# MICROPROPAGATION AND TRANSGENIC PLANT REGENERATION STUDIES USING *AGROBACTERIUM RHIZOGENES* ON TWO MEDICINAL PLANTS

*Thesis submitted to the University of Calicut*
*in partial fulfilment of the requirement*
*for the Degree of*

**DOCTOR OF PHILOSOPHY**
**IN BOTANY**

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DEPARTMENT OF BOTANY
UNIVERSITY OF CALICUT
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*Dedicated to the*
*care*
*love*
*and inspiration*
*of my beloved parents*

# UNIVERSITY OF CALICUT

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## CERTIFICATE

This is to certify that the thesis entitled **"Micropropagation and transgenic plant regeneration studies using *Agrobacterium rhizogenes* on two medicinal plants"** submitted to the University of Calicut by **Martin K P**, for the Degree of **Doctor of Philosophy in Botany**, embodies the results of bonafide 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, assoicateship, fellowship or other similar title or recognition.

**Aleyamma Thomas**

# DECLARATION

I hereby declare that the work presented in this thesis entitled **"Micropropagation and transgenic plant regeneration studies using *Agrobacterium rhizogenes* on two medicinal plants"** is original and was carried out by me in the Department of Botany, University of Calicut and has not been submitted earlier either in part or in full for any degree or diploma of any university.

**Martin K P**

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*Martin KP*

# Abbreviations

| | |
|---|---|
| AA | : Ascorbic acid |
| ABA | : Abscisic acid |
| AC | : Activated Charcoal |
| $AgNO_3$ | : Silver nitrate |
| $B_5$ | : Gamborg *et al.* (1968) medium |
| BAP | : 6-benzylaminopurine |
| $CaCl_2$ | : Calcium chloride |
| CaMV | : Cauliflower Mosaic Virus |
| CH | : Casein Hydrolysate |
| $CoCl_2$ | : Cobalt Chloride |
| CPW | : Cell Protoplast Washing |
| CT | : Coconut Toddy |
| CW | : Coconut Water |
| 2,4-D | : 2,4-dichlorophenoxyacetic acid |
| DMSO | : Dimethyl sulphoxide |
| EDTA | : Ethylene Dimethyl Tetra Acetate |
| FAA | : Formalin-Acetic acid-Alcohol |
| $GA_3$ | : Gibberellic acid |
| ***gfp*** | : green fluorescent protein |
| ***gus*** | : β-glucuronide |
| IAA | : Indole-3-acetic acid |
| IBA | : Indole-3-butyric acid |
| Kin | : Kinetin (6-furfurylaminopurine) |
| MES | : 2-[N-Morpholino]ethanesulphonic acid |
| MS | : Murashige and Skoog (1962) medium |
| NAA | : α-Naphthaleneacetic acid |
| nm | : nanometer |
| *nos* | : nopaline synthase |
| ***npt*** | : neomycin phosphotransferase |
| O.D. | : Optical Density |
| PCM | : Protoplast Culture Medium |
| TBA | : Tertiary Butyl Alcohol |
| TLC | : Thin Layer Chromatography |
| v/v | : volume/volume |
| w/v | : weight/volume |
| X-gluc | : 5-bromo-4-chloro-3-indolyl β-glucuronide |
| YE | : Yeast Extract |
| YM | : Yeast Mannitol medium (Hooykaas *et al.* 1977) |

# CONTENTS

# INTRODUCTION

# INTRODUCTION

Medicinal plants consist of plants related to health, which contain constituents that are used as drugs as per/by Western Standards (Farnsworth and Soejarto 1991). Man, since his origin utilized plants as the most important eco-friendly resource of medicines/pharmacologically active compounds. Nature, the largest and the best combinatorial storehouse of herbal plants provided remedies to cure all mankinds' ailments. The herbal plants utilized by the ancient civilization remains to be valued even now and today more than three quarter of world's population relies on plants and plant extracts. Recent World Health Organization (WHO) studies indicate that over 30% of the worlds plant species has at one time or another been used for medicinal purposes.

Medicinal plants are used for the preparation of a wide spectrum of derivatives ranging from traditional extracts with high standard content of active constituents to chemically pure compounds. These covers phytochemicals such as alkaloids, terpenes, flavonoids, essential oils, glycosides, etc. Moreover, the bioactive curare compounds from the medicinal plants can serve as leads for developing new synthetic drugs in modern medicines. However, some of the plant compounds remains to be unique due to their structural complexity.

Herbal medicines are being used about 80 % of the world population primarily in the developing countries for primary health care (Kamboj 2000). Medicinal plants of India has found clinical application from the Atharva Veda (circa 1500 BC). The use of medicinal herbs progressed from the magico-religious Vedic period to the ***Samhita*** period in ***ayurveda.*** The fascination of our holistic system of medicine especially ayurveda, which relies on the use of more than 7,000 medicinal plants attained popularity not only in India but also abroad.

The advent of modern medicine in the 18th century was a setback to ***ayurveda***, which suffered considerable neglect at the hands of colonial administration. However, the swing back of the pendulum returned the plants to the center in drug development as medicines. The popularity of the herbal drugs is due to its ready acceptance of local populace, its local availability, relative inexpensiveness and high effectiveness with minimal side effects.

Now the traditional herbal medicines, both nationally and internationally are receiving considerable attention from pharmaceutical industries. The traditional herbal remedies or chemical extracts isolated from a wide range of taxa are equally exploited even today by the industrial societies. The scientific study of traditional medicines, derivatives of drugs, through bioprospection and systematic conservation, domestication and cultivation of the concerned medicinal plants thus assumes great importance in today's context when more people need safe and effective medicines at an affordable rate.

The global market for medicinal product claims US $ 50 billion per year excluding the traditional preparations used in countries like India and being promised to grow even further. With immense bioresources and living traditions of her own, India is exceptionally well placed to mount an integrated programme for the multiplication of herbal plants and the development of plant based drugs that are important for public health and for global market.

India's total turnover for ayurvedic and herbal product is 2,300 crore. India, one of the best combinatorial repositories of medicinal and aromatic plants has a pivotal role in global need and can strengthen the economy through export. For the prevention of invasion on our indigenous or folk medicines, it is our duty to study and grow our own plants, especially the needy herbal plants.

The great surge of interest on medicinal plants is based on its availability on a continuous basis at moderate price. Collective gatherings of herbal plants from the wild resulted in the depletion of bioresources and the extinction of rare species. Increasing population, urbanization and industrialization also lead extensive destruction of bioresources. Though awareness of medicinal plant conservation was created by Chiang Mai (Thailand) declaration (1988), it has been not gained a momentum to ensure conservation and increase the quantities for the present and future generations. Pharmaceutical industries are also facing an increased difficulty in securing ample supply of herbal plants because of its significant decrease in propagation.

Conventional vegetative propagation is beset with the problems of scanty and delayed rooting and slow multiplication rate. It takes years to build up the commercial quantities of selected clones. In addition, seed propagation is hampered by the poor and very short span of seed viability and low germination rate. During the last few years tissue culture techniques have been extensively exploited not only for the rapid and large scale propagation but also for the *ex situ* conservation of many medicinal and aromatic plants.

Micropropagation through direct organogenesis has been proved to be the most reliable way for large-scale propagation of genetically stable and true-to-type progenies, which also help the conservation of germplasm of many herbal plants. Somatic embryogenesis is now considered as the useful strategy, for the propagation in bulk and also as a tool in genetic manipulation studies. In addition, it remains as a suitable material for germplasm conservation.

The great advantage of isolated protoplasts is that they can be used to study the cell structures and cell process. Efficient and reproducible protocol for protoplast isolation, culture and subsequent regeneration either by

organogenesis or somatic embryogenesis is prerequisites for gene transfer studies *via* somatic hybridization or genetic engineering.

Germplasm conservation has become a necessity for the future sustainable harvesting systems and which serves as a means to maintain species diversity to prevent genetic erosion. Germplasm conservation includes not only the conservation of the genetic stocks but also the beneficial variations that occurred in due course.

The concept of synseeds and minimal growth as conservation strategies at low cost has recently been attracted considerable interest. Synseeds, in a broad view includes encapsulated vegetative propagules capable of developing into a complete plant. Besides the somatic embryos, vegetative propagules such as shoot meristem, protocorm, apical or axillary buds, callus derived adventitious buds are also used for encapsulation. As seed is the preferred planting vehicle for both cultivation and conservation in all plant species, somatic embryo derived artificial seeds have much relevance due to its ease of production, handling and direct delivery to field. All at seed efficiency, artificial seeds can revolutionize the production of both seed and vegetatively propagated species (Gray 1987).

The local populace should fascinate tissue culture technology only when it is highly productive at low cost. Usually, many media components used for large-scale micropropagation are in purified form (such as sucrose, double distilled water etc.) and hence expensive. In an economic point of view for commercial purpose it is therefore essential to find a way to reduce the cost of these components by using table sugar, tap water, CW, etc.. Moreover, when transplanted out of the culture vessels, the propagated plantlets suffer from severe environmental stress and result in severe loss (Preece and Sutter 1991; van Huylenbroeck and Debergh 1996). Manipulation of acclimatization prior to or upon transplanting usually reduces the loss, but it brings additional expenses

(van Huylenbroeck and Debergh 1996). These constrains are minimized in recent years by the attempts of micropropagation through photoautotrophic culture, a recent spin off of tissue culture, consists of growing cultures without adding any carbon source or organic salts to the medium. Strategy of withdrawal of important nutrients have a manifold advantage both to propagation at low cost and high rate of survival in field transfer as well as for effective germplasm conservation. Elaboration of satisfactory procedures for management of cell and tissue at "near zero" or "minimal growth" will have great value for further experimentation on earth and even in space. *In vitro* culture methods are the best alternative for the propagation and conservation of herbal plants especially the endangered or rare ones. Of the two methods of conservation, *ex situ* is more powerful than *in situ* because it is safer *i.e.* without the impediments of natural hazards.

Large-scale production of secondary metabolites such as alkaloids, terpenes, flavonoids, glycosides, cardenolides etc. from field grown plants has its own limitations due to the dependency of the metabolism to season and environmental constrains during the cultivation. Emergence of secondary metabolite production by cell culture methods fascinated the production of both the existing as well as novel compounds, useful as new drugs availed very much to the pharmaceutical industries. Plant tissue culture system offers the best way to exploit our biorichness of medicinal and aromatic plants and thus to strengthen our economy without affecting the national heritage.

Classical breeding programme is time consuming and inadequate due to the restricted gene pool and incompatibility. Biotechnology, the innovations of the 20th century represent an area of biology for isolating and transferring genes employing recombinant DNA technology to reprogramme the genetic make up of the recipient organism to perform the altered and modified functions. Genetic transformation techniques resulted in the production of transgenic plants with

desired characters. The establishment of efficient regeneration protocols, either through organogenesis or somatic embryogenesis is a fundamental mandatory for the development of transformed plants. ***Agrobacterium*** mediated genetic transformation studies and development of transgenic plants have already been reported in many medicinal plants. Of the two strains of *Agrobacterium*, *A. tumefaciens* harbouring the Tumour inducing (**Ti**) plasmid induces the "crown gall" while *A. rhizogenes* harbouring the Root inducing (**Ri**) plasmid induces "hairy roots". *A. rhizogenes* mediated transformation is highly suitable for the production of genetically stable transformed plants. Induction of hairy root has much importance today in the case of plants in which the root as their officinal part.

Considering all these facts in into account, two important medicinal plants, *Eryngium foetidum* L. and *Holostemma ada-kodien* Schult. with low seed viability and propagation rate and root as the officinal part have been selected for the present studies on micropropagation and transformation.

**IMPORTANCE OF THE PLANT MATERIALS**

## *Eryngium foetidum* L.

*Eryngium*, a South American genus belonging to the family Apiaceae (Umbelliferae) consists of nearly 250 species widely distributed in warm tropical and temperate regions. *Eryngium foetidum* L., one of the three species present in India is an aromatic perennial herb with a rosette of long spathulate spiny toothed leaves. This species, popularly known as "Spiny coriander" or "Stinking sharewort" (also as "fitweed") has became rare and restricted to certain regions only.

The plant is considerably valued for its pharmaceutical properties in Indo-China, Mexico, Nicaragua, Guinea and Brazil. The leaves are reported to posses

anti-inflammatory and analgesic properties (Saenz *et al.* 1997). Root, the officinal part of the herb is sticky, nervine tonic, aphrodisiac, expectorant, diuretic, diaphoretic and stomachic. *Eryngium* is known for its high saponin content and are generally responsible for its biological activity. The genus also contains flavonoids such as Kaempferol and quercetin glycosides (Hohmann *et al.* 1997). The plant remains to be an alternative source for Kaempferol, a product extracted from *Kaempferia galanga*. The plant is strongly aromatic, contains essential oils comprised of about 40 compounds indispensable to perfumery, flavour and pharmaceutical industries (Wong *et al.* 1994; Pino *et al.* 1997).

The "Spiny coriander" is used as a substitute of coriander, *Coriandrum sativum* as a food flavouring agent. The leaves are used as a condiment for culinary purposes in North-East India (Sankat and Maharaj 1996). The leaves are pickled in Sikkim. The plant has been used as a cattle fodder in Java (Burkill 1965).

## ***Holostemma ada-kodien*** Schult.

The genus *Holostemma* of Asclepiadaceae consists of about three species distributed in South-East Asia. *Holostemma ada-kodian* Schult. [Syn. *H. annulare* (Roxb.) K. Schum.], prevalently familiar as "**Jivanti**" or "**Jivani**" (Mal. Adakodien, Adapatiyan) is a perennial, twiny laticiferous shrub distributed in tropical Himalayas, Burma, Western Peninsula, Sri Lanka and China. *H. ada-kodien*, an indigenous species to India has become extremely rare in its natural habit due to extensive collection. The plant is now occasionally encountered over hedges and in open forests.

*Holostemma ada-kodien*, a famed medicinal plant is a requisite raw drug for more than 34 ayurvedic preparations. It is one of the major ingredients of the drug, **"Jivanti"** in the indigenous system of medicine (Kolammal 1979) which

have the property to bequeath health and liveliness. Jivanti is rejuvenative and easy of digestion, promote health and vigour, improve voice, mitigate *tridosa,* cough, dyspnoea and fever, burning sensation, dysentery, night blindness poisonous affections and tuberculosis (Sivarajan and Balachandran 1994). The tuberous roots are sweet, refrigerant, ophthalmic, emollient, alterant, tonic, stimulant, aphrodisiac, expectorant and galactagogue. They are also used in stomachalgia and constipation. The roots made into a paste are applied in opthalmiopathy and orchitis. It is a rich source of vitamin-A. The roots rubbed into a mash are used in cold milk as a curare to diabetes (Kirtikar and Basu 1975).

According to Nair *et al.* (1992) about 150 metric ton of root tubers will be needed per annum for ayurvedic preparations in major South Indian pharmacies. Besides the medicinal virtues, the leaves, flowers and fruits are used as vegetables; the plant is also used as cattle feed. The fiber from the bark is reputed to be used for cordage and papermaking. The latex on drying yields an elastic residue (Santapau 1953).

The plant is reported as rare (Mathew 1983). According to Dan and Shanavaskhan (1991) this is due to the indiscriminate collection of root tubers as the raw material for the ayurvedic drug preparations and other anthropogenic reasons. As per the first red list of medicinal plants of South India, it is a vulnerable species (CAMP 1995). Conventional propagation *via* seed and stem and root cuttings is not at a pace to meet the commercial need.

**OBJECTIVES OF THE PRESENT STUDY**

Considering the high medicinal value and low seed viability and propagation, these two important medicinal plants, *Eryngium foetidum* L. and *Holostemma ada-kodien* Schult. were selected for the *in vitro* studies with the following objectives:

## ***Eryngium foetidum*** L.

- Induction of direct and indirect organogenesis from different explants *viz.* root, stem disc, leaf and scape
- Induction of direct and indirect somatic embryos and evaluation of various factors
- Induction of secondary somatic embryogenesis
- Photoautotrophic micropropagation
- Protoplast isolation, culture and regeneration of plants *via* organogenesis and somatic embryogenesis
- Ontogenic studies of shoots and embryos
- Encapsulation of somatic embryos and germination studies
- Evaluation of conservation *in vitro* by synseeds and photoautotrophic medium
- Induction of roots and successful establishment of plants in field conditions
- Comparative qualitative analysis of secondary metabolites in the root tissues of *in vitro* and field grown plants by TLC
- Induction of hairy roots from leaf explants using *Agrobacterium rhizogenes* (LBA 9402 strain) harbouring the **pGreen** binary plasmid with ***gus/gfp*** construct
- Transgenic plant regeneration studies with the expression of ***gus/gfp*** gene

## ***Holostemma ada-kodien*** Schult.

- Induction of axillary shoot multiplication and indirect organogenesis
- Induction of indirect somatic embryogenesis and evaluation of various factors
- Protoplast isolation, culture and regeneration of plants *via* somatic embryogenesis
- Histological studies of the developmental stages of somatic embryos
- Encapsulation of somatic embryos and embryo-like unipolar shoot buds and its germination studies
- Evaluation of conservation *in vitro* at synseeds and whole plant level
- Induction of roots and successful establishment of plantlets in field condition
- Comparative qualitative analysis of secondary metabolites in the tuberous roots *in vitro* and field grown plants by TLC
- Induction of hairy roots from leaf and internode explants using *Agrobacterium rhizogenes* (LBA 9402 strain) harbouring the **pGreen** plasmid with ***gus/gfp*** construct
- Transgenic plant regeneration studies with the expression of ***gus/gfp*** gene

# REVIEW OF LITERATURE

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Plant tissue culture was conceived and enunciated by Haberlandt's (1902) prophecy of totipotency, the deemed inherent ability in every living cell of all the plants to the genesis of an entire plant. Plant propagation *via* tissue culture technique has been emanated over last 40-50 years as a spin off of *in vitro* studies on differentiation, blossomed into success as a technology without parallel and the progress has been overwhelmed. With its widespread reduction to nominally routine technique and adoption as a common laboratory tool, plant tissue culture *per se* became somewhat less of a methodological propagation that once it was. The elucidation of the facsimile, the inherent totipotency was empowered by the pioneering experiments of Laibach (1925, 1929), Gautheret (1934), White (1934,1937), van Overbeek *et al.* (1941), Skoog (1944), Loo (1945) and Murashige and Skoog (1962).

An endeavour to review the various aspects of *in vitro* studies performed on medicinal plants especially on Apiaceae and Asclepiadaceae with relevance to the present studies on *Eryngium foetidum* and *Holostemma ada-kodien* are curtailed below under the subtitles.

**Micropropagation**

Micropropagation, the most advanced commercial and methodological application of biotechnology exploits the morphogenic potential of existing growing points or meristems within the plants (Giles and Morgan 1987). The exploration of the doctrine of totipotency at chemical as well as at conservation level brought the micropropagation of medicinal plants to the fore, which revitalized pharmaceutical industries. After the pioneer work of Morel (1960) on virus elimination and clonal propagation, much progress have been witnessed in

the large-scale propagation of many medicinal and aromatic plants (Table I), besides the medicinal plants on Apiaceae and Asclepiadaceae (Table II & III).

Acquisition of morphogenic competence can occur with greater or less ease in different tissues. The unique phenomenon of certain plant tissue is their ability to induce *de novo* a range of developmental patterns including embryogenesis. Together with other phytohormones, auxins play an important role in the regulation of morphogenesis at molecular, cellular or organ and whole plant level (Gaspar *et al.* 1996; Dodeman *et al.* 1997; Charriere and Hahne 1998). The process leading to adventitious bud formation is thought to be under the control of growth substances, a relatively high ratio of cytokinin to auxin (Skoog and Miller 1957). According to the critique of Skoog and Miller (1957), *in vitro* morphogenesis is controlled by the balance between auxin and cytokinin added to the culture medium and the same were later well documented by Carman (1990), Gaspar *et al.* (1996), De Klerk *et al.* (1997a), Arockiasamy and Ignacimuthu (1998), Banerjee *et al.* (1999) and Sivakumar and Krishnamurthy (2000).

Morphogenesis *in vitro* falls into two mutually exclusive pathways: organogenesis and somatic embryogenesis (Hicks 1980).

**Organogenesis**

The two basic strategies in organogenesis are direct, in which the competence is already present at the culture onset and indirect *via* an intermediate callus phase, in which the competence is achieved during the *in vitro* culture (Thorpe *et al.* 1991; De Klerk *et al.* 1997a). Direct shoot organogenesis from the primary tissue is more desirable than indirect (Larkin and Scowcroft 1981; Jasrai *et al.* 1999; Sivakumar and Krishnamurthy 2000).

**Table I** Organogenetic studies on some medicinal plants during the recent past

| Name of the plant | Explant | Results | References |
|---|---|---|---|
| *Acorus calamus* | Rhizome buds | Axil. bud multiplication | Harikrishnan & Hariharan 1999 |
| *Allium sativum* | Stem | Indirect organogenesis | Barandiaran *et al.* 1999 |
| *A. sativum* | Stem disc | Direct organogenesis | Ayabe & Sumi 1998 |
| *Alpinia calcarata* | Rhizome buds | Indirect organogenesis | Martin & Hariharan 1999 |
| *A. galanga* | Rhizome buds | Axil. bud multiplication | Borthakur *et al.* 1999 |
| *Asparagus officinalis* | Shed microspore | Indirect organogenesis | Peng & Wolyn 1999 |
| *Azadirachta indica* | Leaf | Direct organogenesis | Eeswara *et al.* 1998 |
| *Bacopa monnieri* | Stem, leaf, flower buds | Indirect organogenesis | Tejavathi & Shailaja 1999 |
| *Boerhaavia diffusa* | Node | Axil. bud multiplication | Phukan *et al.* 1999 |
| *Butea monosperma* | Cotyledonary node | Shoot multiplication | Kulkarni & D'Souza 2000 |
| *Centella asiatica* | Leaf | Direct organogenesis | Banerjee *et al.* 1999 |
| *Cichorium intybus* | Leaf | Direct organogenesis | Pieron *et al.* 1998 |
| *Coleus forskohlii* | Leaf, stem and flower bud | Indirect organogenesis | Suryanarayanan & Pai 1998 |
| *Curcuma aromatica* | Rhizome buds | Indirect organogenesis | Anand & Hariharan 1999 |
| *Dendrobium moschatum* | Stem disc | Direct organogenesis | Kanjilal *et al.* 1999 |
| *Enicostemma axillare* | Leaf | Direct organogenesis | Sudhersan 1998 |
| *Gloriosa superba* | Shoot tip | Shoot multiplication | Sivakumar & Krishnamurthy 2000 |

| | | | |
|---|---|---|---|
| *Houttuynia cordata* | Node | Axil. bud multiplication | Handique & Bora 1999 |
| *Kaempferia galanga* | Rhizome buds | Indirect organogenesis | Vincent *et al.* 1998 |
| *Lavandula* x *intermedia* | Leaf | Direct organogenesis | Dronne *et al.* 1999b |
| *Mentha* spp. | Leaf | Direct organogenesis | Faure *et al.* 1998 |
| *Mentha x piperita* | Protoplasts | Indirect organogenesis | Jullien *et al.* 1998 |
| *Moricandia nitens* | Protoplasts | Indirect organogenesis | Tian & Meng 1999 |
| *Pinus wallichiana* | Seedling | Shoot multiplication | Bastola *et al.* 2000 |
| *Piper longum* | Leaf | Indirect organogenesis | Philip *et al.* 2000 |
| *Pisonia alba* | Node | Axil. bud multiplication | Jagadishchandra *et al.* 1999 |
| *Plumbago zeylanica* | Node | Axil. bud multiplication | Rout *et al.* 1999a |
| " | Stem | Indirect organogenesis | Rout *et al.* 1999b |
| *Sapindus mukorossi* | Seedling | Axil. bud multiplication | Philomina & Rao 1999 |
| *Scabiosa columbaria* | Anther and ovule | Indirect organogenesis | Romeijn and van Lammeren 1999 |
| *Sesamum indicum* | Node | Axil. bud multiplication | Gangopadhyay *et al.* 1998 |
| *Solanum nigrum* | Leaf | Direct organogenesis | Shahzad *et al.* 1999 |
| *Aegle marmelos* | Node | Axil. bud multiplication | Ajithkumar & Seeni 1998 |
| *Tridax procumbens* | Node | Axil. bud multiplication | Sahoo & Chand 1998a |
| *Uraria picta* | Node | Axil. bud mutliplication | Anand *et al.* 1998 |
| *Vetiveria zizanioides* | Mesocotyl | Indirect organogenesis | George & Subramanian 1999 |
| *Vitex negundo* | Node | Axil. bud multiplication | Sahoo & Chand 1998b |
| *Withania somnifera* | Meristem | Indirect organogenesis | Teli *et al.* 1999 |
| " | Stem | Indirect organogenesis | Rani & Grower 1999 |

**Table II** *In vitro* studies reported on **Apiaceae**

| Name of the plant | Results | References |
|---|---|---|
| *Ammi majus* | SE | Sehgal 1964a, '72; Grewal *et al.* 1976 |
| *Anethum graveolens* | SE | Johri & Sehgal 1966; Reinert *et al.* 1966; Ratnamba & Chopra 1974; Williams & Collin 1976; Sehgal 1968, '78; Schafer *et al.* 1986 |
| *Angelica acutiloba* | SE | Ohga *et al.* 1989 |
| *A. sinensis* | SE | Zhang & Cheng 1989; Huang *et al.* 1996; Tsay & Huang 1998 |
| *Apium graveolens* | SE | Reinert *et al.* 1966; Williams & Collin 1976; Al-Abta & Collin 1978; Orton 1984; Altman *et al.*1990; Collin & Issac 1991; Nadel *et al.* 1995; Toth & Lacy 1992; Choi & Soh 1997 |
| *Bunium persicum* | SE | Wakhlu *et al.* 1990; Grewal 1996a, b; Grewal & Rani 1999 |
| *Bupleurum falcatum* | Org. | Kohda *et al.* 1990 |
| | SE | Bang *et al.* 1999* |
| *B. scorzonerifolium* | SE | Xia *et al.* 1992 |
| *Carum carvi* | SE | Ammirato 1973, '77; Furmanowa *et al.* 1991; Schafer *et al.* 1986; Krens *et al.* 1997 |
| *Centella asiatica* | SE | Banerjee *et al.* 1999 |
| *Conium maculatum* | SE | Netien & Raynaud 1972 |
| *Coriandrum sativum* | SE | Steward *et al.* 1970; Sehgal 1972; Schafer *et al.* 1986; Chen *et al.* 1995; Kim *et al.* 1996 |
| " | Org. | Kataeva & Popowich 1993 |
| *Cuminum cyminum* | SE | Jha *et al.* 1983 |
| *Daucus carota* | SE | Reinert 1958, '67; Steward *et al.* 1958; Halperin & Wetherell 1964; Halperin 1967; |

| | | |
|---|---|---|
| | | Sussex & Frei 1968; Grambow *et al.* 1972*; Kameya & Uchimiya 1972*; Chen & Holden 1973; Jones 1974a; Smith & Street 1974; McWilliam *et al.* 1974; Fujimura & Komamine 1975; Haccius 1978; Kamada & Harada 1979a; Kitto & Janick 1985; Nomura & Komamine 1985, '95; Smith & Krikorian 1990; Kamada *et al.* 1989; Roustan *et al.* 1989; Kiyosue *et al.* 1990; Wake *et al.* 1995; Neumann & Grieb 1992; Higashi *et al.* 1998; Osuga *et al.* 1999; Kitamiya *et al.* 2000 |
| *Didiscus coerulea* | SE | Ball & Joshi 1966 |
| *Eryngium foetidum* | Org. | Arockiasamy & Ignacimuthu 1998 |
| | SE | Ignacimuthu *et al.* 1999 |
| *Foeniculum vulgare* | SE | Sehgal 1964b; Johri & Sehgal 1966; Maheswari & Gupta 1965; Hunault 1984; Miura & Tabata 1986*; Miura *et al.* 1987; Hunault *et al.* 1989; Ohga *et al.* 1989; Hunault & Du Manoir 1992; Mathew 1993; Hunault & Maatar 1995; Anzidei *et al.* 1996 |
| " | Axil. Bud | Du Manoir *et al.* 1985; Hunault & Du Manoir 1992 |
| *Glehnia littoralis* | SE | Hiraoka & Oyanagi 1992; Hirai *et al.* 1997 |
| *Heracleum candicans* | Org. | Wakhlu & Sharma 1999 |
| " | SE | Wakhlu & Sharma 1998 |
| *Oenanthe aquatica* | SE | Waris 1959 |
| *Petroselinum hortense* | SE | Vasil *et al.* 1964; Vasil & Hildebrandt 1966 |
| *P. crispum* | SE | Ammirato 1983; Schafer *et al.* 1986 |
| *Peucedanum palustre* | SE | Vuorela *et al.* 1993 |
| *Pimpinella anisum* | SE | Kudielka & Theimer 1983; Schafer *et al.* 1986 |
| *Sium suave* | SE | Ammirato & Steward 1971; Steward *et al.* 1970 |
| *Trachyspermum ammi* | SE | Johri & Sehgal 1966; Sehgal & Abbas 1994 |

- **Protoplasts** Org. - Organogenesis SE - Somatic Embryogenesis Axil. Bud. - Axillary bud multiplication

**Table III** *In vitro* studies reported on **Asclepiadaceae**

| Name of the plant | Results | References |
|---|---|---|
| *Araujia sericifera* | SE | Torne *et al.* 1992, '96, '97 |
| " | Axil. Bud | Tideman & Hawker 1982 |
| *Asclepias curassavica* | Axil. Bud | Pramanik & Datta 1986 |
| *A. erosa* | Axil. Bud | Lee *et al.* 1982; Lee & Thomas 1985 |
| *A. rotundifolia* | Axill. Bud & Org. | Tideman & Hawker 1982 |
| *A. syriaca* | Org. | Tepper & Knapp 1992 |
| | SE | Wilson & Mahlberg 1977 |
| *A. tuberosa* | SE | Zilis 1979 |
| *Calotropis gigantea* | Org. | Roy & De 1990 |
| *Ceropegia* spp. | SE & Org. | Patil 1998 |
| *C. bulbosa* | Axil. Bud | Ramulu & Pullaiah 1999 |
| *Gymnema elegans* | Axil. Bud | Komalavalli & Rao 1997 |
| *G. sylvestre* | Axil. Bud | Reddy *et al.* 1998 |
| *Hemidesmus indicus* | Axil. Bud | Jayanthi & Patil 1995; Sharma & Yelne 1995; Patnaik & Debata 1996 |
| | SE & Org. | Sarasan *et al.* 1994 |
| *Holostemma annulare* | Axil. Bud | Jayanthi & Patil 1995; Sudha *et al.* 1998, 2000** |
| *Hoya* spp. | SE | Maraffa *et al.* 1981 |
| *Pergularia minor* | SE | Prabhudesai & Narayanaswamy 1974 |
| *Tylophora indica* | Axil. Bud | Chattopadhyay *et al.* 1992; Sharma & Chandel 1992a |
| | SE | Rao *et al.* 1970; Rao & Narayanaswamy 1972; Benjamin *et al.* 1979 |
| | SE & Org. | Mhatre *et al.* 1984* |

* **Protoplasts** Org. - Organogenesis SE - Somatic embryogenesis
Axil. Bud - Axillary Bud multiplication ** ***In vitro*** **Root**

Direct regeneration from different explants has become opulent in the literature (Table I). All types of explants are reported to have the potential of direct organogenesis (Table I).

Shoot multiplication from axillary bud is the easy and safe method of obtaining clonal uniformity, which assures faithful production of genetically identical plants. Clonal propagation by axillary shoot multiplication has been reported on many medicinal plants of Apiaceae and Asclepiadaceae (Table II & III) and also on many medicinal plants of other families (Table I). Most of the published data authenticates that cytokinin is the enhancive force for the induction of axillary bud multiplication. Growth and multiplication of axillary shoots has been reported from nodal explants on basal medium itself (Imhoff *et al.* 1996; Siril and Dhar 1997; Kumar *et al.* 1998; Thirunavoukkarasu and Debata 1998) and also either by the synergistic effect of cytokinin and an auxin (Patnaik and Debata 1996; Reddy *et al.* 1998; Ajithkumar and Seeni 1998; Kannan and Jasrai 1998; Anand *et al.* 1999) or with a cytokinin alone. Among the cytokinins, BAP is encountered to be the mightiest one for the induction of axillary bud multiplication on many medicinal plants (Sudha *et al.* 1998; Patil 1998; Wakhlu and Sharma 1999; Handique and Bora 1999) followed by Kin (Pramanik and Datta 1986). The combinations of cytokinin were also reported to be superior for the induction of shoot multiplication (Thirunavoukkarasu and Debata 1998; Kathiravan and Ignacimuthu 1999; Jagadishchandra *et al.* 1999; Sivakumar and Krishnamurthy 2000). Combinations of auxins and cytokinins with $GA_3$/CW/AA etc. were also reported (Sharma and Chandel 1992a; Komalavalli and Rao 1997; Kathiravan and Ignacimuthu 1999).

Indirect regeneration *via* an intermediate callus phase results somaclonal variations, makes the strategy less desirable for large scale clonal multiplication (Thorpe *et al.* 1991). However, it remains as the simple way to variations (Skirvin

*et al.* 1994; Patnaik *et al.* 1999; Barandiaran *et al.* 1999; Al-Zahim *et al.* 1999; Chakrabarty *et al.* 2000). Indirect organogenesis has been reported on many medicinal plants (Table I, II & III).

**Somatic Embryogenesis**

Somatic embryogenesis, the most spectacular achievements in plant tissue culture, was first demonstrated in carrot by Reinert (1958) and Steward *et al.* (1958). The somatic cells embarking on an embryogenic pathway provide great opportunity to explore the windows of the fundamental questions of growth, differentiation and development.

The plants developed through somatic embryogenesis are non-chimeric, which shows genetic uniformity and clonal fidelity (Stefaniak 1994; Merkle *et al.* 1995; Ishii *et al.* 1998). Somatic embryogenesis has been reported in many medicinal plants of Apiaceae and Asclepiadaceae (Table II & III).

Direct somatic embryogenesis from predetermined tissue is associated with greater genetic and cytological fidelity (Binsfeld *et al.* 1999; Choi *et al.* 1999; Iantcheva *et al.* 1999). Direct embryogenesis has been reported in tea (Akula and Dodd 1998), ginseng (Choi *et al.* 1998, 1999), groundnut (Venkatachalam *et al.* 1999) and diploid annual medics (Iantcheva *et al.* 1999).

Indirect embryogenesis has been reported in many medicinal plants like *Bacopa monnieri* (Tiwari *et al.* 1998), *Salvia* spp. (Kintzios *et al.* 1999), *Mesembryanthemum crystallinum* (Cushman *et al.* 2000), *Cuscuta trifolii* (Bakos *et al.* 2000) and *Sapindus mukorossi* (Sinha *et al.* 2000). The callus mediated embryogenesis requires dedifferentiation and acquisition of embryogenic state (Sharp *et al.* 1982).

Most of the reports on embryogenesis in medicinal plants were furnished with MS medium and its modifications (Tsay and Huang 1998; Choi *et al.* 1998, 1999). The efficacy of growth regulators in *in vitro* embryogenesis has been elucidated in many medicinal plants. In most of the cases, 2,4-D was proved as the trusty hormone to bring out the changes leading to somatic embryogenesis (Halperin and Wetherell 1964; Kim *et al.* 1996; Wakhlu and Sharma 1998; Patil 1998; Kitamiya *et al.* 2000). When compared to the efficacy of 2,4-D, NAA also retain an equal status as evidenced by many reports. Combinations of auxins and cytokinins were also reported for embryogenesis. Kin/BAP in association with NAA or 2,4-D has been reported in many medicinal plants of Apiaceae and Asclepiadaceae: *Hoya* sp. (Maraffa *et al.* 1981), *Hemidesmus indicus* (Sarasan *et al.* 1994), *Araujia sericifera* (Torne *et al.* 1997), *Heracleum candicans* (Wakhlu and Sharma 1998), *Eryngium foetidum* (Ignacimuthu *et al.* 1999) and *Bupleurum falcatum* (Bang *et al.* 1999). However, inhibitory effect of cytokinin was also demonstrated (Choi *et al.* 1997). A high amount of an auxin is needed to stimulate the somatic embryogenesis in most of the cases; lower levels in subsequent cultures is reported to improve the morphological and physiological development of the embryos (Hirai *et al.* 1997; Patil 1998; Ara *et al.* 2000a).

Maturation and germination of somatic embryos are the two major steps for the eventual recovery of healthy plants. In most of the cases, the maturation and conversion occurs on transfer of the embryos to the medium with reduced level of salts either without growth regulators (Sarasan *et al.* 1994; Merkle *et al.* 1995; Sunnichan *et al.* 1998; Joseph *et al.* 1999) or with low level of growth regulators (Rao *et al.* 1970; Rao and Narayanaswamy 1972; Sehgal and Abbas 1994; Sinha *et al.* 2000).

Additives, the undefined supplement's role in morphogenesis have been emphasized in the dawn of plant tissue culture history. However, the efficacy of

additives remains as strife especially in somatic embryogenesis. The effectiveness of additives such as CW, CH, YE and AC has been widely investigated in the carrot family. CW continues to be extremely useful in embryo induction and maturation (Rao and Narayanaswamy 1972; Castillo and Smith 1997; Zhao *et al.* 1999; Sinha *et al.* 2000). The role of CH, the reduced nitrogen source in somatic embryogenesis has been emphasized in carrot (Reinert 1958; Ammirato 1977), celery (Reinert *et al.* 1966), *Tylophora* (Rao and Narayanaswamy 1972) and coriander (Chen *et al.* 1995).

**Protoplast**

Protoplast isolation, culture and plant regeneration was reported in many medicinal plants. Most of the reports envisage that the nutritional requirements of the cultured plant cells and protoplasts are similar. As on line with micropropagation, MS medium with or without modification is reported as the most prevalent medium (Benmoussa *et al.* 1997; Zhang *et al.* 1998). A perusal of literature shows the authenticity of the use of an osmotic agent with respect to the isolation and recovery of protoplasts and mannitol is widely explored (Nakano *et al.* 1995; Giri and Reddy 1994, 1998). Plant regeneration from protoplasts has been reported either through organogenesis as in *Gentiana* sp. (Nakano *et al.* 1995), pinellia (He *et al.* 1996), peppermint (Jullien *et al.* 1998) and *Moricandia nitens* (Tian and Meng 1999) or through somatic embryogenesis as in fennel (Miura and Tabata 1986), *Hyoscyamus muticus* (Giri and Ahuja 1990), sweet potato (Dhir *et al.* 1998), *Bupleurum falcatum* (Bang *et al.* 1999) and mango (Ara *et al.* 2000b). Protoplast fusion and efficient regeneration of hybrids of many plants has also been reported (Tamura *et al.* 1998; Dabuza *et al.* 1998; Chen and Adachi 1998; Jarl *et al.* 1999; Kochevenko *et al.* 2000).

**Photoautotrophic micropropagation**

Photoautotrophic micropropagation was first described by Kozai (1991). Endeavours are at realm to develop autotrophic plantlets to make plant tissue culture techniques more economic. The difficulty encountered with the *in vitro* culture is the covert contamination (Leifert *et al.* 1994; Mantell 1998; Kamoun *et al.* 1998). Nevertheless, the photoautotrophic micropropagation minimize the risk of contamination in cultures, which in turn will reduce the production cost abreast to the better acclimatization under *ex vitro* condition (Kozai 1991; Jeong *et al.* 1995; Pospisilova *et al.* 1997; Mitra *et al.* 1998a, b).

**Synseed**

The concept of synseed by Murashige (1978) is brought about by Kitto and Janick (1985) in carrot. According to Gray and Purohit (1991) synthetic seed is a somatic embryo that is engineered to be of practical use in commercial plant production. The term now at the broadest, means an artificially encapsulated vegetative propagule capable to develop into a complete plant *in vitro* and *ex vitro* (Aitken-Christie *et al.* 1995). Artificial seeds, the low cost and high volume system (Redenbaugh 1990) bypass many of the growth and acclimatization steps normally ascertained with micropropagated plants. Encapsulation matrix, potent to serve as a reservoir of nutrients as artificial endosperm and supply of necessary nutrients to the encased embryo or shoot (Pattnaik *et al.* 1995). Synseeds facilitates the exchange of capsules in sterile condition between labs and also for the germplasm conservation with proper techniques (Maruyama *et al.* 1997; Chetia *et al.* 1998; Datta *et al.* 1999; Naidu and Sreenath 1999).

Retrieval of plantlet has been reported either from encapsulated somatic embryos (Wake *et al.* 1995; Castillo *et al.* 1998; Antonietta *et al.* 1999) or shoot buds (Maruyama *et al.* 1997; Standardi and Piccioni 1998; Micheli *et al.* 1998).

Axillary buds of *Morus indica* (Bapat *et al.* 1987) and adventitious buds of *Morus alba* (Machii 1992) have been encapsulated in alginate or agar beads and were stored for 34-80 days at 4°C without loss of viability. Datta *et al.* (1999) stored encapsulated protocorm of *Geodorum densiflorum* at 4°C for 120 days without the loss of viability.

**Conservation *in vitro***

Plant tissue culture offers rapid multiplication of elite and rare plant species and also an alternative method of *ex situ* conservation. There are different strategies for the conservation of germplasm *viz.* (i) cryopreservation and (ii) slow or medium growth system (Monotte 1995). Slow growth strategies, which delay the transfer to fresh medium, range from temperature reduction to manipulation of other conditions of culture environment including chemical constituents of culture media is termed as "medium term storage" (Reed 1995). The enormous potential of applying minimal or zero growth strategies for maintaining non-seed germplasm has been recognized for many years (Maruyama *et al.* 1997; Chetia *et al.* 1998; Datta *et al.* 1999).

Withdrawal of sugar from growing systems can minimize cell proliferation dramatically (Van't Hof 1966). Critical lowering or depletion of a particular nutrient might be useful for cell culture management (Krikorian 1996). Photoautotrophic micropropagation as mentioned earlier is an ample choice of conservation in particular at whole plant level without any jeopardy of contamination (Mitra 1998a,b).

**Secondary metabolites**

Last few years had witnessed manifold explorations of plant tissue culture, which facilitated further studies on biosynthesis, regulation and accumulation of the natural products at commercial level. Unorganized plant cell cultures are

known to synthesize and transform a wide range of secondary metabolites (Bouque *et al.* 1998; Laurain-Mattar *et al.* 1999; Caruso *et al.* 2000). The production of secondary metabolites has been reported in many medicinal plants. For examples, anthraquinones from *Morinda elliptica* (Abdullah *et al.* 1998), anitmicrobial flavonoids from *Glycyrrhiza glabra* (Li *et al.* 1998), indole alkaloids from *Rauwolfia sellowii* (Rech *et al.* 1998), tropane alkaloids from *Datura metel* (Cusido *et al.* 1999), dianthin from *Dianthus caryophyllus* (Messeguer *et al.* 1999), galphimine-b from *Galphima glauca* (Osuna *et al.* 1999), littorine and hyoscyamine from *Datura stramonium* (Zabetakis *et al.* 1999), carnosic acid from *Rosmarinus officinalis* (Caruso *et al.* 2000) and taxane from *Taxus cuspidata* (Son *et al.* 2000). The morphogenic differentiation such as root (especially in the case of root as officinal part) or/and shoot has been reported to enhance production of secondary compounds (Selles *et al.* 1999; Pepin *et al.* 1999). Plant cell cultures are also known to produce secondary metabolites not known to occur in the plant intact (Vanek *et al.* 1999; Furmanowa *et al.* 1999; Yamamoto *et al.* 1999).

**Genetic transformation**

Transformation, the simple way to transgenesis is the cynosure of the ongoing research in tissue culture. It is bewitched by controlled and rapid route to the creation of new characteristics in plants than traditional breeding techniques and it serves to increase the diversity of potential source of germplasm for further recombination and also at the production level. The advent of recombinant DNA (r-DNA) technology hand-in-hand with the cell and tissue culture techniques enabled the researchers for the introduction of alien genes and their expression at whole plant level. Much progress has been made during the last few years in transgenic plant research with a spellbound critique on the **terminator gene technology**.

