the somatic embryos passed through the normal developmental stages to root and shoot differentiation when cultured on MS semisolid medium with or without growth regulators. Frequency of germination was 55% on basal MS medium. Higher frequency (65%) of germination was obtained on MS medium supplemented with 0.5 mg/l Kn. (Plate 7 F,G)

iii) Effect of sucrose

Effect of sucrose concentration on somatic embryogenesis was studied by incorporating varying levels of sucrose (1-5 %) on embryogenic calli inducing medium (MS medium supplemented with 0.5 mg/l 2,4-D and 3.0 mg/l 2 iP.). Nodal explants cultured on the media produced calli within 20 days. The calli were subcultured on MS hormone free liquid medium to find out the number of somatic embryos formed per gram of calli. The level of sucrose significantly affected embryogenesis. Number of somatic embryos per gram of calli increased with an increase in sucrose concentration upto 4 g/l in induction medium (Figure 3). Further increase of sucrose concentration beyond 4% in induction medium showed a decline in number of somatic embryos formed per gram of calli on subsequent cultures.

SYNSEEDS

Among the various levels of sodium alginate (1, 2, 3, 4 and 5%) tested for encapsulation 3% was the most suitable for encapsulation. At lower

concentrations (1-2%) the beads were delicate and were difficult to handle. At higher concentrations (4-5%) beads were too hard which adversely affected germination or development by preventing emergence of root and shoot. The concentration of $CaCl_2(2H_2O)$ solution also affected the formation and germination/development of synseeds. Among the different concentrations tested (25, 50, 75 and 100 mM) 50 mM was best for the formation and subsequent germination/development of synseeds. The encapsulated embryos using 3% sodium alginate and 50 mM $CaCl_2(2H_2O)$ (Plate 8 B) showed signs of germination within 20 days on hormone free MS medium (Plate 8 D). However, the shoot buds encapsulated using the same concentrations of sodium alginate and $CaCl_2$ $(2H_2O)$ (Plate 8 A) did not show any sign of growth when cultured on same medium. But when cultured on MS medium containing 2.0 mg/l BA the encapsulated shoot buds produced an average of 4 shoots (Plate 8 C) within 30 days. Storage of encapsulated embryos/shoot buds for 3-6 months at 4°C did not affect their germination/development.

ROOTING *IN VITRO*

For root induction individual shoots (>3 cm) were excised and cultured on MS medium supplemented with IAA (0.1 – 3.0mg/l) or IBA (0.1 – 3.0mg/l) or NAA (0.1 – 3.0mg/l). Roots were formed on all the combinations tested (with varying frequency) (Plates 1D; 2D; 3D; 4E; 5E; 6C; 8E; 9A) (Table 18). Higher concentrations of auxins (IAA > 2.0 mg/l, IBA >1 mg/l; NAA > 0.5 mg/l)

Table 18. Effect of auxins (IAA, IBA & NAA) on *in vitro* rooting of *H. keralensis*

Growth regulators(mg/l)	% Response	No. of Roots
IAA		
0.1	60	6.3 ± 0.88
0.2	75	9.8 ± 0.18
0.5	80	14.6 ± 0.42
1.0	80	16.4 ± 0.16
2.0	70	9.9 + C ± 0.11
3.0	75	7.2 + C ± 0.27
IBA		
0.1	40	11.8 ± 0.56
0.2	65	16.7 ± 0.12
0.5	90	22.1 ± 0.22
1.0	90	20.4 ± C + 0.63
2.0	85	16.8 + C ± 0.47
3.0	90	13.2 + C ± 0.29
NAA		
0.1	90	4.1 ± 0.15
0.2	85	6.6 ± 0.74
0.5	80	4.3 + C ± 0.19
1.0	80	5.2 + C ± 0.37
2.0	85	3.7 + C ± 0.61
3.0	75	2.9 + C ± 0.23

Data from 20 replicates in two experiments (Mean ±SE)
Growth period 50 days

caused basal callusing and subsequently decreased number of roots/shoot (Table 18). IBA at 0.5 mg/ in MS medium was found as the most effective combination for rooting of *H. keralensis* shoots and produced highest number of roots (25/shoot) with highest frequency (90%) (Table 18).

SECONDARY METABOLITE ANALYSIS

Thin layer chromatography (TLC) performed the comparative analysis of the alkaloids present in the roots of *in vitro* and field grown plants

Root extracts from *in vitro* plants showed 5 compounds (spots) with Rf values 0.025, 0.056, 0.156, 0.263and 0.95. However root extracts from *in vivo* plants showed only three compounds (spots) with Rf values 0.025, 0.056, and 0.956. This indicated that two more compounds were additionally present in the roots of *in vitro* grown plants (Plate 10).

ACCLIMATIZATION AND TRANSFER TO FIELD CONDITIONS

Plantlets with fully expanded leaves and well-developed roots were successfully hardened in the controlled conditions for 20days and eventually established in natural soil. The plantlets with healthy root and shoot system regenerated through various *in vitro* techniques were transferred after washing with distilled water to small plastic pots containing sterile sand. The pots were then covered with polythene bag/glass bottles (Plate 9 B) to maintain high humidity. The plantlets were watered daily. The polythene bags/glass bottles

were removed after 20 days. Plantlets produced new leaves within 10 days after acclimatization (Plates 1E; 2E; 3E; 4F; 5F; 6D; 7H 8F; 9C) then they were transferred to large pots containing sand and soil (1:1 ratio). The potted plants produced new leaves and showed healthy growth (Plate 9 D). Morphologically there was no detectable variation between *in vitro* raised and naturally grown plants. The plantlets developed via direct or indirect shoot regeneration showed high percentage (80%) of survival while that by somatic embryogenesis showed 65% survival only.

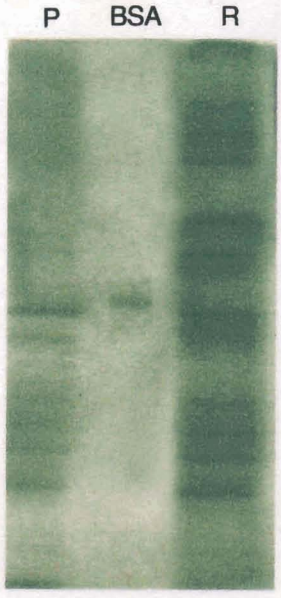
BIOCHEMICAL STUDIES

Biochemical analysis of primary calli (induced on MS medium supplemented with IAA 2mg/l) and regenerating calli (on MS medium supplemented with IAA 0.5 mg/l + BA 2 mg/l) showed that there were marked differences between the two calli in terms of biochemical characteristics. In the regenerating callus cultures of *H.keralensis* metabolites like sugars, proteins and phenolics were high compared to primary callus. Peroxidase activity was also high in regenerating calli (Table 19). The results from the analysis of polypeptide patterns showed more types of polypeptides in the regenerating callus then in the primary callus (Figure 4).

Table 19. Changes in metabolite levels and activity of peroxidase in primary and regenerating callus cultures of *H. keralensis*

Type of Callus	Total sugar %	Total protein %	Total phenolics mg/g Fr. Wt.	Peroxidase Units/ mg Protein
Regenerating callus	14.2	0.31	5.6	40
Primary callus	7.6	0.22	4.2	3.2

Figure 4



SDS -PAGE *Heliotropium keralensis*

P-primary callus
r-regenerating callus

PLATE 1. Micropropagation from shoot tip of *H. keralensis.*

A. Shoots induced on MS + BA (0.5mg/l)+Kn
B. Shoots induced on MS + BA (2mg/l)+Kn (2mg/l)
C. Elongated shoots on MS+ BA (0.5mg/l)(40 days old)
D. Roots induced on MS + IAA (0.5 mg/l)
E. Hardened plantlet in a small pot

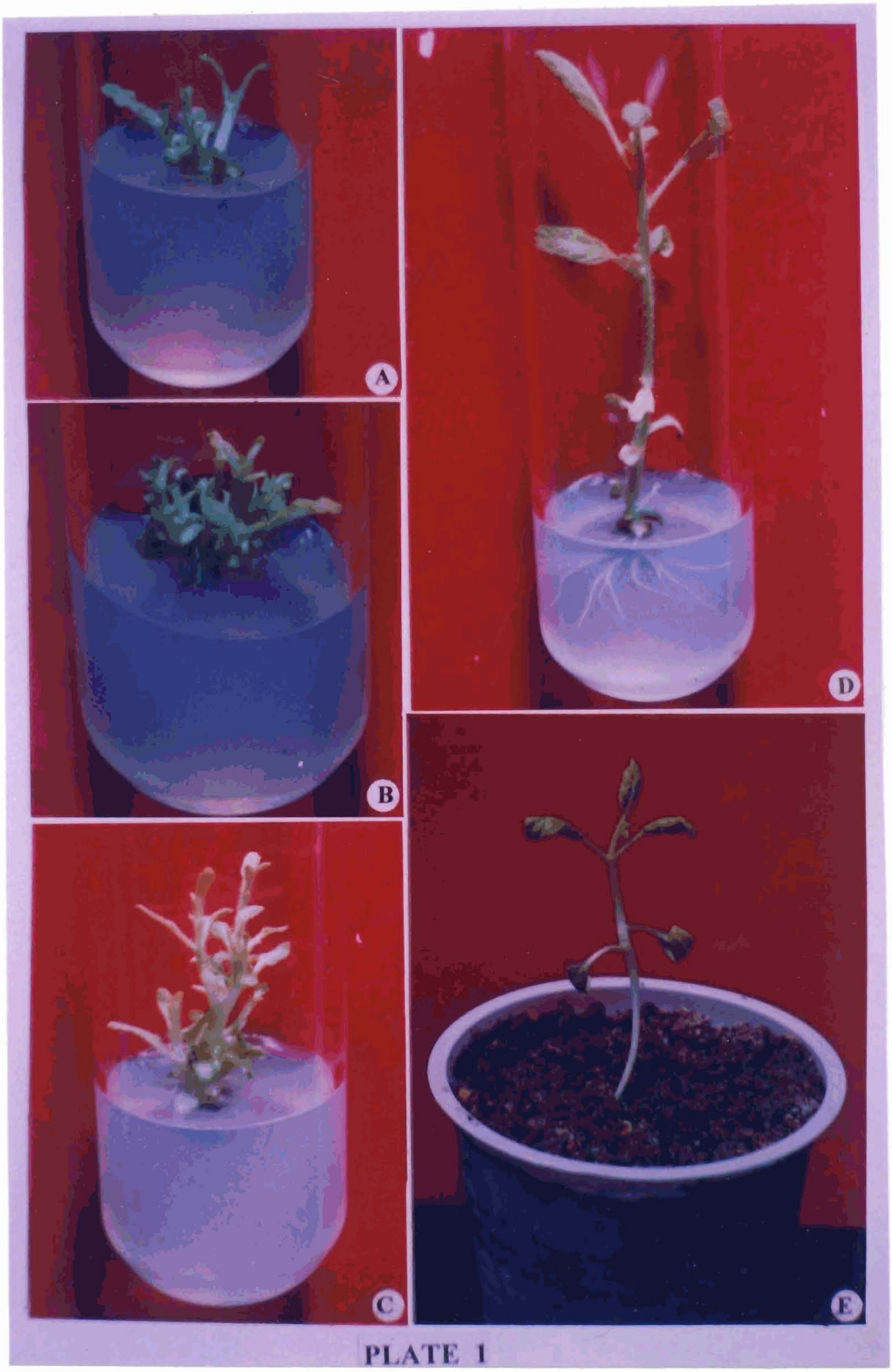


PLATE 1

PLATE 2. Micropropagation from nodes of *H. keralensis.*

A. Shoots induced on MS + IAA (0.5 mg/l)
B. Shoots induced on MS + BA (1.5 mg/l)
C. Shoots induced on MS + BA (0.5 mg/l)+Kn (3mg/l)
D. Roots induced on MS + IAA (1.0 mg/l)
E. Hardened plantlet in a small pot

PLATE 2

PLATE 3. Micropropagation from roots of *H. keralensis*

A. Shoots induced on MS+BA (2mg/l)
B. Shoots induced on MS+BA (4mg/l)+Kn (4mg/l)
C. Elongated shoots (after 40 days of growth)
D. Roots induced on MS+IAA (0.5mg/l)
E. Hardened plantlet in small pot

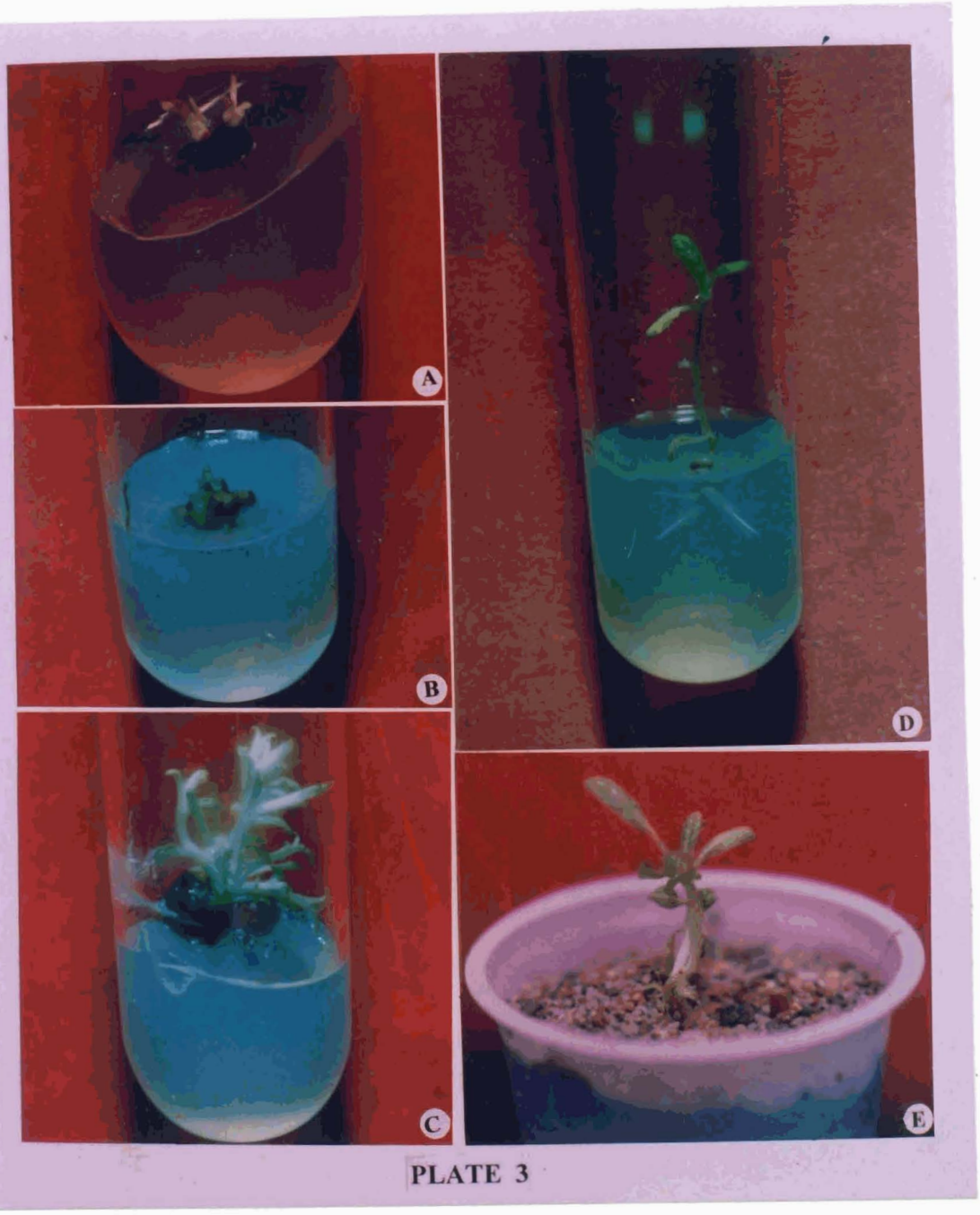


PLATE 3

PLATE 4. Micropropagation through indirect shoot regeneration from internodes of *H.keralensis*

A. Green friable callus induced on MS+BA (3 mg/l) (20 days old).
B. Brown coloured pale white callus induced on MS+2,4-D (0.5mg/l) (40days old)
C. Indirect shoot regeneration on MS+BA (3mg/l)
D. Shoot regeneration on MS+BA (2mg/l)+IAA (0.5mg/l)
E. Roots induced on MS+ NAA (2mg/l)
F. Hardened plantlet in small pot.

