RAPID PROPAGATION AND CONSERVATION OF SELECTED LEGUMINOUS MEDICINAL PLANTS USING *IN VITRO* TECHNIQUES

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DECLARATION

I hereby declare that this thesis entitled **"Rapid propagation and conservation of selected leguminous medicinal plants using *in vitro* techniques"** is a bonafide record of research work done by me during the course of my research and that the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title of any other university or society.

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CERTIFICATE

Certified that this thesis entitled **"Rapid propagation and conservation of selected leguminous medicinal plants using *in vitro* techniques"** is a record of research work done independently by Ms. Deepa S. Nair under my guidance and supervision and that it has not previously formed the basis for the award of any degree, fellowship or associateship to her.

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Deepa

Deepa S. Nair

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LIST OF ABBREVATIONS

2,4-D	2,4- dicholorophenoxyacetic acid
2 iP	2-isopentenyladenine
ABA	abscisic acid
AC	activated charcoal
AdS	adenine sulphate
B5 medium	Gamborg (1968)
BA	N^6-benzyl adenine
CH	casein hydrolysate
CI	callus index
cm	centimeter
CTAB	cetyltrimethylammonium bromide
CW	coconut water
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTPS	deoxynucleotides
EDTA	ethylenediaminotetraacetic acid
g	gram
G	growth score
GA	gibberellic acid
Gl	glutamine
h	hour
H Cl	hydrochloric acid
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
IBPGR	International Board of Plant Genetic Resources

LIST OF ABBREVATIONS CONTINUED

Kn	kinetin (6- furfurylaminopurine)
LN	liquid nitrogen
mg	milligram
min	minute
mM	millimolar
MS	Murashige and Skoog (1962)
Na Cl	sodium chloride
Na OH	sodium hydroxide
NAA	α -naphthaleneacetic acid
nm	nanometer
°C	degree Celsius
PCR	polymerase chain reaction
PVP	polyvinylpyrrollidone
RAPD	random amplified polymorphic DNA
s	second
SDS	Sodiumdodecylsulphate
SH	Schenk and Hildebrandt (1972)
TBGRI	Tropical Botanical Garden and Research Institute
Tris HCl	tris(hydroxymethyl) aminomethanehydrochloride
UV	ultra violet
WPM	woody plant medium (Lloyd and Mc Cown, 1980)
µl	microlitre
µM	micromolar

Introduction

1. INTRODUCTION

India has a treasure of well-recorded and traditionally well practised knowledge on medicinal plants. More than 6000 plants are used in our traditional, folk and herbal systems of medicine. India is endowed with a rich genetic resource of medicinal plants and is rightly called the 'emporium of medicinal plants'.

The rich genetic resource of medicinal plants comprises seven per cent of the world's legume collection, consisting about 1150 taxa under 167 genera (Husain and Kapoor, 1990). The family Fabaceae contains many taxa of pharmaceutical importance. *Clitoria ternatea, Mucuna pruriens* and *Indigofera tinctoria* are important leguminous plants having immense medicinal value. The Plant Genetic Resources Conservation Unit, United States Department of Agriculture has identified them as potential source of useful phytochemicals of pharmaceutical importance (Morris, 1999).

The root of *Clitoria ternatea* is regarded as a good brain tonic and is an ingredient of 'Medhyarasayana', an ayurvedic preparation that increases intelligence quotient (IQ) in children (Jessy, 1995). It is an important constituent of several other commercially manufactured brain tonics. The seeds of *Mucuna pruriens* are a valuable source of L-DOPA (L-3,4dihydroxyphenylalanine), a precursor of dopamine, used in the treatment of Parkinsons' disease world wide (Dymock and Warden, 1980). HP 200, a commercial product with L-dopa as the lead component is effectively used to treat Parkinson's disease (Parikh and Manyam, 1999). *Indigofera tinctoria* is reputed for its property of promoting growth and colour of hair and forms a major ingredient of ayurvedic preparations like 'Nilibhringadi'. Root of the plant has antitoxic property and is used as a good remedy for poisons (Simon *et al.*, 1984). Moreover, it has been found to contain indirubin which is useful for the treatment of chronic myelocytic leukemia (Han, 1994).

These plants bear seeds profusely, which are utilized for propagation and conservation. However, the seeds lose their viability in a short span of time under ambient storage conditions. Seeds being orthodox in nature, can withstand low moisture content and hence long term storage is possible by modern methods such as cryopreservation. However, cryopreservation can be resorted for maintaining only 'base collection' in seed banks. Germplasm conservation calls in for germplasm exchange programmes where seeds are not a preferred entity as there is risk of seed borne pests and pathogens. To overcome the quarantine problems, *in vitro* grown plantlets/cultures are preferred for germplasm exchange.

Biotechnology offers an array of techniques for rapid propagation and conservation of medicinal plants. *In vitro* tissue culture methodology is envisaged as a means for germplasm conservation to ensure the survival of endangered plant species, rapid mass propagation for large-scale revegetation and for genetic manipulation studies (Fay, 1992). The main commercial application of tissue culture technology so far has been in the production of clonal plants at a very rapid rate, compared to conventional methods.

Standardization of *in vitro* propagation system is a pre requisite for *in vitro* conservation. Plantlets have to be maintained without frequent subculturing to maintain genetic stability which is possible by slow growth technique. Slow growth provides for short to medium term storage. Species vary in length of time during which their cultures can be stored. Cryopreservation of meristems, somatic embryos and embryonic axis is a single solution to this problem. Cryopreservation at the temperature of liquid nitrogen (-196°C) offers the possibility for long term storage with maximal phenotypic and genotypic stability (Steponkus, 1985). This method being relatively convenient and economical, large number of genotypes and variants could be conserved and thus maximize the potential for storage of genetically desirable material.

A proper *in vitro* plant regeneration system is foremost and inevitable requisite for successful *in vitro* conservation scheme. *In vitro* propagation protocols provide for large scale multiplication of elite and pathogen free plants. These can be exploited for other biotechnological interventions *viz.,* transformation, protoplast fusion and hybridization.

In this context, it becomes relevant to evolve protocols for rapid propagation and conservation of these medicinally valuable species. The present study 'Rapid propagation and conservation of selected leguminous medicinal plants using *in vitro* techniques' was undertaken with the following objectives:

(i) Standardization of *in vitro* techniques for rapid propagation and conservation of *Clitoria ternatea* L., *Mucuna pruriens* Baker non DC. and *Indigofera tinctoria* L.

(ii) Standardization of culture medium and protocol for short term and medium term conservation

(iii) Attempt on long term conservation of seeds and shoot meristem *via* cryopreservation technique.

Review of Literature

2. REVIEW OF LITERATURE

Plants play a dominant role in the introduction of new therapeutic agents. Drugs from higher plants continue to occupy an important niche in modern medicine (Dev, 1997). Many compounds used in modern medicine have a complex structure. Synthesizing these bioactive chemical compounds at a low cost is not that easy (Shimomura *et al.*, 1997). Pharmaceutical companies depend largely upon materials procured from naturally occurring stands that are being rapidly depleted.

The study 'Rapid propagation and conservation of selected leguminous medicinal plants using *in vitro* techniques', comprised standardization of protocol for rapid propagation and conservation of selected leguminous medicinal plants *viz.* *Clitoria ternatea* L., *Mucuna pruriens* Baker non DC. and *Indigofera tinctoria* L. using *in vitro* techniques.

Clitoria ternatea, Mucuna pruriens and *Indigofera tinctoria* are very valuable plants from the medicinal point of view. These plants form an ingredient of many ayurvedic formulations. Also they are utilized in several allopathic drugs as an important source of bioactive compounds having therapeutic property against several human ailments.

In this chapter, literature on *in vitro* propagation and conservation of medicinal plants has been reviewed. Wherever sufficient literature on medicinal plants is lacking, literature on other plants has been reviewed.

2.1 *IN VITRO* PROPAGATION

In vitro propagation is used widely for the commercial propagation of a large number of plant species, including many medicinal plants (Rout *et al.*, 2000). Legumes are one of the most important groups of crop plants and have been the

subject of efforts to improve desirable traits including *in vitro* culture response. Since legumes are notoriously recalcitrant to regenerate from tissue culture, much effort has been devoted to developing and optimizing efficient *in vitro* regeneration systems to facilitate a variety of technologies (Polanco and Ruiz, 1997). Successful regeneration of legume species has been greatly aided by species-specific determination of critical parameters, such as explant source, genotype and media constituents (Parrott *et al.*, 1992)

Chattopadhayay *et al.* (1995) opined that conventional seed legume breeding programmes can be improved and complemented with *in vitro* genetic manipulation methods if an efficient plant regeneration system is available

According to Murashige (1974) there are three possible routes of *in vitro* propagule multiplication *viz.*, enhanced release of axillary buds, production of adventitious shoots through organogenesis and somatic embryogenesis.

2.1.1 Enhanced Release of Axillary Buds

Axillary shoot proliferation has been the most simple and reliable route for the production of elite clonal plants in many medicinal plants (Dias *et al.*, 2002). Axillary shoot growth is stimulated by overcoming apical meristem dominance (Boxus, 1999).

Preservation of genetic stability in germplasm collections and micropropagation of elite plants is of the utmost importance and propagation of plants through apical or axillary meristem culture allows recovery of genetically stable and true to type progeny (Hu and Wang, 1983; George and Sherrington, 1984).

The shoot proliferation is influenced by age of donor seedling, explant type, culture media, growth regulators and culture conditions.

2.1.1.1 Age of Donor Seedling and Explant Type

The success of any *in vitro* propagation system depends on the right choice of the explant. It depends on the kind of culture to be initiated, the purpose of the proposed culture and the plant species to be used. Orientation of explant also influences axillary shoot proliferation.

Cotyledonary node explants have been found to regenerate shoots in grain legumes, *Glycine max* (Wright *et al.*, 1986), *Phaseolus vulgaris* (Franklin *et al.*, 1991), *Vicia faba* (Selva *et al.*, 1989) , *Pisum sativum* (Jackson and Hobbs, 1990) and *Vigna radiata* (Mahalaxmi *et al.*, 2003)

Gulati and Jaiwal (1994) observed maximum shoot regeneration from explants with both the cotyledons attached to the embryonic axis excised from four day old seedling. Shoot development from cotyledon explant from two day old seedlings of *Glycine max* was studied by Srinivas *et al.* (2000)

Explants, cotyledonary node and *in vitro* developed nodal segments were utilized for mass multiplication of shoots by Dalal and Rai (2004) in *Oroxylum indicum.*

Chattopadhayay *et al.* (1995) opined that multiple shoot regeneration varied with respect to different explants and seedling age in *Mucuna pruriens.* Hypocotyl explants with cotyledons from six to seven day old seedlings gave maximum shoot regeneration compared to epicotyl and hypocotyl explants. Naomita and Rai (2000) studied shoot regeneration from hypocotyls, cotyledons and cotyledonary explants of *Crotalaria lutescens.* These explants exhibited decreasing series in terms of frequency of shoot regeneration.

Nodal explants induced maximum number of shoots in *Aristolochia indica* (Manjula *et al.*, 1997). Shahzad and Siddiqui (2000) achieved multiple shoot regeneration from nodal explant of *Ocimum sanctum.* Nodal explants produced prolific multiple shoot in *Wedelia calendulaceae* (Emmanuel *et al.*, 2000), *Bacopa*

monniera (Tiwari *et al.*, 2000), *Atropa acuminata* (Ahuja *et al.*, 2002), *Baliospermum montanum* (Johnson and Manickam, 2003) and *Baliospermum axillare* (Singh and Sudarshana, 2003).

Lal *et al.* (1988) developed a procedure for clonal multiplication of *Piccorhiza kurroa* through shoot tip culture. Shoot tips were utilized for multiple shoot induction in *Clitoria ternatea* (Kumar *et al.*, 1993), *Vigna mungo* (Das *et al.*, 2002) and *Centella asiatica* (Nath and Buragohain, 2003)

2.1.1.2 Culture Media

Growth and morphogenesis of plant tissues *in vitro* are largely governed by the composition of the culture media. The choice of a particular medium is dictated by the purpose and the plant species or variety to be cultured (Wang and Charles, 1991). The main components of most plant tissue culture media are mineral salts, sugar as carbon source and water. Other components may include organic supplements, growth regulators and a gelling agent, (Gamborg *et al.*, 1968; Gamborg and Phillips, 1995).

A wide variety of media has been devised for *in vitro* culture of plant tissues and organs. White's media is one of the earliest plant tissue culture media formulated. Since 1960 most researchers have been using MS (Murashige and Skoog, 1962), B5 (Gamborg *et al.*, 1968) or SH (Schenk and Hildebrant, 1972) media. MS medium is characterized by high salt concentration. Later woody plant medium (WPM) (Lloyd and Mc Cown, 1980) was developed for *in vitro* culture of woody plants.

Murashige and Skoog medium is the most popular media for *in vitro* propagation of medicinal plants. In medicinal plants *in vitro* plantlet regeneration from nodal explants in MS medium was reported in several species *viz.*, *Aristolochia indica* (Manjula *et al*, 1997), *Atropa acuminate* (Ahuja *et al.*, 2002), *Hemidesmus indicus* (Saha *et al.*, 2003), *Leptadenia reticulata* (Arya *et al.*, 2003),*Baliospermum*

montanum (Johnson and Manickam, 2003), *Wedelia chinensis* (Martin *et al.*, 2003) and *Baliospermum axillare* (Singh and Sudarshana, 2003).

Catapan *et al.* (2001) reported that MS medium was the best for shoot initiation followed by B5, SH, and WPM in *Phyllanthus stipulatus*

Kumar *et al.* (1993) utilized B5 basal media for multiple shoot regeneration in *Clitoria ternatea.* Micropropagation of *Wedelia calendulacea* was carried out using nodal explants on MS medium containing B5 vitamins (Emmanuel *et al.*, 2000). Mechanda *et al.*, (2003) obtained plantlet regeneration on WPM medium in *Echinacea purpurea* plants.

Axillary bud release in *Adhatoda beddomi* was induced on SH medium by Sudha and Seeni (1994).

MS media has been found most suitable medium for shoot proliferation in grain legume, soybean (Saka *et al.*, 1980) and chickpea (Arockiasamy *et al.*, 2000). Selva *et al.* (1989) referred half strength MS medium gave profuse shoot proliferation in *Vicia faba.*

2.1.1.3 Plant Growth Regulators

The growth and morphogenesis *in vitro* are regulated by the interaction and balance between the growth regulators supplied to the medium and the growth substance produced endogenously by the cultured cell. Selection and addition of growth regulators at the optimum level are crucial for successful plant tissue culture.

In axillary shoot proliferation, cytokinin is utilized to overcome apical dominance of shoots and to enhance the branching of lateral buds from each axils (Wang and Charles, 1991). Martin *et al.* (2003) opined that BA has been the most favourable cytokinin for initiation and multiplication of axillary buds. The potentiality of BA over other cytokinin has been well established in medicinal plants (Reddy *et al.*, 1998).

The highest number of shoots per explant was observed on MS medium supplemented with 2.22 µM BA (Johnson and Manickam, 2003). High frequency shoot formation was induced in nodal segments of *Salvadora persica* in MS medium supplemented with BA 4 mg l^{-1}. But the study indicated decline in the number of shoot buds produced per explant in response to higher concentration of BA (Batra *et al.*, 2001).

According to Borthakur *et al.* (1999), multiple shoots obtained with kinetin was inferior to BA with respect to shoot number, shoot length and leaf size in *Houttuynia cordata.* While, promising response due to the combined effect of BA and Kinetin was reported in *Withania somnifera* (Deka *et al.*, 1999) and *Rauvolfia tetraphylla* (Sarma *et al.,* 1999). In contrast to these, Martin *et al.*, 2003 found that addition of kinetin with optimum level of BA did not enhance shoot proliferation in *Wedelia chinensis.*

Auxins alone did not induce any positive impact on shoot multiplication. Synergistic effect of BA and auxins in multiplication and growth of shoots have been well documented in medicinal plants (Sreekumar *et al.*, 2000 and Martin, 2002). IAA along with BA enhanced multiple shoot formation in *Bacopa monnieri* (Yelne *et al.*, 1997) and *Cardiospermum halicacabum* (Jayasheelan and Rao, 1998). Singh and Sudarshana (2003) observed maximum number of shoots per explant on MS medium supplemented with 2 mg l^{-1} BA and 1 mg l^{-1} IBA in *Baliospermum axillare.* The presence of auxins in the culture medium has been shown to improve culture growth (Patnaik and Debata, 1996). However, inhibitory effect on addition of NAA to BA has been reported in *Cedrela fissilis* (Nunes Ed *et al.*, 2002).

Bud break was best when nodes cultured on MS medium were supplemented with 2.6 µM NAA and 4.4 µM BA in *Uraria picta* (Anand *et al.*, 1998). Saha *et al.* (2003) reported that early bud break and maximum shoot regeneration was obtained with BA 2 mgl^{-1} and NAA 0.1mg l^{-1} in *Hemidesmus indicus*

According to Singh and Sudrashana (2003), higher concentration of auxin and cytokinin led to a decrease in shoot number per explant and shoot length due to basal callusing in *Baliospermum axillare*. Pawar *et al.* (2001) opined that higher concentrations of BA supported callus formation in *Withania somnifera*.

Gibberellic acid is a growth regulator which promotes shoot elongation. Addition of GA along with BA was found to be beneficial for the elongation of shoots in *Murraya koenigii* (Bhuyan *et al.*, 1997) and in *Vitex negundo* (Sahoo and Chand, 1998).

2.1.1.4 Culture Conditions

The pH of the culture medium affects the growth and differentiation of tissues in cultures. Plant cells in culture require an acidic pH of 5.5 to 5.8 (Gamborg and Shyluk, 1981).

Murashige (1977) observed that light intensity, light quality and duration affect the growth of *in vitro* grown cultures. He found that optimum light intensity for shoot formation in large number of herbaceous species to be around 1000 lux. The optimum day length was considered to be 16 hours for a wide range of plants.

Yeoman (1986) reported that the usual environment temperature of species should be taken into account for its better performance under *in vitro* conditions. However, most of *in vitro* cultures are grown successfully around $25 \pm 2^{\circ}C$

Air humidity under culture room conditions is most frequently at 70 per cent (Hu and Wang, 1983). Relative humidity is an important factor in hardening and planting out of *in vitro* raised plants.

Maintanence of cultures of *Crotalaria lutescens* under 16 hour light and 8 hour dark cycle was reported by Naomita and Rai (2000).

Cultures were maintained at 24 ± 2°C with a relative humidity of 55 ± 5 per cent for 16 hour photo period at a photon flux density of 15-20 μE $m^{-2}s^{-1}$ in *Spilanthes acmella* (Saritha *et al.*, 2003)

2.1.2 Somatic Organogenesis

Somatic organogenesis may be direct or callus mediated. In direct organogenesis, shoots arise directly from tissues of explant and not from previously formed callus. Indirect somatic organogenesis requires redifferentiation of the differentiated cells leading to callus formation (Ignacimuthu, 2001)

Skoog and Miller (1957) proposed that organ differentiation in plants is regulated by interplay of auxins and cytokinins. Generally, a high concentration of auxin and a low cytokinin promotes abundant cell proliferation with the formation of callus. On the other hand, low auxin and high cytokinin levels in the medium result in the induction of shoot morphogenesis.

The standardization of *in vitro* plant regeneration protocols with intervening callus phase would facilitate mass scale propagation and germplasm conservation *in vitro*. The protocol could also be exploited for generating new genetic variability by somatic hybridization through protoplast fusion as genetic variability has been associated with callus mediated plant regeneration. (Evans *et al.*, 1981 and Arcioni *et al.*, 2001).

2.1.2.1 Explant

Callus can be induced from a variety of explants. Callus formation from explant tissue involves the development of progressively more random plans of cell division, less frequent specialization of cells and loss of organized structures (Thorpe, 1980)

Qurashi *et al.* (1996) suggested that young leaves are the best source of explant for callogenesis in *Cleistanthus collinus*.

Rapid plant regeneration from leaf and stem explant callus was achieved in *Clitoria ternatea* (Kumar *et al.*, 1993), *Centella asiatica* (Patra *et al.*, 1998) and in *Plumbago zeylanica* (Rout *et al.*, 1999).

Roja *et al.*, (1991) reported callus formation from axillary meristem explants in *Withania somnifera* and Anand *et al.*, (1998) from nodal segments in *Uraria picta.* Callus was induced from shoot tips in *Rosmarinus officinalis* (Misra, 2002).

Chattopadhyay *et al.,* (1995) developed a micro propagation system *via* callus using seedling explants, epicotyls, hypocotyls and hypocotyls with cotyledon in *Mucuna pruriens.*

2.1.2.2 Plant Growth Regulators

A high concentration of auxin to cytokinin favours callus formation. However, shoot bud induction from callus is a function of cytokinin activity and relatively high rate of cytokinin to auxin is mandatory in shoot initiation from the callus.

Mukhopadhyay and Ram (1981) referred that the induction of morphogenesis in the callus depends not only on the final regeneration medium but also on the hormonal combination of the initial callus induction medium.

Green globular calli was induced from the leaf segment of *Centella asiatica* in MS medium supplemented with 2 mg l^{-1} Kn and 4 mgl^{-1} NAA. The best results for shoot formation was obtained when it was transferred to regeneration medium augmented with BA, Kn, NAA and adenine sulphate (Patra *et al.*, 1998).

Rani and Grover (1999) observed that axillary leaf explant gave callus proliferation on medium supplemented with 2mg l^{-1} 2, 4-D and 0.2 mg l^{-1} kinetin in *Withania somnifera*. The calli showed maximum shoot multiplication when BA at 2 mg l^{-1} was used.

According to Chattopadhyay *et al.* (1995), multiple shoot regeneration occurred following an initial callus growth on revised Tobacco Medium supplemented with 2.7 µM NAA and 9.8 µ M 2iP using seedling explants in *Mucuna pruriens*.

Optimum callus induction from leaf explants of *Albissia amara* was obtained on MS medium fortified with 3.5 mg l^{-1} NAA and 0.5 mg l^{-1} BA. Regeneration of plantlet was maximum when transferred to medium with higher concentration of BA 1.5 mg l^{-1} and same level of NAA. (Ramamurthy and Savithramma, 2003)

2.1.3 Somatic Embryogenesis

Somatic embryogenesis is a process in which single cell or small group of cells initiate the developmental pathway normally followed only by the pre-dominant embryo within the seed (William, 1987)

Somatic embryogenesis is a non sexual developmental process which produces bipolar embryo-like structures from somatic cells. (Haccius, 1978). It provides for unlimited production of clones with functional shoot, root and the development of the manufactured seed (Redenbaugh *et al.*, 1986).

Sharp *et al.* (1982) reviewed somatic embryogenesis as direct or indirect. Direct embryogenesis proceeds from pre embryogenic determined cells (PEDCs) wherein the embryogenic pathway is predetermined and the cells need only the synthesis of an inducer to express its potential. Indirect embryogenesis, on the other hand, initiates from induced embryogenic determined cells (IEDCs) which require an

induction treatment to the embryogenic state by exposure to specific growth regulators. Once the embryogenic state is reached both cell types proliferate in the same manner.

Somatic embryogenesis in addition to clonal plant multiplication (Jain *et al.*, 1995) favours regeneration of protoplast (Vasil and Vasil, 1980), artificial seed production (Dupuis *et al.*, 1999), germplasm conservation (Zhou *et al.*, 2000 and regeneration of somaclonal variants and metabolite production (Razdan, 2003).

2.1.3.1 Explant

Embryogenic callus were obtained from leaf disc explants in *Thevetia peruviana* (Kumar, 1992), *Medicago sativa* (Meijer and Brown, 1987) and *Clitoria ternatea* (Malabadi and Nataraja 2002).

Embryogenesis from hypocotyl explants are well documented in medicinal plants such as *Ammi majus* (Grewal *et al.*, 1976) and *Trachyspermum ammi* (Jasrai *et al.*, 1992).

Deb (2001) reported somatic embryogenesis and plant regeneration from cotyledonary segments in *Melia azedarach.* Rout *et al.* (1995) observed that in *Acacia catechu,* somatic embryos were formed from cotyledon derived callus.

In *Arachis hypogea*, mature zygotic embryos were cultured for somatic embryogenesis (Gowda *et al.*, 2001) while in *Commiphora wightii*, Kumar *et al.* (2003) found that immature zygotic embryos were the only suitable explant to produce embryonic callus.

Somatic embryogenesis has been reported in cultures initiated from shoot apex in grain legume, *Pisum sativum* (Kysely and Jacobson, 1990)

2.1.3.2 Plant Growth Regulators

According to Hussey (1986), level of growth regulators in the culture medium, particularly lower levels of auxin was ideal for embryo formation. Ammirato (1983) refers the requirement of auxin medium for induction of callus and medium devoid of growth regulators for embryogenesis.

Somatic embryogenesis depended on the type of plant growth regulators in the callus-inducing medium (Beena and Martin, 2003).

Embryo yields and embryo conversion to plantlets were strongly dependant on the 2, 4-D and kinetin concentrations in the induction medium in *Medicago sativa* (Meijer and Brown, 1987)

Ishi *et al.*, (1998) opined that NAA and 2, 4-D are effective growth regulators for the induction of somatic embryogeneis. Barna and Wakulu (1993) found that 2,4-D 5.2 mg l^{-1} was the best for induction of embryogenic callus and embryo initiation from immature leaflets of *Cicer arietinum* and that the addition of Kn or BA to induction medium reduced embryo formation.

The frequency of embryogenic callus formation increased with an increasing concentration of 2, 4-D up to 4.52 μM, but sharply declined at higher concentration of 13.6 μM in *Chelidonium majus* (Kim *et al.*, 1999)

In contrast to this, Mariotti and Arcioni(1983) and Eapen and George (1989), reported the embryogenic potential of callus subjected to higher levels of cytokinins and low levels of auxin in *Coronilia varia* and *Eleucine coracana*, respectively. Higher cytokinin levels counteract residual auxin effects to prevent inhibition of somatic embryogenesis (Wilson *et al.*, 1996) Cytokinins seem to play a key role in embryo induction, proliferation and differentiation (Schuller *et al.*, 2000).

Kumar *et al.* (2003) recorded optimum growth of embryogenic callus on medium supplemented with BA 0.5 mg l^{-1} and IBA 0.1 mg l^{-1} in zygotic embryo explants of *Commiphora wightii*

Ignacimuthu *et al.* (1999) reported that embryogenic callus produced in leaf explants of *Eryngium foetidum* on LS (Linsmaier and Skoog, 1965) medium augmented with 2,4-D 1 mg l^{-1} and BA 1 mg l^{-1}, which when transferred to MS medium supplemented with 2,4-D 0.1 mg l^{-1}, BA 2 mg $l^{-1}$and GA 1 mg l^{-1} induced somatic embryos.

In *Clitoria ternatea*, IAA 5.71 µM in MS basal medium initiated embryogenic callus in leaf explant, while induction of embryo occurred when cultured in the same medium supplemented with Kn 9.3 µM and 5 per cent (v/v) coconut water (Malabadi and Nataraja , 2002). According to Steward and Shantaz (1959), coconut water is very effective in embryo induction and maturation.

According to Chiang and Hsing (1980) addition of GA and cytokinin to the medium stimulates formation of shoots from the embryoids. In *Eryngium foetidum*, conversion of somatic embryos to plantlets occurred on MS medium supplemented with GA 1 mgl^{-1} and BA 0.1 mgl^{-1} (Ignacimuthu *et al.*, 1999).

Arillaga *et al.*, (1987) observed that when cotyledonary embryos of *Digitalis obscura,* when transferred to MS basal medium devoid of growth regulators, regenerated into complete plantlets. Cotyledon derived somatic embryos of *Cajanus cajan* germinated in half strength MS medium (Verma and Chand, 2001)

2.1.4 Rooting

2.1.4.1 In vitro Rooting

The plantlets produced *in vitro* should have a strong and functional root system. Roots are mostly induced in presence of a suitable auxin (Razdan, 2003). IAA, IBA and NAA were found to initiate rooting in plants.

The promotory effect of reducing salt concentrations of MS media on *in vitro* rooting of shoots has been described Upreti and Dhar (1996). According to Martin *et al.* (2003), half strength hormone free medium was superior to full strength for root induction in *Wedelia chinensis.*

In *Vigna radiata*, 1μM IAA in MS basal medium was found best for inducing thick roots with numerous lateral roots (Gulati and Jaiwal, 1994)

Efficacy of IBA at lower concentrations in *in vitro* rooting has been reported in medicinal plants, *Swainsonia salsula* (Yang *et al.*, 2001), *Plumbago* spp. (Das and Rout, 2002) and *Piper longum* (Bhuyan *et al.*, 2000). Saha *et al.* (2003) found that half strength MS with IBA 1.5 mg l^{-1} was best for inducing maximum number of roots in *Hemidesmus indicus.* NAA was found inferior and favoured callus formation at the base.

Rhizogenesis in *Acorus calamus* was inhibited in the presence of high concentrations of NAA and produced short stumpy roots (Anu *et al.*, 2001). In contrast to this, NAA favoured rooting compared to other auxins in *Clitoria ternatea* (Kumar *et al.*, 1993) and *Mucuna pruriens* (Chattppadhyay *et al.*, 1995). Chauhan *et al.* (2003) found that NAA 10.4 μM in quarter strength MS resulted maximum rooting frequency in *Cicer arietinum.*

2.1.4.2 Ex vitro Rooting

The major cost of *in vitro* plant production lies in the rooting and hardening stages. The process of rooting *in vitro* has been estimated to account for approximately 35 to 75 per cent of the total cost of micro-propagation. *Ex vitro* rooting is preferred in many crops to with a view to save time and resources (Maene and Debergh, 1983).

For rooting under *ex vitro* conditions, the shoots for rooting should be handled as microcuttings without using aseptic conditions. Treatment of micro cuttings with root inducing growth substance or commercial rooting powder may be necessary for rooting *ex vitro* (Driver and Suttle, 1987).

John (1996) observed *ex vitro* rooting to be better than *in vitro* rooting in *Holostemma annulare*. Cent percent survival of regenerated plantlets under *ex vitro* conditions was reported in *Wedelia chinensis* (Martin *et al.*, 2003).

2.1.4.3 Hardening and Planting Out

The benefit of any micropropagation system can be fully realized by the successful transfer of plantlets from tissue-culture vessels to the ambient conditions found *ex vitro*. Most species grown *in vitro* require an acclimatization process in order to ensure that sufficient number of plants survive and grow vigorously when transferred to soil (Hazarika, 2003).

Light, temperature and humidity are major factors to be controlled during acclimatization (Hu and Wang, 1983). Standardised rhizosphere environment is necessary for getting better growth of plants (Zimmerman and Fordham, 1985)

The retardation in development of cuticle, epicuticular waxes and functional stomatal apparatus during *in vitro* culture cause high stomatal and cuticular

transpiration rates of leaves in plantlets taken out of the culture vessels (Conner and Conner, 1984). A period of acclimatization is required for newly transferred plantlets to stabilize water status of the plant.

In *Aristolochia indica*, Manjula *et al.* (1997) observed 85-95 per cent survival under green house condition when rooted plantlets were transferred after two-stage hardening in liquid White's medium and vermiculite.

Rooted plantlets of *Celastrus paniculatus* when transferred to 2:1 sand: garden soil wetted with quarter strength WPM and acclimatised in mist chamber at 25°C and 80 per cent relative humidity for weeks gave a survival of 80 per cent (Sharada *et al.*, 2003).

2.2 *IN VITRO* CONSERVATION

Plant *in vitro* technology offers a potential solution to both the long term conservation and mass propagation (*in vitro* propagation) of these leguminous medicinal plants. *In vitro* conservation technologies includes slow growth/ reduced growth storage for short to medium term storage and cryopreservation, at liquid nitrogen temperature (-196 °C) for long term storage.