Recent advances in tissue culture and r-DNA technologies have opened

new avenues for the transformation of higher plants, which in turn produced many transgenic plants with the acquisition of new properties (Tabei *et al.* 1998; Ding *et al.* 1998; Alam *et al.* 1999; Mohapatra *et al.* 1999; Sentoku *et al.* 2000). Various workers have reviewed the boundless uses and applications of transgenic plants (Uchimiya *et al.* 1989; Hammond 1999). Transformation has successfully been accomplished in several plants by different methods, either by vector or non-vector mediated methods (Table IV). *Agrobacterium* spp. mediated genetic transformation of plants has now become a habitual technique. *A. rhizogenes*, the naturally occurring plant pathogen is responsible for the adventitious root formation (hairy roots) at the site of its infection (Mukherjee *et al.* 1995; Giri *et al.* 1997; Sasaki *et al.* 1998; Kifle *et al.* 1999; Yang and Choi 2000; Ohara *et al.* 2000).

Of the different co-cultivation methods involving *A. rhizogenes*, most of the workers adopted the leaf disc transformation method demonstrated by Horsch *et al.* (1985) and which offers a conventional way to obtain large number of clonal transformants of several medicinal plants. At molecular level, the transferred DNA (T-DNA) region of the **root inducing** (Ri) plasmid of *A. rhizogenes* would be integrated into the plant (host) genomic DNA and the gene on the T-DNA be expressed in transformed tissue/plant (Daley *et al.* 1998; Nussbaumer *et al.* 1998; Elliott *et al.* 1999; Kifle *et al.* 1999; Ohara *et al.* 2000; Yang and Choi 2000). Subsequently, the T-DNA would faithfully be transmitted to the descendants of the transformed plant (Nussbaumer *et al.* 1998; Kifle *et al.* 1999; Elliott *et al.* 1999).

One of the major constraints in transformation experiments is the identification of the transformed tissues/plants. Nevertheless, the characterization of different marker as well as reporter genes has made the selection protocols for the transformed tissue/plants much efficient. Among the different reporter genes,

**Table IV** Transformation studies during the last few years

| Name of the plant | Method of Transformation | References |
|---|---|---|
| *Allium sativum* | Biolistics | Barandiaran *et al.* 1998 |
| *Arachis hypogaea* | *A. tumefaciens* | Venkatachalam *et al.* 2000 |
| *Beta vulgaris* | *A. rhizogenes* & *A. tumefaciens* | Kifle *et al.* 1999 |
| *Castanea sativa* | *A. tumefaciens* | Seabra & Pais 1998 |
| *Coleus forskohlii* | *A. rhizogenes* | Sasaki *et al.* 1998 |
| *Crotalaria juncea* | *A. rhizogenes* | Ohara *et al.* 2000 |
| *Daucus carota* | *A. rhizogenes* | Guivarc'h *et al.* 1999 |
| *Datura candida x D. aurea* | *A. rhizogenes* | Nussbaumer *et al.* 1998 |
| *Datura stramonium* | *A. tumefaciens* | Baiza *et al.* 1998 |
| *Digitalis lanata* | *A. rhizogenes* | Pradel *et al.* 1997 |
| *Hyoscyamus muticus* | *A. rhizogenes* | Sevon *et al.* 1997 |
| " | *A. tumefaciens** | Merritt *et al.* 1999 |
| *Hyssopus officinalis* | *A. tumefaciens* | Murakami *et al.* 1998 |
| *Lilium longifolium* | Biolistics | Watad *et al.* 1998 |
| *Lavandula x intermedia* | *A. tumefaciens* | Dronne *et al.* 1999a |
| *Mentha x piperita* | *A. tumefaciens* | Niu *et al.* 1998 |
| *Nicotiana tabacum* | *A. rhizogenes* | Palazon *et al.* 1998 |
| " | Biolistics | Elliott *et al.* 1999 |
| *Panax ginseng* | *A. rhizogenes* | Yang & Choi 2000 |
| *Pinus strobus* | Biolistics * | Tian *et al.* 1997 |
| *P. pinea* | *A. tumefaciens* | Humara *et al.* 1999 |
| *Solanum aviculare* | *A. tumefaciens* | Kittipongpatana *et al.* 1998 |

* Expression of ***gfp***

Others Expression of ***gus***

bacterial ***uid A*** (***gus A***) coding the β-glucuronidase has been explored widely for the identification of transformants (Table IV). However, with the isolation of the gene coding for the green fluorescent protein (***gfp***) from Jellyfish (*Aequorea victoria*) by Chalfie *et al.* (1994), the potential use of ***gfp*** as a universal marker has gained great momentum. Other leading reporter genes in vogue are those coding for luciferase (***luc***) (Owe *et al.* 1986; Raemakers *et al.* 1996; Baruah-Wolff *et al.* 1999) and ***sweetner*** (Barton 1997).

Many medicinal plants, especially dicotyledons have been susceptible to *A. rhizogenes* and thus regenerating transgenic plants (Tepfer 1990; Christey 1997; see Table IV). Development of transgenic plants *via* organogenesis and somatic embryogenesis has well documented with the expression of different reporter and selection cassettes in many plants, as summarized in Table IV.

Unlike *A. tumefaciens*, *A. rhizogenes* mediated transformed plants are reported to have absolute clonal fidelity. The hairy roots potential for secondary metabolite production is an added advantage. Hairy root culture technology thus offers a new route of secondary metabolites production, wherein roots are being used as the officinal part (Giri *et al.* 1997; Pradel *et al.* 1997; Sasaki *et al.* 1998; Li *et al.* 1998; Yang and Choi 2000). Hairy roots induced on many medicinal plants have widely been studied for the *in vitro* production of secondary plant products (Baiza *et al.* 1998; Murakami *et al.* 1998; Tanaka *et al.* 1999; Zarate 1999). Bioreactors for hairy root culture have also been developed for the industrial scale production of commercially important secondary metabolites (Lee *et al.* 1999; Liu *et al.* 1999).

# MATERIALS AND METHODS

# MATERIALS AND METHODS

*In vitro* culture studies such as direct and indirect organogenesis, somatic embryogenesis, protoplast culture, transgenic plant regeneration, etc., have great importance in the propagation of medicinal plants of pharmaceutical relevance. Taking this into account, two medicinally important plants, *Eryngium foetidum* and *Holostemma ada-kodien* were selected for the present investigation.

## CHEMICALS AND CULTURE VESSELS

Analytically pure chemicals and growth regulators (Qualigens, Merck, BDH, Sigma etc.) were used for the preparation of stock solutions of different media. Analytical grade sucrose (BDH) at the range of 0-5 % was used as the carbohydrate source. The trade names were given accordingly in the case of certain other chemicals when used.

Glassware *viz.* culture tubes (25x100 mm, 25x200 mm), conical flasks (100 and 250 ml) of Borosil and Corning glass and jam bottles (500 ml) were used for the *in vitro* studies. Glassware were washed with a detergent (extran) followed by thorough washing with tap water. Cleaned glassware were rinsed with double distilled water and dried in hot air oven at 100°C. The culture tubes and conical flasks were plugged with non-absorbent cotton covered with cotton gauze. The jam bottles were capped with aluminium or polypropylene (autoclavable) closures. Suspension cultures were carried out in 100 ml conical flasks plugged with non-absorbent cotton.

**MEDIA**

Three different media *viz.* Murashige and Skoog (1962), Gamborg's ($B_5$) (Gamborg *et al.* 1968) and White's (White 1963) were tested for the present study (Table V). The basal media were manipulated with different auxins (2,4-D/ NAA/IAA/IBA), cytokinins (BAP/Kin), gibberellins ($GA_3$), $AgNO_3$, $CoCl_2$ and various additives (CW/CH/CT/AA/AC) in different concentrations either singly or in combinations. Photoautotrophic micropropagation was carried out on MS medium without sucrose (hereafter referred as photoautotrophic medium).

**PREPARATION OF MEDIA**

The stock solutions of macro and micronutrients, iron chelates and vitamins of different media were prepared separately. The stock of iron chelates was stored in an amber coloured bottle. Different growth regulators were separately dissolved in respective solvents made up with double distilled water. All the stock solutions were stored in refrigerator at 3 - 4°C.

Required stock solutions were pipetted out and final volume of the medium was made up with double distilled water with or without the addition of carbohydrate source and growth regulators/additives depending on the nature of the experiments. Prior to autoclaving the pH of the medium was adjusted to 5.6-5.8 (for MS and $B_5$) and 5.4-5.6 (for White's) using 1N NaOH or 1N HCl solution. About 10 and 25 ml of the medium was dispensed in the culture tubes and conical flasks (100 ml) respectively. About 40-50 ml medium was dispensed into jam bottles and conical flasks (250 ml). In the case of suspension cultures about 25 ml of medium was transferred to each conical flask. The medium was gelled with 0.8 % agar (w/v) in the case of solid medum. All the media were sterilized at a pressure of 1.06 $Kg/cm^2$ in an autoclave.

**Table V COMPOSITIONS OF CULTURE MEDIA ($mgl^{-1}$)**

| Ingredients | MS | $B_5$ | White's |
|---|---|---|---|
| $(NH_4)_2SO_4$ | | 134 | |
| $(NH_4)NO_3$ | 1,650 | | |
| $KNO_3$ | 1,900 | 2,500 | 80 |
| $(CaNO_3)_2$ | | | 300 |
| $CaCl_2.2H_2O$ | 440 | 150 | |
| $MgSO_4.7H_2O$ | 370 | 250 | 720 |
| $Na_2SO_4$ | | | 200 |
| $KH_2PO_4$ | 170 | | |
| $NaH_2PO_4.H_2O$ | | 150 | 16.5 |
| KCl | | | 65.0 |
| $FeSO_4.7H_2O$ | 27.8 | 27.8 | |
| $Na_2EDTA$ | 37.3 | 37.3 | |
| $Fe_2(SO_4)_3$ | | | 2.5 |
| $MnSO_4.4H_2O$ | 22.3 | | 7.0 |
| $MnSO_4.H_2O$ | | 10.0 | |
| $ZnSO_4.7H_2O$ | 8.6 | 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-pantothenic acid | | | 1.0 |
| Sucrose | 30,000 | 20,000 | 20,000 |
| pH | 5.8 | 5.5 | 5.5 |

## EXPLANTS AND SURFACE STERILIZATION

For the establishment of different cultures, the explants were collected from the field grown plants in Calicut University Botanic Garden. Roots, stem, leaves and peduncle (scape) segments were used as explants in the case of *Eryngium foetidum*. While in the case of *Holostemma ada-kodien*, root, stem and leaf were used as explants. The stem explants in *Holostemma ada-kodien* consists of both nodal and internodal segments. The nodal explants were collected from three different regions of the stem as basal, middle and apical. For photoautotrophic micropropagation, protoplast isolation and transformation studies, the explants were excised from the *in vitro* grown plants.

The source tissues were washed under running tap water followed by a detergent, extran (5 % v/v) for 5-10 min. After thorough washing with double distilled water, surface sterilization of the explants was done with mercuric chloride solution (0.1 % w/v) for 7-10 min. After decanting the sterilant, the materials were washed thoroughly with double distilled water. The sterilized explants were cultured on different medium and all the cultures were incubated at 25±2°C with 16/8 h photoperiod under the white fluorescent tubes (2000 lux), unless otherwise stated.

## PROTOPLAST STUDIES

### (i) Isolation and Purification

Protoplast isolation was attempted with the mesophyll cells of young leaves of *in vitro* grown plants of both *E. foetidum* and *H. ada-kodien*.

Combinations of enzymes Cellulase Onozuka (Yakult) and Macerozyme R-10 (Yakult) at various concentrations were used for the isolation of protoplasts from the leaf mesophyll cells. Table VI and VII represents the composition of

CPW salt solutions (Frearson *et al.* 1973) and enzyme solution respectively. CPW 13 M and CPW 21S solutions were prepared by the addition of 13 % mannitol and 21 % sucrose to the CPW salts.

The fully expanded leaves (near to the apex) were excised from the *in vitro* grown plants for the isolation of the protoplasts. The lower epidermis of the leaves was peeled off by jeweller's/pointed forceps and were cut into small pieces, which later transferred to a 90 mm sterile petridish (Tarson) containing CPW 13 M (1 g/10 ml) The peeled surface was exposed to the solution. After 30 min, the CPW 13 M solution was pipetted off from underneath the leaf pieces and replaced by 10 ml of filtered sterile enzyme solution (through Millipore filter unit, Millex - HA 0.45 µm) by sterile Pasteur's pipette. The petridishes containing the leaf pieces were sealed with parafilm and incubated in dark at 25±2°C and agitated on a rotary shaker at 30-50 rpm. After the incubation period (10-16 h) the protoplast enzyme solution was filtered through a nylon mesh of 64 µ sieve into a sterile plastic centrifuge tube. The protoplast suspension was centrifuged at 80 g for 5-7 min at 1000 rpm. Protoplasts were sedimented at the bottom of the centrifuge tube. The supernatant (enzyme solution) was removed with the help of sterile Pasteur's pipette and transposed with 10-12 ml of CPW 21S solution. The CPW 21S solution with the protoplasts was centrifuged at 100 g for 10 min at 2000 rpm. The protoplasts which formed a green ring/band at the surface of the CPW 21S solution in the centrifuge tube were carefully transferred to fresh sterile tube, to which 10 ml of CPW 13 M was added. The debris sedimented at the bottom of the tube was eliminated. The suspension of the protoplasts in CPW 13 M solution were centrifuged at 80 g for 5-7 min at 1000 rpm and replaced by PCM (consists of MS + 9 % mannitol + 3 % sucrose with different growth regulators as mentioned in text). The protoplasts were counted by using a Haemocytometer.

**Table VI Composition of CPW salt solution** (Frearson *et al.* 1973)

| Components | Concentration ($mgl^{-1}$) |
|---|---|
| $KH_2PO_4$ | 27.2 |
| $KNO_3$ | 101.0 |
| $CaCl_2.2H_2O$ | 1480 |
| $MgSO_4.7H_2O$ | 246 |
| KI | 0.16 |
| $CuSO_4.5H_2O$ | 0.25 |

pH - 5.8

**Table VII Composition of enzyme solution**

| Enzymes and other components | Concentration % (w/v) |
|---|---|
| Cellulase ONOZUKA RS | 0.1 – 1.0 |
| Macerozyme ONOZUKA R10 | 0.3 |
| MES Buffer | 2.0 mM |
| Mannitol | 9.0 |
| CPW salts | as required |

pH - 5.8 - 6.1

### (ii) Protoplast Culture

The protoplasts have been cultured in alginate beads as per the method followed by Giri and Reddy (1994). In alginate bead culture of the protoplasts, equal volumes of 2.4 % (w/v) sodium alginate solution with 9 % mannitol and double strength of protoplast culture medium containing the protoplasts were mixed. The beads were prepared by dropping the alginate solution with the purified protoplasts by a Pasteur's pipette into the solution of 0.7 % $CaCl_2$ + 9 % mannitol (w/v) in a petridish or small beaker. After 10-15 min, the $CaCl_2$ solution was replaced by PCM. The beads with the protoplasts were cultured on corresponding PCM gelled with 0.8 % agar in sterile petridishes. The cultures were incubated in the dark till microcalli appeared in the beads, later transferred to the light.

The protocalli were excised either by dissecting the beads with a sterile scalpel or by dissolving the beads in sterile 0.1 M tri-sodium citrate solution. In the latter case, the callus was washed thrice with sterile double distilled water to remove all the traces of sodium citrate. The calli separated from the beads were cultured for the induction of organogenesis or somatic embryogenesis on MS medium fortified with different growth regulators/additives either alone or in combinations at various concentrations.

### ENCAPSULATION OF EMBRYOS /SHOOT BUDS

Encapsulation studies were conducted by using singulated cotyledonary embryos in *Eryngium foetidum* and with mature embryos and embryo like micropropagated shoot buds (unipolar) in *Holostemma ada-kodien*. Encapsulation matrix was prepared by adding sodium alginate (BDH) at various levels (1.5, 2.0, 2.5 and 4%) in MS medium. The medium was without $CaCl_2$ and also with or without sucrose (1.5%) as specified in the text. The pH was adjusted

to 5.6-5.8. Various levels of $CaCl_2$ (0.5, 0.7 and 1 %) were prepared in double distilled water. The encapsulation matrices and $CaCl_2$ solutions were sterilized in an autoclave and all further steps were carried out under aseptic condition.

For encapsulation, the embryos/shoot buds were blot dried and transferred to the encapsulation matrices. With a sterilized pipette having 0.5-1.0 cm diameter at the tip, the alginate solution with the propagules was drawn and dropped into $CaCl_2$ solution. Each drop was adjusted to enclose a single embryo or shoot bud. They were allowed to remain in the $CaCl_2$ solution for 15-30 min. After the exposure period, the $CaCl_2$ solution was decanted off and the beads were washed thrice with sterile double distilled water.

In the case of *E. foetidum*, to study the seed efficacy of encapsulated embryos, they were cultured *ex vitro* on soilrite + sand (1:1).

**CONSERVATION *IN VITRO***

*In vitro* conservation studies were carried out both at synseed and at whole plant level. Conservation at synseed level was carried out by encapsulating the propagules in different encapsulation matrices and culturing them on germination matrices as specified in text. Two temperature regimes, 4°C and 25±2°C were carried out to study the effect of temperature in preservation virtue. The viability of the propagules was tested at 3, 6, 9 and 12 months. In both the plants, the conservation studies were conducted at the whole plant level. The effect of $AgNO_3$ in conservation was studied with the addition of various levels to half MS with or without auxin.

**SUBCULTURE**

Subculturing of the callus and multiple shoots were carried out at different intervals on solid or liquid medium.

## ROOTING

The excised shoots were transferred onto root induction medium at proper time. The shoots without roots developed in *E. foetidum* were transferred to either full or half MS without hormone. But in *H. ada-kodien*, the well-developed shoots were transferred to either solid or liquid (static) media of full or half MS without hormone or with different auxins (IBA/IAA/NAA) alone, or in combination with cytokinin/CW.

## TRANSPLANTATION AT FIELD CONDITION

The rooted shoots with sufficient size, taken out from the culture vessels were washed thoroughly in running tap water (in the case of plantlets grown on solid medium) and were directly transferred to small pots containing sterile soilrite and sand in the ratio 1:1. The synseeds of *E. foetidum* sown *ex vitro* directly in petridishes containing sterile soilrite and sand (1:1) were ceded to pots later.

## HISTOLOGICAL STUDIES

Different shoot developmental stages and the embryonal stages were fixed in Formalin-Acetic acid-Alcohol (FAA) (Johansen 1940) for the serial microtome section for observing the developmental pattern. The fixed materials were dehydrated through a graded series of Tertiary Butyl Alcohol (TBA), followed by infiltration in paraffin. Embedding of the tissues were done in molten paraffin (58-60°C) with ceresin. Serial sections of 8-10 µ, taken in a rotary microtome (Leitz, Germany) were deparaffined with xylene and dehydrated in ethanol series. The slides were stained with erythrosin/haematoxylin (Delafield's) combination and mounted in DPX mountant.

## STATISTICAL ANALYSIS

All experiments in the present study were conducted in a completely randomized design. The average and standard deviation were calculated according to the method of Misra and Misra (1983). The data represents an average of ten replicates, unless otherwise stated.

## SECONDARY METABOLITE ANALYSIS

The comparative analysis of the alkaloids present in the roots of *in vitro* and field grown plants of both the experimental plants were performed by TLC.

The fresh roots of the field grown plants of both *E. foetidum* and *H. ada-kodien* were collected separately and washed thoroughly in ice cold water. The roots of the *in vitro* growing somatic embryo derived plantlets of *E. foetidum* and *in vitro* formed tuberous roots of *H. ada-kodien* were collected separately. Blot dried root tissues (5 g) of each sample of *E. foetidum* and *H. ada-kodien* were hashed into small pieces and the tissue extractions were made separately with non-polar (petroleum ether- 58-66°C) and polar (distilled ethyl alcohol) solvent. The extracts of both *in vitro* and field grown plants were made in each solvent separately. Acid washed sand was used to grind the tissues properly in 5 g/25 ml solvent. The ground tissue was centrifuged at 2000 rpm for 5 min and the supernatant collected were evaporated and dried. After an addition of 1 ml of the corresponding solvent, they were transferred to small tubes, tightly closed and kept in refrigerator.

Silica gel (G 60) plates were made according to Stahl (1969) by mixing 10 g silica gel (G 60) in 20 ml of double distilled water without trapping air bubbles and was spread on a clean dried TLC plate (20 x 20 cm) with a spreader at a thickness of 0.25 mm. The plates were dried in air and activated at 110°C in hot air oven and allowed to cool.

The samples of each plant in different solvents were applied adjacently on the TLC plate at 5 µl by using a micropipette with the help of a spotting guide. After loading the samples, the plates were allowed to air dry and then kept in a developing tank pre-saturated with chloroform: benzene (1:1). After allowing the solvent to ascent about 15 cm from the sample spot, the plates were taken out and allowed to evaporate the solvent. For visualization of the alkaloids, the plates were sprayed with 5 % conc. $H_2SO_4$ (in ethanol) evenly and allowed to dry, heated at 110°C in hot air oven for 10-15 min for colour development. The charred coloured areas were marked on the plate and the *Rf* value was measured by using the spotting guide.

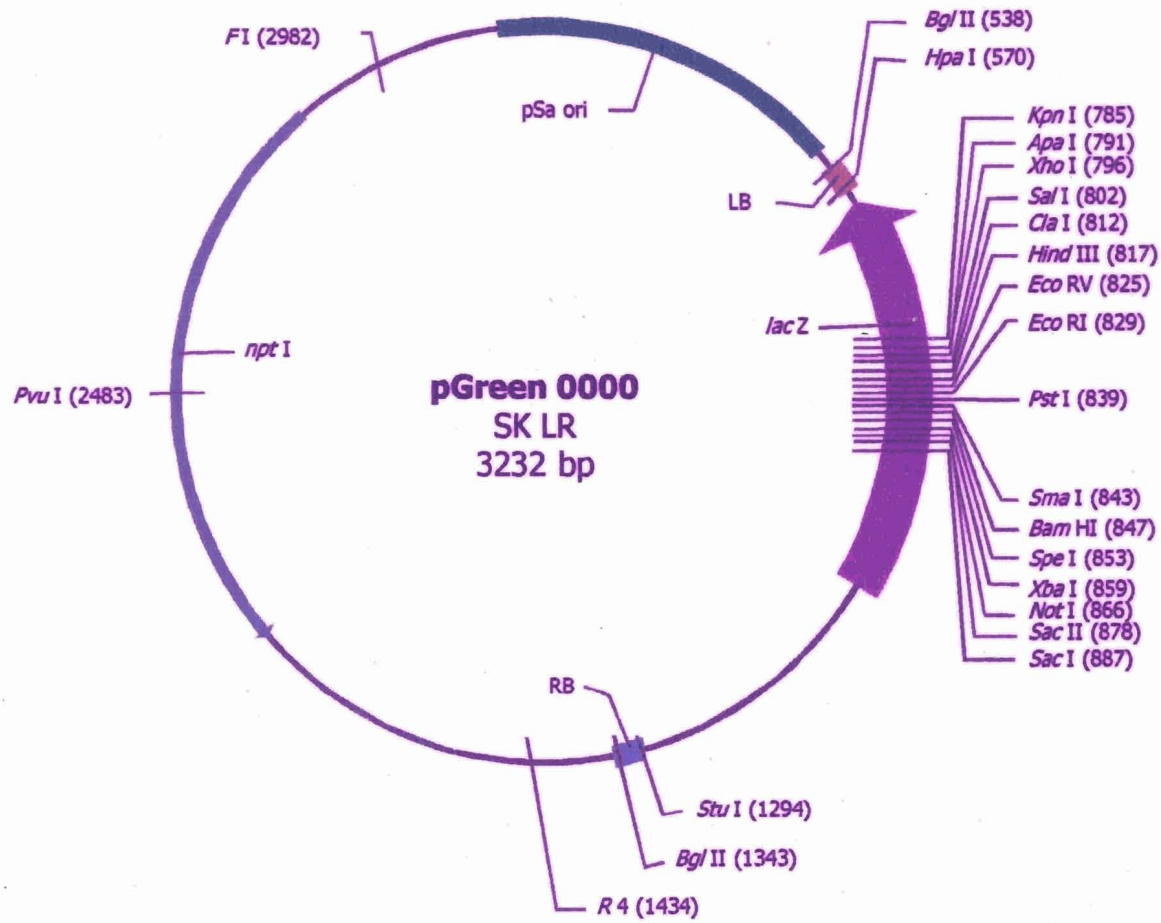
## TRANSFORMATION STUDIES

### *Bacterial strain*

*Agrobacterium rhizogenes* **LBA 9402** (pRi1855), a rifampicin resistant strain harbouring the binary plasmid **pGreen** with ***gus/gfp*** constructs was used for the transformation studies (John Innes Center, UK, has kindly provided the strain as a gift). Schematic representation of the binary plasmid and its constructs are shown in Fig I. To the host cell, the T-DNA confers the gene coding for neomycin phosphotransferase II (***npt II***) driven by the nopaline synthase promoter (***nop***) and an intron coding β-glucuronidase (***gus A***) (Jefferson *et al.* 1987) or ***gfp*** driven by Cauliflower Mosaic Virus (**CaMV**) **35S** promoter. Both ***gus*** and ***gfp*** were used for the assay of the transformants, while ***npt II*** was used as a selection marker of kanamycin sulphate.

For subcultures, one loopful of *A. rhizogenes* master culture was transferred to Yeast Mannitol (YM) liquid medium (Hooykaas *et al.* 1977; Table VIII) in which rifampicin (50 mg $l^{-1}$) and kanamycin (100 mg $l^{-1}$) were added. It was then incubated at 28°C on a rotary shaker (150 rpm). Overnight culture of

Fig 1 Schematic representation of the binary plasmid and the ***gus/gfp*** construct



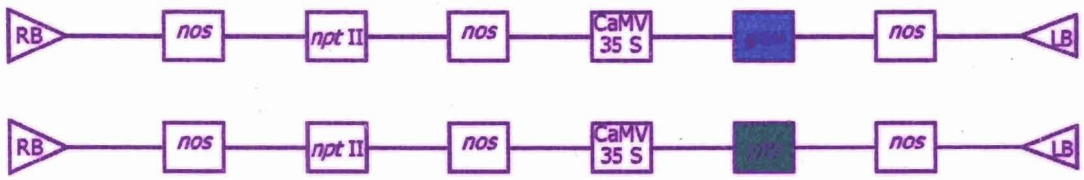



**Table VIII Yeast Mannitol (YM) Medium** (Hooykaas *et al.* 1977)

| Components | Amount (%) w/v |
|---|---|
| Yeast Extract | 0.04 |
| $K_2HPO_4$ | 0.05 |
| $MgSO_4 7H_2O$ | 0.02 |
| NaCl | 0.01 |
| Mannitol | 1.00 |

pH 7.0

the bacteria (OD about 0.5) was used for the co-cultivation. The OD of the suspension was adjusted at 550 nm using a spectrophotometer (Spectronic 20D$^+$, Milton Roy).

***Co-cultivation***

For co-cultivation, leaf segments (from both *E. foetidum* and *H. ada-kodien*) and internode (only from *H. ada-kodien*) were excised (with incisions here and there) from the *in vitro* grown plants and placed on hormone free MS medium in petri-plates. Bacterial suspension of 20 µl was dropped on the explants, using a micropipette (Glaxo) and then the petri-plates were sealed with parafilm. After 3 days of incubation in dark, the explants were washed (5 min) with a solution of antibiotic, cefotaxime (500 mg $l^{-1}$) and dried with No.I sterile Whatmann filter paper and then transferred to hormone free MS medium, supplemented with cefotaxime (500 mg $l^{-1}$). For the complete elimination of *A. rhizogenes*, the co-cultivated explants were transferred thrice in succession to fresh medium in an interval of 4 days, containing the same antibiotic.

**Selection and Culture of Transformed Roots**

Transformed (hairy) roots were selected from the roots developed on co-cultivated explants, upon transfer to MS medium with different levels of kanamycin sulphate (25 - 150 mg $l^{-1}$). Such hairy roots obtained from *E. foetidum* cultures were excised and transferred to the callus inducing medium (MS + 1.5 mg $l^{-1}$ BAP), while that of *H. ada-kodien* were transferred to the medium containing MS + 1.0 mg $l^{-1}$ 2,4-D. The calli so obtained were subcultured for organogenesis and somatic embryogenesis on the corresponding medium standardized for each, as described elsewhere.

In all experiments, the antibiotic was individually added to the sterile medium, using a Millipore unit (Millex - HA 0.45 µm).

### *Gus/gfp assay*

Histochemical assay of ***gus*** gene product was carried out according to the method of Jefferson *et al.* (1987) using X-gluc stain. One ml of X-gluc stain (5-bromo 4-chloro 3-indolyl-β-glucuronide salt) was prepared by adding 500 µl 2x buffer to 50 µl of X-gluc stock solution and was made up to 1 ml with sterile double distilled water. X-gluc stock was prepared by adding 960 µl of dimethyl sulphoxide (DMSO) to 10 mg of X-gluc powder. The 2x buffer was prepared by mixing 200 µl of 0.5 M ethylene dimethyl tetra acetate (EDTA) and 100 µl of Triton X-100 and was made up to 5 ml with 50 mM $NaH_2PO_4$ (pH 7). The tissues such as plantlet, leaf and embryo were incubated overnight in the X-gluc solution at 37°C, for the visual observation of the blue colour. The leaf tissues were bleached with several washes in 70 % ethyl alcohol. Expression of ***gfp*** has been observed by exposing the callus directly to UV radiation for 24 h keeping on the laminar-air-flow chamber using a UV lamp (Philips 40 W).

## PHOTOGRAPHY

The photographs of the present studies were taken using a Pentax K1000/Nikon camera. The photomicrographs of the histological specimens were taken using a microscope with inbuilt camera (Leitz, Germany). The photographs of the isolated protoplasts were taken using a dark field Phase-contrast microscope (Nikon, Japan).

# OBSERVATION

# OBSERVATION

Present investigations of micropropagation and transgenic plant regeneration were carried out on two medicinally important plants *viz. Eryngium foetidum* (Apiaceae) and *Holostemma ada-kodien* (Asclepiadaceae).

## *ERYNGIUM FOETIDUM*

For establishing an efficient protocol for organogenesis and somatic embryogenesis on *E. foetidum*, the explants such as root, stem (discs, 0.3 - 0.5 cm thick), leaf and peduncle (scape) isolated from field grown plants were cultured on different media supplemented with varied hormonal combinations.

### DIRECT ORGANOGENESIS

Attempts were made for the initiation of direct shoot regeneration with different basal media and the media augmented with varied hormonal combinations.

#### Effect of Basal Media

For the induction of multiple shoots, different explants were cultured on MS, half MS, $B_5$ and White's media without any hormone. Of the three media tested, MS medium was found superior to the other two, showed an average of 3 shoots/stem disc, followed by $B_5$ medium (Table IX, Fig II A). Shoots were initiated within 15 days of culture and attained 5-6 leaves within 40 days with two or three roots from the basal end. Roots were initiated directly from the explants also. Induction of shoots was late for three weeks on $B_5$ medium and the shoots remained very small with two or rarely three narrow leaves (Fig II B). No root

formation obtained even after 40 days on $B_5$ medium. On White's medium, the stem discs induced only roots.

The leaf (petiolar) explants responded only on MS medium, induced shoots (one, rarely two) and more than five roots directly from the explant (Fig II C).

In the case of root explants, both MS and half MS were effective to produce more or less same number of shoots (2-4) within 25 days (Fig II D) followed by White's and $B_5$ media.

The scape explants also induced a more or less same number of shoots as in stem disc (Table IX). As MS basal medium was found superior to the other two media, the same was used for further studies.

**Effect of Growth Regulators**

Effect of different growth regulators was also studied with different explants of *E. foetidum* for direct shoot induction.

**Effect of Cytokinins**

MS medium supplemented with BAP or Kin either singly or in combination or with auxins (NAA/IAA) was used to establish direct organogenesis on different explants.

**Effect of BAP/Kin**

For the initiation of multiple shoots on different explants, MS medium fortified with various concentrations of BAP/Kin (0.1–5.0 mg $l^{-1}$) was tested (Table IX). Of the different concentrations of BAP tested, it was found that BAP at 3.0 mg $l^{-1}$ was more effective for multiple shoot formation. Maximum number of shoot formation was achieved from scape explants (Table IX; Fig II E). The

**Table IX** Effect of basal media and growth regulators/CW in the induction of direct shoots from different explants of *Eryngium foetidum*

| Media | Growth Regulators (mg $l^{-1}$) BAP | Kin | NAA | IAA | CW (%) | Per cent of response R | STD | L | SC | Average No. of shoots ± SD R | STD | L | SC |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| White's | Hormone free | | | | 40 | 0 | 0 | 0 | 0.7±0.2 | 0 | 0 | 0 | |
| $B_5$ | Hormone free | | | | 0 | 60 | 30 | 50 | 0 | 1.9±0.8 | 0.5±0.2 | 1.3±0.4 | |
| MS | Hormone free | | | | 90 | 100 | 80 | 100 | 2.5±0.3 | 2.9±0.2 | 1.2±0.5 | 2.7±0.3 | |
| Half MS | Hormone free | | | | 90 | 90 | 80 | 90 | 2.8±0.5 | 2.1±0.4 | 0.9±0.3 | 2.2±0.4 | |
| " | 0.5* | | | | 100 | 100 | 90 | 90 | 2.1±0.4 | 2.8±0.3 | 1.7±0.3 | 2.9±0.5 | |
| " | 1.0** | | | | 100 | 100 | 90 | 100 | 2.9±0.4 | 3.6±0.5 | 3.1±0.4 | 3.4±0.4 | |
| " | 1.5***** | | | | 100 | 100 | 90 | 100 | 3.1±0.5 | 3.7±0.6 | 3.3±0.3 | 3.8±0.7 | |
| " | 2.0*** | | | | 100 | 100 | 90 | 100 | 3.3±0.4 | 3.9±0.4 | 3.5±0.5 | 4.1±0.5 | |
| " | 2.5** | | | | 100 | 100 | 80 | 100 | 4.1±0.5 | 4.8±0.7 | 4.3±0.6 | 4.9±0.4 | |
| " | 3.0* | | | | 100 | 100 | 90 | 100 | 4.7±0.7 | 8.9±0.9 | 5.3±0.8 | 9.4±0.9 | |
| " | 4.0 | | | | 80 | 100 | 80 | 100 | 3.4±0.3 | 4.9±0.7 | 4.5±0.7 | 7.1±0.6 | |
| " | 5.0 | | | | 80 | 100 | 80 | 90 | 2.8±0.5 | 3.6±0.4 | 3.2±0.5 | 5.7±0.9 | |
| " | | 0.5 | | | 70 | 90 | 70 | 80 | 3.7±0.6 | 4.5±0.7 | 4.2±0.6 | 4.7±0.8 | |
| " | | 1.0 | | | 80 | 90 | 80 | 90 | 5.8±0.9 | 8.7±0.8 | 7.9±0.9 | 9.8±0.7 | |
| " | | 1.5 | | | 90 | 100 | 80 | 90 | 8.9±1.2 | 14.3±1.6 | 10.3±1.8 | 6.5±1.5 | |
| " | | 2.0 | | | 80 | 100 | 70 | 90 | 6.2±0.9 | 11.5±1.7 | 8.6±1.3 | 13.1±1.7 | |
| " | | 2.5 | | | 70 | 90 | 60 | 80 | 5.7±0.6 | 9.8±0.8 | 7.1±0.5 | 10.0±1.2 | |
| " | | 3.0 | | | 80 | 100 | 70 | 90 | 4.9±1.1 | 8.6±0.7 | 6.4±0.9 | 8.9±0.8 | |

Contd.

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| " | | 4.0 | | | | | | 70 | 90 | 70 | 90 | 3.9±0.5 | 5.7±0.8 | 4.9±0.9 | 6.1±0.6 |
| " | | 5.0 | | | | | | 90 | 100 | 80 | 90 | 3.3±0.7 | 5.1±0.5 | 4.1±0.8 | 5.8±0.4 |
| " | | 0.5 | 0.5* | | | | | 80 | 100 | 80 | 90 | 7.8±1.3 | 11.9±1.1 | 6.3±1.3 | 13.0±1.4 |
| " | | 1.0 | 0.5 | | | | | 80 | 100 | 80 | 90 | 10.1±2.6 | 38.6±4.8 | 12.5±3.9 | 13.0±1.4 |
| " | | 0.5 | 1.0 | | | | | 80 | 100 | 80 | 90 | 14.6±2.3 | 51.8±4.2 | 19.2±3.9 | 57.1±4.8 |
| " | | 0.5 | | 2.0 | | | | 70 | 100 | 70 | 80 | 11.2±2.7 | 34.5±3.7 | 17.3±4.7 | 38.4±5.3 |
| " | | 0.5 | | 1.0 | 0.1* | | | 90 | 90 | 80 | 80 | 8.3±1.4 | 23.6±2.9 | 5.7±3.6 | 26.3±3.2 |
| " | | 0.5 | | 1.0 | 0.5* | | | 90 | 100 | 80 | 100 | 6.3±1.2 | 12.3±2.1 | 10.2±1.9 | 17.1±2.3 |
| " | | 0.5 | | 1.0 | 1.0** | | | 80 | 100 | 80 | 90 | 4.8±0.9 | 10.7±1.6 | 6.6±1.1 | 11.3±1.5 |
| " | | 0.5 | | 1.0 | | 0.1 | | 90 | 100 | 80 | 100 | 7.9±1.2 | 19.9±2.3 | 13.4±1.9 | 21.2±3.7 |
| " | | 0.5 | | 1.0 | | 0.5* | | 70 | 100 | 80 | 90 | 5.8±0.9 | 9.8±1.8 | 8.4±1.3 | 14.7±3.2 |
| " | | 0.5 | | 1.0 | | 1.0** | | 80 | 100 | 80 | 100 | 5.2±1.1 | 11.6±1.4 | 7.2±1.2 | 12.5±3.3 |
| " " | | | | | | | 5.0 | 70 | 90 | 80 | 80 | 2.9±0.4 | 3.8±0.6 | 2.7±0.3 | 4.3±0.5 |
| " | | | | | | | 10.0 | 80 | 90 | 80 | 90 | 4.1±0.6 | 5.3±0.4 | 3.6±0.8 | 5.6±0.5 |
| " | | | | | | | 15.0 | 70 | 80 | 80 | 90 | 3.9±0.7 | 4.8±0.6 | 3.1±0.3 | 5.1±0.3 |
| " | 0.5 | 1.0 | | | | | 10.0 | 90 | 90 | 80 | 80 | 13.3±2.5 | 52.4±5.3 | 19.3±4.1 | 57.3±5.1 |
| " | 1.0* | 0.5 | | | | | 10.0 | 80 | 100 | 80 | 90 | 9.7±1.3 | 30.2±4.2 | 12.5±1.7 | 35.7±5.9 |

Data represents an average of 10 replicates
Growth period 40 days
R – Root STD – Stem Disc L – Leaf SC – Scape
* Amount of callus

stem and scape explants cultured with BAP (3.0 mg $l^{-1}$) showed their first response as a green ring in the region mid way between the epidermis and pith, within 10 days. In the case of leaf explants, small round green nodules - "meristemoids" originated first at the midrib and further extended to other regions. These "meristemoids" later developed into shoots (Fig II F). The formation of meristemoids and shoot development was observed from the cut ends of root explants also (Fig II G).

When the concentration of BAP was reduced below the optimal level (3.0 mg $l^{-1}$), the rate of shoot induction was reduced, but exhibited a tendency for callus formation. BAP at 1.5 mg $l^{-1}$ was found more effective for the induction and proliferation of callus. The callus was hard and green, but the region in contact with the medium became brown or black (Fig II H). Later 3-5 shoots were originated from the callus depending on the nature of the explant and hormonal concentrations; the scape explants showed an average of 4 shoots within 40 days (Fig II H).

Among the various concentrations of Kin tested, Kin at 1.5 mg $l^{-1}$ was found effective to induce maximum number of shoots on all types of explants. More than eight numbers of shoots were induced; the number of shoots was more on scape explants (Table IX; Fig III A-C). The shoots originated directly from the explants. The shoot induction was declined with the increase or decrease in Kin concentration beyond the optimal level (1.5 mg $l^{-1}$).

Of the two cytokinins tested individually, it was observed that Kin was more effective than BAP for the shoot and root induction.

**Effect of BAP+Kin**

Explants cultured on MS medium containing Kin (1.0 mg $l^{-1}$) + BAP (0.5 mg $l^{-1}$) produced more number of shoots on all explants (Table IX; Fig III D-G),

The scape explants induced an average of 57 shoots within 40 days (Table IX; Fig III H). The shoots induced roots from the base. Further increase in the number of shoots and roots was also noticed by prolonged culture without subculturing. However, subculturing of the shoots showed further multiplication and these shoots were healthy too, bearing 5-12 leaves of deep green (Fig III I).

**Effect of Coconut Water**

On MS medium with an addition of CW at 10 or 15 %, the stem and scape explants induced an average of 5 shoots (Table IX). The root explants produced an average of 4 shoots. The shoots produced 3-5 leaves within 30 days and roots were also developed in the same medium within 40 days. Compared to the hormone free MS medium, an addition of CW was found stimulative to some extent.

Addition of CW (10 %) along with Kin (1.0 mg $l^{-1}$) + BAP (0.5 mg $l^{-1}$) was not much stimulatory (Table IX).

**Effect of Cytokinins with Auxins**

Addition of auxins (NAA/IAA) to MS medium with Kin (1.0 mg $l^{-1}$) + BAP (0.5 mg $l^{-1}$) was found inferior when compared to MS containing Kin (1.0 mg $l^{-1}$) + BAP (0.5 mg $l^{-1}$) for the induction of shoots (Table IX).

**CALLUS STUDIES**

Callus induction from different explants of *E. foetidum* was established on MS medium fortified with various concentrations and combinations of auxins and cytokinins/CW (Table X).

**Effect of NAA/IAA**

The effect of NAA/IAA at different concentrations used for the induction of callus from different explants is given in Table X. Stem discs, scape and leaf explants induced callus, shoot and root on MS medium containing NAA/IAA up to 2.0 mg $l^{-1}$ (Table X; Fig IV A-C). The root explants induced callus and root only. The callus developed at the cut ends of the explants, which was not in contact with the medium, was hard green (Fig IV C). While the callus obtained from the portions of the explants, which was in contact with the medium was friable and grey white and later became brown/black (Fig IV C). The roots initiated from the explants were green above the level of the medium (Fig IV B, C). The developed roots induced callus at the region of contact with the medium (Fig C, E). The number of shoots varied (2-7) according to the nature of explants; stem discs and scape explants were more responsive than the other explants in inducing shoots (Fig IV D). The shoots were initiated either from the green callus or from the base of the roots developed on the explant. The number of shoots exhibited a decline as to the increase of NAA/IAA concentration. The shoots started to produce roots from their base after 25 days of culture. When the concentration of NAA/IAA was on a reduced rate (0.1-0.5 mg $l^{-1}$), an enhanced number of shoot formation occurred with a reduction in the number of roots and an amount of callus especially on stem discs.

Compared to NAA, shoot, callus and root induction was delayed on IAA containing medium. Nevertheless, at lower concentrations of IAA, the number of shoots was more than that of the same concentrations of NAA.