PLATE 4

PLATE 5. Micropropagation through indirect shoot regeneration from leaves and nodes of *H. keralensis.*

A. Green callus induced from leaf on MS+2,4-D (0.5mg/l) +BA (1mg/l) (10days)

B. White friable callus induced from node on MS+2,4-D (2mg/l)

C. Brown coloured white friable callus induced from node on MS+2,4-D(3mg/l) (50 days old).

D. Shoot regeneration from callus on MS+BA (3mg/l) + IAA (0.5mg/l)

E. Roots induced on MS+IAA (0.5mg/l)

F. Hardened plantlet in small pot.

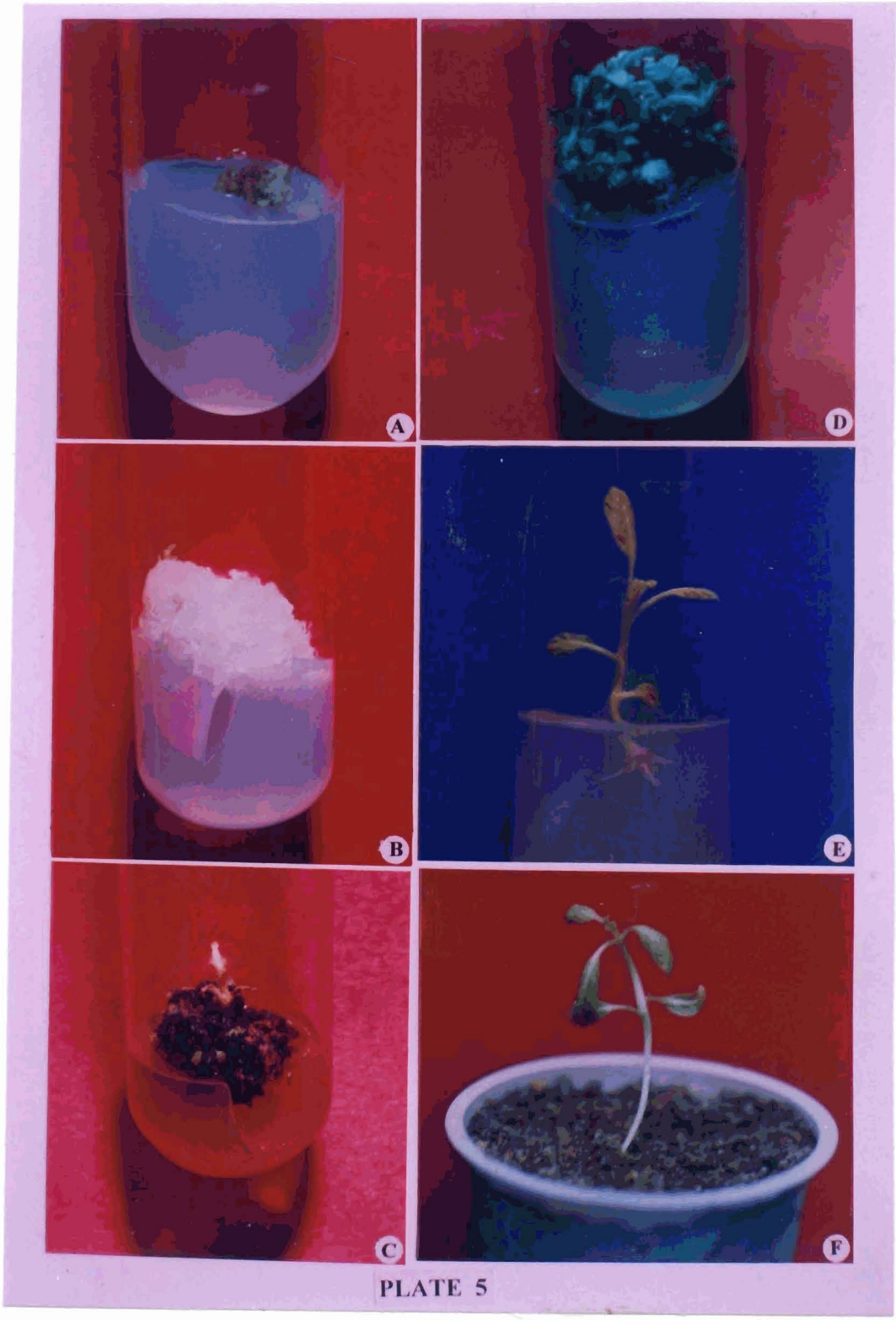


PLATE 5

PLATE 6. Micropropagation through indirect shoot regeneration from roots of *H. keralensis*

A. Callus induced on MS+2,4-D (0.5 mg/l)+ 2,iP (2mg/l) (10days old).
B. Shoot regeneration from callus on MS +BA (3mg/l)
C. Roots induced on MS+IBA (0.2mg/l)
D. Hardened plantlet in small pot.

PLATE 6

PLATE 7. Plant regeneration via. Somatic embryogenesis of *H. keralensis.*

A. Non embryogenic callus induced on MS+IAA (0.5mg/l)
B. Embryogenic callus induced on MS+IAA (0.5mg/l) +2iP(2mg/l).
C. Globular embryos induced from embryonic callus from nodal explants on MS basal medium
D. Somatic embryoids showing different stages of development.
E. Mature somatic embryo (Torpedo stage)
F. Somatic embryo showing germination (10 days).
G. Somatic embryo germinated plantlet.
H. Hardened plantlet in small pot.

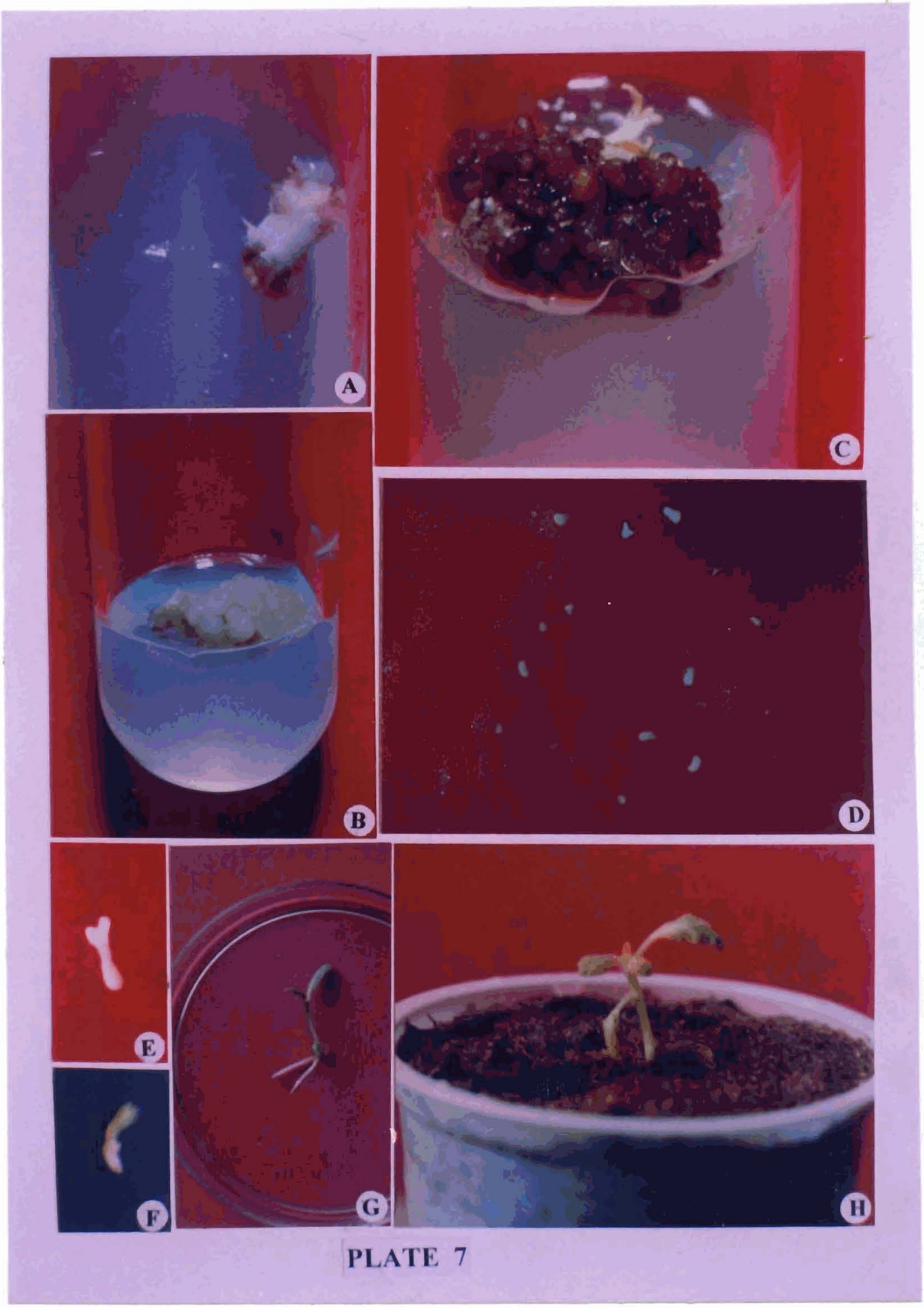


PLATE 7

PLATE 8. Encapsulation and germination of somatic embryos/shoot buds of *H. keralensis.*

A. Encapsulated shoot tips
B. Encapsulated somatic embryos
C. Multiple shoots developed from encapsulated shoot bud on MS+BA (2mg/l) (25 days old.)
D. Germination of encapsulated somatic embryos on MS basal medium.
E. Plantlet developed from synseed.
F. Hardened plantlet in small pot.

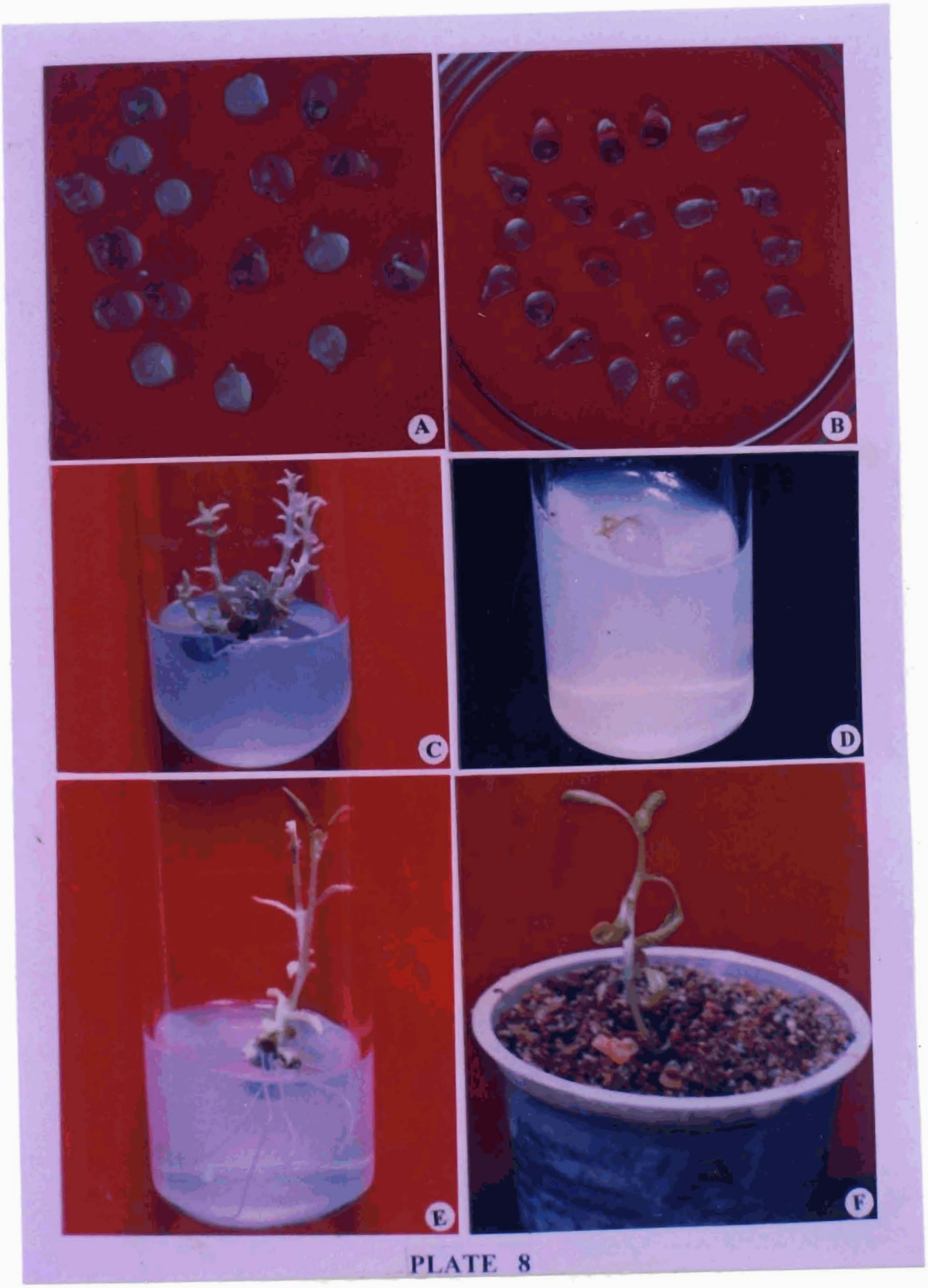


PLATE 8

PLATE 9. Steps in hardening and field transfer of *H. keralensis*.

A. Plantlet with well developed shoot and root system.

B. Plantlet under hardening

C. Hardened plantlet in small pot.

D. Hardened plant under field conditions in pot.

PLATE 9

PLATE 10. TLC analysis (for secondary metabolites) of roots from *in vitro* and field grown plants.

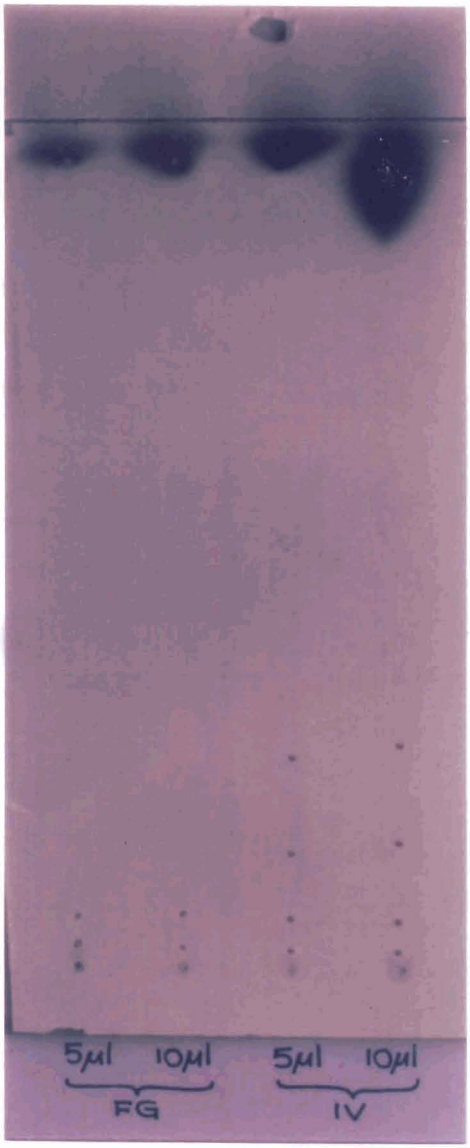


PLATE 10

NAREGAMIA ALATA

For establishing an efficient protocol for micropropagation of *N. alata,* various explants such as shoot tips, nodes, internodes, leaves, petals and roots of naturally grown plants were cultured on different media fortified with different combinations and concentrations of growth regulators. The experiments resulted inefficient and reproducible protocols for micropropagation through organogenesis and somatic embryogenesis of this important medicinal plant.

Among the different concentrations of various surface sterilizing agents (sodium hypochlorite, ethanol and mercuric chloride) tested, 0.1%, $HgCl_2$ treatment for 10 min. produced best results for explants of shoot system (shoot tip, leaf, node, internode and petal) and 0.1% $HgCl_2$ treatment for 15 min. gave best results for root explants.

DIRECT SHOOT REGENERATION

Attempts were made for establishing direct shoot regeneration with different media augmented with different concentrations and combinations of growth regulators. In the present studies direct shoot regeneration was obtained from shoot tip, node and leaf explants of *N. alata.*

i) Multiple shoot formation from shoot tip explants

Three different basal media (MS, B_5, White's) were tested for selecting an appropriate culture medium for *N. alata*. Shoot tips showed elongation (60%) within 10 days on MS basal medium. Shoot tips cultured on B_5 and White's basal media did not show any response even after 20 days. On the basis of the above observation MS medium was selected for further experiments.

Multiple shoots were formed directly from shoot tip explant on MS medium supplemented with varying combinations and concentrations of growth regulators. (Table 20)

Effect of BA

Murashige and Skoog's medium containing BA (0.5 – 3.0 mg/l) was found effective in producing multiple shoots from shoot tips. BA at 2 mg/l was found as the optimal level in which shoot multiplication was high. Further increase in BA (>2mg/l) concentration resulted in reduction in the number of shoots (Table 20).

Effect of Kn

Murashige and Skoog's medium containing Kn (0.5 – 2.0 mg/l) was ineffective in inducing multiple shoots from shoot tip explants of *N. alata*. However, shoot tips elongated on Kn supplemented MS medium (Table 20).

Table 20. Effect of growth regulation on shoot induction from shoot tip explants on *N. alata*

Growth regulators (mg/l)	No. of shoots/ culture	Shoot length	% of Response
BA			
0.5	9.3 ± 0.76	4.43 ± 0.22	30
1.0	12.3 ± 0.45	4.43 ± 0.18	60
2.0	21.9 ± 0.57	4.1 ± 0.07	65
3.0	21.1 ± 0.63	4 ± 0.1	60
Kn			
0.5	SS elongated	3.1 ± 0.15	20
1.0	SS elongated	3.6 ± 0.18	40
2.0	SS elongated	3.8 ± 0.1	45
NAA + BA			
0.5 0.5	9.9 ± 0.49	4.8 ± 0.1	70
0.5 1.0	13.6 ± 0.39	4.82 ± 0.1	75
0.5 2.0	21.2 ± 0.57	4.55 ± 0.13	80
0.5 3.0	27.4 ± 0.52	4.4 ± 0.12	85
0.5 4.0	25.2 ± 0.31	4.12 ± 0.26	85
IAA + BA			
0.5 0.5	10.2 ± 0.14	4.2 ± 0.26	70
0.5 1.0	13.6 ± 0.72	3.9 ± 0.18	75
0.5 2.0	18.0 ± 0.32	3.83 ± 0.47	75
0.5 3.0	17.4 ± 0.51	3.82 ± 0.62	80

Data from 20 replicates in two experiments (Mean ±SE)
Growth period 50 days
SS= Single shoot

Effect of NAA + BA

Murashige and Skoog's medium supplemented with NAA (0.5 mg/l) and BA (0.5 – 4.0 mg/l) was found as the most effective combination for shoot multiplication of *N. alata* (Plate 11 A,B). Shoot induction was observed within 15 days in all the combinations tested. The optimal concentration of both NAA (0.5 mg/l) and BA (3.0 mg/l) produced highest number (27) of shoots (Plate 11A). At higher concentrations of BA (> 3.0 mg/l) both shoot multiplication as well as shoot elongation were declined (Table 20).

Effect of IAA + BA

Murashige and Skoog's medium fortified with IAA (0.5 mg/l) and BA (0.5-3.0 mg/l) combination also produced multiple shoots from shoot tip explants. However, the combination was less effective when compared to NAA + BA combination in MS medium for shoot induction as well as shoot elongation. At the optimal concentrations IAA (0.5mg/l) + BA (2.0 mg/l) combination produced an average of 18 shoots per shoot tip.

ii) Direct shoot regeneration from nodal explants

Murashige and Skoog's medium augmented with growth regulators (BA, Kn, IAA & NAA) either singly or in combinations was used to induce multiple shoots from nodal explants of *N. alata* (Table 21).