In vitro technology has a specialized and potentially important role to play in the conservation of biological diversity. *In vitro* conservation techniques vary according to the storage duration required (Engelmann, 1991). Short and medium term *in vitro* conservation can be achieved by inducing slow growth and long-term storage by cryopreservation.

In vitro conservation provides for saving in field space. In such systems, plants are withdrawn into a protected environment, where they are less likely to be damaged or killed by pathogens and predators. Individual genotypes could be multiplied rapidly when required and the collections can be maintained at a low cost.

In addition to these, genetic stocks would be in a suitable condition for international exchange.

2.2.1 Slow Growth

Slow growth provides for short to medium term storage. It is based on the manipulation of culture conditions/ culture media to allow the cultures to remain viable but with a slow growth rate. In this technique, culture deterioration can be detected visually and therefore loss of viability can be avoided.

In vitro cultures require periodic transfer to fresh media, increasing chances of contamination, resulting in complete loss of the material. Moreover regular subculturing is both expensive and labour intensive. In case of shoot cultures, there is tendency towards the development of juvenility with the number of subcultures (Hausman *et al.*, 1994).

To avoid periodic transfer or to increase the interval of subcultures, slow growth method is resorted to. Withers (1990) noted that success in slow growth has extended the subculturing interval to two years in some crops. Since the germplasm is conserved in a viable but slow-growing state, it could be grown into plants on immediate demand (Kartha, 1985)

Subjecting the cultures to reduced temperature, applying growth retardants, use of osmotically active additives, restricting nutrient supply, reduced oxygen tension, super cooling and reducing light intensity are various means of achieving slow growth (Staritsky *et al.*, 1986).

2.2.1.1 Explant

The type of explant as well as the physiological state is very important. There is a minimal size for the explants. Slow growth storage with organized cultures in the form of shoots gave better results compared to unorganized cultures in the form of

callus. Hensahw *et al.* (1980) employed shoot cultures to study the effect of growth retardants.

Hiraoka and Kodama (1984) revealed that over a period of less than one year, callus of secondary product synthesizing cultures suffer serious effects manifested by poor recovery, growth and erratic yields of secondary products. Shoots on the other hand are genetically more stable and have faster regeneration potential into plants than callus.

Westcott *et al.* (1977) stored nodal cuttings of potato for 12 months under 6 per cent mannitol and got a survival rate of 68 per cent.

The presence of root system increases the survival capacities, as observed by Kartha *et al.* (1981) with *Coffea arabica* plantlets.

2.2.1.2 Culture Vessel

The type of culture vessel can play a very important role. Test tubes containing 10-20 ml media are routinely used, which allows an increase in the number of replicates of each genotype and to limit the incidence of contamination.

Roca *et al.* (1984) indicated that, when storing cassava plant lets in 50 x 140 mm bottles instead of 25 x 150 mm tubes, the rate of shoot elongation in larger vessels doubled, leaf fall diminished and culture viability greatly increased. In addition, leaves and roots remained healthier in the large vessel than in small vessels.

Ng and Ng (1991) is also of the view that the plantlet storage duration can also be prolonged by increasing the volume of the culture medium and storing the plantlet in a bigger culture vessel. The closure of the vessel can influence the condition of stored cultures (Grout, 1990)

2.2.1.3 Culture Medium

A slow growth medium is formulated to allow the explant to develop and grow at a slow rate. It is usually accomplished by using a medium with lower salt

concentration like Whites Medium or Hellers medium, by reducing the strength of normal MS basal medium, by increasing or decreasing the sucrose concentration or by addition of osmotica, cryoprotective substances and growth retardants in culture medium. Medium composition influences plant survival during storage (Orlikowska, 1992). The positive effect of rooting medium containing auxins compared to proliferation medium containing cytokinins on survival per cent is well established in slow growth studies (Hansen and Kristiansen, 1997)

2.2.1.3.1 Reducing Mineral Salt Concentration

A normal culture medium would consist of a mineral salt mixture, such as that in MS salt mixture, sucrose 2 to 3 per cent, vitamins and growth hormones. Reducing the concentration of mineral salt in the medium will retard the growth of cultures (Ng and Ng, 1991).

In slow growth studies with *Janakia arayalpathra*, half strength MS medium could support better survival at 27°C and with *Geophila reniformis*, use of half strength medium was the best at 20°C and 27°C with or without the addition of osmotica (TBGRI, 1997)

2.2.1.3.2 Addition of Osmotica

Growth limitation caused by osmotic concentration is due to reduction of water and nutrients absorbed from the medium. At high concentration, sucrose acts osmotically and it is highly metabolized. Non metabolized osmotics, such as mannitol and sorbitol are more effective than sucrose in culture growth limitation.

According to Schenk and Hildebrandt (1972), high level of sucrose can be used to maintain cultures in dormant conditions for longer periods as sucrose concentration above 4 to 5 per cent had an inhibitory but non-toxic effect on plant cell growth.

The effect of sucrose on the growth of tomato and carnation plantlets were studied by Schnapp and Preece (1986). The medium that gave maximum growth reduction contained 5 per cent sucrose in both tomato and carnation plantlets.

El-gizaway and Ford-Lloyd (1987) investigated the effect of sucrose at two temperature regimes. They found that a survival rate of 72 per cent was achieved at 4°C and 36 per cent at 26°C with 10 per cent sucrose level in *Allium sativum.*

Meristem cultures of *Beta vulgaris* could be stored with 10 per cent sucrose in the medium at 4 to 5°C (Ng and Ng, 1991). MS medium containing 7 per cent sucrose was better for reduced growth and high survival rate of *Holostemma adakodien* (TBGRI, 1997).

Lizarraga *et al.* (1992) studied the effect of sorbitol on the growth of sweet potato *in vitro*. Growth reduction was obtained with 3 per cent for sweet potato. Some enhancement in growth was seen with sweet potato at lower concentration of sorbitol. Same effect was observed in cassava cultures by Roca *et al.* (1984).

With taro, 3 per cent sorbitol was effective in reducing growth at 20°C and light conditions (George and Sherrington, 1984). Growth reduction at higher levels of sorbitol included plant height, node number and leaf size and conversely, root growth seemed to be enhanced in presence of high concentration of sorbitol.

Henshaw *et al.* (1980) stored potato varieties for a period of one year under 3 per cent and 6 per cent mannitol. The growth of potato shoots at 25°C is reduced by the addition of 4 per cent mannitol (Espinoza *et al.*, 1986)

Lizarraga *et al.* (1992) studied the effect of mannitol (0.2-1 per cent) on cultures of *Ipomoea batatus.* At these concentrations mannitol had little effect in reducing growth. It enhanced the growth in all treatments; plant height and node number exceeded that of control. However, long term use of 1 per cent mannitol resulted in 'abnormal growth' characterized by small leaves and short internodes. Roca *et al.* (1982) observed the deterioration of cassava shoots in presence of even 0.1 per cent mannitol with a storage temperature lower than 20°C

The work by Bessembinder *et al.* (1993) showed that the addition of mannitol to the storage medium had a positive effect on survival and growth when the temperature regime was 28°C in *Colocasia esculenta.* In this study mannitol significantly suppressed growth under light conditions but shoots retained a healthy green colour indicating mannitol had a positive effect on survival.

Xanthosoma sagittifolium could be stored for three years on a medium with 3 per cent mannitol without losses (Zandavoort *et al.*, 1994)

2.2.1.3.3 Addition of Cryoprotectants

According to Engelmann (1991), addition of cryoprotective substances to the culture medium could retard the growth of cultures. Exogenously supplied proline has a cryoprotective function (Withers and King, 1979; Van Swaaji *et al.*, 1985; Duncan and Widholm, 1987; Songstad *et al.*, 1990; Santarius, 1992) in higher plant cells.

2.2.1.3.4 Addition of Antioxidants

Phenolic substances and its oxidation products are known to inhibit enzyme activities (Andersen and Sowers, 1968) and to adversely affect plant tissue culture (Gupta *et al.*, 1980). PVP (polyvinylpyrrolidone) was used to promote tissue culture and embryogenesis by removing phenolic substances so to reduce tissue browning (Gupta *et al.*, 1980; Tyagi *et al.*, 1981; Zhong *et al.*, 1995). The effect of this antioxidant on slow growth of garlic was studied by Wasswa (1991).In garlic, on

addition of PVP (6, 8, 10 and 12 per cent) to the culture medium, initial establishment obtained was 75-100 per cent. There was no significant effect on plant growth with increasing concentration of PVP.

2.2.1.3.5 Addition of Growth Retardants

Westcott (1981) and Roca *et al.* (1982) used abscisic acid in order to reduce the growth of shoots of potato. However, these authors indicate that ABA is detrimental to some varieties. Negash (1990) investigated the effect of ABA on garlic. He found that a higher concentration of 15 mg l^{-1} gave better results in terms of survival and reduced growth.

Abscisic acid was effective in inhibiting the growth of sweet potato plants in culture at a concentration of 10 mg l^{-1} (Jarret and Gawel, 1991). Efficacy of growth suppression varied with genotype. In this study, explants showed little or no extension and development at concentration equal to or greater than 1 mgl^{-1}. At these concentrations explants remained green and viable and on culture to medium lacking in ABA normal growth was resumed.

George and Sherrington (1984) observed that ABA induces dormancy in plant meristems. In a study on *Solanum tuberosum* by Powell and Caligari (1989), 5 mg l^{-1} ABA was found most effective in reducing growth.

2.2.2 Cryopreservation

Cryopreservation offers the only safe and cost-effective option for the long term conservation of genetic resources. This technique involves suspension of metabolic activities of a specimen by subjecting to ultra-low temperature (-150 to -196°C). Conventionally LN is used as the coolant to achieve the temperature of –196°C in liquid phase and –150°C in vapour phase.

As only very small pieces of biological material can survive the freezing stress, plant parts used for cryopreservation include axillary buds, pollen grains,

pieces of callus and aliquots of cell suspension cultures. Before immersion and storage in liquid nitrogen, the explants need preparation and pre-conditioning to withstand desiccation and freezing at ultra-low temperature. Many biological materials tolerate extensive desiccation, such as some seeds and pollen, which are not injured when dried and then put directly in LN (Styles *et al.*, 1982; Akihama and Omura, 1986).

Cryopreservation is a reliable method for long term storage of the germplasm of endangered species (Bramwell, 1990). Several medicinal plant species have been successfully cryopreserved (Bajaj, 1995; Naik, 1998).

2.2.2.1 Cryopreservation of Seeds

Germplasm of many plants are stored in the form of seeds. Seeds are grouped according to their desiccation response. Orthodox seeds retain their viability even after drying to very low moisture content (4-7 per cent water on fresh weight basis), where as recalcitrant seeds lose viability after drying below relatively higher water content (20 to 30 per cent water).

Orthodox seeds in the dry state are easily preserved at low temperatures (Towill, 1991). According to Cromarty *et al.* (1982), temperature for storing seeds range from 10°C to -20°C. When dried many seeds are stable for long periods of time under these conditions, but seed deterioration in storage does occur, depending on species, temperature and moisture level. A technique which may overcome this problem is cryostorage in liquid nitrogen. At this temperature, biological processes are brought to a halt and it is presumed that the deterioration which can occur in seeds at higher temperatures will not take place, making very long term storage possible (IBPGR, 1982)

Stanwood *et al.* (1986) referred cryopreservation of orthodox seeds as an economic and reliable technique, an alternative to widely used electrical refrigeration

procedures involving storage at -20°C. Given a reliable electricity supply, the latter technique can be convenient because of the ready availability of suitable refrigeration equipment but, particularly with large diverse collections, the advantage may be outweighed in economic terms. The need for regular monitoring of seed viability and regeneration of the seed stocks as viability declines, can add very considerably to the costs.

The advantage of storing at liquid nitrogen temperature is that it is low enough to prevent damage to cells by ice-crystal growth during long term storage. Further as long as liquid nitrogen supply is guaranteed, the storage facilities that are required are relatively simple and economical in use. (Henshaw and Blakesley, 1996) suggested cryopreservation technique as highly reliable in the sense that at this temperature no physico-chemical changes occur, with the result that storage life theoretically is indefinite.

Stanwood (1980) opined that the development of simple cryostorage protocol for orthodox seeds has allowed cryopreservation of a large number of commercial species at low cost, significantly reducing seed deterioration in storage. With regular use of such storage systems for seeds of commercially important species, wild and endangered species can be preserved (Touchell and Dixon, 1994).

Decruse *et al.* (1999) reported the effect of cryostorage of seeds of medicinal plants, *Andrographis paniculata, Abrus precatorious, Coleus forskohlii, Dipteracanthus prostratus, Hemidesmus indicus, Embelia ribes, Ocimum gratissimum, Rauvolfia micrantha and Tylophora indica.* Seeds of these medicinal plants except that of *R. micrantha* and *E. ribes* when desiccated to 4-7 per cent moisture exhibited 80-95 per cent germination which was not affected significantly by one week storage in LN. The seeds of *R. micrantha* on desiccation to 5-7 per cent moisture level and cryopreservation showed 73 per cent germination comparable to that of the desiccation control. However, seeds of *E. ribes* when desiccated down to

4 per cent moisture, germinated well under ambient conditions but failed to germinate after storage in LN.

Iriondo *et al.* (1992) studied the influence of LN preservation of seeds of two legumes, *Onobrychis eriophora and Spartium junceum* under different conditions of seed moisture and different periods of LN exposure time in. In the former, desiccation of seed did not improve seed survival after LN storage. Germination per cent in desiccated seeds did not significantly differ from non-desiccated seeds. While in the later seed desiccation decreased germination per cent in LN stored as well as control seeds.

According to Stanwood and Bass (1981), moisture content is the single most important factor affecting the ability of germplasm to be stored in LN. Orthodox seed generally tolerate desiccation below 10 per cent moisture content and do not exhibit much reduction in germination after LN storage. A seed moisture content of 4-10 per cent has proved to be better for safe storage of several crop and wild species (Gonazalez-Benito *et al.*, 1995)

Vertucci and Roos (1993) opined that the optimum moisture content for storage at -150°C for peanut, soybean and pea ranged from 6.7-14 per cent. Chaudhury *et al.* (1991) is of view that seeds that are shed with high moisture content show a considerable decline in viability below a certain level of moisture and increased seed moisture content leads to a decline in the survival after LN storage.

Experiments subjecting seeds to LN exposure have shown that many species survive cryo storage if they are exposed in the dry state *i.e.*, moisture content ranging from 2.2 to 17.5 per cent (Stanwood , 1980)

Ospina *et al.* (2000) studied the effect of moisture content on *Passiflora ligularis* seed viability after immersion in liquid nitrogen. *P. ligularis* seeds exhibited

orthodox behaviour; the higher total viability moisture content values were around 8 per cent and showed a progressive decrease with lower moisture content values.

Wang *et al.*, (1994) collated information on the cryopreservation of orthodox tree seeds. They suggested that moisture contents between 3.8 and 11 per cent are safe for at least short periods of time (from 4 days to 3 years).

One possible cause of problems with LN seed storage is the requirement to optimize both the rate of cooling and the subsequent rate of warming; these must be determined empirically (Meryman and Williams, 1985). Pre-treatment with cryoprotectants sometimes improves seed survival in liquid nitrogen, but in certain circumstances it can be damaging too (Touchell and Dixon, 1993). Mechanical injury to seed by direct immersion into LN causing loss in viability has also been reported for some species (Wang *et al.*, 1994).

Mandal (2000) opined that although cryopreservation of orthodox seed is considered to be a straight forward method, real data on amenability of seeds of many species to freeze preservation are not available. Cryopreservation of seeds of many small seeded crop species is important, as it requires very little space and seeds can be conserved for indefinite periods. At NBPGR, New Delhi, a cryobank with 1243 accessions of various orthodox seed species of cereals, millets, pulses, vegetables, medicinal and aromatic plants has been established.

2.2.2.2 Cryopreservation of Embryo/ Embryonic Axis

Seeds of many species whose seeds are large to be frozen directly, embryos/ embryonic axes is a logical alternative (Pence, 1992). Moreover in wide-scale hybridization programmes, especially those dealing with intergeneric crosses, which are incompatible due to regeneration or abortion of the embryos, they can be dissected out at immature stages and employed (Meryman and Williams, 1985).

Cryopreservation of zygotic embryos has been reported to be successful for a number of tree species that show orthodox, intermediate, or recalcitrant seed storage behavior (Engelmann *et al.*, 1995). For successful cryopreservation, excised embryos or embryonic axes must survive desiccation below the threshold freezable moisture content of about 18 to 33 per cent (Hor *et al.*, 1990), below which value there is no freezable water for ice formation in ultra-low temperatures.

According to Meryman and William (1985), young and relatively small embryos are easy to manipulate and show higher survival rate than mature embryos on cryopreservation. In contrast to this Vertucci *et al.* (1991), observed that mature embryonic axes of *Landolphia kirkii* were more tolerant to cryopreservation than immature axes.

2.2.2.2.1 Cryopreservation by Simple Desiccation

Rapid desiccation of excised embryonic axes has been achieved in a laminar air flow cabinet in matter of few hours, the time taken depends on the axes size and maximum degree of desiccation tolerated or required. Fast drying may allow excised embryos to survive desiccation to lower moisture contents than those dried more slowly within intact seeds (Berjak *et al.*, 1984).Excised embryos or embryonic axes can be dried quickly in a laminar airflow cabinet at room temperature (Normah *et al.*, 1986).

After flash drying of embryonic axes extracted from mature seeds of *Landolphia kirkii,* where moisture content was reduced from 67 per cent to 23 per cent in 30 min, 90 per cent of the excised embryonic axes were able to germinate (Pammenter *et al.*, 1991). Fast drying with silica gel or with an aseptic air current has allowed excised embryonic axes to survive desiccation to a lower value.

Urugami *et al.* (1990) opined that in dehydration/drying method of cryopreservation, the induction or modification of drought tolerance may be the main

factor for successful cryopreservation. Successful cryopreservation by dehydration in a laminar air flow was obtained with embryonic axes of seeds of intermediate nature like coffee (Abdel-Esquivel *et al.*, 1992); oil palm (Grout *et al.*, 1983) and black pepper seeds (Chaudhury and Chandel, 1994).

According to Touchell and Walters (2000), *Zizania palustris*, an aquatic grass had highest survival when dried to between 0.36g water/g dry weight and 0.56 g water/g dry weight. Exposing embryos with high moisture content resulted in freeze damage. Viability was also reduced when embryos with water contents lower than 0.36g water/g dry weight were exposed to liquid nitrogen, suggesting increased desiccation stress with lower temperatures.

2.2.2.2.2 Cryopreservation by Encapsulation –Dehydration

The encapsulation-dehydration technique is based on the technology developed for the production of synthetic seeds (Redenbaugh, 1993). Zygotic embryos are encapsulated in a bead of alginate and pregrown for various duration in liquid medium with high sucrose concentration. Beads are then partially dehydrated under the air current of laminar flow cabinet or using silicagel, down to water content of about 20 per cent. Freezing is usually rapid by direct immersion in liquid nitrogen. For recovery, samples are usually placed directly under standard culture conditions. Survival rates are high and growth recovery of the cryopreserved material is generally rapid and direct without callus formation.

Marzalina *et al.* (1994) used this technique for successful cryopreservation of the zygotic embryos of *Swetenia macrophylla* . Uragami *et al.* (1993) applied encapsulation-dehydration procedure to cryopreserve the embryos of oilseed rape.

2.2.2.3 Cryopreservation of Shoot Meristem

Shoot meristem has become an indispensable tool in clonal propagation. Since the constituent cells of the shoot meristem are less differentiated and has more uniform ploidy than those of mature tissues, plants regenerated by the *in vitro* culture of the shoot meristem without the callus–mediated process or organogenesis would be true to type as opposed to other means of propagation. Further more, the shoot meristem has a greater ability to regenerate the whole plant than do callus–cultured cells. Therefore cryopreservation of isolated shoot meristem in LN is potentially suitable and reliable means of plant germplasm conservation (Sakai, 1985).

2.2.2.3.1 Cryopreservation by Encapsulation –Dehydration

According to Engelmann (1997), shoot apices can be effectively cryopreserved by encapsulation dehydration procedure. This technique has been applied to apices of numerous species of both temperate and tropical origin.

According to Sakai and Matsumoto (1996) encapsulation- dehydration procedure is promising for cryopreservation of meristems. Shoot tips of pear (Dereuddre *et al.*, 1990) and *Solanum* (Fabre and Dereuddre, 1991) were cryopreserved using the same technique.

Manipulation of micropropagation medium or incubation environment may be necessary to have a suitable *in vitro* plant for meristem isolation. Apical shoot tips and axillary shoot tips from the entire length of *in vitro* plants of potato and mint responded equally well after LN exposure (Towill, 1991). In contrast to this, a study by Dereuddre *et al.* (1988) in carnation showed that terminal and axillary shoot tips from the top portion of carnation survived in greater percentages than those from lower portions of the plant.

Kartha (1982) isolated shoot tips of size 0.4-0.5 mm of *Pisum sativum* and strawberry for successful cryopreservation. Ogawa *et al.* (1997) used 1-2 mm shoot primordia of melon (*Cucumis melo*) for cryopreservation. Axillary buds of 0.5 -1 mm in length were dissected from proliferating shoot cultures for encapsulation of *Eucalyptus grandis* x *E. camaldulensis* hybrid by Blakesley and Kiernan (2001).

Actively growing tissues like that of apices require special treatments before they could withstand freezing stresses (Kartha *et al.*, 1979). Influence of ammonium nitrate in the pre-freeze and post-freeze culture medium and 2 or 30 day preconditioning in the presence of 0.5 M sucrose on regeneration of shoot tips of *Holostemma annulare* following cryopreservation using an encapsulation-dehydration protocol was studied by Decruse and Seeni (2002). A long preconditioning phase of 30 days significantly reduced tissue water and improved post-freeze recovery of shoot tips. It also revealed the importance of reducing ammonium nitrate in the culture medium to get maximum recovery of cryopreserved shoot tips of *Holostemma annulare.*

Shoot apices and axillary buds are encapsulated to form alginate beads. Sakai and Matsumoto (1996) suspended shoot apices of *Wasabi japonica* and *Lilium japonicum* in calcium free half MS medium supplemented with 3 percent sodium alginate solution and 0.4 M sucrose. The mixture was dispensed with a sterile disposable syringe into liquid culture medium containing 100 mM calcium chloride plus 0.4 M sucrose and held in half MS to form beads. Blakesley and Kiernan (2001) treated axillary buds of *Eucalyptus grandis* x *E. camaldulensis* hybrids in the same manner to form alginate beads with the exception that 10.1 M sucrose was used instead of 0.4 M sucrose.

In a novel encapsulation technique developed for the production of artificial seeds by Patel *et al.* (2000), involves suspending shoot tips of *Solanum tuberosum* in a solution containing 1.5 per cent carboxymethylcellulose solution and 1 per cent

calcium chloride and then dipping into 0.8 per cent sodium alginate solution to form beads.

In encapsulation dehydration technique, tolerance to dehydration and deep freezing could be induced by preculturing the encapsulated apices in sucrose enriched medium. According to Uragami *et al.* (1990), dried axillary buds from plantlets of *Asparagus officinalis* grown *in vitro* were successfully cryopreserved following pre culture on solidified culture medium supplemented with 0.7 M sucrose at 25°C for 2 days. Best results on cryopreservation of encapsulated shoot apices of *Wasabi japonica* and *Lilium japonicum* obtained on preculturing on 0.8 M sucrose for 24 h (Sakai and Matsumoto, 1996).

Touchell *et al.* (1992) suggested that shoot tips of *Grevillea scapigera* cryostored using encapsulation-dehydration methods required cryoprotection with DMSO to survive freeze-thaw cycles. Dehydration injury causes cell shrinkage, the formation of membrane invaginations and the possible loss of plasma membrane to the cytoplasm (Morris *et al.,* 1988). Further more, during dehydration increased concentration of solutes within the cell can be toxic. These potentially damaging events can be reduced by the application of penetrating solutes (Meryman and Williams, 1985).

DMSO is commonly used as a cryoprotectant. It is very effective as it permeate the cell. According to Dereuddre *et al.* (1988) pre culture of carnation shoot tips with 0.25 - 0.75 M sucrose and 5 per cent DMSO enhanced their survival after LN treatments. Again this is the findings of Touchell and Dixon (1996) that encapsulation dehydration is a simplified method that avoids the necessity of chemical cryoprotection.

Blakesley and Kiernan (2001) encapsulated axillary buds of *Eucalyptus grandis* x *E. camaldulensis* hybrids were subjected to evaporative dehydration and

found that dehydration to 4 h (19 per cent moisture content) gave best result after exposure to LN.

Rapid warming or thawing is required to retain viability in shoot tips. Warming is accomplished by immersing cryovials containing encapsulated beads into a water bath at 35-40°C for a few seconds (Towill, 1991). After thawing encapsulated meristems are transferred to suitable growth medium and culture environment. Growth regulator concentrations require adjustment in order to enhance survival and growth of shoot tips (Withers *et al,* 1988). However, callus production must be avoided to reduce the frequency of somaclonal variation after shoot initiation. Sakai and Matsumoto (1996) when transferred encapsulated shoot apices of *Wasabi japonica* and *Lilium japonicum* on to a recovery medium of half MS with 0.1 mg l^{-1} BA showed good regeneration. Shoot formation was recorded as per cent of total number of apices forming normal shoots 21 days after planting.

2.2.2.3.2 Rapid Freezing with Addition of Cryoprotectants

Cryoprotectants enhance the survival of hydrated tissues after exposure to low temperatures (Finkle *et al.*, 1985). The most effective cryoprotectant must permeate the cell. DMSO, ethylene glycol and propylene glycol have this property. Some very low molecular weight compounds such as sucrose, glucose, glycerol, proline and mannitol do not permeate or do so very slowly, but are effective when added with a permeating cryoprotectant. Successful cryopreservation of meristems has also been reported in several species, such as *Pisum sativum, Fragaria ananassa* (Kartha et *al.,* 1979, 1980), *Cicer arietinum* (Bajaj 1979; Kartha 1985) and *Arachis hypogoea* (Bajaj, 1983). In most of these cases, DMSO, sucrose or glycerol, either alone or in combination have been used as cryoprotectants and percent survival of cultures varied from 16 to 80.

2.2.3 Genetic Stability Assessment of Cryopreserved Materials Using RAPD Technique

Cryopreservation is a multi-component procedure which involves tissue culture, pre growth, cryoprotection, freezing, thawing, recovery and plant regeneration (Harding and Benson, 1994). Cryopreservation, freezing and thawing are tightly coupled events and success of each phase is integrally linked to the overall success of cryopreservation. Each aspect of cryopreservation procedure may affect the recovery of tissues and predispose cells to genetic change.

The freezing of cells may induce genetic damage. The rate of freezing and thawing process can result in the formation of intracellular ice crystals. The resultant damage to cellular components has the potential to disrupt the nucleus, chloroplast and mitochondrion. Dehydration injury causes cell shrinkage, the formation of membrane invaginations and the possible loss of plasma membrane to the cytoplasm. Hence, the long term genetic consequences of dehydration and freezing injury for *in vitro* conservation need to be assessed (Harding, 1996).

According to Harding and Benson (1995), molecular analysis of plant DNA is ideal for stability assessments. The DNA sequence is essentially the same in all cells in all tissues of the plant, and DNA can be extracted and stored for long periods of time by freezing. Moreover, this technique provides results of greater resolution.

PCR and RAPD technology are highly relevant to stability assessments of plants derived from *in vitro* conservation. Where a gene sequence is known, the PCR can amplify a specific region of the genome defined by a pair of primers. A single oligonucleotide primer of arbitrary nucleotide sequence or arbitrarily primed oligonucleotides in combination with PCR produce randomly amplified polymorphic DNA (RAPDs) fragments are useful markers for the detection of genetic change (William *et al.*, 1991).

The RAPD technique is considered to be a fast, simple and efficient method for evaluating genetic stability of cryopreserved material, which can be used rapidly after the completion of a freezing experiment and will efficiently complement other genetic stability evaluation methods. This technique has been successfully used to study the genetic stability of cryopreserved materials of *Arachis* species (Gagliardi *et al.*, 2003), *Diascorea bulbifera* (Dixit *et al.*, 2003), *Vitis vinefera* (Zhai *et al.*, 2003).

Materials and Methods

3. MATERIALS AND METHODS

The present study, 'Rapid propagation and conservation of selected leguminous medicinal plants using *in vitro* techniques' was carried out at the Department of Plantation Crops and Spices and at the Department of Plant Biotechnology, College of Agriculture, Vellayani during the period from 2001-2004. The experiments on cryopreservation were done at the Division of Biotechnology, Tropical Botanical Garden and Research Institute, Palode.

Investigations were carried out in two phases *viz.*, *in vitro* propagation and *in vitro* conservation on three important leguminous medicinal plants, *Clitoria ternatea* L., *Mucuna pruriens* Baker non DC. and *Indigofera tinctoria* L. (Plate 1). Propagation studies were carried out by enhanced release of axillary buds, somatic organogenesis and somatic embryogenesis. *In vitro* conservation was attempted using slow growth and cryopreservation techniques.

The details of materials and methods adopted for the study are presented in this chapter.

3.1 PHASE I: *IN VITRO* PROPAGATION

3.1.1 Collection and Preparation

Seeds of superior accessions of *Clitoria ternatea*, *Mucuna pruriens* and *Indigofera tinctoria*, identified from previous studies in the Department of Plantation Crops and Spices, College of Agriculture, Vellayani, were used for this study. Details and description of the accessions are given in Table 1.

Plate 1. Plant species selected for the study

a. *Clitoria ternatea* L. : Butterfly Pea (Sanghupushpam)

b. *Mucuna pruriens* Baker non DC. :Velvet Bean (Naikurna)

c. *Indigofera tinctoria* L. : Indian Indigo (Neela amari)

Plate 1. Plant species selected for the study

3.1.2 Surface Sterilization

Mature ripe pods, indicated by brown colour in *Clitoria ternatea* and *Indigofera tinctoria* and deep black colour pods in *Mucuna Pruriens* were harvested and seeds extracted were washed in running tap water. Then the seeds are immersed in water with a few drops of wetting agent, labolene for half an hour, followed by washing several times with distilled water. They were then transferred to a sterile beaker and treated with mercuric chloride (0.08 per cent) for 15 min. This was followed by washing five times with sterile distilled water.

3.1.3 *In vitro* Seed Germination

Seeds of selected accessions of *Clitoria ternatea*, *Indigofera tinctoria* and *Mucuna pruriens* were germinated *in vitro*, in MS basal medium. Explants were selected from *in vitro* raised seedlings.

3.1.4 Inoculation and Incubation

The glasswares and tools required for inoculation were washed thoroughly in tap water, rinsed with distilled water, covered with aluminium foil and autoclaved at 121°C and 1.06 kg cm^{-2} pressure for 45 minutes. Horizontal type autoclave ('NAT' Steel) was used for autoclaving. The closure of the culture vessel was removed before inoculation, the rim of vessel flamed before and after inoculation and closure replaced.

Inoculation operations were carried out in a laminar air flow chamber (Klenzaids, Model 1104). The closure of the culture vessels was removed before inoculation, the rim of the culture vessels was flamed before and after inoculation and closure replaced.

Surface sterilized seeds were soaked in sterile distilled water for 1-3 days, depending on the plant species. The seed coat was removed and the cotyledons were

dissected out longitudinally into two equal halves. A transverse cut was made on each half. The cotyledons with or without embryonic axis were inoculated in the culture medium.

Explants, other than cotyledons were obtained from *in vitro* germinated seedlings. They were excised / cut using sterile surgical blade. Single nodal cuttings (0.5 - 0.8 cm) with a single axillary bud were inoculated in the culture medium.

The cultures were then incubated in a culture room maintained at light intensity, 3000 lux using white fluorescent tubes for 16 h. The temperature of the room was regulated using an air conditioner at 20 ± 2°C at a relative humidity (RH) of 60 per cent.