**Effect of 2,4-D**

Different concentrations of 2,4-D were tested for the induction of callus (Table X). The callus obtained at 1.0 mg $l^{-1}$ 2,4-D was creamy yellow and

**Table X** Morphogenetic response of different explants of *Eryngium foetidum* on MS medium with auxins alone or in combination with cytokinin/CW

| Growth Regulators (mg $l^{-1}$) | | | | | CW | Nature of response | | | | Amount of callus | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| NAA | IAA | 2,4-D | Kin | BAP | (%) | R | STD | L | SC | R | STD | L | SC |
| 0.5 | | | | | | R,C | S,R,C | S,R,C | S,R,C | 0 | + | + | + |
| 1.0 | | | | | | R,C | S,R,C | S,R,C | S,R,C | + | + | + | + |
| 1.5 | | | | | | R,C | S,R,C | S,R,C | S,R,C | + | ++ | ++ | ++ |
| 2.0 | | | | | | R,C | S,R,C | S,R,C | S,R,C | + | +++ | +++ | ++ |
| 3.0 | | | | | | R,C | S,R,C | S,R,C | S,R,C | ++ | ++ | ++ | ++ |
| | 0.5 | | | | | R,C | S,R,C | S,R,C | S,R,C | 0 | + | + | + |
| | 1.0 | | | | | R,C | S,R,C | S,R,C | S,R,C | + | + | + | + |
| | 1.5 | | | | | R,C | S,R,C | S,R,C | S,R,C | + | ++ | ++ | ++ |
| | 2.0 | | | | | R,C | S,R,C | S,R,C | S,R,C | ++ | ++ | ++ | ++ |
| | 3.0 | | | | | R,C | S,R,C | S,R,C | S,R,C | ++ | +++ | +++ | +++ |
| | | 0.1 | | | | C | R,C | C | C | 0 | ++ | ++ | ++ |
| | | 0.5 | | | | C | C | C | C | + | ++ | ++ | + |
| | | 1.0 | | | | C | C | C | C | ++ | +++ | +++ | +++ |
| | | 1.5 | | | | C | C | C | C | +++ | ++++ | ++++ | ++++ |
| | | 2.0 | | | | C | C | C | C | ++ | +++ | +++ | +++ |
| | | 3.0 | | | | C | C | C | C | ++ | ++ | ++ | ++ |
| 0.5 | | | 0.5 | | | R,C | S,R,C | S,R,C | S,R,C | + | ++ | ++ | ++ |
| 1.0 | | | 0.5 | | | S,R,C,E | S,R,C | S,R,C | S,R,C | ++ | +++ | +++ | +++ |
| 1.0 | | | 1.0 | | | R,C | S,R,C | S,R,C | S,R,C | ++ | +++ | ++ | +++ |
| 1.5 | | | 0.5 | | | R,C | S,R,C | S,R,C | S,R,C | +++ | +++ | +++ | +++ |
| 1.5 | | | 1.0 | | | R,C | S,R,C | S,R,C | S,R,C | ++ | +++ | +++ | +++ |
| 2.0 | | | 0.5 | | | R,C | S,R,C | S,R,C | S,R,C | ++ | ++ | ++ | ++ |
| 2.0 | | | 1.0 | | | R,C | S,R,C | S,R,C | S,R,C | ++ | ++ | ++ | ++ |
| 2.0 | | | 2.0 | | | R,C | S,R,C | S,R,C | S,R,C | ++ | ++ | ++ | ++ |
| 1.0 | | | | 0.5 | | R,C | S,R,C | S,R,C | S,R,C | ++ | ++ | +++ | +++ |
| 2.0 | | | | 0.5 | | R,C | S,R,C | S,R,C | S,R,C | ++ | ++ | +++ | +++ |

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2.0 | | | | 1.0 | | R,C | S,R,C | S,R,C | S,R,C | ++ | +++ | +++ | +++ |
| 2.0 | | | | 2.0 | | R,C | S,R,C | S,R,C | S,R,C | ++ | +++ | +++ | +++ |
| | 1.0 | | 0.5 | | | R,C | S,R,C | S,R,C | S,R,C | + | ++ | ++ | ++ |
| | 1.0 | | 1.0 | | | R,C | S,R,C | S,R,C | S,R,C | + | ++ | ++ | ++ |
| | 1.5 | | 0.5 | | | R,C | S,R,C | S,R,C | S,R,C | ++ | +++ | +++ | +++ |
| | 1.5 | | 1.0 | | | R,C | S,R,C | S,R,C | S,R,C | + | ++ | ++ | ++ |
| | 2.0 | | 0.5 | | | R,C | S,R,C | S,R,C | S,R,C | ++ | +++ | +++ | +++ |
| | 2.0 | | 1.0 | | | R,C | S,R,C | S,R,C | S,R,C | ++ | ++ | ++ | ++ |
| | 2.0 | | 2.0 | | | R,C | S,R,C | S,R,C | S,R,C | ++ | ++ | ++ | ++ |
| | 1.0 | | | 0.5 | | R,C | S,R,C | S,R,C | S,R,C | + | ++ | ++ | ++ |
| | 2.0 | | | 0.5 | | R,C | S,R,C | S,R,C | S,R,C | ++ | +++ | +++ | +++ |
| | 2.0 | | | 1.0 | | R,C | S,R,C | S,R,C | S,R,C | ++ | +++ | +++ | +++ |
| | 2.0 | | | 2.0 | | R,C | S,R,C | S,R,C | S,R,C | ++ | +++ | +++ | +++ |
| | | 0.5 | 0.5 | | | R,C | S,R,C | S,R,C | S,R,C | + | ++ | ++ | ++ |
| | | 0.5 | 1.0 | | | R,C | S,R,C | S,R,C | S,R,C | + | + | + | + |
| | | 1.0 | 0.5 | | | C | C | C | C | +++ | ++++ | ++++ | ++++ |
| | | 1.0 | 1.0 | | | C | R,C | R,C | R,C | ++ | +++ | +++ | +++ |
| | | 1.5 | 1.0 | | | C | R,C | R,C | R,C | ++ | +++ | +++ | +++ |
| | | 2.0 | 0.5 | | | C | C | C | C | ++ | +++ | +++ | +++ |
| | | 2.0 | 1.0 | | | C | C | C | C | ++ | +++ | +++ | +++ |
| | | 2.0 | 2.0 | | | C | R,C | R,C | R,C | + | ++ | ++ | ++ |
| | | 1.0 | | 0.5 | | C | C | C | C | ++ | +++ | +++ | +++ |
| | | 2.0 | | 0.5 | | C | C | C | C | ++ | +++ | +++ | +++ |
| | | 2.0 | | 1.0 | | C | C | C | C | ++ | +++ | +++ | +++ |
| | | 2.0 | | 2.0 | | C | C | C | C | ++ | +++ | +++ | +++ |
| 1.0 | | | | | 10.0 | R,C | S,R,C | S,R,C | S,R,C | ++ | +++ | +++ | +++ |
| 2.0 | | | | | 10.0 | R,C | S,R,C | S,R,C | S,R,C | ++ | +++ | +++ | +++ |
| | 2.0 | | | | 10.0 | R,C | S,R,C | S,R,C | S,R,C | ++ | +++ | +++ | +++ |
| | | 1.0 | | | 10.0 | C | C | C | C | ++ | +++ | +++ | +++ |
| | | 2.0 | | | 10.0 | C | C | C | C | ++ | +++ | +++ | +++ |

Data represents an average of 10 replicates. Growth period 40 days

S-Shoot R-Root C-Callus E-Embryos STD-Stem Disc L-Leaf 0 - Very little ++ - Little +++ - Moderate ++++ - Profuse

globular in nature (Fig IV F-H). The globular structures later showed a tendency to develop into roots. Further increase in the concentration of 2,4-D, globular nature of the callus was reduced and the callus became friable and soft. Lower concentration of 2,4-D caused a reduction in the rate of callus formation and the callus was pale green (Fig IV I). On leaf explants, callus formation was observed at both adaxial and abaxial surfaces but was more on the abaxial side (Fig IV G). No shoot formation was observed on any of the explant cultured at any concentration of 2,4-D.

**Effect of NAA/IAA+Kin/BAP**

Of the different concentrations of NAA/IAA tested with Kin, NAA/IAA at 1.0 mg $l^{-1}$ + Kin at 0.5 mg $l^{-1}$ was found as the optimal concentration, which induced both friable and hard green callus, shoots and roots on stem discs, leaf and scape explants (Table X; Fig V A-C). The root explants induced root and callus only. However, the root explants immediately following the stem portion cultured on NAA (1.0 mg $l^{-1}$) + Kin (0.5 mg $l^{-1}$) induced embryoids in addition to shoot and callus (page 41 - direct embryogenesis). The friable callus was originated at the region where the explant was in contact with the surface of the medium, especially at the cut ends. The callus induced from the roots at the point of contact to the medium was also friable and greyish, which became brown or black (Fig V A, B). An enhanced formation of green callus was observed on scape explants when it was placed vertically (Fig V C). Shoots were also initiated either from the green callus or from the base of the roots (originated from the explant) in the same combination. Number of shoots was more on scape explants followed by stem discs and leaf explants. Root initiation took place at the base of the shoot in the same medium, which also induced friable brown callus at the point of contact to the medium (Fig V B).

Combination of NAA/IAA with BAP at different concentrations (Table X) showed a similar response but was characterized by an increased amount of green callus along with a reduction in the number of shoots and roots.

**Effect of 2,4-D+Kin/BAP**

Different concentrations of 2,4-D in combination with Kin/BAP were also tested to study the induction of callus from different explants (Table X). Combination of 2,4-D (1.0 mg $l^{-1}$) with Kin (0.5 mg $l^{-1}$) started to induce globular structures after 7 days (Fig V D; Fig VI A, B). These globular structures were developed on both adaxial and abaxial surfaces of leaf explants (Fig VI A).

A combination of 2,4-D (0.1 mg $l^{-1}$) with Kin (0.5 mg $l^{-1}$) resulted in the formation of 2-4 small shoots on root explants along with friable callus. Small green roots were developed on proximal end of the leaf explant; while at distal end very small shoots (7-9) were formed (Fig VI C); out of which one or two were grown prominently and produced 2-5 leaves. These shoot later developed roots from the base. An increase in the amount of 2,4-D increased the amount of friable callus, but an increase of Kin enhanced root formation. However, globular structures and friable creamy callus were formed later.

Combinations of BAP (0.5 mg $l^{-1}$) with 2,4-D (1.0 mg $l^{-1}$) resulted many globular like structures, which were not similar as found on 2,4-D+Kin combination. Higher concentration of BAP to 2,4-D resulted in the formation of green and hard callus.

**Effect of Auxins with CW**

The results obtained on MS medium supplemented with various auxins (NAA/IAA/2,4-D) with different volumes of CW (5 - 15 %) for the establishment of callus cultures in *E. foetidum* were given in Table X. Response of all types of

explants with auxins and CW (10 %) were inferior to the treatments of auxins with Kin (Table X).

## INDIRECT ORGANOGENESIS

Indirect organogenesis was established by subculturing of both hard green and friable calli developed on different explants. Subculturing of the green callus on full MS medium without any growth regulator induced an average of four shoots. However, MS medium fortified with Kin (1.0 mg $l^{-1}$) + BAP (0.5 mg $l^{-1}$) were more effective, resulted in an average of 25 shoots within 40 days (Fig VI D). Combination of BAP (1.0 mg $l^{-1}$) + Kin (0.5 mg $l^{-1}$) resulted in lower number of shoot formation (an average of 20/culture) along with callus proliferation. A further increase in shoot formation was noticed, when the cultures were kept for a long period. Root induction was also obtained in the same medium. The roots were elongated and much branched. Compared to the hard green callus, the efficacy of friable callus was inferior for the induction of shoots (Fig VI E). The callus retained the morphogenetic potential more or less at the same rate as the initial cultures even after 400 days.

## PHOTOAUTOTROPHIC MICROPROPAGATION

Photoautotrophic micropropagation was established from stem, leaf and root explants excised from the *in vitro* raised shoots, hard green callus and also with the plantlets obtained from different cultures on MS medium with sucrose and growth regulators (Heterotrophic medium).

On photoautotrophic medium (MS without sucrose), different explants showed a delayed shoot induction with less number of shoots compared to heterotrophic medium. The shoots were developed at the cut ends of the explants. The stem discs produced an average of four shoots followed by root and leaf explants (Fig VII A-C). The petiolar region of the leaf explants induced 2

- 4 meristemoids, of which one or two developed in to shoots (Fig VII C). The shoots exhibited a slow growth, compared to the shoots on heterotrophic medium.

Subculturing of hard green callus developed on different cultures to photoautotrophic basal medium induced an average of 4 shoots (Fig VII D, E). The callus did not show proliferation. These shoots remained short with small leaves even after six months (Fig VII D). However, subculturing of these shoots to fresh photoautotrophic basal medium revived normal growth (Fig VII E).

Transfer of plantlets, developed on different cultures to photoautotrophic basal medium showed multiplication of shoots with dark green leaves (Fig VII F). However, the plantlets showed very slow growth. The plantlets induced new roots in addition to the roots at the time of transfer to photoautotrophic medium.

## SOMATIC EMBRYOGENESIS

Direct and indirect somatic embryogenesis and subsequent plant regeneration were established from different explants of *E. foetidum*.

### Direct Somatic Embryogenesis

Direct somatic embryogenesis was established on root explants (excised from the portion immediately following the stem) on MS medium fortified with NAA (1.0 mg $l^{-1}$) + Kin (0.5 mg $l^{-1}$). About 40 % of the cultured explants showed positive response of direct somatic embryogenesis. Embryoids of globular to torpedo stages were developed at the cut ends of the root explants (Fig VIII A). The embryos rarely developed to cotyledonary stage on the same medium. However, upon transfer to half MS supplemented with 10 % CW/CT these embryos underwent conversion into plantlets. In addition to the embryo

formation, less amount of hard green callus and one or two shoot buds were also occurred at the central portion of the cut end (Fig VIII A).

**Indirect Embryogenesis**

Somatic embryogenesis in *E. foetidum* was established either through **spontaneous** origin or by repeated subculture of the callus (here after called as "**indirect**" embryogenesis).

**Spontaneous Embryogenesis**

Hard green and friable grey white callus was obtained from different explants of *E. foetidum* cultured on full MS medium containing NAA/IAA+Kin.

Roots originated directly from the explant as well as from the base of the *in vitro* shoots were green in colour above the level of the medium. These roots produced friable callus at the point of contact with the medium, which containing NAA+Kin (2.0 mg $l^{-1}$ each). The callus, which initiated after 25 days of culture, was grey white and later became brown/black. These calli started to induce embryoids after 75 days of culture (without subculture) (Fig VIII B-D). The embryoids passed through the sequential developmental stages of globular to cotyledonary through heart and torpedo. The embryoids with 2-7 cotyledons looked like small white flowers (Fig VIII E, F). These embryoids, whether it was dicotyledonary or multicotyledonary, exhibited a distinct root pole. None of the embryoids undergone conversion even after 120 days of culture on the same medium. Secondary somatic embryogenesis was also observed from the primary embryos (see page 45). The spontaneous induction of embryoids was delayed on IAA+Kin compared to the NAA+Kin.

The embryos exhibited conversion on half MS containing 10 % CW/CT.

## Indirect Embryogenesis

The friable callus obtained on MS medium containing auxins (NAA/2,4-D) and Kin was found suitable for the induction of somatic embryogenesis. The type of auxin and its concentration used in the medium significantly influenced the induction of embryoids.

### MS + NAA + Kin

Subculturing of the brown calli (Fig IX A) developed on MS with NAA (1.0 - 2.0 mg $l^{-1}$) + Kin (0.5 - 1.0 mg $l^{-1}$) to reduced levels of the same hormones induced somatic embryos. Though the embryoids were formed at the first subculture itself, subsequent transfer showed an enhanced frequency of embryoids. Half MS with NAA at 0.5 mg $l^{-1}$+ Kin at 0.25 mg $l^{-1}$ was found more suitable, in which embryos developed at the rate of about 200 embryos/10 mg callus (Fig IX B). The embryos were with 2 - 7 cotyledons, which remained as such even after 90 days without any conversion (Fig IX B). Further, proliferation of the embryogenic callus was also occurred on the same medium.

### MS + 2,4-D + Kin

The creamy yellowish friable callus developed on MS medium containing 2,4-D (1.0 mg $l^{-1}$) + Kin (0.5 mg$l^{-1}$) (Fig IX C) was found superior to the brown friable callus obtained on NAA+Kin medium for the induction of somatic embryogenesis.

The calli upon transfer to suspension and static cultures of half or full MS with reduced levels of 2,4-D and Kin showed proliferation of embryogenic callus and induction of embryos (Fig IX D). Subsequent cultures on half MS hormone free medium resulted in the development of roots abreast to the maturation of few embryos. Nevertheless, the transfer of the embryogenic callus from 2,4-D+Kin to

suspension cultures of half MS with 10% CW/CT showed proliferation of embryogenic callus and development of embryos up to cotyledonary stage. The mature embryos upon transfer to static cultures of half MS + CW/CT resulted in conversion under light condition (Fig IX E). The embryos were developed in to plantlets on the same medium (Fig IX F). Ten per cent CW/CT was found as optimal (Fig X A). About 10 mg callus produced around 500 mature embryos (Fig X A).

The level of sucrose also has significant role in the induction, proliferation and maturation of embryos. The optimum level of sucrose for somatic embryogenesis was 1.5 - 3.0 per cent. Higher or lower concentration of sucrose showed a reduction in the induction and proliferation of embryos (Fig X B). However, sucrose had no significant role with respect to conversion. Transfer of the mature embryos to photoautotrophic basal medium resulted in conversion, but was rather slow.

In general, the embryos passed through the normal developmental stages: from globular to plantlet (Fig XI A-E). The multicotyledonary embryos were also showed sequential development and underwent conversion (Fig XI F-H). The plantlets grew well with dark green leaves and produced numerous roots

Suspension cultures were found better for the proliferation of embryogenic callus, induction and proliferation of embryoids and its development to maturity. While with respect to conversion, solid medium under light was found apposite. The mature embryos developed small green cotyledons and well developed root pole in suspension cultures of half MS+CW/CT.

Of the three media tested for the induction of somatic embryogenesis, MS medium was found superior to White's medium, followed by $B_5$.

## SECONDARY SOMATIC EMBRYOGENESIS

Secondary somatic embryogenesis of **direct** and **indirect** origin was observed from the primary developed embryos.

### Direct Secondary Somatic Embryogenesis

The primary embryos developed spontaneously from the roots of the *in vitro* developed plants induced few numbers of secondary somatic embryos (3-7) directly from the shoot apical region (Fig XII A, B). Two to seven numbers of cotyledons were observed on secondary somatic embryos, which were cream to white in colour looked like white flowers (Fig XII A, B). But, the cotyledons of the primary embryos became dark green (Fig XII A, B).

The secondary embryos developed from the hypocotyl region showed growth up to early cotyledonary stage (Fig XII C). The embryos with dormant plumular region and long root pole developed on different combinations (half MS + NAA 0.5 mg $l^{-1}$ + Kin 0.25 mg $l^{-1}$ or half MS + 10% CW/CT) in suspension culture, induced globular embryos by consuming the shoot apex (Fig XII D).

Transfer of the germinated embryos to half MS medium fortified with NAA (0.5 mg $l^{-1}$) + Kin (0.25 mg $l^{-1}$) developed accessory embryos from the roots (Fig XII E).

The secondary embryos passed through various embryonal (globular to cotyledonary) stages. These secondary embryos were also with 2-7 numbers of white cotyledons (Fig XII A, B, E). Though the embryos underwent maturation, no conversion occurred on the same medium. The embryos showed conversion on half MS medium supplied with 10 % CW/CT.

**Indirect Secondary Somatic Embryogenesis**

The mature primary somatic embryos induced callus from the shoot apex on half MS medium containing NAA + Kin/CW (Fig XII F). The callus later induced numerous globular embryos (Fig XII F). On half MS medium containing NAA + Kin/CW, the roots of the germinated embryos also induced callus, which was brown/black and friable (Fig XII G). The callus on the same medium later induced globular to cotyledonary embryos (60 days) (Fig XII G). The accessory embryos underwent conversion on half MS + CW/CT (10 %).

**PROTOPLAST STUDIES**

Protoplast isolation studies on *E. foetidum* was done using leaf mesophyll cells of *in vitro* grown plants. Different concentrations of enzymes were tested for the isolation of protoplasts (Table XI). Of the enzymes at various concentrations tested, Cellulase Onozuka (0.6%) in combination with Macerozyme R10 (0.3 %) was found effective for the isolation of protoplasts. Higher concentration of cellulase resulted bursting of the protoplasts and at lower concentration, the yield was low. The incubation at dark (25±2°C) with shaking at 30-50 rpm was found better for the isolation of protoplasts. The protoplasts from leaf mesophyll cells started to release after 4 h incubation. The optimum incubation period for better releasing of protoplasts from leaf mesophyll was 12-14 h. The yield of the protoplast at optimum incubation period was 3 - 6 x $10^6$ /g fresh weight of leaf. Though the protoplast yield increased with longer period of incubation, the protoplasts showed bursting.

Two types of protoplasts were observed: large vacuolated ones and small ones with dense cytoplasm (Fig XIII A). The protoplast with dense cytoplasmic streaming was considered as viable.

**Table XI** Effect of enzymes concentrations in the yield of protoplasts from leaf mesophyll cells of *Eryngium foetidum*

| Cellulase (%) | Macerozyme (%) | Protoplasts yield/g leaf tissue |
|---|---|---|
| 0.1 | 0.3 | $0.6 \times 10^6$ |
| 0.6 | 0.3 | $3 - 6 \times 10^6$ |
| 1.0 | 0.3 | $0.5 - 1 \times 10^6$ |

Incubation time: 12 - 14 h

**Protoplast Culture**

The protoplasts were encapsulated as the method given in **Materials and Methods**. Microcolonies in the alginate beads had been visible to the naked eye as brown/black spots within 30 days (Fig XIII B) and for further growth, the protocalli were isolated and transferred to MS medium with different growth regulators.

**Organogenesis**

The microcalli (protocalli) developed from protoplasts within the beads made up with MS + Kin (1.0 mg $l^{-1}$) + BAP (0.5 mg $l^{-1}$) showed regeneration after 80 days of incubation under light conditions (Fig XIII C). The regeneration potential was low within the bead. However, the calli separated and cultured on MS medium supplemented with Kin (1.0 mg $l^{-1}$) + BAP (0.5 mg $l^{-1}$) showed higher per cent of shoot induction.

**Somatic Embryogenesis**

The brown/black calli developed from protoplasts encapsulated within MS + 2,4-D (1.0 mg $l^{-1}$) + Kin (0.5 mg $l^{-1}$) when excised and subcultured on MS medium with the same combinations of growth regulators resulted proliferation of callus. The newly formed callus was creamy to light brown, which later induced embryoids of various stages: globular, heart, torpedo and cotyledonary (Fig XIII D, E). The number of cotyledons varied from 1-7 and was white to slightly creamy in colour. The embryos underwent conversion on half MS with 10 % CW and the plantlets were healthy with green leaves (Fig XIII F).

## HISTOLOGICAL STUDIES

Developmental studies showed that the shoots were originated from phloem cells of the different explants *viz.*, root, stem, leaf and scape (Fig XIV A, B).

In the case of direct somatic embryogenesis, the embryos originated from the cortical cells of root explants (Fig XIV C). Indirect embryogenesis occurred from the callus developed from the cortical cells of root, stem and scape explants. In the case of leaf explants, the callus originated from the mesophyll cells especially from the spongy cells below the vascular traces (Fig XIV D-F)).

The embryogenic cells were small with more cytoplasmic contents (Fig XV A -1). The embryos were originated from the peripheral as well as internal cells of the callus. The embryogenic cells became two celled by a transverse division (Fig XV A -2,3,4). Of the two cells, the cell with dense cytoplasm (upper cells) divided by an oblique periclinal division and become three-celled (Fig XV A -5). By further periclinal and anticlinal divisions of the upper cells globular to cotyledonary embryos were formed (Fig XV B-L). The embryos showed a distinct epidermal layer (Fig XV C-L).

## SYNSEEDS

The singulated cotyledonary embryos were encapsulated in half MS + CW/CT (10 %) containing sodium alginate of different concentrations. The synseeds thus obtained were cultured on semisolid half MS basal medium.

Of the different concentrations of sodium alginate tested, beads made up with alginate at 2.5 % were found more effective, considering the germination potential of synseeds (Fig XVI A). Higher percentage of alginate (above 2.5 %) adversely affected the germination frequency of synseeds (Fig XVI A). Of the

different concentrations of $CaCl_2$ tested (0.5, 0.7 and 1 %), 0.7 % was found suitable for the formation of good beads with respect to germination (Fig XVI B). The synseeds made in 2.5 % sodium alginate with 10% CW, exposed to 0.7 % $CaCl_2$ 100% showed conversion within 15 days (Fig XVII A, B).

**Synseeds at seed efficiency**

The potential of synseeds at seed efficiency was also tried. The encapsulated beads (made in half MS +10 % CW/CT with 2.5 % alginate) cultured *ex vitro* on soilrite+sand (1:1) showed 100 % conversion (Fig XVII C). Though the conversion was high, the major impediment was the contamination caused by fungi and bacteria.

**CONSERVATION *IN VITRO***

*In vitro* conservation studies were conducted at synseed and whole plant level (by photoautotrophic cultures). The main conservation strategy adopted in the present study aims the growth at minimal rate.

**(i) Conservation by Synseeds**

The singulated mature embryos encapsulated using 2.5% alginate either in half MS or in double distilled water (both with sucrose at 1.5 %) were used for the conservation studies. The two temperature regimes adopted for the conservation of germplasm was 4°C (in refrigerator) and 25±2°C (usual culture conditions).

The growth of the synseeds prepared in double distilled water with sucrose was very slow compared to that on half MS+sucrose. The singulated embryos had taken 2 months for conversion and showed slow growth (Fig XVII D, E).

The growth of the synseeds preserved at 4°C was inferior to those at 25±2°C. The viability of the synseeds stored at 4°C showed a positive correlation to the period of storage *i.e.* as the storage period prolongs the viability reduced. The viability of the synseeds after 12 months was 40 per cent. The synseeds cultured at 25±2°C, need not be tested for the viability at different intervals, which showed a survival rate of 70 per cent after 12 months.

(ii) **Conservation by Photoautotrophic cultures**

Conservation by photoautotrophic cultures was carried out with the transfer of well-developed plantlets to photoautotrophic medium. The plantlets transferred into culture bottles covered with aluminium or polypropylene closures remained at minimal growth without being subcultured even after 12 months (Fig XVII F). Though the leaves of the plantlets were dark green, they showed a slight reduction in size: both in breadth and length. The plantlets at minimal growth developed new roots also.

**ROOTING *IN VITRO***

The regenerated shoots produced roots from the base of the shoot in regeneration/multiplication medium itself (an average of ten roots/shoot). The cultures showed an increased tendency of root formation as the days of the culture passed on. The shoots without roots were excised and transferred to either full or half MS hormone free medium for root induction. Of which, half MS hormone free medium was found effective and an average of 16 roots/shoot was induced (Fig XVIII A). In all cases the roots were long and much branched.

**TRANSFER TO FIELD CONDITIONS**

Well-grown plantlets transferred directly to small pots containing sterile soilrite+sand (1:1), which revived growth within 15 days (Fig XVIII B). The

plantlets developed *via* photoautotrophic micropropagation and somatic embryogenesis exhibited 100 per cent survival, while that by organogenesis showed 80 % survival. The leaves became dark green with serrated margins.

## SECONDARY METABOLITE ANALYSIS

In the present study TLC was performed to compare the alkaloids present in the roots of *in vitro* (*via* somatic embryogenesis) (Fig XVIII C) and field grown plants.

Petroleum ether extracts of root tissues of *in vitro* and field grown plants showed two spots each of *Rf* values 0.56 and 0.8 upon spraying with the reagent (conc. 5 % $H_2SO_4$ in ethyl alcohol) (Fig XVIII D). However, ethanolic extracts revealed major differences in the chromatographic pattern. The *in vitro* roots showed six compounds with *Rf* values 0.1, 0.18, 0.3, 0.4, 0.6 and 0.8, where as the samples from field grown plants gave only two identifiable components, with *Rf* values of 0.56 and 0.8 (Fig XVIII D).

Fig II A-H Direct organogenesis from different explants of *Eryngium foetidum* on different media

A. Shoots developed from stem disc on MS basal medium
B. Shoots developed from stem disc on $B_5$ basal medium
C. Shoots developed from leaf (petiolar) explant on MS basal medium
D. Shoots developed from root explant on hormone free MS medium
E. Shoots developed from stem disc on MS+ BAP (3.0 mg $l^{-1}$)
F. Shoots developed from leaf explant on MS+ BAP (1.5 mg $l^{-1}$)
G. Shoots developed from root explant on MS+ BAP (3.0 mg $l^{-1}$)
H. Shoots developed from scape explant on MS+ BAP (1.5 mg $l^{-1}$)

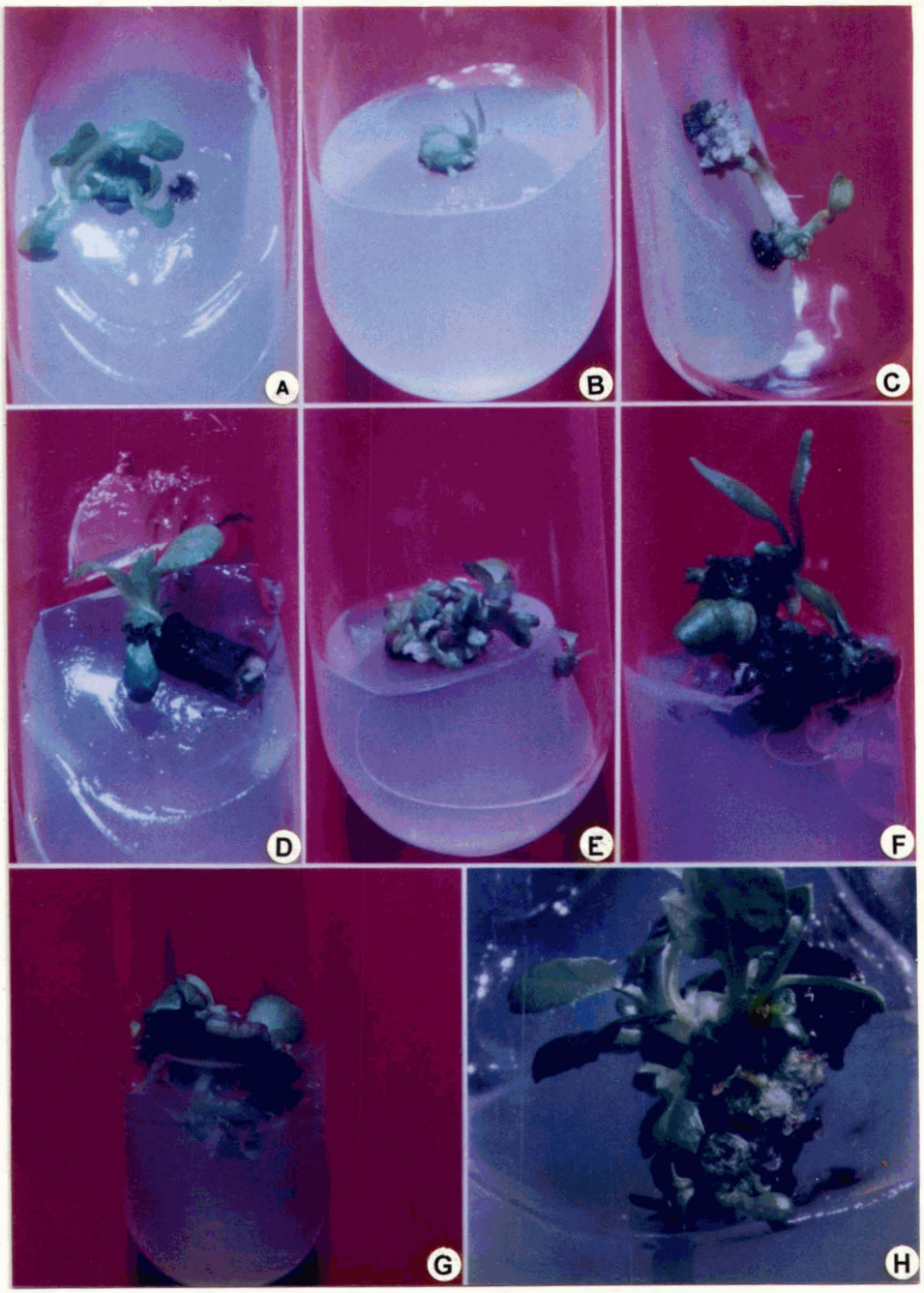



Fig II

Fig III A-I Direct organogenesis from different explants of *Eryngium foetidum* on MS medium with different growth regulators

A. Shoots induced from root explant on MS+ Kin (1.5 mg $l^{-1}$)
B. Shoots induced from stem explant on MS+ Kin (1.5 mg $l^{-1}$)
C. Shoots induced from scape explant on MS+ Kin (1.5 mg $l^{-1}$)
D. Shoots induced from root explant on MS+ Kin (1.0 mg $l^{-1}$) + BAP (0.5 mg $l^{-1}$)
E. Shoots induced from leaf (petiolar) explant on MS+ Kin (1.0 mg $l^{-1}$) + BAP (0.5 mg $l^{-1}$)
F. Shoots induced from stem disc on MS+ Kin (1.0 mg $l^{-1}$) + BAP (0.5 mg $l^{-1}$)
G. Shoots induced from scape explant on MS+ Kin (1.0 mg $l^{-1}$) + BAP (0.5 mg $l^{-1}$)
H. Shoots on above medium (35 days old)
I. Subculture showing shoot multiplication (30 days)


Fig III

Fig IV A-I Morphogenic response of different explants of *Eryngium foetidum* on MS medium with different auxins

A. Shoot, root and callus developed from stem disc on MS+ NAA (2.0 mg $l^{-1}$)

B Shoot, root and callus developed from leaf explant on MS+ NAA (2.0 mg $l^{-1}$) (after 20 days)

C. Shoot, root and callus developed from scape explant on MS+ NAA (2.0 mg $l^{-1}$)

D. Shoot and callus developed from scape explant on MS+ IAA (2.0 mg $l^{-1}$)

E. Root and callus developed from leaf explant on MS+ IAA (3.0 mg $l^{-1}$)

F. Callus developed from root explant on MS+ 2,4-D (1.0 mg $l^{-1}$)

G. Callus developed from leaf (petiolar) explant on MS+ 2,4-D (1.0 mg $l^{-1}$)

H. Callus developed from scape explant on MS+ 2,4-D (1.0 mg $l^{-1}$)

I. Callus developed from stem disc on MS+ 2,4-D (0.5 mg $l^{-1}$)

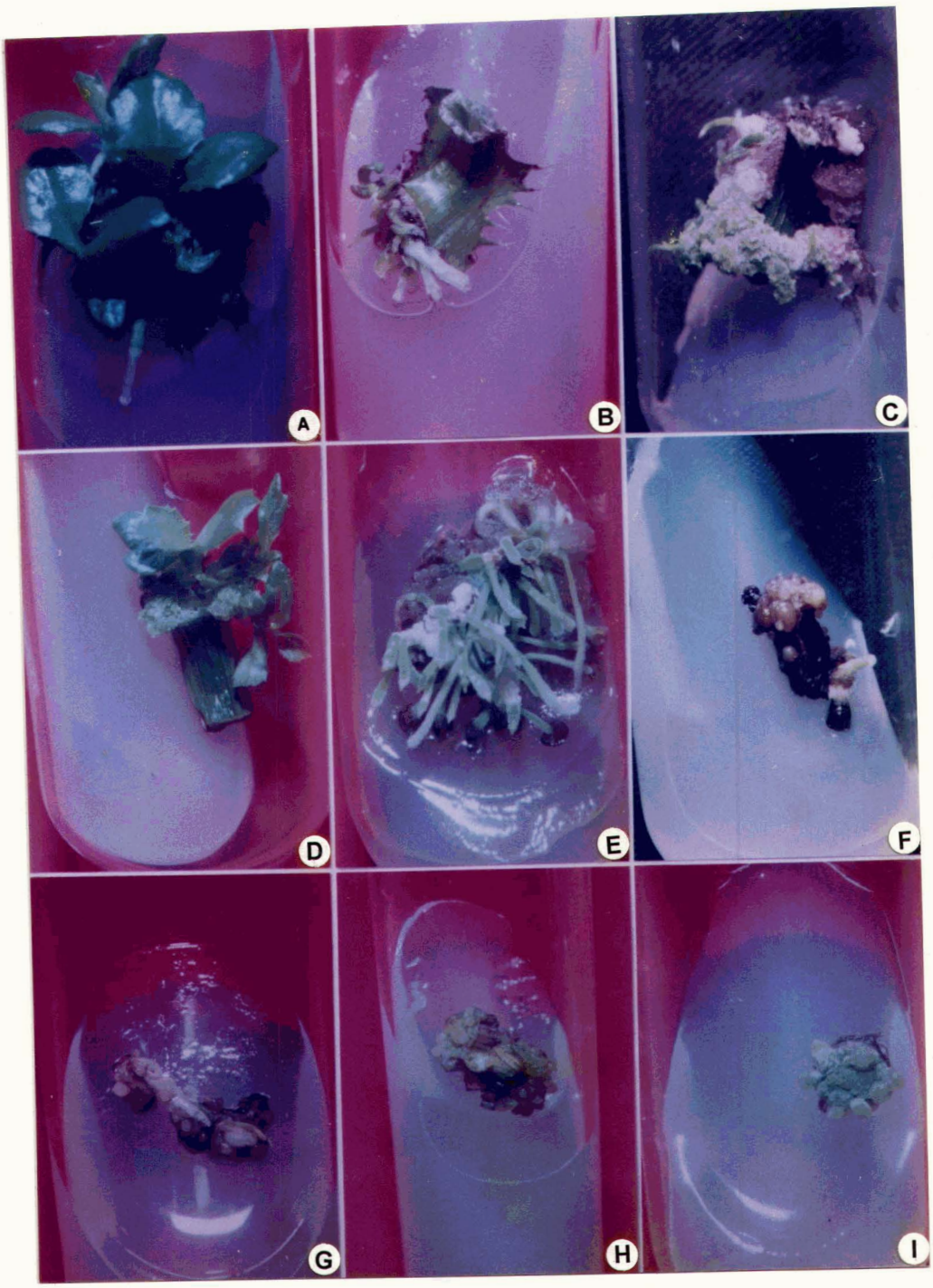



Fig IV

Fig V A-D Morphogenic response of different explants of *E. foetidum* on MS medium with auxins and cytokinins

A. Shoot, root and callus from leaf explant on MS + NAA (1.0 mg $l^{-1}$) + Kin (0.5 mg $l^{-1}$)

B. Shoot, root and callus from stem disc on MS + NAA (1.0 mg $l^{-1}$) + Kin (0.5 mg $l^{-1}$)

C. Shoot, root and callus from scape explant on MS + NAA (1.0 mg $l^{-1}$) + Kin (0.5 mg $l^{-1}$)

D. Cream friable callus from root explant on MS + 2,4-D (1.0 mg $l^{-1}$) + Kin (0.5 mg $l^{-1}$)

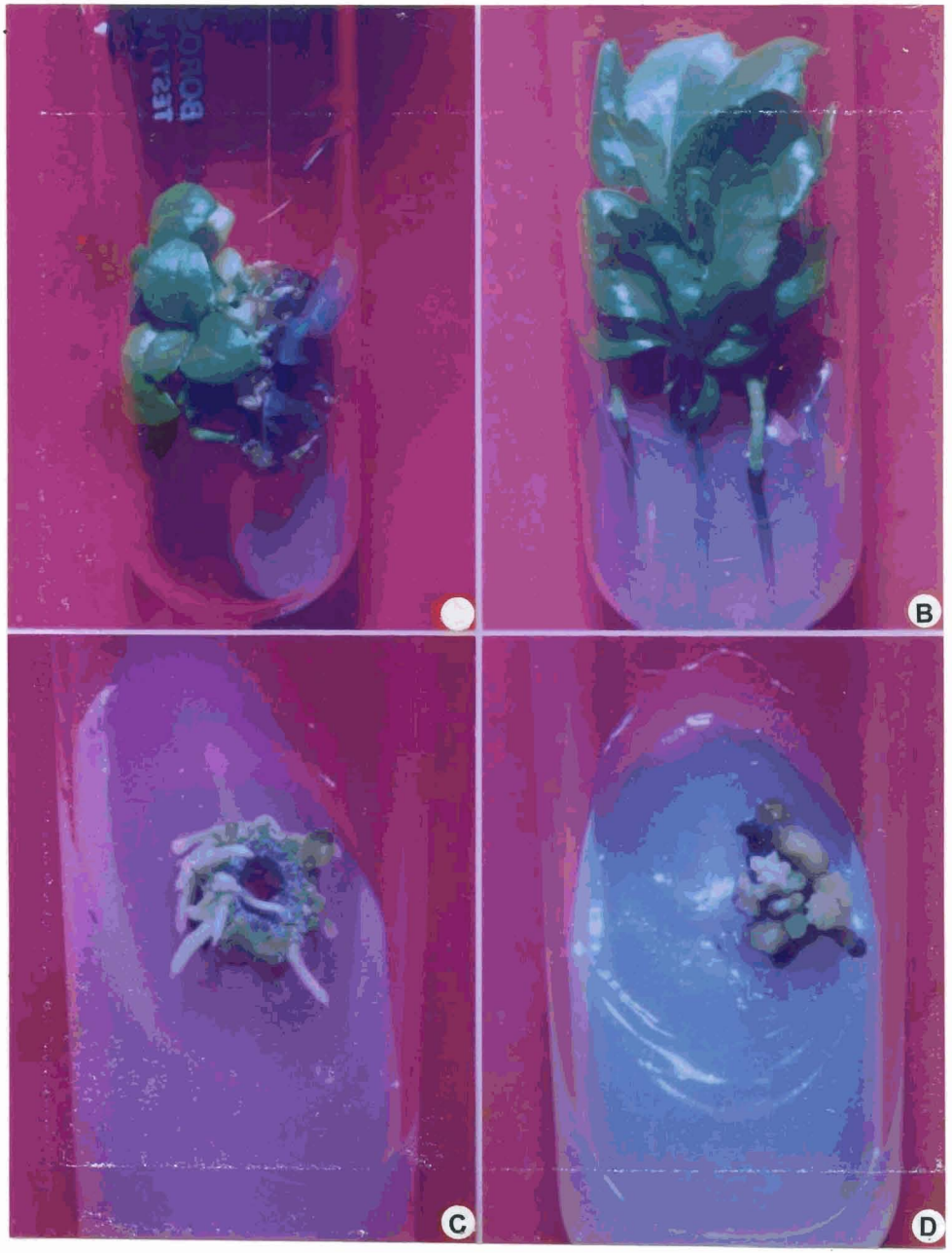



Fig V

Fig VI A-C Morphogenic response of different explants of *Eryngium foetidum* on MS medium with 2,4-D + Kin

A. Callus from leaf (petiolar) explant on MS + 2,4-D (1.0 mg $l^{-1}$) + Kin (0.5 mg $l^{-1}$)

B. Callus with globular structures from scape explant on MS + 2,4-D (1.0 mg $l^{-1}$) + Kin (0.5 mg $l^{-1}$)

C. Shoots, root and callus from leaf explant on MS + 2,4-D (0.1 mg $l^{-1}$) + Kin (0.5 mg $l^{-1}$)

D & E Indirect organogenesis in *Eryngium foetidum*

D. Shoots developed from hard green callus on MS + Kin (1.0 mg $l^{-1}$) + BAP (0.5 mg $l^{-1}$)

E. Shoot developed from friable brown callus on MS + Kin (1.0 mg $l^{-1}$) + BAP (0.5 mg $l^{-1}$)

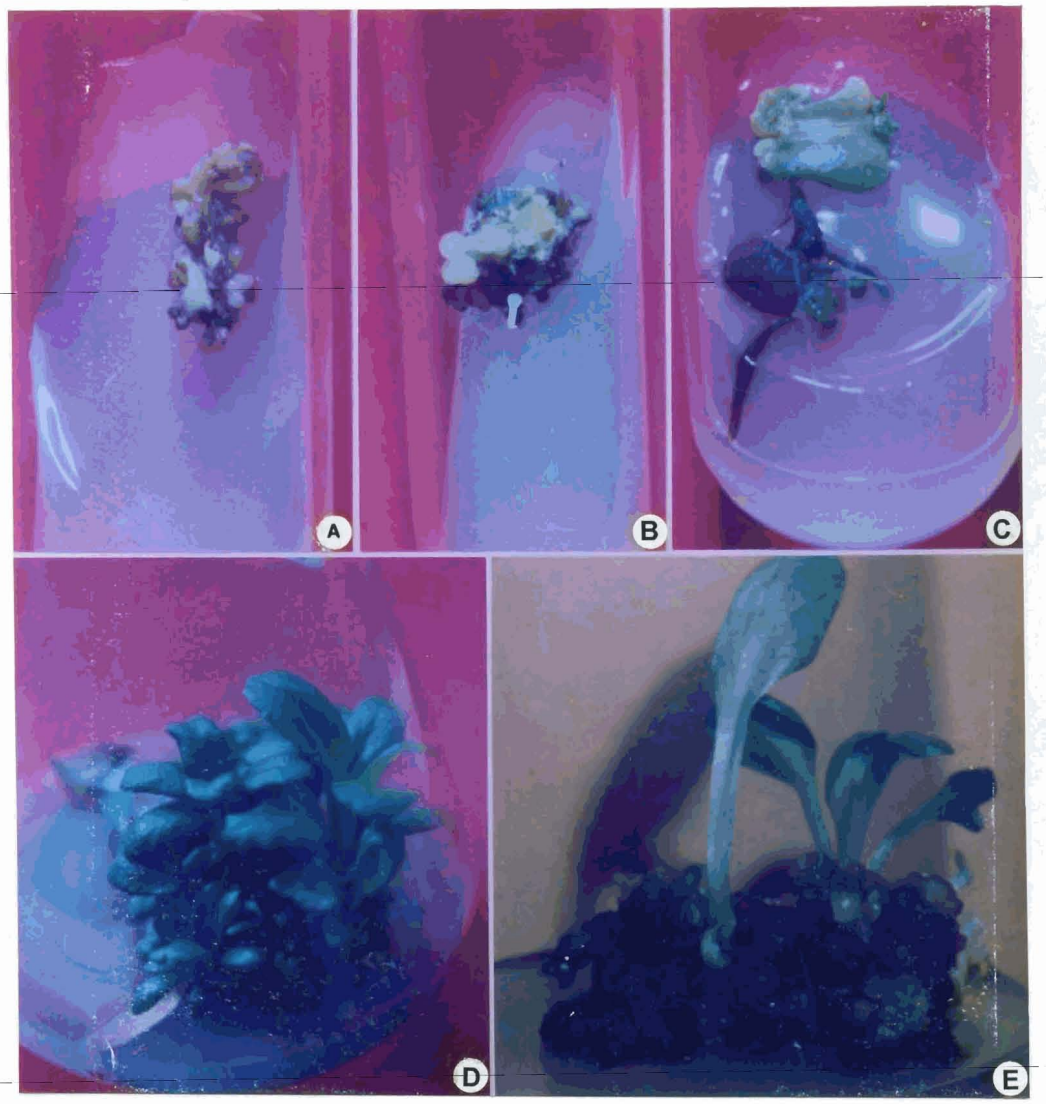



Fig VI

Fig VII A-F Micropropagation from different explants of *Eryngium foetidum* on photoautotrophic medium (MS basal medium without sucrose)