Table 21. Effect of Growth Regulators on Shoot Induction from Nodal Explants on *N. alata*

Growth regulators (mg/l)	No. of shoots/ culture	Shoot length	% of Response
BA			
0.5	7.89 ± 0.39	5.3 ± 0.16	45
1.0	9.9 ± 0.76	4.5 ± 0.25	60
1.5	13.42 ± 0.62	4.33 ± 0.18	70
3.0	17.8 ± 0.38	3.86 ± 0.11	75
Kn(mg/l)			
0.5			
1.0	SS + C	3.45 ± 0.21	50
2.0	SS + C	3.6 ± 0.25	45
NAA + BA			
0.5 0.5	9.2 ± 0.65	6.27 ± 0.2	70
0.5 1.0	9.64 ± 0.7	5.5 ± 0.27	70
0.5 ± 1.5	16.3 ± 0.7	5.13 ± 0.1	80
0.5 2.0	20.4 ± 0.7	4.22 ± 0.13	85
0.5 3.0	24.4 ± 0.5	3.96 ± 0.1	85
0.5 4.0	24.3 ± 0.43	3.92 ± 0.21	85
1.0 3.0	13.2 ± 0.72 +C	3.89 ± 0.22	85
IAA + BA			
0.5 0.5	9.2 ± 0.42	5.5 ± 0.2	75
0.5 1.0	10.2 ± 0.76	5.2 ± 0.08	75
0.5 2.0	21.8 ± 0.82	4.53 ± 0.12	80
0.5 3.0	21.2 ± 0.61	4.54 ± 0.36	75

Data from 20 replicates in two experiments (Mean ±SE)
Growth period 50 days
SS-Single shoot
C- Callusing

Effect of BA

Effective multiple shoot induction from nodal explants was obtained on MS medium supplemented with BA at different concentrations (0.5-3.0 mg/l). The simulating effect of BA in the bud break and multiple shoot production was highest (20/ explant) at an optimal concentration (2mg/l). The percentage of multiple shoot induction was declined with the increase in BA concentration beyond the optimal level of 2mg/l .

Effect of Kn

Kinetin at varied concentrations (0.5 – 2.0 mg/l) on MS medium failed to produce multiple shoots from nodal explants. However, single shoot formation was observed at higher concentrations of Kn($\geq$ 1.0 mg/l) (Plate 12 A).

Effect of NAA + BA

Nodal explants showed highest shoot multiplication on MS medium supplemented with a combination of NAA+BA combinations. The combination also promoted shoot elongation. NAA (0.5 mg/l) and BA (3.0 mg/l) produced maximum number of shoots (24) per nodal explant within 20-25 days (Plate 12 B). Increase or decrease of NAA or BA concentrations from the optimal levels adversely affected shoot multiplication.

Effect of IAA + BA

Murashige and Skoog's medium with combination of IAA (0.5 mg/l) and BA (0.5 – 3.0 mg/l) was also effective in inducing multiple shoots from nodal explants. However, the combination was less effective when compared to NAA + BA combination. At the optimal concentrations of IAA (0.5 mg/l) and BA (2.0 mg/l) the nodal explants produced an average of 22 shoots. (Table 21)

iii) Direct shoot regeneration from leaf explants

Different levels of cytokinins (BA and Kn) either singly or in combination were tried to establish direct shoot regeneration from leaf explants. Among these, MS medium fortified with BA (0.1-3mg/l) and BA (0.1-3mg/l) + Kn (0.1-3mg/l) was effective for direct multiple shoot regeneration from leaf explants.

Direct shoot formation from the explant without any callusing was obtained only on MS medium supplemented with 0.1 mg/l BA. About 40% of the leaf explants produced single shoots on the above medium(Plate 13 A,B) (Table 22). At higher concentrations (>0.5mg/l) of BA multiple shoots were formed with varying frequency . However, along with direct adventitious shoot formation little amount of callusing was also observed on leaf explants.

Murashige and Skoog's medium containing BA (0.1 – 3.0mg/l) and Kn (0.1 – 3.0mg/l) was most effective combination for direct shoot regeneration. Optimal concentration of BA (2.0mg/l) and Kn (2.0mg/l) on MS medium

Table 22. Effect of growth regulators on shoot induction from leaf explants on *N. alata*

Growth Regulators (mg/l)		No. of shoots/ culture	Shoot length	% of Response
BA				
0.1		1.1 ± 0.15	3.48 ± 0.26	30
0.5		3.25 ± 0.52 +C	3.4 ± 0.31	40
1.0		11.6 ± 0.27 + C	3.14 ± 0.18	45
2.0		19.14 ± 0.61 + C	2.98 ± 0.27	65
3.0		18.2 ± 0.17 +C	2.92 ± 0.62	65
BA	**+ Kn**			
0.1	0.1	2.31 ± 0.24	3.7 ± 0.28	50
0.5	0.5	4.2 ± 12 +C	3.32 ± 0.47	60
1.0	1.0	13.6 ± 0.27 +C	3.14 ± 0.65	75
2.0	2.0	27.1 ± 0.41 + C	2.96 ± 0.14	75
3.0	3.0	24.3 ± 0.31 + c	2.87 ± 0.23	70

Data from 20 replicates in two experiments (Mean ±SE)
Growth period 50 days
C = callusing

enhanced proliferation and induced highest number (27/leaf) of shoots from leaf explants (Plate 13 C).

iv) Effect of nutrient media

To select most suitable medium for shoot multiplication of *N. alata* shoot tips were cultured on various media (MS, B_5 and White's) supplemented with 0.5mg/l NAA and 3mg/l BA. MS medium was found as the most effective one on which highest number of shoots (27) were developed followed by B_5 (24) and White's (3) media (Figure 5).

iv) Effect of sucrose concentration

Direct shoot proliferation and growth were greatly influenced by the level of sucrose added in the culture medium. To find out the optimal level of sucrose, shoot tip explants were cultured on MS medium containing varying concentrations of sucrose (1-5%) along with 0.5 mg/l NAA + 3.0 mg/l BA. Sucrose at 3% gave significant result compared to other concentrations (Figure 6).

INDIRECT SHOOT REGENERATION

For the establishment of indirect shoot regeneration of *N. alata*, various explants viz., leaves, internodes, petals, and roots were cultured on MS medium fortified with different growth regulators. When the explants were cultured on MS medium supplemented with auxin only callusing was observed. However,

Figure 5. Effect of nutrient media on shoot induction from shoot tip explants on *N. alata*

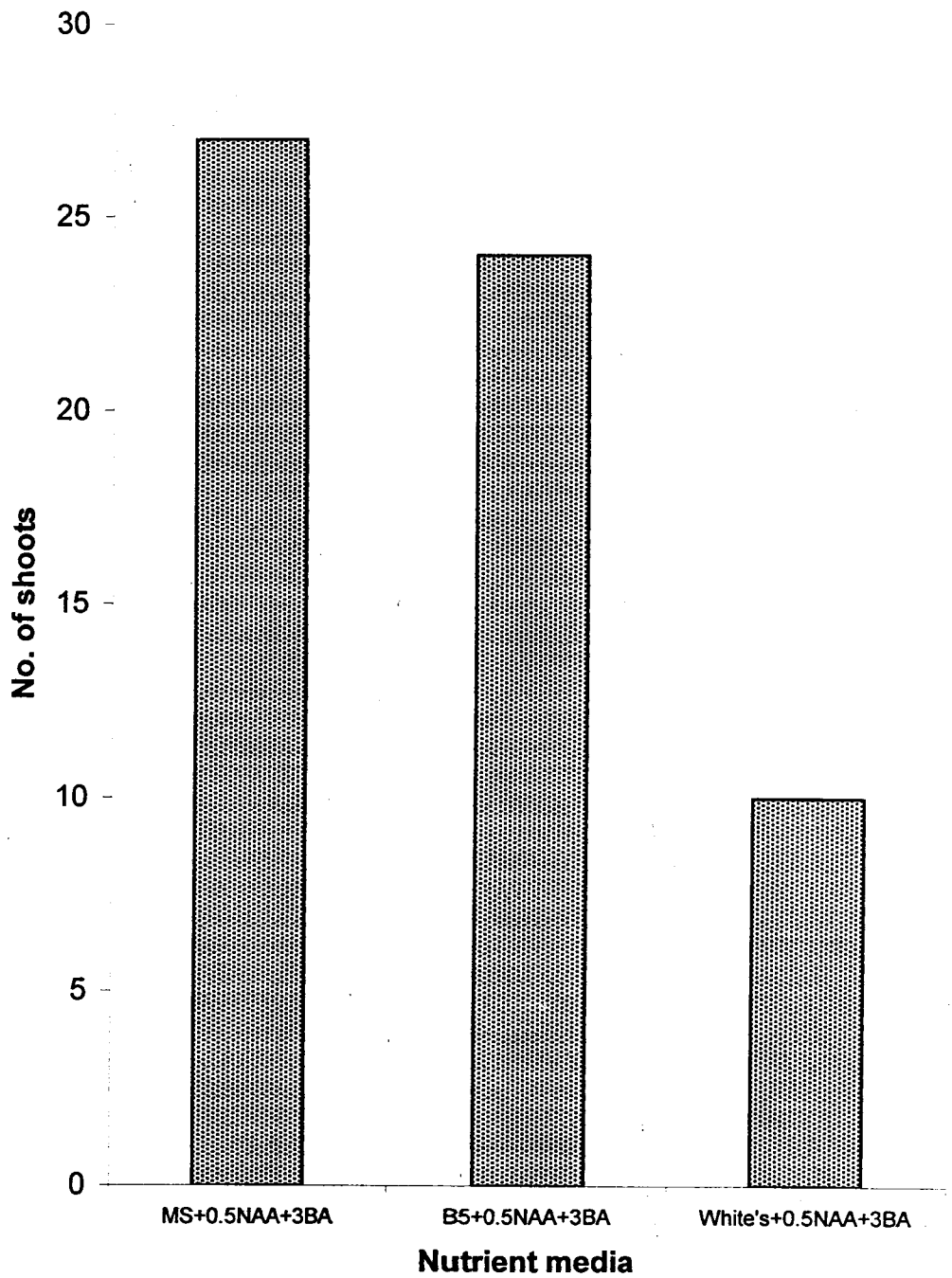
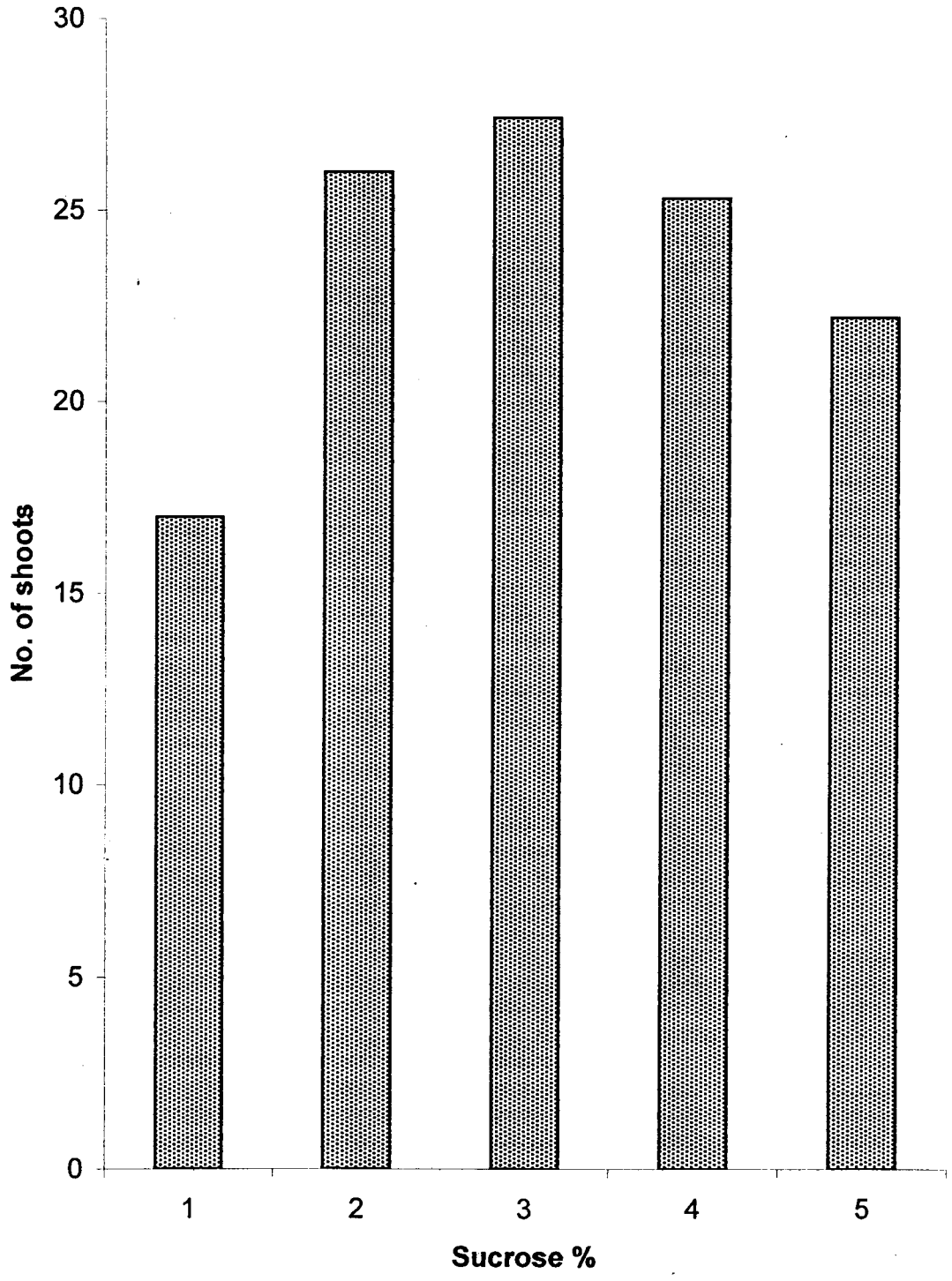


Figure 6. Effect of sucrose on shoot induction from shoot tips of *N. alata* (Cultured on MS with 0.5mg/l NAA +3mg/l BA)



when explants were cultured on MS medium supplemented with combination of BA and an auxin, (IAA or NAA) induction as well as regeneration of shoots from calli were observed (Table 23).

Effect of NAA + BA

Different explants when cultured on MS medium supplemented with NAA (0.5 mg/l) + BA (0.5 - 4.0 mg/l) induction of calli and regeneration of shoots occurred on the same medium (Plate 17 D). In the case of root explants, combination containing at least 1.0 mg/l BA was necessary for regeneration of shoots from the calli. The calli induced on this combination were green and friable with nodular structures (Plate 15 B) and regeneration was observed within 30 days (Plate 17 C). 0.5 mg/l NAA + 3.0 mg/l BA in MS medium was the optimal concentrations for indirect shoot regeneration for most of the explants(Plates 14 C; 15 D; 16 E). Among the different explants tested leaf explants produced highest number of shoots (11) in the optimal combination (Table 23).

Effect of IAA + BA

MS medium supplemented with IAA (0.5 – 1.0 mg/l) + BA (0.5 – 4.0 mg/l) was also effective for indirect shoot regeneration from various explants of *N. alata*. But root explants required a higher concentration of BA (≥1.0 mg/l)

Table 23. Combined effect of auxins & cytokinins on callus induction and regeneration from internode, leaf,root & petal explants of *N. alata*

Growth Regulators (mg/l)		Internode	Leaf	Root	Petal
NAA	**BA**				
0.5	0.5	3.2 ± 0.12	3.8 ± 0.37	C Only	2.9 ± 0.61
0.5	1.0	4.23 ± 0.67	4.6 ± 0.46	2.3 ± 0.23	3.6 ± 0.14
0.5	2.0	8.33 ± 0.49	9.1 ± 0.22	4.9 ± 0.49	6.9 ± 0.22
0.5	3.0	10.8 ± 0.14	11.2 ± 0.19	8.2 ± 0.17	7.3 ± 0.66
0.5	4.0	10.5 ± 0.29	11.1 ± 14.5	5.4 ± 0.11	7.3 ± 0.66
1.0	0.5	C Only	C Only	C only	C only
IAA	**BA**				
0.5	1.0	3.4 ± 0.24	3.2 ± 0.72	C only	2.1 ± 0.71
0.5	2.0	4.2 ± 0.13	4.1 ± 0.39	2.4 ± 0.32	3.9 ± 0.18
0.5	3.0	6.7 ± 0.38	6.5 ± 0.64	5.2 ± 0.19	6.5 ± 0.28
0.5	4.0	8.3 ± 0.33	8.5 ± 0.55	5.2 ± 0.14	7.1 ± 0.33
0.5	5.0	8.29 ± 0.41	8.2 ± 0.12	5.1 ± 0.27	7.2 ± 0.41
1.0	0.5	2.1 ± 0.22	2.2 ± 0.11	1.7 ± 0.36	1.8 ± 0.34
NAA	**Kn**				
0.5	3.0	C Only	C only	C only	C only
IAA	**Kn**				
0.5	3.0	C only	C only	C only	C only

Data from 20 replicates in two experiments (Mean ±SE)
Growth period 50 days
C= Callus

for regeneration of shoots. Leaf explants showed best response for regeneration in comparison with other explants (Table 23).

Callusing was established within 8-10 days (Plate 15 A) and shoot multiplication occurred only after one month. The calli were white and friable initially (Plate 14 B) later it turned green (Plate 16 B) and meristematic and shoots were emerged out (Plate 16 D). The best response and higher percentage of shoot multiplication was obtained on the optimal combination of IAA (0.5mg/l) and (4.0 mg/l)

Effect of NAA + Kn

Combinations of NAA (0.5 mg/l) + Kn (0.5-4mg/l) in MS medium also induced calli from all explants tested. The calli were pale white and friable but no shoot formation occurred in the combination (Plate 16 A)(Table 23).

Effect of IAA + Kn

Murashige and Skoog's medium with IAA + Kn combinations induced calli from all the explants (Table 23). However, the calli did not regenerate shoots on the same medium. The calli obtained were pale white and friable (Plate 17 A,B).

Effect of NAA

Murashige and Skoog's medium with NAA at the range 0.5 – 3.0 mg/l effectively induced callus within 12 days from explants of shoot system (leaf,

petal, internode) and within 25 days from root explants. The calli were pale white and friable (Plate 16 C). 2.0 mg/l was the optimum concentration of NAA for induction and proliferation of calli from various explants of *N. alata*. Root formation was also observed from the calli (Table 24).

Effect of IAA

Murashige and Skoog's medium with IAA at the range of 0.5 – 4.0 mg/l induced callusing from various explants of *N. alata* within 20 days from leaf, internode and petal explants. However, callus induction was delayed on root explants for 10 more days. The calli were white and friable. Optimum concentration of IAA for callus induction was 3.0 mg/l for all explants cultured (Plate 14 A)(Table 24).

Effect of 2,4-D

It was found that 2,4-D was the most effective auxin for callus induction from various explants of *N. alata.* Calli were induced within 10 days on all the explants tested (Table 24). Callus induction and proliferation were progressively increased on 2,4-D concentrations and found 2.0 mg/l 2,4-D was the optimal concentration for callus induction. The calli were pale yellow and friable (Plate 15C).