3.1.5 Culture Media

The basal media used for the study were either MS (Murashige and Skoog, 1962), B5 (Gamborg *et al.*, 1968), SH (Schenck and Hildebrandt, 1972) and WPM (Lloyd and McCown, 1980) depending on the objective of the experiment. The chemicals used in the culture media were of analytical grade from Sisco Research Laboratories (Mumbai), Merck (Mumbai) and British Drug House (Mumbai).

Standard procedures were followed for the preparation of basal medium (Thorpe, 1980). Stock solutions of major and minor nutrients, organic supplements and plant growth substances were prepared by dissolving the required quantity of chemicals in specific volume of distilled water/ethylalcohol/0.1 N HCl or NaOH, depending on the chemical and were stored under refrigerated conditions (4°C).

The glasswares used were washed with dilute liquid detergent (labolene) and rinsed with single distilled water. Specific quantities of the stock solutions were pipetted out into 1000 ml beaker. Sucrose, casein hydrolysate, adenine sulphate and inositol were added fresh in required quantity, weighed using an electronic balance (Sartorius analytic A120S) and dissolved by constant stirring. The pH of the medium was adjusted between 5.6 and 5.8 using an electronic pH meter (Philips, model PP9046). Agar was added to the medium

and final volume made up to 1000 ml, using a volumetric flask. Agar was melted using a heating mantle, stirring thoroughly while heating for uniform mixing. Activated charcoal, when needed was added in the medium at this stage. The melted medium was dispensed into pre-sterilised culture vessels such as test tubes (25 x 150 mm) and Erlenmeyer flasks (100 ml). Measure of culture medium in test tubes and flasks were 15 and 50 ml, respectively. The culture vessels containing medium were plugged firmly with non absorbent cotton wool. They were then autoclaved at 121°C and 1.06 kg cm^{-2} pressure for 20 minutes using NAT horizontal autoclave.

3.1.6 Explant

Nodal segments and leaves excised from *in vitro* raised seedling and seed cotyledons were tried for enhanced release of axillary buds and cotyledons, stem segment and leaves were used as explants for induction of somatic organogenesis and somatic embryogenesis in *Clitoria ternatea and Mucuna pruriens.* In case of *Indigofera tinctoria*, only nodal segments and leaves from *in vitro* raised seedlings were used as explants for enhanced release of axillary buds and for somatic organogenesis and somatic embryogenesis, stem segment and leaves were tried (Table 2).

3.1.7 Enhanced Release of Axillary Buds

3.1.7.1 Plant Growth Substances

Cytokinins and Auxin

Explants were subjected to treatments with different combinations of cytokinins (BA and Kn) and auxin (IAA) with MS as the basal medium. Treatments involved different levels of BA (0.1 to 1.0 mg l^{-1}), Kn (0.1 to 1.0 mg l^{-1}) and IAA (0.05, 0.1 mg l^{-1}) (Table 3). The treatments were replicated six times. In the case of nodal segments, observations were recorded on number of days for bud initiation, number of shoots, length of the longest shoot and number of leaves.

Table 1.Details and description of the accessions of *Clitoria ternatea*, *Mucuna pruriens* and *Indigofera tinctoria* selected for the study

Plant	Accession No.	Botanical description	Yield attributes
Clitoria ternatea L.	MP-73	Fast-growing perennial climber with pinnate leaves; leaflets mostly 5-9, one terminal, ovate-oblong, obtuse, about 3 cm long,; flowers solitary on pedicels and creamy white, yellow at base, seeds rounded about 6-7 mm wide	Shoot yield 1982.22 kg/ha and root yield 162.22 kg/ha (Reshmi and Reghunath, 2003a,b)
Mucuna pruriens Baker non DC.	MP-51	Twinning annual; Leaves opposite, lanceolate, trifoliate; Flowers dark purple flowers occur in drooping racemes; Fruits are curved, 4–6 seeded; Pod longitudinally ribbed thick and leathery, densely covered with grey trichomes; Seeds are creamy white, reniform and 12 mm long.	Pod yield 2187 kg/ha (Nirmalatha, Personal communication)
Indigofera tinctoria L.	MP-100	Perennial shrub ; leaves pinnate; flowers red or purple in cluster ; pods longitudinally ribbed , 1.5 to 2.0 cm long with 4-5 small seeds	shoot yield 9900 kg/ha and root yield 1800 kg/ha (Sarada and Reghunath, 2003)

Table 2.Explants tried for *in vitro* propagation studies in *Clitoria ternatea*, *Mucuna pruriens* and *Indigofera tinctoria*

Plants	Explants	
	Enhanced release of axillary buds	Somatic organogenesis & embryogenesis
Clitoria ternatea	Nodal segments, leaves, cotyledons	Cotyledons, stem segment and leaves
Mucuna pruriens	Nodal segments, leaves, cotyledons	Cotyledons, stem segment and leaves
Indigofera tinctoria	Nodal segments, leaves	Stem segment and leaves

Table 3. Plant growth substances tried for shoot proliferation *via* enhanced release of axillary buds from various explants of *Clitoria ternatea*, *Mucuna pruriens* and *Indigofera tinctoria*

Treatment	Plant growth regulator(s) (mg l^{-1})
P1	BA 0.10
P2	BA 0.50
P3	BA 1.00
P4	Kn 0.10
P5	Kn 0.50
P6	Kn 1.00
P7	BA 0.50 + Kn 0.10
P8	BA 0.50 + Kn 0.50
P9	BA 0.50 + Kn 1.00
P10	BA 1.00 + Kn 0.10
P11	BA 1.00 + Kn 0.50
P12	BA 1.00 + Kn 1.00
P13	BA 0.50 + IAA 0.05
P14	BA 0.50 + IAA 0.10
P15	BA 1.00 + IAA 0.05
P16	BA 1.00 + IAA 0.10
P17	Kn 0.50 + IAA 0.05
P18	Kn 0.50 + IAA 0.10
P19	Kn 1.00 + IAA 0.05
P20	Kn 1.00 + IAA 0.10
P21	BA 0.50 + Kn 0.50 + IAA0.05
P22	BA 0.50 + Kn 0.50 +1AA 0.10

Culture medium: MS + Inositol (100 mg l^{-1}) + Sucrose (30 g l^{-1}) + Agar (7 g l^{-1})

Gibberellic Acid

Effect of GA_3 on shoot proliferation was studied using varying levels of GA_3 (1, 2, 3 mg l^{-1}) in combination with the best treatment of other growth substances as per Table 3. The treatments were replicated six times. Observations were recorded on number of shoots, length of the longest shoot and number of leaves.

3.1.7.2 Basal Media

The best treatment (growth regulator as per Table 3) was later tried on quarter strength MS, half strength MS, MS + activated charcoal, SH, B5 and WPM to assess its effect on shoot proliferation (Table 4). The treatments were replicated six times. Observations were recorded on number of shoots, length of the longest shoot and number of leaves.

3.1.7.3 Carbon Source

Sucrose

Varying levels of sucrose (20, 30, 40, 50 g l^{-1}) were tried to study their effect on shoot proliferation (Table 5). The treatments were replicated six times. Observations were recorded on number of shoots, length of the longest shoot and number of leaves.

Glucose

The effect of glucose at different levels (20, 30, 40, 50 g l^{-1}) were tried to study their effect on shoot proliferation (Table 5). The treatments were replicated six times. Observations were recorded on number of shoots, length of the longest shoot and number of leaves.

3.1.7.4 Ethylene Inhibitor

The effect of cobaltous chloride (ethylene inhibitor) at two different levels (5 and 10 mg l^{-1}) on shoot proliferation was studied (Table 5). The treatments were replicated six times. Observations were recorded on number of shoots, length of the longest shoot and number of leaves.

3.1.7.5 Gelling Agent

Different levels of agar (5 to 8 g l^{-1}) were tried to study their effect on shoot proliferation (Table 5). The treatments were replicated six times. Observations were recorded on number of shoots, length of the longest shoot and number of leaves.

3.1.8 Somatic (Indirect) Organogenesis

3.1.8.1 Callus Initiation

Explants of *Clitoria ternatea, Mucuna pruriens* and *Indigofera tinctoria* were subjected to different treatments for callus initiation. The treatments tried included different levels of 2, 4-D (0.1 to 2.0 mg l^{-1}), NAA (0.5 to 2.0 mg l^{-1}) and BA (0.1, 0.5 mg l^{-1}) (Table 6). The treatments were replicated six times. Observations were recorded on number of cultures initiating callus from the explant. Callus index (CI) was computed by multiplying per cent cultures initiating callus with growth score (G). Growth of the callus was assessed based on a visual rating (with score 1.0 to the smallest and score 4.0 to the largest). The mean score was expressed as growth score, 'G' (Poor 1, Medium 2, Good 3, Profuse 4).

3.1.8.2 Shoot Regeneration

The callus was inoculated into medium with varying levels of auxins, cytokinins and adenine sulphate. Different concentrations of BA (1, 2 mg l^{-1}), Kn (1, 2 mg l^{-1}), NAA (0.5, 1 mg l^{-1}) adenine sulphate (20 mg l^{-1}) and their combinations were tried to assess the

Table 4.Effect of basal media on shoot proliferation *via* enhanced release of axillary buds from nodal explants of *Clitoria ternatea, Mucuna pruriens* and *Indigofera tinctoria*

Treatment	Basal medium
M1	¼ MS
M2	½ MS
M3	MS
M4	MS+AC (0.5 mg l^{-1})
M5	SH
M6	B5
M7	WPM

Culture medium: Basal medium + Inositol (100 mg l^{-1}) + Sucrose (30 g l^{-1}) + Agar (7 g l^{-1}) + plant growth regulator(s) (best treatment from Table 3)

Table 5. Different levels of carbon source, ethylene inhibitor and gelling agent tried for shoot proliferation *via* enhanced release of axillary buds from nodal explants of *Clitoria ternatea, Mucuna pruriens* and *Indigofera tinctoria*

Carbon source:	Sucrose (g l^{-1})
S1	20
S2	30
S3	40
S4	50
	Glucose (g l^{-1})
T1	20
T2	30
T3	40
T4	50
Ethylene inhibitor:	Cobaltous chloride (mg l^{-1})
CC1	05
CC2	10
Gelling agent:	Agar (g l^{-1})
AG1	05
AG2	06
AG3	07
AG4	08

Culture medium: MS + Inositol (100 mg l^{-1}) + Sucrose (30 g l^{-1}) + Agar (only in case of treatments not involving agar) 7 g l^{-1}+ plant growth regulators (best treatment from Table 3)

Table 6. Plant growth substances tried for callus initiation from various explants of *Clitoria ternatea*, *Mucuna pruriens* and *Indigofera tinctoria*

Treatment	Plant growth regulator(s) (mg l^{-1})
C1	2,4-D 0.10
C2	2,4-D 0.50
C3	2,4-D 1.00
C4	2,4-D 2.00
C5	BA 0.10
C6	BA 0.50
C7	NAA 0.50
C8	NAA 1.00
C9	NAA 2.00
C10	2,4-D 0.10 + BA 0.10
C11	2,4-D 0.50 + BA 0.10
C12	2,4-D 1.00 + BA 0.10
C13	2,4-D 2.00 + BA 0.10
C14	2,4-D 0.10 + BA 0.50
C15	2,4-D 0.50 + BA 0.50
C16	2,4-D 1.00 + BA 0.50
C17	2,4-D 2.00 + BA 0.50
C18	2,4-D 0.50 + NAA 0.50
C19	2,4-D 1.00 + NAA 0.50
C20	2,4-D 2.00 + NAA 0.50
C21	2,4-D 1.00 + NAA 1.00
C22	2,4-D 2.00 + NAA 1.00
C23	NAA 0.50 + BA 0.10
C24	NAA 1.00 + BA 0.10
C25	NAA 2.00 + BA 0.10
C26	NAA 0.50 + BA 0.50
C27	NAA 1.00 + BA 0.50
C28	NAA 2.00 + BA 0.50

Culture medium: MS + Inositol (100 mg l^{-1}) + Sucrose (30 g l^{-1}) + Agar (7 g l^{-1})

effect of these plant growth substances on regeneration of shoot from callus (Table 7). The treatments were replicated six times. Observations were taken on number of cultures developing shoots, number of shoots and length of the longest shoot.

3.1.9 Somatic Embryogenesis

3.1.9.1 Callus Initiation

The basal medium used was MS for all the three plants included in the study. The combination of plant growth substances tried were the same as in Table 6.

Embryogenic callus identified as granular/nodular, friable and fast growing callus were transferred to the same medium after three weeks of culture for further proliferation of the callus.

3.1.9.2 Induction of Somatic Embryos

The embryogenic calli were transferred to culture medium with different levels and combinations of BA (0.5 to 3.0 mg l^{-1}), GA (0.1, 0.5,1,5 mg l^{-1}), NAA (5 mg l^{-1}), glutamine (400 mg l^{-1}), coconut water (10 to 20 per cent), casein hydrolysate (600 mg l^{-1}), 2, 4-D (0.5 mg l^{-1}) and activated charcoal (250 mg l^{-1}). The treatments were replicated six times (Table 8). The percent cultures inducing somatic embryos in each treatment and the nature of embryos were recorded.

3.1.9.3 Maturation and Germination

Somatic embryos at different stages of growth were inoculated on to maturation and germination medium, MS medium devoid of hormones and supplemented with GA (0.1, 0.5, 1 mg l^{-1}). The treatments were replicated six times. The per cent germination of embryos was estimated.

Table 7. Plant growth regulators tried for shoot regeneration from the callus derived from various explants of *Clitoria ternatea*, *Mucuna pruriens* and *Indigofera tinctoria*

Treatment	Plant growth regulator(s) (mg l^{-1})
SR1	BA 1.00
SR2	BA 2.00
SR3	Kn 1.00
SR4	Kn 2.00
SR5	BA 1.00 + Kn 1.00
SR6	BA 1.00 + Kn 2.00
SR7	BA 2.00 + Kn 1.00
SR8	BA 2.00 + Kn 2.00
SR9	BA 1.00 + NAA 0.50
SR10	BA 1.00 + NAA 1.00
SR11	BA 2.00 + NAA 0.50
SR12	BA 2.00 + NAA 1.00
SR13	Kn 1.00 + NAA 0.50
SR14	Kn 1.00 + NAA 1.00
SR15	Kn 2.00 + NAA 0.50
SR16	Kn 2.00 + NAA 1.00
SR17	BA 1.00 + Kn 1.00 + NAA 0.50
SR18	BA 1.00 + Kn 1.00 + NAA 1.00
SR19	BA 2.00 + Kn 1.00 + NAA 0.50
SR20	BA 2.00 + Kn 1.00 + NAA 1.00
SR21	BA 1.00 + Kn 1.00 + Ad S 20.00 + NAA 1.00
SR22	BA 1.00 + Kn 2.00 + Ad S 20.00 + NAA 1.00
SR23	BA 2.00 + Kn 1.00 + Ad S 20.00 + NAA 1.00
SR24	BA 2.00 + Kn 2.00 + Ad S 20.00 + NAA 1.00

Culture medium: MS + Inositol (100 mg l^{-1}) + Sucrose (30 g l^{-1}) + Agar (7 g l^{-1})

Table 8. Plant growth regulators tried for somatic embryo induction in callus cultures of *Clitoria ternatea*, *Mucuna pruriens* and *Indigofera tinctoria*

Treatment	Plant growth regulator(s)(mg l^{-1})
I1	BA 0.50
I2	BA 1.00
I3	GA 0.10
I4	GA 0.50
I5	GA 1.00
I6	BA 0.50 + NAA1.00
I7	BA 1.00 + NAA 1.00
I8	GA 0.50 + NAA 1.00
I9	GA 1.00 + NAA 1.00
I10	BA 1.00 + CW 10 %
I11	BA 1.00 + CW 15%
I12	BA 1.00 + CW 20%
I13	BA 2.00 + CW 10%
I14	BA 2.00 + CW 15%
I15	BA 2.00 + CW 20%
I16	BA 3.00 + CW 10%
I17	BA 3.00 + CW 15%
I18	BA 3.00 + CW 20%
I19	BA 1.00 + GA 5.00 + Gl 400 + CH 600 + CW 20%+2,4-D 0.5 + AC 250
I20	BA 1.00 + GA 5.00 + Gl 400 + CH 600 + CW 20 % +2,4-D 0.5 + ABA5.00+ AC 250

Culture medium: MS + Inositol (100 mg l^{-1}) + Sucrose (30 g l^{-1}) + Agar (7 g l^{-1})
CW: coconut water; Gl: glutamine; CH: casein hydrolysate; AC: activated charcoal

3.1.9.4 Acclimatization and Plant Regeneration

The complete plants with root and shoot system were transferred to basal MS medium for elongation. After 3 - 4 weeks, fully developed plantlets 3 - 4 cm in length with 2 - 3 leaves were removed from cultures and transferred to pots containing sterilized sand for acclimatization. Acclimatization was accomplished by covering the pots with a polythene cover and maintaining them in a growth chamber for two weeks.

3.1.10 Rooting

3.1.10.1 In vitro Rooting

Well developed shoots having 3-5 cm length were separated and subjected to different rooting treatments. The shoots were inoculated into medium with varying levels of IBA (0.5 to 2.5 mg l^{-1}), IAA (1 to 2.5 mg l^{-1}) and NAA (0.5 to 2 mg l^{-1}) (Table 9). Each treatment was replicated six times. Observations were taken on per cent cultures initiating roots, number of days for root initiation, number of roots and length of roots.

3.1.10.2 Ex vitro Rooting

Well developed shoots having 3-8 cm length were separated and were given different hormone treatments. Both quick dip (pulsing) and slow dip methods were tried. In case of quick dip, 1000 mg l^{-1}IBA and 500 mg l^{-1} IBA were tried for 20 s. For slow dip, plantlets were kept in IBA 100 mgl^{-1} solution for 20 h. The treated plantlets were planted out in different potting media (sand, soilrite, soilrite (2): sand (1)). Each treatment was replicated twelve times. Observations were taken on per cent survival of plantlets, four weeks after planting out.

3.1.10.3 Planting Out and Acclimatization

Plantlets that showed normal growth *in vitro* were planted out in plastic pots containing potting media. The cotton plug of the culture vessels were removed, sterile water added to the vessels and kept as such for 10-15 min. Then rooted plantlets were taken out from culture vessels with the help of forceps. The agar adhering to the roots was completely removed by thorough washing with running tap water. The plantlets were treated with Indofil M -45 (0.3 per cent) solution for 30 min before planting out. The effect of potting media on *ex vitro* establishment of the plants was tried by providing different media (sand, soilrite, soilrite (2): sand (1)). Each treatment was replicated twelve times. Per cent survival of plantlets after four weeks was recorded.

3.1.11 Statistical Analysis

Completely randomized design (Panse and Sukhatme, 1985) was followed for statistical analysis, wherever applicable.

3.2 PHASE II: *IN VITRO* CONSERVATION

3.2.1 Slow Growth

Slow growth for short to medium term storage was tried in *Clitoria ternatea, Mucuna pruriens* and *Indigofera tinctoria.*

3.2.1.1 Culture Vessel, Culture Medium and Explant

Standard Borosil testubes (25 x150 mm) were dispensed with 25 ml of the media for slow growth experiments.

Nodal cuttings with single axillary bud were selected as explant. Rooting medium consisting of MS or half strength MS with and without IBA 0.5 mg l^{-1} were tried for

selecting the basal culture media for slow growth experiments in the three plant species. The treatments were replicated six times. The observations were recorded after 16 weeks of incubation. Culture medium which recorded the lowest plant height and late or no senescence was selected as the suitable medium. Rooting percentage was also recorded in each treatment.

3.2.1.2 Addition of Osmoticum, Cryoprotectant, Antioxidant and Growth Retardant

Osmoticum *viz.*, sucrose (6, 8, 10, 12 and 15 per cent), sorbitol (0.5, 1, 1.5, 2 and 3 per cent) and mannitol (0.5, 1, 1.5, 2 and 3 per cent), cryoprotectant *viz.*, proline (5, 10, 15, 20 and 30 mM), antioxidant *viz.*, PVP (2, 4, 6, 8 and 10 per cent) and growth retardant *viz.*, ABA (10, 15, 20, 25 and 30 mg l^{-1}) were tried for short to medium term storage by inducing slow growth (Table 10). Each treatment was replicated 12 times.

Observations were made at four weeks interval. Plant survival rate and plant height (represented the average height of live plants) during this period were recorded. Rate of growth at each four week period was also estimated. When more than 75 per cent reduction in the volume of culture medium was observed, plants from the treatments which showed more than fifty per cent survival were subcultured to regeneration / shoot proliferation medium. Nodal cuttings from each treatment were inoculated on to the standardized proliferation medium to estimate the rate of regeneration. This experiment was replicated six times. Cultures in those treatments which gave more than 50 per cent regeneration on proliferation medium were subcultured again to the slow growth medium to reassess the efficacy of the medium to induce slow growth. Regeneration was estimated for cultures in treatments which gave more than 50 per cent survival after 12 weeks of first subculture (ie., total period of a storage of an explant is (28 +12) 40 weeks.

Observations on plant height, number of shoots and number of nodes were recorded from six cultures in each treatment just before first subculture and from surviving plants after 12 weeks of first subculture. Presence of callus and roots was also recorded at these two stages.

Table 9. Plant growth regulators tried for *in vitro* rooting of shoots of *Clitoria ternatea, Mucuna pruriens* and *Indigofera tinctoria*

Treatment	Plant growth regulator(s) (mg l^{-1})
R1	IBA 0.50
R2	IBA 1.00
R3	IBA 1.50
R4	IBA 2.00
R5	IBA 2.50
R6	IAA 1.00
R7	IAA 1.50
R8	IAA 2.50
R9	NAA 0.50
R10	NAA 1.00
R11	NAA 1.50
R12	NAA 2.00

Culture medium: ½ MS + Inositol (100 mg l^{-1}) + Sucrose (30 g l^{-1}) + Agar (7 g l^{-1})

Table 10. Effect of different osmotica, cryoprotectant, antioxidant and growth retardant on slow growth induction of cultures of *Clitoria ternatea, Mucuna pruriens* and *Indigofera tinctoria*

Osmoticum	Levels	Other additives	Levels
Sucrose	(%)	Cryoprotectant (Proline)	(mM)
Su T1	6.0	Pr T1	05
Su T2	8.0	Pr T2	10
Su T3	10.0	Pr T3	15
Su T4	12.0	Pr T4	20
Su T5	15.0	Pr T5	30
Sorbitol	(%)	Antioxidant (PVP)	(%)
SbT1	0.5	Pvp T1	02
Sb T2	1.0	Pvp T2	04
Sb T3	1.5	Pvp T3	06
Sb T4	2.0	Pvp T4	08
Sb T5	3.0	Pvp T5	10
Mannitol	(%)	Growth retardant (ABA)	(mg l^{-1})
MnT1	0.5	Aba T1	10
MnT2	1.0	Aba T2	15
Mn T3	1.5	Aba T3	20
Mn T4	2.0	Aba T4	25
Mn T5	3.0	Aba T5	30

Culture medium: ½ MS + IBA (0.5 mg l^{-1}) + Inositol (100 mg l^{-1}) + Sucrose (only in case of treatments not involving sucrose) 30 g l^{-1} + Agar (7gl^{-1})

3.2.1.3 Statistical Analysis

Completely randomized design (Panse and Sukhatme, 1985) was followed for statistical analysis, wherever applicable.

3.2.2Cryopreservation

The materials and methods tried for cryopreservation in *Clitoria ternatea*, *Mucuna pruriens* and *Indigofera tinctoria* is presented in Table 11.

3.2.2.1 Seeds

Seeds of *Clitoria ternatea*, *Mucuna pruriens* and *Indigofera tinctoria* were collected fresh from the plants and were kept at ambient temperature while the experiments were being performed. After cleaning a batch of 10-100 seeds, depending on the plant's seed size was subjected to moisture determination gravimetrically using an electronic balance (Mettler PM 200) by constant temperature oven method (103°C for 17 h) as recommended by International Seed Testing Association (ISTA, 1985).

Seeds were dried over charged silicagel at ambient temperature (29 ± 3°C) to varying moisture content of 4-8 per cent. Depending on the size of the seed, samples each containing 10-100 seeds were placed in 4 ml cryovials, and plunged in liquid nitrogen in Cryocan (MVE cryogenics, LAB 30). Effect of cryostorage for varying periods *viz.*, 1 day, 1 week, 3 weeks, 6 weeks and 9 weeks on germination was studied. Corresponding desiccation control consisted of equal number of seeds stored at ambient temperature.

Rewarming (thawing) of the liquid nitrogen preserved seeds was carried out at room temperature (29 ± 3°C) to equilibrium. Desiccation control seeds and rewarmed seeds were transferred to petri dishes (6 cm) containing two sheets of filter paper, adding sufficient quantity of distilled water for germination. All seeds except that of *Mucuna pruriens* were given mechanical scarification using sand paper before transferring to petri

dishes. This was incubated at ambient conditions (29 ± 3°C; 12 h photoperiod with a light intensity of 170 ± 95 lux). Water was replenished regularly at two-day interval. Three replicates of 10-100 seeds were used. Germination was determined by noting radicle emergence. Final observation on germination was recorded 20 days after incubation.

3.2.2.2 Zygotic Embryos

3.2.2.2.1 Simple Desiccation

Seeds of *Mucuna pruriens* were surface sterilized with 1 per cent sodium hypochlorite solution for 10 min, and soaked overnight for the easy removal of seed coat. Excised embryos were rapidly dried in laminar air flow for different lengths of time, 0 – 3 h at 30 min interval. The moisture content of the embryos was determined by gravimetric comparison of fresh and dry weight. Dry weight of embryos was measured after two hour exposure at 103°C. After one day in liquid nitrogen, embryos were rewarmed in a 40°C water bath. Control as well as cryopreserved desiccated embryos were then placed on basal MS semi-solid medium without hormones for germination. Cultures were incubated at 25°C in the dark. Embryo survival was scored when the coleoptile elongated to more than half the length of the embryo. Three replicates of ten embryos were used for each trial.

3.2.2.2.2 Rapid Freezing with Addition of Cryoprotectant

Excised embryos of *Mucuna pruriens* were treated with 1 ml of cryoprotectant solution (Table 12) and kept for 30 min and then immersed directly in liquid nitrogen. Cryopreserved embryos were thawed by rapid transfer of cryotubes in a water bath at 37°C. After thawing, cryoprotectant solutions were drained from the cryotubes and replaced with a medium containing 30 per cent (w/v) of sucrose and kept for 20 min. Embryos were than transferred to MS medium free of hormones for germination. Cultures were incubated at 25°C in the dark. Embryo survival was scored when the coleoptile elongated to more than half the length of the embryo. Three replicates of ten embryos were used for the trial.

Table 11. Materials and methods tried for conservation by cryopreservation of *Clitoria ternatea*, *Mucuna pruriens* and *Indigofera tinctoria*

Plant	Materials and Methods
Clitoria ternatea	seeds: direct immersion in liquid nitrogen meristem:encapsulation dehydration; rapid freezing with addition of cryoprotectant somatic embryos: encapsulation dehydration
Mucuna pruriens	Seeds: direct immersion in liquid nitrogen zygotic embryo: simple desiccation; rapid freezing with addition of cryoprotectant meristem:encapsulation dehydration; rapid freezing with addition of cryoprotectant
Indigofera tinctoria	seed : direct immersion in liquid nitrogen meristem:encapsulation dehydration; rapid freezing with addition of cryoprotectant

Table12. Cryoprotectants tried for rapid freezing of embryo and meristem of *Clitoria ternatea*, *Mucuna pruriens* and *Indigofera tinctoria*

Treatment	Cryoprotectant
Cp1	Glycerol 0.7 M
Cp2	DMSO 10 %
Cp3	Mannitol 0.5 M
Cp4	Ethylene glycol 5 %
Cp5	Proline 6 %
Cp6	Glucose 0.7 M
Cp7	Suc rose 0.7 M
Cp8	Glycerol0.7 M + DMSO 5 %
Cp9	Glucose 0.7 M + DMSO 5 %
Cp10	Sucrose 0.7 M + DMSO 5 %

3.2.2.3 Shoot Meristem

3.2.2.3.1 Encapsulation Dehydration

3.2.2.3.1.1 Pre conditioning

Meristems or axillary buds of *Clitoria ternatea*, *Mucuna pruriens* and *Indigofera tinctoria* were inoculated on to half MS medium (devoid of glycine) supplemented with 0.5 M sucrose and 0.65 per cent agar. This was incubated at 25 $\pm$ 2°C under 12 h photoperiod at a photon flux intensity of 30-50 µmol $m^{-2}s^{-2}$ for three weeks.

3.2.2.3.1.2 Encapsulation

Pre conditioned embryos were suspended in calcium free half MS medium (devoid of glycine) supplemented with 2.5 per cent sodium alginate (Sigma, St.Louis, USA) and 0.5 M sucrose. This mixture was dispensed with a 5 ml micropipette into half strength liquid MS medium supplemented with 0.1 M calcium chloride and 10 per cent sucrose.

3.2.2.3.1.3 Pre culture

After 30 min, beads were pre cultured in modified half MS liquid medium supplemented with 0.75 M sucrose and 3 per cent DMSO dispensed into 100 ml Erlenmeyer flasks (50 beads in 25 ml medium) for one day without agitation. Beads were then transferred to fresh medium of same composition and incubated in darkness at 4°C (in a refrigerator) for three days.

3.2.2.3.1.4 Dehydration

To determine the optimum drying time, beads after pre culture were desiccated for 0-5 h in a sterile air laminar flow cabinet. Liquid nitrogen tolerance was tested for each drying time at one hour interval. Moisture content of the beads after every one hour was

determined on a fresh weight basis from three replicates of beads dehydrated prior to oven drying at 103°C for two hours.

3.2.2.3.1.5 Cryopreservation and Recovery

Dehydrated beads were transferred to 4 ml cryovial and directly immersed in liquid nitrogen where they were stored at least for one hour. On rewarming, cryotubes were removed from liquid nitrogen and transferred to water under constant circulation in a water bath maintained at 40°C for 30-60 s. The rewarmed shoot tips were transferred to recovery medium (half MS + + GA 0.5 mg l^{-1} + BA 0.2 mg l^{-1} sucrose 3 per cent + agar 0.6 per cent) and incubated in culture room under diffused light intensity for one week and then under bright light. Each treatment consists of 10 beads and was repeated thrice. The results were expressed as survival percent and / or regeneration into shoot or callus. Survival was estimated as per cent of treated shoots remaining green and showing early symptoms of *in vitro* response (swelling / shoot or callus initiation). Regeneration was estimated as percentage of nodal segments differentiated either into shoots or callus after four weeks of post- culture.

3.2.2.3.2 Rapid Freezing with Addition of Cryoprotectant

Nodal segments excised from 1 to 2 month old plantlets regenerated *in vitro* were cut into segments of about 2 mm long and treated with 1ml of cryoprotectant solution (Table 12) and kept for 30 min and then immersed directly into liquid nitrogen. Each treatment consisted of ten beads and was replicated thrice.

Cryopreserved nodal segments were thawed by rapid transfer of cryotubes in a water bath at 37°C. After thawing, cryoprotectant solutions were drained from the cryotubes and replaced with a medium containing 30 per cent (w/v) sucrose and kept for 20 min.

Nodal segment explants were transferred on to recovery medium (half strength MS + GA 0.5 mg l^{-1} + BA 0.2 mg l^{-1} + sucrose 3 per cent + agar 0.6 per cent) and cultured at 25°C under a 16 h photoperiod. Examination of the explants for survival was performed at weekly intervals. Survival was estimated as per cent of treated shoots remaining green and showing early symptoms of *in vitro* response (swelling / shoot or callus initiation). Regeneration was estimated as percentage of nodal segments differentiated either into shoots or callus after four weeks of post- culture.