A. Shoots initiated on stem disc explant
B. Shoots initiated on root explant
C. Shoots initiated on leaf (petiolar) explant
D. Shoot initiation from hard green callus
E. Shoot showing revived growth after subculture
F. Shoot multiplication, shoots showing well developed green leaves

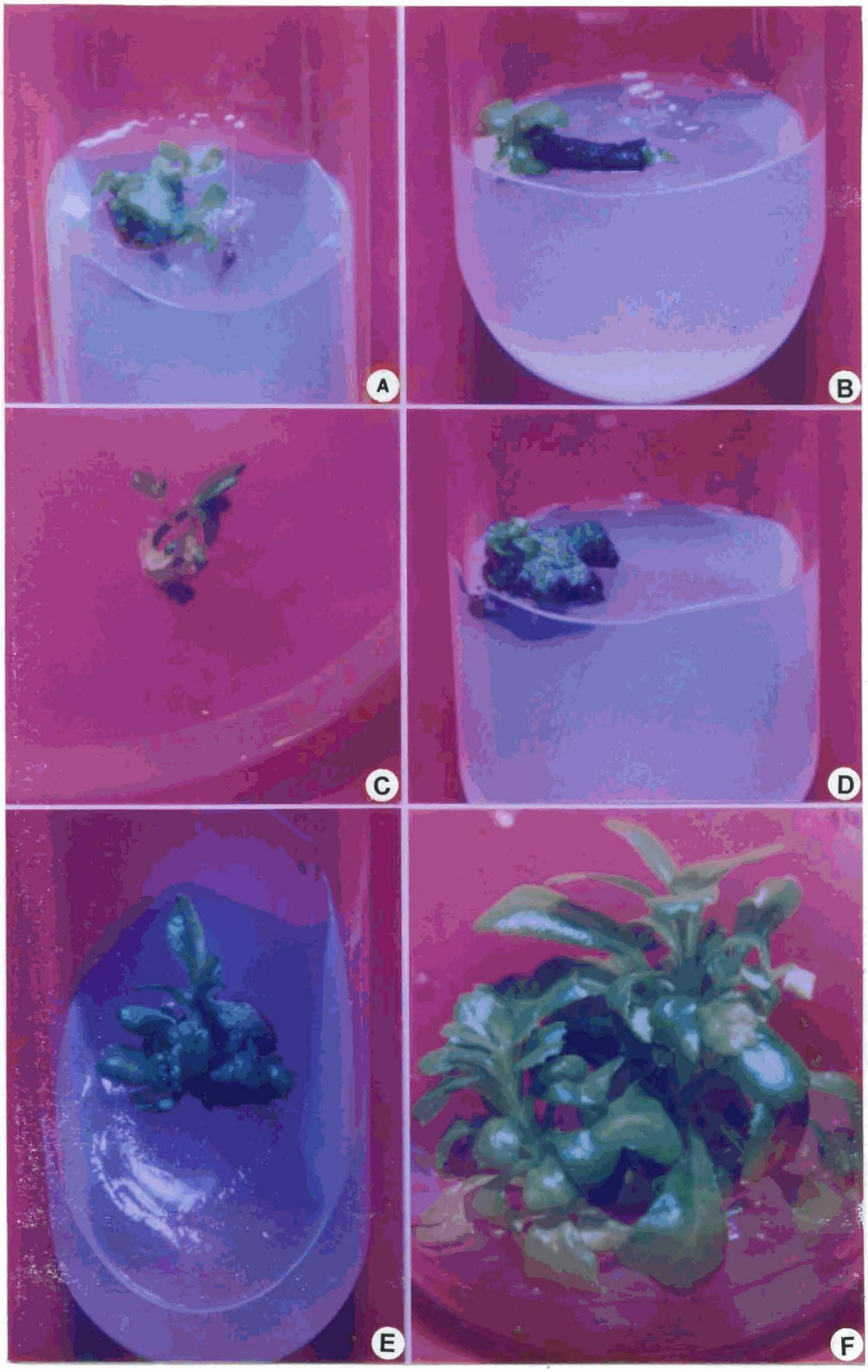



Fig VII

Fig VIII A-F Somatic embryogenesis from different explants of *Eryngium foetidum* on MS medium

A. Shoot and direct somatic embryos from root explants on MS + NAA (1.0 mg $l^{-1}$) + Kin (0.5 mg $l^{-1}$)

B. Leaf explant showing roots, callus shoots and embryos (embryos originated spontaneously from the callus developed on roots from the explant)

C. Spontaneous formation of embryos from the callus on roots of *in vitro* growing plantlets on MS + NAA (2.0 mg $l^{-1}$) + Kin (2.0 mg $l^{-1}$)

D. Enlarged somatic embryos

E,F Clusters of embryos with 2-7 numbers of cotyledons

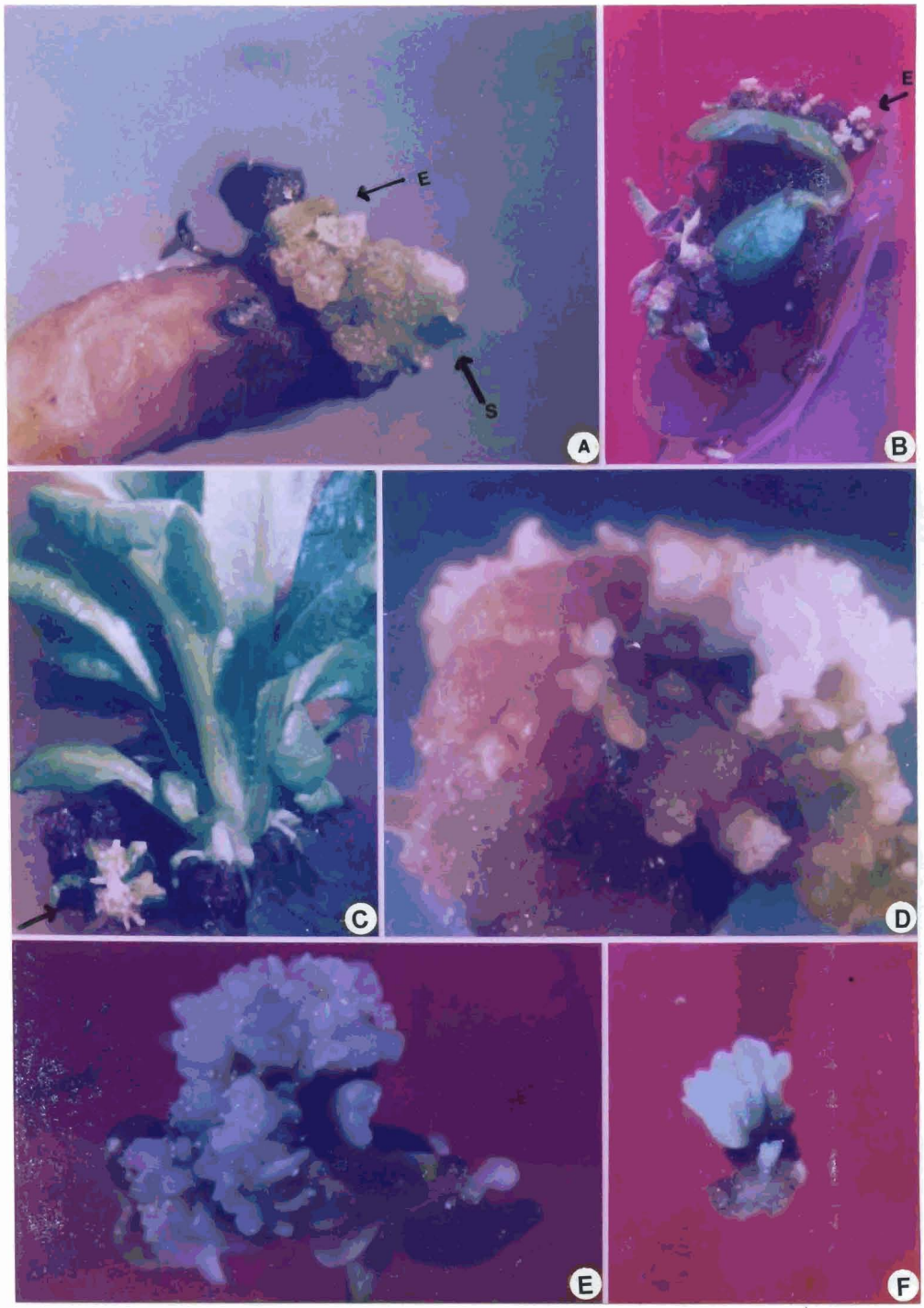



Fig VIII

Fig IX A-F Indirect somatic embryogenesis from callus originated from different explants of *Eryngium foetidum*

A. Friable brown embryogenic callus developed from root explant and *in vitro* developed root on MS + NAA (1.0 mg $l^{-1}$) + Kin (0.5 mg $l^{-1}$)

B. Embryos developed on half MS + NAA (0.5 mg $l^{-1}$) + Kin (0.25 mg $l^{-1}$)

C. Creamy friable embryogenic callus on MS + 2,4-D (1.0 mg $l^{-1}$) + Kin (0.5 mg $l^{-1}$)

D. Proliferated embryogenic callus with different stages of embryos

E. Conversion of embryos on half MS +10 % CW

F. Plantlets developed from embryos on half MS + 10 % CW

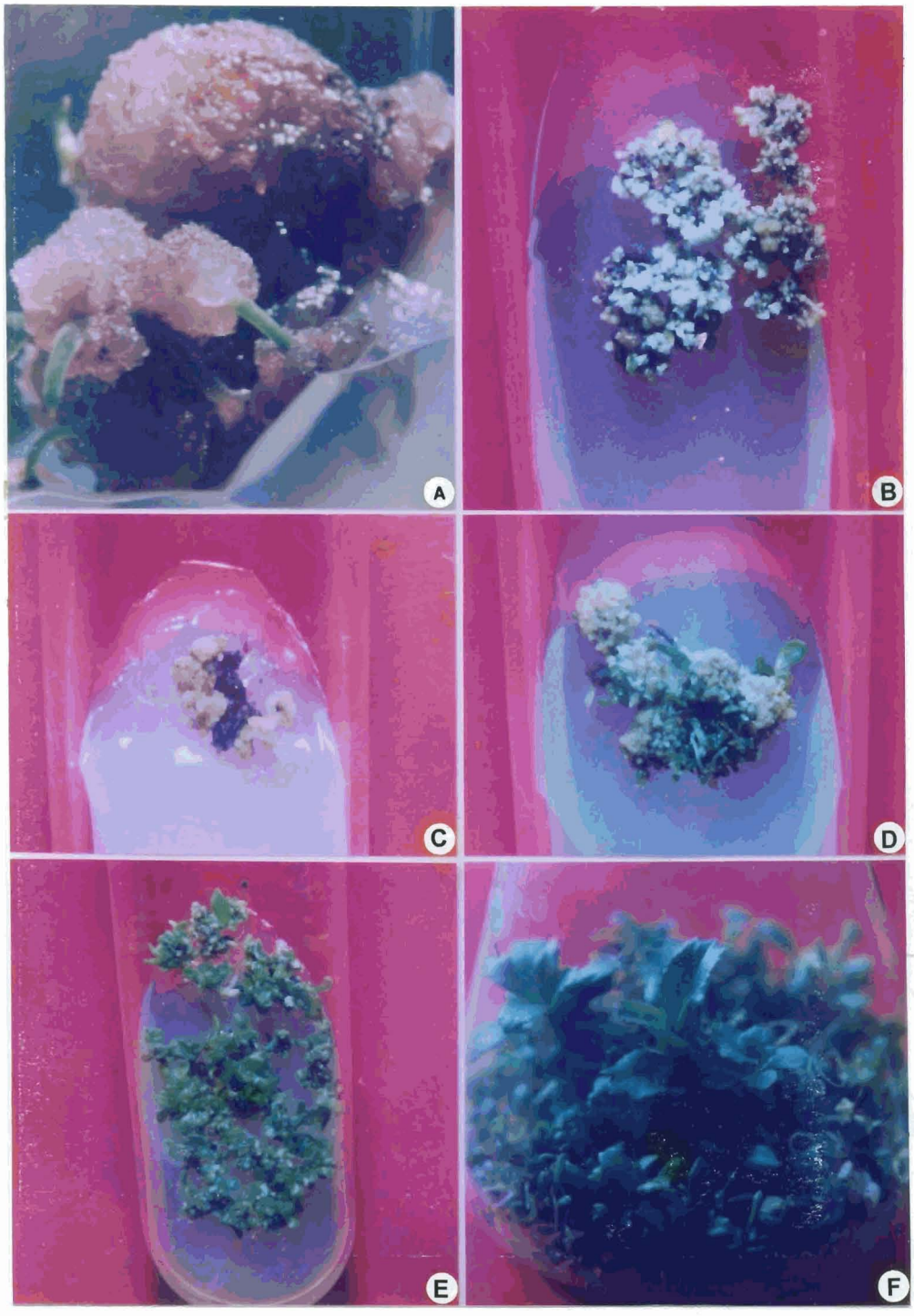


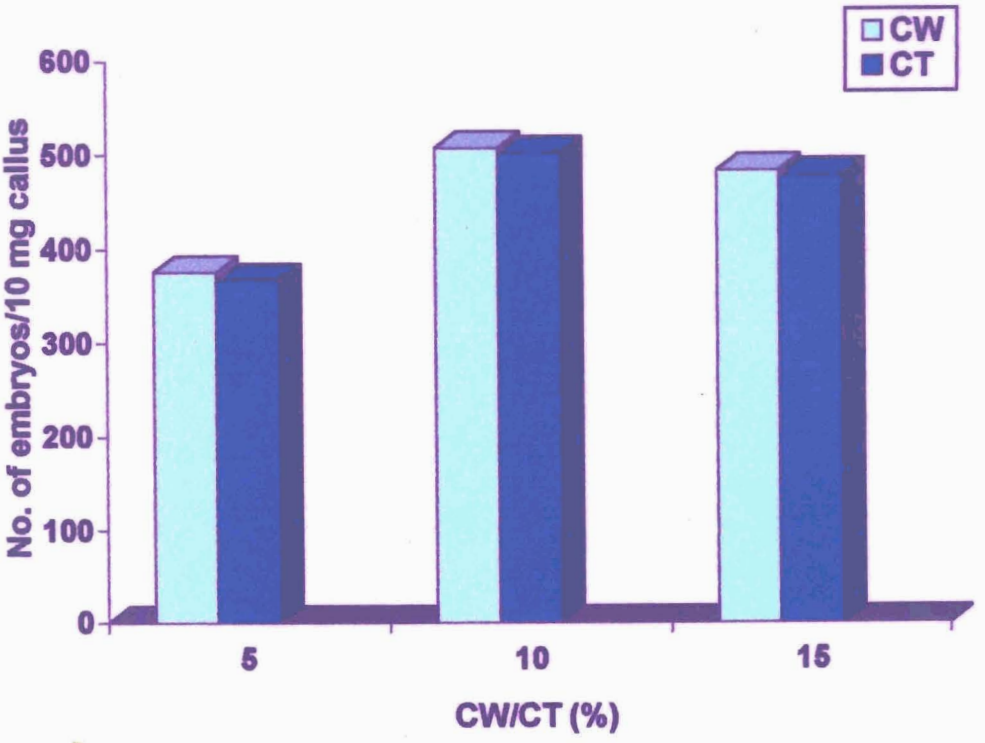

Fig IX

Fig. X A Effect of CW/CT in the induction of somatic embryos on *E. foetidum* on half MS medium with 1.5 % sucrose

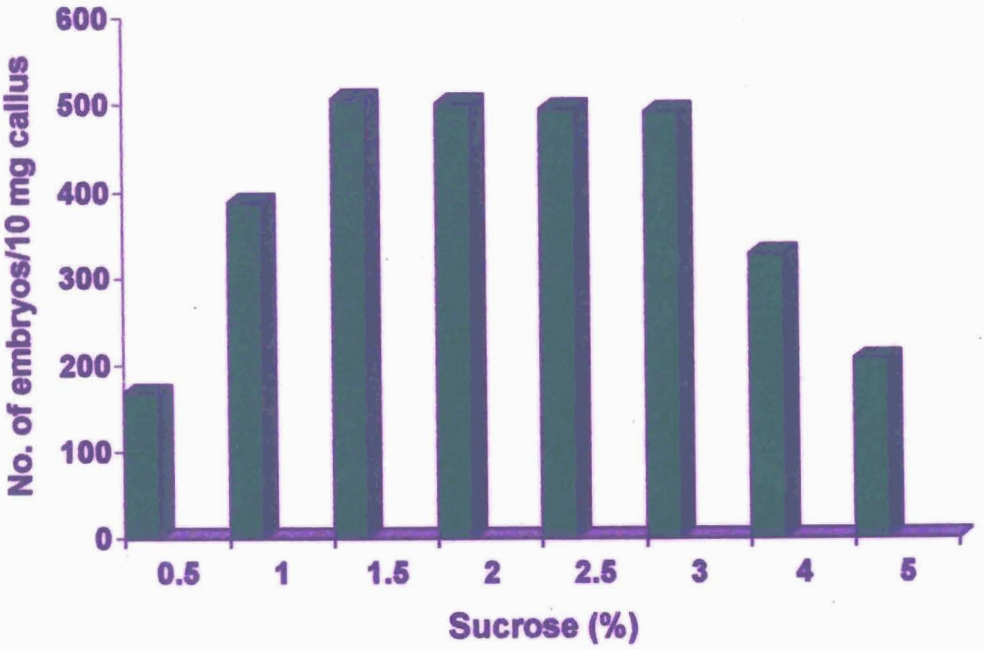
(Data represents an average of 5 replicates)

Fig. X B Effect of sucrose in the induction of somatic embryo on *E. foetidum* half MS medium with 10 % CW

(Data represents an average of 5 replicates)


CW
CT
600
500
400
300
200
100
0
No. of embryos/10 mg callus
5
10
15
CW/CT (%)

A


600
500
400
300
200
100
0
No. of embryos/10 mg callus
0.5
1
1.5
2
2.5
3
4
5
Sucrose (%)

B

Fig X

Fig XI A-H Somatic embryos of *Eryngium foetidum* with different developmental stages

A. Cluster of embryos up to torpedo

B. Early cotyledonary embryo

C. Dicotyledonary mature embryo

D. Germinating embryo

E. Embryo derived plantlet

F. Polycotyledonary mature embryo

G,H Sequential development of polycotyledonary embryo

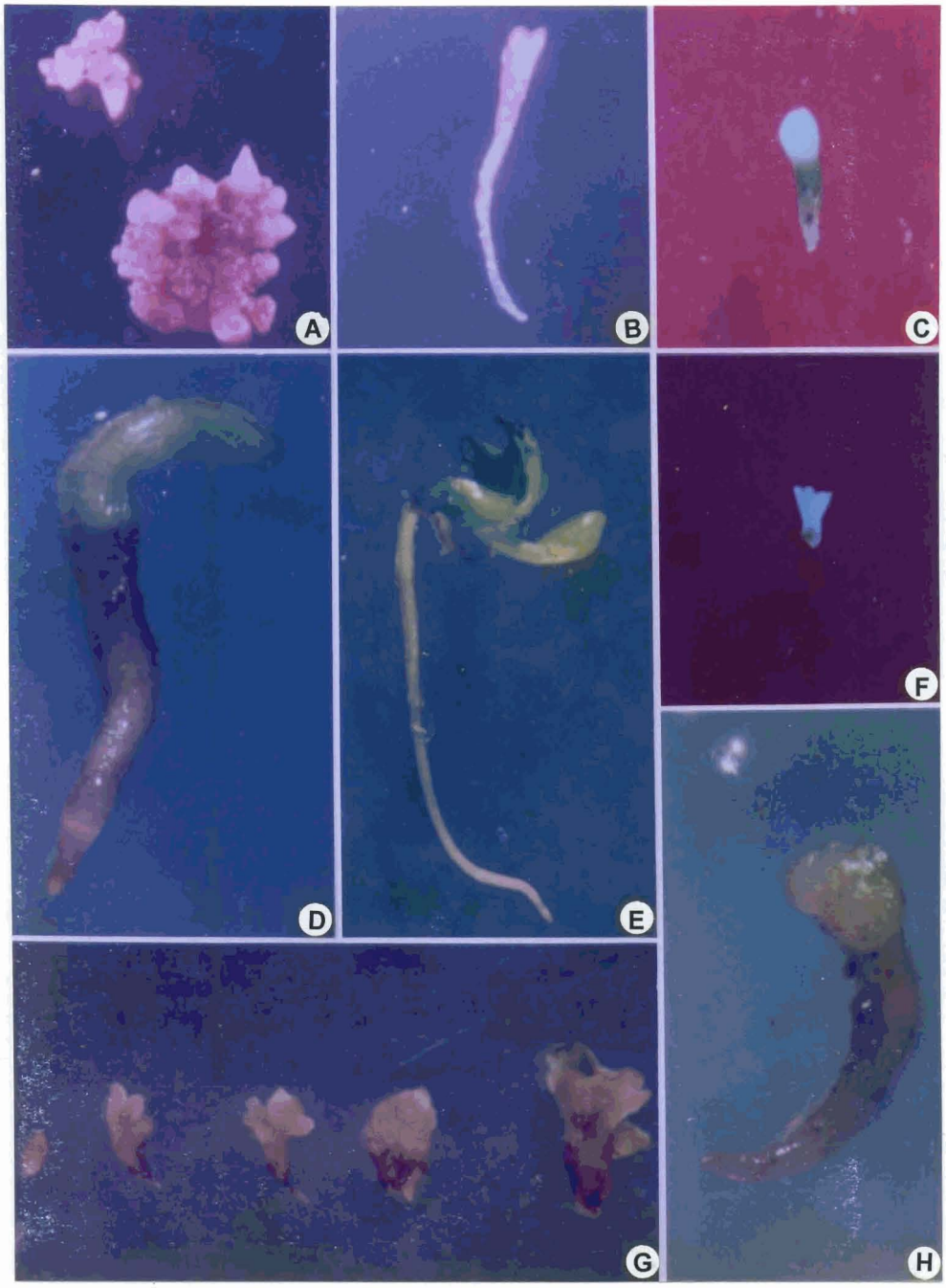



Fig XI

Fig. XII A-G Induction of secondary somatic embryos in *Eryngium foetidum*

A. Direct secondary somatic embryos in clusters induced on the apex of primary embryos
B. An enlarged secondary embryo
C. Direct secondary somatic embryos just below the shoot apex of primary embryo
D. Direct secondary somatic embryos in cluster around the shoot apex
E. Direct secondary somatic embryos in cluster on root of primary embryo
F. Indirect secondary somatic embryos on callus developed from shoot apex of primary embryos
G. Indirect secondary somatic embryos from callus developed on the root of primary embryos

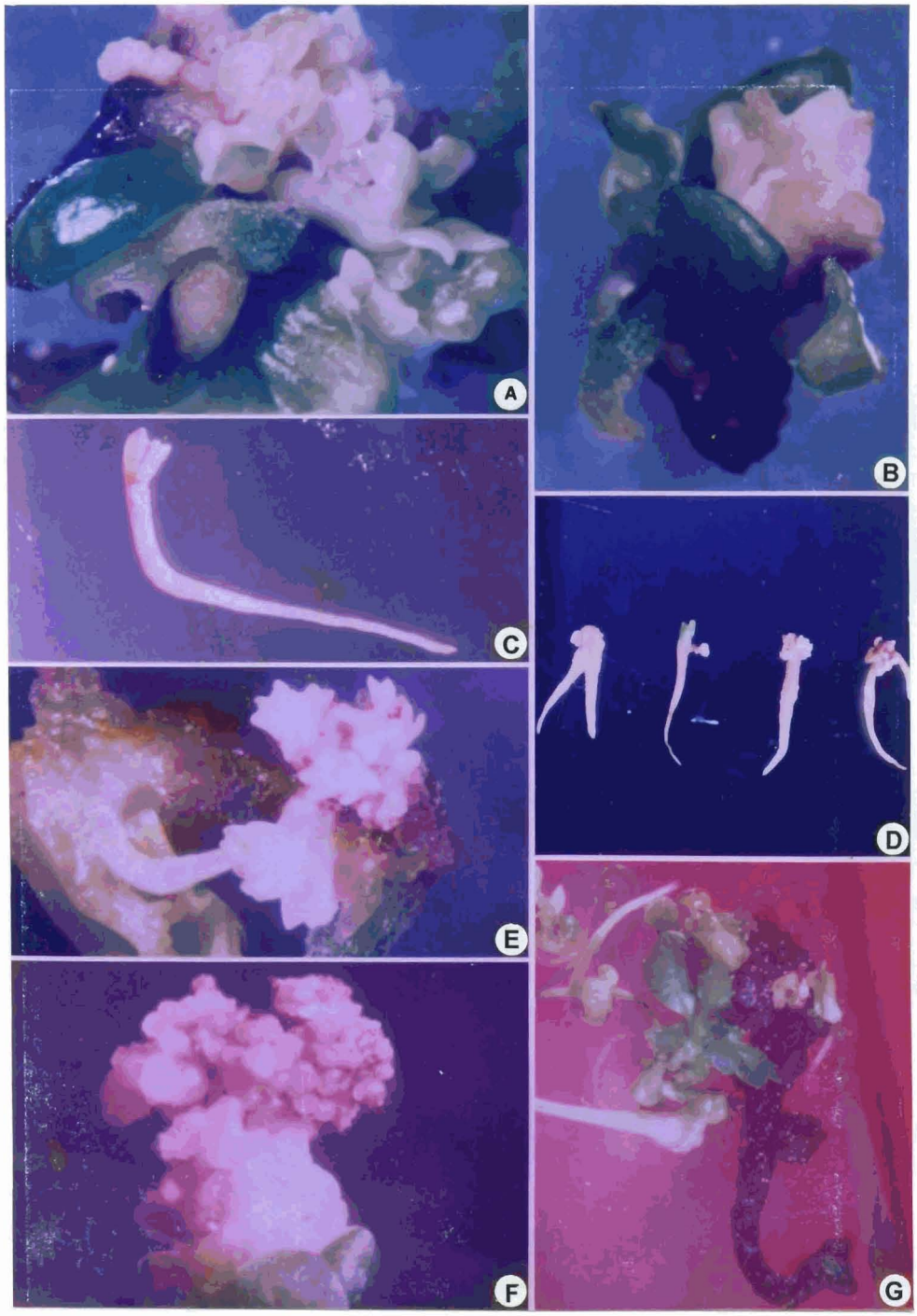



Fig XII

Fig XIII A-F Protoplast isolation, culture and regeneration of *E. foetidum*

A. Isolated protoplasts from leaf mesophyll cells

B. Protocalli (brown spots) developed from protoplast encapsulated in alginate beads in MS+ Kin (1.0 mg $l^{-1}$) + BAP (0.5 mg $l^{-1}$)

C. Shoots developed from alginate beads on above concentrations (80 days)

D,E Somatic embryos induced from protocalli [isolated from alginate beads of MS + 2,4-D (1.0 mg $l^{-1}$) + Kin (0.5 mg $l^{-1}$)] on half MS + 10 % CW

F. Well-developed plantlets *via* protocalli mediated somatic embryogenesis on half MS + 10% CW

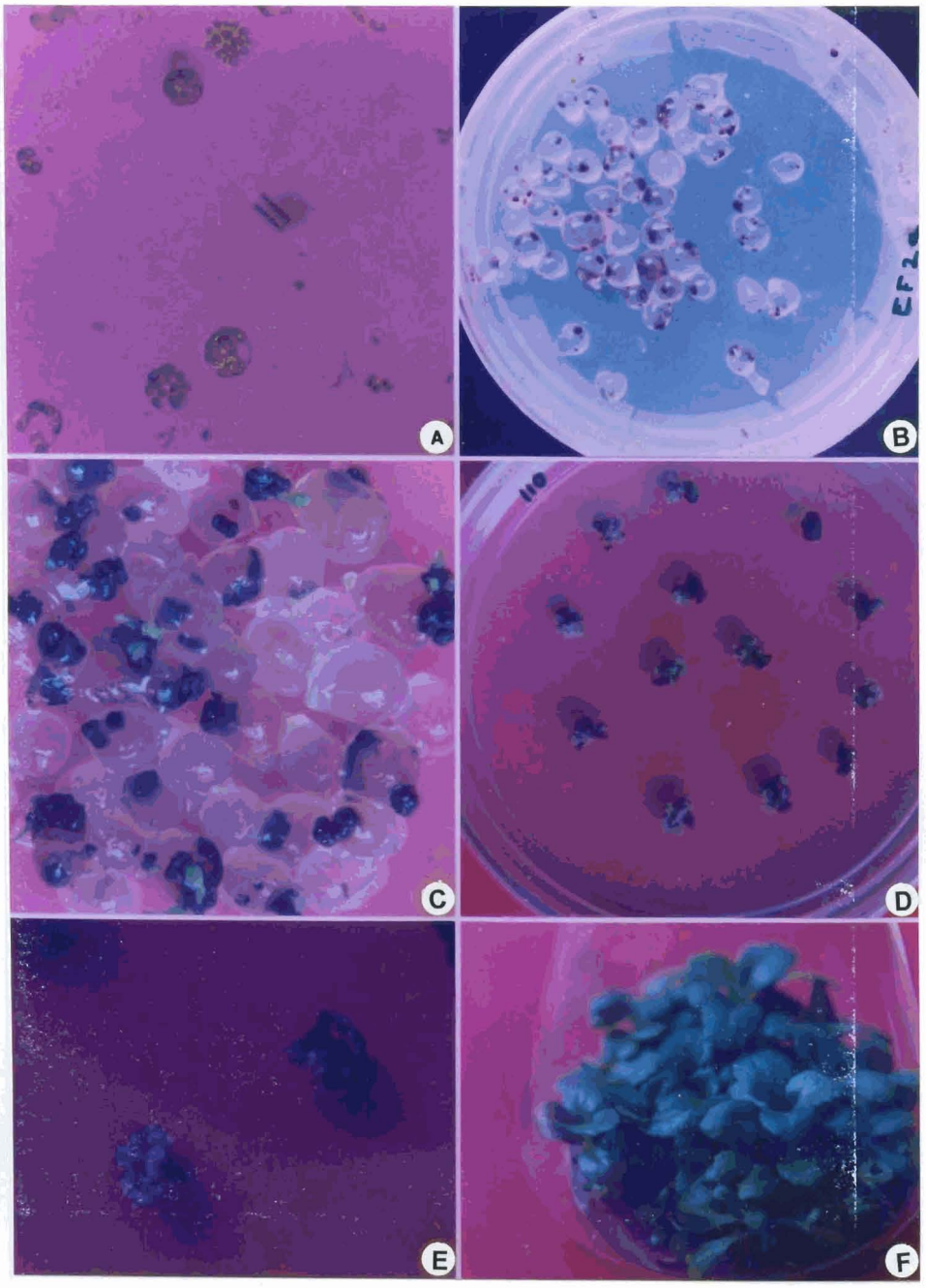



Fig XIII

Fig. XIV A-F Ontogenic studies of shoot and somatic embryos of *E. foetidum*

A. Direct shoot formation from phloem cells of leaf explant (x 10)
B. Developed shoot bud (x 10)
C. Direct somatic embryo (late globular) from root cortical cells of root (x 10)
D. Division of mesophyll cells just below the vascular traces (after 5 days) (x 10)
E. Formation of callus (after 20 days) (x 10)
F. Formation of embryogenic calli from both the sides of leaf (x 10)

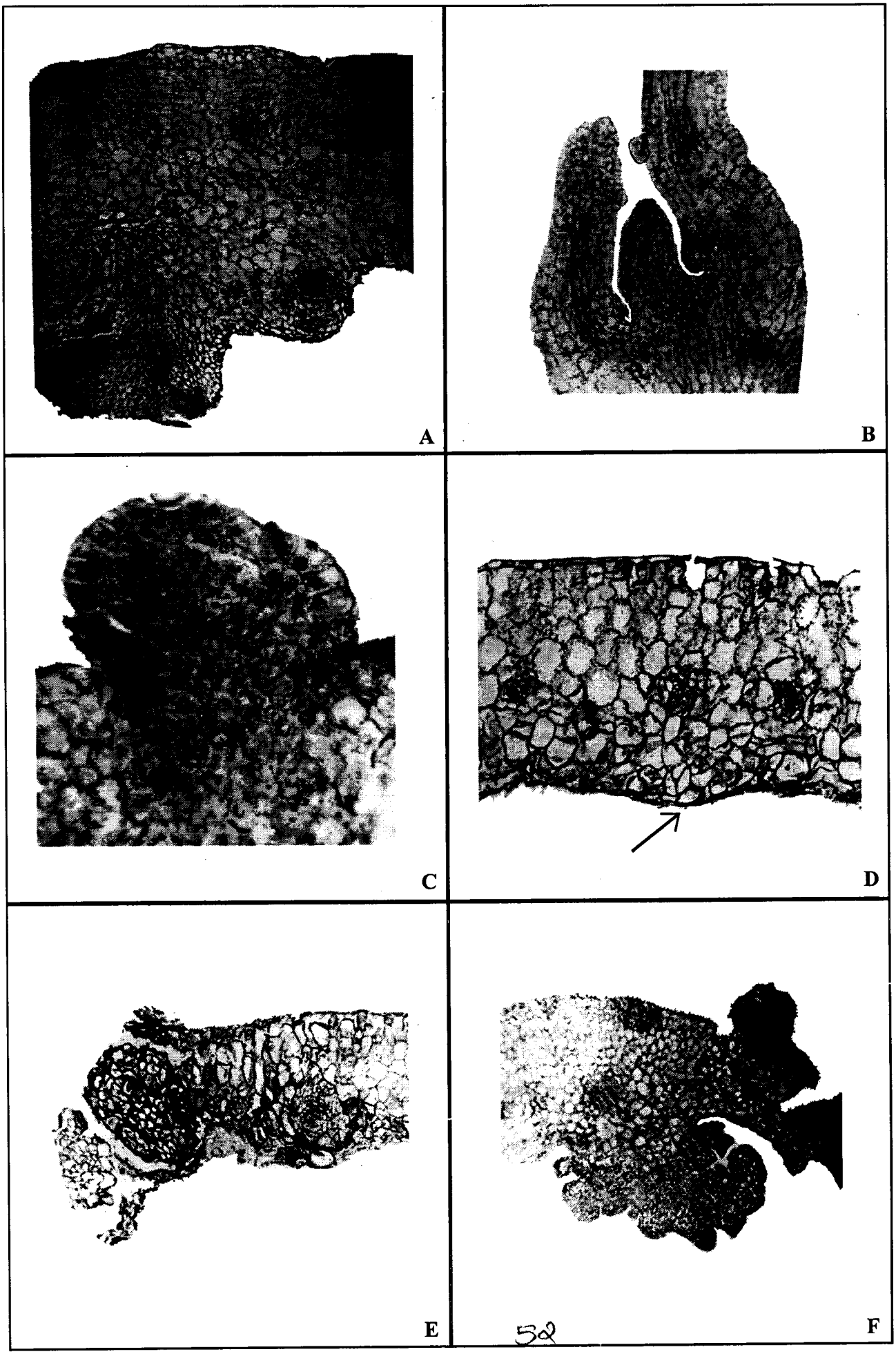



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Fig XIV

Fig. XV A-O Developmental stages of somatic embryo of *E. foetidum* (x 20)

A. 1. Embryonal cell just before division
2,3 Embryonal cell at transverse division
4. Two celled embryo
5. Three celled embryo

B. 1. Embryo at 5 celled stage
2. Embryo at 7 celled stage
3. Embryo at 9 celled stage

C-E Embryos at early globular stage

F. Globular embryo

G. Early heart shaped embryo

H. Heart shaped embryo

I. Early torpedo embryo

J. Late torpedo embryo

K. Embryo just before cotyledonary stage

L. Cotyledonary stage embryo with root pole

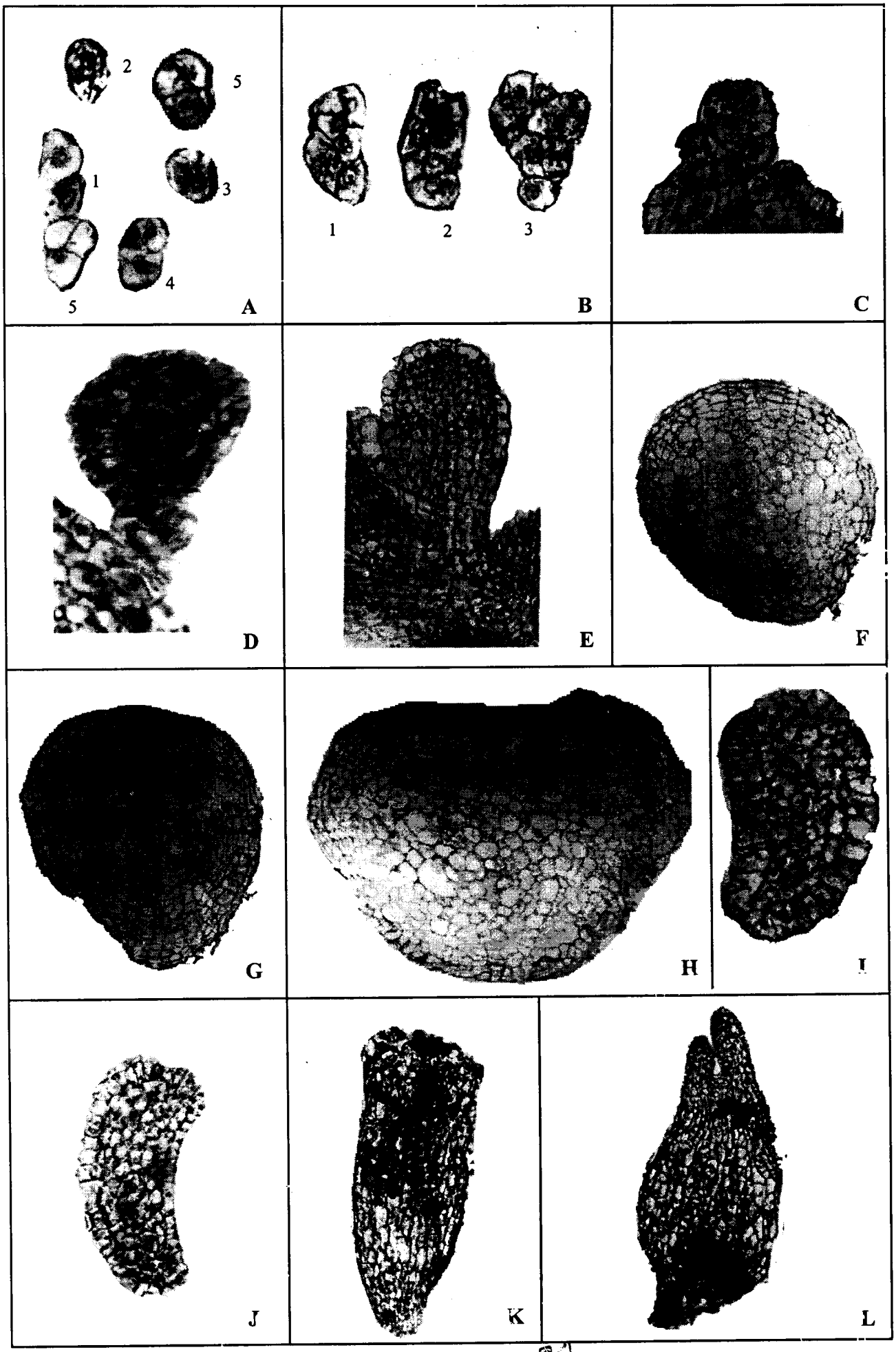
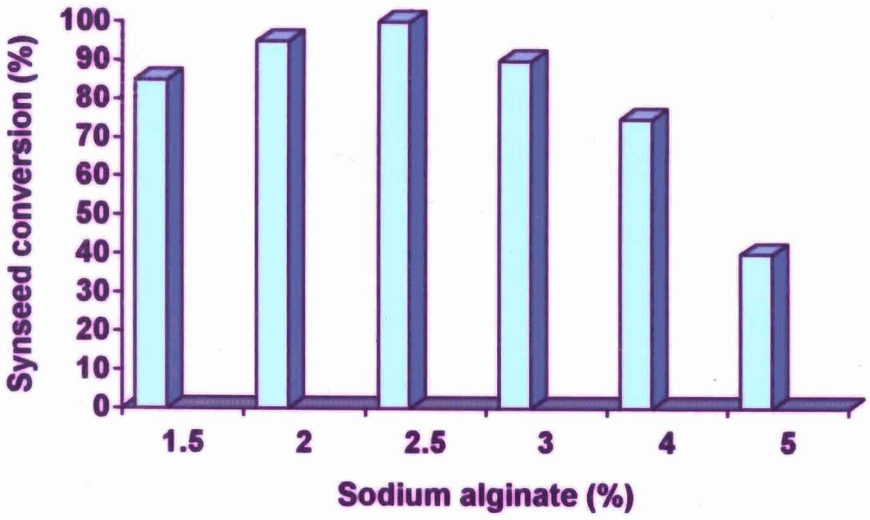


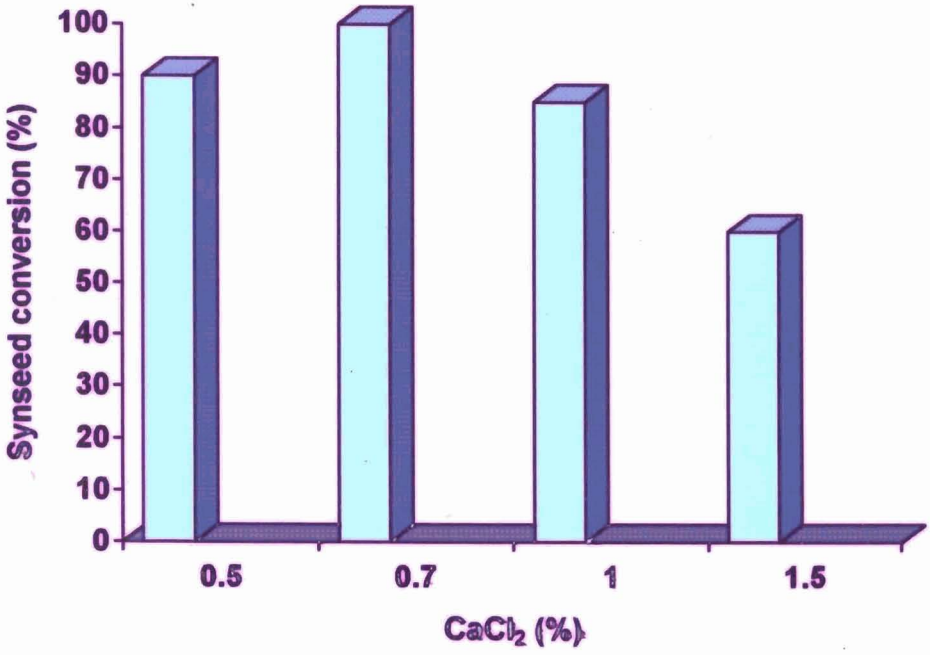

Fig XV

Fig XVI A & B Effect of sodium alginate and $CaCl_2$ in the conversion of synseeds of *E. foetidum*