The calli obtained from various explants on MS medium supplemented with auxin or auxin + kinetin were tested for their regenerative potential by

Table 24. Effect of auxins (NAA, IAA & 2,4-D) on callus induction from various explants of *N. alata*

Growth Regulators (mg/l)	Internode	Leaf	Root	Petal
NAA				
0.5	+ & R	+	-	+
1.0	++ & R	++ & R	-	+
2.0	+++ & R	+++ & R	++ & R	++
3.0	++ & R	+++ &R	++ & R	++
IAA				
0.5	+	+		+
1.0	+	+	+	+
2.0	+++	++	++	++
3.0	+++	+++	++	++
4.0	+	+	+	+
2,4-D				
0.1	+	+		+
0.5	++	++	+	++
1.0	++	++	+	++
2.0	++++	++++	++	+++
3.0	+++	+++	++	+++

+ =Very slight; ++ =Little; +++ =Moderate ; ++++= Profuse
Data from 20 replicates in two experiments
Growth period 50 days

subculturing on MS medium with 0.5 mg/l NAA + 3mg/l BA. All the calli regenerated shoots when cultured on the above combination.

SOMATIC EMBRYOGENESIS

Somatic embryos of *N. alata* were formed within 60 days upon subculturing of the embryogenic calli obtained from various explants on MS basal medium. Direct somatic embryogenesis and subsequent plant regeneration were also established from leaf explants.

i) Direct somatic embryogenesis

Direct somatic embryogenesis was obtained from leaf explants of *N. alata* on MS medium supplemented with 0.1 mg/l 2,4-D (Plate 18 A, B). The mode of placing the explant on the nutrient medium showed marked influence on the embryogenic response of the explant. When the adaxial side of the leaf was touching the medium only direct embryoids were formed on the other side (Plate 18 B). But when abaxial side was touching the medium, direct somatic embryos and embryogenic calli were formed on the other side (Plate 18 A). At higher concentrations of 2,4-D ($\geq$ 0.5 mg/l) only embryogenic calli were formed instead of direct somatic embryos (Table 25).

ii) Indirect somatic embryogenesis

Indirect somatic embryogenesis of *N. alata* was established by subculturing embryogenic calli on MS basal medium

Table 25. Effect of 2,4-D IAA, NAA, BA & Kn on somatic embryogenesis from leaf explants of *N. alata*

Growth Regulators (mg/l)	Response	% of Response	No. of embryos found/from cali
2,4-D			
0.1	Direct embryos and Embryogenic Calli	80	4.3 ± 0.27
0.5	EC	85	6.7 ± 0.33
1.0	EC	85	10.2 ± 0.62
2.0	EC	80	8.2 ± 0.24
3.0	EC	70	8.4 ± 0.22
NAA			
0.1	NC	70	-
0.5	NC	65	-
1.0	NC	70	-
2.0	NC	75	-
2,4-D+ Kn			
0.5 0.5	NC	40	-
0.5 1.0	NC	70	-
2,4-D+ BA			
0.5 0.5	NC	60	-
0.5 1.0	NC	65	-

Data from 20 replicates in two experiments (Mean ±SE)
Growth period 50 days
EC-Embryogenic calli
NC-Non embryogenic calli

a. Induction of embryogenic calli

Different explants of *N. alata* were cultured on MS medium supplemented with various growth regulators for the induction of embryogenic calli.

Effect of growth regulators

Leaf explants were cultured on MS medium supplemented with various growth regulators for the induction of embryogenic calli. The type of the auxin and its concentration used in the medium significantly influenced the formation of embryogenic callus (Table 25). Among the various combinations and concentrations of growth regulators tested (2,4-D, NAA, 2,4-D+Kn and 2,4-D+BA), MS medium supplemented with 2,4-D (0.1-3.0 mg/l) only produced embryogenic calli from the explants (Table 25). The calli obtained were glossy and pale white (Plate 18 C) initially, which later turned yellow. (The embryogenic potential was later recorded by observing response of the calli on MS basal medium in 60 days). Addition of BA or Kn along with 2,4-D favoured callus proliferation. However, the calli were non embryogenic.

Effect of various explants

Among different explants cultured (leaf, internode, root and petal) on MS medium supplemented with 1.0 mg/l 2,4-D, leaf explants produced calli with

highest embryogenic potential (the embryogenic potential was later recorded by observing response of calli on MS hormone free medium) (Figure 7).

Effect of sucrose

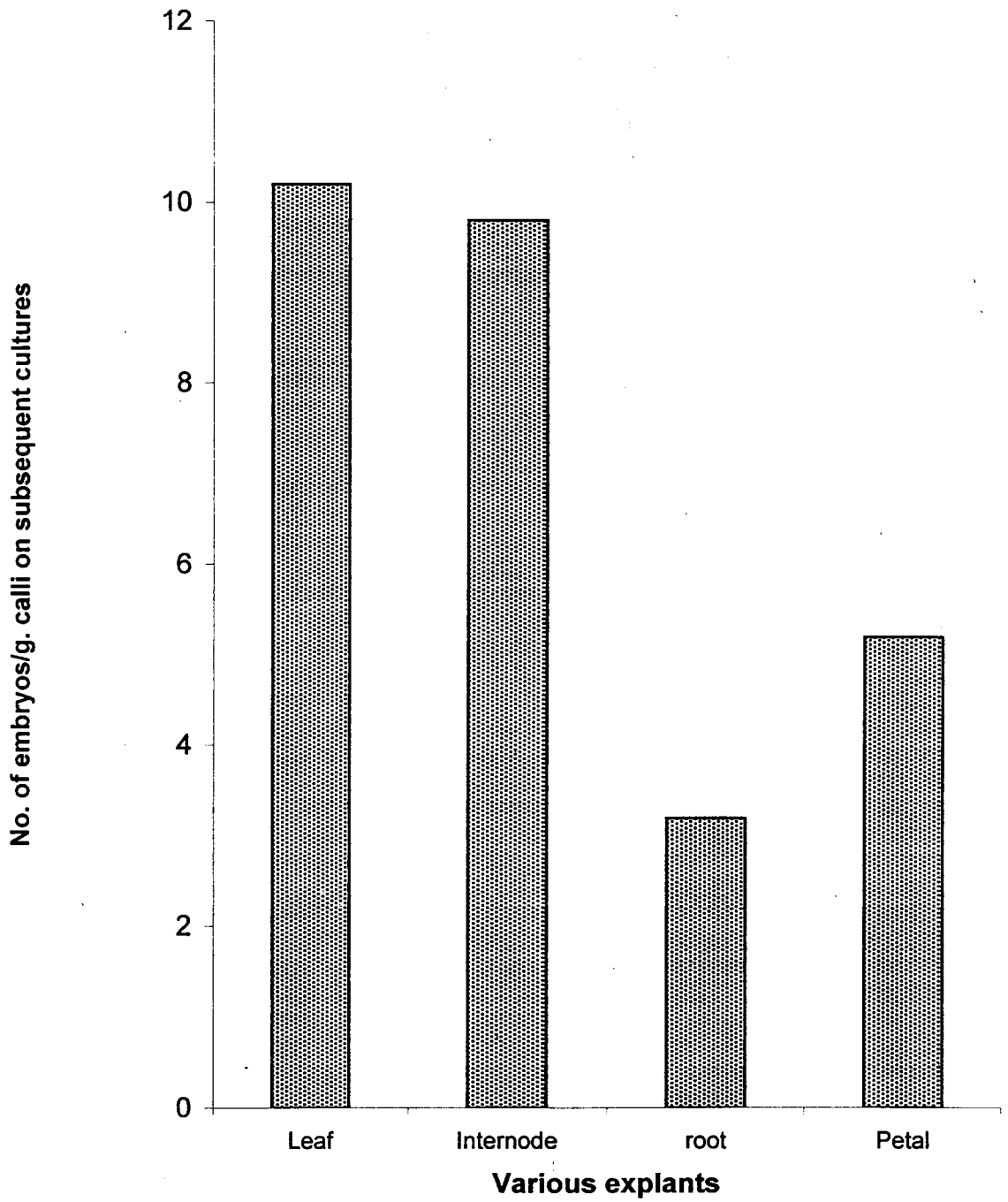
The level of sucrose also had significant effect in inducing embryogenic calli. Among the different concentrations of sucrose (2-6%) tested in MS medium supplemented with 1.0 mg/l 2,4-D, medium containing 5% sucrose produced calli with highest embryogenic potential (Figure 8).

b) Induction and maturation of somatic embryos

Somatic embryos were formed when embryogenic calli were cultured on MS hormone free semi-solid (Plate 18 D) or liquid medium (Plate 18 E,F,G) within 60 days. Liquid medium was superior to semi-solid medium for the induction of somatic embryos from embryogenic calli. The embryogenic calli obtained from leaf explants on MS medium supplemented with 1.0 mg/l 2,4-D produced an average of 10 somatic embryos /g calli in liquid medium whereas only 8 embryos were formed/g calli when cultured on semi-solid medium.

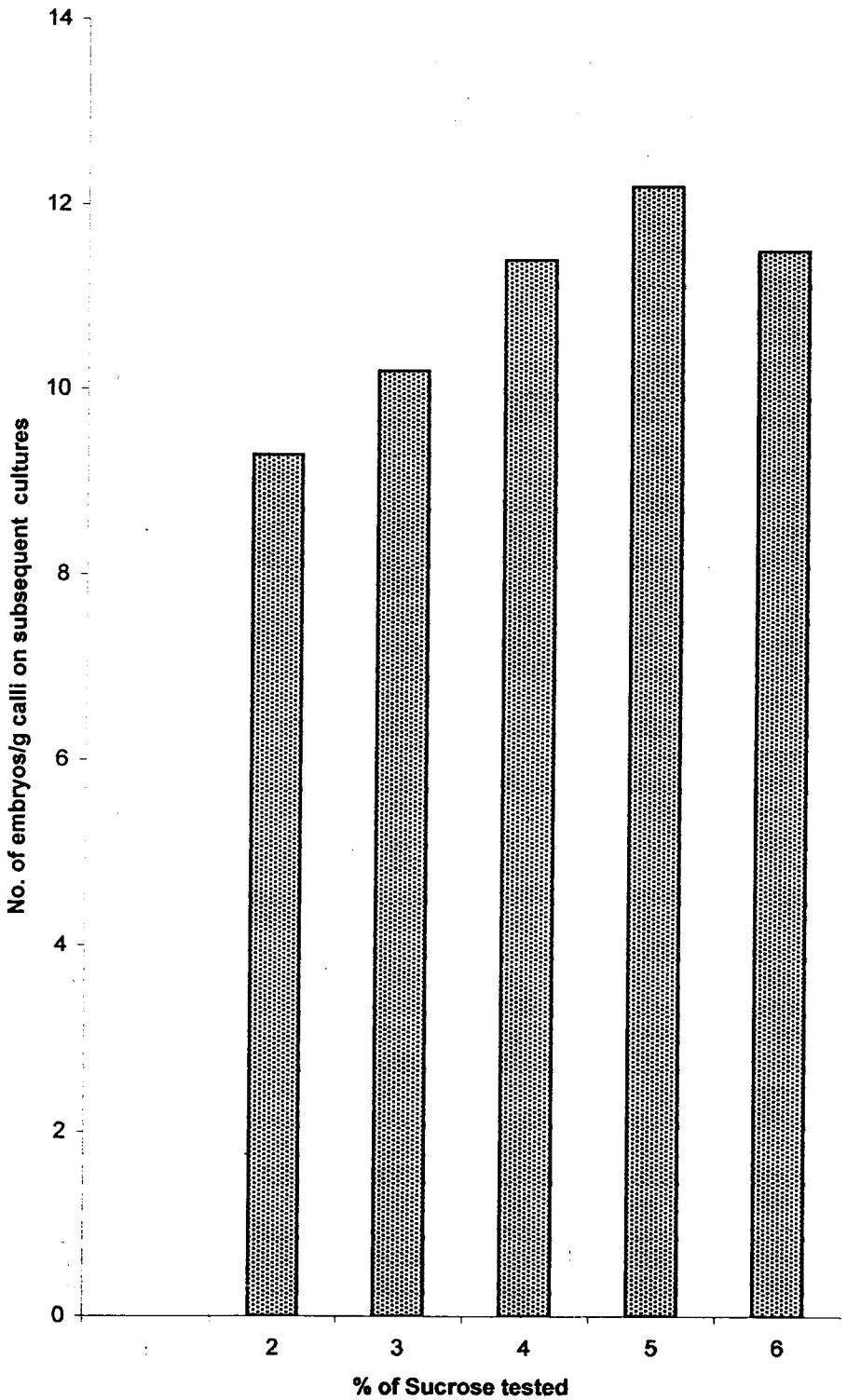
In general, the embryos passed through the normal developmental stages (globular, heart, torpedo) (Plate 18 E) and underwent germination (Plate 18 H) when cultured on MS basal semi- solid medium with a frequency of over 70%. The plantlets derived through somatic embryogenesis were similar to normal field grown plants.

Figure 7. Effect of various explants on somatic embryogenesis of *N. alata* cultured on MS + 1mg/l 2,4-D



60

Figure 8. Effect of sucrose concentration on somatic embryogenesis of *N. alata* (Cultured on MS + 1mg/l 2,4-D)



SYNSEEDS

Three percent sodium alginate and 50 mM $CaCl_2(2H_2O)$ solutions were found to be the best for the encapsulation and subsequent germination /development of somatic embryos/shoot buds of *N. alata* (Plate 19 A, B).

Encapsulated embryos were germinated within 30 days on MS basal medium at a frequency of 60%. Encapsulated shoot buds did not show any sign of growth on MS basal media even after 20 days of transfer. However, about 70% encapsulated shoot buds showed signs of development within 15 days when cultured on MS medium supplemented with 1.0 mg/l Kn. Storage at 4°C for 6 months did not affect the germination/development (Plate 19 C) of the encapsulated propagules.

ROOTING *IN VITRO*

For root induction individual shoots (> 3cm) were excised and cultured on half or full strength MS medium supplemented with various growth regulators. Half strength MS medium was superior to full strength medium for root induction of *in vitro* raised shoots of *N. alata*. Among the three auxins (NAA, IAA and IBA) tested NAA was superior to other two for rooting of *N.alata* shoots (Table 26) (Plates 11 C;12 C 13 D;14 D; 15 E; 16 F; 17 E;19 D; 20 A). Optimal concentration (0.5mg/l) of NAA on half strength MS medium induced highest number of roots from *in vitro* raised shoots (Plate 20 A).

Table 26. Effect of ½ and full strength MS media & auxins (IAA, NAA & 2,4-D) on *in vitro* rooting of *N. alata*

Growth Regulators mg/l	% of Response	No. of roots	Root length
NAA			
0.1	40	3.12 ± 0.24	1.6 ± 0.71
0.2	50	4.3 ± 0.18	1.9 ± 0.8
0.3	70	8.7 ± 0.36	3.4 ± 0.12
0.4	85	9.2 ± 0.11	4.2 ± 0.62
0.5	90	13.1 ± 0.21	4.1 ± 0.11
1.0	90	11.7 ± 0.24	3.8 ± 0.23
IBA			
0.1	-		
0.5	70	4.2 ± 0.3	2.2 ± 0.14
1.0	80	8.7 ± 0.51	2.6 ± 0.27
2.0	85	7.3 ± 0.49	3.1 ± 0.32
IAA			
0.1	-	-	-
0.5	70	2.4 ± 0.43	3.7 ± 0.8
1.0	70	5.6 ± 0.62	3.9 ± 0.14
MS full strength			
NAA			
0.5	85	9.5 ± 0.22	4.0 ± 0.14
IBA			
1.0	70	4.9 ± 0.46	3.7 ± 0.22
IAA			
1.0	80	6.7 ± 0.26	3.4 ± 0.26

Data from 20 replicates in two experiments (Mean ±SE)
Growth period 50 days

Table 26. Effect of ½ and full strength MS media & auxins (IAA, NAA & 2,4-D) on *in vitro* rooting of *N. alata*

Growth Regulators mg/l	% of Response	No. of roots	Root length
NAA			
0.1	40	3.12 ± 0.24	1.6 ± 0.71
0.2	50	4.3 ± 0.18	1.9 ± 0.8
0.3	70	8.7 ± 0.36	3.4 ± 0.12
0.4	85	9.2 ± 0.11	4.2 ± 0.62
0.5	90	13.1 ± 0.21	4.1 ± 0.11
1.0	90	11.7 ± 0.24	3.8 ± 0.23
IBA			
0.1	-		
0.5	70	4.2 ± 0.3	2.2 ± 0.14
1.0	80	8.7 ± 0.51	2.6 ± 0.27
2.0	85	7.3 ± 0.49	3.1 ± 0.32
IAA			
0.1	-	-	-
0.5	70	2.4 ± 0.43	3.7 ± 0.8
1.0	70	5.6 ± 0.62	3.9 ± 0.14
MS full strength			
NAA			
0.5	85	9.5 ± 0.22	4.0 ± 0.14
IBA			
1.0	70	4.9 ± 0.46	3.7 ± 0.22
IAA			
1.0	80	6.7 ± 0.26	3.4 ± 0.26

Data from 20 replicates in two experiments (Mean ±SE)
Growth period 50 days

Optimal concentration (0.5mg/l) of NAA on half strength MS medium induced highest number of roots from *in vitro* raised shoots (Plate 20 A).

SECONDARY METABOLITE ANALYSIS

In the present studies Thin layer chromatography (TLC) was performed to compare the alkaloids present in the roots of *in vitro* and field grown plants.

Root extracts from *in vitro* plants showed 4 compounds (spots) with Rf values 0.031, 0.056, 0.0462 and 0.975. However, root extracts from *in vivo* plants showed only 3 compounds (spots) with Rf values 0.031, 0.56 and 0.975. This indicates the presence of an additional compound in *in vitro* roots compared to *in vivo* roots (Plate 21)

ACCLIMATISATION AND TRANSFER TO FIELD CONDITIONS

Plantlets derived from different cultures with sufficient root and shoot system were successfully hardened under laboratory conditions. The plantlets were transferred after washing with sterilized distilled water into small plastic pots containing sterile sand and soil in 1:1 ratio and were covered with polythene bags or glass bottles (Plate 20 B) for acclimatization. After 20-25 days they were eventually established in natural conditions (Plates 11D; 12 D; 13 E; 14 E, 17 F; 18 I; 19 E). These plantlets were successfully transferred to large pots/field showed high percentage of (85%) survival (Plate 20 C).