3.2.2.4 Somatic Embryos

Germinated somatic embryos were preconditioned, encapsulated and precultured as described in meristem encapsulation-dehydration. To determine the optimum drying time, precultured encapsulated beads were air desiccated for 0-5 h in a laminar flow cabinet. Liquid nitrogen tolerance was tested for each drying time at one hour interval. Moisture content of the beads after every one hour was determined on a fresh weight basis from three replicates of air dehydrated beads prior to oven drying at 103°C for two hours.

Dehydrated beads were transferred to 4 ml cryovial and directly immersed in liquid nitrogen where they were stored at least for one hour. On rewarming, cryotubes were removed from liquid nitrogen and transferred to water under constant circulation in a water bath maintained at 40°C for 30-60 s. The rewarmed shoot tips were transferred to recovery medium (half MS + sucrose 3 % + GA 0.5 mg l^{-1} + BA 0.2 mgl^{-1} + agar 0.6 %) and incubated in culture room under diffused light intensity for one week and then under bright light. Each treatment consists of ten beads and was repeated thrice. The results were expressed as survival per cent (per cent embryos retaining green colour) and regeneration per cent (per cent embryos with visible elongation of shoot and root).

3.2.2.5 Statistical Analysis

Completely randomized design (Panse and Sukhatme, 1985) was followed for statistical analysis, wherever applicable.

3.2.3 Estimation of Genetic Stability of Cryopreserved Materials Using RAPD Technique

3.2.3.1 Isolation of Genomic DNA

In the three plants, *Clitoria ternatea*, *Mucuna pruriens* and *Indigofera tinctoria*, CTAB protocol (Murray and Thompson., 1980) was adopted with slight modification for DNA extraction.

Young leaves from the cryopreserved and regenerated plants as well as control plants (seedlings maintained at ambient conditions) were used for DNA extraction. Leaf material (0.5 g) was first washed in running tap water and later in distilled water two to three times after chopping the leaves. The leaves were then dried by spreading on tissue paper. The leaves were then pulverized in liquid nitrogen in a pre-cooled mortar and pestle by rapid grinding to a fine powder. Dry powder was transferred to 2 ml centrifuge tube and 1ml extraction buffer (0.7 N NaCl, 1 % CTAB, 50 mM Tris- HCl (pH 8.0) and 10 mM EDTA) was added to it. Added 100 µl PVP, 50 µl SDS (sodiumdodecylsulphate) and 20 µl β mertcaptoethanol to centrifuge tube and mixed thoroughly and was incubated in water bath at 65°C for one h. The mixture was then centrifuged at 15000 rpm for 10 min at 4°C. After collecting the upper phase, chloroform: isoamyl alcohol (24:1) extraction was repeated until the interphase disappeared. For *Mucuna pruriens,* upper phase was first extracted with phenol: chloroform: isoamyl alcohol (25:24:1) and later repeated with chloroform: isoamyl alcohol (24:1) extraction until the interphase disappeared. Then supernatant is collected; one tenth volume of 3.0 M sodium acetate followed by double volume of chilled absolute isopropyl alcohol was added to it. This was kept in the refrigerator at 4°C for 30 min and then centrifuged at 10000 rpm for 10 min at 4°C to pellet the DNA. The supernatant was discarded. The pellet was washed in 70 per cent alcohol and centrifuged at 10000 rpm for 5 min at 4°C. This step was repeated once again for *Mucuna pruriens.* The supernatant was again discarded and the pellet was air dried for 20 min. Then the pellet was dissolved in 0.5 ml of 1x Tris EDTA buffer (10mM Tris HCl, 1mM EDTA- pH 8.0) and stored at -20°C.

3.2.3.2 Quantification of DNA

The quantification of DNA is essential before it is subjected to amplification by PCR. DNA quantification was carried out with the help of UV- vis Spectrophotometer (Spectronic Genesys 5).

The buffer in which the DNA was already dissolved was taken in a cuvette to calibrate the spectrophotometer at 260 and 280 nm wavelength. The optical density (OD) values of the DNA samples dissolved in the buffer were recorded at both 260 and 280 nm.

The quantity of DNA in the sample was estimated using the following formula:

Amount of DNA (ng/µl) = A_{260} x 50 x dilution factor /1000,
where A_{260} is absorbance at 260 nm

The quality of DNA could be judged from the ratio of the OD values recorded at 260 and 280 nm. A A_{260}/A_{280} between 1.8 and 2.0 indicates good quality of DNA, where A_{280} is the absorbance at 280 nm.

3.2.3.3 Agarose Gel Electrophoresis

Agarose gel electrophoresis was carried out in a horizontal gel electrophoresis unit (Bangalore Genei). The required amount of agarose was weighed out (0.7 % for visualizing the genomic DNA and 1% for amplified products) and melted in 1 x TAE buffer (0.04 Tris acetate, 0.001 M EDTA (pH 8.0) by boiling. After cooling to about 50°C, ethidium bromide was added to a final concentration of 0.5 µg ml^{-1}.The mixture was then poured to a preset template with appropriate comb. After solidification, the comb and sealing tapes were removed and the gel was mounted in an electrophoresis tank filled with 1 x TAE buffer. The gel was completely covered on the surface by the buffer. The DNA sample was mixed with required volume of gel loading buffer (6.0 x loading dye 40 per cent sucrose, 0.25 per cent bromophenol blue). Each well was loaded with 20 µl of sample.

One of the wells was loaded with 5.0 µl of molecular weight marker along with required volume of gel loading buffer. Electrophoresis was performed at 75 volts until the loading dye reached three fourth of the length of the gel. The gel was visualized using an ultra violet visible (UV-Vis) trans illuminator (Appligene Oncor, France).

3.2.3.4 Random Amplified Polymorphic DNA (RAPD) Analysis

DNA amplification was done using arbitrarily designed primers (Operon Inc., CA, USA) adopting the procedure of William *et al.* (1990) with required modifications.

Polymerase chain reactions of genomic DNA were performed in 12.5 µl reaction mixture containing 1.25 µl 10x PCR Buffer, 5pM primer, 200 µM each of dNTPs, 0.75 units of Taq and 20ng genomic DNA. Amplification was performed in a programmable thermal controller (MJ Research, Inc.) for an initial denaturation at 95°C for three min followed by 45 cycles of denaturation at 95°C for one minute, annealing at 40°C for one minute and extension at 72°C for 2 min. The synthesis step of final cycle was extended further by 5 min. Finally products of amplification were cooled to 4°C. The DNA fragments produced and the PCR molecular weight markers were visualized in a agarose gel electrophoresis, stained with ethidium bromide and photographed with the help of gel doc system.

The genetic fidelity assessment of cryopreserved materials (seeds, meristems, zygotic and somatic embryos) of the three plant species under study was done by comparing the RAPD banding pattern with that of the parental source plant/ explant, as suggested by Zhai *et al.* (2003). The RAPD patterns of plantlets regenerated from cryopreserved seeds were compared with RAPD bands of field grown control plants and that of meristems, zygotic and somatic embryos were compared with RAPD bands of *in vitro* grown plantlets.

Results

4. RESULTS

Investigations were carried out for standardizing *in vitro* techniques for rapid propagation and conservation of medicinal plants, *Clitoria ternatea*, *Mucuna pruriens* and *Indigofera tinctoria* at the Department of Plantation Crops and Spices and at the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2001-2003. The cryopreservation experiments were conducted at the Division of Biotechnology, Tropical Botanical Garden and Research Institute, Palode during 2003-2004. The results of the study are presented in this chapter.

4.1 *IN VITRO* PROPAGATION

4.1.1 Enhanced Release of Axillary Buds

4.1.1.1 Explant(s)

Nodal segments and leaves excised from *in vitro* raised seedlings and seed cotyledons were used as explants in *C. ternatea* and *M. pruriens*. In *I. tinctoria*, leaf and nodal segments were tired. But leaves and cotyledonary explants did not give rise to shoots in any of the three cases.

4.1.1.2 Plant Growth Substances

Cytokinins and Auxin

Twenty treatments involving various combinations of plant growth substances (BA, Kn and IAA) were tried to study their effect on shoot proliferation from nodal explants in the three plant species. Result of the study is presented in Table 13.

Table 13. Effect of cytokinins and auxin on enhanced release of axillary buds from nodal explants of *Clitoria ternatea, Mucuna pruriens* and *Indigofera tinctoria*

Treatment No.	Plant growth regulators (mgl^{-1})			*Clitoria ternatea*					*Mucuna pruriens*					*Indigofera tinctoria*				
	BA	Kn	IAA	SP(%)	DBI	SpC	LLS(cm)	LpS	S.P(%)	DBI	SpC	LLS(cm)	LpS	S.P(%)	DBI	SpC	LLS(cm)	LpS
P1	0.10	-	-	100	4.67	4.00	3.50	3.61	100	7.67	2.33	3.30	2.58	100	6.00	2.50	3.45	2.90
P2	0.50	-	-	100	4.50	4.50	4.32	3.81	100	7.33	2.67	3.78	3.14	100	5.67	3.50	4.35	3.15
P3	1.00	-	-	100	4.17	4.17	4.07	3.75	100	7.17	2.67	3.82	2.94	100	5.50	2.67	4.52	3.45
P4	-	0.10	-	100	5.17	2.33	4.10	4.08	100	8.50	2.00	2.55	2.39	100	6.17	1.50	2.98	3.00
P5	-	0.50	-	100	5.17	2.50	4.33	3.86	100	8.17	2.50	2.57	1.93	100	6.33	1.67	3.22	3.08
P6	-	1.00	-	100	5.33	2.67	4.25	3.83	100	7.83	2.17	2.45	1.86	100	6.67	1.33	3.53	3.17
P7	0.50	0.10	-	100	4.83	4.67	6.03	4.44	100	6.67	3.00	3.82	2.79	100	5.67	2.00	4.43	3.58
P8	0.50	0.50	-	100	4.83	6.33	7.00	4.69	100	6.00	5.17	4.83	3.07	100	5.17	3.67	4.50	3.39
P9	0.50	1.00	-	100	4.83	4.17	4.70	3.86	100	7.33	3.33	3.52	2.31	100	6.33	2.33	4.63	3.64
P10	1.00	0.10	-	100	5.00	4.33	4.85	4.11	100	6.50	3.50	3.98	2.68	100	5.17	2.67	4.70	3.58
P11	1.00	0.50	-	100	4.83	5.50	5.10	3.85	100	6.00	3.50	5.10	3.33	100	7.00	2.00	4.00	2.75
P12	1.00	1.00	-	100	5.00	5.17	4.37	3.68	100	6.00	3.00	4.42	3.04	100	4.83	2.50	5.15	3.83
P13	0.50	-	0.05	100	4.17	3.83	3.83	3.51	100	8.00	2.17	3.33	2.56	100	6.17	2.33	3.27	2.56
P14	0.50	-	0.10	100	4.33	3.50	3.62	2.86	100	8.33	2.50	3.55	2.64	100	6.50	2.17	2.92	2.54
P15	1.00	-	0.05	100	4.33	4.00	3.70	2.98	100	7.50	2.00	3.75	2.86	100	6.33	2.50	4.23	2.86
P16	1.00	-	0.10	100	4.50	3.67	3.52	2.92	100	7.00	1.50	3.17	2.92	100	5.00	4.67	5.18	4.03
P17	-	0.50	0.05	100	4.67	2.17	2.30	2.47	100	8.33	1.67	2.47	2.00	100	7.00	1.33	2.90	2.50
P18	-	0.50	0.10	100	5.00	2.00	2.18	2.42	100	8.50	2.17	2.38	1.81	100	7.33	1.17	2.57	2.25
P19	-	1.00	0.05	100	4.67	2.33	1.97	2.25	100	8.83	2.00	2.13	1.63	100	7.00	1.50	2.88	2.36
P20	-	1.00	0.10	100	4.17	2.17	1.83	2.17	100	9.00	2.00	2.08	1.61	100	7.67	1.50	2.78	2.17
P21	0.50	0.50	0.05	100	4.00	4.67	4.58	3.70	100	7.67	3.00	3.47	2.40	100	6.50	2.50	3.02	2.67
P22	0.50	0.50	0.10	100	4.50	3.83	4.47	3.44	100	8.00	2.17	3.38	2.11	100	6.67	2.33	2.88	2.53
Control	-	-	-	100	5.67	1.00	2.00	2.83	100	7.33	1.00	4.52	3.33	100	7.83	1.00	2.75	3.17
CD (5%)	-	-	-	-	0.86	0.82	0.91	0.65	-	1.31	0.89	1.10	0.71	-	1.23	0.90	1.20	0.76
FV(5 %)	-	-	-	-	1.85	19.81	15.68	8.98	-	3.63	7.14	4.70	4.41	-	3.54	7.33	3.97	3.74

The data represent mean of six replications; Culture medium: MS

SP: Survival; DBI: Days for bud initiation; SpC: Shoot /culture; LLS: Length of longest shoot; LpS: leaves / shoot

All the treatments with plant growth substances registered cent per cent survival rate showing growth and multiple shoot production from the node in all the three plant species. In all the three plant species, though survival was 100 per cent, control plants, in the medium with no growth regulators produced only one shoot per culture in all the replications.

In *C. ternatea*, there was not much variation between different treatments with regard to number of days for bud initiation. Bud initiation was earliest (4 days) when cultured in P21 (BA 0.5 mgl^{-1} + Kn 0.5 mgl^{-1} + IAA0.05 mgl^{-1}) and late (5.67 days) in control plants.

With regard to number of shoots, *C. ternatea* produced maximum number of shoots (6.33) per culture in P8 (BA 0.5 mg l^{-1} + Kn 0.5 mg l^{-1}). The least number of shoots (2.00) per culture was recorded by P18 (Kn 0.5 mg l^{-1} and IAA 0.1 mgl^{-1}) which was on par with P4, P5, P6, P17, P19 and P20.

Maximum length of the longest shoot (7.00 cm) was recorded by P8 (BA 0.5 mg l^{-1} + Kn 0.5 mg l^{-1}) followed by P7 in *C. ternatea*. The length of the longest shoot was minimum (1.83 cm) in P20 (Kn 1.0 mg l^{-1} + IAA 0.1 mg l^{-1}) which was on par with P17, P18, P19 and control. When BA was supplemented in the medium length of the longest shoot showed increasing tendency upto 0.5 mg l^{-1} but decreased with higher concentration. Kn also showed same trend with regard to shoot length.

Treatment P8 (BA 0.5 mg l^{-1} and Kn 0.1 mgl^{-1}) produced maximum number of leaves (4.69) per shoot and was on par with P4, P7, and P10 in *C. ternatea*. Least number of leaves (2.17) per shoot was recorded in P20 (Kn 1.00 mg l^{-1} + IAA 0.10 mg l^{-1}) which was on par with P17, P18 and P19.

In *M. pruriens* bud initiation was earliest (6 days) in cultures P8 (BA 0.5 mg l^{-1} + Kn 0.5 mgl^{-1}), P11 (BA1.00 mgl^{-1} + Kn 0.50 mgl^{-1}) and P12 (BA 1.0 mgl^{-1} + Kn 1.0 mgl^{-1}) which were on par with P3, P7, P10 and P16. Bud initiation was late (9

days) in P20 (Kn 1.0 mgl^{-1} + IAA 0.1 mgl^{-1}). P4, P5, P6, P13, P14, P17, P18, P19 and P22 were found to be on par with P20.

In *M. pruriens* also P8 (BA 0.5 mg l^{-1} and Kn 0.5 mg l^{-1}) recorded maximum (5.17) shoot proliferation. Minimum number of shoot (1.5) per culture was obtained in P16 (BA 1.0 mg l^{-1} + IAA 0.1 mg l^{-1}) which was on par with P1, P4, P6, P13, P15, P17, P18, P19, P20 and P22.

In *M. pruriens*, P11 (BA 1.0 mg l^{-1} and Kn 0.5 mg l^{-1}) recorded maximum length of the longest shoot (5.1 cm) which was on par with P8, P12 and control. Length of the longest shoot was least (2.08 cm) in P20 (Kn 1.0 mg l^{-1} + IAA 0.1 mg l^{-1}) which was on par with P4, P5, P6, P16, P17, P18 and P19.

P11 (BA 1.0 mg l^{-1} + Kn 0.5 mg l^{-1}) recorded maximum number of leaves (3.33) per shoot in *M. pruriens* and was on par with P2, P3, P7, P8, P10, P11, P12, P14, P15, P16 and control. Number of leaves per shoot was minimum (1.61) in P 20 (Kn 1.0 mg l^{-1} + IAA 0.1 mg l^{-1}). This was on par with P5, P6, P9, P17, P18, P19 and P22.

In *I. tinctoria* earliest bud initiation (4.83 days) was obtained in culture P12 (BA 1.0 mgl^{-1} + Kn 1.0 mgl^{-1}) which was on par with P1, P2, P3, P7, P8, P10 and P16. Control plants (medium with out any growth regulators) registered late (7.83 days) bud initiation. This was on par with P6, P11, P17, P18, P19, P20 and P22.

In *I. tinctoria* number of shoots per culture was highest (4.67) in P16 (BA 1.0 mg l^{-1} + IAA 0.1 mg l^{-1}) followed by P2 (BA 0.5) and lowest (1.17) in P18 which was on par with P4, P5, P6, P7, P16, P18, P19 and P20.

I. tinctoria recorded maximum length of the longest shoot (5.18 cm) in P16 (BA 1.0 mg l^{-1} + IAA 0.1 mg l^{-1}) which was on par with P2, P3, P7, P8, P9, P10, P11, P12 and P15. While length of the longest shoot was minimum (2.57 cm) in P18 (Kn

0.5 mg l^{-1} + IAA 0.1 mg l^{-1}). This was on par with P1, P4, P5, P6, P13, P14, P17, P18, P19, P20, P21, P22 and control.

In *I. tinctoria* maximum number of leaves (4.03) per shoot was recorded in treatment P16 (BA 1.0 mg l^{-1} + IAA 0.1 mg l^{-1}) which was on par with P3, P7, P8, P9, P12 and P16. P20 (Kn 1.0 mg l^{-1} + IAA 0.1 mg l^{-1}) recorded lowest number of leaves (2.17) per shoot and was on par with P1, P11, P13, P14, P15, P17, P18, P19, P21 and P22.

In *C. ternatea* and *I. tinctoria* bud initiated within a week in most of the cultures. In *M. pruriens* bud initiation took 6-9 days. Bud initiation is indicated by swelling of axillary buds, which later develops into shoot (Plates 2a, 2b and 2c).

P8 (BA 0.5 mg l^{-1} and Kn 0.5 mg l^{-1}) recorded maximum shoot proliferation in *C. ternatea* and *M. pruriens*. P16 (BA 1.00 mg l^{-1} + IAA 0.10 mg l^{-1}) recorded maximum number of shoots per culture in *I. tinctoria* but least in *M. pruriens*.

In all the three species, number of shoots per culture, length of the longest shoot and number of leaves per shoot were less in the treatments with Kn alone or Kn in combination with IAA. Least value with regard to all the above parameters were recorded in cultures where Kn is present in combination with IAA.

The effect of various treatments on enhanced release of axillary buds in *C. ternatea, M. pruriens* and *I. tinctoria* are illustrated in Figs. (1, 2 and 3) and Plate 2.

Gibberellic Acid

The effect of three different levels of gibberellic acid were also studied in the three plant species taking the best treatment in Table (13) as control. Results of the study are presented in Table 14.

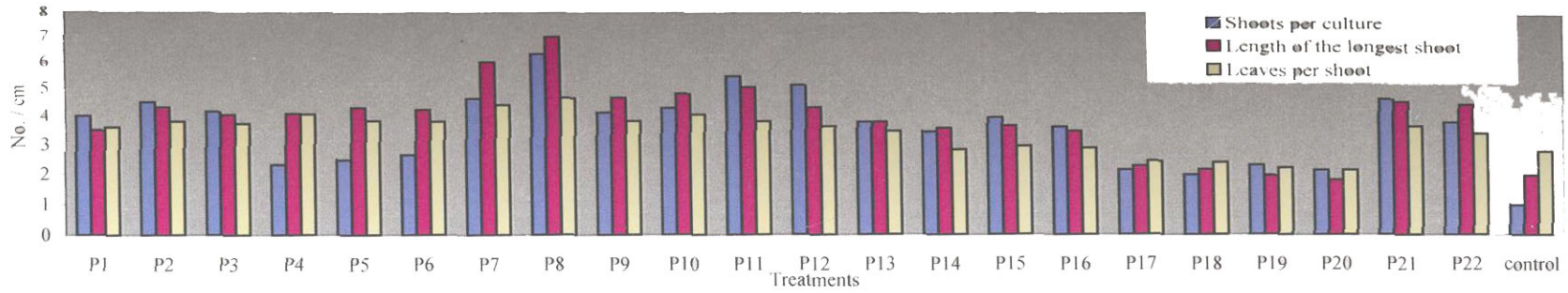


Fig 1. Effect of cytokinins and auxin (IAA) on enhanced release of axillary buds from nodal explants of *Clitoria ternatea*

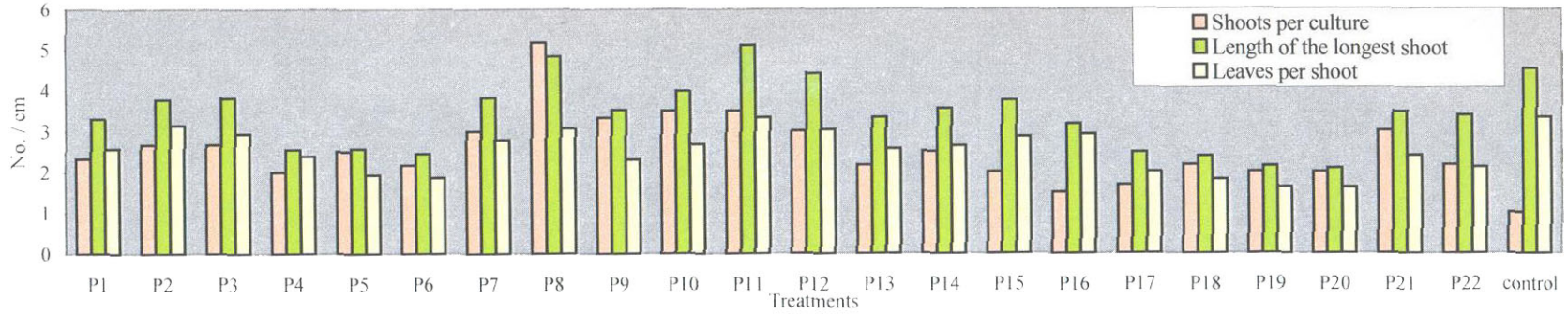


Fig 2. Effect of cytokinins and auxin (IAA) on enhanced relaese of axillary buds from nodal explants of *Mucuna pruriens*

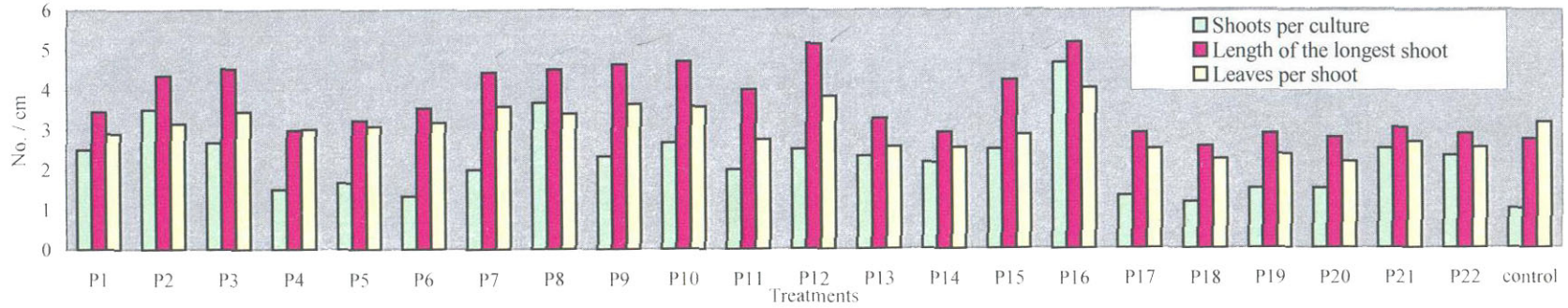


Fig 3. Effect of cytokinins and auxin (IAA) on enhanced relaese of axillary buds from nodal explants of *Indigofera tinctoria*

Plate 2. Enhanced release of axillary buds from nodal explants of *Clitoria ternatea*, *Mucuna pruriens* and *Indigofera tinctoria*

a. Bud break in *C. ternatea*

b. Bud break in *M. pruriens*

c. Bud break in *I. tinctoria*

d. Shoot proliferation in *C.ternatea* in culture medium MS + Kn 0.5 mg l^{-1}

e. Shoot proliferation in *C.ternatea* in culture medium MS + BA 0.5 mg l^{-1}

f. Shoot proliferation in *C.ternatea* in culture medium MS + BA 0.5 mg l^{-1} + Kn 0.5 mg l^{-1}

g. Shoot proliferation in *M. pruriens* in culture medium MS + Kn 0.5 mg l^{-1}

h. Shoot proliferation in *M. pruriens* in culture medium MS + BA 0.5 mg l^{-1}

i. Shoot proliferation in *M. pruriens* in culture medium MS + BA 0.5 mg l^{-1} + Kn 0.5 mg l^{-1}

j. Shoot proliferation in *I. tinctoria* in culture medium MS + Kn 0.5 mg l^{-1}

k. Shoot proliferation in *I. tinctoria* in culture medium MS + BA 0.5 mg l^{-1} + Kn 0.5 mg l^{-1}

l. Shoot proliferation in *I. tinctoria* in culture medium MS + BA 1.0 mg l^{-1} + IAA 0.1 mg l^{-1}

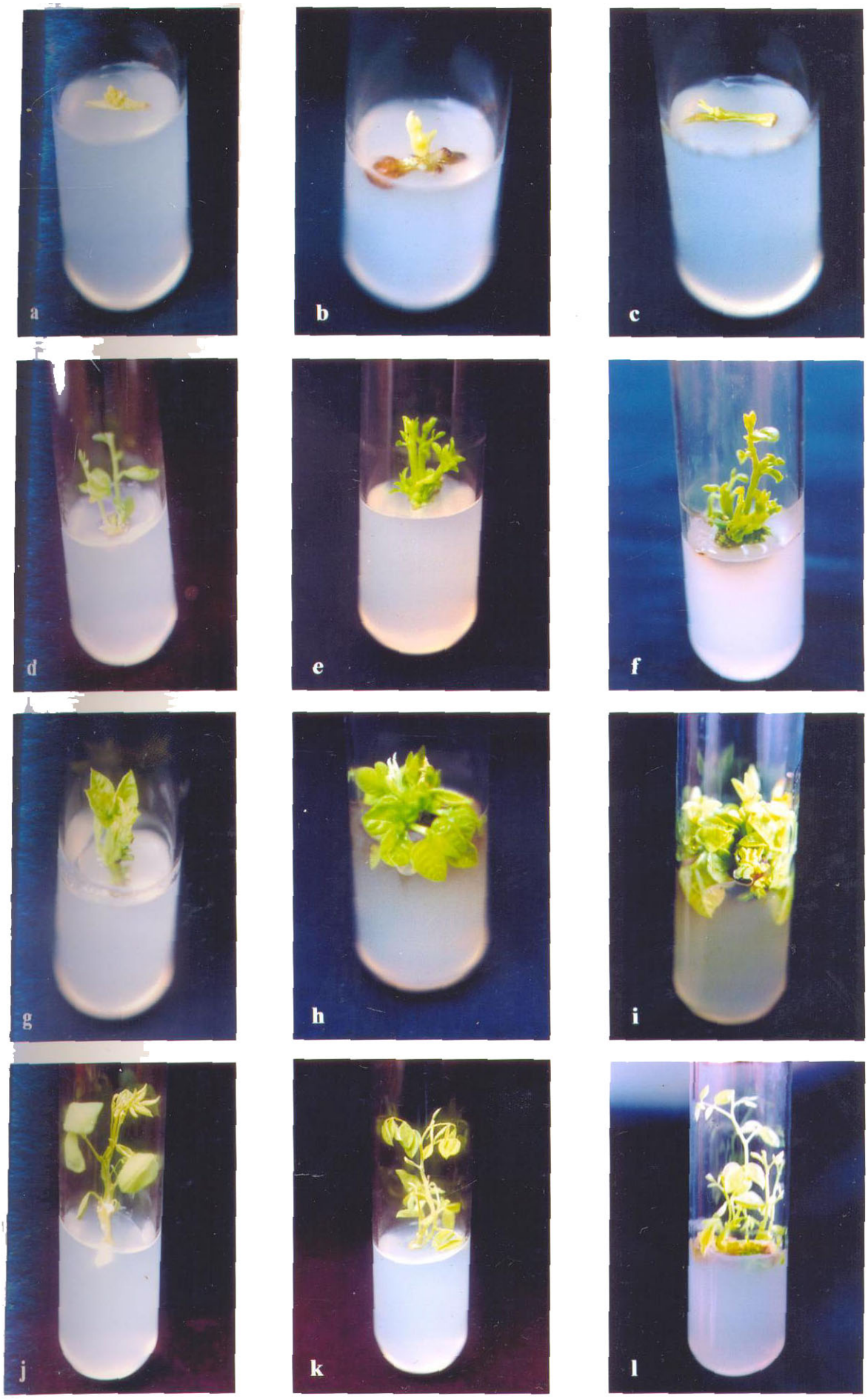


Plate 2. Enhanced release of axillary buds from nodal explants of *Clitoria ternatea*, *Mucuna pruriens* and *Indigofera tinctoria*

It was observed that in *C. ternatea* gibberellic acid did not have any significant influence on number of shoots. Length of the longest shoot was maximum (7.38 cm) in G3 (GA 3.0 mg l^{-1}) which was on par with G2 (GA 2.0 mg l^{-1}) and control. G1 (GA 1 mg l^{-1}) was significantly inferior to all other treatments which recorded the lowest shoot length (6.38 cm). With regard to number of leaves per shoot, control recorded maximum number (4.69) which was significantly superior to all other levels of gibberellic acid tried. G1 recorded the lowest number of leaves per shoot and was found on par with G3.

In *M. pruriens* and *I. tinctoria*, gibberellic acid did not have any significant influence on the number of shoots, length of longest shoot and number of leaves per shoot when compared to the control. There was increase in plant height with increase in levels of GA in the medium. In all the three species, control recorded maximum leaf number. This indicates that GA caused internodal elongation.

4.1.1.3 Basal Medium

Basal media such as MS (quarter, half, full, full + activated charcoal), SH, B5 and WPM were compared to assess their effect on shoot proliferation in the three plant species. Results of the study are given in Table 15.

Full MS was found to be significantly superior to others with respect to number of shoots, length of the longest shoot and number of leaves per shoot in *C. ternatea* (Fig. 4). Maximum number of shoots (6.33) was produced by cultures in full strength MS whereas WPM, B5, SH, quarter MS, half MS and MS with activated charcoal were significantly inferior to MS but on par with each other. Maximum value for length of the longest shoot (7 cm) and number of leaves (4.69) per shoot was recorded by the cultures in MS and minimum in B5 (1.47 cm and 1.67 respectively) which is on par with quarter MS, half MS and MS with activated charcoal.