Data represent an average of 20 synseeds


Synseed conversion (%)
100
90
80
70
60
50
40
30
20
10
0
1.5
2
2.5
3
4
5
Sodium alginate (%)

A


Synseed conversion (%)
100
90
80
70
60
50
40
30
20
10
0
0.5
0.7
1
1.5
CaCl2 (%)

B

Fig XVI

Fig XVII A-C Encapsulation of somatic embryos of *Eryngium foetidum* and its germination

A. Somatic embryos encapsulated in alginate + half MS with 10 % CW

B. Embryos in alginate beads showing germination (10 days)

C. Germination of synseeds on soilrite+sand (25 days)

D,E Germinated somatic embryo encapsulated in sodium alginate (water with 1.5 % sucrose) after 6 and 9 months

F. Plantlets on photoautotrophic medium (12 months)

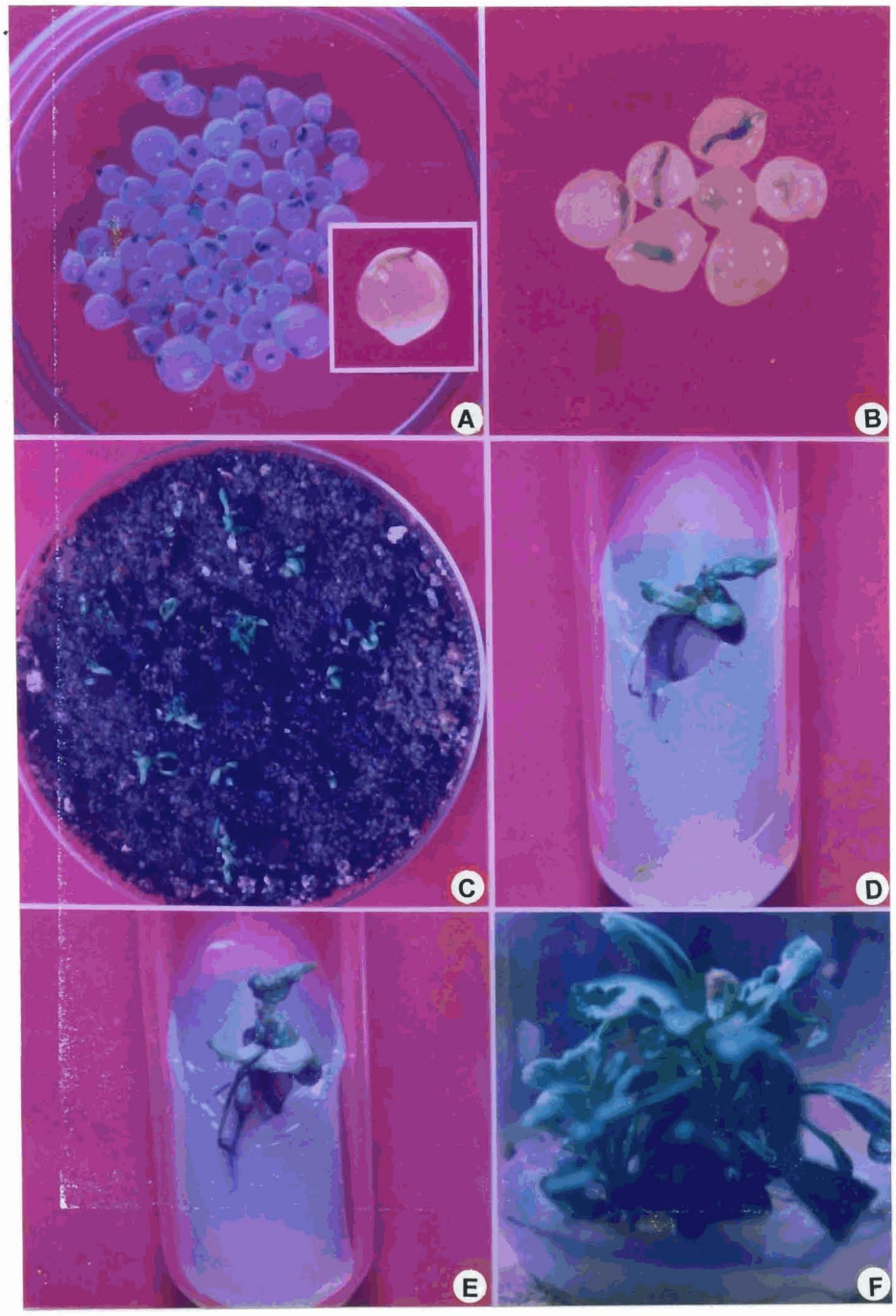



Fig XVII

Fig XVIII A-D *In vitro* rooting of *Eryngium foetidum* and secondary metabolite analysis

A. Rhizogenesis from shoots on half MS without hormone
B. Plantlet in small pot (20 days old)
C. Roots developed on somatic embryo derived plantlets used for extraction for TLC analysis
D. TLC plate showing secondary metabolite at different *Rf* values

(EtOH - Ethyl alcohol)
(PE - Petroleum ether)
*(I V - in vitro)*
(FG - Field grown)

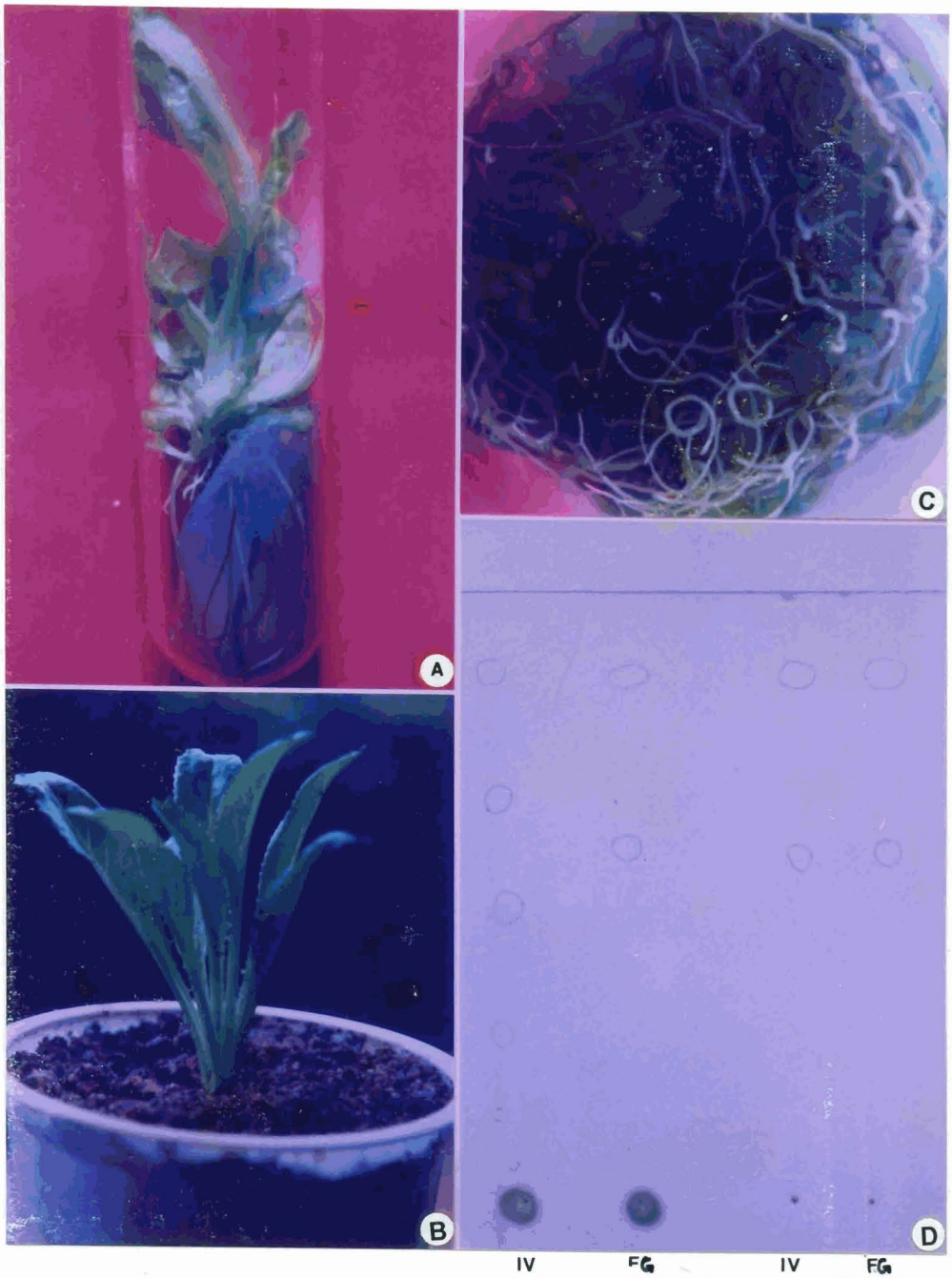



Fig XVIII

# *Holostemma ada-kodien*

*In vitro* cultures were established in *Holostemma ada-kodien* by culturing different explants such as node, internode, leaf and root on different media (MS, $B_5$ and White's). Either, the basal media were used as such or the media fortified with different hormones or additives as specified in respective sections.

## AXILLARY BUD MULTIPLICATION

The experiments were initiated with the culture of nodal explants excised from different regions of the plant (basal, middle and apical) on different basal media. An average of two shoots was induced within 10 days from the basal nodal explants on MS basal medium (Table XII; Fig XIX A). On $B_5$ medium, the nodal explants exhibited an initiation of only one (rarely two) shoot. White's medium did not show any response. Hence, further studies were carried out with basal nodal segments on MS medium supplemented with cytokinins, auxins and additives either singly or in combinations as described below.

### Effect of BAP

The nodal explants were cultured on MS medium fortified with various concentrations of BAP for the induction of multiple shoots (Table XII). Of the different concentrations of BAP tested, BAP at 1.5 mg $l^{-1}$ was found effective, which induced an average of 4 shoots from basal nodal explants within 40 days (Table XII). An increase in the concentrations of BAP beyond the optimal level (1.5 $mgl^{-1}$), the shoots failed to elongate. In all the concentrations of BAP, creamy green/pale green, compact nodular callus was also developed at the basal cut end of the nodal explants.

Subculturing of the nodal explants excised from *in vitro* grown shoots, on MS medium with the same concentrations of BAP produced an average of 7

**Table XII** Effect of basal media and growth regulators/CW in axillary bud multiplication of *Holostemma ada-kodien*

| Media | Growth Regulators (mg $l^{-1}$) | | | | | CW (%) | % of response | Average No. of shoots ±SD |
|---|---|---|---|---|---|---|---|---|
| | BAP | Kin | IBA | IAA | NAA | | | |
| White's | | | | | | | 0 | 0 |
| $B_5$ | | | | | | | 70 | 0.9±0.1 |
| MS | | | | | | | 90 | 2.3±0.4 |
| " | 0.5 | | | | | | 90 | 2.7±0.3 |
| " | 1.0 | | | | | | 100 | 3.2±0.5 |
| " | 1.5 | | | | | | 100 | 4.3±0.4 |
| " | 2.0 | | | | | | 100 | 3.8±0.2 |
| " | 3.0 | | | | | | 80 | 2.3±0.4 |
| " | 4.0 | | | | | | 80 | 2.0±0.3 |
| " | 5.0 | | | | | | 70 | 1.9±0.2 |
| " | | 0.5 | | | | | 70 | 2.2±0.3 |
| " | | 1.0 | | | | | 90 | 2.8±0.5 |
| " | | 1.5 | | | | | 100 | 3.3±0.6 |
| " | | 2.0 | | | | | 100 | 3.2±0.4 |
| " | | 3.0 | | | | | 90 | 2.4±0.3 |
| " | | 5.0 | | | | | 70 | 1.8±0.4 |
| " | 1.5 | 0.5 | | | | | 100 | 3.1±0.6 |
| " | 1.5 | 1.0 | | | | | 90 | 2.7±0.3 |
| " | 1.5 | 2.0 | | | | | 80 | 2.1±0.5 |
| " | 2.0 | 0.5 | | | | | 80 | 3.6±0.2 |
| " | 2.0 | 1.0 | | | | | 100 | 2.9±0.5 |
| " | | | | | | 5.0 | 70 | 2.3±0.3 |
| " | | | | | | 10.0 | 90 | 2.9±0.2 |
| " | | | | | | 15.0 | 80 | 2.2±0.4 |
| " | 1.5 | | | | | 10.0 | 100 | 3.2±0.2 |
| " | 2.0 | | | | | 10.0 | 100 | 3.5±0.5 |
| " | 1.5 | | 0.5 | | | | 100 | 6.3±0.3 |
| " | 2.0 | | 0.1 | | | | 100 | 6.7±0.5 |
| " | 2.0 | | 0.5 | | | | 100 | 7.3±0.7 |
| " | 2.0 | | 1.0 | | | | 100 | 4.9±0.8 |
| " | 2.0 | | | 0.1 | | | 100 | 4.9±0.5 |
| " | 2.0 | | | 0.5 | | | 90 | 4.5±0.8 |
| " | 2.0 | | | 1.0 | | | 90 | 3.7±0.7 |
| " | 2.0 | | | | 0.1 | | 100 | 4.8±0.5 |
| " | 2.0 | | | | 0.5 | | 100 | 3.4±0.7 |
| " | 2.0 | | | | 1.0 | | 90 | 2.4±0.6 |

Data represents an average of 10 replicates. Growth period 40 days

shoots with in 40 days (Fig XIX B). Cotyledonary embryo like shoots (without root pole - unipolar) was developed in subsequent cultures. Subsequent cultures showed an increased number of shoots, but failed to elongate (Fig XIX C). The shoots developed later showed premature abscission of leaves and shoot tips. However, the shoots survived with the initiation of axillary buds just below the abscissional node.

On second subculture, the callus developed at the base of the nodal explants showed a tendency to develop "meristemoids" within 35-40 days. These meristemoids developed into shoots in prolonged culture. An increased number of shoots were developed (25 shoots) without subculturing (Fig XIX D).

**Effect of Kin**

Of the various concentrations of Kin added to MS medium, Kin at 1.5 mg $l^{-1}$ was found optimal for shoot induction (an average of 3 shoots) from the basal nodal explants (Table XII). Compared to the shoots developed on BAP medium, the shoots on Kin containing medium was more elongated. Higher or lower concentration of Kin other than the optimal resulted in a decline in the number of shoots.

Culture of the nodal explants excised from the primary cultures showed the same rate of shoot multiplication in Kin containing medium. In all the cases, the basal cut ends of the explants induced nodular hard creamy callus inside the medium. As in BAP containing medium, the shoots showed abscission of leaf and shoot tips as the duration of culture extends; but was in less extent and delayed.

**Effect of BAP + Kin**

MS medium with the combination of BAP and Kin at various concentrations was also tested for the induction of axillary shoot multiplication (Table XII). Among the different concentrations tested, BAP at 2.0 mg $l^{-1}$ + Kin at 0.5 mg $l^{-1}$ developed an average of 3 shoots only. The shoots were more elongated than on the medium containing BAP alone. Higher concentration of BAP decreased the number of shoots as well as shoot elongation. Where as, higher amount of Kin, resulted the elongation of the shoots with a reduction in the number of shoots. The basal cut end of the nodal explants induced callus, which was pale green to creamy depending on the BAP: Kin ratio.

**Effect of CW**

MS medium containing CW either alone or in combination with BAP was tested for the induction of multiple shoots from the basal nodal explants. Of the various levels tested, CW at 10% was found more effective; induced an average of 3 shoots (Table XII; Fig XIX E). The initiated axillary buds were swollen at the base (Fig XIX E). Combination of BAP and CW was found inferior to the medium with BAP alone. In all the cases, MS medium with CW alone or in combination with BAP, the nodal explants in both primary and in subcultures induced calli from the basal cut end. The callus was creamy to pale green and did not induce any shoot.

**Effect of BAP + Auxins**

The morphogenic response of nodal explants to various concentrations of auxins with the combinations of BAP was also studied (Table XII). MS medium fortified with BAP (2.0 mg $l^{-1}$) and IBA (0.5 mg $l^{-1}$) showed a high frequency of multiplication; induced an average of 7 shoots along with basal callus formation (Fig XIX F). Some of the shoots failed to elongate. The induction of shoots

declined with the increase or decrease in auxin/cytokinin ratio. Of the three auxins (NAA, IAA and IBA) used in combination with BAP, IBA was found more effective than the other two (Table XII).

Subculturing of the nodal explants from the *in vitro* derived shoots on MS medium with the same concentrations of BAP + IBA showed more shoot proliferation; an average of 18 shoots (Fig XX A). However, some of the shoots remained short (Fig XX A). The developed shoots exhibited abscission of leaves and young shoot tips. The callus formed at the basal cut end of the explants was nodular and pale green. The amount of callus formation was dependent on the concentrations of auxins used and showed a positive response with the increase of auxin concentrations. The basal callus cultured on the same concentrations of BAP + IBA showed a tendency to develop meristemoids and shoot buds (Fig XX B).

**Nodal explants from different positions**

Axillary bud initiation and shoot multiplication was performed with the nodal explants excised from three different position of the source plant *viz.*, the basal, mid and terminal (nodes just below the first one or two pair of leaves). The basal nodal explants of both field grown and *in vitro* grown, showed better response of inducing more number of shoots followed by mid and terminal. Nevertheless, the basal nodal explants of field grown source were confined with more contamination than that with mid and terminal. In subsequent cultures, the basal stumpy region developed more number of shoots, along with callus formation. The callus showed an increased tendency to induce shoot regeneration *de novo.*

## CALLUS STUDIES

Different explants of *H. ada-kodien viz.*, leaf (petiolar, mid and tip portions), internodes and roots were cultured on different media (MS, $B_5$ and White's) devoid of growth regulators for the induction of callus (Table XIII). Callogenesis was obtained within 10 days on MS basal medium from the internodal and leaf explants especially from the petiolar region. In all explants, the callus was confined at the cut ends only. Of the different explants cultured, the internodal explants developed more amount of callus on MS medium (Fig XX C). The callus was creamy and friable, which later became semi-hard and pale green. In addition to callus formation, rhizogenesis were also occurred especially on leaf explants. The callus initiation and proliferation was at an enhanced rate under dark than light. Of the three media tested, MS medium was superior to the other two. For further studies of callus induction, only MS medium was used. Callus subcultured on MS hormone free medium showed proliferation at an enhanced rate.

### Effect of Growth Regulators

MS medium augmented with varied hormonal combinations were used to study the effect of growth regulators for the induction and proliferation of callus (Table XIII).

### Effect of NAA/IAA

The potential of callus induction and proliferation from different explants obtained on MS medium fortified with various concentrations of NAA/IAA were presented in Table XIII. Of the various concentrations of NAA/IAA tested, 2.0 mg $l^{-1}$ was found effective, which induced creamy and friable callus, which later became semi-hard. At lower concentrations of NAA/IAA (0.5 mg $l^{-1}$), in addition to callus formation numerous small roots were induced, especially on the petiolar

explants (Fig XX D). Increased concentrations of NAA/IAA showed an enhanced callus development with a decline of rhizogenesis. Subsequent cultures of the callus on the same medium showed proliferation of callus with an increased rate.

**Effect of 2,4-D**

Different explants cultured on MS medium supplemented with various concentrations of 2,4-D (0.1-3.0 mg $l^{-1}$) exhibited creamy or pale green friable callus within a week (Table XIII). Concentrations of 2,4-D at 1.0-2.0 mg $l^{-1}$ were found effective for the induction and proliferation of callus. Lower concentrations induced small roots (1-4) abreast to the callus formation and the callus showed a tendency to be semi-hard and pale green. An increased rate of callus proliferation without root formation were characterized by subculturing the callus.

**Effect of NAA/IAA+ Kin/BAP**

MS medium supplemented with the combinations of auxins and cytokinins were also tested to study their effect on callus induction and proliferation (Table XIII). Of the different combinations and concentrations tested, NAA/IAA (1.0-2.0 mg $l^{-1}$) and Kin/BAP (0.5 mg $l^{-1}$) were found suitable for the induction and proliferation of callus on different explants (Fig XX E). On leaf explants abreast to the callus formation, rhizogenesis were also occurred at 1.0 mg $l^{-1}$ NAA/IAA + 0.5 mg $l^{-1}$ Kin/BAP. The texture and colour of the callus varied depending on the auxin/cytokinin ratio. Increase of NAA/IAA made the callus more friable, while increase of Kin/BAP made the callus nodular and semi-hard or hard. At higher BAP:NAA ratio, the callus was pale green to green, whereas on higher Kin:NAA ratio, the callus was creamy or yellowish and hard. The callus, which grew below the level of the medium, became hard and nodular. Subculturing of the callus on the medium with the same concentrations of NAA/IAA with Kin showed an enhanced rate of proliferation.

**Table XIII** Efficacy of basal media and growth regulators in the induction of callus from different explants of *H. ada-kodien*

| Media | Growth Regulators (mg $l^{-1}$) | | | | | % of response | | | Amount of callus | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | NAA | IAA | 2,4-D | Kin | BAP | R | IN | L | R | IN | L |
| MS | | | Hormone free | | | 40 | 90 | 70 | + | ++ | + |
| $B_5$ | | | Hormone free | | | 30 | 60 | 50 | 0 | + | 0 |
| White's | | | Hormone free | | | 40 | - | - | + | | |
| MS | 0.5 | | | | | 60 | 90 | 90 | + | ++ | + |
| " | 1.0 | | | | | 80 | 100 | 100 | + | +++ | ++ |
| " | 1.5 | | | | | 90 | 100 | 100 | ++ | +++ | +++ |
| " | 2.0 | | | | | 90 | 100 | 100 | +++ | ++++ | ++++ |
| " | 3.0 | | | | | 80 | 90 | 90 | ++ | +++ | +++ |
| " | | 0.5 | | | | 60 | 90 | 90 | + | ++ | + |
| " | | 1.0 | | | | 80 | 100 | 100 | + | ++ | ++ |
| " | | 1.5 | | | | 90 | 100 | 100 | + | ++ | ++ |
| " | | 2.0 | | | | 90 | 100 | 100 | ++ | +++ | +++ |
| " | | 3.0 | | | | 80 | 90 | 90 | ++ | ++ | ++ |
| " | | | 0.01 | | | 90 | 100 | 90 | + | ++ | ++ |
| " | | | 0.10 | | | 100 | 100 | 80 | + | ++ | ++ |
| " | | | 0.50 | | | 60 | 90 | 90 | ++ | +++ | ++ |
| " | | | 1.00 | | | 80 | 100 | 100 | +++ | ++++ | +++ |
| " | | | 1.50 | | | 90 | 100 | 100 | +++ | ++++ | ++++ |
| " | | | 2.00 | | | 90 | 100 | 100 | ++++ | +++++ | ++++ |
| " | | | 3.00 | | | 80 | 90 | 90 | +++ | ++++ | +++ |
| " | | | | 0.5 | | 60 | 90 | 90 | + | ++ | ++ |
| " | | | | 1.0 | | 80 | 100 | 100 | + | +++ | ++ |

Contd.

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| " | | | | 2.0 | | | 80 | 90 | 90 | ++ | +++ | ++ |
| " | | | | | 0.5 | | 60 | 90 | 90 | + | ++ | + |
| " | | | | | 1.0 | | 80 | 100 | 100 | + | +++ | ++ |
| " | | | | | 2.0 | | 90 | 100 | 100 | +++ | ++++ | ++++ |
| " | | | | | 3.0 | | 80 | 90 | 90 | ++ | +++ | +++ |
| " | 2.0 | | | 0.1 | | | 90 | 100 | 100 | +++ | ++++ | ++++ |
| " | 2.0 | | | 0.5 | | | 100 | 100 | 90 | +++ | +++++ | ++++ |
| " | 2.0 | | | 1.0 | | | 80 | 90 | 90 | ++ | ++++ | +++ |
| " | 3.0 | | | 0.5 | | | 80 | 90 | 80 | ++ | ++++ | ++++ |
| " | 2.0 | | | | 0.5 | | 90 | 100 | 90 | +++ | +++++ | ++++ |
| " | 2.0 | | | | 1.0 | | 80 | 100 | 90 | ++ | ++++ | +++ |
| " | | 2.0 | | 0.1 | | | 90 | 100 | 100 | ++ | +++ | +++ |
| " | | 2.0 | | 0.5 | | | 100 | 100 | 90 | +++ | ++++ | +++ |
| " | | 2.0 | | 1.0 | | | 80 | 90 | 90 | ++ | +++ | ++ |
| " | | 2.0 | | | 0.5 | | 90 | 90 | 80 | +++ | ++++ | ++++ |
| " | | 2.0 | | | 1.0 | | 90 | 90 | 80 | ++ | +++ | +++ |
| " | | | 0.5 | 0.5 | | | 90 | 100 | 90 | ++ | +++ | ++ |
| " | | | 1.0 | 0. 5 | | | 90 | 100 | 100 | +++ | ++++ | +++ |
| " | | | 1.5 | 0.5 | | | 100 | 100 | 100 | +++ | +++++ | ++++ |
| " | | | 1.0 | 1.0 | | | 90 | 100 | 100 | ++ | ++++ | +++ |
| " | | | 2.0 | 0.5 | | | 90 | 100 | 100 | ++ | +++++ | ++++ |
| " | | | 2.0 | 1.0 | | | 80 | 100 | 90 | ++ | ++++ | +++ |
| " | | | 3.0 | 0.5 | | | 80 | 100 | 100 | ++ | ++++ | +++ |
| " | | | 1.0 | | 0.5 | | 90 | 100 | 90 | ++ | ++++ | +++ |
| " | | | 1.5 | | 0.5 | | 90 | 100 | 100 | +++ | +++++ | ++++ |
| " | | | 2.0 | | 0.5 | | 80 | 100 | 90 | +++ | +++++ | ++++ |

Data Represnents an average of 10 replicates

Growth period 40 days 0 - No response + - Very little ++ - Little ++++ - Moderate +++++ - Profuse

**Effect of 2,4-D+Kin/BAP**

Callus induction and proliferation was obtained from different explants on MS medium fortified with various concentrations of 2,4-D in combination with Kin/BAP (Table XIII). Of which, 2,4-D at 1.0 mg $l^{-1}$ and Kin/BAP at 0.5 mg $l^{-1}$ was found optimal for the induction of callus. In all the types of explants, especially in the root and internode the whole surface of the explants was consumed for callus formation (Fig XXI A, B). Depending on the auxin: cytokinin ratio, the texture and colour of the callus were varied. At higher concentrations of auxin, the callus became friable and creamy in colour and at higher concentrations of cytokinin, compact nodular callus was induced. Subculturing of the callus showed an enhanced rate of proliferation.

**Effect of Cytokinins**

Callus formation was obtained on all explants, nodal (alluded earlier), internodal, leaf and root explants on MS medium containing various concentrations of BAP/Kin (Table XIII). When the cytokinins used individually it was more effective at 1.5 mg $l^{-1}$. The callus was hard and nodular in all the cases (Fig XXI C). At higher concentrations of BAP, the callus was more green and nodular; while with Kin, the callus was creamy and nodular. Subculturing of the callus especially on BAP medium showed proliferation with the tendency to become more hard and nodular.

In general, all types of explants exhibited callus initiation at the cut ends, which further extended to the other regions. Callus formation was observed on the veins and vein tips of leaves also. Of the different portions of leaf cultured, induction of more callus was observed at the petiolar regions, followed by mid and tip. Proliferation of callus occurred on both adaxial and abaxial sides and

induction of callus was faster under darkness than in light. The callus was more friable under dark.

**INDIRECT ORGANOGENESIS**

Indirect shoot morphogenesis was accomplished from the callus obtained from nodal and internodal cultures.

Nodular pale green or green callus developed from the lower cut end of the nodal explants cultured on MS+BAP (1.5 mg $l^{-1}$) started to develop numerous meristemoids after 25 days (Fig XXI D). Later these meristemoids developed into shoots. Combination of an auxin (IBA/NAA) with BAP resulted in the formation of more nodular callus with reduction in the formation of meristemoids.

Subculturing of the nodular callus on BAP medium showed an enhanced rate of proliferation of meristemoids and a high frequency of shoot development (Fig XXI E). An average of 55 shoots was developed on MS medium containing BAP (1.5 mg $l^{-1}$) with more number of meristemoids (Fig XXI F). However, this shoots later showed necrosis and premature abscission of leaves and shoot tips. Nevertheless, the shoot revived growth with the emergence of axillary buds (Fig XXI G).

The callus developed from the internodal and proximal end of the petiolar explants became nodular when cultured on MS+BAP (2.0 mg $l^{-1}$) medium. The same upon subculturing to the medium with the same concentration of BAP induced an average of 5 shoot buds (Fig XXII A). Subculturing of the shoot buds showed more meristemoids (Fig XXII B). The meristemoids later developed into shoots (Fig XXII C). Compared to nodal callus (80 %), the percentage of response was low in the internodal callus (30%).

In the present investigation, it was found that BAP was the potent cytokinin for the indirect organogenesis.

**Effect of $AgNO_3/CoCl_2$**

To prevent the leaf and shoot tip abscission, the shoot multiplication medium (MS+ 1.5 mg $l^{-1}$ BAP) was modified by adding various concentrations of $AgNO_3$ (1.0 - 5.0 mg $l^{-1}$) or $CoCl_2$ (10.0 - 100 mg $l^{-1}$). Of the various concentrations of $AgNO_3$ tested, 3.0 - 5.0 mg $l^{-1}$ was found beneficial, which controlled the abscission of leaves and shoot tips. Though the size of the leaves and growth were increased on the medium with $AgNO_3$, the shoot regeneration/multiplication rate was hindered (Fig XXII D).

Addition of $CoCl_2$ at 20.0 - 50.0 mg $l^{-1}$ was found effective in the present study and the result was similar to that of $AgNO_3$. An increased concentration of $CoCl_2$ was found lethal.

**TUBEROUS ROOTS *IN VITRO***

Tuberous roots were induced by subculturing the callus on MS medium containing different concentrations of CW (10 %) alone or BAP (2.0 mg $l^{-1}$)+NAA (0.5 mg $l^{-1}$)+AA (100 mg $l^{-1}$)+$AgNO_3$ (3.0 mg $l^{-1}$) (Fig XXIII A-D). Compared to the MS+CW (10 %), more numbers of tuberous roots were developed on the medium with the combinations of BAP+NAA+AA+$AgNO_3$ (Fig XXIII A, B).

**SOMATIC EMBRYOGENESIS**

The potential of somatic embryogenesis in *H. ada-kodien* was studied using the friable callus obtained from different explants on MS medium containing various growth regulators (2,4-D/NAA/IAA) either individually or in combinations with BAP/Kin. The friable calli obtained on MS medium with reduced concentrations of NAA/IAA exhibited a tendency to become hard or to develop

into roots. Hence for the establishment of somatic embryogenesis, the callus obtained on 2,4-D (0.5 or 1.0 mg $l^{-1}$) containing medium was used.

Friable calli developed on MS+2,4-D (1.0 mg $l^{-1}$) under darkness were serially subcultured in suspension and static MS (full or half) without or with reduced levels of 2,4-D. Though the callus underwent proliferation, no embryoids were observed during the first subculture (Fig XXIV A). Subculturing on static medium with 2,4-D (0.1 mg $l^{-1}$) induced few globular embryoids (Fig XXIV B). However, the callus induced embryoids up to torpedo stages after 10 days of second subculturing in suspensions of half or quarter MS containing 0.05 - 0.1 mg $l^{-1}$ 2,4-D (Fig XXIV C-F). Few of the embryos developed into cotyledonary stages in quarter MS, but the cotyledons remained fused. Though the development of embryos was asynchronous in both half and quarter MS, the cultures were encountered with more number of globular embryoids. During the development, the globular embryos showed a small suspensor like structure, which persisted up to heart stage. The embryos in small clusters were produced at an average of 40/5 mg callus. Rarely the primary embryoids developed accessory embryoids especially from the heart and torpedo stages. In certain cultures, the embryoids underwent dedifferentiation when the culture was kept for a long time in 2,4-D medium.

Transfer of the embryoids of different stages to suspensions of 1/4 MS without any growth regulator promoted maturation (Fig XXIV G). On static cultures under light, the cotyledons became green and showed splitting off into two or three (Fig XXIV H). However, the plumular apices of the mature embryoids remained undeveloped on static culture of the same medium.

Conversion of the embryoids were studied by transferring them into 1/10 MS basal medium or it with the addition of various levels of cytokinins (BAP/Kin), $GA_3$ and additives (CH/CW/CT/AC). Conversion of embryoids occurred on 1/10

MS with low sucrose level (1.5 %) on static medium under light conditions (Fig XXIV I). However, only 40 % of embryos underwent conversion.

Of the various levels of sucrose added to the medium (half or quarter MS + 0.05 - 0.1 mg $l^{-1}$ 2,4-D), 1.5 to 3 % showed no significant difference in the frequency of embryoid development. However, high concentration above 3 % and lower than 1.5 % inhibited somatic embryogenesis.

The callus developed under dark condition showed better efficacy for the induction of somatic embryogenesis. The embryoid formation was less under light conditions and the mature embryos showed a tendency to dedifferentiate (Fig XXV A-C). But, conversion of embryos occurred only under light conditions.

The suspension cultures were found superior to static cultures. A large number of embryoids were formed in suspension cultures, in which embryo maturation also occurred. On static cultures a delayed embryo formation was found and the embryos not developed beyond the globular stage.

**Effect of Different Media**

Regarding the efficacy of different media tested for somatic embryogenesis, it was found that suspension cultures of half or quarter MS was found superior to full MS, $B_5$ and White's. Somatic embryogenesis was delayed on White's medium supplemented with 0.1 mg $l^{-1}$ 2,4-D compared to half or quarter MS; and the embryoids developed only by third subculture (120 days). No embryoids were formed on full MS and $B_5$ even by third subculture.

**PROTOPLAST STUDIES**

Protoplast isolation and cultures were attempted with the leaf mesophyll cells of *in vitro* grown plants of *H. ada-kodien.* The enzymes, Cellulase Onozuka (0.6%) in combination with Macerozyme R10 (0.3 %) was found effective for the

isolation of protoplasts (Table XIV). Higher concentration of cellulase beyond the optimal level (0.6 %) found bursting of the protoplasts and lower concentration was encountered with low yield. Incubation under darkness at 25±2°C on a rotary shaker (at 30-50 rpm) was found suitable for the isolation of large numbers of protoplasts from the leaf mesophyll cells. An incubation period of 14-16 h showed a good yield of protoplasts (2.5 - 3.5 x $10^6$ /g fresh weight of leaf).

Two types of protoplasts were observed: large vacuolated ones and small ones with dense cytoplasm (Fig XXV C). The latter with good cytoplasmic streaming was considered as viable.

The protoplasts encapsulated in alginate with MS + 2,4-D (1.0 mg $l^{-1}$) were incubated under dark. The protocalli in the beads as microcolonies were observed after one month to the naked eye as small creamy spots (Fig XXV E). As the days passed on, the microcalli exhibited browning. The microcalli isolated and transferred on half or quarter MS containing 2,4-D (0.05-0.1 mg $l^{-1}$) produced few globular embryoids after 3 months (Fig XXV F). The transfer of embryogenic callus into suspension showed embryo induction at higher frequency. The development of embryos was asynchronous and developed up to cotyledonary stage in quarter MS containing 0.05 mg $l^{-1}$ 2,4-D. Transfer of the mature embryos on static 1/10 MS medium without growth regulator promoted conversion under light.

**HISTOLOGICAL STUDIES**

Histological studies showed the development of the embryos from globular to cotyledonary stages (Fig XXVI A-F). The embryos were with distinct epidermis and vascularization. The meristematic cells at the region of the shoot apices were richly cytoplasmic (Fig XXVI E).

**Table XIV** Effect of enzymes concentrations in the yield of protoplasts from leaf mesophyll cells of *Holostemma ada-kodien*

| Cellulase (%) | Macerozyme (%) | Protoplasts yield /g leaf tissue |
|---|---|---|
| 0.1 | 0.3 | $0.2 - 0.5 \times 10^6$ |
| 0.6 | 0.3 | $2.5 - 3.5 \times 10^6$ |
| 1.0 | 0.3 | $0.6 - 1.2 \times 10^6$ |

Incubation time: 14 - 16 h

## SYNSEEDS

Encapsulation studies were carried out with mature embryoids and embryo like shoot buds without root (unipolar) initiated from the axillary buds. Both the propagules were encapsulated in various levels of sodium alginate (1.5, 2.0, 2.5 and 4.0 %); it was found that 2.5 % were suitable for conversion (Fig XXVII A). Of the different concentrations of $CaCl_2$ tested (0.5, 0.7 and 1.0 %), the beads formed in 0.7 % was found ideal for high rate of conversion (Fig XXVII B).

The mature somatic embryos, encapsulated in 2.5 % alginate prepared in 1/10 MS hormone free medium (with 1.5 % sucrose) (Fig XXVIII A) cultured under light on static cultures of the same medium showed about 25 % conversion.

The unipolar shoot buds (Fig XXVIII B) encapsulated in 2.5 % alginate solution prepared in MS+ BAP (1.5 mg $l^{-1}$) showed 90 % conversion. The encapsulated buds showed conversion within 15 days and exhibited proliferation at an average of 12 shoots in clusters from the base (Fig XXVIII C). Some of the shoots later showed elongation (Fig XXVIII D). Subculturing of the shoot buds showed further multiplication.

## CONSERVATION *IN VITRO*

*In vitro* conservation studies on *H. ada-kodien* carried out at synseed and whole plant level as described below.

### (i) Conservation by Synseeds

*In vitro* conservation of germplasm by synseeds was conducted with the encapsulation of unipolar shoot buds and singulated mature embryos in 2.5 % alginate prepared either in half MS or double distilled water with sucrose (1.5 %). They were cultured on half MS or water gelled with agar.

The growth of the unipolar shoot buds and embryos in the beads prepared with sterile double distilled water was very slow compared to that of half MS with 1.5 % sucrose. The shoot buds encapsulated in double distilled water with sucrose took 2 months to emerge out and attained a size of 3 cm with three nodes and small leaves with in a period of 12 months (Fig XXVII E). Small roots were also developed. About 80% of survival were observed on photoautotrophic medium.

Of the two temperature regimes tested, the synseeds kept at 4°C showed very slow growth with below 30 % viability compared to that at 25±2°C. The present observation showed conservation at 25±2°C was feasible compared to that at 4°C. At 25±2°C, 80 % of the shoot bud derived synseeds survived even after 12 months.

(i) **Conservation at Shoot bud/plantlet level/shoot multiplication level**

The shoots with 2-4 nodes transferred on half MS +1.5 %sucrose + low levels of IBA/IAA (0.01 - 0.1 mg $l^{-1}$) and with or without $AgNO_3$ exhibited slow growth, which remained even after 12 months (in culture bottles). The shoots in cotton plugged culture tubes needed subculturing after six months due to the exhaustion of the medium. The internodes were short and the shoots bore small leaves. The shoots developed roots with little callus induction. Addition of $AgNO_3$ at 5.0 mg $l^{-1}$ along with auxins not made any noticeable change in growth.

The embryo like shoot buds (unipolar) initiated from the axillary buds upon culture on half MS + IBA/IAA (0.01- 0.05 mg $l^{-1}$) with or without $AgNO_3$ (3.0 - 5.0 mg $l^{-1}$) remained without any noticeable change even after 12 months (Fig XXVIII F, G). The medium supplemented with IBA/IAA and $AgNO_3$ (5.0 mg $l^{-1}$) was

found more feasible (Fig XXVIII F, G). Upon transfer to the shoot multiplication medium (BAP), an average of 12 shoots was developed.

Addition of $AgNO_3$ to shoot multiplication medium (MS+1.5 mg $l^{-1}$ BAP) curtailed the growth and was also found feasible in conservation of germplasm even after 12 months.

**ROOTING *IN VITRO***

The shoots developed either through axillary bud multiplication or through indirect organogenesis never induced roots on the shoot multiplication medium.

Well-developed shoots with 3-6 nodes developed on different media were excised and cultured for root induction on full or half MS with hormone free solid or liquid medium. Half strength MS medium was effective in inducing roots compared to full MS (Table XV). The results obtained in both liquid and solid medium were similar.

An enhanced root induction was observed, on half MS medium fortified with lower levels of auxin alone. Of the three auxins tested (NAA/IAA/IBA), IBA at 0.05 mg $l^{-1}$was most effective; induced an average of 7 roots/shoot (Table XV) (Fig XXIX A). Addition of NAA produced callus from the base of the shoot and was inferior to IBA and IAA. The number of roots declined with the increase in auxin concentration, but showed a tendency to induce callus. Addition of BAP/Kin (0.05-0.5 mg $l^{-1}$)/CW (10%) along with IBA showed a decrease in root number but characterized by an enhanced callus formation from the base of the shoots.

Though the result observed in both the liquid and solid medium was almost similar, the roots developed in liquid medium were more elongated (Fig

**Table XV** *In vitro* rooting studies on *Holostemma ada-kodien*

| Medium | Growth Regulators (mg $l^{-1}$) IBA | NAA | IAA | Kin | CW (%) | Per cent of response | Average No. of roots/shoot ±SD |
|---|---|---|---|---|---|---|---|
| MS | | Hormone free | | | | 80 | 2.1±0.4 |
| 1/2 MS | | Hormone free | | | | 90 | 3.5±0.5 |
| " | 0.01 | | | | | 80 | 5.2±0.7 |
| " | 0.05 | | | | | 80 | 7.1±0.6 |
| " | 0.10 | | | | | 90 | 6.5±0.8 |
| " | 0.50 | | | | | 100 | 3.7±0.5 |
| " | 1.00 | | | | | 100 | 3.1±0.3 |
| " | | 0.05 | | | | 80 | 3.6±0.5 |
| " | | 0.10 | | | | 100 | 2.9±0.6 |
| " | | 1.00 | | | | 100 | 2.4±0.5 |
| " | | | 0.05 | | | 90 | 6.8±0.7 |
| " | | | 0.10 | | | 100 | 6.1±0.5 |
| " | | | 1.00 | | | 100 | 2.9±0.4 |
| " | 0.05 | | | 0.1 | | 80 | 3.9±0.7 |
| " | 0.05 | | | 0.5 | | 90 | 2.9±0.5 |
| " | 0.05 | | | | 5.0 | 90 | 3.5±0.2 |
| " | 0.05 | | | | 10.0 | 90 | 2.6±0.4 |
| 1/2MS* | 0.05 | | | | | 100 | 6.9±0.7 |

Data represents an average of 10 replicates
Growth period 30 days
* Liquid

XXIX B). In addition, the shoot showed a faster growth when compared to that on solid medium (Fig XXIX B).

## TRANSFER TO FIELD CONDITIONS

Plantlets with well-developed roots were transferred directly to small pots containing sterile soilrite+sand (1:1) which revived growth within 15 days (Fig XXIX C). The plantlets developed *via* different ways exhibited 90 % survival in the soil.

## SECONDARY METABOLITE ANALYSIS

Comparative qualitative analyses of the alkaloids present in the tuberous roots of *in vitro* and field grown plants was performed by TLC.

The root samples of field grown plants showed five components which were identical in *Rf* values (0.07, 0.14, 0.44, 0.57 and 0.7) to those from the *in vitro* samples (Fig XXIX D). This indicated that two components (*Rf* values 0.1 and 0.22) additionally present in the *in vitro* tissues were qualitatively distinct (Fig XXIX D). Using polar solvent for extraction, the two tissue samples showed similarities in *Rf* values of 3 components (*Rf* 0.14, 0.44 and 0.6). The component with *Rf* value 0.1 being an additional entity in *in vitro* root tissues (Fig XXIX D).