BIOCHEMICAL STUDIES

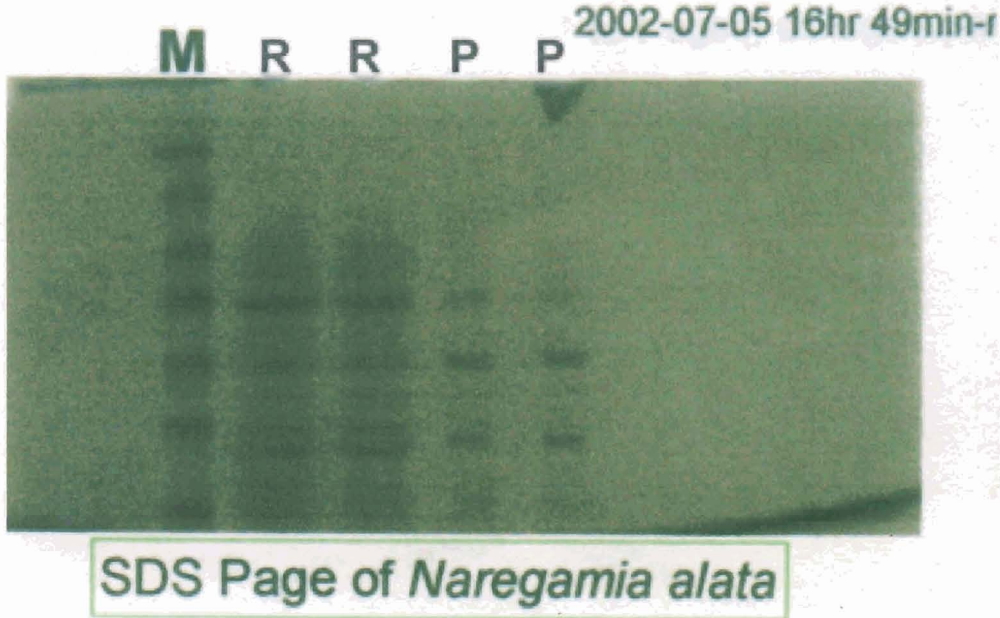
Biochemical analysis of primary calli (induced on MS medium supplemented with IAA 2mg/l) and regenerating calli (on MS medium supplemented with IAA 0.5 mg/l + BA 2 mg/l) showed that there were marked differences between the two calli in terms of biochemical characteristics. Metabolites like sugars, proteins and phenolics were found to be high in the regenerating callus than in the primary callus of *N. alata*. Peroxidase activity was also high in the regenerating callus than in the primary callus (Table 27). Polypeptide pattern analysis of the two calli showed more types of polypeptides in the regenerating callus then in the primary callus (Figure 9).

Table 27. Changes in metabolite levels and activity of peroxidase in primary and regenerating callus cultures of *N. alata.*

Type of Callus	Total sugar %	Total protein %	Total phenolics mg/g Fr. Wt.	Peroxidase Units/ mg Protein
Regenerating callus	18.3	0.44	3.9	50
Primary callus	10.6	0.34	3.6	12

Data represents an average of 4 replicates scored in 50 days old callus.

Figure 9.



M-Molecular marker
R-Regenerating callus
P-Primary callus

PLATE 11. Micropropagation of *N. alata* from shoot tip.

A. Multiple shoots induced on MS+NAA (0.5 mg/l) +BA (3mg/l)
B. Multiple shoot showing elongation.
C. Root induction on ½ MS+NAA (0.2mg/l)
D. Hardened plantlet in small pot.

PLATE 11

PLATE 12. Micropropagation of *N. alata* from nodes.

A. Shoot induction on MS +Kn (2mg/l)
B. Multiple shoots induced on MS +NAA (0.5mg/l) + BA (3mg/l)
C. Roots induced on ½ MS+NAA (0.5mg/l)
D. Hardened plantlet in small pot.

PLATE 12

PLATE 13. Micropropagation of *N. alata* from leaf explant.

A. Shoot induction on MS +BA (0.1mg/l) (10 days old)
B. Shoot induction on MS +BA (0.1mg/l) (15 days old)
C. Multiple shoots induced on leaf on MS +BA (2mg/l)+Kn (2mg/l)
D. Roots induced on ½ MS + IAA (2mg/l)
E. Hardened Plantlet in small pot.

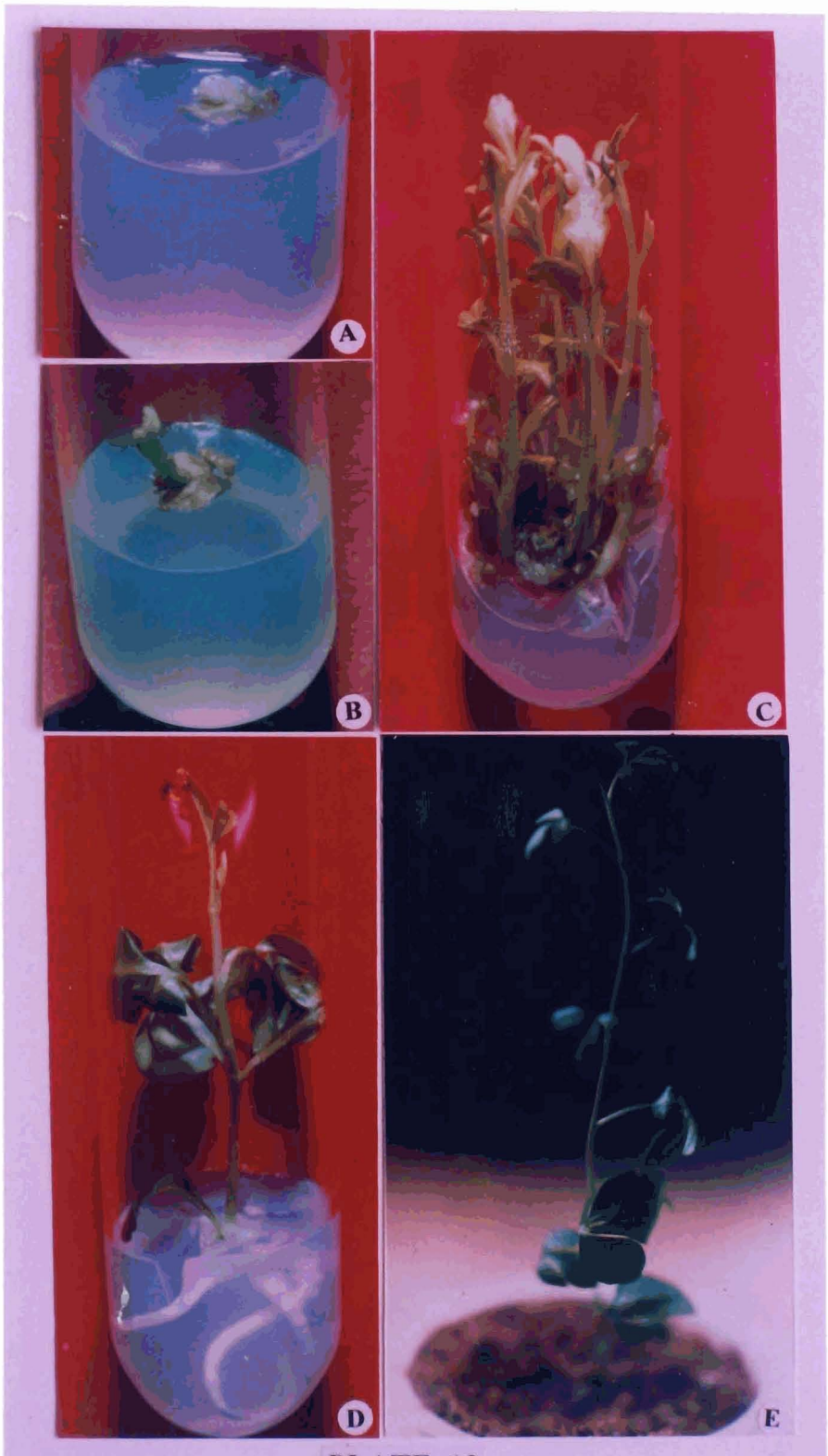


PLATE 13

PLATE 14. Micropropagation through indirect shoot regeneration of *H.keralensis* from internodes.

A. Callus induced on MS+IAA (0.5mg/l)
B. Callus induced on MA+ NAA (0.5mg/l)+BA (1mg/l)
C. Callus regeneration on MS +NAA (0.5mg/l) +BA (4mg/l)
D. Roots induced on ½ MS +IAA (0.5mg/l)
E. Hardened plantlet in small pot.

PLATE 14

PLATE 15. Indirect organogenesis from leaves of *N. alata*

A. Callus induced on MS+ IAA (0.5mg/l) + BA (2mg/l)
B. Callus induced on MS+NAA (0.5mg/l)+ BA (3mg/l)
C. Callus induced on MS +2,4-D (2mg/l)
D. Shoot regeneration from callus on MS+ NAA (0.5mg/l)+BA (3mg/l) (50 days old)
E. Roots induced on ½ MS+ IAA (0.5mg/l)

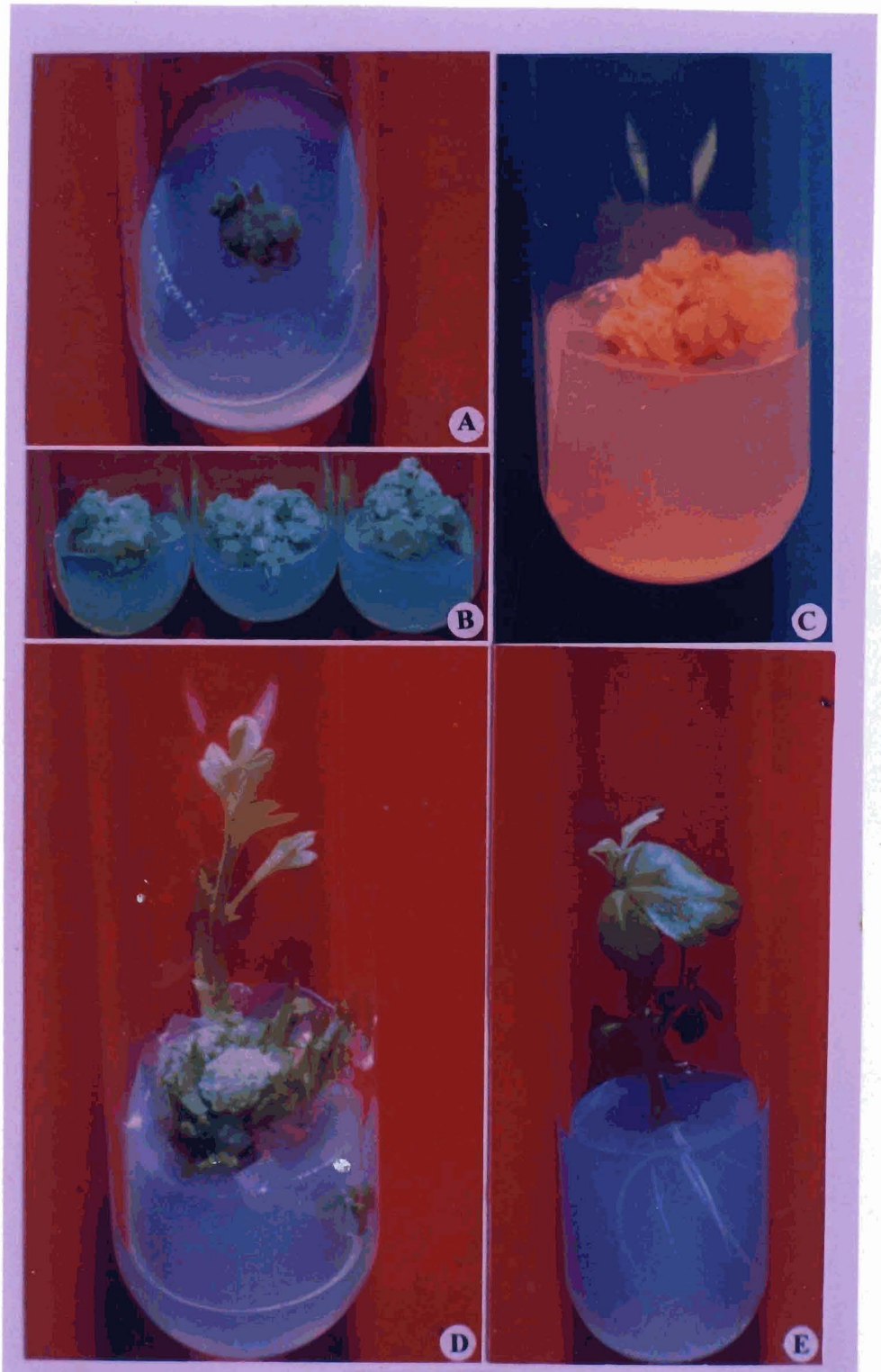


PLATE 15

PLATE 16. Indirect organogenesis from petals of *N. alata*.

A. Callus induced on MS + IAA (0.5 mg/l).
B. Callus induced on MS + IAA (0.5mg/l)+ BA (1mg/L) (20 days old)
C. Callus induced on MS + NAA (0.5 mg/l) + BA (1mg/l) (20 days)
D. Indirect shoot regeneration on MS + IAA (0.5 mg/l) + BA (2mg/l) (40 days).
E. Rooting on MS + IBA (1 mg/l)

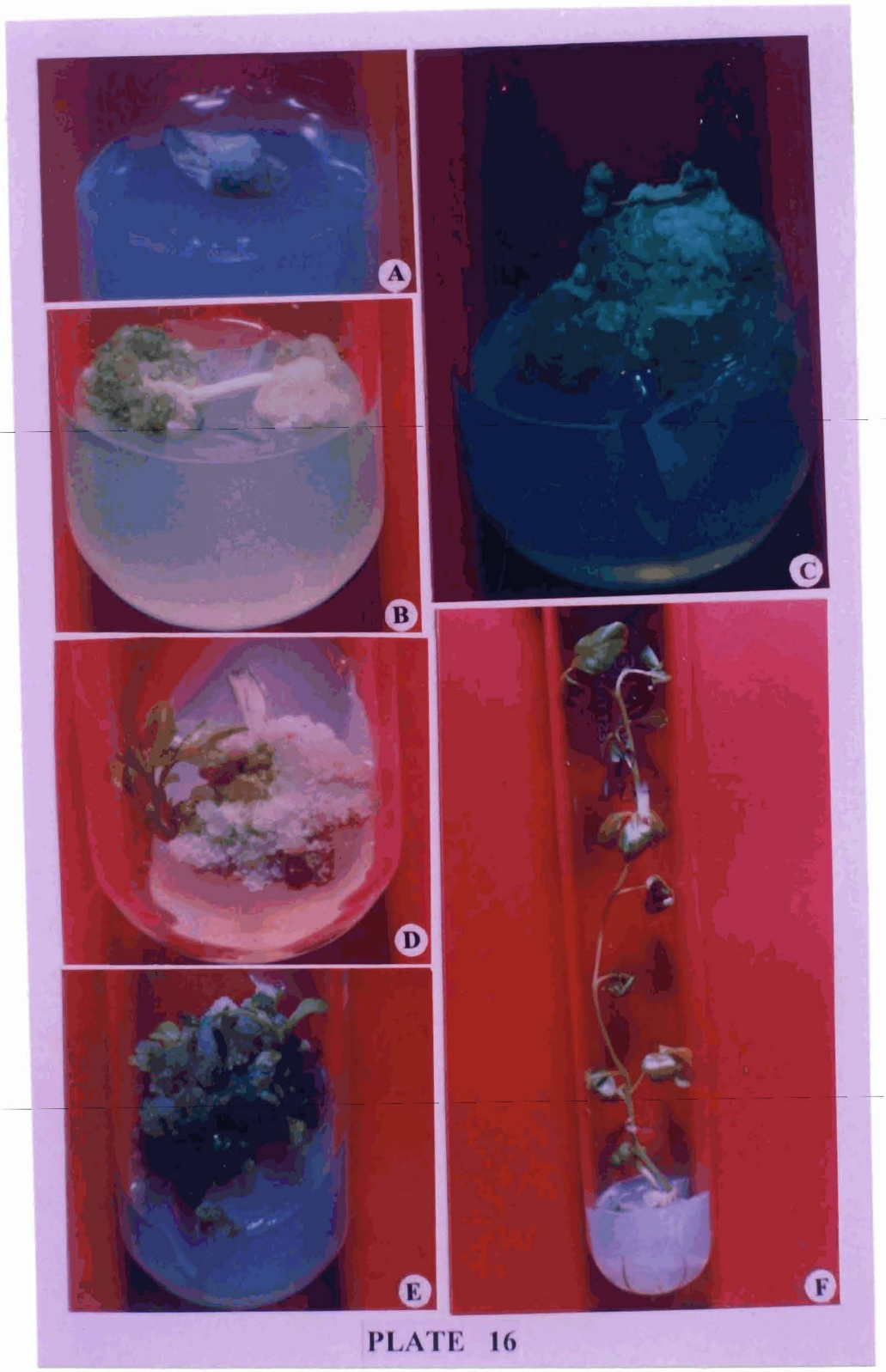


PLATE 16

PLATE 17. Microproparation through indirect shoot regeneration from roots of *N. alata.*

A. Callus induced on MS + IAA (1mg/l) (10 days).
B. Callus induced on MS + IAA (1mg/l) (25 days).
C. Indirect shoot regeneration on MS + NAA (0.5 mg/l) + BA (1 mg/l)
D. Indirect shoot regeneration on MS + NAA (0.5mg/l) + BA (4 mg/l)(40 days)
E. Roots induced on ½ MS + NAA (0.1 mg/l)
F. Hardened plantlet obtained through indirect shoot regeneration in small pot.