Table 14. Effect of gibberellic acid on enhanced release of axillary buds from nodal explants of *Clitoria ternatea, Mucuna pruriens and Indigofera tinctoria*

Treatment No.	GA(mgl^{-1})	*Clitoria ternatea*			*Mucuna pruriens*			*Indigofera tinctoria*		
		SpC	LLS	LpS	SpC	LLS	LpS	SpC	LLS	LpS
G1	1	6.00	6.38	3.11	5.00	4.90	2.69	4.50	5.20	3.61
G2	2	6.33	7.08	3.42	5.33	4.93	2.72	4.67	5.22	3.71
G3	3	5.83	7.38	3.69	4.83	4.95	2.73	4.50	5.43	4.00
Control	-	6.33	7.00	4.69	5.17	4.83	3.07	4.67	5.18	4.03
CD (5%)	-	NS	0.51	0.55	NS	NS	NS	NS	NS	NS
FV (5%)	-	NS	5.90	13.54	NS	NS	NS	NS	NS	NS

The data represent mean of six replications; Culture medium: MS+ best treatment in Table 13
SpC: Shoot / culture; LLS: Length of longest shoot; LpS: Leaves / shoot

Table 15. Effect of basal media on enhanced release of axillary buds from nodal explants of *Clitoria ternatea, Mucuna pruriens and Indigofera tinctoria*

Treatment No.	BM	*Clitoria ternatea*			*Mucuna pruriens*			*Indigofera tinctoria*		
		SpC	LLS	LpS	SpC	LLS	LpS	SpC	LLS	LpS
M1	¼ MS	1.33	1.57	1.83	2.00	2.42	2.20	2.50	3.32	3.45
M2	½ MS	1.83	1.98	2.00	2.17	3.57	2.72	3.00	3.65	3.74
M3	MS	6.33	7.00	4.69	5.17	4.83	3.07	4.67	5.18	4.03
M4	MS+AC	1.67	2.33	2.53	3.00	3.10	2.21	3.17	2.82	3.36
M5	SH	1.33	2.67	2.81	2.50	2.80	2.08	4.00	3.18	3.40
M6	B5	1.33	1.47	1.67	2.83	3.55	2.53	3.00	2.83	3.32
M7	WPM	1.33	3.17	3.33	2.67	2.98	2.29	2.33	3.13	3.42
CD(5%)	-	0.71	1.10	0.95	0.87	1.05	0.52	0.95	1.28	NS
FV (5%)	-	55.91	25.10	10.12	12.16	4.51	3.81	6.24	3.04	NS

The data represents mean of six replications; Culture medium: MS+ best treatment in Table 13
SpC: Shoot / culture; LLS: Length of longest shoot; LpS: Leaves / shoot

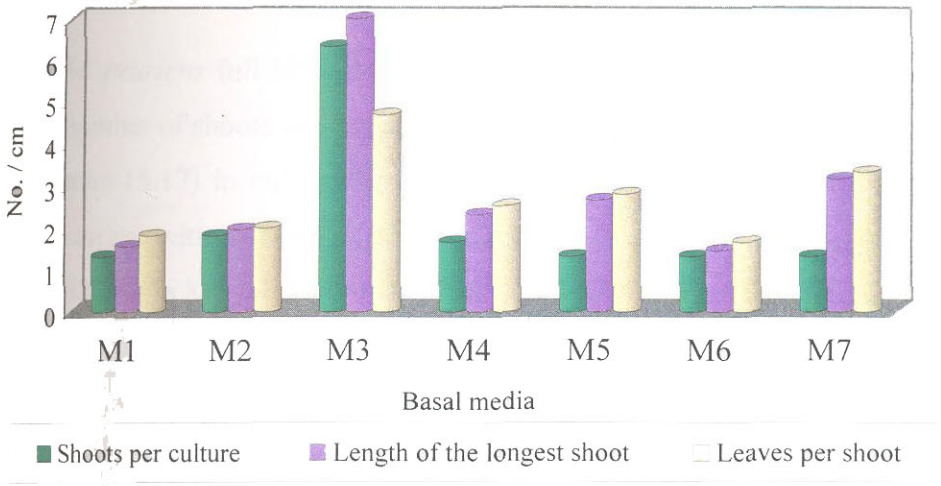


Fig 4. Effect of basal media on enhanced release of axillary buds from nodal explants of *Clitoria ternatea*

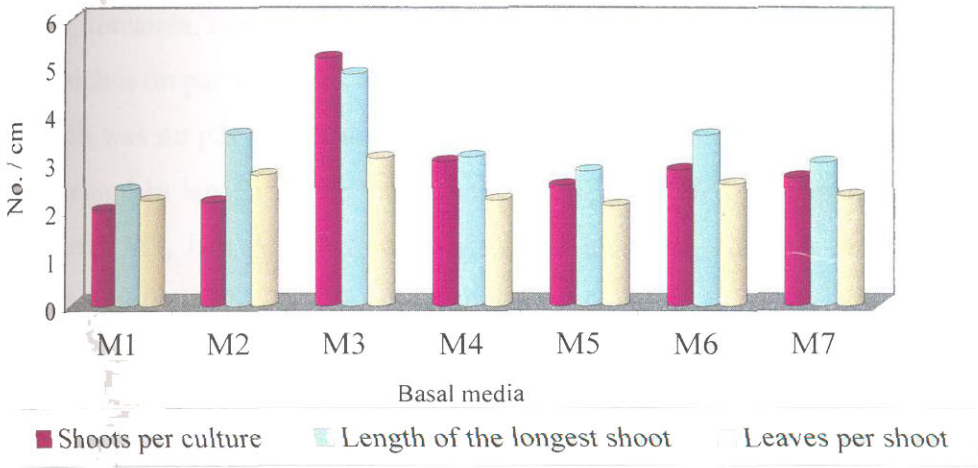


Fig 5.Effect of basal media on enhanced release of axillary buds from nodal explants of *Mucuna pruriens*

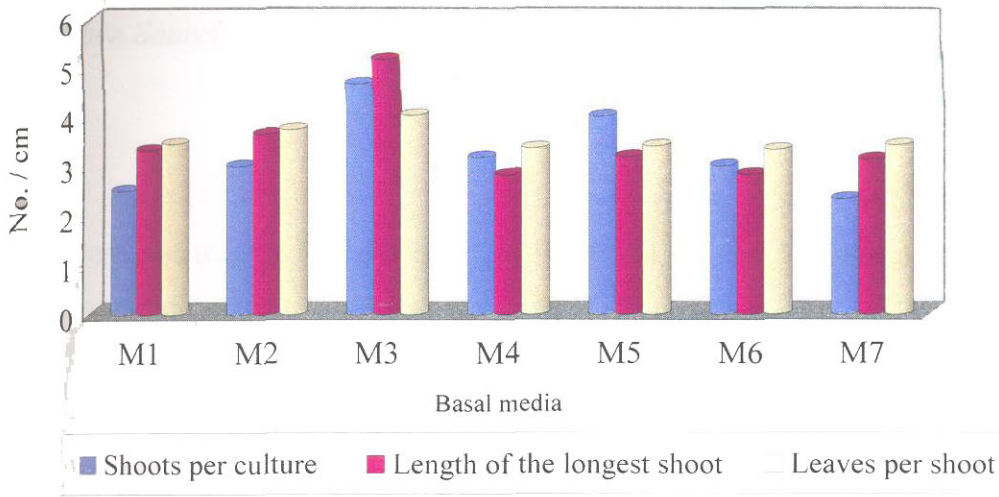


Fig 6. Effect of basal media on enhanced release of axillary buds from nodal explants of *Indigofera tinctoria*

In *M. pruriens* full MS was found to be significantly superior to others with respect to number of shoots and length of the longest shoot (Fig 5). Number of shoots was maximum (5.17) in cultures grown in MS and minimum (2.0) in quarter MS which was on par with half MS, SH, B5 and WPM. Length of the longest shoot was highest (4.83 cm) in MS whereas quarter MS, MS with activated charcoal, SH, WPM were found to be significantly inferior to MS but on par with each other. Maximum number of leaves per shoot was recorded by cultures in MS which was on par with half MS. Quarter MS, MS with activated charcoal, SH, B5 and WPM were significantly inferior to MS but on par with each other.

In *I. tinctoria*, maximum number of shoots (4.67) was recorded by cultures in full MS which is on par with SH. Minimum number of shoots (2.33) were recorded in WPM which was on par with quarter MS, half MS, MS with activated charcoal and B5. With regard to length of the longest shoot, MS recorded maximum value (5.18 cm). Quarter MS, half MS, MS with activated charcoal, SH, B5 and WPM was significantly inferior to MS but on par with each other (Fig. 6). Whereas, basal media did not significantly influence the number of leaves per shoot in *I. tinctoria.*

Full MS was found to be the most suitable media for shoot proliferation in *C. ternatea, M. pruriens* and *I. tinctoria.*

4.1.1.4 Carbon Source

Sucrose

Sucrose was tried at four different levels (20, 30, 40 and 50 g l^{-1}) to assess its effect on shoot proliferation. Results are presented in Table 16.

There was significant variation among different levels of sucrose with regard to number of shoots per culture, length of the longest shoot and number of leaves per shoot in *C. ternatea.* Maximum number of shoots (6.33) per culture was recorded by

S2 (30 g l^{-1}) which was on par with S3 (40 g l^{-1}) and S4 (50 g l^{-1}). S1 (20 g l^{-1}) recorded least number of shoots (4.0) per culture and was significantly inferior to all others. With regard to length of the longest shoot, S2 recorded maximum value (7.0 cm) and is on par with S3 and minimum value (5.33 cm) recorded by S1 and is on par with S4. In case of number of leaves per shoot, S2 was found significantly superior (4.69) to all other levels of sucrose. Least number of leaves (3.54) per shoot was recorded in S4 which was on par with S1 and S3.

In *M. pruriens*, among the four different levels of sucrose, S3 recorded maximum number of shoots (5.5) per culture and was on par with S2 and S4. S1 was significantly inferior to all other levels of sucrose. Different levels of sucrose did not evoke any significant difference with regard to length of the longest shoot and number of leaves per shoot.

In case of *I. tinctoria*, there was no significant difference in number of shoots produced per culture, length of the longest shoot and number of leaves per shoot among the different levels of sucrose tried.

Glucose

The results of the trial conducted to study the effect of four different levels of glucose (20, 30, 40 and 50 gl^{-1}) on shoot proliferation in *C. ternatea, M. pruriens* and *I. tinctoria* are presented in Table 16.

In *C. ternatea*, glucose at various levels were found to be significantly inferior to control having sucrose at 30 gl^{-1} in the medium with regard to number of shoots per culture, length of the longest shoot and number of leaves per shoot.

In case of *M. pruriens*, control recorded maximum number of shoots (5.17) per culture which was on par with T3. T1 registered minimum number (2.33) of shoots per culture which was on par with T2 and T4. Different levels of glucose did

not show any significant difference with that of control with regard to length of the longest shoot and number of leaves per shoot.

In *I. tinctoria*, there was no significant difference in number of shoots produced per culture, length of the longest shoot and number of leaves per shoot among the different levels of sucrose tried. However, maximum number of shoots per culture, length of the longest shoot and number of leaves per shoot was registered on control plants.

4.1.1.5 Ethylene Inhibitor: Cobaltous chloride

Cobaltous chloride tried at two levels 10 mg l^{-1} and 15 mg l^{-1} did not produce any significant difference with regard to number of shoots and length of the longest shoot in *C. ternatea* (Table 16). With regard to number of leaves per shoot, control plant recorded maximum value and was significantly superior to two levels of cobaltous chloride, while the two levels of cobaltous chloride were on par.

Two levels of cobaltous chloride did not evoke any significant variation with regard to number of shoots, length of the longest shoot and number of leaves per shoot in *M. pruriens* and *I. tinctoria* (Table 16).

4.1.1.6 Gelling Agent: Agar

Agar was tried at four different levels (5, 6, 7 and 8 g l^{-1}) to assess its effect on shoot proliferation in the three plant species. Results of the study are presented in Table 16.

In *C. ternatea*, there was significant variation with regard to number of shoot per culture. Maximum number of shoots (6.67) per culture was obtained in AG1 (agar 5 g l^{-1}) which was on par with AG 2 (agar 6 g l^{-1}) and AG3 (agar 7 g l^{-1}) whereas AG4 (agar 8 g l^{-1}) recorded minimum number of shoots (4.67). There was no

Table 16. Effect of carbon source, ethylene inhibitor and gelling agent on enhanced release of axillary buds from nodal explants of Clitoria ternatea, Mucuna pruriens and Indigofera tinctoria

Treatment			*Clitoria ternatea*			*Mucuna pruriens*			*Indigofera tinctoria*		
Carbon source			SpC	LLS	LpS	SpC	LLS	LpS	SpC	LLS	LpS
Sucrose (gl^{-1})	S1	20	4.00	5.33	3.64	3.17	4.10	2.55	4.17	4.47	3.69
	S2	30	6.33	7.00	4.69	5.17	4.83	3.07	4.67	5.18	4.03
	S3	40	6.00	6.68	3.87	5.50	4.90	3.11	4.67	4.68	3.75
	S4	50	5.50	5.55	3.54	4.67	4.07	2.52	5.17	4.85	3.27
	CD (5%)		1.23	0.97	0.79	1.33	NS	NS	NS	N	NS
	FV (5%)		6.12	6.30	3.83	5.20	NS	NS	NS	NS	NS
Glucose (gl^{-1})	T1	20	3.17	4.18	2.97	2.33	4.22	2.76	2.67	3.73	3.17
	T2	30	3.83	4.72	3.12	3.00	4.38	2.89	3.17	4.35	3.75
	T3	40	3.67	4.80	2.95	4.00	4.45	2.97	3.50	4.70	3.76
	T4	50	2.67	4.15	2.67	3.33	4.18	2.64	3.67	3.17	3.33
	Control	-	6.33	7.00	4.69	5.17	4.83	3.07	4.67	5.18	4.03
	CD (5%)		0.94	1.19	0.62	1.20	NS	NS	NS	NS	NS
	FV (5%)		19.23	8.30	14.35	6.83	NS	NS	NS	NS	NS
Ethylene inhibitor(mgl^{-1})											
Cobaltous chloride (mgl^{-1})	CCl1	5	6.00	6.38	3.71	5.00	4.77	2.86	4.50	5.08	3.81
	CCl2	10	5.67	6.30	3.70	4.67	4.70	2.81	4.67	5.05	3.92
	Control	-	6.33	7.00	4.69	5.17	4.83	3.07	4.67	5.18	4.03
	CD (5%)		NS	NS	0.66	NS	NS	NS	NS	NS	NS
	FV (5%)		NS	NS	6.75	NS	NS	NS	NS	NS	NS
Gelling agent											
Agar (gl^{-1})	AG1	5	6.67	6.38	4.70	5.5	5.07	3.15	5.33	5.70	4.09
	AG2	6	6.50	6.71	4.73	5.33	4.88	3.08	5.17	5.37	4.08
	AG3	7	6.33	7.00	4.69	5.17	4.83	3.07	4.67	5.18	4.03
	AG4	8	4.67	6.30	4.42	3.33	4.42	3.06	4.00	4.53	4.02
	CD (5%)		0.83	NS	NS	1.04	NS	NS	NS	NS	NS
	FV (5%)		10.84	NS	NS	8.15	NS	NS	NS	NS	NS

The data represents mean value of six replications; Culture medium: MS+ best treatment in Table 13
SpC: Shoot / culture; LLS: Length of the longest shoot; LpS: Leaves / shoot

significant variation among the treatments with regard to length of the longest shoot and number of leaves per shoot.

Significant variation was observed with regard to number of shoots per culture among various levels of agar tried in *M. pruriens*. Maximum number (5.5) was registered in AG1 which is on par with AG2 and AG3; while AG4 recorded minimum number (3.33). No significant variation was observed among treatments for length of the longest shoot and number of leaves per shoot.

In *I. tinctoria*, there was no significant difference in number of shoots produced per culture, length of the longest shoot and number of leaves per shoot, among the different levels of agar tried.

4.1.2 Somatic (Indirect) Organogenesis

4.1.2.1 Callus Initiation

Twenty eight treatments with different combinations of plant growth substances (2, 4-D, NAA and BA) were tried to assess its effect on callus initiation, using various explants of *Clitoria ternatea*, *Mucuna pruriens* and *Indigofera tinctoria*. Stem, leaf and cotyledon were used as explants in *C. ternatea* and *M. pruriens* and in *I. tinctoria* stem and leaf were used as explants. The effect of various treatments on callus initiation in the three plant species is illustrated in Plate 3.

The effect of plant growth substances on callus initiation from leaf, stem and cotyledonary explants of *C. ternatea* are presented in Table 17 .Cent per cent cultures initiated callus with stem explant of *C. ternatea* in treatments, C4, C7, C8, C17, C18 and C28. But C7, C8 and C18 registered low growth score. Highest growth score (3.67) was registered by C28 (NAA 2.0 mg l^{-1} + BA 0.5 mg l^{-1}) followed by C17 (2,4-D 2.0 mg l^{-1} + BA 0.5 mg l^{-1}). The lowest growth score (1.00) was registered by C1 (2, 4-D 0.1 mg l^{-1}), C10 (2,4-D 0.1 mg l^{-1} + BA 0.1 mg l^{-1}) , C18 (2,4-D 0.5 mg l^{-1}

Table 17. Effect of plant growth substances on callus initiation from stem, leaf and cotyledonary explants of Clitoria ternatea

Treatment	Plant growth regulators (mgl^{-1})			Stem				Leaf				Cotyledon			
No.	2,4-D	NAA	BA	CIC(%)	G	CI	NC	CIC(%)	G	CI	NC	CIC(%)	G	CI	NC
C1	0.10	-	-	33.33	1.00	33.33	Y.F	66.67	1.00	66.67	Y.F	50.00	1.00	50.00	Cw
C2	0.50	-	-	33.33	2.00	66.67	Y.F	66.67	1.25	83.34	Y.F	50.00	1.33	66.50	Cw
C3	1.00	-	-	83.33	1.60	133.33	Y.F	66.67	1.25	83.34	Y.F	66.67	1.00	66.67	Cw
C4	2.00	-	-	100.00	2.50	250.00	Y.F	33.33	2.50	83.33	Y.F	66.67	1.50	68.17	Cw
C5	-	-	0.10	*	*	*	*	*	*	*	*	*	*	*	*
C6	-	-	0.50	*	*	*	*	*	*	*	*	*	*	*	*
C7	-	0.50	-	100.00	1.33	133.00	Lb.C	*	*	*	R	100.00	1.33	133.00	Y.R
C8	-	1.00	-	100.00	1.50	150.00	Lb.C	*	*	*	R	100.00	1.50	150.00	Y.R
C9	-	2.00	-	83.33	1.60	133.33	Lb.C	*	*	*	R	83.33	1.40	116.66	Y.R
C10	0.10	-	0.10	33.33	1.00	33.33	Y.C	33.33	1.00	33.33	Y.C	33.33	1.00	33.33	Lb
C11	0.50	-	0.10	50.00	3.00	150.00	Y.C	100.00	2.83	283.00	Y.G.F	33.33	1.50	50.00	Lb
C12	1.00	-	0.10	33.33	2.00	66.66	Y.C	33.33	2.00	66.66	Y.C	33.33	1.50	50.00	Lb
C13	2.00	-	0.10	83.33	1.20	100.00	Y.C	66.67	1.25	83.34	Y.C	66.67	2.75	183.34	Lb
C14	0.10	-	0.50	50.00	2.00	100.00	Y.C	50.00	2.00	100.00	Y.C	33.33	1.00	33.33	Lb
C15	0.50	-	0.50	33.33	3.00	99.99	Y.C	33.33	1.50	50.00	Y.C	33.33	1.00	33.33	Lb
C16	1.00	-	0.50	50.00	1.33	66.50	Y.C	100.00	1.50	150.00	Y.C	66.67	2.00	133.34	Lb.Gb
C17	2.00	-	0.50	100.00	3.50	350.00	Y.C	83.33	1.80	150.00	Y.C	100.00	2.67	267.00	Lb.Gb
C18	0.50	0.50	-	100.00	1.00	100.00	Lb	33.33	2.00	66.66	Y.C	50.00	2.00	100.00	Cw.C
C19	1.00	0.50	-	66.67	1.50	100.01	Lb	33.33	2.00	66.66	Y.C	50.00	2.33	116.50	Cw.C
C20	2.00	0.50	-	83.33	1.00	83.33	Lb	83.33	1.40	116.66	Y.C	66.67	2.25	150.01	Cw.C
C21	1.00	1.00	-	83.33	1.00	83.33	Lb	83.33	1.80	150.00	Y.C	66.67	2.00	133.34	Cw.C
C22	2.00	1.00	-	83.33	1.00	100.00	Lb	83.33	2.00	166.66	Y.C	83.33	1.60	133.33	Cw.C
C23	-	0.50	0.10	66.67	1.25	83.34	Lb	66.67	1.25	83.34	W.C	66.67	1.00	66.67	Y.C
C24	-	1.00	0.10	33.33	2.00	66.67	Lb	66.67	1.50	100.01	W.C	66.67	1.00	66.67	Y.C
C25	-	2.00	0.10	83.33	1.00	83.33	Lb	83.33	1.40	116.66	W.C	83.33	1.00	83.33	Y.C
C26	-	0.50	0.50	50.00	2.00	100.00	Lb	83.33	1.60	133.33	W.C	66.67	1.25	83.34	Y.C
C27	-	1.00	0.50	83.33	2.00	166.67	Lb.Gb	100.00	3.00	300.00	W.C	83.33	1.00	83.33	Y.C
C28	-	2.00	0.50	100.00	3.67	367.00	Lb.Gb	66.67	1.50	100.01	W.C	83.33	1.20	100.00	Y.C
Control	-	-	-	*	*	*	*	*	*	*	*	*	*	*	*

The data represents mean of six replications; Culture medium: MS; * indicates no callus formation

CIC: cultures initiating callus; G: Growth score; CI: callus index; NC: nature of callus; Y.F: yellow friable; Y.G.F: Yellow granular friable; Lb light brown compact; Y.R- yellow callus with root initials; Y.N: Yellow nodular W.C: white compact; Y.C: yellow compact; Cw: creamy whit root directly from cut ends; Lb Gb: light brown callus with green buds

+ NAA 0.5 mg l^{-1}), C20 (2,4-D 2.0 mg l^{-1} + NAA 0.5 mg l^{-1}) , C21 (2,4-D 1.0 mg l^{-1} + NAA 1.0 mg l^{-1}), C22 (2,4-D 2.0 mg l^{-1} + NAA 1.0 mg l^{-1}) and C25 (NAA 2.0 mg l^{-1} + BA 0.1 mg l^{-1}). Treatment C28 recorded the highest callus index (367) followed by C17 (350). Lowest callus index (33.33) was recorded by C1 and C10.

Using leaf explant of *C. ternatea*, treatments C11, C16 and C27 recorded cent per cent callus initiation. The highest growth score (3.0) was registered in C27 (NAA 1.0 mg l^{-1} + BA 0.5 mg l^{-1}) followed by C 11 (2, 4-D 0.5 mg l^{-1} + BA 0.1 mg l^{-1}). Growth score was least (1.0) in treatments C1 (2, 4-D 0.1) and C10 (2,4-D 0.1 mg l^{-1} + BA 0.1 mg l^{-1}). Maximum callus index (300) was registered by treatment C27 followed by C11 (283). C10 recorded minimum callus index (33.33).

In case of cotyledonary explant of *C. ternatea* C7, C8 and C17 recorded cent per cent callus initiation. But C7 and C8 recorded low growth score. The highest growth score (2.67) was registered by C17 (2,4-D 2.0 mg l^{-1} + BA 0.5 mg l^{-1}) and least 1.0 by C1, C3, C10, C14, C15, C23, C24 and C25.

Among the three different explants tried for callus initiation in *C. ternatea*, stem explant recorded maximum growth score and callus index. Cotyledonary explant recorded lowest growth score and callus index.

Nature of callus varied with different explants and different treatment combinations (Table 17). In case of leaf explant, root initials were produced from the cut ends directly in cultures with NAA alone; while yellow compact and white compact callus were obtained in cultures with NAA in combination with 2, 4-D and NAA in combination with BA respectively.

The effect of plant growth substances on callus initiation from leaf, stem and cotyledonary explants of *M. pruriens* are presented in Table 18. With regard to stem explant of *M. pruriens*, in C13 (2, 4-D 2.0 mg l^{-1} + BA 0.1 mg l^{-1}) and C21 (2, 4-D 1.0 mg l^{-1} + NAA 1.0 mg l^{-1}) 83.33 per cent of cultures initiated callus. None of the

Table 18. Effect of plant growth substances on callus initiation from stem, leaf and cotyledonary explants of Mucuna pruriens

Treatment	Plant growth regulators (mgl^{-1})			Stem				Leaf				Cotyledon			
No.	2,4-D	NAA	BA	CIC(%)	G	CI	NC	CIC(%)	G	CI	NC	CIC(%)	G	CI	NC
C1	0.10	-	-	50.00	1.33	66.58	B.C	33.33	2.00	66.66	Lg.F	*	*	*	*
C2	0.50	-	-	33.33	2.50	83.33	B.C	83.33	1.80	149.99	Lg.F	50.00	1.33	66.50	B.C
C3	1.00	-	-	50.00	2.33	116.50	B.C	66.67	2.75	183.34	Lg.F	66.67	2.25	150.01	B.C
C4	2.00	-	-	50.00	2.33	116.50	B.C	33.33	2.50	83.33	Lg.C	50.00	2.33	116.50	B.C
C5	-	-	0.10	*	*	*	*	*	*	*	*	*	*	*	*
C6	-	-	0.50	*	*	*	*	33.33	2.00	66.67	G.C	*	*	*	*
C7	-	0.50	-	33.33	1.00	33.33	Lb	*	*	*	*	*	*	*	*
C8	-	1.00	-	33.33	1.50	50.00	Lb	33.33	2.00	66.66	G.C	*	*	*	*
C9	-	2.00	-	33.33	1.50	50.00	Lb	33.33	2.00	66.66	G.C	*	*	*	*
C10	0.10	-	0.10	33.33	2.50	83.33	G.C	50.00	2.00	100.00	Lg.C	66.67	1.00	66.67	W.C
C11	0.50	-	0.10	50.00	1.67	83.50	G.C	50.00	2.33	116.50	Lg.C	50.00	2.00	100.00	W.C
C12	1.00	-	0.10	50.00	2.00	100.00	G.C	33.33	3.50	116.66	Lg.C	50.00	2.33	116.50	W.C
C13	2.00	-	0.10	83.33	2.40	199.99	G.C	33.33	1.50	50.00	Lg.C	66.67	2.50	166.68	W.C
C14	0.10	-	0.50	33.33	2.50	83.33	G.C	33.33	2.00	66.66	Lg.C	50.00	1.33	66.50	W.C
C15	0.50	-	0.50	50.00	2.67	133.50	G.C	33.33	1.50	50.00	Lg.C	50.00	1.67	83.50	W.C
C16	1.00	-	0.50	66.67	2.50	166.68	G.C	33.33	1.50	50.00	Lg.C	66.67	1.75	116.67	W.C
C17	2.00	-	0.50	66.67	2.50	166.68	G.C	66.67	1.00	66.67	Lg.C	83.33	2.80	233.32	W.C
C18	0.50	0.50	-	33.33	1.00	33.33	B.C	33.33	2.50	83.33	G.C	50.00	2.00	100.00	B.C
C19	1.00	0.50	-	33.33	2.00	66.66	B.C	66.67	1.50	100.01	G.C	50.00	1.67	83.50	B.C
C20	2.00	0.50	-	33.33	2.50	83.33	B.C	66.67	1.00	66.67	G.C	66.67	1.00	66.67	B.C
C21	1.00	1.00	-	83.33	1.00	83.33	B.C	50.00	1.33	66.50	G.C	66.67	1.00	66.67	B.C
C22	2.00	1.00	-	66.67	2.00	133.34	B.C	66.67	1.25	83.34	G.C	50.00	1.33	66.50	B.C
C23	-	0.50	0.10	33.33	1.50	50.00	Lb.C	50.00	2.67	133.50	Yg.C	*	*	*	*
C24	-	1.00	0.10	33.33	2.00	66.66	Lb.C	66.67	2.25	150.01	Yg.C	*	*	*	*
C25	-	2.00	0.10	50.00	1.67	83.50	Lb.C	66.67	2.50	166.68	Yg.C	*	*	*	*
C26	-	0.50	0.50	50.00	2.00	100.00	Lb.C	66.67	2.00	133.34	Yg.C	*	*	*	*
C27	-	1.00	0.50	66.67	1.75	116.67	Lb.C	83.33	2.40	199.99	Yg.C	*	*	*	*
C28	-	2.00	0.50	50.00	2.33	116.50	Lb.C	100.00	2.83	283.00	Yg.C	*	*	*	*
Control	-	-	-	*	*	*	*	*	*	*	*	*	*	*	*

The data represent mean of six replications; Culture medium: MS; *indicates no callus formation
CIC: cultures initiating callus; G: Growth scrore; CI: callus index; NC: nature of callus; Yg.F-yellowish green & fragile; Yg.C: yellowish green & compact; Lb C-light brown compact; W.C-white compact; G.C-green compact; B.C- brown compact; Lb C: light brown compact

treatments recorded cent per cent callus initiation. C13 recorded highest growth score of 2.67 and callus index of 199.99. Lowest growth score (1.0) was recorded by treatments C7 (NAA 0.5 mg l^{-1}), C18 (2, 4-D 0.5 mg l^{-1} + NAA 0.5 mg l^{-1}) and C21 (2, 4-D 1.0 mg l^{-1} + NAA 1.0 mg l^{-1}) and lowest callus index by C7 and C18.

In case of leaf explant of *M. pruriens*, treatment C28 (NAA 2.0 mg l^{-1} + BA 0.5 mg l^{-1}) recorded cent per cent callus initiation with a high growth score and callus index of 2.83 and 283 respectively. This was followed by C27 (NAA 1.0 mg l^{-1} + BA 0.5 mg l^{-1}) with a callus initiation per cent of 83.33 per cent, growth score of 2.40 and callus index of 199.99. C20 (2, 4-D 2.0 mg l^{-1} + NAA 0.5 mg l^{-1}) recorded minimum growth score (1.0) and minimum callus index (66.67).

In case of cotyledonary explants of *M. pruriens*, none of the treatments gave cent per cent response. Maximum callus initiation per cent (83.33 per cent) was registered by C17 (2,4-D 2.0 mg l^{-1} + BA 0.5 mg l^{-1}) with the highest growth score (2.83) and callus index (233.32). C10 (2, 4-D 1.0 mg l^{-1} + BA 0.1 mg l^{-1}) C20 (2,4-D 2.0 mg l^{-1} + NAA 0.5 mg l^{-1}) and C21(2,4-D 2.0 mg l^{-1} + NAA 1.0 mg l^{-1}) registered least growth score (1.0) and least callus index (66.67). Treatments with BA and NAA alone or in combination did not evoke any callus initiation with cotyledonary explants.

Among the different explants tried for callus initiation in *M. pruriens*, leaf explant registered maximum growth score and callus index and least by cotyledon.

The effect of plant growth substances on callus initiation from stem and leaf explants of *I. tinctoria* is presented in Table 19. In treatments, C16 and C17 cent per cent cultures initiated callus with stem explant of *I. tinctoria*. C17 recorded maximum growth score (2.5) and maximum callus index (250). Minimum growth score was recorded by C1, C7, C8, C9, C10, C21, C23, C24 and C26. Least callus index was recorded by C8, C9, C10, C23, C24 and C26.