# TRANSFORMATION STUDIES

## Induction of Hairy roots

The leaf explants of both *E. foetdium* and *H. ada-kodien* and internodes of *H. ada-kodien* co-cultivated on hormone free MS medium with *A. rhizogenes* LBA 9402 (pRi 1855) harbouring the binary plasmid **pGreen** either with ***gus*** or ***gfp*** reporter gene induced hairy roots and the per cent response is shown in Fig XXX A. The hairy roots were started to emerge after 12 days of co-cultivation both from the cut surfaces and the incisions made on the explants. The hairy roots were thick with tufts of white hairs (Fig XXXI A-D).

For control, the explants of the plants under study were cultured (without co-cultivation) on hormone free MS medium. In both the cases, normal roots were induced, but with reduced frequency; in comparison to those obtained with co-cultivation. About 20% of the leaf explants of *E. foetidum* produced 1-3 roots, but in *H. ada-kodien*, the percentage of root (1-3, as above) induction was varied with explants: 30 % for leaf and 20 % for internode. Compared to the hairy roots, the induction of root in the control was late and started to appear after 20 days of culture. The roots on the control explants of both the materials were creamy, long, slender and without tufts of hairs (Fig XXXI E, F). In addition to the roots, callus induction from the cut ends of the explants was also noticed and was more pronounced on the internodal explants of *H. ada-kodien* (Fig XXXI E, F).

## Transgenic plant regeneration studies

Transformed roots containing the marker gene, ***gus/gfp*** were selected by its subsequent culture on hormone free MS medium, supplemented with different levels of kanamycin sulphate. During control studies, the concentration of kanamycin sulphate in the MS basal medium, above 25 mg $l^{-1}$ was found lethal,

while the transformed roots showed survival, even in the presence of 150 mg $l^{-1}$ kanamycin.

Detailed regeneration studies were conducted in both the transformed roots containing the marker, ***gus***.

The segments of hairy roots of *E. foetidum,* obtained after co-cultivation induced hard green callus from its cut ends, when subcultured on MS medium fortified with BAP (1.5 mg $l^{-1}$). The green callus upon transfer to MS medium supplemented with Kin (1.0 mg $l^{-1}$) + BAP (0.5 mg $l^{-1}$) induced 3-7 shoots. The shoots showed stunted growth with wrinkled leaves in rosette fashion (Fig XXXII A).

Contrary to above, the segments of kanamycin resistant hairy roots of *H. ada-kodien* induced friable callus on MS medium, fortified with 2,4-D (1.0 mg $l^{-1}$). For embryogenic studies, the calli were subcultured in 1/4 strength liquid MS medium with 2,4-D (0.1 mg $l^{-1}$). Upon 3$^{rd}$ subculture - after 120 days of callus initiation - somatic embryos were induced and developed up to torpedo stage. Further developments were arrested, even when transferred to the maturation medium (hormone free1/10 MS).

Segments of the kanamycin resistant transformed roots of both the plants were cultured individually on the callus induction medium, standardized for each material. The transformed roots of *E. foetidum* and *H. ada-kodien* containing the marker ***gfp*** induced callus on MS + BAP (1.5 mg $l^{-1}$) and MS + 2,4-D (1.0 mg $l^{-1}$), respectively (Fig XXXII B). The callus developed from transformed root segments of *H. ada-kodien* was friable and creamy, but that of *E. foetidum* was green. But the region of callus in contact with the medium was pale-brown in colour and the same was used for the confirmation of ***gfp*** expression.

***gus/gfp* Assay**

For the confirmation of the ***gus*** gene expression, X-gluc stain was used. Upon treatment, the leaves of the regenerated plantlets from the callus of transformed root segments of *E. foetidum* and the underdeveloped embryos of *H. ada-kodien* showed deep blue colour, the indication of ***gus*** gene expression (Fig XXXII C-E).

For the confirmation of the ***gfp*** gene expression, the calli induced from the transformed root segments of both plants were exposed to UV light for 24 h, which showed green fluorescent colour, the indication of ***gfp*** gene expression (Fig XXXII F).

The scope of further studies of this piece of work should be aimed at the large scale induction of hairy roots, qualitative and quantitative analyses of the secondary metabolites in comparison to its production by the intact plants, regeneration of healthy transgenic plants in particular with the expression of ***gfp***, multiplication of the same *en masse* and finally, the successful establishment of the transgenic plants at commercial basis.

Fig XIX A-F Shoot formation from nodal explants of *Holostemma ada-kodien*

A. Axillary bud initiated on MS hormone free medium
B. Shoot multiplication on MS +BAP (1.5 mg $l^{-1}$)
C. Shoots showing stunted growth in subsequent culture
D. Shoots developed from the basal nodal callus on MS+BAP (1.5 mg $l^{-1}$)
E. Shoots initiated on MS+10 % CW
F. Axillary bud multiplication on MS+BAP (2.0 mg $l^{-1}$) + IBA (0.5 mg $l^{-1}$)

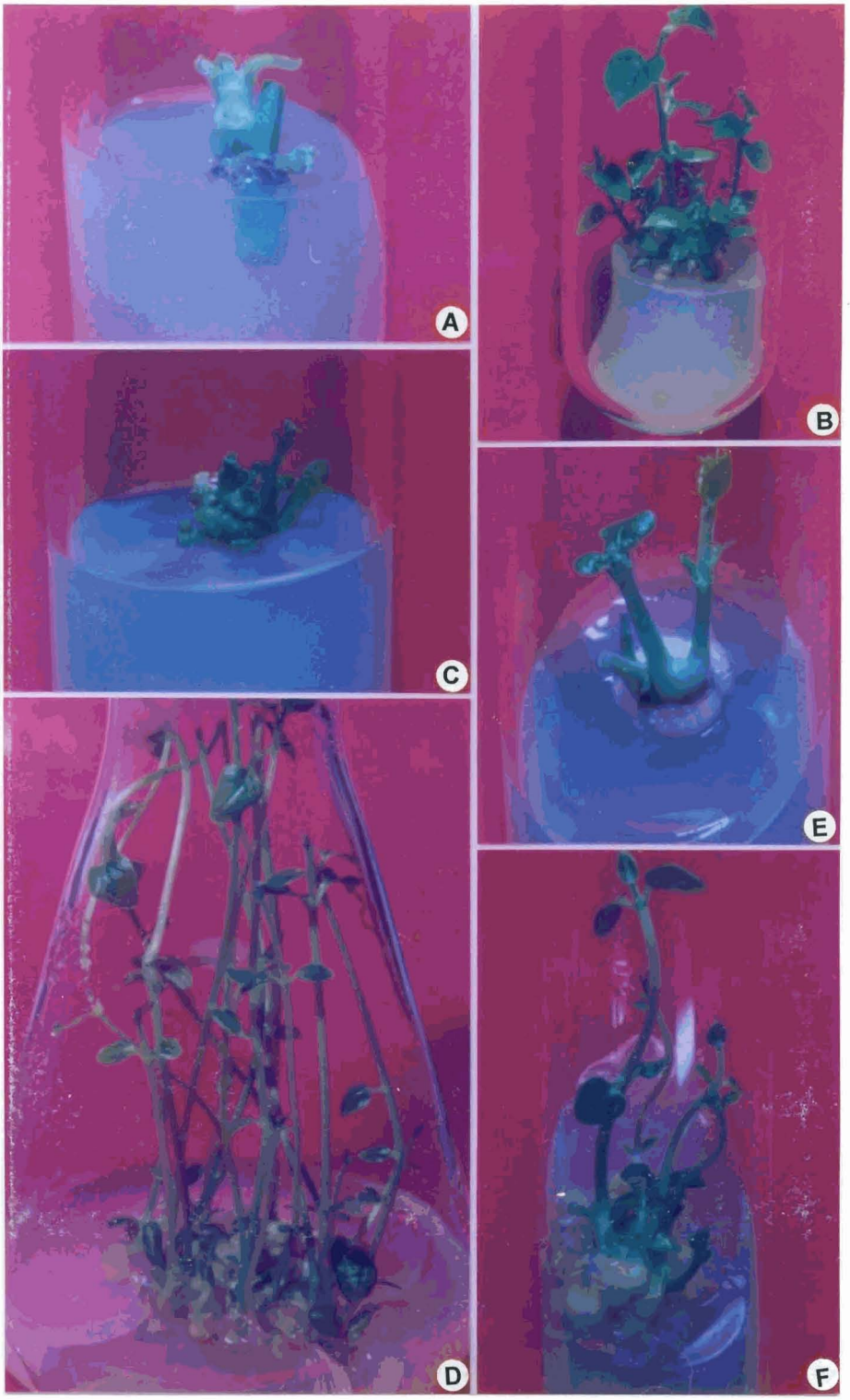



Fig XIX

Fig. XX A - E Shoot and callus induction from different explants of *H. ada-kodien*

A. Shoots developed in subsequent culture on MS+ BAP (2.0 mg $l^{-1}$) IBA (0.5 mg $l^{-1}$)
B. Meristemoids developed from the basal callus on MS+BAP (2.0 mg $l^{-1}$) + IBA (0.5 mg $l^{-1}$)
C. Callus induction from internodal explant on MS hormone free medium
D. Callus induction from leaf explants on MS+NAA (0.5 mg $l^{-1}$)
E. Callus induction from internode on MS+NAA (1.0 mg $l^{-1}$) + BAP (0.5 mg $l^{-1}$)

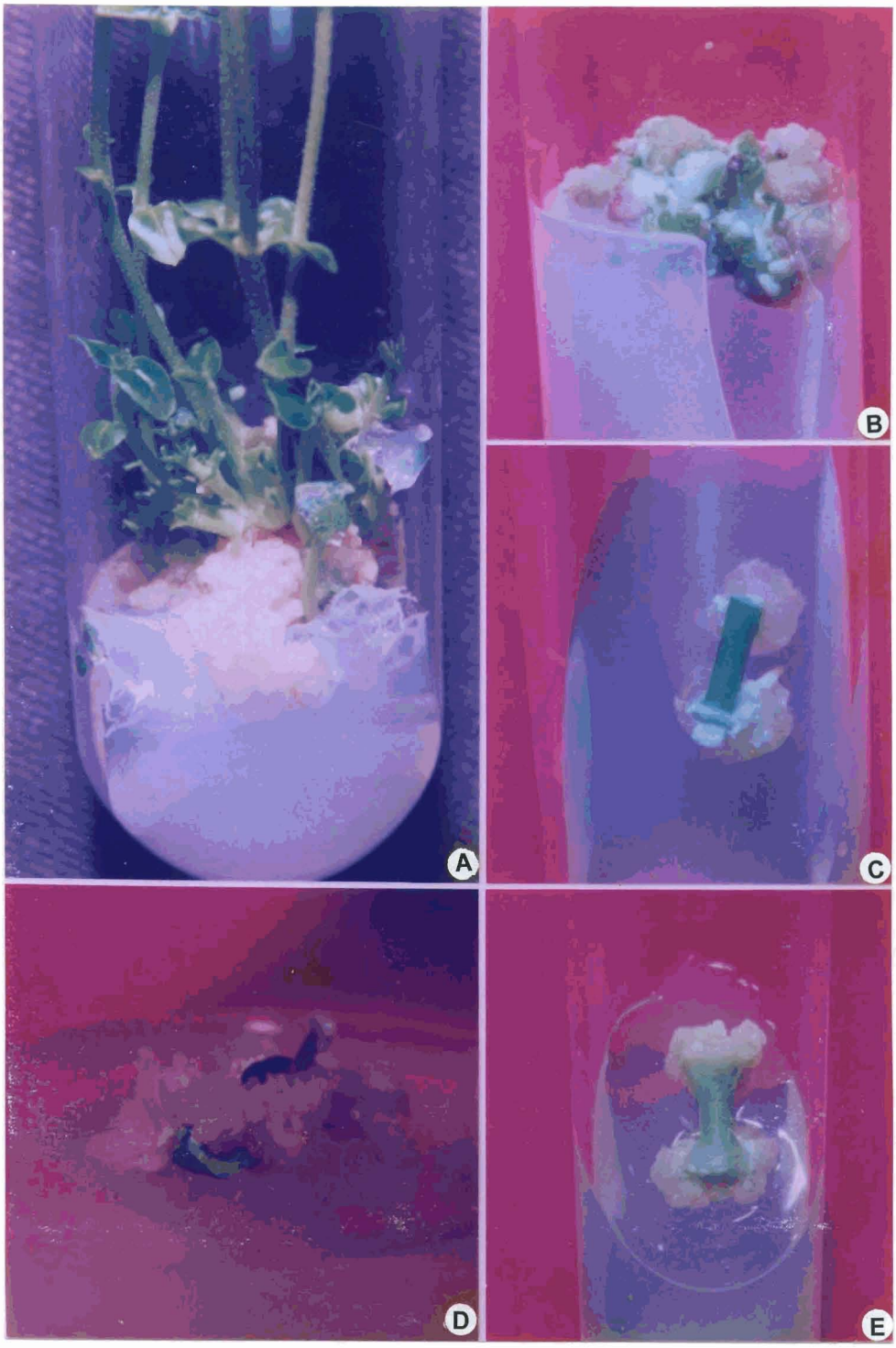



Fig XX

Fig. XXI A-G Callus induction and indirect organogenesis on *H. ada-kodien*

A. Callus induction from root explant on MS+2,4-D (1.0 mg $l^{-1}$)+Kin (0.5 mg $l^{-1}$)
B. Callus induction from internode on MS+2,4-D (1.0 mg $l^{-1}$)+Kin (0.5 mg $l^{-1}$)
C. Callus induction from internode on MS+BAP (1.5 mg $l^{-1}$)
D. Nodular callus from the basal cut ends of nodal explants. Callus developed inside the medium (MS+BAP 1.5 mg $l^{-1}$)
E. Meristemoids developed during subculture on MS+BAP (1.5 mg $l^{-1}$)
F. Shoots developed from the meristemoids (MS+BAP 1.5 mg $l^{-1}$)
G. Shoots showing abscission of leaves and shoot tips and emergence of new shoots

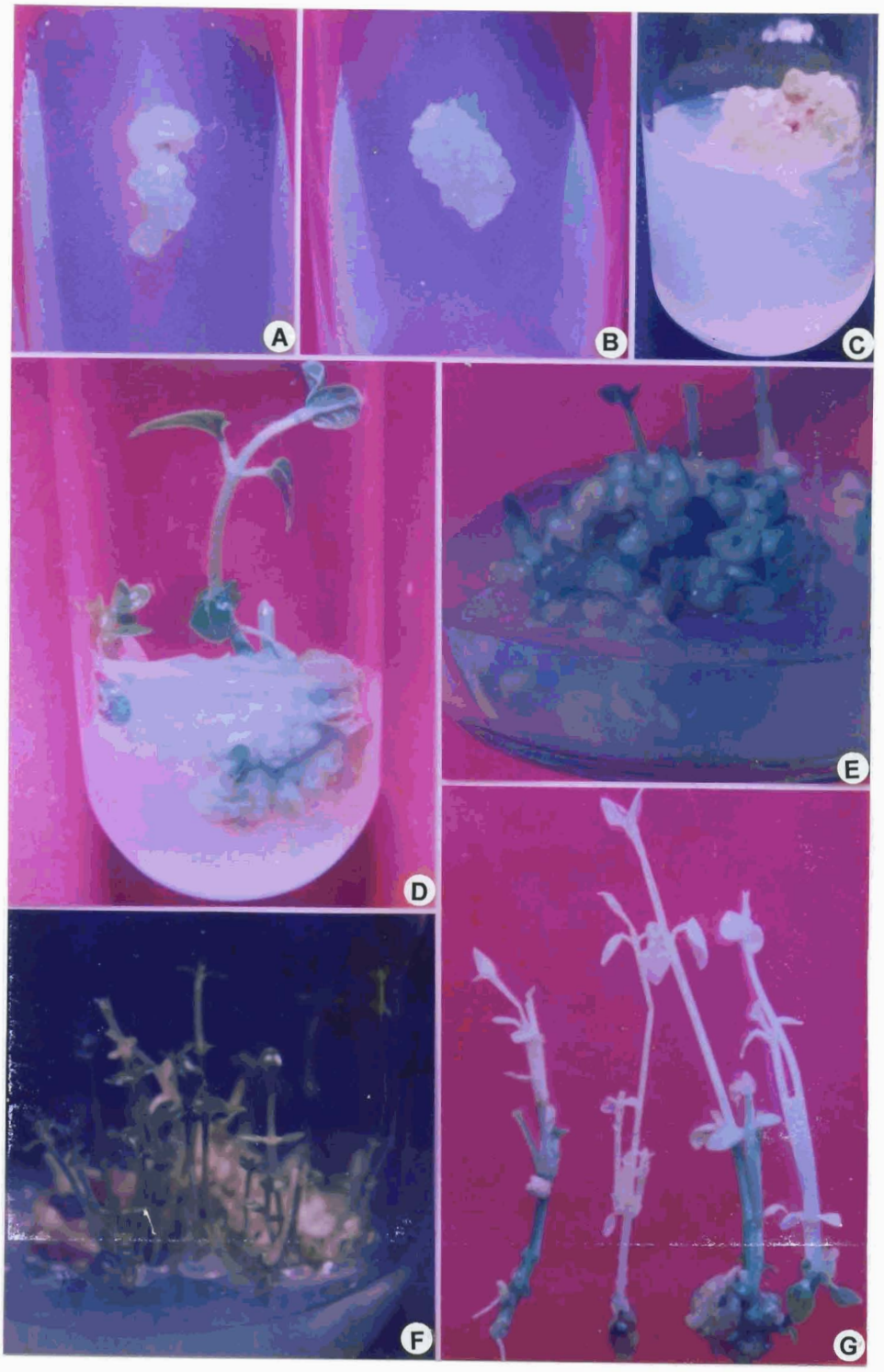



Fig XXI

Fig XXII A-D Indirect organogenesis from internodal callus of *H. ada-kodien*

A. Meristemoids developed from the internodal callus by subculturing on MS+BAP (2.0 mg $l^{-1}$)
B. Shoot buds on MS+BAP (2.0 mg $l^{-1}$)
C. Well-grown shoots with basal callus (MS+2.0 BAP mg $l^{-1}$)
D. Shoots with green well developed leaves on MS+BAP (1.5 mg $l^{-1}$) + $AgNO_3$ (3.0 $mgl^{-1}$)

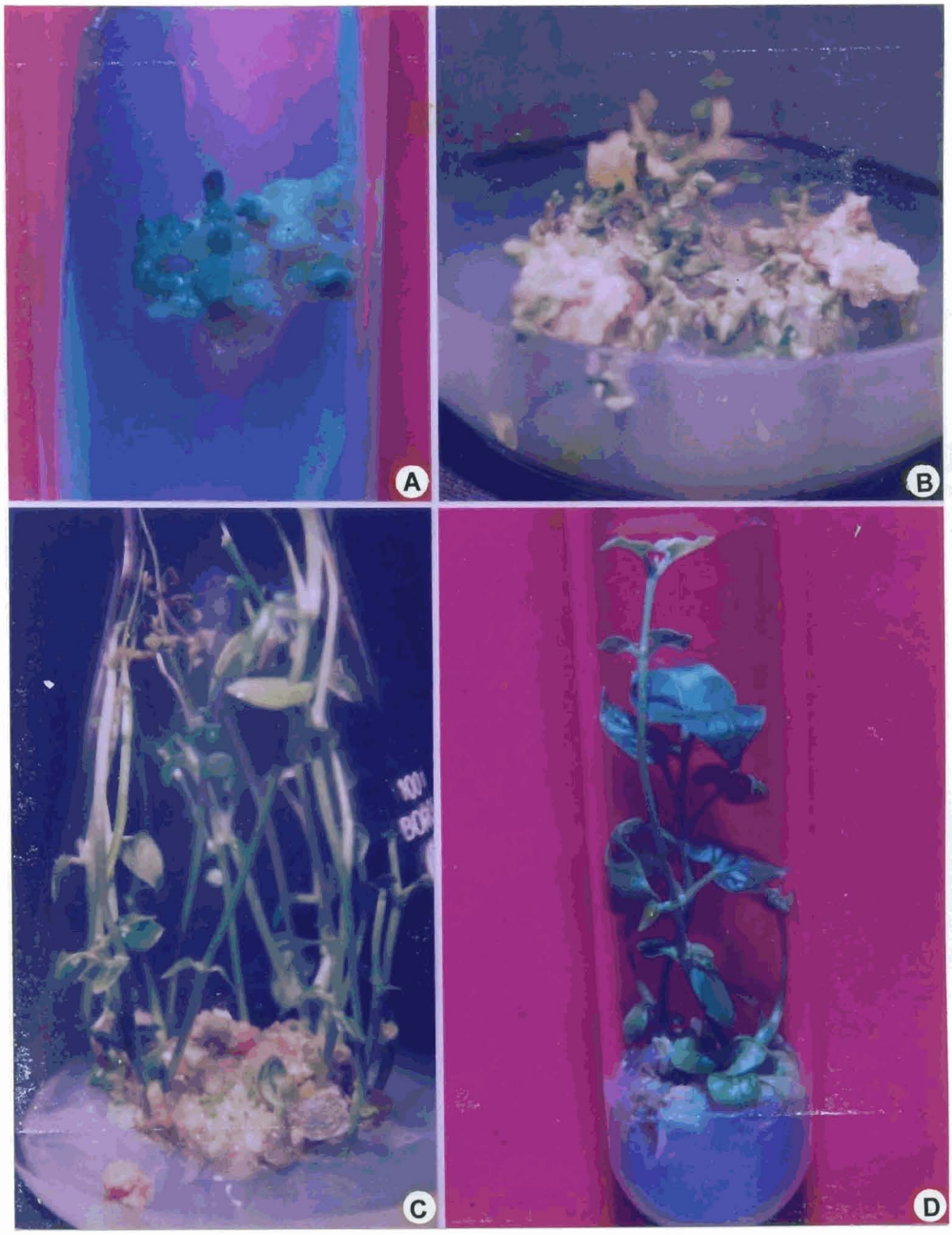



Fig XXII

Fig XXIII A-D Tuberous roots *in vitro* developed from callus of *H. ada-kodien*

A. Tuberous roots developed on MS + 10 % CW
B. Tuberous roots developed on MS + BAP (2.0 mg $l^{-1}$) + NAA (0.5 mg $l^{-1}$) + AA (100 mg $l^{-1}$) + $AgNO_3$ (3.0 mg $l^{-1}$)
C. A view from the bottom side
D. An isolated tuberous root

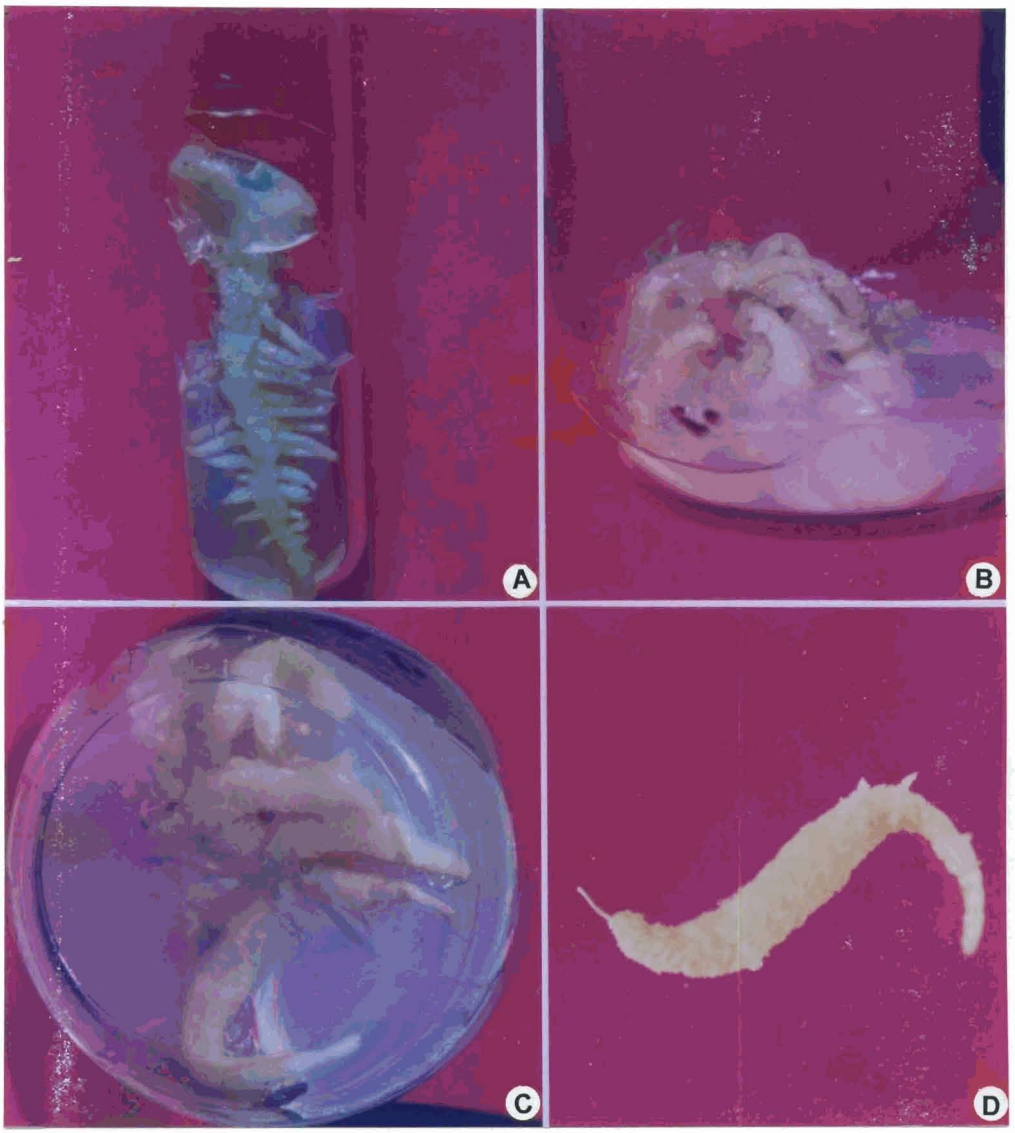



Fig XXIII

Fig XXIV A-I Somatic embryogenesis on *Holostemma ada-kodien*

A. Embryogenic callus on half MS + 2,4-D (0.1 mg $l^{-1}$)

B. Globular embryos (on solid half MS + 2,4-D (0.1 mg $l^{-1}$)

C - F Different stages of embryos developed on half MS liquid medium with 2,4-D (0.1 mg $l^{-1}$)

G. Cotyledonary embryo

H. Embryo with split cotyledon

I. Germinating embryo

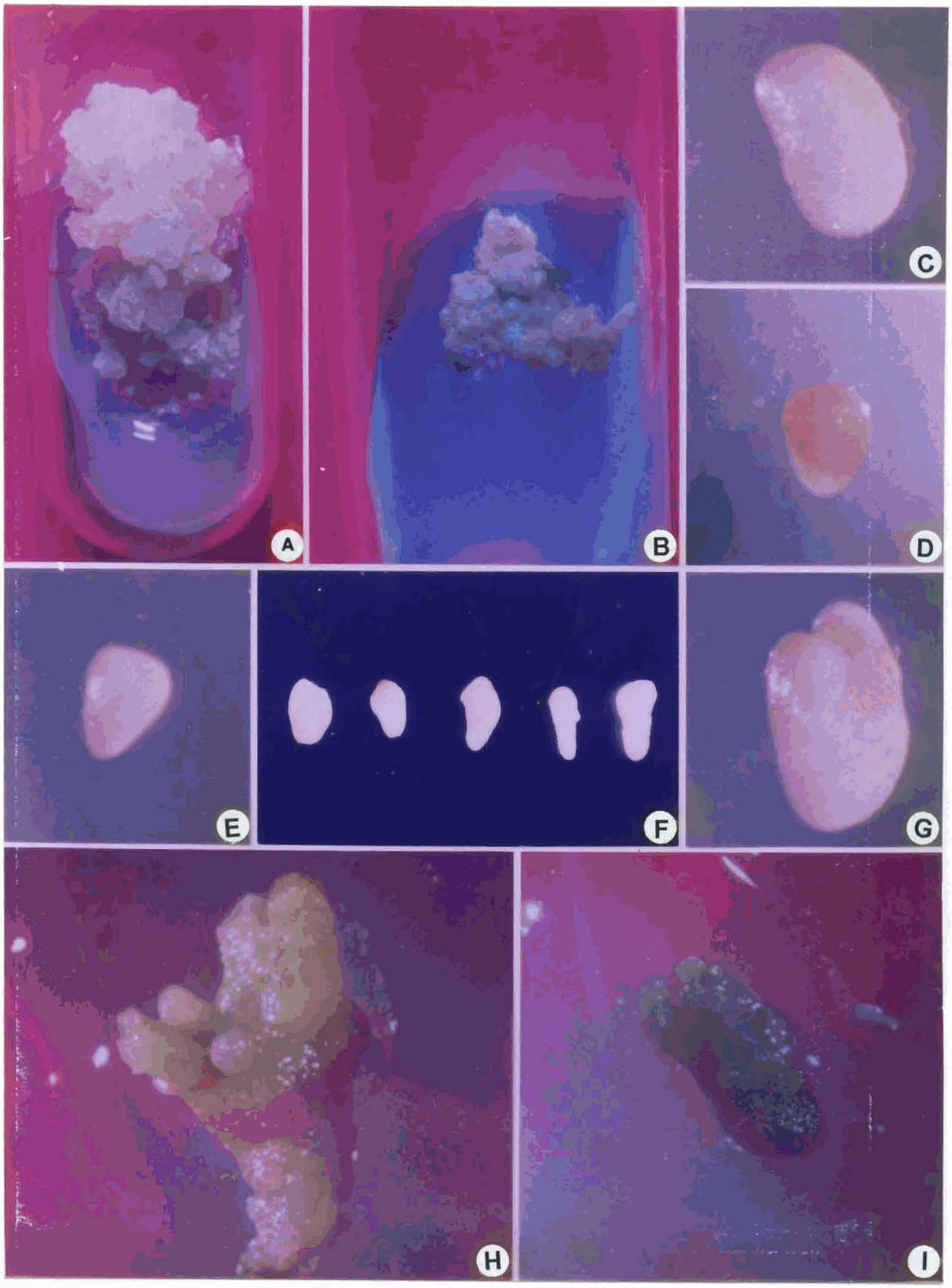



Fig XXIV

Fig. XXV A-C Different stages of embryos *H. ada-kodien* showing dedifferentiation

D-F Protoplast culture

D. Isolated protoplasts from leaf mesophyll cells

E. Protocalli developed from protoplasts encapsulated in alginate beads ( MS + 1.0 mg $l^{-1}$ 2,4-D)

F. Proliferated protocalli on half MS + 2,4-D (0.1 mg $l^{-1}$)

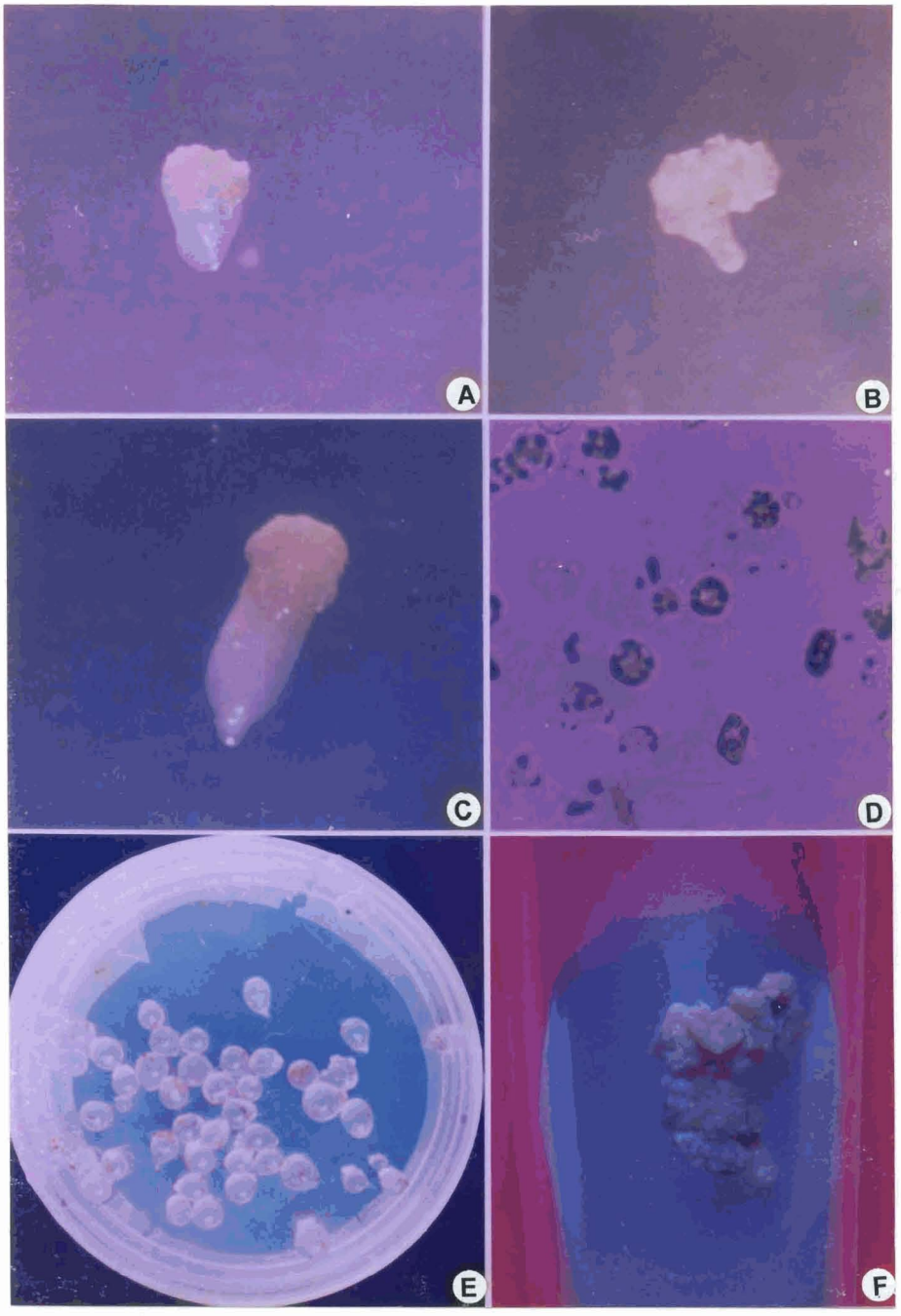



Fig XXV

Fig. XXVI A-F Developmental stage of somatic embryos of *H. ada-kodien*

A. Globular embryo (x 20)
B. Late globular embryo (x 20)
C. Early heart shaped embryo (x 20)
D. Heart shaped embryo (x 20)
E. Embryo with cotyledonary and shoot primordia (x 20)
F. Embryo with cotyledonary, shoot and root primordia (x 20)

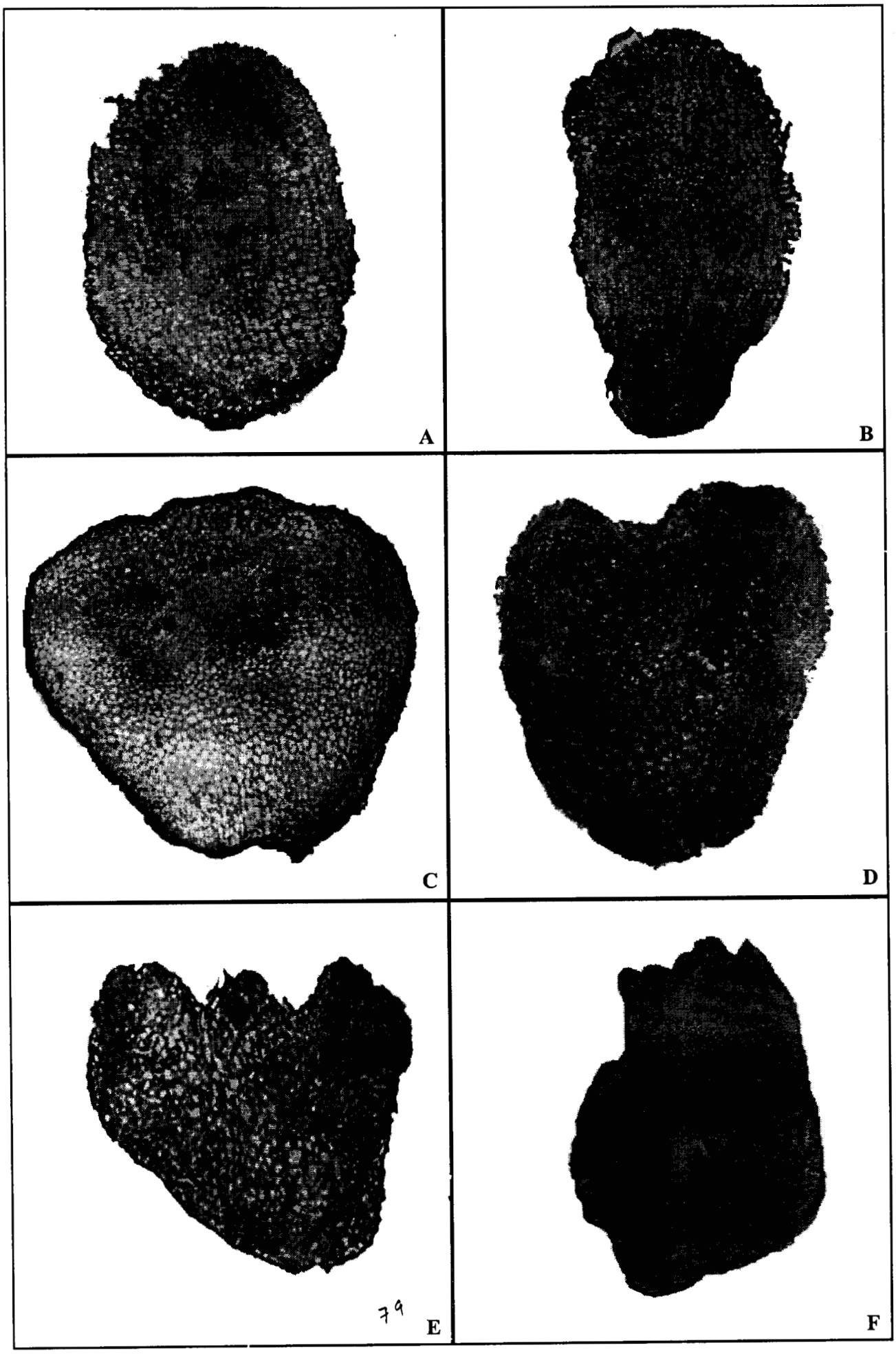



Fig XXVI

Fig XXVII A Effect of sodium alginate in the conversion of synseeds of *Holostemma ada-kodien*

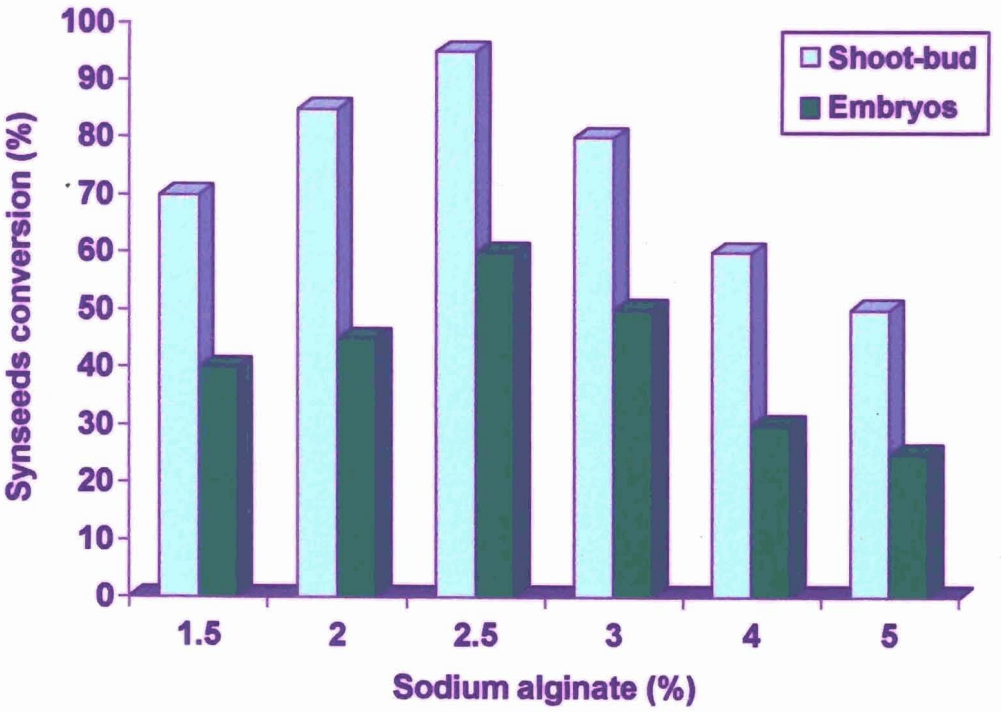
Encapsulation of shoot buds: Alginate in MS+BAP (1.5 mg $l^{-1}$) with sucrose (1.5 %)
Encapsulation of embryos: Alginate in 1/10 hormone free MS +Sucrose (1.5 %)
Data represents an average of 20 synseeds

Fig XXVII B Effect of $CaCl_2$ in the conversion of synseeds of *H. ada-kodien*

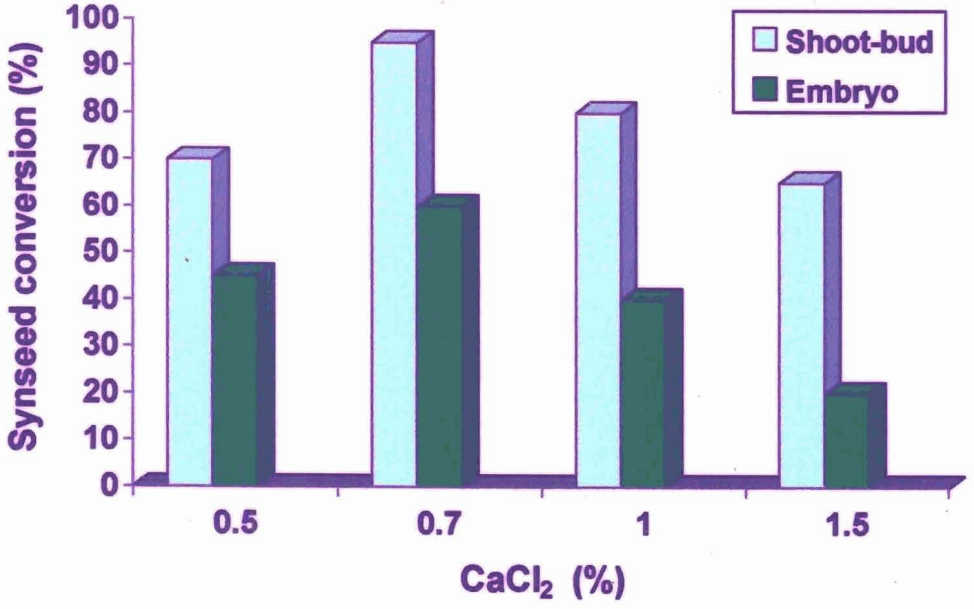
Encapsulation of shoot buds: Alginate (2.5 %) in MS+BAP (1.5 mg $l^{-1}$) with sucrose (1.5 %)

Encapsulation of embryos: Alginate (2.5 %) in 1/10 hormone free MS +sucrose (1.5 %)

Data represents an average of 20 synseeds


Synseeds conversion (%)
100
90
80
70
60
50
40
30
20
10
0
Shoot-bud
Embryos
1.5
2
2.5
3
4
5
Sodium alginate (%)

A


Synseed conversion (%)
100
90
80
70
60
50
40
30
20
10
0
Shoot-bud
Embryo
0.5
0.7
1
1.5
CaCl2 (%)

B

Fig XXVII

Fig. XXVIII A-G Encapsulation and conservation studies on *H. ada-kodien*

A. Embryos encapsulated in sodium alginate with 1/10 MS hormone free medium
B. Shoot bud used for encapsulation
C. Germination of shoot bud derived synseeds made in alginate with MS + BAP (1.5 mg $l^{-1}$)
D. Growth of shoots from synseeds (after 30 days)
E. Growth of shoot buds encapsulated in alginate with water + sucrose (1.5 %) after 12 month ( **x** 10)
F. Shoot bud on half MS + IBA (0.05 $mgl^{-1}$) + $AgNO_3$ (5.0 $mgl^{-1}$) containing medium (after 3 months)
G. The shoot bud on the same medium (12 months)