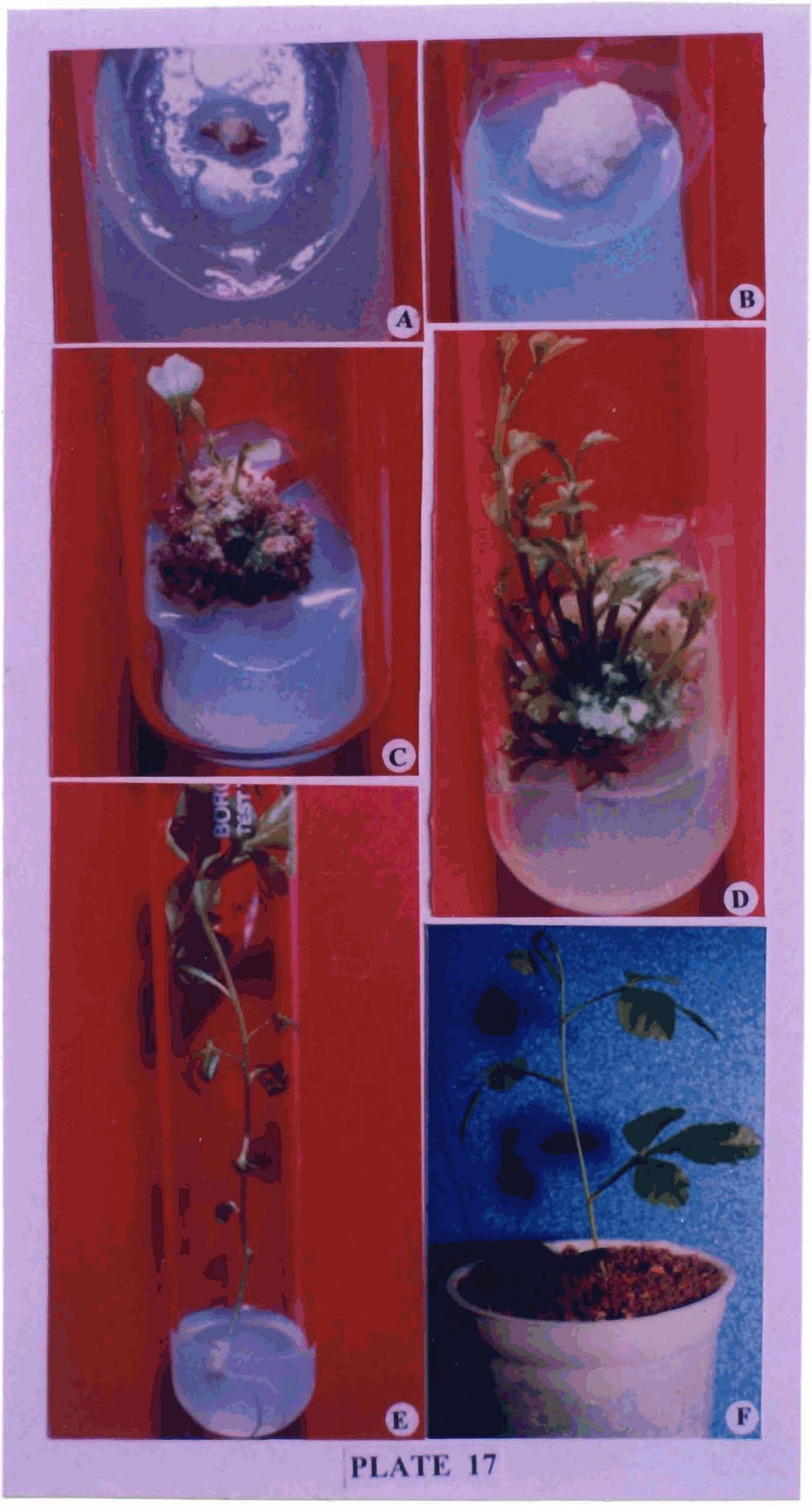


PLATE 17

PLATE 18. Plant regeneration via. Somatic embryogenesis of *N. alata.*

A. Induction of direct somatic embryos and embryogeneic callus from leaf on MS + 2,4-D (0.1 mg/l) (abaxial side in contact with the medium).
B. Induction of direct globular somatic embryos from leaf on MS + 2,4-D (0.1 mg/l) (adaxial side in contact with medium)
C. Embryogenic callus induced from internode on MS + 2,4-D (1 mg/l)
D. Globular embryos developed from embryogenic callus on MS basal medium.
E. Somatic embryos showing different stages of development.
F. Globular somatic embryos formed in suspension culture.
G. Heart shaped somatic embryos.
H. Embryo germinated plantlet.
I. Hardened plantlet in small pot.

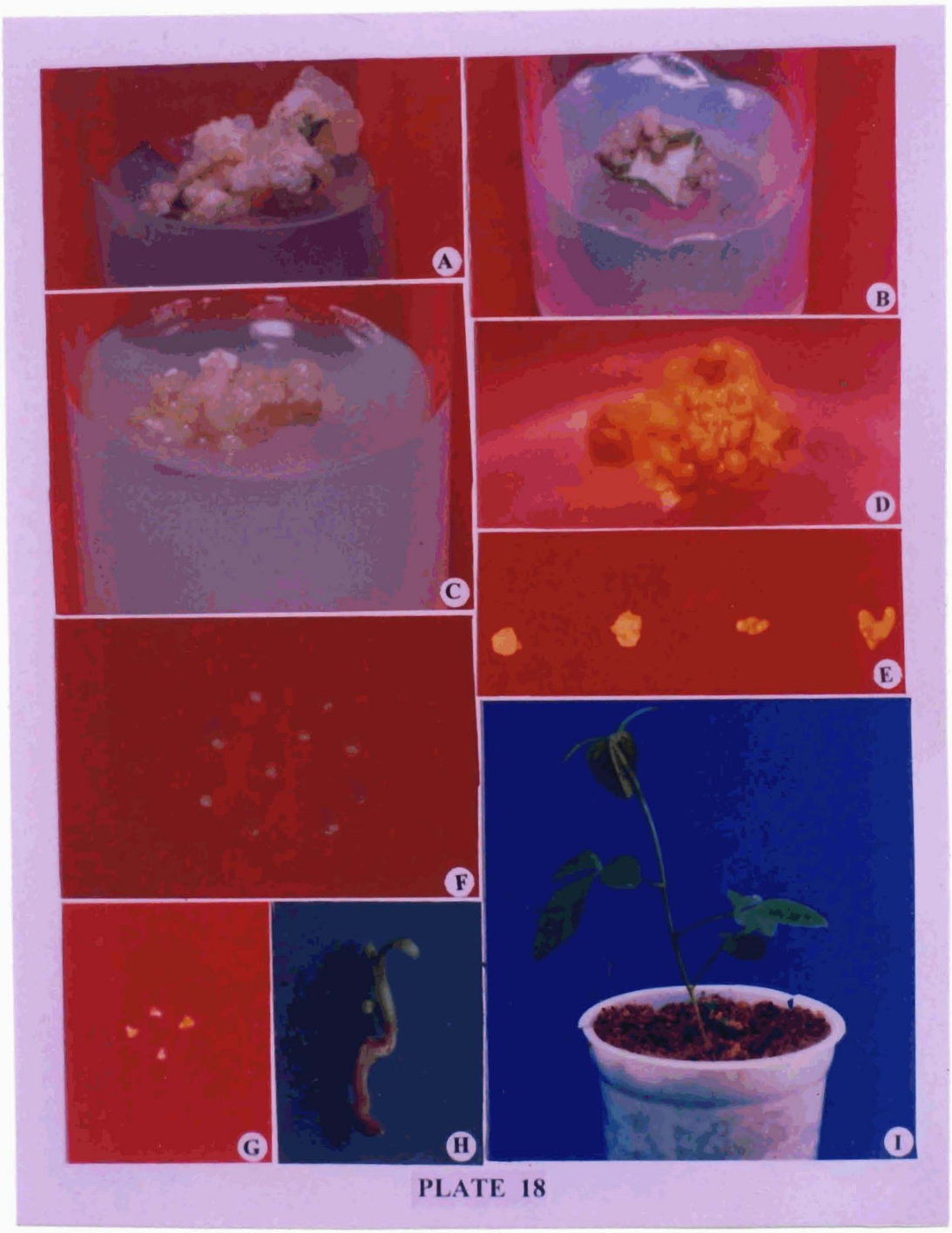


PLATE 18

PLATE 19. Encapsulation and germination of somatic embryos/ shoot buds of *N. alata.*

A. Encapsulated shoot buds.
B. Encapsulated somatic embryos
C. Germinating synseed.
D. Plantlet developed from synseed.
E. Hardened plantlet in small pot.

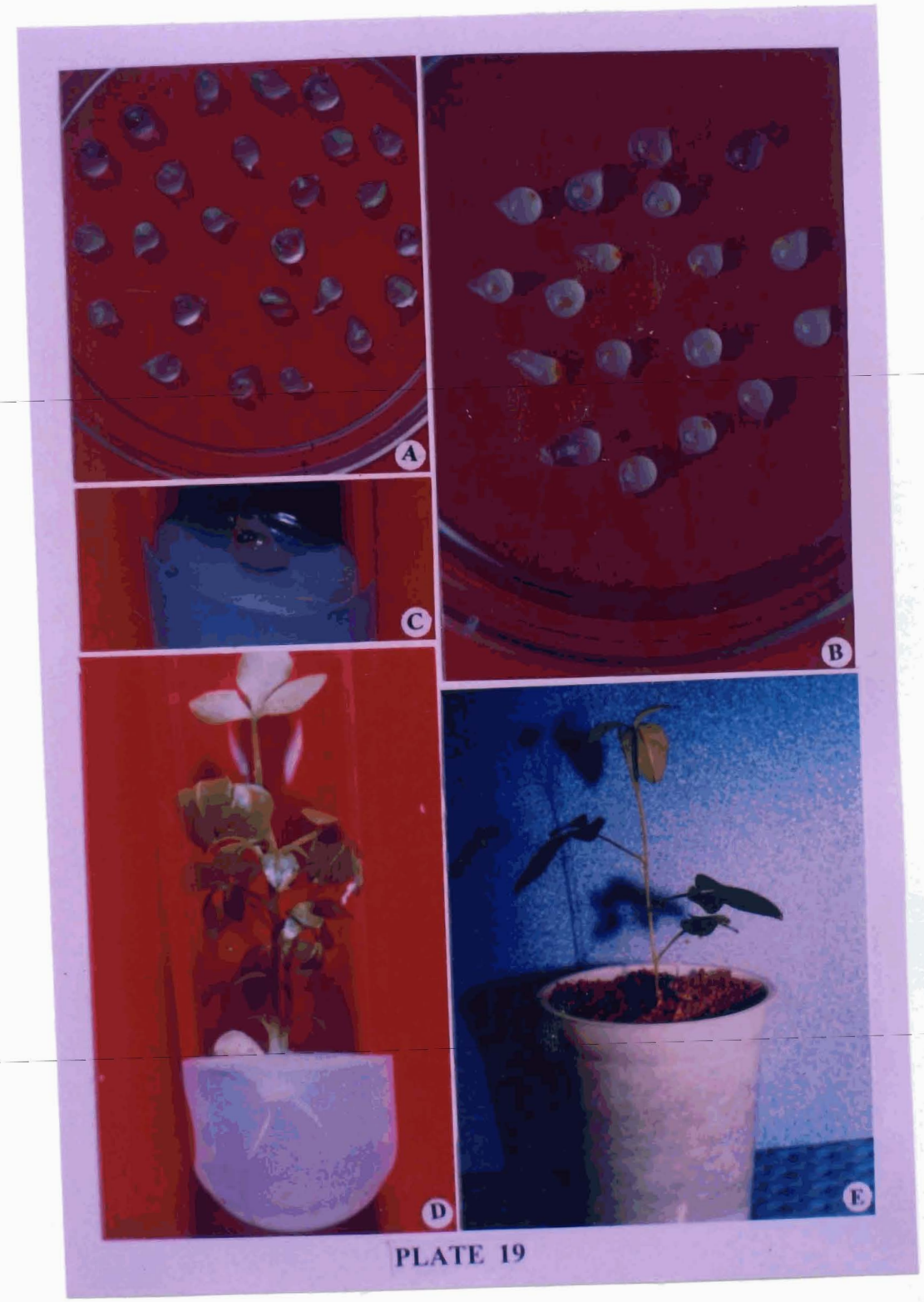


PLATE 19

PLATE 20. Steps in hardening and field transfer of *N. alata*.

A. Plantlet with well developed shoot and root system.

B. Plantlet under hardening

C. Hardened plant under field conditions in pot.

PLATE 20

PLATE 21. TLC analysis (for secondary metabolites) of roots from *in vitro* and field grown plants.

PLATE 21

DISCUSSION

Medicinal plants, the oldest source of pharmaceutically active compounds, still remains as an important source of useful compounds for therapeutic uses. Most of the medicinal plants used in the preparation of medicines have been collected from their natural habitats. Such collection practices have augmented the natural extinction process of these plants. Realizing the importance of conservation of important medicinal plants and extreme scarcity of phyto drugs faced by phyto based pharmaceutical industry, the present studies on two important medicinal plants viz. ***Heliotropium keralensis*** and ***Naregamia alata*** were undertaken as a model system in formulating effective protocols for mass propagation and conservation. The work was mainly concentrated on direct and indirect shoot regeneration, somatic embryogenesis and synthetic seed production of the above mentioned plant species. The important observations and results obtained are discussed here in the light of relevant recent literature.

HELIOTROPIUM KERALENSIS

Different explants of *H. keralensis* were subjected to surface sterilization for establishing various cultures. For the success of well established culture removal of external microorganisms from plant tissues is most important because, microorganisms (bacteria and fungi) present on plant surface are able

to grow readily on culture media which contain organic compounds such as sugars, amino acids and vitamins (George 1993). Selection of surface sterilizing agent, its concentration, treatment time etc depends on nature of explants, extent of surface microflora and sensitivity of the explant tissue to various sterilants and should be determined by trial and error methods. In the present studies 0.1% $HgCl_2$ treatment was found to be very effective for the surface sterilization of different explants of *H.keralensis*. The efficacy of 0.1% $HgCl_2$ solution in surface sterilization was reported earlier in many medicinal plants like *Panax ginseng* (Tang 2000), *Centella asiatica* (Tiwari *et al.*2000) *Cephaelis ipecacunha* (Rout *et al.*2001), *Vitex negundo* (Thiruvengadan and Jayabalan 2001), *Swainsona salsula* (Yang *et al.* 2001) and *Plumbago* species (Das and Rout 2002).

In the present studies on *H. keralensis,* subculturing of the tissue to fresh medium of the same composition was found to be very effective to overcome the problem of phenolic oxidation and subsequent death of tissues in culture. Efficacy of periodic transferring of explants to fresh medium of same composition to overcome the problem associated with phenolic oxidation was reported earlier in *Pisonia alba* (Jagadischandra *et al.,*1999) and *Punica granatum* (Naik *et al.,* 1999).

Addition of activated charcoal in the culture medium retarded growth of tissues in culture in the present studies on *H.keralensis*. According to Gallo-

Meagher and Green, (2002) this retardation of growth in culture might be due to the adsorption of plant growth regulators by activated charcoal. Weatherhead *et al.,(*1979) and Komalavalli and Rao (2000) also reported growth inhibiting activity of activated charcoal and other additives in culture.

DIRECT SHOOT REGENERATION

i) From shoot tip explants

Shoot tip culture is preferred for micropropagation to produce large number of genetically identical clones, due to low levels of somaclonal variation generated during the process (Bajaj and Dhankju, 1979; Grattapaglia and Machado 1990). Multiple shoot formation from shoot apices has been reported in many medicinal plants like *Phyllanthus amarus* (Bhattacharyya and Bhattacharya, 2001), *Lippia junelliana* (Juliani *et al.,*1999) and *Anthemis nobilis* (Echeverrigaray *et al.*, 2000a). Most of these reports suggest the requirement of an exogenous supply of cytokinins for direct shoot regeneration from shoot tip explants. Shoot multiplication as observed in the present studies on *Heliotropium keralensis* was also the functional activity of cytokinins. Of the two cytokinins (BA and Kn) tested in the present studies, BA was found as the single effective growth regulator. The simulative effect of BA over Kn in direct shoot regeneration was reported earlier in many medicinal plants like *Ocimum* spp (Pattnaik and Chand, 1996) *Vitex negundo* (Sahoo and Chand, 1998b) *Lippia junelliana* (Juliani *et al.*, 1999) and *Bacopa monniera* (Tiwari *et al.,*

2001). However, a combination of BA +Kn in the culture medium was found to be most effective for direct shoot regeneration in the present studies on *H. keralensis*. Similar results showing the efficacy of BA+Kn combination in direct shoot regeneration were reported in many medicinal plants like *Gymnema elegans* (Komalavalli and Rao,1997), *Kaempferia galanga* (Vincent *et al.*,1998), *Pisonia alba* (Jagadishchandra *et al.,* 1999) and *Gymnema sylvestre* (Komalavalli and Rao 2000). This enhancement of BA+Kn combination in direct shoot regeneration might be due to the synergistic interaction of BA and Kn. A decline in shoot multiplication was observed with an increase in the concentrations of cytokinins higher than the optimal level (3.0mg/l BA +3.0 mg/l Kn). This result obtained in the present studies on *H.keralensis* was in conformity with the reports of Kukreja *et al.* (1990), Sen and Sharma (1991), Vincent *et al.,*(1992), Sahoo and Chand (1998b), Juliani *et al.,(*1999) and Bhattacharyya and Bhattacharya (2001).

In the present studies on *H. keralensis* addition of auxins along with cytokinins in the medium was less effective for the shoot multiplication when compared to the medium containing cytokinins alone (BA+Kn), similar results were reported in *Zingiber officinale* (Hosoki and Sagawa 1977), *Picrorrhiza kurroa* (Upadyay *et al.,*1989), and *Wasabia japonica* (Hosokawa *et al.,* 1999). However Komalavalli and Rao (2000), Tiwari *et al.,* (2000) and Casado *et al.,*(2002) reported synergistic auxin cytokinin interaction in shoot multiplication

of *Gymnema sylvestre, Centella asiatica* and *Santolina canescens* respectively. Promotive effect of auxins in shoot elongation, as observed in the present studies was reported earlier in *Petasites hybridus* (Wildi et al., 1998) and *Hybanthus enneaspermus* (Prakash *et al.*, 1999).

ii) From Nodal explant

Direct multiple shoot regeneration from nodal explants has been reported in many medicinal plants (Sahoo and Chand, 1998b, Nobre *et al.*, 2000;Tiwari *et al.,* 2000; Casado *et al.,* 2002). Among the different factors, exogenous cytokinin concentration was the most important one, which affected shoot multiplication from nodal explants in most of the reports. These observations were in conformity with the results of present experiments on *H.keralensis.* In the present studies BA was highly effective than Kn for shoot multiplication from nodal explants of *H.keralensis.* This result was in concurrence with the reports of Girija *et al.*,(1999) and Tiwari *et al.,(*2001). The ineffectiveness of Kn in shoot multiplication might be due to the specificity of the explant to certain growth regulators. Synergistic interaction of BA and Kn has been reported in the shoot multiplication of *Kaempferia galanga* (Vincent *et al.,* 1992a), *Alpinia calcarata (* Agretious *et al.,* 1996) *Vitex negundo* (Sahoo and Chand 1998b) and *Eleocarpus robustus* (Roy *et al.,* 1998). These reports were in corroborative with the results obtained in the present experiments on *H.kerlensis.* Vincent (2001), Hosoki and Sawaga(1977) etc., reported a

decrease in shoot multiplication when auxins were added in the multiplication medium. These reports were in consensus with the results of present experiments on *H.keralensis*. However, the addition of auxins in the multiplication medium promoted elongation of shoots of *H.keralensis* in the present experiments. Such enhancement of auxins in shoot elongation was reported earlier in *Eucalyptus grandis* (Luis et al., 1999) and *Coleus forskohlii* (Reddy et al., 2001) .

iii) From root explants

According to George (1993) unlike other cultures, root cultures exhibit a high degree of genetic stability. Hence direct shoot regeneration from root explants is highly desirable for the clonal multiplication of medicinal plants. Direct shoot regeneration and subsequent micropropagation from root explants has been reported in many plants like *Piper colubrinum* (Kelker and Krishnamurthy, 1998), *Swertia chirata* (Wawrosch *et al.,* 1999), and *Azardiracta indica*(Salvi *et al.*, 2001).