Table 19. Effect of plant growth substances on callus initiation from stem and leaf explants of Indigofera tinctoria

Treatment No.	Plant growth regulators (mgl^{-1})			Stem				Leaf			
	2,4-D	NAA	BA	CIC(%)	G	CI	NC	CIC(%)	G	CI	NC
C1	0.10	-	-	50.00	1.00	50.00	Y.C	33.33	1.00	33.33	B.C
C2	0.50	-	-	50.00	1.67	83.50	Y.C	50.00	2.33	116.50	B.C
C3	1.00	-	-	50.00	1.67	83.50	Y.C	66.67	1.25	83.34	B.C
C4	2.00	-	-	66.67	2.50	166.68	Y.C	66.67	1.50	100.01	B.C
C5	-	-	0.10	50.00	2.00	100.00	B.C	50.00	1.00	50.00	B.C
C6	-	-	0.50	66.67	1.50	100.01	B.C	50.00	1.00	50.00	B.C
C7	-	0.50	-	50.00	1.00	50.00	B.C	*	*	*	*
C8	-	1.00	-	33.33	1.00	33.33	B.C	*	*	*	*
C9	-	2.00	-	33.33	1.00	33.33	B.C	*	*	*	*
C10	0.10	-	0.10	33.33	1.00	33.33	Y.C	*	*	*	*
C11	0.50	-	0.10	66.67	1.50	100.01	Y.C	*	*	*	*
C12	1.00	-	0.10	50.00	1.67	83.50	Y.C	50.00	1.00	50.00	B.C
C13	2.00	-	0.10	66.67	1.50	100.01	Y.C	66.67	1.25	83.34	B.C
C14	0.10	-	0.50	50.00	1.67	83.50	Y.C	66.67	1.50	100.01	B.C
C15	0.50	-	0.50	50.00	2.00	100.00	Y.C	66.67	1.67	111..34	B.C
C16	1.00	-	0.50	100.00	2.00	200.00	Y.C	66.67	2.00	133.34	B.C
C17	2.00	-	0.50	100.00	2.50	250.00	Y.C	50.00	1.00	50.00	B.C
C18	0.50	0.50	-	66.67	1.25	83.34	Y.C	33.33	1.50	50.00	B.C
C19	1.00	0.50	-	66.67	1.50	83.50	Y.C	33.33	2.00	66.66	B.C
C20	2.00	0.50	-	50.00	2.00	100.00	Y.C	50.00	1.33	66.50	B.C
C21	1.00	1.00	-	50.00	1.00	50.00	Y.C	50.00	1.00	50.00	B.C
C22	2.00	1.00	-	66.67	1.50	100.01	Y.C	66.67	1.00	66.67	B.C
C23	-	0.50	0.10	33.33	1.00	33.33	B.C	33.33	1.00	33.33	B.C
C24	-	1.00	0.10	33.33	1.00	33.33	B.C	50.00	1.67	83.50	B.C
C25	-	2.00	0.10	50.00	1.33	66.50	B.C	50.00	1.00	50.00	B.C
C26	-	0.50	0.50	33.33	1.00	33.33	B.C	33.33	1.00	33.33	B.C
C27	-	1.00	0.50	33.33	1.50	50.00	B.C	50.00	2.33	116.50	B.C
C28	-	2.00	0.50	66.67	2.00	133.34	B.C	50.00	2.00	100.00	B.C
Control	-	-	-	*	*	*	*	*	*	*	*

The data represent mean of six replications. Culture medium: MS; * indicates no callus formation
CIC: cultures initiating callus; G.S: Growth scrore; C.I: callus index; NC: nature of callus; Y.C-Yellow compact; B.C- Brown compact callus

Plate 3. Callus initiation from various explants of *Clitoria ternatea, Mucuna pruriens* and *Indigofera tinctoria*

a. Callus initiation from stem explant of *C. ternatea* in culture medium MS + 2,4-D 2.0 mg l^{-1} + BA 0.5 g l^{-1}

b. Callus initiation from leaf explant of *C. ternatea* in culture medium MS + NAA 2.0 mg l^{-1}

c. Callus initiation from cotyledon explant of *C. ternatea* in culture medium MS + NAA 1.0 mg l^{-1}

d. Callus initiation from cotyledon explant of *C. ternatea* in culture medium MS + 2,4-D 2.0 mg l^{-1} + BA 0.5 mg l^{-1}

e. Callus initiation from stem explant of *M. pruriens* in culture medium MS + 2,4-D 2.0 mg l^{-1} + NAA 1 mg l^{-1}

f. Callus initiation from leaf explant of *M. pruriens* in culture medium MS + 2,4-D 1.0 mg l^{-1} + NAA 0.5 mg l^{-1}

g. Callus initiation from leaf explant of *M. pruriens* in culture medium MS + 2,4-D 1.0 mg l^{-1} + BA 0.1 mg l^{-1}

h. Callus initiation from cotyledon explant of *M. pruriens* in culture medium MS + 2,4-D 2.0 mg l^{-} + BA 0.1 mg l^{-1}

i. Callus initiation from stem explant of *I. tinctoria* in culture medium MS + 2,4-D 2.0 mg l^{-1}

j. Callus initiation from stem explant of *I. tinctoria* in culture medium MS + NAA 2.0 mg l^{-1}

k. Callus initiation from stem explant of *I. tinctoria* in culture medium MS + NAA 2.0 mg l^{-1} + BA 0.5 mg l^{-1}

l. Callus initiation from leaf explant of *I. tinctoria* in culture medium MS + 2,4-D 1.0 mg l^{-1} + BA 0.5 mg l^{-1}

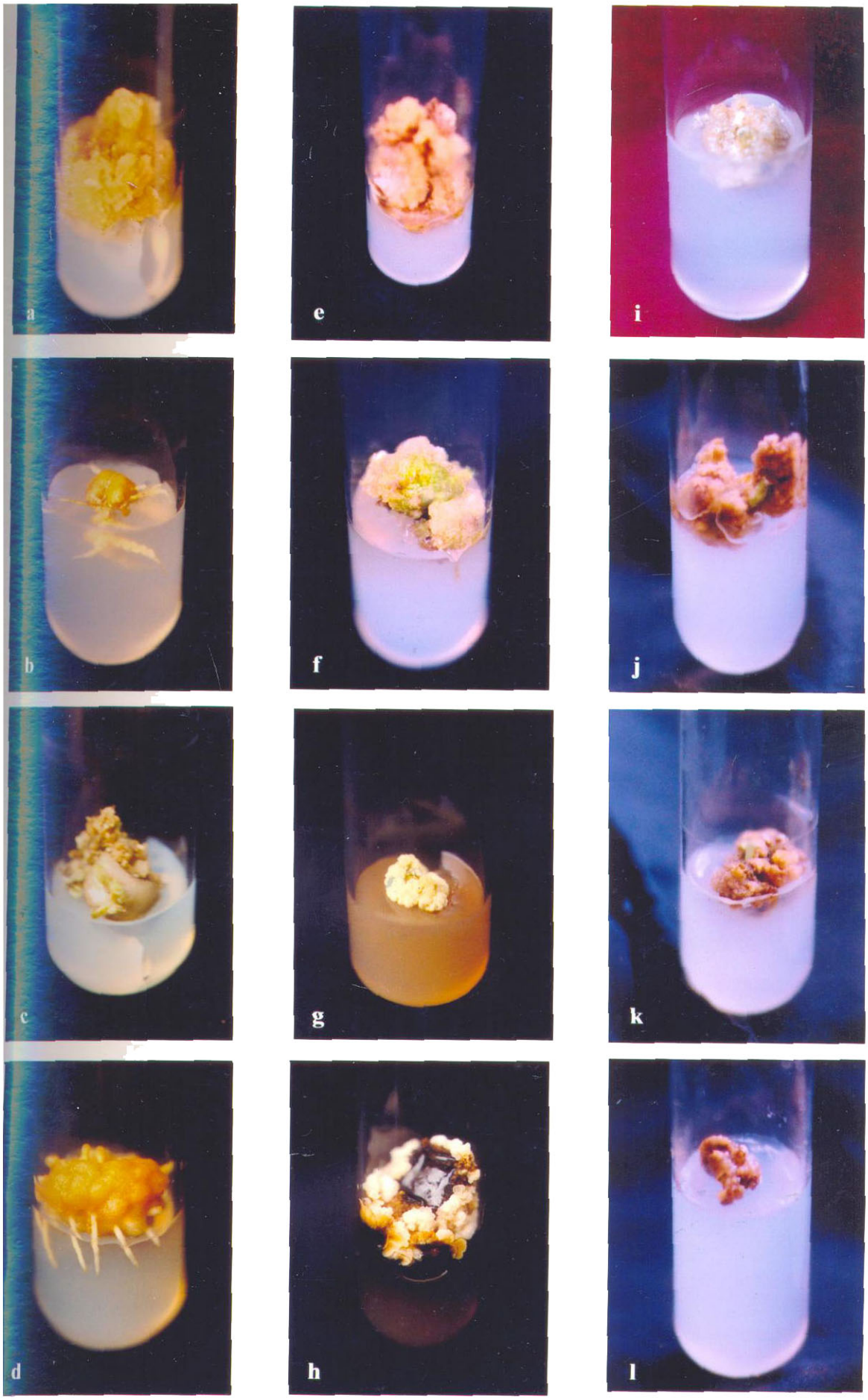


Plate 3. Callus initiation from various explants of *Clitoria ternatea*, *Mucuna pruriens* and *Indigofera tinctoria*

In case of leaf explant of *I. tinctoria*, callus initiation was much less with various treatments tried. Highest rate of callus initiation per cent obtained was 66.67 per cent with treatments C3, C4, C12, C13, C14, C15, C16 and C22. Highest growth score (2.33) was recorded by C2, C16 and C27 of which C16 (2,4-D 1.0 mg l^{-1} + BA 0.5 mg l^{-1}) registered highest callus index (155.34). Lowest growth score was recorded by C1, C5, C6, C12, C21, C22, C23, C25 and C26 of which C1, C23 and C26 registered lowest callus index (33.33).

Between the two explants tried for callus initiation in *I. tinctoria*, stem explant recorded maximum growth score and callus index. Nature of callus differed with different treatment combinations with stem explant but with leaf explant brown compact callus was obtained in all treatments.

In all the three plant species, no callus initiation was obtained in control treatments (without any plant growth substances).

4.1.2.2 Shoot Regeneration

The calli obtained in the initiation media of the three plant species were subcultured into 24 different treatments with various growth regulators such as cytokinins (BA and Kn), auxin (NAA) and adenine sulphate. The rate of shoot regeneration from calli cultures varied widely (0 to 100 per cent) in all the three species with different explants tried. Results of the study in *C. ternatea*, *M. pruriens* and *I. tinctoria* are presented in Tables 20, 21 and 22 respectively. The shoot regeneration from various explants of *C. ternatea*, *M. pruriens* and *I. tinctoria* are illustrated in Plates 4, 5 and 6 respectively.

4.1.2.2.1 Shoot Regeneration from Stem Derived Callus of Clitoria ternatea

Cent per cent cultures differentiated into shoots in the treatments SR17, SR18, SR19, SR20 and SR23. SR1 (BA 1.0 mg l^{-1}), SR3 (Kn1.0 mg l^{-1}), SR4 (Kn1.0 mg l^{-1})

Table 20. Effect of plant growth substances on shoot regeneration from the stem, leaf and cotyledon derived callus of Clitoria ternatea

Treatment No	Plant growth substances (mg l^{-1})				Stem			Leaf			Cotyledon		
	Ba	Kn	NAA	AdS	SRP	SpC	LLS	SRP	SpC	LLS	SRP	SpC	LLS
SR1	1.00	-	-	-	*	*	*	33.33	1.00	2.15	83.33	1.00	1.25
SR2	2.00	-	-	-	33.33	2.00	1.80	50.00	1.67	1.93	50.00	1.33	2.80
SR3	-	1.00	-	-	*	*	*	*	*	*	*	*	*
SR4	-	2.00	-	-	*	*	*	*	*	*	*	*	*
SR5	1.00	1.00	-	-	33.33	1.50	1.85	33.33	1.50	2.00	33.33	1.50	3.40
SR6	1.00	2.00	-	-	33.33	1.00	1.55	33.33	1.00	1.75	33.33	1.00	3.10
SR7	2.00	1.00	-	-	33.33	2.00	2.65	33.33	2.00	1.90	66.67	1.50	2.13
SR8	2.00	1.00	-	-	33.33	2.50	2.60	33.33	1.00	1.50	50.00	1.00	1.97
SR9	1.00	-	0.5	-	66.67	2.25	2.33	100.00	1.50	1.70	100.00	1.17	1.97
SR10	1.00	-	1.00	-	50.00	2.00	1.83	83.33	1.40	1.70	86.67	1.00	2.00
SR11	2.00	-	0.50	-	66.67	2.50	2.25	100.00	2.00	3.70^{+}	100.00	1.50	2.73
SR12	2.00	-	1.00	-	66.67	2.25	1.98	83.33	1.60	2.04	83.33	1.00	2.00
SR13	-	1.00	0.50	-	16.66	2.00	1.50	33.33	1.50	1.65	33.33	1.50	2.05
SR14	-	1.00	1.00	-	33.33	1.50	2.05	33.33	1.00	1.20	33.33	1.00	1.94
SR15	-	2.00	0.50	-	33.33	1.00	1.55	*	*	*	50.00	1.30	1.80
SR16	-	2.00	1.00	-	33.33	1.00	1.00	*	*	*	50.00	1.00	1.50
SR17	1.00	1.00	0.50	-	100.00	1.83	1.73	50.00	1.33	1.47	100.00	1.67	3.33
SR18	1.00	1.00	1.00	-	100.00	1.33	1.50	33.33	1.00	1.20	100.00	1.50	3.18
SR19	2.00	1.00	0.50	-	100.00	2.00	2.03	33.33	1.50	1.85	100.00	3.50^{+}	5.62^{+}
SR20	2.00	1.00	1.00	-	100.00	1.50	1.75	33.33	1.00	1.80	100.00	2.17	3.95
SR21	1.00	1.00	1.00	20.00	83.33	1.80	2.92	66.67	1.00	1.53	50.00	2.00	4.03
SR22	1.00	2.00	1.00	20.00	66.67	1.75	3.55	50.00	1.00	1.27	33.33	1.50	3.35
SR23	2.00	1.00	1.00	20.00	100.00	2.33	5.17^{+}	66.67	1.50	1.83	*	*	*
SR24	2.00	2.00	1.00	20.00	50.00	2.00	4.30^{+}	50.00	1.33	1.87	*	*	*
Control	-	-	-	-	*	*	*	*	*	*	*	*	*
FV (5%)						NS	6.43		NS	6.01		4.99	6.42

The data represents mean of regenerated plants in each treatments; Culture medium: MS;
* no shoot regeneration; + & - indicates highest and lowest on par values
SRP- % shoot regeneration; SpC: shoots / culture; LLS: Length of the longest shoot

Plate 4. Shoot regeneration from stem, leaf and cotyledon derived callus of *Clitoria ternatea*

a. Stem derived callus in culture medium MS + NAA 2.0 mg l^{-1} + BA 0.5 mg l^{-1}

a1. Shoot regeneration from stem derived callus in culture medium MS + BA 2.0 mg l^{-1} + Kn 1.0 mg l^{-1} + NAA 1.0 mg l^{-1} + AdS 20 mg l^{-1}

b. Leaf derived callus in culture medium MS + NAA 1.0 mg l^{-1} + BA 0.5 mg l^{-1}

b1. Shoot regeneration from leaf derived callus in culture medium MS + BA 2.0 mg l^{-1} + NAA 0.5 mg l^{-1}

c. Cotyledon derived callus in culture medium MS + 2,4-D 2.0 mg l^{-1} + BA 0.5 mg l^{-1}

c1. Shoot regeneration from cotyledon derived callus in culture medium MS + BA 2.0 mg l^{-1} + Kn 1.0 mg l^{-1} + NAA 0.5 mg l^{-1}

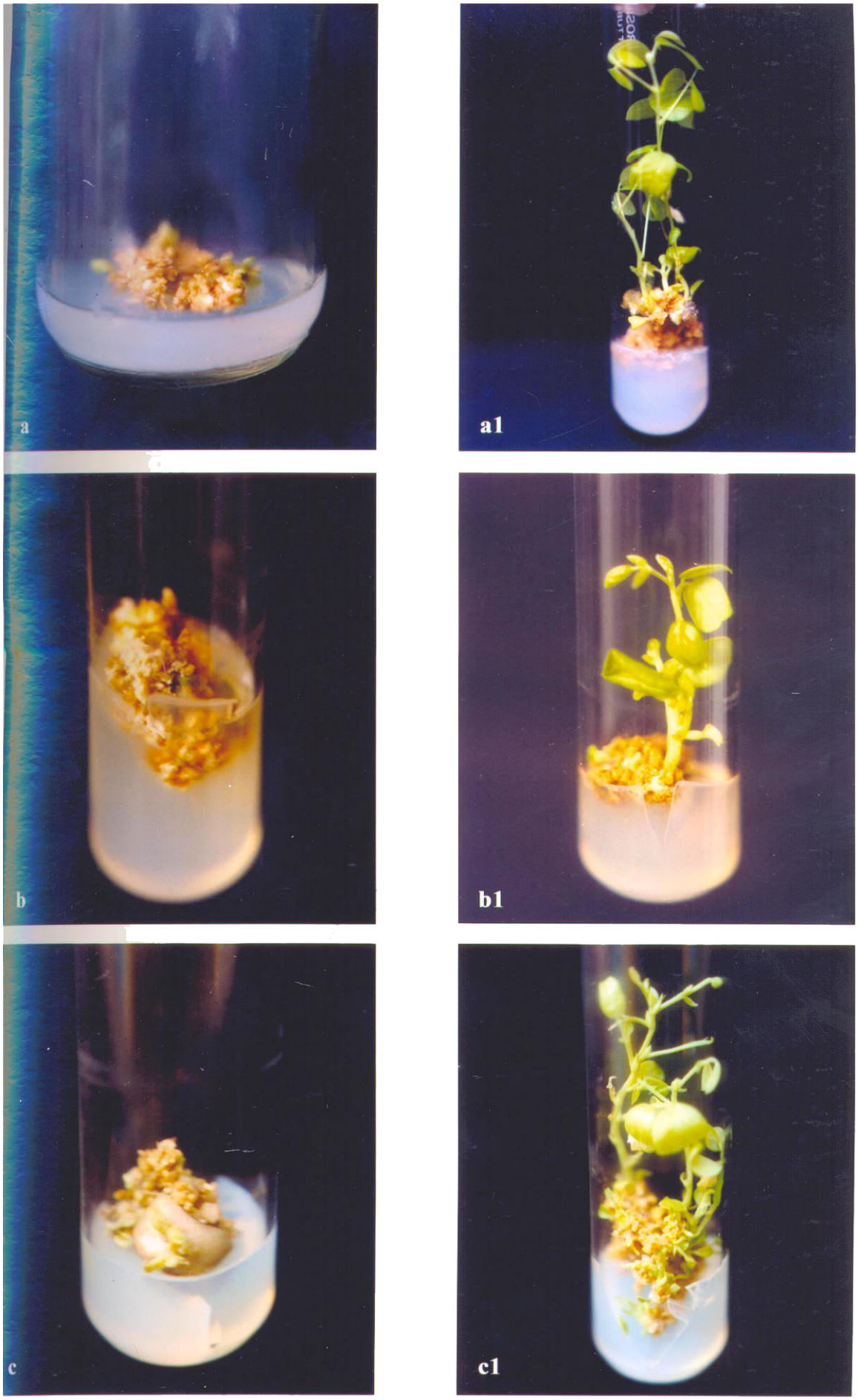


Plate 4. Shoot regeneration from stem, leaf and cotyledon derived callus of *Clitoria ternatea*

and control failed to develop shoots. There was no significant variation with regard to number of shoots per culture. However the length of the longest shoot was maximum (5.17 cm) in SR23 (BA 2.0 mg l^{-1} + Kn 1.0 mg l^{-1} + NAA 1.0 mg l^{-1} + adenine sulphate 20.0 mg l^{-1}) and least (1.0 cm) in SR16 (Kn 2.0 mg l^{-1} + NAA 1.0 mg l^{-1}).

4.1.2.2.2 Shoot Regeneration from Leaf Derived Callus of Clitoria ternatea

SR9 and SR11 registered cent per cent shoot regeneration. SR3, SR4, SR15, SR16 and control did not develop shoots. There was no significant variation with regard to number of shoot per culture. Maximum length of the longest shoot (3.7 cm) was recorded by SR11 (BA 2.0 mg l^{-1} + NAA 0.5 mg l^{-1}) and minimum (1.2 cm) by SR14 (Kn 1.0 mg l^{-1} + NAA 1.0 mg l^{-1}) and SR18 (BA 1.0 mg l^{-1} + Kn 1.0 mg l^{-1} + NAA 1.0 mg l^{-1}).

4.1.2.2.3 Shoot Regeneration from Cotyledon Derived Callus of Clitoria ternatea

SR9, SR11, SR17, SR18, SR19, SR20 and SR21 registered cent per cent shoot regeneration from cotyledon derived callus. SR3, SR4, SR23, SR24 and control failed to develop shoots. Significant variation was observed with regard to number of shoots per culture and the length of longest shoot. Maximum number (3.5) of shoots per culture was recorded by SR 19 (BA 2.0 mg l^{-1} + Kn 1.0 mg l^{-1} + NAA 0.5 mg l^{-1}) and least value (1.0) by SR1, SR6, SR8, SR10, SR12, SR14 and SR16. SR 19 registered maximum length of the longest shoot (5.62) and SR1 (BA 1.0 mg l^{-1}) recorded minimum length (1.25 cm).

4.1.2.2.4 Shoot Regeneration from Stem Derived Callus of Mucuna pruriens

Shoot regeneration was cent per cent in SR11, SR12, SR13 and SR17, while SR5, SR6, SR7, SR8, SR15, SR16 and control did not develop shoots. No significant variation was observed with regard to number of shoots per culture. Maximum

Table 21 .Effect of plant growth substances on shoot regeneration from the stem, leaf and cotyledon derived callus of *Mucuna pruriens*

Treatment	Plant growth substnces (mg l^{-1})				Stem			Leaf			Cotyledon		
.No	Ba	Kn	NAA	AdS	SRP	SpC	LLS	SRP	SpC	LLS	SRP	SpC	LLS
SR1	1.00	-	-	-	33.33	1.50	1.35^{-}	66.67	1.00^{-}	3.78	*	*	*
SR2	2.00	-	-	-	33.33	2.00	1.90^{-}	83.33	1.40^{-}	4.00	*	*	*
SR3	-	1.00	-	-	33.33	1.00	2.10^{-}	*	*	*	*	*	*
SR4	-	2.00	-	-	33.33	1.00	1.75^{-}	*	*	*	*	*	*
SR5	1.00	1.00	-	-	*	*	*	33.33	2.00^{+}	3.10	*	*	*
SR6	1.00	2.00	-	-	*	*	*	33.33	1.50^{-}	4.05	*	*	*
SR7	2.00	1.00	-	-	*	*	*	50.00	2.00^{+}	3.27	*	*	*
SR8	2.00	1.00	-	-	*	*	*	66.67	1.25^{-}	3.38	*	*	*
SR9	1.00	-	0.50	-	50.00	1.67	2.27	83.33	1.40^{-}	3.22	83.33	1.60	0.82
SR10	1.00	-	1.00	-	66.67	1.25	2.03^{-}	66.67	1.50^{-}	3.90	33.33	2.00	0.70
SR11	2.00	-	0.50	-	100.00	1.33	1.72^{-}	100.00	2.50^{+}	5.38	100.00	2.33	1.08
SR12	2.00	-	1.00	-	100.00	1.50	1.82^{-}	100.00	1.67^{-}	3.88	50.00	1.67	0.83
SR13	-	1.00	0.50	-	100.00	2.17	3.13^{+}	100.00	1.00^{-}	3.72	*	*	*
SR14	-	1.00	1.00	-	83.33	1.40	2.12^{-}	50.00	1.00^{-}	2.87	*	*	*
SR15	-	2.00	0.50	-	*	*	*	33.33	1.50^{-}	3.80	*	*	*
SR16	-	2.00	1.00	-	*	*	*	33.33	1.00^{-}	3.10	*	*	*
SR17	1.00	1.00	0.50	-	100.00	1.33	1.58^{-}	100.00	1.33^{-}	3.85	*	*	*
SR18	1.00	1.00	1.00	-	83.33	1.20	1.78^{-}	100.00	1.00^{-}	3.37	*	*	*
SR19	2.00	1.00	0.50	-	33.33	1.50	1.85^{-}	66.67	1.50^{-}	2.80	*	*	*
SR20	2.00	1.00	1.00	-	33.33	1.50	2.40^{+}	33.33	2.00^{+}	2.75	*	*	*
SR21	1.00	1.00	1.00	20.00	33.33	1.00	1.60^{-}	*	*	*	*	*	*
SR22	1.00	2.00	1.00	20.00	33.33	1.00	1.50^{-}	*	*	*	*	*	*
SR23	2.00	1.00	1.00	20.00	66.67	1.50	2.05^{-}	*	*	*	*	*	*
SR24	2.00	2.00	1.00	20.00	33.33	1.00	1.90^{-}	*	*	*	*	*	*
Control	-	-	-	-	*	*	*	*	*	*	*	*	*
FV (5%)						NS	3.09		3.50	NS		NS	NS

The data represents mean of regenerated plants in each treatments; Culture medium : MS
SRP- % shoot regeneration; SpC: shoots / culture; LLS: Length of the longest shoot
(--) represents not added; (*) no shoot regeneration; ($^{+}$) & ($^{-}$)indicates highest and lowest on par values

length (3.13 cm) of longest shoot was obtained in treatment SR13 (Kn 1.0 mg l^{-1} + NAA 0.5 mg l^{-1}) and least (1.35 cm) in SR1 (BA 1.0 mg l^{-1}).

4.1.2.2.5 Shoot Regeneration from Leaf Derived Callus of Mucuna pruriens

SR11, SR12, SR13, SR17 and SR18 recorded cent per cent shoot regeneration, while SR3, SR4, SR21, SR22, SR23, SR24 and control failed to develop shoots. There was significant variation with regard to number of shoot per culture. Number of shoots per culture (2.5) was the highest in SR11 (BA 2.0 mg l^{-1} + NAA 0.5 mg l^{-1}) and the lowest in treatments SR1, SR13, SR14, SR16 and SR18. No significant variation was observed in case of length of the longest shoot. However, SR11 recorded maximum length (5.38 cm) of longest shoot and SR20 (BA 2.0 mg l^{-1} + Kn 1.0 mg l^{-1} + NAA 1.0 mg l^{-1}) recorded minimum length (2.75 cm).

4.1.2.2.6 Shoot Regeneration from Cotyledon Derived Callus of Mucuna pruriens

Among the 24 treatments tried, only four treatments *viz.*, SR9, SR10, SR11 and SR12 gave shoot regeneration. Cent per cent regeneration was obtained with SR11. However, there was no significant variation with respect to number of shoots per culture and length of the longest shoot.

4.1.2.2.7 Shoot Regeneration from Stem Derived Callus of Indigofera tinctoria

The callus derived only from the stem explant differentiated into shoots in *I. tinctoria*. Cent per cent shoot regeneration was recorded by treatments SR11 and SR12, while SR3, SR4, SR16, SR21, SR22, SR23 and SR24 failed to produce shoots. Significant variation was observed with respect to number of shoot per culture. Maximum number of shoots (3.0) per culture was recorded by SR11 (BA 2.0 mg l^{-1} + NAA 0.5 mg l^{-1}) and minimum (1.00) by SR6 (BA 1.0 mg l^{-1} + Kn 2.0 mg l^{-1}) and SR14 (Kn 1.0 mg l^{-1}+ NAA 1.0 mg l^{-1}). Different treatments tried did not have any significant influence on the length of the longest shoot.

Table 22. Effect of plant growth substances on shoot regeneration from the stem derived callus of *Indigofera tinctoria*

Treatment No.	Plant growth substances (mg l^{-1})				Stem		
	BA	Kn	NAA	AdS	SRP	SpC	LLS
SR1	1.00	-	-	-	33.33	1.50^{-}	2.30
SR2	2.00	-	-	-	50.00	1.33^{-}	2.47
SR3	-	1.00	-	-	*	*	*
SR4	-	2.00	-	-	*	*	*
SR5	1.00	1.00	-	-	33.33	1.50^{-}	3.30
SR6	1.00	2.00	-	-	33.33	1.00^{-}	3.50
SR7	2.00	1.00	-	-	50.00	1.67^{-}	3.63
SR8	2.00	1.00	-	-	*	1.60^{-}	3.24
SR9	1.00	-	0.50	-	83.33	1.50^{-}	2.68
SR10	1.00	-	1.00	-	66.67	2.50^{+}	3.17
SR11	2.00	-	0.50	-	100.00	3.00^{+}	5.15
SR12	2.00	-	1.00	-	100.00	2.00^{-}	3.17
SR13	-	1.00	0.50	-	50.00	1.50^{-}	3.75
SR14	-	1.00	1.00	-	33.33	1.00^{-}	3.35
SR15	-	2.00	0.50	-	33.33	2.00^{-}	3.43
SR16	-	2.00	1.00	-	*	*	*
SR17	1.00	1.00	0.50	-	50.00	1.67^{-}	2.50
SR18	1.00	1.00	1.00	-	50.00	2.00^{-}	3.78
SR19	2.00	1.00	0.50	-	66.67	1.30^{-}	3.10
SR20	2.00	1.00	1.00	-	50.00	1.50^{-}	2.20
SR21	1.00	1.00	1.00	20.00	*	*	*
SR22	1.00	2.00	1.00	20.00	*	*	*
SR23	2.00	1.00	1.00	20.00	*	*	*
SR24	2.00	2.00	1.00	20.00	*	*	*
Control	-	-	-	-	*	*	*
FV (5 %)					-	2.22	NS

The data represent mean of regenerated plants in each treatments; Culture medium: MS;

* no shoot regeneration; + & - indicates highest and lowest on par values

SRP- % shoot regeneration; SpC: shoots / culture; LLS: Length of the longest shoot

Plate 5. Shoot regeneration from stem, leaf and cotyledon derived callus of *Mucuna pruriens*

a. Stem derived callus in culture medium MS + 2,4-D 2.0 mg l^{-1} + BA 0.1 mg l^{-1}

a1. Shoot regeneration from stem derived callus in culture medium MS + Kn 1.0 mg l^{-1} + NAA 0.5 mg l^{-1}

b. Leaf derived callus in culture medium MS +NAA 2.0 mg l^{-1} + BA 0.5 mg l^{-1}

b1. Shoot regeneration from leaf derived callus in culture medium MS + BA 2.0 mg l^{-1} + NAA 0.5 mg l^{-1}

c. Cotyledon derived callus in culture medium MS + 2,4-D 2.0 mg l^{-1} + BA 0.5 mg l^{-1}

c1. Shoot regeneration from cotyledon derived callus in culture medium MS + BA 2.0 mg l^{-1} + NAA 0.5 mg l^{-1}

Plate 6. Shoot regeneration from stem derived callus of *Indigofera tinctoria*

a. Stem derived callus in culture medium MS + 2,4-D 2.0 mg l^{-1} + BA 0.5 mg l^{-1}

a1. Shoot regeneration from stem derived callus in culture medium MS + BA 2.0 mg l^{-1} + NAA 0.5 mg l^{-1}

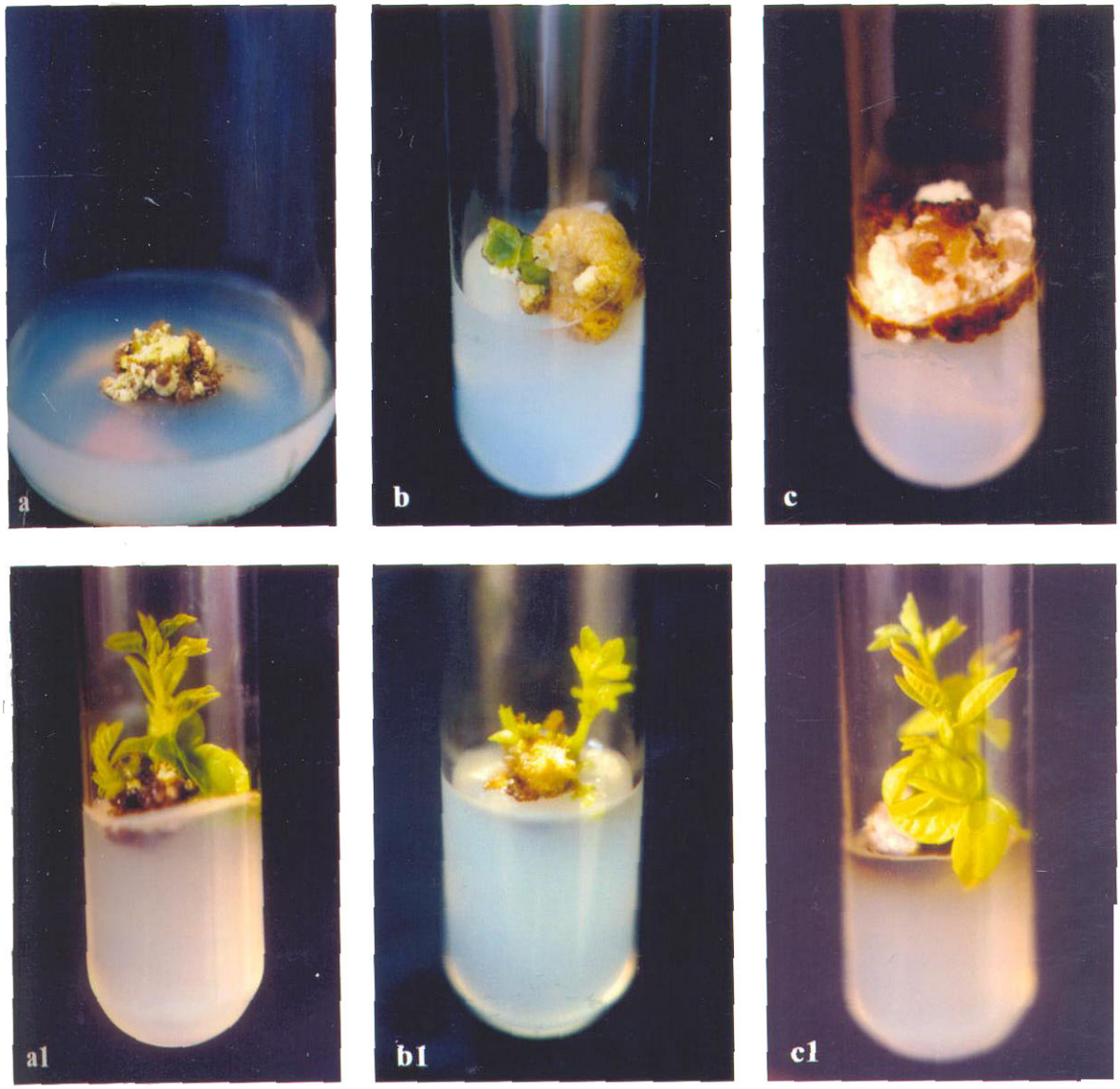


Plate 5. Shoot regeneration from stem, leaf and cotyledon derived callus of *Mucuna pruriens*

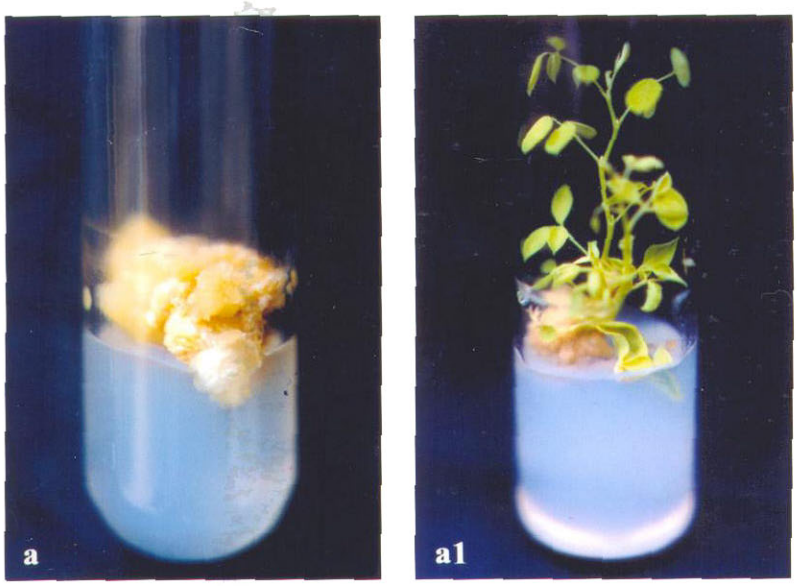


Plate 6. Shoot regeneration from stem derived callus of *Indigofera tinctoria*

4.1.3 Somatic Embryogenesis

4.1.3.1 Callus Initiation

The combinations of plant growth substances tried were the same as those tried for callus initiation in the case of indirect somatic organogenesis in all the three plant species. After four weeks of culture, the calli were transferred to the same medium for further proliferation.