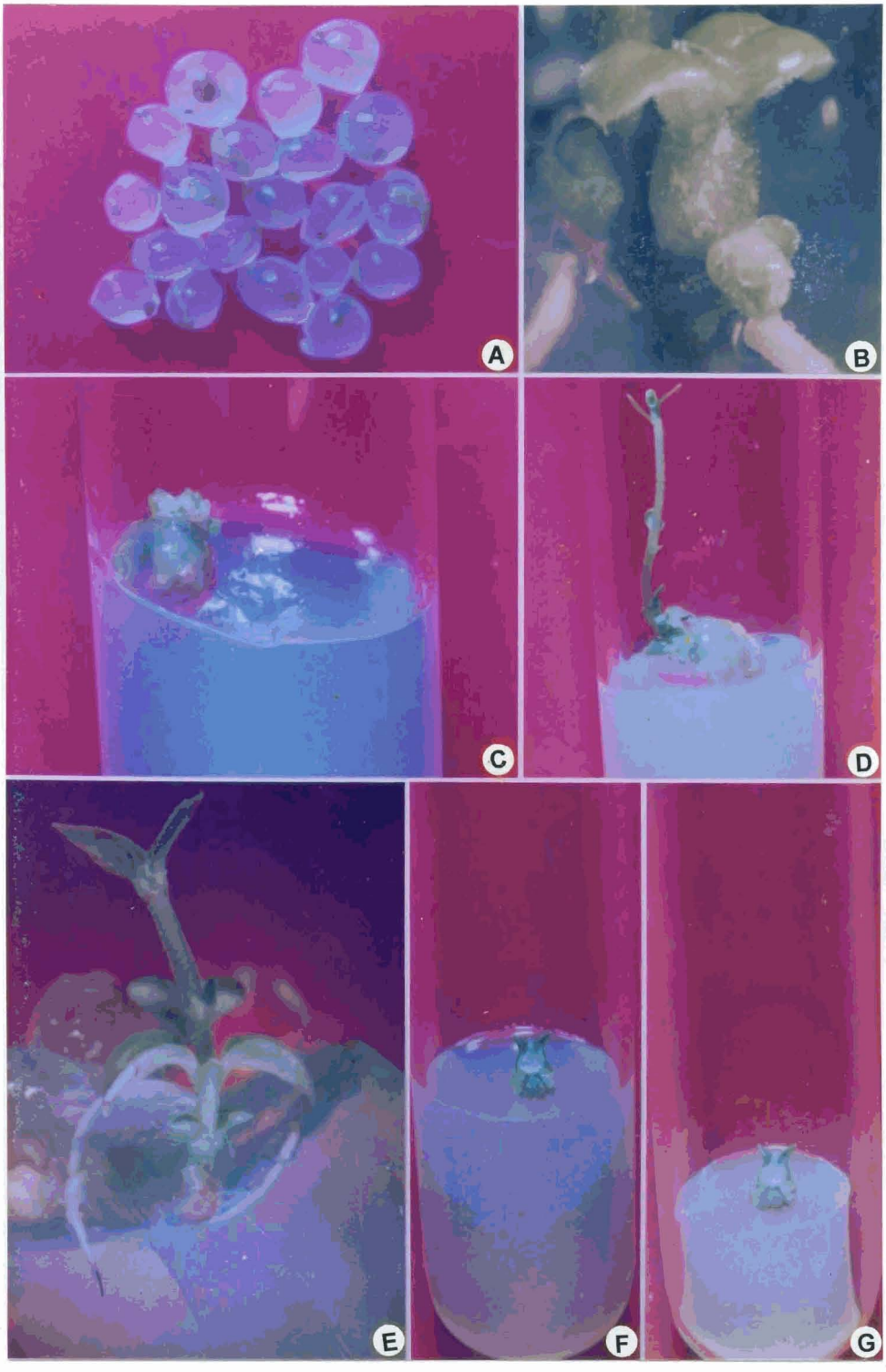



Fig XXVIII

Fig. XXIX A-D Rhizogenesis *in vitro* and secondary metabolite analysis of *Holostemma ada-kodien*

A. Roots developed on solid half MS + IBA (0.05 mg $l^{-1}$)
B. Roots developed in liquid half MS + IBA (0.05 mg $l^{-1}$)
C. Plantlet in small pots (after 20 days)
D. TLC analysis of secondary metabolites of tuberous roots *in vitro* and field grown plants

(EtOH - Ethyl alcohol)
(PE - Petroleum ether)
(I V - *in vitro)*
(F G - Field grown)

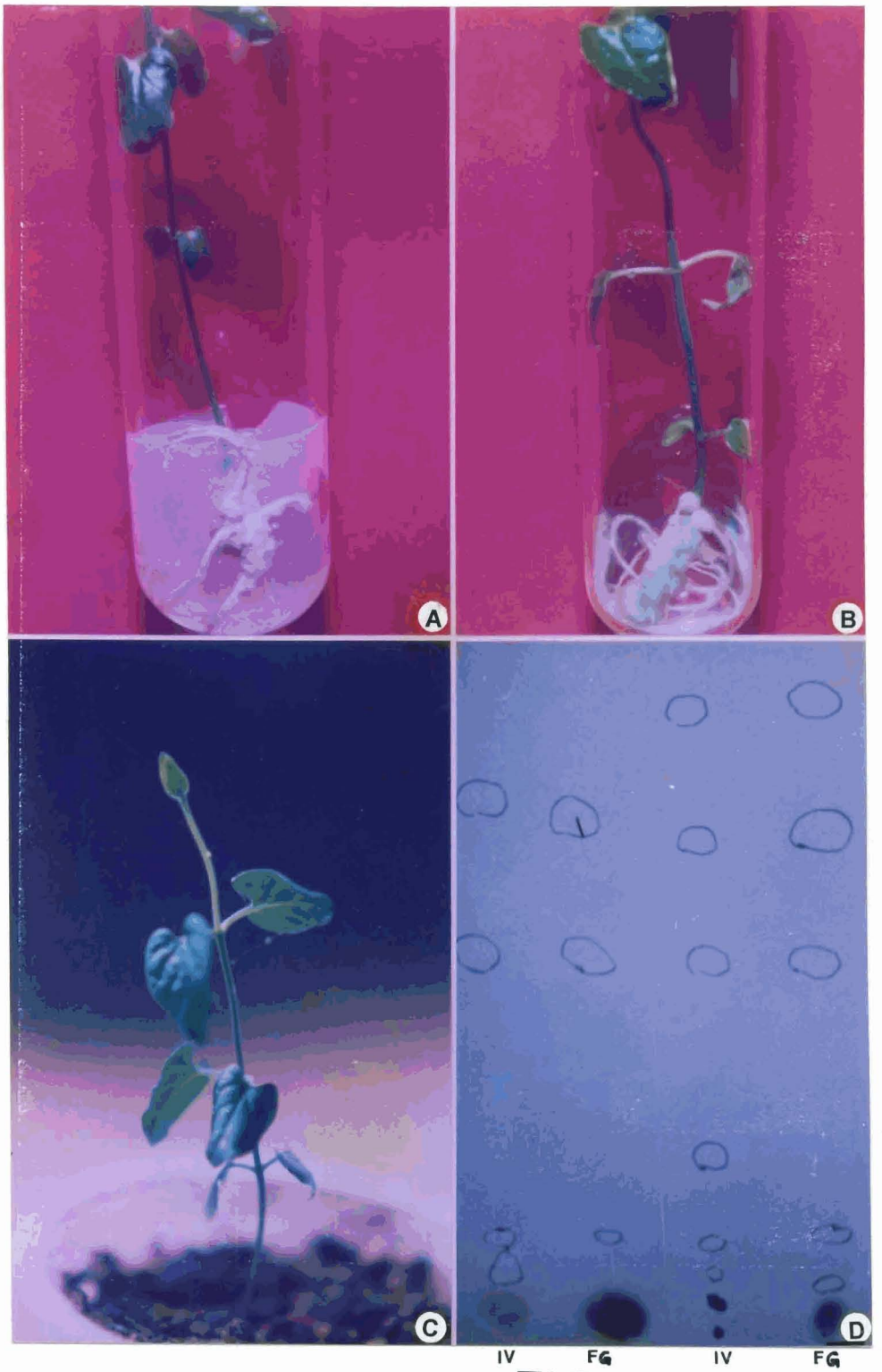



Fig XXIX

Fig XXX Per cent of explants induced hairy roots from explants of *Eryngium foetidum* and *Holostemma ada-kodien* after infection with *Agrobacterium rhizogenes* harbouring the binary plasmid **pGreen** with ***gus/gfp*** reporter genes

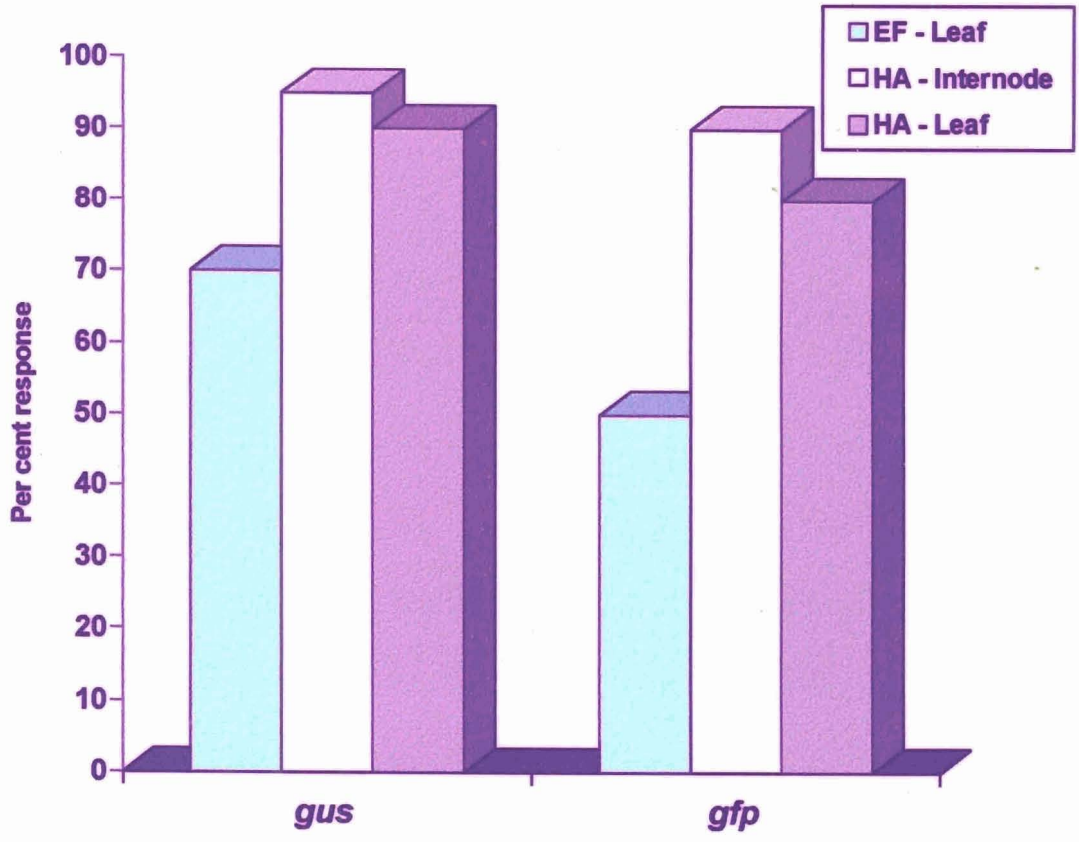

EF - Leaf
HA - Internode
HA - Leaf
Per cent response
100
90
80
70
60
50
40
30
20
10
0
gus
gfp

Fig XXX

Fig XXXI Induction of roots from co-cultivated explants and control

A. Hairy roots developed on leaf explants of *Eryngium foetidum* co-cultivated with *Agrobacterium rhizogenes* harbouring the binary plasmid **pGreen** with ***gus*** construct

B. Hairy roots developed on leaf explants of *Holostemma ada-kodien* co-cultivated with *Agrobacterium rhizogenes* harbouring the binary plasmid **pGreen** with ***gus*** construct

C. Hairy roots developed on internode explants of *Holostemma ada-kodien* co-cultivated with *Agrobacterium rhizogenes* harbouring the binary plasmid **pGreen** with ***gus*** construct

D. Initiation of hairy roots on internode explant of *Holostemma ada-kodien* co-cultivated with *Agrobacterium rhizogenes* harbouring the binary plasmid **pGreen** with ***gfp*** construct

E. Control explants of *Eryngium foetidum* on MS basal medium

F. Control explants of *Holostemma ada-kodien* on MS basal medium

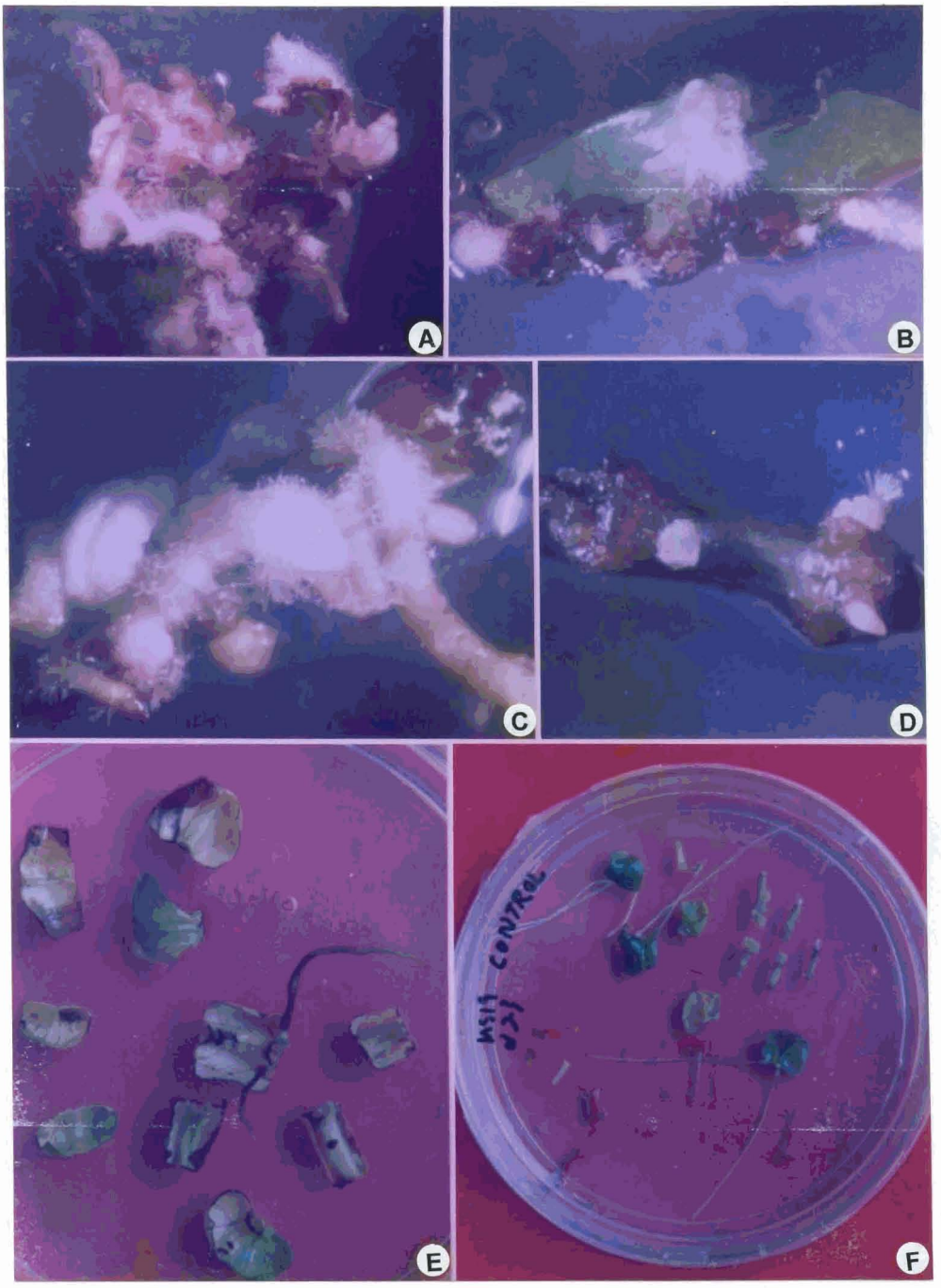



Fig XXXI

Fig XXXII Transgenic plant regeneration and assay of transformation

A. Transformed shoots regenerated from the callus induced on hairy roots developed from co-cultivated explants of *Eryngium foetidum* with *Agrobacterium rhizogenes* harbouring the binary plasmid **pGreen** with ***gus*** construct

B. Callus induced on hairy roots developed from co-cultivated explants of *Holostemma ada-kodien* with *Agrobacterium rhizogenes* harbouring the binary plasmid **pGreen** with ***gfp*** construct

C. ***gus*** gene expression of transformed plants of *Eryngium foetidum*

D. ***gus*** gene expression of leaves of transformed plants of *Eryngium foetidum*

E. ***gus*** gene expression in somatic embryos induced from transformed callus developed from hairy roots of *Holostemma ada-kodien*

F. ***gfp*** expression in transformed callus induced from hairy roots of *Holostemma ada-kodien*

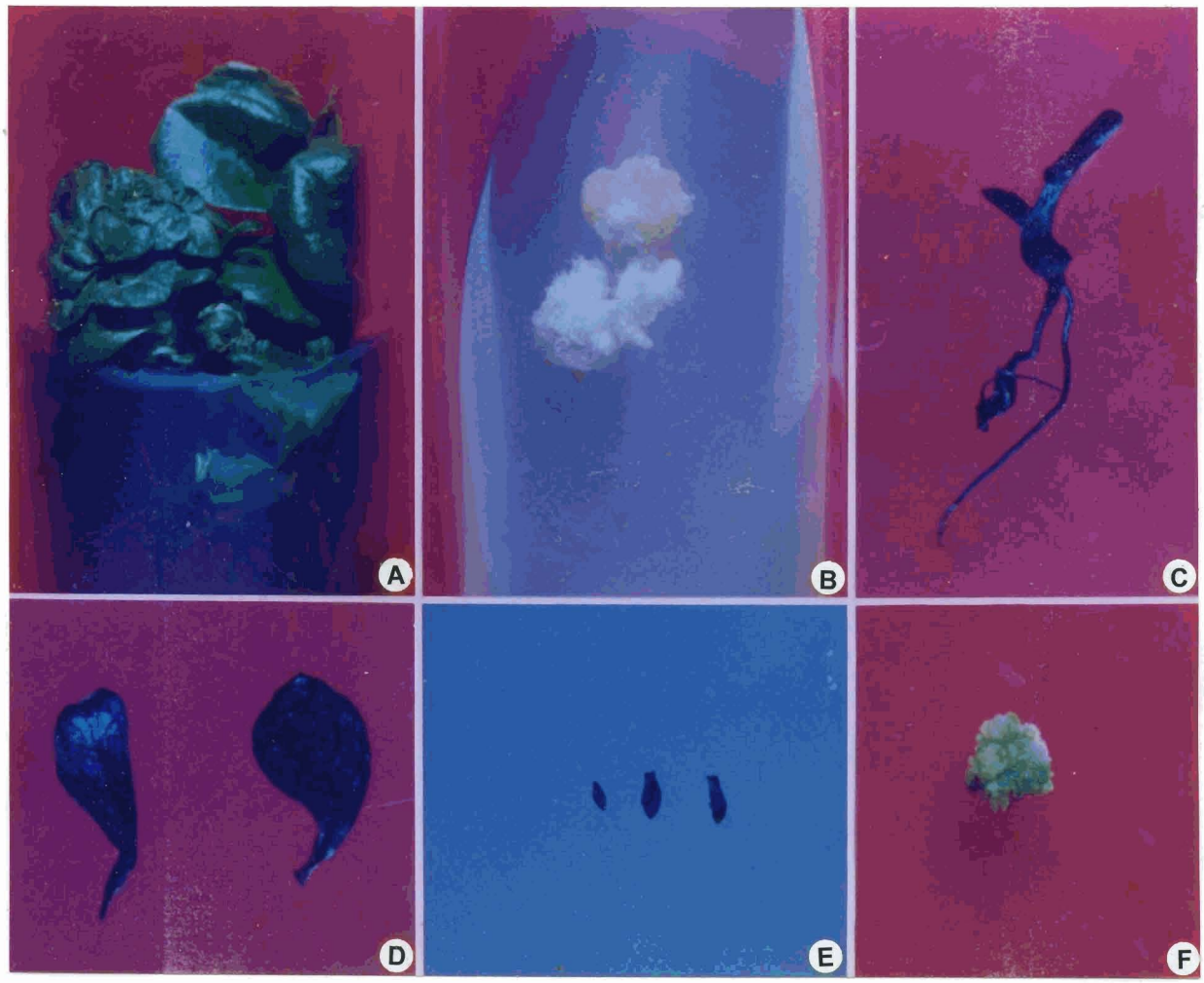



Fig XXXII

# DISCUSSION

# DISCUSSION

Plant morphogenesis *in vitro* consists of three distinct analogous phases: (i) "acquisition of competence" or "dedifferentiation" (during which the tissue becomes competent to respond to the organogenic/embryogenic stimulus) (ii) "induction" (during which the cells become determined to form either a root, a shoot or an organ or an embryo) and (iii) "realization" (out growth to an organ or an embryo) (De Klerk *et al.* 1997a). The first phase usually involve a period of callus growth, however the cells present in the explant become competent at the culture onset without cell division or without cell division at a large scale (Thorpe *et al.* 1991; De Klerk *et al.* 1997a).

Last few years have witnessed much progress in rapid propagation, conservation and genetic modification of medicinal and aromatic plants especially the endangered species. The present experimental work is also focussed on the same aspects of two medicinal plants *viz. Eryngium foetidum* and *Holostemma ada-kodien.*

## *ERYNGIUM FOETIDUM*

### Direct organogenesis

During the present investigation on *Eryngium foetidum* direct shoot regeneration was established from different explants on different media either with or without hormones.

Eapen and Gill (1986) reported direct shoot regeneration from different cultivars of *Vigna aconitifolia* on hormone free MS medium. Spontaneous regeneration of shoots without an exogenous trigger has been reported on

different explants: internodes of *Torenia fournieri* (Kamada and Harada 1979b) and *Jatropha curcas* (Nigam *et al.* 1997) and leaf and internode of *Bacopa monnieri* (Mathur and Kumar 1998). According to Wilson and van Staden (1990), the signals related to wounding play a vital role during regeneration. This may be due to the trigger of endogenous hormones (factors) or the ratio of ions present in the medium. The ability of shoot induction on root explants on full or half MS and White's medium compared to high salt $B_5$ in the present experiment clarifies the role of ions. The reduction in the number of shoots in leaf and root explants may be because of the difference in regeneration potential among different explants, which is attributed by the physiological state, age and cellular differentiation among the constituent cells (Murashige 1974).

**Effect of growth regulators**

Present studies on *E. foetidum* were envisaged with an enhanced direct shoot formation from different explants in the presence of growth regulators supplemented to the medium. Of the two cytokinins (BAP/Kin) tested in the present study, Kin was found as the single effective growth regulator. The stimulative effect of Kin over BAP has been reported in *Hemidesmus indicus* (Patnaik and Debata 1996). However, a combination of Kin+BAP was found as more effective for the induction of direct shoots. The efficacy of the combination of both BAP and Kin has been reported in many medicinal plants as in *Thevetia peruviana* (Kumar and Kumar 1995), *Elaeocarpus robustus* (Roy *et al.* 1998), *Kaempferia galanga* (Vincent *et al.* 1998) and *Pisonia alba* (Jagadishchandra *et al.* 1999). According to them it is due to the synergistic effect of both cytokinins. The result obtained in the present study was not true *in toto* to the report on *E. foetidum* (Arockiasamy and Ignacimuthu 1998). According to them, BAP in combination with IAA (in the case of leaf) and BAP (2.0 mg/l) alone (in the case

of root explants) was efficient to induce direct shoot formation but at low frequency compared to the present results.

The present study showed induction of direct shoot formation from different explants in response to the growth regulators. Direct shoot formation in response to different growth regulators has been reported on various types of explants: root, stem and leaf of *Piper colubrinum* (Kelker and Krishnamurthy 1998); leaf and stem of *Bacopa monnieri* (Tiwari *et al.* 1998); leaf of *Enicostemma axillare* (Sudhersan 1998), peppermint and spearmint (Faure *et al.* 1998), *Azadirachta indica* (Eeswara *et al.* 1998), *Lavandula* (Dronne *et al.* 1999b), *Solanum nigrum* (Shahzad *et al.* 1999) and *Centella asiatica (*Banerjee *et al.* 1999); stem disc of *Allium sativum* (Ayabe and Sumi 1998) and *Dendrobium moschatum* (Kanjilal *et al.* 1999).

In addition to the direct shoot formation on BAP medium, the cultures were characterized with the induction of callus from different explants. This is in consonance with the report on *E. foetidum* (Arockiasamy and Ignacimuthu 1998), where BAP was found as the effective growth regulator for the induction of callus. During the present study, when the concentration of BAP exceeds Kin resulted in more callus formation. Reduction in the number of shoots accompanied by the formation of callus as to the ratios of cytokinins may be due to the significant changes in the organization and modification of DNA *i.e.* by the activation of the genetic mechanism for plant regeneration by high amount of Kin or its suppression by the high concentration of BAP. The changes in the physiological and biochemical nature of the cells depending on the ratio of the two cytokinins may also be a possible reason.

**Callus studies**

During the present studies on *E. foetidum*, stem disc, leaf and scape explants induced shoot, root and callus on MS medium containing NAA/IAA, while the root explants induced root and callus only. The root formation was followed by shoot formation. Induction of root, shoot and calli on auxin alone or at high auxin: cytokinin ratio has been reported in many plants like *Limnophila chinensis* (Sangwan *et al.* 1976), *Lobularia maritima* (Khanna and Chopra 1977), *Torenia fournieri* (Kamada and Harada 1979b) and three grain legumes (Kunjumon *et al.* 1996). Arockiasamy and Ignacimuthu (1998) reported the formation of roots and callus in *E. foetidum*, but no shoots. The formation of shoots by auxins alone is in contrary to the well known concept of Skoog and Miller (1957) *i.e.* shoot formation as a result of high exogenous cytokinin: auxin ratio may be attributed by the net result of the balance between the endogenous and exogenous growth regulator/factor(s). Moreover, root and callus formation is considered as the functions of auxins. Being the concept of auxin as root and callus inducive, their formation cause a reduction in the amount of auxin in the medium, which facilitates a high cytokinin:auxin ratio and hence shoot induction.

**Indirect organogenesis**

Callus can undergo re-differentiation in to a variety of organs under appropriate culture milieu. Organogenesis from somatic cells or tissues is conceived to be under the control of phytohormones (Skoog and Miller 1957).

In the present studies on *E. foetidum*, two types of calli (friable brown and hard green compact) showed organogenesis. Indirect organogenesis has been reported in many medicinal plants like *Eryngium foetidum* (Arockiasamy and Ignacimuthu 1998), *Heracleum candicans* (Wakhlu and Sharma 1999), *Bacopa monnieri* (Shrivastava and Rajani 1999), *Vetiveria zizanioides* (George and

Subramanian 1999) and *Allium sativum* (Barandiaran *et al.* 1999). The hard green compact callus showed high potential of shoot regeneration compared to the friable callus. The low frequency of shoot formation in the latter may be due to the utilization of media factors to the elicitation and synthesis of secondary metabolites. The compactness facilitates cell to cell transfer of shoot induction factor(s) effectively, which may be the reason behind the high frequency of regeneration from hard compact callus. In contrast to this, in the case of friable callus localization of shoot inducing factors to certain cells at large amounts and its interaction with the secondary metabolites may also be a reason for the low potential of regeneration.

**Photoautotrophic micropropagation**

During the present studies on *E. foetidum*, the explants from the *in vitro* grown plants were capable to induce direct shoots on MS basal medium devoid of sucrose (Photoautotrophic medium). The callus obtained on different medium, when subcultured on sucrose free medium also developed shoots.

Carbohydrate source is considered as of prime importance in *in vitro* morphogenesis (Romano *et al.* 1995), which play a pivotal role in growth and development not only for functioning as substrates but also for affecting the differentiation (Koch 1996). Nevertheless, the wide spread exploitation of micropropagation, the challenging and exciting area commercially is restricted by production cost mainly by the media cost (sucrose and gelling agent constitute about 80% of the cost) and by the covert contamination losses and also due to the low survival rates during acclimation (Mitra *et al.* 1997, 1998a, b). Usually the photoautotrophic cultures have been established under $CO_2$ enrichment as in *Chrysanthemum* (Mitra *et al.* 1998a), *Dendrobium* (Mitra *et al.* 1998b) and *Coffea arabusta* (Nguyen *et al.* 1999). Photoautotrophic cultures use $CO_2$ as their sole source like higher plants. Environmental factors such as $CO_2$ concentration, temperature, humidity etc. play the major role towards the development of

photoautotrophic plants grown without sugar in the medium (Kozai 1991; Jeong *et al.* 1995; Pospisilova *et al.* 1997; Mitra *et al.* 1997,1998a, b). However, in the present study no enrichment of $CO_2$ has been carried out.

According to Hohmann *et al.* (1995), the genus *Eryngium* is characteristic of quercetin glycosides. Besides this, Hohmann *et al.* (1997) reported the presence of d-glucose and L-rhamnose, mannitol and quercetin glycosides in another species, *E. campestris*. Considering this, the potential of photoautotrophic micropropagation of *E. foetidum* may be due to the presence of mannitol, glucose and glycosides in sufficient or due to the presence of $CO_2$ enrichment in the culture vessels by culture milieu or by both. In addition, the histological studies with respect to the shoot development showed that they were originated from the phloem cells. The sugars and minerals are transported through the phloem cells. Hence, one of the feasible reasons behind the shoot induction on sugar free medium is the accumulation of sugars and minerals in the phloem cells. The induction of more shoots from stem discs and root explants than that from leaf explants clarify the role of sugars and minerals present in the phloem cells.

Microbial contamination is the most important cause of loss in commercial as well as scientific culture labs. (Leifert *et al.* 1994; Mantell 1998; Kamoun *et al.* 1998). During the present study, photoautotrophic micropropagation was not encountered with microbial contamination. According to Jeong *et al.* (1995), in addition to more expenditure, the carbon source (sucrose) makes the culture susceptible to microbial contamination. Photoautotrophic micropropagation thus minimize the contamination abreast to the production cost (Kozai *et al.* 1991; Mitra *et al.* 1998a, b).

## SOMATIC EMBRYOGENESIS

Somatic embryogenesis has been accomplished in many medicinal plants following the pioneer observations of Reinert (1958) and Steward *et al.* (1958). According to Sharp *et al.* (1982) somatic embryos can be arose from cells present in the explant tissue and was referred them as Pro-Embryogenic Determined Cells (PEDCs). Somatic embryos may also arise after several to many cell divisions in culture regimes that cause dedifferentiation and negation of previously active developmental pathways and was referred them as Induced Embryogenic Determined Cells (IEDCs). The present studies on *E. foetidum*, both PEDCs (direct) and IEDCs (indirect) types of embryogenesis were obtained.

### Direct somatic embryogenesis

Direct somatic embryos were formed on root explants from the portion immediately following the stem on MS medium containing NAA (1.0 mg $l^{-1}$)+Kin (0.5 mg $l^{-1}$). According to Engelbrecht (1974) root is the synthesizing site of cytokinin. The potential of direct somatic embryogenesis from the peripheral region of the root explants, is attributed by the balance between the growth regulators supplied exogenously and the endogenous cytokinin present in root. Direct somatic embryogenesis have been reported from cotyledons of ginseng (Choi *et al.* 1998, 1999); from leaves of tea (Kato 1996), neem (Murthy and Saxena 1998) and *Dianthus* (Yantcheva *et al.* 1998); from nodes of tea clone TRI-2025 (Akula and Dodd 1998) and from flower buds of onion (Luthar and Bohanec 1999).

Abreast to the somatic embryos from the peripheral region, the root explants induced unipolar shoots from the central region. Histological studies confirmed the origin of somatic embryos from the cortical cells and shoots from the green callus originated from the phloem cells. As in the present study,

somatic embryos and shoots from different tissues of the same explant have been reported in cultured petiole of carrot, an another member of Apiaceae (Neumann and Grieb 1992). According to Arnholdt-Schmitt *et al.* (1995) cells of different tissues of carrot plants are characterized by specific methylation patterns. Hence the formation of different morphogenetic patterns from different tissues of the same explant may be due to the specific DNA methylation of the different tissues.

**Indirect somatic embryogenesis**

During the present studies on *E. foetidum*, somatic embryos were formed spontaneously from the callus obtained from the roots on the explants and *in vitro* grown plants on MS medium with NAA+Kin (2.0 mg $l^{-1}$ each). Spontaneous origin of somatic embryos from root explants has been demonstrated in *Peucedanum palustre*, an another member of Apiaceae (Vuorela *et al.* 1993). However they could obtain the embryoids after subculturing the callus. In contrast to their result, in the present study the embryos developed without subculture and showed maturity too.

MS medium with 2,4-D + Kin was found effective for the induction of friable embryogenic callus followed by NAA+Kin. However, according to Ignacimuthu *et al.* (1999) a combination of 2,4-D+BAP+$GA_3$ was efficient for the induction of indirect somatic embryogenesis in the same species. The effectiveness of 2,4-D+Kin for the induction of indirect somatic embryogenesis has been well established among the Apiacean members *viz.* celery (Williams and Collin 1976; Toth and Lacy 1992) and fennel (Hunault 1984; Miura *et al.* 1987; Hunault and Du Manoir 1992). In contrast to the present study, superior effect of NAA+Kin over 2,4-D+Kin was reported in coriander (Chen *et al.* 1995). However, efficacy of 2,4-D+BAP has been reported in celery (Nadel *et al.* 1989) and *Heracleum candicans* (Wakhlu and Sharma 1998).

During the present study, the transfer of the embryos to full or half MS medium without growth regulator resulted in the transformation of the embryos into roots (unipolar). According to Carman (1988, 1990) some exogenous auxin is generally needed to prevent somatic embryos from converting into unipolar structures.

In the case of *E. foetidum*, the embryos underwent maturation and conversion at high frequency on half MS medium supplemented with CW/CT. However, the previous report of embryogenesis on *E. foetidum* (Ignacimuthu *et al.* 1999) was encountered with low frequency of induction and conversion. The effectiveness of additives such as CW, CH, YE and AC in somatic embryogenesis has been widely investigated in many members of Apiaceae. The present study was in agreement with the reports on carrot (Steward 1963), *Ranunculus* (Konar and Nataraja 1965) and *Tylophora indica* (Rao and Narayanaswamy 1972). According to them CW is not a general requisite, but was beneficial for post-embryo development. However, CW continues to be extremely useful in embryo induction and maturation (Castillo and Smith 1997; Ishii *et al.* 1998; Zhao *et al.* 1999; Sinha *et al.* 2000). However, inhibition of germination of embryos has also been reported (Carman 1988). One of the conceivable reasons behind the effectiveness of CW in the present investigation on *E. foetidum* is the presence of auxins, cytokinins, amino acids, gibberellins, sugars etc. in an appropriate ratio facilitating the development of the somatic embryos.

**Effect of sucrose**

In the present study, for the induction of somatic embryos, the optimal concentration of sucrose was 1.5-3.0 %. Beyond the optimal concentration, it was found inhibitory for the induction of somatic embryogenesis. Efficacy of sucrose at 1.5-3 % has been reported in *Tylophora indica* (Rao *et al.* 1970; Rao and Narayanaswamy 1972), *Araujia sericifera* (Torné *et al.* 1997), *Angelica*

*sinensis* (Tsay and Huang 1998) and *Hemidesmus indicus* (Sarasan *et al.* 1994). Inhibitory effect at higher concentrations of sucrose has been reported in *Trachyspermum ammi* (Sehgal and Abbas 1994) and spinach (Komai *et al.* 1996). Nevertheless, 5.0-6.0 % of sucrose has been reported as effective in zonal geranium (Wilson *et al.* 1996), *Glehnia littoralis* (Hirai *et al.* 1997) and *Tagetes erecta* (Bespalhok and Hattori 1998). Sucrose (10 %) has been reported to facilitate the mass production of somatic embryos in *Panax ginseng* (Asaka *et al.* 1994) and *P. notoginseng* (Shoyama *et al.* 1997). Finer (1987) observed that sucrose at 15 % was effective for the induction of embryos. In *Azadirachta indica* (Su *et al.* 1997), though induction of somatic embryos was enhanced by 5.0 % sucrose, conversion of embryos occurred only by lowering its concentration up to 1.0 %.

**Effect of different media**

During the present studies of organogenesis on *E. foetidum*, it was found that MS medium was more suitable for the induction of shoots on all types of explants except root, followed by $B_5$ medium. White's medium was found inferior to both. The efficacy of MS medium has been reported in many medicinal plants. In the case of root explants, half MS and White's media were effective, followed by $B_5$. Root as the organ of absorption, the acquisition of high levels of mineral elements in the roots is the conceivable reason behind the effectiveness of half MS and White's, the low salt media compared to $B_5$.

During the present studies of somatic embryogenesis on *E. foetidum* MS and half MS medium was found effective followed by White's medium. $B_5$ medium was inferior to both. The effectiveness of half MS has been reported in plants like *Angelica sinensis* (Tsay and Huang 1998) and *Iris hollandica* (Hida *et al.* 1999). Hirai *et al.* (1997) reported the effectiveness of half MS, Nitsch and White's medium in *Glehnia littoralis*. The efficacy of White's medium in

somatic embryogenesis has been reported in celery (Ammirato 1973) and *Tylophora indica* (Rao and Narayanaswamy 1972; Prabhudesai and Narayanaswamy 1974). According to Hirai *et al.* (1997) the efficacy of MS or half MS and White's medium is due to the resemblance in the composition of their nitrogen sources *i.e.*, $NO^{3-}$: $NH^{4+}$. However, Vuorela *et al.* (1993) reported somatic embryogenesis from roots of milk parsley on $B_5$ medium.

The embryogenic callus exhibited acquisition of competence to somatic embryogenesis almost at the same frequency even after 360 days in *E. foetidum*. The retention of embryogenic potential without reduction has been reported in *Urginea indica* (Jha and Sen 1986). In contrast to the present study, Lambe *et al.* (1999) reported progressive loss of embryogenic potential with increase of subculture in *Pennisetum glaucum*.

**Secondary somatic embryogenesis**

During the present studies on *E. foetidum*, secondary somatic embryos were formed either directly or indirectly from the primary embryos. Formation of direct secondary somatic embryo has been reported in *Quercus suber* (Puigderrajols *et al.* 1996) and *Acanthopanax koreanum* (Choi *et al.* 1997). Indirect formation of secondary somatic embryos has been reported in celery (Hunault 1984; Nadel *et al.* 1990), *Haworthia* spp. (Mycock *et al.* 1997), onion (Saker 1998), *Myrtus communis* (Parra and Amo-Marco 1999) and *Bunium persicum* (Grewal and Rani 1999).

**Protoplast culture and regeneration**

Somatic hybridization and direct gene transfer are now become a habitual tool to introduce desired and novel traits in plants, especially in plants where sexual incompatibility prevails. To achieve this goal, an efficient and reproducible protocol of plant regeneration through protoplast culture is a prerequisite.

During the present studies on *E. foetidum*, the enzyme treatment facilitated isolation of the protoplasts *en masse* and was cultured by alginate bead techniques. The efficacy of alginate bead techniques has been reported in plants like saffron (Isa *et al.* 1990), *Gentiana* (Nakano *et al.* 1995) and rice (Giri and Reddy 1994, 1998). According to Giri and Reddy (1994, 1998) alginate encapsulation culture method was more effective than agarose and having higher protoplast stability. More over alginate encapsulation enable the protoplasts to plate without temperature shock and the excision through liquification by the addition of a chelating agent or tri-sodium citrate facilitates recovery of developing cell colonies for transfer to other media (Giri and Reddy 1994, 1998).

During the present investigation, the protoplasts of *E. foetidum* showed both direct and indirect organogenesis. Direct organogenesis from protoplasts has been reported in peppermint (Jullien *et al.* 1998) and *Moricandia nitens* (Tian and Meng 1999). The frequency of direct regeneration was low, may be due to the inhibitory effect of the osmoticum as the growth is going on. High frequency of shoot regeneration was observed from this protocalli upon transfer to fresh medium. Indirect organogenesis from protoplast has also been reported in many medicinal species like *Gentiana* (Nakano *et al.* 1995), *Pinellia ternata* (He *et al.* 1996), *Nicotiana africana* (Rakosy-Tican and Menczel 1998), peppermint (Jullien *et al.* 1998), *Bupleurum falcatum* (Bang *et al.* 1999) and *Isatis indigotica* (Hu *et al.* 1999).

In the present investigation, somatic embryos were also produced indirectly from the protoplast. Somatic embryogenesis from protocalli has been reported in many plants such as fennel (Miura and Tabata 1986), *Hyoscyamus muticus* (Giri and Ahuja 1990), *Astragalus adsurgens* (Luo and Jia 1998), sweet potato (Dhir *et al.* 1998), *Medicago truncatula* (das Neves *et al.* 1999) and mango

(Ara *et al.* 2000b). An efficient protocol for the production of plants from protoplast opens the windows of genetic manipulation at single cell level.

**Histological studies**

Histological studies done with different explants of *E. foetidum* showed that the shoots were originated directly from the phloem cells. Direct shoot Induction from different tissues of the explant has been reported: from vascular cambium (Wenzel and Brown 1991), phloem-cambium cell (Caruso 1971) cortex (Singh and Chandra 1984; Arai *et al.* 1997) and epidermis or subepidermal in stem explants (Chlyah 1974; Creemers-Molenaar *et al.* 1994); cortical, subcortical (Twyford and Mantell 1996) or epidermis or cortex of root (Knoll *et al.* 1997); cotyledon (Gaba *et al.* 1999) and leaf epidermis (Lo *et al.* 1997).

Histological studies showed that the induction of direct somatic embryos was occurred from the cortical cell of the root explants. Somatic embryogenesis has been reported from epidermal cells (Choi *et al.* 1998, 1999; Gaba *et al.* 1999) or subjacent cell layers (Choi *et al.* 1999). There are reports of the induction of somatic embryos from mid rib or tip of the leaves, basal region adjacent to the petiole and leaf margin (Pedroso and Pais 1993).

The origin of somatic embryos in *E. foetidum* from single cell is in consonance with the report of many workers (Steward *et al.* 1964; Haccius 1978; Nomura and Komamine 1985; Choi *et al.* 1998; 1999). However, initiation of embryos from a cluster of embryogenic cells (Sussex 1972; Puigderrajols *et al.* 1996; Loiseau *et al.* 1998) or also by both ways (Vasil and Hildebrandt 1966; Pence *et al.* 1980; Maheswaran and Williams 1985) has also been reported. According to Johansen (1950), the early segmentation pattern of the zygotic embryos of the *Eryngium* is of the Solanad type. Difference in embryo development *in vitro* has been reported in carrot (McWilliam *et al.* 1974).

According to them, the cell division sequences of early somatic embryos in suspension culture of carrot correspond to the Crucifer type, while the early segmentation pattern of zygotic embryos was of Solanad type. Celery, an another member of the carrot family showed Solanad type than to the Crucifer or Asterad types (Choi and Soh 1997).

During the present investigation, somatic embryos passed through the normal developmental stages (globular to cotyledonary) with epidermal, cortical and vascular tissues as reported in many medicinal plants like *Glehnia littoralis* (Hirai *et al.* 1997), *Bacopa monnieri* (Tiwari *et al.* 1998) and *Bunium persicum* (Grewal and Rani 1999). The somatic embryos shared the gross morphology together with its zygotic counterparts *i.e.* analogous with the stages of development from globular to cotyledonary (Torne *et al.* 1997; Tiwari *et al.* 1998; Wakhlu and Sharma 1998; Sagare *et al.* 1999; Sihna *et al.* 2000; Cushman *et al.* 2000).

**Synseeds**

Encapsulation of embryos or shoot buds improves the success of *in vitro* derived plant delivery to field or green house. It is also advantageous in the preservation virtue of elite germplasm.