Wawrosch *et al.,*(1999) reported that in comparison with Kn, BA was highly effective for direct shoot induction from root explants of *Swertia chirata*. This report was in consonant with the results of present experiments on *H.keralensis*. A combination of BA and Kn was most effective for the direct shoot regeneration from root explants of *H.keralensis*. Similar results showing enhancement of shoot multiplication in BA+Kn combination was reported in

many plants like *Anacardium occidentale* (Das *et al.*,1996) and *Gymnema elegans* (Komalavalli and Rao, 1997).

Addition of auxins in the shoot multiplication medium decreased number shoots/culture. However, the addition promoted shoot elongation. Similar results were reported in *Picrorrhiza kurroa* (Upadhyay *et al.*, 1989) and *Wasabia japonica* (Hosokawa *et al.*, 1999). The promotive effect of auxin in shoot elongation as observed in the present studies was reported earlier in *Eucalyptus grandis* (Luis *et al.*, 1999) and *Coleus forskohlii* (Reddy *et al.*, 2001).

iv) Effect of media

Media formulations have extensive influence on the growth and proliferation of tissues cultured *in vitro*, hence one of the most important factors affecting establishment of cultures is the composition of nutrient medium, and MS medium is the most widely used medium for the tissue culture of higher plants (Mc Cown and Selimer 1987; George 1993). Echeverrigaray *et al.*(2000a) reported that MS medium was the most effective one followed by B_5 and White's for the multiple shoot production from the apical or axillary buds of *Anthemis nobilis*. The results obtained in the present experiment on *H.keralensis* were in corroborative with results on *Anthemis nobilis*. Efficacy of MS medium over other media formulations has also been reported in many medicinal plants such as *Gymnema sylvestre* (Komalavalli and Rao 2000), *Vitex*

negundo (Sahoo and Chand 1998b) and *Swertia chirata* (Wawrosch *et al.,* 1999). The efficacy of MS medium for shoot multiplication and growth indicates high salt requirements for the growth and multiplication of *H.keralensis*.

v) Effect of sucrose concentration

In the present studies an exogenous supply of 3% sucrose was found most effective for direct shoot regeneration/multiplication from shoot tip explants of *H.keralensis*. Three percent as the optimal concentration of sucrose has been reported in many medicinal plants like *Gymnema elegans* (Komalavalli and Rao 1997), *Vitex negundo* (Sahoo and Chand 1998b), *Crossandra infundibuliformis* (Girija *et al.*,1999), and *Gymnema sylvestre* (Komalavalli and Rao 2000). Morphogenesis is an energy requiring process and sucrose in the medium mainly acts as an energy source ((Murashige and Nakano 1968; George 1996). Hence in the present studies, decrease in shoot multiplication observed at lower sucrose concentrations ($\leq$ 2%) might be due to insufficiency of sucrose for the production of required energy for morphogenesis and inhibitory effect of higher concentration of sucrose on shoot multiplication might be due to the inability of explants to utilize sucrose at higher concentrations or due to the effect of sucrose on osmotic potential of cells.

vi) Subculturing

There was no significant increase in shoot multiplication when *in vitro* raised shoot tips were cultured, compared with the shoot tips from field grown plants. This result obtained in the present experiment on *H. keralensis* was in corroborative with the results obtained in the experiments on *Plumbago rosea* (Harikrishnan 1999). Similar response of *in vitro* and *in vivo* shoot tips in culture indicates that there was no significant differences in endogenous growth regulators of *in vitro* and *in vivo* shoot tips of *H. keralensis.*

INDIRECT SHOOT REGENERATION.

Callus can undergo redifferentiation into a variety of organs under appropriate culture media. Organogenesis from somatic cells or tissues is conceived to be under the control of phytohormones (Skoog and Miller 1957).

It has been reported that shoot organogenesis from callus cultures i.e. indirect shoot regeneration can be used as an effective method for the multiplication of medicinal plants (Suryanarayan and Pai 1998; Reddy *et al.*, 2001; Koroch *et al.,* 2002).

Callus induction which is an essential pre-requisite for indirect shoot regeneration, involves initiation of cell division continued proliferation of cells and subsequent structural and physiological differentiation (Gresshoff 1978). In the present studies on *H.keralensis,* nature and proliferation of the induced calli

varied with the growth regulators supplemented in the media. This observation was in corroborative with the reports of Prakash *et al.*,(1999), Lin *et al.*,(2000) and Koroch *et al.,* (2002). Effectiveness of auxin - cytokinin interaction for callus induction and proliferation was reported in *Echinaceae purpurea* by Koroch *et al.,* (2002). This report was in consonant with the results obtained in the present experiments on *H. keralensis*. Similar results has also been reported in many other medicinal plants like *Hybanthus enneaspermus* (Prakash *et al.,* 1999) *Solanum nigrum* (Shahzad *et al.*, 1999), and *Vitex negundo* (Thiruvengadan and Jayabalan 2001).

The differences in cultural requirements exist among different explants collected from the same plant may be attributed to the various levels of endogenous plant growth regulators present in the explant (Ghosh and Sen 1994). The differential response in callus formation observed from different explants (nodal explants produced highest amount of calli) in the present studies on *H. keralensis* might be due to the difference in the amount of endogenous growth regulators in the explants, as reported in *Echinaceae purpurea* (Choffe *et al.,* 2000 ; Koroch *et al.,* 2002)

Callus initiated on one medium needs to be transferred to another with a different composition of growth regulators (a regeneration medium), for shoot initiation to occur in most plants (George 1993). In the present experiments on *H. keralensis* also a separate regeneration medium was found

to be necessary for the regeneration of calli. Development of shoots from callus involve a delicate balance of auxin - cytokinin ratio in nutrient medium (Narayanaswami 1994). Prakash *et al.,* (1999); Thiruvengadan and Jayabalan (2001); Reddy *et al.,* (2001) and Koroch *et al.,* (2002) reported effectiveness of auxin- cytokinin combination in callus regeneration of many medicinal plants. These reports were in consonant with the results of present experiments on *H. keralensis.*

In the present studies on *H. keralensis,* brown coloured calli failed to regenerate shoots. This failure in shoot regeneration from brown coloured calli or might be due to the presence of inhibitory substances (oxidised phenolics) in the calli. Non-regenerative nature of some calli was reported earlier in the medicinal plant *Hybanthus enneaspermus* (Prakash *et al.,* 1999).

MS medium containing BA was effective in inducing multiple shoots from callus of *H. keralensis.* Superior effect of BA over Kn in callus regeneration as observed in the present study was reported earlier in *Alpinia calcarata.* (Martin and Hariharan 1999) and *Kaempferia galanga* (Vincent 2001).

SOMATIC EMBRYOGENESIS.

Somatic embryogenesis and organogenesis are rapidly becoming acceptable techniques for the clonal propagation of superior plant species. (Gary and Brent 1986; Choi *et al.,* 1998b). Somatic embryogenesis depends on

the concentration of auxins and cytokinins used during culture (Korac and Neskovic 1999; Rout *et al.,* 2000). In the present studies explants and concentration of growth regulators significantly influenced somatic embryogenesis of *H. keralensis*.

In the present study on *H. keralensis,* embryogenic calli were developed on MS medium supplemented with a combinations of an auxin (IAA) and cytokinin (2iP). The synergistic effect of auxin - cytokinin combination in the induction of embryogenic calli has been reported in many medicinal plants like *Thevetia peruviana* (Kumar 1992); *Lithospermun erythrorhizon* (Ju *et al.,* 1997); *Eschscholzia californica* (Park and Facchini 1999); *Cephaelis ipecacuanha* (Rout *et al.*, 2001); *Simmondsia chinenesis* (Hamama *et al.,* 2001); *Gymnema sylvestre* (Kumar *et al.,* 2002). Kumar *et al.,(* 2002) and Gallo-Meagher and Green (2002) reported the efficiency of 2iP in the induction of somatic embryogenesis in *Calliandra tweedi* and *Seriona repens* respectively. In the present studies on *H.keralensis* also 2iP was found to be very effective for somatic embryogenesis. According to Trigiano *et al.,* (1988) 2,4-D is the most widely used auxin for somatic embryogenesis. However 2,4-D was found to be ineffective for the somatic embryogenesis of *H. keralensis*. Kumar *et al.,* (2002) also reported the ineffectiveness of 2,4-D for the induction of somatic embryogenesis in *Calliandra tweedi.*

In *Vicia narbodensis* somatic embryos were formed when the embryogenic calli cultured on media containing auxins and cytokinins were transferred to media with low concentration or without growth regulators (Albrecht and Kohlenbach 1989). This report was in corroborative with the result of the present experiment on *H. keralensis.* Kumar (1992); Rout *et al.,* (2001) and Kumar *et al.,* (2002) reported maturation and germination of somatic embryos on MS basal medium. These reports were in consensus with the results of present experiment on *H. keralensis.* However, higher frequency of germination of somatic embryos of *H. keralensis* was observed on MS medium supplemented with Kn. Park and Facchini (1999), Hammama *et al.,* (2001) and Kumar *et al.,* (2002) also reported enhancement in the germination of somatic embryos in the presence of cytokinins in the medium.

In the present study on *H. keralensis*, 4% was found to be the optimal concentration of sucrose in the embryogenic callus induction medium for the production of highest number of embryos/g calli on subsequent cultures. Efficacy of optimal concentration of sucrose for somatic embryogenesis has also been reported in many plants like *Hemidesmus indicum* (Sarasan *et al.*, 1994); *Panax ginseng* (Asaka *et al.*, 1994) and *Azadiracta indica* (Su *et al.,* 1997) .

SYNSEEDS

The synthetic seed technology is designed to combine the advantages of clonal propagation with those of seed propagation (Ara *et al.*, 2000).

Encapsulation of somatic embryos or shoot buds and subsequent retrieval of complete plantlets have been reported in many species (Redenbaugh *et al.*, 1986; Bapat *et al.*, 1987; Ghosh and Sen 1994; George and Eapen 1995; Pattnaik *et al.*, 1995; Vincent 2001). In the present studies the encapsulation of somatic embryos and shoot buds in 3% alginate was found to be optimal for the conversion of encapsulated propagules. Similar reports of highest plantlet regeneration frequency with an optimal level of 3% sodium alginate were reported earlier in *Solanum melongena* (Rao and Singh 1991), *Eleusine coracana* (George and Eapen 1995) *Camellia japonica* (Janeiro *et al.*, 1997) and *Kaempferia galanga* (Vincent 2001).

Germination of encapsulated somatic embryos on MS basal medium as observed in the present studies was in consonant with the reports on *Asparagus cooperi* (Ghosh and Sen 1994); *Elettaria cardamomum* (Ganapathy *et al.*, 1994); *Alpinia galanga* and *Kampferia rotunda* (Anand 2000) and *Kampferia galanga* (Vincent 2001). The exogenous cytokinin requirement for the development of encapsulated shoot buds of *H. keralensis* might be due to the presence of sub-optimal level of cytokinins for the development in the encapsulated shoot buds.

Reed *et al.*, (1998) reported that low temperature storage at 4°C was effective for long-term maintenance (more than 2 years) of viability of

encapsulated propagules of pear. In the present studies on *H. keralensis* also synseeds stored at 4°C retained viability.

ROOTING *IN VITRO*

Success of micropropagation mainly depends upon *in vitro* root induction, hence, rooting of *in vitro* shoots is one of the most critical steps in micropropagation of plants. Most of the reports on *in vitro* rooting envisage a separate rooting medium for the rooting of *in vitro* shoots (Echeverrigaray *et al.*, 2000a; Dhar *et al.,* 2000; Tiwari *et al.,* 2001; Fracaro and Echeverrigaray 2001; Das and Rout 2002).

In the present studies on *H.keralensis* highest number of roots were formed when the shoots were cultured on MS medium supplemented with 0.5mg/l IBA. Efficacy of IBA for the rooting of *in vitro* shoots has been reported in many medicinal plants like *Vitex negundo* (Sahoo and Chand1998b); *Anthemis nobilis* (Echeverrigaray *et al.,* 2000a); *Pittosporum napaulensis* (Dhar *et al.*, 2000); *Bacopa monnira* (Tiwari *et al.,* 2001); *Cunila galioides* (Fracaro and Echeverrigaray 2001) and *Plumbago sps* (Das and Rout 2002). Callus formation and subsequent reduction in root number at higher concentrations of IBA observed in the present studies on *H. keralensis* were in agreement with the reports on *Plumbago sps.* (Das and Rout 2002) and *Pittosporum napaulensis* (Dhar *et al.*, 2000).

ACCLIMATIZATION AND FIELD TRANSFER

Survival of micropropagated plants depends upon their ability to withstand water loss and carryout photosynthesis. This can be greatly enhanced by gradual acclimatization and hardening (George 1996). In the present, studies plantlets developed by direct or indirect shoot organogenesis showed 80% survival while that by somatic embryogenesis showed only 65 % survival. This variation in survival percentage might be due to the difference in the age of plant, time of transfer, number of roots present at the time of transfer etc.

SECONDARY METABOLITE ANALYSIS

Plant cell cultures also produced secondary metabolites not known to occur in the plant intact. (Furmanowa *et al.*, 1999; Yamamoto *et al.*, 1999). Comparative analysis of both *in vitro* and *in vivo* roots showed differences in compounds in *H. keralensis.* Secondary metabolite analysis showed 5 compounds in the extracts of *in vitro* roots and 3 compounds in the extracts of *in vivo* roots. This increase in the types of secondary metabolites in the *in vitro* roots might be due to the production of new compounds in the cultures or some secondary metabolites present in sub-detectable quantity in *in vivo* plants might have synthesized in large quantities in the cultured tissues due to difference in growth conditions.

BIOCHEMICAL STUDIES

Accumulation of metabolites like sugars, proteins, and phenolics was observed in the regenerating callus cultures of *H. keralensis*. Similar results showing accumulation of metabolites in regenerating calli was reported earlier in many plants (Kavikishor, 1987; Fett *et al*, 1992 Yadav *et al*, 1995, Puroht *et al*, 1996, Choi and Kim, 1997). According to Thorpe and Murashige (1970) accumulation of more metabolites in regenerating callus suggests that the process of morphogenesis requires energy and reducing power. Rawal and Mehta (1982) reported that high peroxidase activity was associated with shoot formation in callus cultures of tobacco. This observation was in consonant with the results of present experiments on *H. keralensis*. Choi and Kim (1997) and Omokolo *et al.* (1997) also reported an increase in peroxidase activity during morphogenesis.

In the present studies on *H.keralensis* regenerating and primary calli showed difference in polypeptide patterns. Such differences in polypeptide patterns in different types of calli were reported earlier in *Camelli japonica* (Pedroso and Pais 1995).

NAREGAMIA ALATA

Last few years have witnessed much progress in micropropagation and germplasm conservation of medicinal/ aromatic species. The major objectives of the present investigations on *Naregamia alata* also was to develop an efficient and reproducible protocol for mass propagation and germplasm conservation of this important medicinal plant through tissue culture techniques.

Plants grown in the field or in glass house would be affected with microorganisms and surface contaminants. Hence, the excised plant parts should be surface sterilized or disinfected by chemical means (Narayanaswamy 1994; Murashige 1974). In the present studies on *N. alata* 0.1 % $HgCl_2$ solution was found as the most effective surface sterilant. John *et al.,* (1997) and Daniel *et al.,*(1999) also reported efficacy of 0.1 % $HgCl_2$ solution in removing surface contaminants of *N. alata.* Roots and other underground plant parts are highly contaminated hence a longer duration of surface sterilant treatment was needed for disinfecting the underground structures (George 1993). In the present studies on *N. alata* also a longer period of surface sterilant treatment was needed for disinfecting the root explants, compared to the explants of shoot system.

DIRECT SHOOT REGENERATION

i) From shoot tip explants.

Direct regeneration of plants from shoot tips are more desirable than indirect (Battacharyya and Bhattacharya 2001; Echeverrigaray *et al.,* 2000). In the present experiments on *N. alata* multiple shoots were developed from shoot tips on MS medium supplemented with BA singly or in combination with auxins (NAA + BA and IAA +BA). The results indicate that BA as the most important constituent for the induction of multiple shoots. Daniel *et al.*, (1999) and John *et al.,* (1997) also reported that BA was inevitable for the induction of multiple shoots of *N. alata.* Efficacy of BA as the most important constituent in direct shoot regeneration from shoot tip explants had also been reported in medicinal plants like *Anthemis nobilis* (Echeverrigaray *et al.,* 2000a) and *Phyllanthus amarus* (Bhattacharyya and Bhattacharya 2001). Kinetin was inefficient to induce multiple shoots from shoot tip explant, this result indicates growth regulator specificity of the species.

Murashige and Skoog's medium supplemented with a combination of auxin (NAA) and cytokinin (BA) the best response was elicited. The efficacy of auxin - cytokinin combination in shoot multiplication has been reported in many medicinal plants like *Iphigenia indica* (Mukhopadhyay *et al.,* 2002); *Kaempferia galanga* (Shirin *et al.,* 2000) and *Plumbago* species (Das and Rout 2002) . The

enhancement of shoot multiplication in auxin- cytokinin combination might be due to the synergistic interaction of the two growth regulators.

ii) From node.

Nodal culture is highly advantageous for large-scale clonal propogation as there is minimum chances for mutation and is the simplest of all known micropropagation techniques (George 1993). Nodal cultures have been widely used for the clonal propagation of many medicinal plants (Sahoo and Chand 1998; Komalavalli and Rao 2000; Nobre *et al.,* 2000; Tiwari *et al.,* 2000 and Dias *et al.,* 2002).

In the present studies on *N. alata* multiple shoots were developed from nodal explants on MS medium supplemented with BA, NAA +BA and IAA + BA. Hence, the results indicate that BA was inevitable for the induction of multiple shoots from nodal explants of *N. alata.* Efficacy of BA in multiple shoot induction from nodal explant has been reported in many medicinal plants like *Gymnema sylvestre* (Komalavalli and Rao 2000); *Centella asiatica* (Tiwari *et al.,* 2000;).*Lavendula viridis* (Dias *et al.,* 2002), *Bacopa monniera* (Tiwari *et al.,* 2001); *Vitex negundo* (Sahoo and Chand 1998b), *Viburnum tinus* (Nobre *et al.,* 2000) and *Santolina canescens* (Casado *et al.,* 2002).