4.1.3.2 Induction of Somatic Embryos

Twenty treatments with various combinations of growth regulators were tried for induction of somatic embryo. Calli derived from various explants did not have embryogenic potential in any of the three species except for yellow granular and friable leaf derived callus obtained in C11 (2, 4-D 0.5 mg l^{-1} + BA 0.1 mg l^{-1}) in *C. ternatea*. The embryogenic response of leaf derived callus of *C. ternatea* is presented in Table 23 and Plate 7. Different stages of the embryo *viz.*, globular, heart and torpedo shaped embryos were formed in treatments I1 (BA 0.50 mg l^{-1}) and I2 (BA 1.00 mg l^{-1}) within 30 - 45 days. Somatic embryos were formed in 91.67 per cent of cultures in treatment I2 followed by I1. I8 (GA 0.5 mg l^{-1} + NAA 1.0 mg l^{-1}) and I9 (GA 1.0 mg l^{-1} + NAA 1.0 mg l^{-1}) developed globular and heart shaped embryos while I6, I7, I10, I11 and I12 developed only globular shaped embryos. No embryogenesis was obtained in treatments with GA alone or higher concentration of BA in combination with CW or BA in combination with GA, 2, 4-D and various supplements (Gl, CH, CW, AC and ABA).

4.1.3.3 Maturation and Germination of Somatic Embryos

Torpedo shaped embryos when detached and inoculated on to germination medium, developed shoot and root initials; while the other stages of embryos when transferred to the same medium did not give further development. The effect of different levels of GA (0.1, 0.5 and 1.0 mg l^{-1}) on germination of somatic embryos

Table 23. Effect of plant growth substances on embryogenic response from leaf callus of *Clitoria ternatea*

T.t No.	Plant growth substances			CW (%)	CEF (%)	Remarks
	BA (mg l^{-1})	GA (mg l^{-1})	NAA (mg l^{-1})			
I1	0.50	-	-	-	83.33	Callus with globular, heart and torpedo shaped embryos
I2	1.00	-	-	-	91.67	Callus with globular, heart and torpedo shaped embryos
I3	-	0.10	-	-	-	No embryogenesis
I4	-	0.50	-	-	-	No embryogenesis
I5	-	1.00	-	-	-	No embryogenesis
I6	0.50	-	1.00	-	50.00	Callus with globular shaped embryos
I7	1.00	-	1.00	-	58.33	Callus with globular shaped embryos
I8	-	0.50	1.00	-	75.00	Callus with globular and heart shaped embryos
I9	-	1.00	1.00	-	50.00	Callus with globular and heart shaped embryos
I10	1.00	-	-	10	16.67	Callus with globular shaped embryos
I11	1.00	-	-	15	25.00	Callus with globular shaped embryos
I12	1.00	-	-	20	25.00	Callus with globular shaped embryos
I13	2.00	-	-	10	-	No embryogenesis
I14	2.00	-	-	15	-	No embryogenesis
I15	2.00	-	-	20	-	No embryogenesis
I16	3.00	-	-	10	-	No embryogenesis
I17	3.00	-	-	15	-	No embryogenesis
I18	3.00	-	-	20	-	No embryogenesis
I19*	1.00	5.00	-	20	-	No embryogenesis
I20**	1.00	5.00	-	20	-	No embryogenesis
Control	-	-	-	-	-	No embryogenesis

Culture medium: MS; CEF: cultures showing embryo formation; CW coconut water; Gl:glutamine; CH: casein hydrolysate; AC: activated charcoal; ABA : Abscisic acid

*Supplements to the treatment : Gl 400 mg l^{-1} + CH 600 mg l^{-1} + 2,4-D 0.5 mg l^{-1} + AC 250 mg l^{-1}

** Supplements to the treatment: Gl 400 mg l^{-1} + CH 600 mg l^{-1} + 2,4-D 0.5 mg l^{-1} + ABA 5.00 mg l^{-1} + AC 250 mg l^{-1}

Treatment replication: 12

Table 24. Effect of GA on germination of somatic embryos in *Clitoria ternatea*

Treatment No.	GA (mg l^{-1})	Germination percent
Gn1	0.10	85.07 (67.24)
Gn2	0.50	85.23 (67.37)
Gn3	1.00	87.62 (69.38)
Control	0.00	77.67 (61.79)
CD (5 %)	-	NS

Data in parentheses indicate transformed value (arc transformation)

Plate 7. Somatic embryogenesis from leaf callus of *Clitoria ternatea*

a. Yellow friable callus in culture medium MS + 2,4-D 0.5 mg l^{-1} + BA 0.1 mg l^{-1}

b. Callus differentiation and initiation of embryogenesis in culture medium MS + BA 1.0 mg l^{-1}

c. Heart shaped and torpedo shaped embryo formation in culture medium MS + BA 1.0 mg l^{-1}

d. Different stages of embryogenesis leading to whole plant

e. *Ex-vitro* plant acclimatization

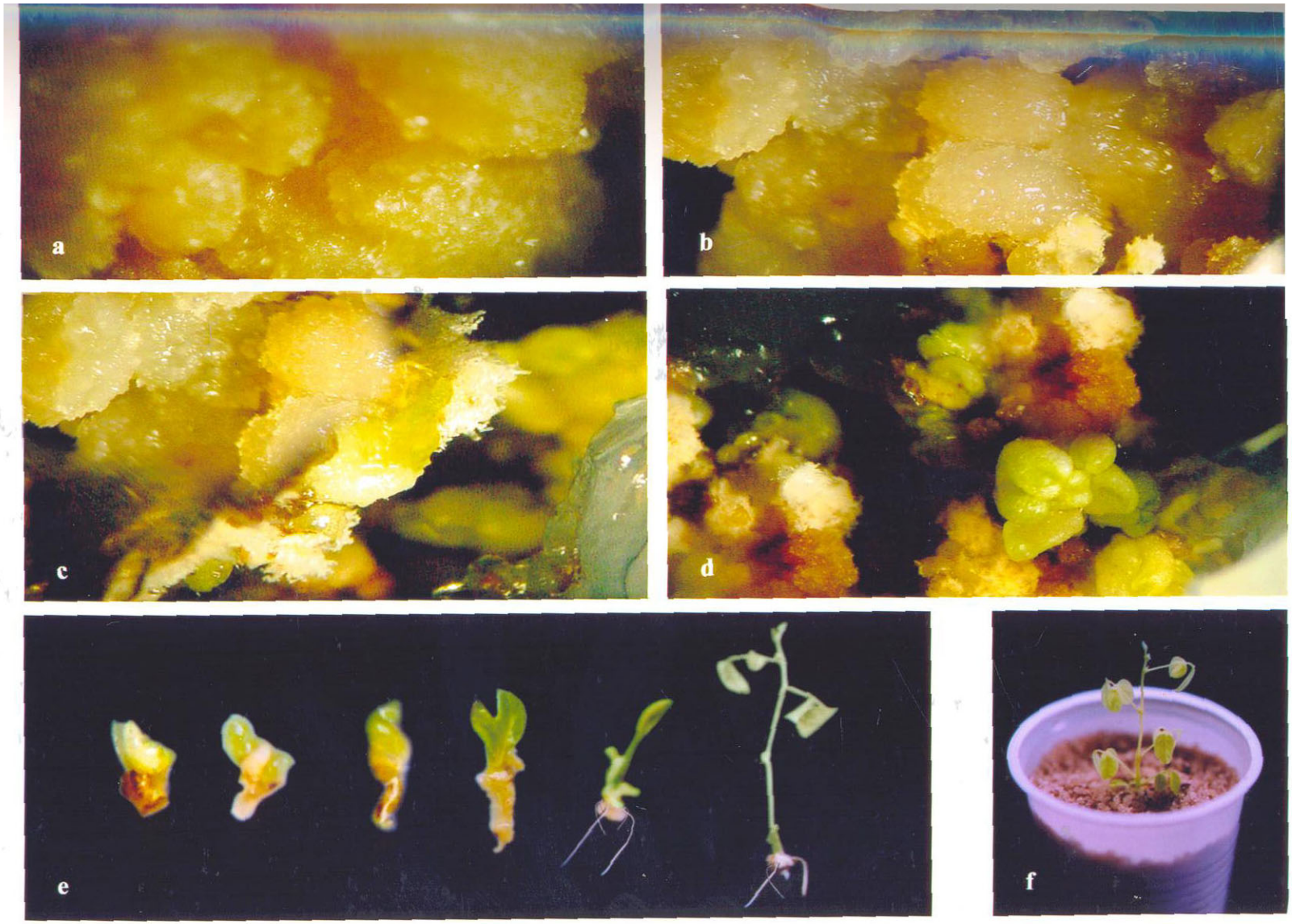


Plate 7. Somatic embryogenesis from leaf callus of *Clitoria ternatea*

are presented in Table 24. There was no significant variation with regard to germination rate of somatic embryos. However, maximum germination (87.62 per cent) was obtained with Gn3 (GA1.00 mg l^{-1}). There was a progressive increase in germination per cent with increase in GA level.

4.1.3.4 Acclimatization and Plant Regeneration

From one gram of embryogenic callus, an average of 60 to 70 plantlets was obtained. The fully developed plantlets when transferred to pots containing sterilized sand for acclimatization and 40 per cent survival frequency was recorded. No difference in morphology was observed among regenerated plantlets.

4.1.4. Rooting

4.1.4.1 In vitro Rooting

Twelve treatments with various levels of auxins (IBA, IAA and NAA) alone were tried for *in vitro* rooting in the three plant species. Results of the study are presented in Table 25 and Figs. (7, 8 and 9)

In *C. ternatea*, maximum root initiation per cent (66.67 per cent) was obtained in R10 (NAA 1.0 mg l^{-1}) (Plate 8a). No rooting was observed in control treatments with no growth regulators. There was significant variation with regard to days for root initiation, number of roots and mean length of roots. Earliest (12 days) root initiation was recorded by R10 followed by R11 (13.67 days) and late (18days) by R2 (IBA 1.0 mg l^{-1}), R6 (IAA 1.0 mg l^{-1}) and R8 (IAA 2.5 mg l^{-1}). R10 recoded maximum number of roots (3.5) and root length (3.03 cm).

Root initiation per cent varied from 0 to 66.67 per cent in *M. pruriens*. No rooting was observed in control treatments. R5 (IBA 2.5 mg l^{-1}) recorded maximum (66.67 per cent) root initiation (Plate 8b). Different levels of auxins significantly influenced days for root initiation, number of roots per culture and mean root length. Earliest (16.5 days) rooting was registered in R5 followed by R10. Root initiation was late (23.5 days) in R1 (IBA 0.5 mg l^{-1}). Number of roots per culture (5.25) was

Table 25. Effect of auxins on *in vitro* rooting of *Clitoria ternatea, Mucuna pruriens and Indigofera tinctoria*

Treatment No.	Auxins (mgl^{-1})	*Clitoria ternatea*				*Mucuna pruriens*				*Indigofera tinctoria*			
		R.I.P(%)	DRI	NoR	MLR(cm)	R.I.P(%)	DRI	NoR	MLR(cm)	R.I.P(%)	DRI	No R	MLR(cm)
R1	IBA 0.50	50.00	16.33^{+}	1.00^{-}	0.90^{-}	33.33	23.50^{-}	2.00^{-}	1.45^{-}	16.67	15.00	1.00^{-}	1.50^{-}
R2	IBA 1.00	16.67	18.00^{+}	1.00^{-}	0.90^{-}	33.33	21.00	2.33^{-}	1.50^{-}	16.67	17.00	1.00^{-}	1.90^{-}
R3	IBA 1.50	50.00	16.33^{+}	1.33^{-}	0.98^{-}	33.33	23.00^{-}	2.00^{-}	3.41^{+}	50.00	16.00	2.00^{-}	1.76^{-}
R4	IBA 2.00	33.33	16.00^{+}	1.50^{-}	1.05^{-}	50.00	19.33	2.00^{-}	1.55^{-}	50.00	15.67	2.33^{-}	2.21^{+}
R5	IBA 2.50	50.00	16.00^{+}	1.50^{-}	0.88^{-}	66.67	16.50^{+}	5.25^{+}	2.02^{-}	50.00	15.00	3.33^{-}	2.14^{+}
R6	IAA 1.00	16.67	18.00^{+}	1.00^{-}	1.00^{-}	33.33	21.00	1.50^{-}	1.10^{-}	33.33	15.00	2.00^{-}	0.92^{-}
R7	IAA 1.50	50.00	15.67^{+}	1.33^{-}	1.08^{-}	50.00	19.67	1.00^{-}	1.35^{-}	66.67	12.75	4.25^{+}	2.85^{+}
R8	IAA 2.50	16.67	18.00^{+}	1.00^{-}	0.80^{-}	50.00	21.33	1.50^{-}	1.58^{-}	50.00	13.33	2.00^{-}	1.61^{-}
R9	NAA 0.50	50.00	14.00^{+}	1.67^{-}	1.30^{-}	50.00	19.33	1.00^{-}	1.40^{-}	33.33	15.50	2.50^{-}	1.18^{-}
R10	NAA 1.00	66.67	12.00^{-}	3.50^{+}	3.03^{+}	50.00	19.00^{+}	2.67	1.83^{-}	50.00	15.67	1.67^{-}	1.23^{-}
R11	NAA 1.50	50.00	13.67^{-}	1.67^{-}	1.48^{-}	33.33	20.50	1.50^{-}	1.25^{-}	16.66	15.00	2.00^{-}	1.15^{-}
R12	NAA 2.00	16.67	15.00^{+}	1.00^{-}	1.50^{-}	33.33	21.00	1.00^{-}	1.43^{-}	33.33	15.00	2.00^{-}	1.35^{-}
Control	-	00.00	00.00	0.00	0.00	00.00	00.00	0.00	0.00	33.33	12.75	2.00^{-}	0.90^{-}
FV (5%)			2.61	5.06	8.50		3.32	6.05	6.76		NS	2.70	3.04

The data represent mean of rooted plants in each treatment; Culture medium:1/2MS; R.I.P: % root initiation ; DRI: days for root initiation; NoR: no of roots; MLR: mean root length;+ & - indicates highest and lowest on par values

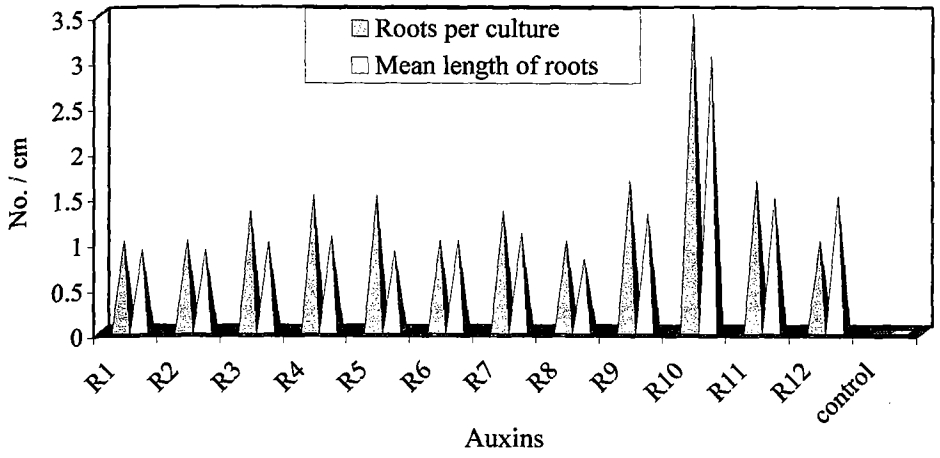


Fig 7. Effect of auxins on *in vitro* rooting of *Clitoria ternatea*

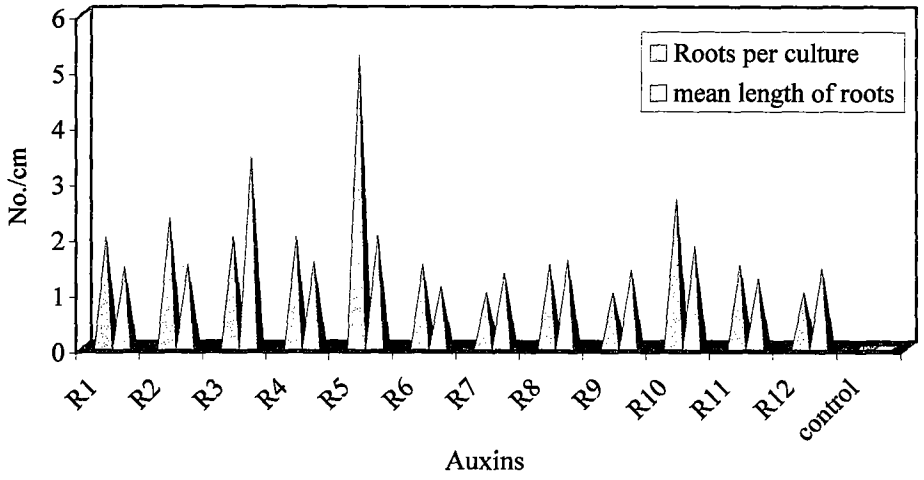


Fig 8. Effect of auxins on *in vitro* rooting of *Mucuna pruriens*

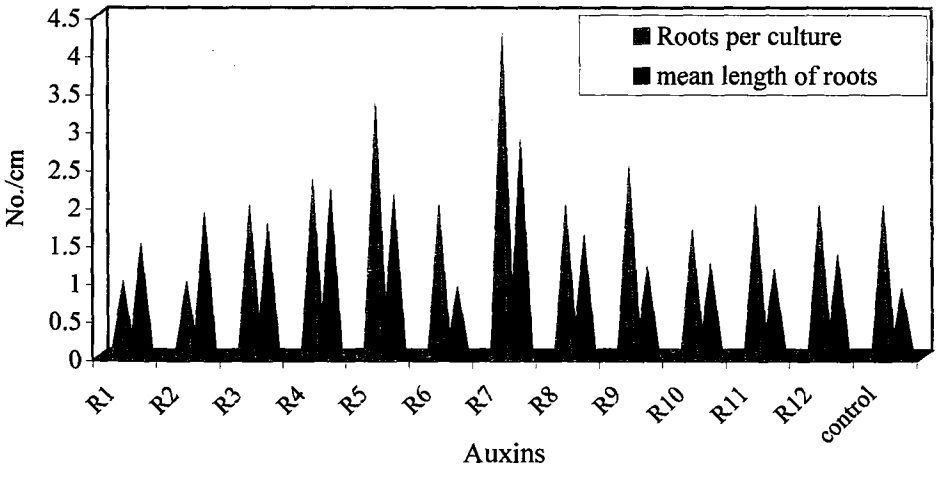


Fig 9. Effect of auxins on *in vitro* rooting of *Indigofera tinctoria*

maximum in R5 and minimum (1.0) in R2 (IBA 1.0 mg l^{-1}), R4 (IBA 2.0 mg l^{-1}), and R12 (NAA 2.0 mg l^{-1}). Mean root length was highest (3.41 cm) in treatment R8 (IAA 2.5 mg l^{-1}) and lowest (1.1 cm) in R1.

In *I. tinctoria* root initiation per cent varied from 16.67 to 66.67 per cent. Maximum root initiation (66.67 per cent) was registered in treatment R7 (IAA 1.5 mg l^{-1}) (Plate 8c). Control treatments with no growth regulator produced roots in 33.33 per cent cultures. No significant variation was observed with regard to days for root initiation. It varied between 12.57 to 17.00 days. Number of shoots per culture and root length exhibited significant variation at different levels of auxins. Maximum number (4.25) of roots and root length (2.85 cm) was recorded by R7. Least number (1.0) of roots was registered by R1 (IBA 0.5 mg l^{-1}) and R2 (IBA 1.0 mg l^{-1}) and root length by (0.9 cm) by control plants.

4.1.4.2 Ex vitro Rooting

The effect of pre-treatments with IBA was recorded after four weeks of culture in *C. ternatea*, *M. pruriens* and *I. tinctoria* (Table 26).

Among the pre-treatments given with IBA to the unrooted micro-shoots maximum survival rate (83.33 per cent) was obtained in ER1 (IBA 1000 mg l^{-1} for 20 s) in *C. ternatea* and least (50 per cent) in ER3 (IBA 100 mg l^{-1} for 20 h).

In *M. pruriens*, ER2 (IBA 500 mg l^{-1} for 20 s) recorded maximum survival rate (83.33 per cent) and least (58.33 per cent) in ER3 (IBA 100 mg l^{-1} for 20 h).

ER1 (IBA 1000 mg l^{-1} for 20 s) registered maximum survival rate (75 per cent) and ER3 (IBA 100 mg l^{-1} for 20 h) the minimum (41.67 per cent) after four weeks in *I. tinctoria*.

4.1.4.3 Planting Out and Acclimatization

The *in vitro* rooted plants were carefully removed from the culture vessels and planted out in different potting media (sand, soilrite and soilrite: sand (2:1)) to study the effect on *ex vitro* establishment. The survival rate of plants was estimated after

Plate 8. *In vitro* rooting and *ex vitro* establishment in *Clitoria ternatea*, *Mucuna pruriens* and *Indigofera tinctoria*

a. Rooting of shoots of *C. ternatea in vitro* in culture medium MS + NAA 1.0 mg l^{-1}

b. Rooting of shoots of *M. pruriens in vitro* in culture medium MS + IBA 2.5 mg l^{-1}

c. Rooting of shoots of *I. tinctoria in vitro* in culture medium MS + IAA 1.5 mg l^{-1}

d. *Ex vitro* establishment of *in vitro* rooted *C. ternatea* plants

e. *Ex vitro* establishment of *in vitro* rooted *M. pruriens* plants

f. *Ex vitro* establishment of *in vitro* rooted *I. tinctoria* plants

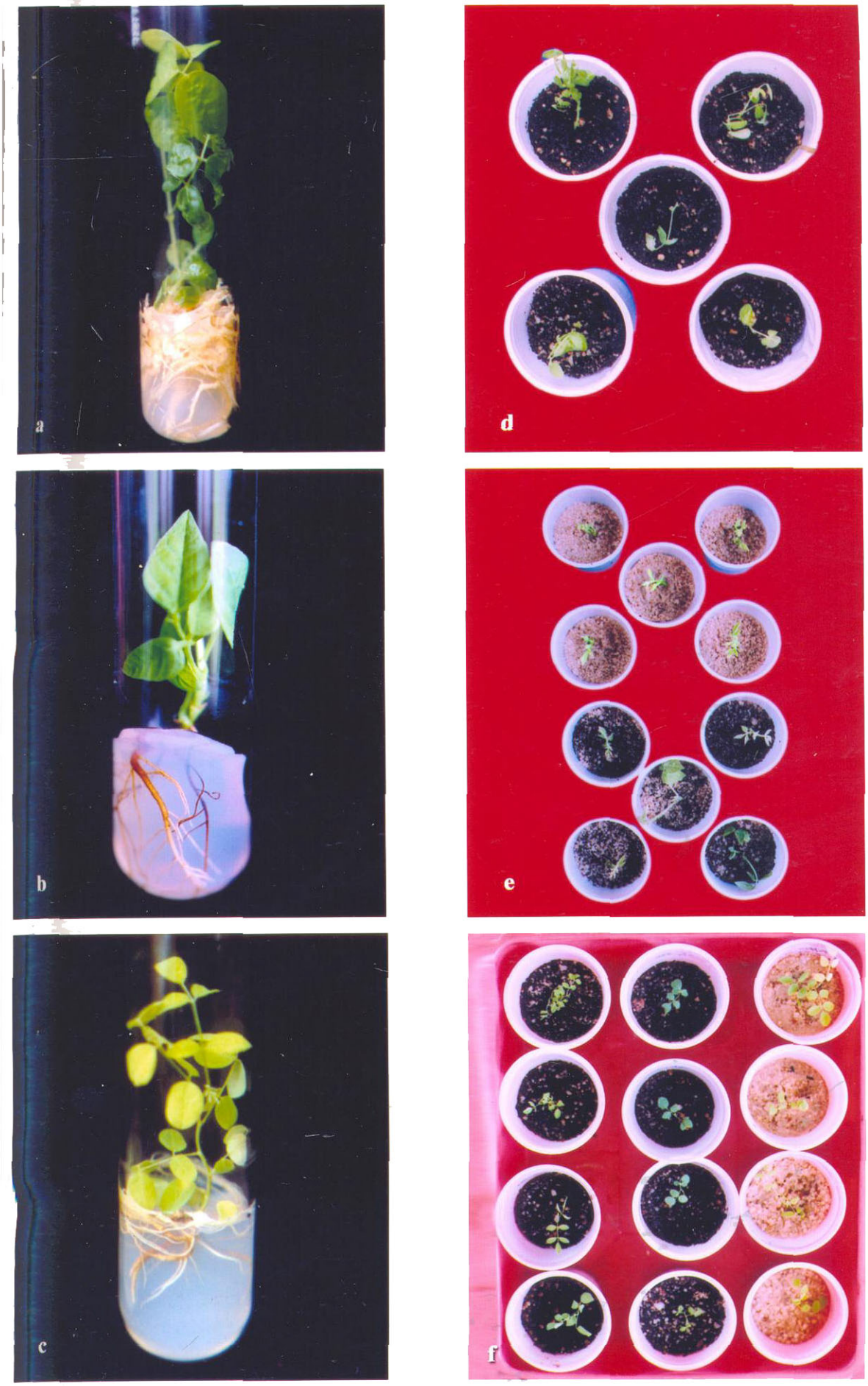


Plate 8. ***In vitro*** **rooting and** ***ex vitro*** **establishment in** ***Clitoria ternatea*****,** ***Mucuna pruriens*** **and** ***Indigofera tinctoria***

Table 26. Effect of IBA pretreatments on ex vitro rooting of Clitoria ternatea, Mucuna pruriens and Indigofera tinctoria

Treatment No.	IBA ($mg\ l^{-1}$)	Pretreatment duration	Survival rate (%) after four weeks of treatment		
			Clitoria ternatea	*Mucuna pruriens*	*Indigofera tinctoria*
ER1	1000	20 s	83.33	75.00	75.00
ER2	500	20 s	75.00	83.33	66.67
ER3	100	20 h	50.00	58.33	41.67
Control	-	-	-	-	-

The data represents mean of 12 replications

Table 27. Effect of potting media on *ex vitro* establishment of *Clitoria ternatea, Mucuna pruriens* and *Indigofera tinctoria*

Treatment No.	Potting media	Survival rate (%) after four weeks of treatment		
		Clitoria ternatea	*Mucuna pruriens*	*Indigofera tinctoria*
PM1	Sand	83.33	75.00	91.67
PM2	Soilrite	75.00	50.00	50.00
PM3	Soilrite:Sand(2:1)	58.33	58.33	66.67

The data represents mean of 12 replications

Table 28. Effect of MS medium and IBA in inducing slow growth* in *Clitoria ternatea, Mucuna pruriens* and *Indigofera tinctoria*

T.No.	Media	*Clitoria ternatea*			*Mucuna pruriens*			*Indigofera tinctoria*		
		PH (cm)	**S (wks)	Remarks	PH (cm)	**S (wks)	Remarks	PH (cm)	**S (wks)	Remarks
MS1	½ MS	5.08	13	Rooting in 33.33 % cultures	3.62	13	No rooting	5.77	12	Rooting in 33.33 % cultures
MS2	½ MS + IBA($0.5 mgl^{-1}$)	4.48	-	Rooting in all cultures	3.33	-	Rooting in all cultures	5.50	-	Rooting in all cultures
MS3	MS	5.35	10	No rooting	4.18	11	No rooting	6.13	9	Rooting in 16.67 % cultures
MS4	MS + IBA($0.5 mgl^{-1}$)	5.23	12	Rooting in all cultures	4.10	13	Rooting in all cultures	6.00	11	Rooting in all cultures
CD Value (5 %)		0.61	-	-	0.55	-	-	0.44	-	-
F Value		3.46	-	-	4.68	-	-	3.43	-	-

*Observations were recorded after 16 weeks of incubation; PL: plant height; S: senescence
** Senescence indicated by wilting of leaves

four weeks of culture (Table 27). PM1 (sand) recorded maximum survival percentage in all the three plant species, *C. ternatea* (83.33 per cent), *M. pruriens* (75.00 per cent) and *I. tinctoria* (91.67 per cent) and least survival rate (58.33) was recorded by PM3 (soilrite: sand (2:1)) in *C. ternatea* and survival rate (50 per cent) in PM2 (soilrite) in *M. pruriens* and *I. tinctoria.* The *ex vitro* establishment of *C. ternatea, M. pruriens* and *I. tinctoria* are illustrated in Plates 8d, 8e and 8f, respectively.