Encapsulation of embryos in alginate at 2.5 % was found optimal for the conversion of the encapsulated propagules. Castillo *et al.* (1998) elucidated the significance of concentration of alginate with respect to the synseed conversion in papaya and reported that 2.5 % of sodium alginate as the suitable concentration for high frequency of conversion. A reduction in conversion of embryos was observed beyond the optimal level (2.5 %). However, Ghosh and Sen (1994) achieved maximum conversion of embryos in *Asparagus cooperi* encapsulated at 3.5 % sodium alginate. The rate of conversion is also affected by the

concentration of $CaCl_2$. In the present study, of the different concentrations of $CaCl_2$ tested, beads formed at 0.7 % $CaCl_2$ were with high frequency of conversion.

Synseeds at seed competence is at domain with or without the addition of anti-microbial agents into the encapsulation matrix. During the present study the transfer of synseeds directly to the field were also showed successful germination. Field delivery and germination with different clef of success has been reported in many plants *viz. Daucus carota* (Sakamoto *et al.* 1992), *Asparagus cooperi* (Ghosh and Sen 1994), *Spathoglottis plicata* (Nayak *et al.* 1998) and *Geodorum densiflorum* (Datta *et al.* 1999).

**Conservation *in vitro***

Conservation of elite germplasm of rare and endangered species by *in vitro* methods has gained many strides in the recent time. As in the present study, conservation by synseed method has been reported in many plants *viz. Rauvolfia serpentina* (Sharma and Chandel 1992b) and coriander (Chen *et al.* 1995). Of the two temperature regimes tested for the storage of germplasm, the normal culture temperature (22±2°C) was found superior to 4°C. However, effectiveness of storage at low temperature was also opulent. Reed *et al.* (1998) preserved the germplasm of pear in dark at 4°C for more than 2¾ years. Brodelius *et al.* (1982) suggested that growth immobilization or zero growth in alginate beads might be attributed to a reduction in the respiration process.

**Conservation by photoautotrophic culture (plantlet level)**

During the present study on *E. foetidum*, the shoots developed on photoautotrophic medium exhibited slow growth and were found amicable for the conservation of germplasm to more than 12 months without subculture and free of contamination.

Removal of important nutrients like sugar from growing system can reduce cell proliferation dramatically (Van't Hof 1966). According to Krikorian (1996) lowering or depletion of particular nutrients will be useful for cell culture management. The conservation of elite germplasm of the valuable medicinal plants by photoautotrophic methods has much pertinence, considering the reduction of cost by the withdrawal of sucrose and with high rate of survival without the peril of contamination.

**Rooting *in vitro***

Success of micropropagation mainly depends upon *in vitro* root induction and thereby the high percentage of survival in the field condition. Most of the reports envisage the need of a separate rooting medium (Usha and Swamy 1998; Sunnichan *et al.* 1998; Choi *et al.* 1999; Jagadishchandra *et al.* 1999). Nevertheless, the present studies on *E. foetidum* showed sufficient number of roots in the shoot multiplication medium itself. However, Arockiasamy and Ignacimuthu (1998) could not observe root formation in the same species. Root induction in the shoot multiplication medium itself were also reported in ginger (Sharma and Singh 1997), *Kaempferia galanga* (Vincent *et al.* 1998), *Alpinia calcarata* (Martin and Hariharan 1999) and *A. galanga* (Borthakur *et al.* 1999). The shoots without roots transferred on half MS basal medium produced enormous amount of roots. Arockiasamy and Ignacimuthu (1998) reported root induction only on an auxin + $GA_3$ supplemented medium in *E. foetidum*. During the present studies on *E. foetidum*, the roots produced in all cultures comes into contact with the medium become brown/black, which may be due to the acquisition of biochemical potential by the factors present in the medium. Arockiasamy and Ignacimuthu (1998) have also envisioned the same in *E. foetidum*.

**Transplantation to field conditions**

One of the hindrances in the micropropagation is the low survival rate of the plantlets in the field, which increase the production cost further. However, during the present investigation on *E. foetidum* the plantlets developed on the photoautotrophic medium showed 100 % survival in field condition. According to Kozai *et al.* (1988) high rate of survival in photoautotrophically microproagated plants is due to the high photosynthetic or photoautotrophic competence of plants *in vitro*. The low photosynthetic ability of the plants propagated otherwise is due to the sole dependence of organic carbon supplied in the medium. When transplanted out of the culture vessels they suffer from severe environmental stress; hence substantial loss may occur (Preece and Sutter 1991; van Huylenbroeck and Debergh 1996). In photoautotrophic micropropagation, the plants use $CO_2$ as their sole carbon source like higher plants, which makes them less susceptible to water loss when transplanted out (Kozai 1991; Pospisilova *et al.* 1997; Mitra *et al.* 1998a, b).

**Secondary metabolite analysis**

Comparative analysis of both *in vitro* and *ex vitro* root tissues, showed the presence of new compounds in *in vitro* derived tissue extracts.

The ethanolic extracts of root tissues of *in vitro* grown plants showed more number of compounds (six) compared to the field grown plant extract (two). In contrast, Gokul and Tejavathi (1999) reported more compounds in the field grown tissues compared to the *in vitro* tissues of *Cissampelos pareira*. Similarly, lack of certain compounds in *in vitro* derived tissues when compared to the plant intact has been reported in *Artemisia pallens* (Benjamin *et al.* 1990) and in *Rauwolfia sellowii* (Rech *et al.* 1998). In the present study, the difference in the number of compounds may be due to the differential growth stage of the roots *i.e.*, the root

tissue of the *in vitro* grown plants are from plantlet just after embryonal stages. Gokul and Tejavathi (1999) also exemplified the influence of supplements in the medium to the synthesis of secondary compounds. It may also be a reason for the difference in the number of compounds.

## *Holostemma ada-kodien*

### Axillary bud multiplication

### Effect of Basal Media

During the present studies on *Holostemma ada-kodien*, the nodal explants excised from the basal region induced an average of two shoots on MS basal medium. Sudha *et al.* (1998) reported the initiation of only one axillary bud in the same species. Axillary bud initiation and multiplication on basal medium has been reported in *Sapium sebiferum* (Siril and Dhar 1997), *Gmelina arborea* (Thirunavoukkarasu and Debata 1998) and *Sesamum indicum (*Gangopadhyay *et al.* 1998). However, the nodal explants have not responded with the basal medium in the culture of *Hemidesmus indicus* (Patnaik and Debata 1996), *Gymnema elegans* (Komalavalli and Rao 1997) and *Tridax procumbens* (Sahoo and Chand 1998a). The explants remained green and then exhibited necrosis. The initiation of axillary bud, which is very common, is demonstrated to be the function of endogenous cytokinin. In the present study, axillary bud multiplication occurred in the basal medium, which may be due to the effect of the endogenous cytokinin present in the nodal explants or it is due to the culture milieu.

### Effect of growth regulators

Culture studies on *Holostemma ada-kodien*, showed that BAP was superior to Kin for the initiation and multiplication of axillary buds. The superior effect of BAP over Kin was reported in the same species by Jayanthi and Patil (1995) and Sudha *et al.* (1998) and also in many other medicinal species like *Sapium sebiferum* (Siril and Dhar 1997), *Artemisia annua* (Usha and Swamy 1998), *Sterculia urens* (Sunnichan *et al.* 1998) and *Ceropegia* spp. (Patil 1998). In the case of *Asclepias curassavica* (Pramanik and Datta 1986) and *Ceropegia bulbosa* (Ramulu and Pullaiah 1999), the effect of Kin and BAP is equal for the

induction of axillary bud multiplication. However, Patnaik and Debata (1996) reported Kin as superior to BAP for shoot multiplication in *Hemidesmus indicus*.

Higher concentration of cytokinin was reported to inhibit shoot elongation (Hu and Wang 1983). During the studies on *H. ada-kodien,* higher levels of BAP above the optimum level (1.5 mg $l^{-1}$) caused a suppression of shoot elongation, which resulted in stunted shoots with smaller leaves. Stunted growth with small leaves has also been reported in *Hemidesmus indicus* (Patnaik and Debata 1996), *Tridax procumbens* (Sahoo and Chand 1998a) and *Gymnema sylvestre* (Reddy *et al.* 1998). According to Yi *et al.* (1992) this was due to an exorbitant accretion of BAP.

During the present investigation on *H. ada-kodien,* combination of BAP with Kin was not effective for axillary bud multiplication. The ineffectiveness of BAP+Kin has also been reported in another member of the Asclepiadaceae, *Gymnema elegans* (Komalavalli and Rao 1997). However, an enhanced shoot multiplication in the combination of BAP and Kin has been reported in *Pisonia alba* (Jagadishchandra *et al.* 1999) and *Canavalia virosa* (Kathiravan and Ignacimuthu 1999).

In the present study higher frequency of shoot regeneration was observed in the combination of BAP with an auxin (IBA). According to Sudha *et al.* (1998) BAP in combinations with NAA was more effective in the same species. Synergistic effect of BAP in combination with an auxin was reported in many medicinal plants including members of Asclepiadaceae: BAP + NAA in *Sapium sebiferum* (Siril and Dhar 1997), *Vitex negundo* (Kannan and Jasrai 1998), *Gymnema sylvestre* (Reddy *et al.* 1998), *Aegle marmelos* (Ajithkumar and Seeni 1998), *Psoralea corylifolia* (Saxena *et al.* 1998) and *Boerhaavia* (Phukan *et al.* 1999); BAP + IBA in *Actinidia deliciosa* (Kumar *et al.* 1998). All these reports exemplified that low concentrations of an auxin can positively modify the shoot

induction response when combined with a cytokinin. However, a decrease in axillary bud multiplication in the presence of BAP with an auxin has been reported in *Gmelina arborea* (Thirunavoukkarasu and Debata 1998) and *Artemisia annua* (Usha and Swamy 1998).

An enhanced shoot multiplication obtained by subsequent cultures in the present investigation is in accordance with the report on same species (Sudha *et al.* 1998) and also in other medicinal plants like *Gmelina arborea* (Thirunavoukkarasu and Debata 1998), *Psoralea corylifolia* (Saxena *et al.* 1998) and *Aegle marmelos* (Ajithkumar and Seeni 1998). However, according to Patnaik and Debata (1996) subsequent cultures were not promotive in the induction of shoots in *Hemidesmus indicus*.

Callus formation was also observed at the basal cut ends of the nodal segments in *H. ada-kodien* is in concomitant with the report of Sudha *et al.* (1998) in the same species. Callus formation from the basal cut ends of nodal explants has been reported in *Tylophora indica* (Sharma and Chandel 1992a), *Hemidesmus indicus* (Sharma and Yelne 1995), *Uraria picta* (Anand *et al.* 1998) and *Psoralea corylifolia* (Saxena *et al.* 1998) also. According to Marks and Simpson (1994) this may be due to the action of auxin accumulated at the basal cut ends, which stimulates cell proliferation, especially in the presence of cytokinins (Tao and Verbelen 1996). According to Preece *et al.* (1991), the formation of basal callusing is frequent in shoot cultures of species with strong apical dominance.

Of the nodal explants of *H. ada-kodien* cultured from the three different regions, explants from the basal region close to the root showed an enhanced shoot multiplication followed by middle and tip. This result is in concomitant with the report on same species by Sudha *et al.* (1998) and on *Maytenus ilicifolia* by Pereira *et al.* (1995). The high efficacy of shoot multiplication in the basal nodal

segments over the other two may be attributed by the increase of endogenous growth regulator, which differ from one region to another (Okubo *et al.* 1991) or due to the difference in nutrient availability (Norton and Norton 1986). According to Raghavaswamy *et al.* (1992) the difference between the physiological status of the explants is also a reason for it.

In the present study, the micropropagated shoots exhibited leaf and shoot tip abscission. Similar phenomenon has also been demonstrated during the *in vitro* multiplication of *Hemidesmus indicus* (Patnaik and Debata 1996) and *Psoralea corylifolia* (Saxena *et al.* 1998). The necrosis and abscission of leaves and shoots were due to the accumulation of ethylene and this has been alleviated by the addition of $AgNO_3$ and $CoCl_2$ - ethylene inhibitors.

During the present study the effect of ethylene has been minimized by the addition of inhibitors such as $AgNO_3$ and $CoCl_2$, however, it resulted in the reduction of number of shoots. Success has been reported in the alleviation of the inhibitory effect of ethylene in many plant species. Leaf abscission frequency in *Annona squamosa* was hindered by the use of $AgNO_3$ (Lemos and Blake 1996a) but Silver thiosulphate (STS) has been shown to be more effective (Lemos and Blake 1996b). The effectiveness of $AgNO_3$ has been reported in many plants: *Heracleum candicans* (Wakhlu and Sharma 1998), cucumber (Mhatre *et al.* 1998) and sunflower (Potdar *et al.* 1999). Recently Paul and Sikdar (1999) reported the potency of $AgNO_3$ in the regeneration of transgenic plants from *Brassica*. The efficiency of cobalt has been reported in carrot (Roustan *et al.* 1989), cucumber (Mhatre *et al.* 1998) and sunflower (Potdar *et al.* 1999). Roustan *et al.* (1989) and (Mhatre *et al.* 1998) have also reported the effectiveness of cobalt chloride in carrot and cucumber respectively.

## Callus Studies

Callus was established from root, internode and leaf explants of *H. ada-kodien* on MS hormone free medium. According to Kahl (1983), the callus formation without an exogenous growth regulator is due to the wound reaction, where the cells at the cut ends undergoes mitosis, which leads to callus formation. There are many reports about callus induction in the absence of an exogenous stimulus (Yeoman and Macleod 1977). Pérez-Francés *et al.* (1995) reported callus formation from *Erysimum scoparium* on basal medium. The hormonal trigger for the dedifferentiation is probably an auxin (De Klerk *et al.* 1997a). In view of Wilson and van Staden (1990), the signals related to wounding may also play a role during dedifferentiation. A decline in cytokinin contents and an increase in auxin level were also reported in the *in vitro* culture of *Actinidia deliciosa* (Centeno *et al.* 1996). In addition, several studies have shown that (de)differentiation is related to DNA methylation (Palmgren *et al.* 1991). According to some authors, DNA amplification during the first hours after inoculation proceeds mitotic activity and this may be related to wounding, activation of the cell cycle and/or initiation of dedifferentiation (Arnholdt-Schmitt *et al.* 1995).

During the present study callus formation was occurred on cytokinin enriched medium also. Induction of callus has been reported from Dutch elm hybrid 'Commelin' on cytokinin containing medium (Jouira *et al.* 1998).

## Indirect organogenesis

Indirect organogenesis was observed from internodal and nodal callus developed on BAP containing medium. Regeneration of shoots from calli developed at the basal cut end of the nodal explants has been reported in certain members of Asclepiadaceae such as *Tylophora indica* (Sharma and Chandel

1992a) and *Hemidesmus indicus* (Sharma and Yelne 1995). The internodal callus in the present study exhibited a low frequency of regeneration. Recently Sudha *et al.* (2000) has reported direct shoot regeneration from *in vitro* derived roots of the same species. Indirect organogenesis from internodal and leaf callus has also been reported in *Hemidesmus indicus* (Sarasan *et al.* 1994) and *Ceropegia* spp. (Patil 1998). The potency of nodal callus compared to the internodal callus may be due to the clairvoyance of some internal components that are essential to evoke regeneration.

**Tuberous roots *in vitro***

The callus cultured on MS with CW (10-15 %) or BAP+NAA+AA+$AgNO_3$ induced tuberous roots *in vitro*. The $AgNO_3$ medium induced more number of roots. The formation of underground organs as in plants *ex vivo* has been demonstrated in many medicinal plants as in *Dioscorea composita* (Alizadeh *et al.* 1998), *Bunium persicum* (Grewal 1996b), *Helianthus tuberosus* (Gamburg *et al.* 1999), *Lachenalia* (Slabbert and Niederwieser 1999) and in crop plants like potato (Villafranca *et al.* 1998; Gopal *et al.* 1998). Sudha *et al.* (1998) reported the formation of tuberous roots in *H. ada-kodien* only after 2 months in the field. Tuberous roots being the officinal part in *H. ada-kodien*, the induction of tuberous roots *in vitro* has of much pertinence.

**Somatic embryogenesis**

Somatic embryogenesis in *Holostemma ada-kodien* was of the IEDCs type.

Embryoids were developed only from friable callus obtained on 2,4-D (1.0-2.0 $mgl^{-1}$) containing medium. Most of the protocols for somatic embryogenesis use a strong auxin such as 2,4-D in the primary culture medium, which support both cell proliferation and induction of embryoids. According to Zimmerman

(1993) the pro-embryogenic mass in an auxin containing medium generally synthesize all genes necessary to complete the globular stage of embryos. The superior effect of 2,4-D in inducing somatic embryos has been reported in many medicinal plants as in *Tylophora indica* (Rao and Narayanaswamy 1972), *Glehnia littoralis* (Hirai *et al.* 1997) and *Ceropegia* spp. (Patil 1998). However, the efficacy of 2,4-D in combination with BAP as in *Heracleum candicans* (Wakhlu and Sharma 1998); and with Kin as in *Hemidesmus indicus* (Sarasan *et al.* 1994) and *Acanthopanax koreanum* (Choi *et al.* 1997) or cytokinin alone as in zonal geranium (Wilson *et al.* 1996) and *Araujia sericifera* (Torné *et al.* 1997) for the induction of somatic embryogenesis were also reported. The effectiveness of NAA/IAA individually or in combination with cytokinin was also reported as in *Araujia sericifera* (Torné *et al.* 1992) and *Azadirachta indica* (Su *et al.* 1997).

Reduced levels of salt have been reported to be of a recondite effect on the successive development of the embryos. During the present study on *H. adakodien* subsequent transfer of embryogenic calli to ½ MS and ¼ MS with low levels or without 2,4-D resulted in the development of embryoids up to cotyledonary stage. Sunnichan *et al.* (1998) reported effectiveness of ¼ MS in *Sterculia urens*. Efficacy of reduced salt strength for the development of embryoids has also been reported in *Glehnia littoralis* (Hirai *et al.* 1997), *Ceropegia* spp. (Patil 1998), *Piper nigrum* (Joseph *et al.* 1999) and *Salvia* spp. (Kintzios *et al.* 1999). Inhibitory effect of high concentration of inorganic elements on embryo development has been reported in *Hemidesmus indicus* (Sarasan *et al.* 1994). This may be due to the influence of salt concentration as a whole or due to inhibitory effect by increased concentration of a particular element or $NO^{3-}$ : $NH^{4+}$ ratio.

During the present study, the transfer of the embryos to 1/4 MS supplemented with auxins (at low level)/BAP/Kin/$GA_3$/AC/CH/CT/CW either alone

or in combinations resulted in dedifferentiation. The embryos showed conversion only on 1/10 basal medium, but it was at low frequency. However, conversion of embryos has been reported among the members of Asclepiadaceae on medium supplemented with CW as in *Hemidesmus indicus* (Sarasan *et al.* 1994) and BAP as in *Araujia sericifera* (Torne *et al.* 1997) and *Ceropegia* spp. (Patil 1998).

During the present investigation on *Holostemma ada-kodien*, as in *Eryngium foetidum* high frequency of embryogenesis has been observed in suspension cultures. Effectiveness of suspension culture over static has been reported in *Hemidesmus indicus* (Sarasan *et al.* 1994), *Tylophora indica* (Rao and Narayanaswamy 1972), *Piper nigrum* (Joseph *et al.* 1999) and neem (Murthy and Saxena 1998). Somatic embryos formed in liquid medium did not develop further unless it was transferred to static cultures.

Transfer of the embryos to solid medium containing cytokinin /auxins/additives resulted in dedifferentiation under light. Mycock *et al.* (1997) and Potdar *et al.* (1999) reported dedifferentiation respectively in *Haworthia* spp. and sunflower. Rao *et al.* (1970) also observed dedifferentiation of embryoids but on a medium with high concentration of auxin. Dedifferentiation is reported to be related to DNA methylation (Palmgren *et al.* 1991). The activation of genes facilitating callus formation or by the inactivation of the genes governing the embryogenesis by light may also be a reason.

Somatic embryos developed in *H. ada-kodien* showed germination but at low frequency on 1/10 MS hormone free medium. According to Stuart *et al.* (1985), the embryos exposed to auxin during development fails to accumulate storage protein, which result in low frequency germination. Removal of auxin from the culture is therefore considered to be essential for the inactivation of several genes or synthesis of new gene-products for completion of embryo development (Zimmerman 1993). The low frequency of germination may be due

to the persistence of auxin and thereby the failure of accumulation of storage products. Artificially induced dormancy by culture environments was also proposed by Gray and Purohit (1991).

During the present study, the embryos of *H. ada-kodien* exhibited a suspensor like structure. The presence of suspensor has been demonstrated in *Hemidesmus indicus* (Sarasan *et al.* 1994), celery (Choi and Soh 1997) and chickpea (Sagare *et al.* 1999). However, the presence of suspensor is still in contention. According to Swamy and Krishnamurthy (1981) and Krishnamurthy (1999), the so-called suspensor is not in pact with true suspensor of zygotic embryos in function.

As in *Eryngium foetidum,* in the case of *Holostemma ada-kodien* also sucrose at 1.5-3 % was found optimal and the effect of sucrose has been already discussed under the subheading, **Effect of Sucrose** in *Eryngium foetidum* (page 79).

Light was considered to be effective on somatic embryogenesis through its effect on induction (Verhagen and Wann 1989) and also on some morphological characteristics of differentiation (Halperin 1966; Ammirato and Steward 1971). In the present studies on *H. ada-kodien*, the callus developed in dark induced high frequency of embryos compared to that from light. D'Onofrio *et al.* (1998) reported an inhibitory effect of somatic embryogenesis under white fluorescent light and high frequency of embryogenesis under red light in quince leaves. In contrast to the present observation, induction of somatic embryogenesis under high light intensity has been reported in tobacco (Haccius and Lakshman 1965). Dark conditions reduced the frequency of embryoids on petal explants of *Araujia sericifera* (Torné *et al.* 1997).

**Protoplast culture and regeneration**

In the case of *H. ada-kodien*, the protocalli formed from protoplasts encapsulated in alginate beads showed browning, which hindered further proliferation of protocalli. Cell browning has been reported in the protoplast culture of *Gentiana* embedded in gellan gum and also in liquid culture (Nakano *et al.* 1995). The browning may be due to the inhibitory effect of the osmoticum as reported in many plants. Plant regeneration either *via* organogenesis or somatic embryogenesis from callus derived protoplasts of *Tylophora indica* (Mhatre *et al.* 1984) was the only one report among the members of the Asclepiadaceae. An efficient protocol of protoplast to plant opens the windows of genetic manipulation of these plants at single cell level.

**Histological studies**

Histological studies of somatic embryos as in *Eryngium foetidum*, passed through discernible developmental stages (globular to cotyledonary) with normal epidermal, cortical and vascular tissues as reported in many medicinal plants: *Glehnia littoralis* (Hirai *et al.* 1997), *Araujia sericifera* (Torne *et al.* 1997), *Bacopa monnieri* (Tiwari *et al.* 1998), *Ceropegia* spp. (Patil 1998), *Hevea brasiliensis* (Jayasree *et al.* 1999) and *Sapindus mukorossi* (Sinha *et al.* 2000).

**Synseeds**

The encapsulated shoot buds (unipolar) showed high rate of conversion compared to the encapsulated embryos. Though less attention has been given to the encapsulation of non-embryogenic propagules, since 1990's it was widely explored especially in those with no or less conversion frequency of somatic embryos. Somatic embryo derived synseeds was hurdled by the conversion, the notable relic of the synseed technology, which limits its practical use and its commercial exploration (Kozai *et al.* 1991; Tautorus and Dunstan 1995).

However, the encapsulation of shoot buds offers an alternative to the embryo-derived synseed and also to the preservation of elite germplasm. Successful encapsulation of vegetative propagules has been documented with promising results in sandalwood and mulberry (Bapat 1993) and six woody species (Piccioni and Standardi 1995).

**Conservation *in vitro***

**(i) Synseed level**

Germplasm conservation through alginate encapsulation of shoot buds has been reported in many plant species. Axillary buds of *Morus indica* (Bapat *et al.* 1987) and adventitious buds of *Morus alba* (Machii 1992) have been encapsulated in alginate or agar beads and were stored for 34-80 days at 4°C without loss of viability. Kinoshita and Saito (1992) reported that the encapsulated axillary buds of *Betula platyphylla* var. japonica stored at 4°C for more than 80 days without loss of viability. Microcuttings of *Eucalyptus grandis* X *E. urophylla* encapsulated in alginate beads kept on a nutrient agar medium were stored for 10 months at 30/25°C with a plant recovery rate of 52 % after storage (Yuehua and Wenming 1994). The shoot tips of *Cedrela odorata, Guazuma crinita* and *Jacaranda mimosaefolia* in alginate stored at 12,15 and 20°C was reported to show viability after 12 months (Maruyama *et al.* 1997). According to Maruyama *et al.* (1997) the apt temperature for storage was 12°C. Chetia *et al.* (1998) stored encapsulated protocorm at 4°C for 40 days without the loss of viability.

**(ii) Plantlet/shoot bud level**

The growth of plant cells and tissues cultured *in vitro* depends upon exogenously added growth substances (Krikorian 1996; Lopez-Delgado *et al.* 1998; Babu *et al.* 1999; Shiota *et al.* 1999). During the present study, the shoots

and embryo-like shoot buds (unipolar) cultured on half MS with 1.5 % sucrose and low levels of auxins either with or with out $AgNO_3$ showed slow growth or "zero growth" even after 12 months. Use of minimal medium and growth retardant such as ABA as well as the growing of cultures on a sucrose-free medium was reported to make delay the subculture intervals (Jones 1974b; Shiota *et al.* 1999). The storage at nonfreezing temperature (2-8°C) has been successfully applied to a large number of species (Aitken-Christie and Singh 1987). The effectiveness of acetyl salicylic acid added to the medium has been reported in the preservation of potato microplants (Lopez-Delgado *et al.* 1998). Babu *et al.* (1999) reported the preservation of cardamom germplasm on a minimal medium with 1.5 % sucrose and mannitol. Conservation of elite germplasm at whole plant or shoot bud level, especially from the shoots originated from the axillary buds has much pertinence in the conservation of germplasm without the possibility of variation.

Sudha *et al.* (1998) exemplified the feasibility of cultures in bottles for short-term conservation (eight months) in the same species, without the addition of $AgNO_3$. Addition of $AgNO_3$ extended the period of preservation of the germplasm further.

According to Felle (1989) exogenously added auxin can affect the pH of the medium most often by lowering it. As per the evidences in literature, lowering of pH can change many major metabolic processes, but it depends on the developmental and physiological state of the tissue, cell or organ. Thus in the present study retarded growth may be due to the lowering of pH by the auxin alone or by an interaction of auxin and $AgNO_3$. The efficacy of $AgNO_3$ either alone or in combination with auxin is not elucidated so far.

In short, conservation at whole plant level was found superior to that of the encapsulated propagules. However, to circumvent the problems of cryopreservation *in toto*, a fine refinement for the resuscitation at feasible rate

after long term storage of elite germplasm is needed to avoid the capitalization of cryopreservation.

**Rooting *in vitro***

Auxins play a vital role in root initiation, elongation and lateral root formation (De Klerk *et al.* 1997b). In the present studies on *Holostemma ada-kodien,* half MS medium with IBA was found more suitable for root induction followed by IAA and NAA. Effectiveness of IBA in rooting was reported in many medicinal plants like *Vitex negundo* (Kannan and Jasrai 1998), *Gmelina arborea* (Thirunavoukkarasu and Debata 1998) and *Gloriosa superba* (Sivakumar and Krishnamurthy 2000). According to De Klerk *et al.* (1997b), the rate of uptake of auxin was different. Uptake of NAA was six times faster than IAA (Peeters *et al.* 1991) and IBA was four times faster than IAA (van der Krieken *et al.* 1993). Slow movement and slow degradation results in the localization of the IBA near the site of application (Nickell 1982). The efficacy of IAA was demonstrated in *Aegle marmelos* (Ajithkumar and Seeni 1998) and ginseng (Choi *et al.* 1999). The low efficacy of NAA in root induction may be due to the diversification of it for other metabolic pathways leading to calli formation. However, effectiveness of NAA was also been reported in medicinal plants as in *Cinnamomum camphora* (Huang *et al.* 1998) and *Gloriosa superba* (Sivakumar and Krishnamurthy 2000).

During the present study, half MS medium with cytokinin/CW induced callus along with few numbers of roots. The inhibitory effect of cytokinin has been reported in *Gymnema sylvestre* (Reddy *et al.* 1998). But Patnaik and Debata (1996) reported effectiveness of IBA along with cytokinin (Kin) for root induction in *Hemidesmus indicus*. In certain cases, addition of cytokinin favoured rhizogenesis (Martin and Perez 1995). Roots were induced on both solid and liquid medium. The shoots rooted in the liquid medium showed faster growth and better vigour. The better growth of roots as well as shoots may be due to the

higher gas diffusion and higher oxygen concentration or due to the better diffusion of nutrients or by the synergistic effect of both. In addition, rooting in liquid medium avoids removal of agar etc., which cause contamination and loss during the acclimatization.

**Secondary metabolite analysis**

Comparative TLC analysis of the secondary metabolites showed the presence of more compounds in the *in vitro* tuberous roots compared to the *ex vitro*. The balanced physical and chemical conditions in *in vitro* in contrast to the field grown is the possible reason behind the presence of new compounds. The activation of genes responsible for the synthesis of compounds coupled with the silencing of genes for the compounds occurring in plants due to the culture milieu may also be the reason behind the additional compounds.

The preliminary result obtained here has of much pertinence considering the unequivocal acuity on the identification of alkaloids present in *Holostemma ada-kodien* as well as in *Eryngium foetidum*. However, qualitative analysis along with quantitative in particular should be performed to confirm and to exploit the real potential of the production of secondary metabolites present in these plants.

The present investigation has resulted in a protocol that could be used for true-to-type mass propagation and *ex situ* conservation of both *E. foetidum* and *H. ada-kodien*, two medicinal plants of much pharmaceutical relevance.

# TRANSFORMATION STUDIES

Plant transformation studies, using *Agrobacterium* spp. and direct gene transfer techniques have now been proved as challenging tools for the introduction of alien genes to desired plants, especially of economically significant ones such as ornamentals, crop and medicinal plants. The Ri binary vector system of *A. rhizogenes* provides a powerful tool for the development of transgenic plants, especially on dicotyledonous plants. Transfer of Ri-mediated foreign genes into the target plant cells and their subsequent integration and expression play a key role in the field of transgenic plant research.

Transformation studies with *A. rhizogenes* harbouring the binary plasmid **pGreen** with ***gus/gfp*** induced hairy roots on the co-cultivated explants of *E. foetidum* and *H. ada-kodien*. The efficacy of this constructed plasmid, as a novel binary vector for the transformation of plants has well been documented by Hellens *et al.* (1999). During the present study, successful regeneration of plants from transformed tissues of *E. foetidum* was obtained. Induction of callus from the transformed hairy roots and subsequent plant regeneration have been reported in many crop and medicinal plants such as *Scoparia dulcis* (Yamazaki *et al.* 1996), *Aconitum heterophyllum* (Giri *et al.* 1997), *Eucalyptus camaldulensis* (Ho *et al.* 1998), mexican lime (Perez-Molphe-Balch and Ochoa-Alejo 1998), *Crotalaria juncea* (Ohara *et* al. 2000) and *Panax ginseng* (Yang and Choi 2000),

The shoots regenerated from the transformed roots *via* callus showed abnormalities like stunted growth with rosette leaves. In their studies with transformed *Scoparia dulcis*, Yamazaki *et al.* (1996) had observed morphological abnormalities such as shortening of internodes, small and wrinkled leaves etc. Similar effects have also been reported in transformed plants of snapdragon (Hoshino and Mii 1998), *Brassica carinata* (Babic *et al.* 1998) and tobacco (Palazon *et al.* 1998; Daley *et al.* 1998). The morphological abnormalities noticed

in the present study could be attributed to the accumulation of the auxins produced by the *rol* genes present in the T-DNA, being integrated into the plant genome.

During the present study, though the callus from explants of *H. ada-kodien* induced somatic embryogenesis, no further developments were noticed. Considering the 30% conversion of somatic embryos in to plantlets from the callus obtained from different explants without co-cultivation, lack of conversion of the embryos obtained from the callus of hairy roots may be due to the silencing of the genes facilitating somatic embryo conversion, by the integration of the foreign DNA in to the plant genome.

Faithful identification of the transformed tissues/plants is a crucial factor in the transformation experiments. Of the different reporter genes, bacterial ***uid A*** (***gus A***) coding β-glucuronidase has widely been explored for the identification of transformants. The isolation of ***gfp*** from Jellyfish as reporter gene and its subsequent explorations have made the identification of transformed tissues easy, in a non-invasive way (Chalfie *et al.* 1994). The ***gfp*** encoded protein absorbs UV or blue light and fluoresces it in to green. Its advantage over ***gus***, as reporter gene is that the expression can directly be detected in living cells without the requirement of substrate and cofactors.

The callus induced from transformed hairy roots of *E. foetidum* and *H. ada-kodien* clearly proved the expression of the reporter gene, ***gfp***. Expression of ***gfp*** has been reported in many transformed plants like maize (Sheen *et al.* 1995), arabidopsis (Sheen *et al.* 1995; Haseloff and Amos 1995), pine (Tian *et al.* 1997) and tobacco (Elliott *et al.* 1999).

Induction of hairy roots *en masse* and the exploitation of its higher potential for secondary metabolite production, compared to the roots of plant

intact have been reported in many medicinal plants, especially in plants having root as officinal part such as *Hyssopus officinalis* (Murakami *et al.* 1998), *Glycyrrhiza glabra* (Li *et al.* 1998), *Coleus forskohlii* (Sasaki *et al.* 1998) and *Panax ginseng* (Yang and Choi 2000). In this context, considering root as the officinal part of the present study materials, mass growing of the hairy root has much pertinence, coupled with regeneration, multiplication and successful establishment of the transgenic plants. The outcome of these preliminary studies reflects that both the plants under study are much amenable to genetic transformation and subsequent development of transgenic plants. Further, a detailed investigation aimed at the elucidation of the structure and biosynthetic mechanism involved in the metabolites produced by these plants has to be initiated, employing the modern tools of genetic engineering and molecular biology.

# SUMMARY

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Medicinal plants are the most eco-friendly combinatorial source of many biodynamic curare compounds, which serves as the blue print for the preparation of new drugs, essential to mankind. The great surge of interest on medicinal plants is its availability throughout at less cost. Exploration of the potential of secondary metabolites brought the medicinal plants to the fore, which resulted the extirpation of the wild bioresources and extinction of very rare and endangered plant species especially in countries like India with high bio-richness. To overcome this situation, at present, propagation and conservation by tissue culture methods are in practice. The judicious utilization of various tissue culture techniques on medicinal plants enables the propagation, conservation and extraction of secondary metabolites and development of transgenic plants with acquisition of new characters. Clonal propagation by tissue culture reduces the mean generation time in addition to the advantage of propagation independent of season. Potential of somatic embryogenesis enables rapid propagation and accelerates the advantage of synthetic seed technology and conservation of selected genotypes. An efficient protocol of morphogenesis *in vitro* is a prerequisite for the manifold exploration of genetic transformation. *Agrobacterium rhizogenes* harbouring the Ri plasmid induce "hairy roots". Considering the correlation of biosynthesis of secondary metabolites to the morphogenesis as root, induction of hairy root has much intrigue today in the case of plants in which the root is the officinal part. In the light of these, two experimental plants, *Eryngium foetidum* and *Holostemma ada-kodien,* with low propagation rate and seed viability and root as medicinal part have been selected for the present work.

## *Eryngium foetidum* L.

*Eryngium foetidum* (Apiaceae), commonly known as "Spiny coriander" is a rare aromatic perennial herb restricted to certain regions of India. The plant is known for its saponin contents and is generally responsible for its biological activity and it also contains flavonoids and quercetin glycosides. It is strongly aromatic, contains essential oils comprised of 40 compounds. The aromatic essential oil is indispensable to perfumery, flavour and pharmaceutical industries.

Direct and indirect organogenesis were established from different explants (leaf, stem disc, root and scape) of *E. foetidum* cultured on MS medium with or without growth regulators. MS medium was found superior for the induction of shoots. The stem and scape explants induced an average of 3 shoots on MS basal medium.

MS medium with cytokinins alone or in combination with auxins was tested for direct shoot multiplication. Of the two cytokinins (BAP/Kin) tested individually, Kin was found superior for direct shoot induction. Nevertheless, the combination of Kin with BAP was found more effective, which induced an enhanced number of shoots. Of the different combinations tested, Kin at 1.0 mg $l^{-1}$ and BAP at 0.5 mg $l^{-1}$ facilitated an average of 57 shoots on scape explants within 40 days. The shoots developed numerous well-developed roots from the basal region.

Shoot, root and calli (hard green and friable brown) were established from stem disc, leaf and scape explants on MS medium supplemented with NAA or IAA alone or in combination with BAP or Kin. Only roots and callus were induced from root explants. The leaf explants produced 3-5 shoots on MS medium containing 2.0 mg $l^{-1}$ NAA/IAA. Addition of Kin or BAP with IAA or NAA showed a

more or less same mode of response as the auxins used singly, but with slight increase in the number of shoots and in the amount of callus.

Callus formation was obtained on MS medium containing 1.0 mg $l^{-1}$ 2,4-D. Callus was friable and creamish yellow. Among the combinations of 2,4-D with Kin or BAP, 2,4-D (1.0 mg $l^{-1}$) with Kin (0.5 mg $l^{-1}$) was found better for the induction of callus.

MS medium fortified with BAP (1.5 mg $l^{-1}$) singly was more effective for the induction of callus than the auxins used alone or in combination with cytokinins.

For the induction of indirect organogenesis, hard green callus developed on BAP containing medium was subcultured on MS + Kin (1.0 mg $l^{-1}$) + BAP (0.5 mg $l^{-1}$). An average of 25 shoots was developed with in 40 days. The shoots developed *in vitro* induced numerous roots in the same medium.

The different explants excised from the *in vitro* grown plants cultured on MS basal medium without sucrose (photoautotrophic medium) induced 1-4 shoots. Subculturing of the callus on photoautotrophic medium induced an average of 4 shoots.

Embryos of cotyledonary stage were formed spontaneously from the brown coloured callus developed on roots of regenerated plants grown on MS medium fortified with NAA + Kin (2.0 mg $l^{-1}$ each).

Friable callus developed from different explants on MS medium containing 2,4-D (1.0 mg $l^{-1}$) + Kin (0.5 mg $l^{-1}$), when transferred to the medium with the same concentrations or with reduced level, embryogenic calli were formed. These embryogenic calli when transferred to liquid half MS containing 10 % CW/CT, somatic embryos of cotyledonary stage were developed at the rate of 500 embryos/10 mg callus. These embryos with 2-7 cotyledons underwent

conversion also. Sucrose 1.5-3 % was found optimal for the induction, maturation and conversion of embryos. Suspension cultures were superior to static cultures for the induction of embryos. Light was a requisite for the conversion of mature embryos at high frequency.

The isolated protoplasts showed regeneration *via* organogenesis and somatic embryogenesis.

Histological studies showed that, the shoots were originated from the phloem cells of the explant. The embryos originated directly from the cortical cells of root explants. The embryogenic calli were originated from the cortex of the different explants, while on leaf it was from mesophyll cells.

Mature embryos, encapsulated in half MS containing 10 % CW with 2.5 % sodium alginate upon transfer to hormone free half MS medium showed 100 per cent conversion. The field transfer of the synseeds showed germination *ex vitro* at equal rate but was impeded by microbial contamination.

Conservation through photoautotrophic culture was found superior to synseeds. The plantlets showed slow growth on photoautotrophic medium and remained healthy and free of contamination even for more than 12 months without being subcultured.

Root formation occurred on shoots in the shoot induction medium itself. The shoots without roots showed rapid induction of numerous roots following the transfer to hormone free half MS medium and the plantlets were successfully established in the soil.

Thin Layer Chromatographic analysis of secondary metabolites showed the presence of more compounds in the root extracts of the *in vitro* grown plantlets compared to that of the field grown plants.

## ***Holostemma ada-kodien*** Schult.

*H. ada-kodien*, a member of Asclepiadaceae is known as "Jivanti", an indigenous laticiferous, perennial plant to India. As per the red list of medicinal plants, it is a vulnerable species. Tuberous root is the officinal part of the plant and about 150 metric ton of tuberous roots are needed per annum for ayurvedic preparations in South Indian pharmacies.

Of the two cytokinins tested individually for the axillary bud multiplication, BAP was found superior to Kin. Albeit, among the different concentrations and the combinations of growth regulators used, a combination of BAP (2.0 mg $l^{-1}$) + IBA (0.5 mg $l^{-1}$) has shown to be more advantageous for the rapid shoot induction. Subsequent cultures showed an enhanced number of shoots. The nodal explants induced callus from the basal cut ends on all concentrations of BAP tested.

Callus induction was established from different explants such as root, stem (node and internode) and leaf cultured on MS medium supplemented with different growth regulators either alone or in combinations. The cytokinins used individually were also effective for callus induction from different explants especially from internodal and nodal segments.

An average of 55 shoots along with numerous meristemoids was developed from the callus induced from basal cut end of the nodal explants on MS medium containing BAP (1.5 mg $l^{-1}$). The callus developed from the internodal segments on MS+BAP (2.0 mg $l^{-1}$) upon transfer on fresh medium with the same concentration of BAP showed shoot regeneration. But the percentage of response was less compared to that from the nodal callus.

The callus cultured on MS medium with CW or BAP+NAA+AA+$AgNO_3$ induced tuberous roots.

The callus obtained on MS medium fortified with 2,4-D (1.0mg $l^{-1}$), upon transfer to half or quarter liquid MS with reduced levels of 2,4-D (0.01- 0.5 mg $l^{-1}$) induced somatic embryos of globular to torpedo stages. The embryos in $1/10^{th}$ MS hormone free medium promoted maturation. Upon transfer to solid $1/10^{th}$ MS hormone fee medium only 40 % of the embryos underwent conversion. Besides growth regulators, light/dark conditions and sucrose also influenced somatic embryogenesis. Dark and suspension cultures were found as superior to light and static cultures in terms of frequency of embryo induction and maturation. The optimum sucrose concentration was 1.5-3 %. The embryos passed through all the discernible stages of development with distinct epidermis.

The protoplasts isolated from mesophyll cells were cultured and showed plant regeneration *via* somatic embryogenesis.

The embryo-like shoot bud derived synseeds exhibited 90 per cent conversion and was found superior to the embryo-derived synseeds.

MS medium supplemented with $AgNO_3$ was efficient for the conservation of the germplasm *in vitro* than that by the synseeds.

Rooting of the excised shoots was best on MS medium fortified with IBA (0.5 mg $l^{-1}$) and the plantlets were successfully established *ex vitro*.

TLC analysis of the extracts of tuberous roots showed the presence of more compounds in *in vitro* developed roots compared to that of the field grown plants.

## Transformation studies

Co-cultivation of leaf explant of *Eryngium foetidum* and leaf and internode of *Holostemma ada-kodien* with *Agrobacterium rhizogenes* harbouring the binary plasmid **pGreen** either with ***gus*** or ***gfp*** construct induced hairy roots with tuft of white hairs. The control explants induced only 1–3 roots and without much root hairs. The transformed roots were selected by culturing them on MS basal medium supplemented with high amount of kanamycin (150 mg $l^{-1}$). Concentration of kanamycin above 25 mg $l^{-1}$ was found lethal to control roots.

The kanamycin resistant hairy root segments induced callus. The callus induced from the hairy roots developed on explants of *E. foetidum* infected with the plasmid harbouring ***gus*** construct developed shoots on MS+ Kin (1.0 mg $l^{-1}$) + BAP (0.5 mg $l^{-1}$). The developed shoots showed stunted growth with wrinkled leaves and were rosette in appearance. The callus induced from the hairy roots developed on explants of *H. ada-kodien* infected with the plasmid harbouring ***gus*** construct developed embryos in subsequent cultures. The embryos developed up to the late torpedo stage; but failed to undergo conversion. The regenerated transformed plants and leaves of *E. foetidum* and embryos of *H. ada-kodien* showed deep blue colour after incubation for an overnight of the same in X-gluc solution, which confirmed the transformed nature.

The callus induced from the hairy roots developed on explants of *E. foetidum* and *H. ada-kodien* infected with the plasmid harbouring ***gfp*** construct showed green fluorescence after 24 h exposure to UV light, also confirmed the transformation. However no transformed plants were obtained with the expression of ***gfp*** gene.

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*** Originals not seen**