A combination of auxin (NAA) and cytokinin (BA) in MS medium produced highest shoot proliferation from nodal explants of *N. alata.* Synergistic

interaction of the two growth regulators (NAA and BA) in shoot multiplication from nodal explants has been reported in many medicinal plants like *Vitex negundo*, *Tridax procumbens* (Sahoo and Chand 1998a,b); *Centella asiatica* (Tiwari *et al.*, 2000) and *Santolina canescens* (Casado *et al.*, 2002).

iii) From leaves

Protocols for micropropagation from leaf explants are highly desirable as genetic manipulation can be done easily and large number of explants can be obtained from a single plant (Tiwari *et al.,* 2001).

In the present studies on *N. alata* adventitious shoots were formed from leaf explants on MS medium supplemented with BA or a combination of BA and Kn. John *et al.*, (1997) also reported adventitious shoot formation from leaf explant on the same species on MS medium containing BA. Kinetin was not effective in inducing multiple shoots from the explants when supplemented singly in culture medium. Efficacy of BA over Kn was reported earlier in many medicinal plants like *Bacopa monniea*, (Tiwari *et al.,* 2001) and *Plumbago* species, (Das and Rout 2002).

At higher concentrations of BA ($\geq$ 0,5 mg/l) along with the formation of adventitious shoots little amount of calli were also formed from leaf explants of *N. alata*. However, John *et al.*, (1997) reported adventitious shoot formation without any visible callusing from leaf explants of the same species on MS

medium supplemented with a combination of BA and gibberellic acid. This difference in morphogenic response of the same species might be due the different experimental set up used.

In the present studies MS medium supplemented with a combination of BA and Kn was most effective for the production of adventitious shoots from leaf explant. The synergistic effect of two growth regulators as observed in the present study was reported earlier in many plants like *Pisonia alba* (Jagadishchandra *et al.,* 1999) and *Canavalis virosa* (Kathiravan and Ignachimuthu 1999). However, in *Gymnema elegans,* BA + Kn combination was not effective to induce shoot multiplication (Komalavalli and Rao 1997)

iv) Effect of Sucrose

In the present studies on *N. alata* sucrose at 3% was found to be the optimal level for shoot multiplication. Sucrose at 3% as the optimal concentration was reported in many medicinal plants like *Vitex negundo* (Sahoo and Chand 1998b), *Crossandra infundibuliformis* (Girija *et al.*,1999) and *Gymnena sylvestre* (Komalavalli and Rao 2000). Morphogenesis is an energy requiring process and sucrose in the medium mainly acts as an energy source (Murashige and Nakano 1968; George 1996). Hence, in the present studies a decreased shoot multiplication rate observed at lower concentrations of sucrose might be due to the insufficiency of sucrose for the production of required energy for morphogenesis. Inhibitory effect of higher concentrations of sucrose

in shoot multiplication might be due to the inability of the explants to utilize sucrose at higher concentrations or due to the effect of sucrose on osmotic potential of cells.

v) Effect of nutrient media

Success of micropropagation is greatly influenced by the constituents of the culture media used (George 1993). Echeverrigaray *et al.*,(2000) reported that MS was superior to other media like B5 and White's in shoot multiplication from apical/ axillary buds of *Anthemis nobilis* . This report was in agreement with the results of present experiments on *N. alata*.

Efficacy of MS medium over other media formulations was also reported in many medicinal plants like *Swertia chirata* (Wawrosch et al., 1999), ,*Vitex negundo* (Sahoo and Chand 1998b) and *Gymnena sylvestre* (Komalavalli and Rao 2000).

The efficacy of MS medium over other media in shoot multiplication, observed in the present studies on *N. alata* indicates high salt requirement for the shoot multiplication of the species.

INDIRECT SHOOT REGENERATION

Indirect organogenesis can be used as an effective method for micropropagation of important medicinal plants (Suryanarayanan and Pai 1998; Reddy *et al.*, 2001; Koroch *et al.*, 2002).

Among the various auxins (NAA, IAA, 2,4-D) tested, 2,4-D was found as the most effective auxin for the callus induction from various explants of *N. alata*. Efficacy of 2,4-D in callus induction was reported in many plants (Muccirelli *et al.*, 1993; Manickavasagam and Ganapathi 1998; Coker and Comper 2000).

Auxin (IAA or NAA) – cytokinin (BA) combination in MS medium was found to be effective for induction of calli from various explants of *N. alata* the combination was also effective for the regeneration of shoots from the calli. The result indicates synergistic effect of auxin-cytokinin interaction in callus induction as well as regeneraton of *N. alata*.

John *et al.*, (1997) and Daniel *et al., (*1999) on *N. alata* reported the efficacy of BA in shoot induction /regeneration. This was in consensus with the results of present experiments on the same species.

SOMATIC EMBRYOGENESIS

Somatic embryogenesis is a simple and very efficient method for regenerating large number of intact plants from tissue culture (Zimmermann 1993). According to Sharp *et al.*, (1982) somatic embryogenesis may be direct (without callusing) or indirect (through callus phase).

i) Direct somatic embryogenesis

Direct somatic embryogenesis from leaf explants has been reported in many plants like *Camellia japonica* (Pedroso and Pais 1995), tea (Kato 1996), neem (Murthy and Sexena 1998) and *Dianthus* (Yantcheva *et al.,* 1998). In the present studies on *N. alata,* somatic embryos were established directly from leaf explants when cultured on MS medium supplemented with low concentration (0.1mg/l) of 2,4-D. Formation of somatic embryos in a medium containing low concentration/no growth regulators was reported earlier in many plants. (Albrecht and Kohlenbach 1989; Kim *et al.,* 2000; Choi *et al.*, 2002; Tawfik and Noga 2002).

In the present studies on *N. alata* abaxial and adaxial surfaces of the leaf explants showed slight difference in the morphogenic response in culture. Difference in morphogenic response of abaxial and adaxial leaf surfaces was reported in plants like *Bryophyllum diagremontianum* (Bigot 1976) and *Vanda* species (Tanaka *et al.,* 1975). The difference in morphogenic response showed by the two sides of the same explant might be due to the difference in the level of endogenous growth regulators.

ii)Indirect embryogenesis

Embryogenic calli of *N. alata* were formed when the explants were cultured on MS medium supplemented with 2,4-D. Similar results showing

efficacy of 2,4-D in the induction of embryogenic calli has been reported in many medicinal plants (Cushman *et al.*, 2000; Kim *et al.*2000; Tang 2000Choi *et al.*, 2002)

In the present studies on *N. alata* leaf explants produced calli with highest embryogenic potential compared to any other explant cultured. The difference in embryogenic potential of various explants might be due to the difference in endogenous growth regulator levels in the explant sources or different tissue sensitivities to the exogenous growth regulators.

Four percentage sucrose in the medium produced calli with highest embryogenic potential from leaf explants of *N. alata*. Efficacy of optimal sucrose concentration in inducing the pathway that leads to somatic embryogenesis was reported earlier in many plants (Sarasan *et al.,* 1994; Asaka *et al.*, 1994, Su *et al.,* 1997).

In the present studies on *N. alata,* suspension cultures yielded more number of somatic embryos (10/g calli) than semisolid culture (8/g calli). Efficacy of suspension cultures in somatic embryogenesis as observed in the present studies has been reported in many plants (Choudhary and Singh 1995; Anbazhagan and Ganapathi 1999; Monteiro *et al.*, 2002).

Albrecht (1989) and Choi *et al.,* (2002) reported the induction and subsequent germination of somatic embryos from embryogenic calli when

cultured on MS basal media. Theses reports were corroborative with the results obtained in the present experiments on *N. alata*. Rout *et al.,* (1995); Anbazhagan and Ganapathi (1999); Cushman *et al.*, (2000); Kim *et al.*, (2000) and Kumar *et al.*, (2002) also reported germination of somatic embryos in the medium without plant growth regulators.

SYNSEEDS

Synseeds are artificially encapsulated vegetative propagules capable of developing in to complete plants *in vitro* and *ex vitro* (Aitken-Christie *et al.*, 1995). Synseeds facilitate the exchange of capsules in sterile condition between laboratories and also for the germplasm conservation with proper techniques (Maruyama *et al.,* 1997; Chetia *et al.,* 1998; Datta *et al.,* 1999)

Rao and Singh (1991); George and Eapen (1995); Janeiro *et al.*, (1997) and Vincent (2001) reported, 3% as the optimal concentration of sodium alginate for encapsulation and subsequent plant development of vegetative propagules. These reports were corroborative with the results of present experiments on *N. alata*. The low conversion frequency of synseeds at higher concentrations of sodium alginate might be due to the extreme hardness of the beads.

In the present studies on *N. alata* encapsulated somatic embryos germinated on MS basal semi-solid medium where as encapsulated shoot buds

required an exogenous supply of cytokinins for development. This requirement for an exogenous supply of cytokinin might be due to sub- optimal level of endogenous cytokinin in the shoot buds for development.

ROOTING *IN VITRO.*

Rooting of *in vitro* shoots is one of the most critical steps in micropropagation, as subsequent survival of the plantlets in the field depends on well developed of root system.

John *et al.,* (1997) and Daniel *et al.,* (1999) obtained a maximum of 8 roots / shoot of *N. alata* when cultured on MS medium supplemented with 2.0mg/l IBA. However, in the present studies on the same species an average number of 13 roots / shoot were obtained when cultured on half- strength MS medium supplemented with 0.5 mg/l NAA. Hence it was evident that growth regulator as well as nutrients in the medium influenced rooting. Half -strength MS medium was more effective than full strength MS for the induction of roots on *in vitro* shoots. Efficacy of half strength MS medium in root induction has been reported in many plants (Bhattacharya *et al.,* 1990; Sarasan *et al.*, 1994; Reddy *et al.*, 2001; Yang *et al.*, 2001; Sahoo and Chand 1998b).

ACCLIMATIZATION AND TRANSFER TO FIELD CONDITIONS.

Desiccation due to the absence of waxy cuticle or open stomata is some major problems when introducing micropropagated plants into field (Braineed

and Fucchigami 1982; Donnelly and Vidaver 1984). Daniel *et al.*, (1999) recorded 90% and John *et al.,* (1997) recorded 90-96% survival of micropropogated plants of *N. alata* in field. However, in the present studies on the same species the survival frequency of micropropagated plants in the field was about 85%. This variation in survival percentage might be due to the difference in the age of plant, time of transfer etc.

SECONDARY METABOLITE ANALYSIS

Secondary metabolite analysis of *in vivo* and *in vitro* roots of *N. alata* showed one additional compound in the *in vitro* roots compared to *in vivo* roots. But in Issampelos *pereira* more number of compounds were found in *in vivo* tissues compared to *in vitro* tissues (Gokul and Thejavathi 1999). This increase in number of compounds in *in vitro* roots observed in the present studies on *N. alata* might be due to the production of new compounds in culture or might be due to the synthesis of large quantities of a secondary metabolite in culture which occurs in sub- detectable quantity in the *in vivo* roots due to the difference in growth conditions.

BIOCHEMICAL STUDIES

In the present studies on *N. alata* total proteins, total sugars, total phenolics, peroxidase activity and number of polypeptide bands were higher in regenerating callus than in primary callus. Presence of higher levels of

metabolites in regenerating callus suggest that the process of morphogensis requires energy and reducing power (Thorpe and Meier, 1974). Similar observations were reported earlier in many plants (Yadav *et al* 1995; Fett *et al*, 1992; Kavikishor, 1987). Purohit *et al,* (1996) and Lal *et al,* (1988) reported high peroxidase activity during morphogensis. These reports were in consonant with the results obtained in the present experiments on *N. alata.*

Regenerating calli had more number of polypeptide bands than primary calli in the present studies on *N. alata.* Similar results showing difference in polypeptide patterns of different types of calli were reported earlier in *Camellia japonica* (Pedroso and Pais 1995). The additional polypeptides observed in the regenerating callus might have some important role in morphogenesis.

SUMMARY AND CONCLUSION

HELIOTROPIUM KERALENSIS

Heliotropium keralensis (Boraginaceae) is an important medicinal plant of Kerala. The plant has been used for the treatment of asthma, cough, anaemia, scorpion and snake poisoning etc.

Direct shoot regeneration of *H.keralensis* was established from shoot tip, node and root explants by culturing on MS medium supplemented with various cytokinins (BA and Kn). BA was found to be effective in multiple shoot induction from various explants of *H. keralensis* when used singly. However, a combination of BA and Kn was found more effective than using BA alone. Of the different combinations tested 3.0 mg/l BA + 3.0 mg/l Kn induced highest number of shoots from shoot tip and nodal explants. However, 4mg/l BA + 4mg/l Kn on MS medium induced highest number of shoots from root explants. Among the different media formulations (MS, B_5 and White's) tested, MS was found most suitable for the induction of multiple shoots of *H. keralensis*. Of the different concentrations of sucrose tested, 3% was the optimal level for the induction of multiple shoots of *H. keralensis.*

Enormous amount of calli, having regenerative potential, were formed from various explants when cultured on MS medium supplemented with BA or BA + 2,4-D or 2,4-D + 2iP. MS medium with 0.5 mg/l 2,4-D + 3.0 mg/l 2iP was the most effective combination for the induction and proliferation of calli

from various explants. Indirect shoot regeneration of *H. keralensis* was achieved by culturing calli on MS medium supplemented with 0.5 mg/l BA or 0.5 mg/l IAA + 0.5-4.0 mg/l BA. Of the different concentrations and combinations tested 0.5 mg/l IAA + 3.0 mg/l BA produced highest number of shoots from the calli. Multiple shoot formation from calli was a function of cytokinin-auxin activity.

Somatic embryogenesis of *H. keralensis* was achieved by sub culturing the embryogenic calli induced on MS medium supplemented with 0.5 mg/l IAA + 1.0- 4.0 mg/l 2iP. Among the various combinations and concentrations of growth regulators tested 0.5 mg/l IAA + 3.0 mg/l 2iP produced calli with highest embryogenic potential. Suspension cultures were more effective than semi- solid cultures for the induction of somatic embryos from embryogenic calli of *H. keralensis*. The embryoids passed through the normal developmental stages (globular to cotyledonary). Germination of somatic embryos was achieved on semi-solid MS medium.

Encapsulation of embryoids/shoot buds (synseeds) was achieved in 3% sodium alginate and 50mM calcium chloride. Encapsulated somatic embryos or shoot buds germinated /developed when cultured on MS medium with or without BA (2.0 mg/l) even after storage at 4°C for six months.

In vitro raised shoots of *H. keralensis* were rooted when cultured on MS medium supplemented with IAA (0.1-3.0 mg/l) or IBA (0.1 – 3.0 mg/l) or NAA (0.1 – 3.0 mg/l).

Thin layer chromatographic (TLC) analysis of secondary metabolites showed the presence of two additional compounds in the root extracts of *in vitro* grown plantlets compared to that of the of field grown plants.

Biochemical analysis of regenerating and primary callus showed accumulation of metabolites in the regenerating callus.

NAREGAMIA ALATA

Naregamia alata W&A is an important medicinal plant belongs to the family Meliaceae. The plant is used in the treatment of asthma, bronchitis, rheumatism, acute dysentery etc.

Direct multiple shoot regeneration of *N. alata* was achieved by culturing shoot tip and nodal explants on MS medium supplemented with BA (0.5 mg/l) or BA(0.5-4.0 mg/l)+ NAA (0.5 –1.0 mg/l) or BA (0.5 –3.0 mg/l) + IAA (0.5 mg/l). Among the various combinations and concentrations of growth regulators tested, 0.5 mg/l NAA + 3.0 mg/l BA on MS medium produced highest number of shoots from shoot tip and nodal explants. Direct shoot regeneration was occurred on leaf explants of *N. alata* when cultured on MS medium

supplemented with BA (0.1-3.0 mg/l) or BA (0.1-3.0 mg/l) and Kn (0.1-3.0 mg/l). A combination of 2mg/l BA + 2mg/l Kn on MS medium produced highest number of shoots from leaf explants. Sucrose at 3% level produced highest number of shoots from shoot tips compared to other concentrations.

Indirect shoot regeneration of *N. alata* was achieved from the calli obtained on various explants - internode, leaf, root and petal explants on MS medium supplemented with IAA (0.5 –1.0 mg/l)+ BA (0.5-4.0mg/l) or NAA (0.5-1.0 mg/l) + BA (0.5-4.0 mg/l). 0.5 mg/l NAA + 3.0 mg/l BA in MS medium was the best combination for the induction and subsequent regeneration of shoots from the calli.

Direct somatic embryogenesis of *N. alata* was obtained from leaf explant when cultured on MS medium supplemented with 0.1 mg/l 2,4-D. Indirect somatic embryogenesis of *N. alata* was achieved by culturing embryogenic calli (induced from various explants on MS medium supplemented with 0.1-3.0 mg/l 2,4-D) on MS basal medium. Suspension cultures were more effective than semisolid cultures for the induction of somatic embryos from the calli. Maturation and germination of somatic embryos occurred when cultured on MS basal semi- solid medium.

Encapsulated somatic embryos or shoot buds of *N. alata* were germinated / developed when cultured on MS medium with or without Kn (2 mg/l) even after storage at 4°C for 6 months.

In vitro shoots were rooted when cultured on full or half strength MS medium supplemented with IAA (0.5 7 1.0 mg/l) or IBA (0.5-2.0 mg/l0 or NAA (0.1-1.0 mg/l). Half strength MS medium with 0.5mg/l of NAA was the best combination for the induction of roots on *in vitro* shoots of *N. alata.* Micropropagated plants survived in the natural soil following the transfer from the laboratory condition.

Thin layer chromatographic analysis of secondary metabolites showed the presence of an additional compound in the root extracts of *in vitro* plantlets compared to the root extracts of *in vivo* plants.

Biochemical analysis showed accumulation of metabolites in regenerating callus than in the primary callus.

CONCLUSION

The protocols developed in the present experiments for direct and indirect organogenesis, somatic embryogenesis and synthetic seed production of *Heliotropium keralensis* and *Naregamia alata* could be used for rapid propagation and *ex situ* conservation of these two important medicinal plants of Kerala.

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APPENDIX

List of Publications

1. **Delse P. Sebastian** and Molly Hariharan 2002. Micropropagation of *Rotala aquatica* Lour. An important medicinal Plant. Phytomorphology, 52: 137-144.

2. Molly Hariharan, **Delse P. Sebastian**, Sailas Benjamin , and Preshy P 2002 . Somatic embryogenesis in *Leptadenia reticulata* L. A Medicinal Plant. Phytomorphology, 52: 155-160.