4.2 *IN VITRO* CONSERVATION

4.2.1 Slow Growth

4.2.1.1 Culture Medium and Explant

Half and full MS with and without the hormone IBA (0.5 mg l^{-1}) were tried to select the best basal culture medium for slow growth experiments in *C. ternatea, M. pruriens* and *I. tinctoria.* There was significant variation in the height of plants in the media tried (Table 28). In all the three plant species, MS2 (half MS with IBA (0.5 mg l^{-1}) gave the lowest plant height. This was on par with MS1 (half MS without IBA). But senescence indicated by wilting of leaves, initiated comparatively late in MS2 in all the three plant species.

In *C. ternatea, M. pruriens* and *I. tinctoria,* MS2 gave lowest plant height (4.48 cm, 3.33 cm and 5.50 cm, respectively) which is on par with MS1and highest (5.35 cm, 4.18 cm and 6.13 cm, respectively) in MS3 (full MS without IBA) which is on par with MS4 (full strength MS with IBA (0.5 mg l^{-1})). Though MS1 and MS2 were on par, senescence did not occur up to 16 weeks in MS2 in all the three plant species. But in MS1, senescence initiated at 13 weeks in *C. ternatea* and *M. pruriens* and 12 weeks in *I. tinctoria.* In *C. ternatea* cent per cent rooting was obtained in treatments supplemented with IBA; while with half MS, rooting was obtained though at a low per cent of 33.33 per cent. In *M. pruriens*, only treatments supplemented with IBA initiated roots. While in *I. tinctoria*, all the treatments initiated roots

but in those without IBA, root initiation per cent was much less (16.67 to 33.33 per cent).

The study also indicated that shoot development in terms of plant height was low in those treatments supplemented with IBA compared to the ones without it in all the three plant species.

As such, half MS with IBA (0.5 mg l^{-1}) was selected as the base medium for slow growth experiments in *C. ternatea, M. pruriens* and *I. tinctoria.* Single node cuttings of *in vitro* raised seedlings were used are used as explant in all the three plant species.

4.2.1.2 Slow Growth Treatments

Osmoticum *viz.*, sucrose (6, 8, 10, 12 and15 per cent), sorbitol (0.5, 1, 1.5, 2 and 3 per cent) and mannitol (0.5, 1, 1.5, 2 and 3 per cent), cryoprotectant *viz.*, proline (5, 10, 15, 20 and 30 mM), antioxidant *viz.*, PVP (2, 4, 6, 8 and 10 per cent) and growth retardant *viz.*, ABA (10, 15, 20, 25 and 30 mgl^{-1}) were tried to study their effect in slowing down the growth of cultures. Sucrose at 3 per cent was used as control in all the treatments. All the cultures were maintained without subculture upto 28 weeks under ambient culture conditions, at which stage regeneration was estimated on transfer to proliferation medium. Observations on plant height, shoot number and node number was recorded in selected treatments, which gave 50 per cent or more regeneration to identify the treatment with maximum plant inhibition. These cultures were stored up to 40 weeks by transferring to fresh slow growth medium.

Clitoria ternatea

4.2.1.2.1 Addition of Osmotica: Sucrose, Sorbitol and Mannitol

Among the different levels of sucrose tried, the lowest plant height was recorded by SuT5 (sucrose at 15 per cent) and the highest by SuT1 (sucrose at 6 per cent). But the plant height recorded was low in all levels of sucrose compared to control at all

stages of incubation. The rate of plant growth in relation to plant height is presented in Table 29. In SuT1 and control plants showed an increasing trend up to eight weeks of incubation, then decreased; while in all the other treatments rate of growth showed a decreasing trend from eight weeks onwards in *C. ternatea*. Mannitol and sorbitol also showed same trend with regard to plant height. Higher the concentration of additives, lower the plant height. As such SbT1 (sorbitol 0.50 per cent) and MnT1 (mannitol 0.5 per cent) recorded the highest plant length and the lowest by SbT5 (sorbitol 3 per cent) and MnT5 (mannitol 3 per cent). Both in mannitol and sorbitol supplemented medium, decreasing trend in growth rate was observed with regard to plant height from eighth week onwards.

Cent per cent survival rate was obtained with all the levels of sucrose and sorbitol up to 28 weeks after inoculation while with mannitol only MnT1, MnT2 and Mn T3 gave cent per cent survival and MnT4 and MnT5 gave 91.66 per cent survival. When transferred to regeneration medium after 28 weeks, cent per cent regeneration was obtained in cultures SbT3, MnT1, MnT2, MnT3 and MnT4. SuT5 and SbT5 did not gave regeneration at all. All treatments which showed more than 50 per cent regeneration were further maintained till 40 weeks by transferring the cultures to same slow growth medium, to study its effect on further retardation of growth. The rest of the cultures not showing any growth symptoms were discarded. The control plants gave only very low regeneration of 16.67 per cent (Table 30).

There was significant variation with regard to plant height and number of nodes among different levels of sucrose, sorbitol and mannitol after 28 weeks of incubation (Table 31). Among the different levels of various osmoticum tried, SuT1 (sucrose at 6 per cent) gave maximum plant height (4.58 cm) and minimum (1.07 cm) by MnT5 (mannitol at 3 per cent) which is on par with MnT4 (1.60). SbT1 recorded highest node number (7.17) whereas the lowest number (1.17) was recorded by SuT4 (sucrose at 12 per cent), which was on par with SuT3, SbT3, SbT4 and MnT4. SbT4 recorded maximum growth inhibition and a regeneration of 66.67 per cent after 28 weeks of incubation. Among those which gave cent per cent survival and regeneration, MnT3 recorded maximum growth inhibition. All treatments recorded

lower plant height and node number compared to control. There was no significant variation with regard to shoot number among different levels of osmoticum tried.

Among *C. ternatea* cultures stored up to 40 weeks, SuT1, SuT2, SuT3, all levels of sorbitol, MnT2, MnT3 and MnT4 recorded cent per cent survival. When inoculated on to regeneration medium cent per cent regeneration could be achieved in MnT1, MnT2 and MnT3. Among these MnT3 recorded the lowest plant height, shoot number and node number, after 40 weeks of incubation; hence identified as the best osmoticum in slow growth medium.

4.2.1.2.2 Addition of Cryoprotectant: Proline

In *C. ternatea,* higher the proline levels lower the plant height. PrT1 (proline 5 mM) recorded maximum height at all stages of incubation while PrT5 (30 mM), the lowest (Table 29) among the different levels tried. But all levels recorded a plant height that is lower than the control. Rate of growth showed a decreasing trend from eight week onwards at all levels of proline tried.

Cent per cent survival was recorded up to 28 weeks of incubation in all the five levels tried. But PrT5 (proline 30 mM) recorded very low rate of survival (16.67 per cent) and hence discarded from further maintenance in slow growth medium (Table 30). PrT1, PrT2 and PrT3 recorded cent per cent regeneration and PrT4 recorded 83.33 per cent. Among these, there was significant variation with regard to plant height and number of nodes (Table 31). Lowest plant height (0.83 cm) and node number (1.33) was recorded by PrT4 but it gave only 50 per cent regeneration, when subcultured and stored up to 40 weeks. After 40 weeks of incubation only PrT3 recorded 66.67 per cent survival with a plant height (1.70 cm) and node number (2.50). When cultured on to regeneration medium, it recorded cent per cent regeneration.

Table 29. **Effect of slow growth treatments on plant height and rate of growth of *Clitoria ternatea* cultures at different lengths of incubation *in vitro***

Tr. No.	Additives		Mean plant height (cm)							Rate of growth (cm/4 wks)						
			4wks	8wks	12wks	16wks	20wks	24wks	28wks	4wks	8wks	12wks	16wks	20wks	24wks	28wks
SuT1	Sucrose (%)	6.00	1.30	2.66	3.46	4.04	4.29	4.45	4.58	1.30	1.36	0.80	0.58	0.25	0.16	0.13
SuT2		8.00	0.61	1.20	1.49	1.66	1.74	1.81	1.82	0.61	0.59	0.29	0.17	0.08	0.07	0.01
SuT3		10.00	0.48	0.82	0.98	1.03	1.07	1.10	1.12	0.48	0.34	0.16	0.05	0.04	0.03	0.02
SuT4		12.00	0.30	0.46	0.50	0.54	0.58	0.60	0.61	0.30	0.16	0.04	0.04	0.04	0.02	0.01
SuT5		15.00	0.30	0.45	0.47	0.48	0.49	0.50	0.51	0.30	0.15	0.02	0.01	0.01	0.01	0.01
SbT1	Sorbitol (%)	0.50	0.84	1.58	2.28	2.83	3.27	3.53	3.72	0.84	0.74	0.70	0.55	0.44	0.26	0.19
SbT2		1.00	0.73	1.43	1.84	1.97	2.08	2.16	2.21	0.73	0.70	0.41	0.13	0.11	0.08	0.05
SbT3		1.50	0.52	0.73	0.93	1.04	1.13	1.20	1.24	0.52	0.21	0.20	0.11	0.09	0.07	0.04
SbT4		2.00	0.43	0.76	0.94	1.04	1.13	1.19	1.23	0.43	0.33	0.18	0.10	0.09	0.06	0.04
SbT5		3.00	0.37	0.55	0.71	0.81	0.90	0.95	0.98	0.37	0.18	0.16	0.10	0.09	0.05	0.03
MnT1	Mannitol (%)	0.50	1.15	1.77	2.25	2.60	2.88	3.08	3.22	1.15	0.62	0.48	0.35	0.28	0.20	0.14
MnT2		1.00	1.13	1.48	1.80	2.07	2.33	2.52	2.65	1.13	0.35	0.32	0.27	0.26	0.19	0.13
MnT3		1.50	1.03	1.33	1.57	1.73	1.83	1.87	1.90	1.03	0.30	0.24	0.16	0.10	0.04	0.03
MnT4		2.00	0.85	1.07	1.25	1.40	1.52	1.58	1.60	0.85	0.22	0.18	0.15	0.12	0.06	0.02
MnT5		3.00	0.43	0.63	0.82	0.97	1.02	1.05	1.07	0.43	0.20	0.19	0.15	0.05	0.03	0.02
PrT1	Proline (mM)	5.00	1.80	2.53	3.23	3.88	4.23	4.49	4.66	1.80	0.73	0.70	0.65	0.35	0.26	0.17
PrT2		10.00	1.13	1.83	2.08	2.32	2.50	2.60	2.68	1.13	0.70	0.25	0.24	0.18	0.10	0.08
PrT3		15.00	0.90	1.18	1.40	1.55	1.63	1.66	1.69	0.90	0.28	0.22	0.15	0.08	0.03	0.03
PrT4		20.00	0.43	0.58	0.66	0.73	0.77	0.81	0.83	0.43	0.15	0.08	0.07	0.04	0.04	0.02
PrT5		30.00	0.42	0.52	0.58	0.63	0.65	0.66	0.67	0.42	0.10	0.06	0.05	0.02	0.01	0.01
PvpT1	PVP (%)	2.00	0.98	1.68	2.20	2.55	2.72	2.81	2.85	0.98	0.70	0.52	0.35	0.17	0.09	0.04
PvpT2		4.00	0.60	0.88	1.10	1.30	1.47	1.53	1.55	0.60	0.28	0.22	0.20	0.17	0.06	0.02
PvpT3		6.00	0.58	0.83	1.05	1.24	1.30	1.35	1.37	0.58	0.25	0.22	0.19	0.06	0.05	0.02
PvpT4		8.00	0.57	0.81	1.02	1.18	1.23	1.26	1.28	0.57	0.24	0.21	0.16	0.05	0.03	0.02
PvpT5		10.00	0.53	0.76	0.96	1.10	1.18	1.21	1.22	0.53	0.23	0.20	0.14	0.08	0.03	0.01
Control	Sucrose (%)	3.00	1.63	3.33	4.07	4.76	5.31	5.56	5.66	1.63	1.70	0.74	0.69	0.55	0.25	0.10

The data represent mean of only live plants; Culture medium: ½ MS + IBA 0.5 mg l^{-1}

Table 30. Effect of slow growth treatments on survival (Sl) and regeneration(R) of Clitoria ternatea cultures at different lengths of incubation in vitro

Tr. No.	Additives		4 wks	8wks	12wks	16wks	20wks	24wks	28 wks		32wks	36wks	40 wks	
			S (%)	Sl (%)	Sl (%)	Sl (%)	Sl (%)	Sl (%)	Sl (%)	R (%)	Sl (%)	Sl (%)	Sl (%)	Rl (%)
SuT1	Sucrose (%)	6.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	66.67^{S}	100.00	100.00	100.00	50.00
SuT2		8.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	83.33^{S}	100.00	100.00	100.00	66.67
SuT3		10.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	83.33^{S}	100.00	100.00	100.00	66.67
SuT4		12.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	83.33^{S}	100.00	83.33	66.67	50.00
SuT5		15.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	00.00^{D}	-	-	-	-
SbT1	Sorbitol (%)	0.50	100.00	100.00	100.00	100.00	100.00	100.00	100.00	83.33^{S}	100.00	100.00	100.00	83.33
SbT2		1.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	83.33^{S}	100.00	100.00	100.00	83.33
SbT3		1.50	100.00	100.00	100.00	100.00	100.00	100.00	100.00	83.33^{S}	100.00	100.00	100.00	50.00
SbT4		2.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	66.67^{S}	100.00	100.00	100.00	50.00
SbT5		3.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	00.00^{D}	-	-	-	
MnT1	Mannitol (%)	0.50	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00^{S}	100.00	100.00	83.33	100.00
MnT2		1.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00^{S}	100.00	100.00	100.00	100.00
MnT3		1.50	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00^{S}	100.00	100.00	100.00	100.00
MnT4		2.00	91.66	91.66	91.66	91.66	91.66	91.66	91.66	100.00^{S}	100.00	100.00	100.00	83.33
MnT5		3.00	91.66	91.66	91.66	91.66	91.66	91.66	91.66	83.33^{S}	83.33	66.67	66.67	50.00
PrT1	Proline (mM)	5.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00^{S}	100.00	100.00	100.00	66.67
PrT2		10.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00^{S}	100.00	66.67	66.67	50.00
PrT3		15.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00^{S}	100.00	83.33	66.67	100.00
PrT4		20.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	83.33^{S}	100.00	66.67	50.00	50.00
PrT5		30.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	16.67^{D}	-	-	-	-
PvpT1	PVP (%)	2.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00^{S}	100.00	66.67	66.67	100.00
PvpT2		4.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00^{S}	100.00	100.00	100.00	100.00
PvpT3		6.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00^{S}	100.00	100.00	100.00	100.00
PvpT4		8.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00^{S}	100.00	100.00	83.33	40.00^{D}
PvpT5		10.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	83.33^{S}	100.00	83.33	83.33	00.00^{D}
Aba T1	ABA (mg l^{-1})	10.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00^{D}	-	-	-	
Aba T2		15.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00^{D}	-	-	-	
Aba T3		20.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00^{D}	-	-	-	
Aba T4		25.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00^{D}	-	-	-	
Aba T5		30.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00	00.00^{D}	-	-	-	
Control	Sucrose(%)	3.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	16.67^{D}	-	-	-	

S: subcultured; D: discarded; Regeneration medium: MS + BA 0.5 mg l^{-1} + Kn 0.5 mg l^{-1}

Table 31. Effect of effective slow growth treatments on morphological parameters in ***Clitoria ternatea*** **cultures after 28 and 40 weeks of incubation** *in vitro*

Tr. No.	Additives		*28 weeks after incubation				**40 weeks after incubation			
			Plant height (cm)	No.of shoots	No of nodes	Remarks	Plant height (cm)	No.of shoots	No of nodes	Remarks
SuT1	Sucrose	6.00	4.58	1.50	5.67	Cp, Rp	4.68	1.50	5.83	Cp, Rp
SuT2	(%)	8.00	1.82	1.33	2.00	Cp, Rp	1.92	1.33	3.00	Cp, Rp
SuT3		10.00	1.12	1.00	2.83	Cp, Rp	1.18	1.00	2.00	Cp, Rp
SuT4		12.00	0.61	1.00	1.17	Cp, Ra	0.63	1.00	1.33	Cp, Ra
SbT1	Sorbitol	0.50	3.72	1.67	7.17	Cp, Rp	3.85	1.67	7.17	Cp, Rp
SbT2	(%)	1.00	2.21	1.33	4.17	Cp, Rp	2.32	1.33	4.17	Cp, Rp
SbT3		1.50	1.24	1.17	1.83	Cp, Rp	1.28	1.17	1.83	Cp, Rp
SbT4		2.00	1.23	1.00	1.67	Ca, Rp	1.23	1.00	1.67	Ca, Rp
MnT1	Mannitol	0.50	3.22	1.83	3.67	Cp, Rp	3.22	1.83	3.80	Cp, Rp
MnT2	(%)	1.00	2.65	1.33	3.33	Cp, Rp	2.70	1.33	3.33	Cp, Rp
MnT3		1.50	1.90	1.33	3.17	Cp, Ra	1.90	1.33	3.17	Cp, Ra
MnT4		2.00	1.60	1.33	2.83	Cp, Ra	1.60	1.33	2.83	Cp, Ra
MnT5		3.00	1.07	1.00	2.00	Cp, Ra	1.08	1.00	2.00	Cp, Ra
PrT1	Proline	5.00	4.66	1.67	6.83	Cp, Rp	4.71	1.67	6.83	Cp, Rp
PrT2	(mM)	10.00	2.68	1.50	3.17	Cp, Ra	2.70	1.50	3.17	Cp, Ra
PrT3		15.00	1.69	1.50	2.33	Cp, Ra	1.70	1.50	2.50	Cp, Ra
PrT4		20.00	0.83	1.50	1.33	Cp, Ra	0.87	1.50	1.33	Cp, Ra
PvpT1	PVP	2.00	2.85	1.67	3.50	Cp, Ra	2.88	1.67	3.50	Cp, Ra
PvpT2	(%)	4.00	1.55	1.67	2.33	Cp, Ra	1.58	1.67	2.33	Cp, Ra
PvpT3		6.00	1.37	1.50	2.17	Cp, Ra	1.37	1.50	2.17	Cp, Ra
PvpT4		8.00	1.28	1.33	2.00	Cp, Ra	1.30	1.33	2.00	Cp, Ra
PvpT5		10.00	1.22	1.33	1.50	Cp, Ra	D	D	D	-
Control	Sucrose (%)	3.00	5.66	1.83	7.33	Cp, Rp	D	D	D	-
CD			0.53	NS	0.90	-	-	-	-	-
F VALUE			53.19	NS	33.71	-	-	-	-	-

*The data represent mean of six replications; ** The data represent mean of live plants in each treatment
Cp: callus present; Ca: callus absent, Rp: root present; Ra: root absent; D: discarded

4.2.1.2.3 Addition of Antioxidant: PVP

Addition of PVP also resulted in slowing down the growth of cultures in *C. ternatea;* with higher levels, plant height decreased. Highest plant height was obtained in PvpT1 (PVP 2 per cent) and lowest at PvpT5 (PVP 10 per cent) at all stages of incubation among the different levels of PVP tried. Rate of growth also showed a decreasing trend from eighth week onwards (Table 29).

After 28 weeks of culture, cent per cent survival and regeneration was recorded at all levels of PVP, except Pvp T5, where the regeneration was slightly reduced (83.33 per cent), due to contamination (Table 30). At this stage, Pvp T1 recorded maximum plant length (2.85 cm) and node number (3.50) and the least (1.22 cm and 1.50 respectively) by PvpT5 (PVP 10 per cent), which was on par with PvpT2, PvpT3 and PvpT4 (Table 31). But after subculturing and storing for 40 weeks, PvpT5 did not regenerate at all, while PvpT2 and PvpT3 gave cent per cent regeneration. Maximum growth inhibition among these was given by PvpT3. PvpT4 gave only 40 per cent regeneration after 40 weeks of storage.

4.2.1.2.4 Addition of Growth Retardant: ABA

The different levels of ABA tried had to be discarded as none of the plants survived at these concentrations in *C. ternatea.*

Among the different levels of various additives tried, all recorded lower plant height compared to that in control, at all stages of incubation. Effect of slow growth additives on rate of plant growth of *Clitoria ternatea* cultures at different lengths of incubation *in vitro* is illustrated in Fig 10. Without any subculture, the cultures of *C. ternatea* could be maintained for 28 weeks. More than 75 per cent reduction in the volume of culture medium was observed at this stage. The effect of different levels of osmotica, cryoprotectant, antioxidant and growth retardant on plant growth of *C.*

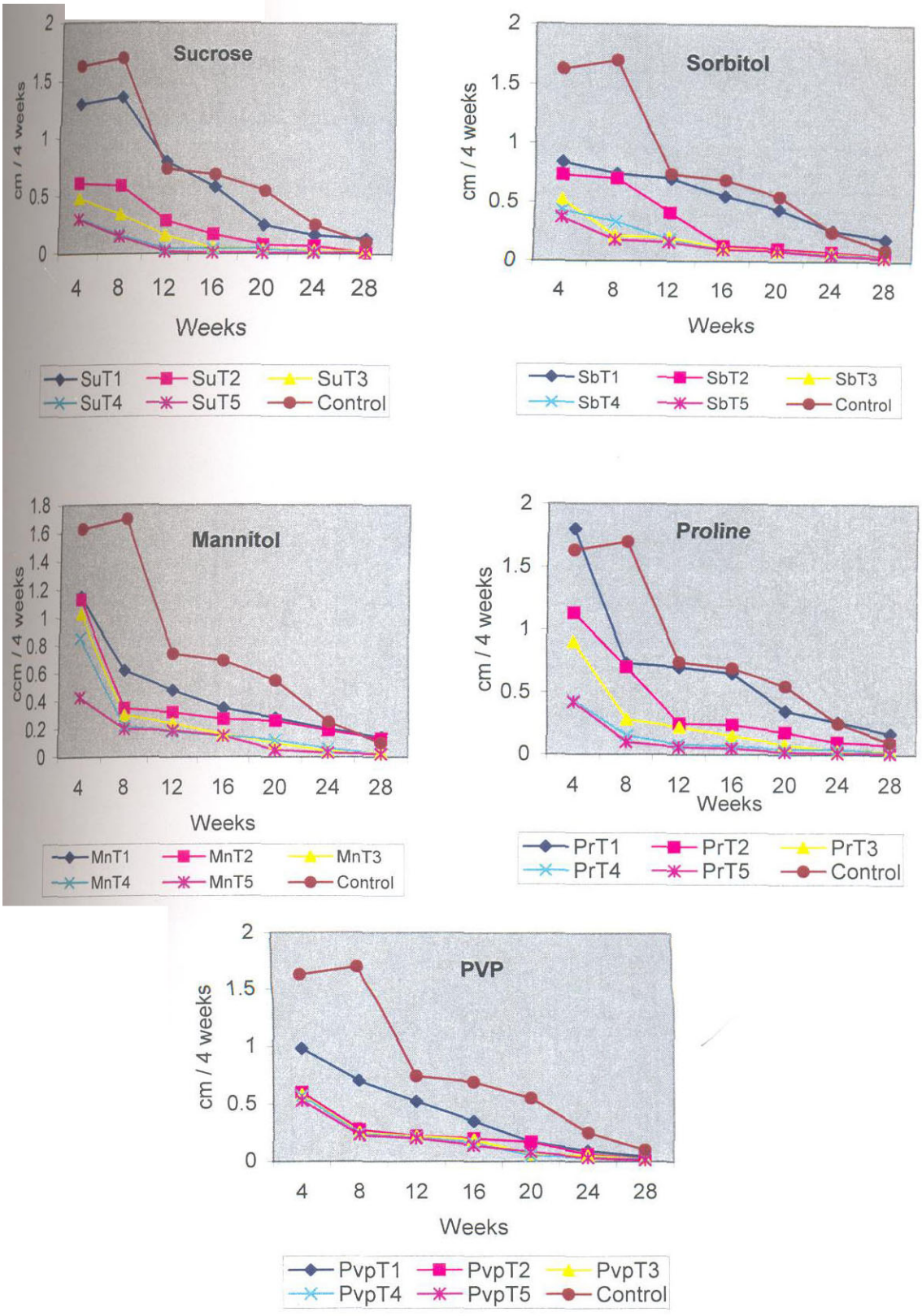


Fig 10. Effect of slow growth additives (osmotica, cryoprotectant and antioxidant) on rate of growth of *Clitoria ternatea* cultures at different lengths of incubation *in vitro*

Plate 9. Effect of different levels of osmotica, cryoprotectant, antioxidant and growth retardant on plant growth of *Clitoria ternatea* cultures after 28 weeks of incubation

a. Plant growth attained by control and different levels of sucrose (6, 8, 10, 12 and 15 %) added to the culture medium (1/2 MS + IBA 0.5 mg l^{-1})

b. Plant growth attained by control and different levels of sorbitol (0.5, 1.0, 1.5, 2.0 and 3.0 %) added to the culture medium (1/2 MS + IBA 0.5 mg l^{-1})

c. Plant growth attained by control and different levels of mannitol (0.5, 1.0, 1.5, 2.0 and 3.0 %) added to the culture medium (1/2 MS + IBA 0.5 mg l^{-1})

d. Plant growth attained by control and different levels of proline (5, 10, 15, 20 and 30 mM) added to the culture medium (1/2 MS + IBA 0.5 mg l^{-1})

e. Plant growth attained by control and different levels of PVP (2, 4, 6, 8 and 10 %) added to the culture medium (1/2 MS + IBA 0.5 mg l^{-1})

f. Plant growth attained by control and different levels of ABA (10, 15, 20, 25 and 30 mg l^{-1}) added to the culture medium (1/2 MS + IBA 0.5 mg l^{-1})

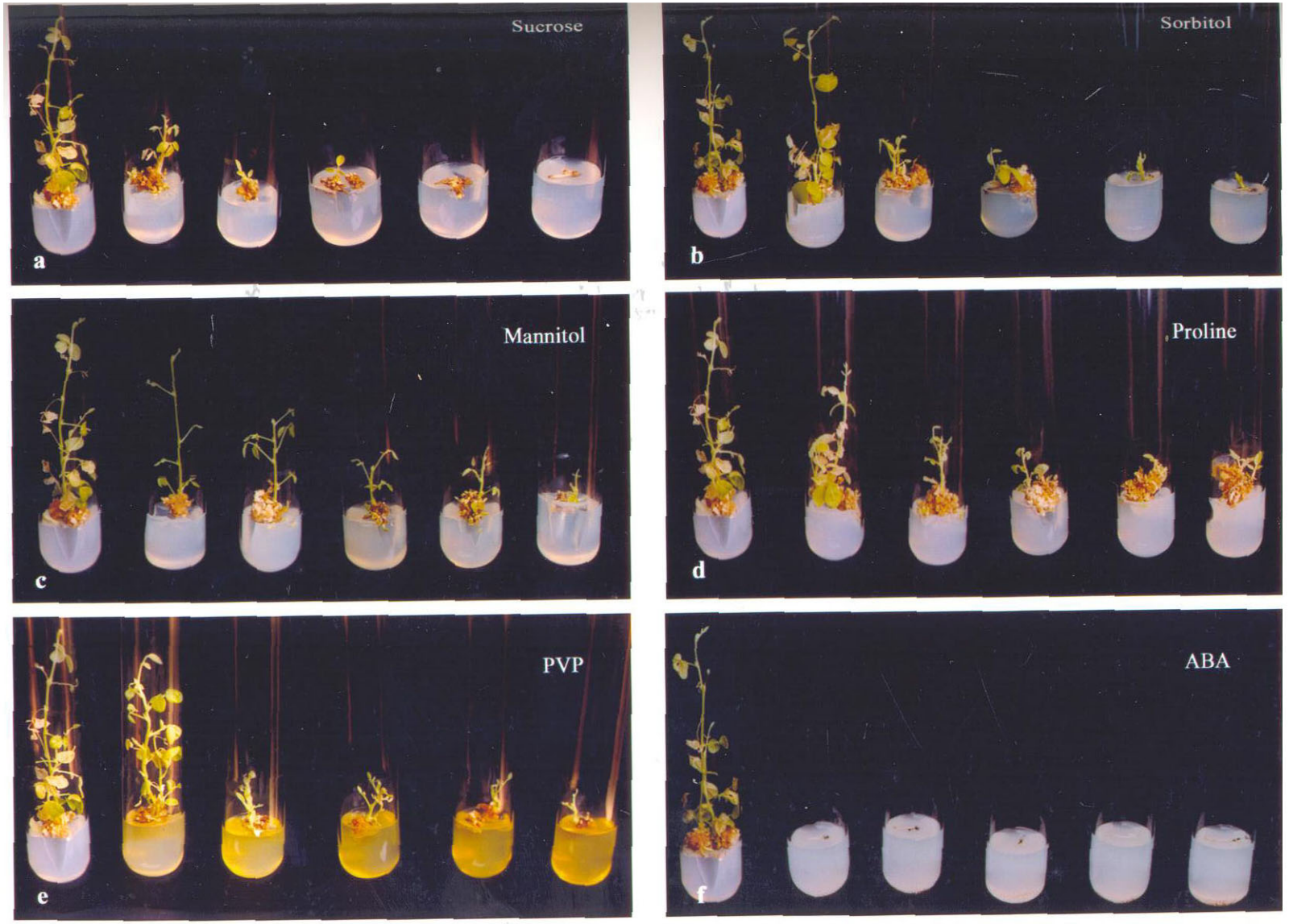


Plate 9. Effect of different levels of osmotica, cryoprotectant, antioxidant and growth retardant on plant growth of *Clitoria ternatea* cultures after 28 weeks of incubation

Plate 10. Shoot regeneration in *Clitoria ternatea* after imposing slow growth treatments for 28 weeks

a. Shoot regeneration in culture medium MS + BA 0.5 mg l^{-1} + Kn 0.5 mg l^{-1} from single nodes excised from slow growth cultures with different levels of sucrose (6, 8, 10 and 12 %) as additives

b. Shoot regeneration in culture medium MS + BA 0.5 mg l^{-1} + Kn 0.5 mg l^{-1} from single nodes excised from slow growth cultures with different levels of sorbitol (0.5, 1.0, 1.5 and 2.0 %) as additives

c. Shoot regeneration in culture medium MS + BA 0.5 mg l^{-1} + Kn 0.5 mg l^{-1} from single nodes excised from slow growth cultures with different levels of mannitol (0.5, 1.0, 1.5, 2.0 and 3.0 %) as additives

d. Shoot regeneration in culture medium MS + BA 0.5 mg l^{-1} + Kn 0.5 mg l^{-1} from single nodes excised from slow growth cultures with different levels of proline (5, 10, 15 and 20mM) as additives

e. Shoot regeneration in culture medium MS + BA 0.5 mg l^{-1} + Kn 0.5 mg l^{-1} from single nodes excised from slow growth cultures with different levels of PVP (2, 4, 6, 8 and 10 %) as additives

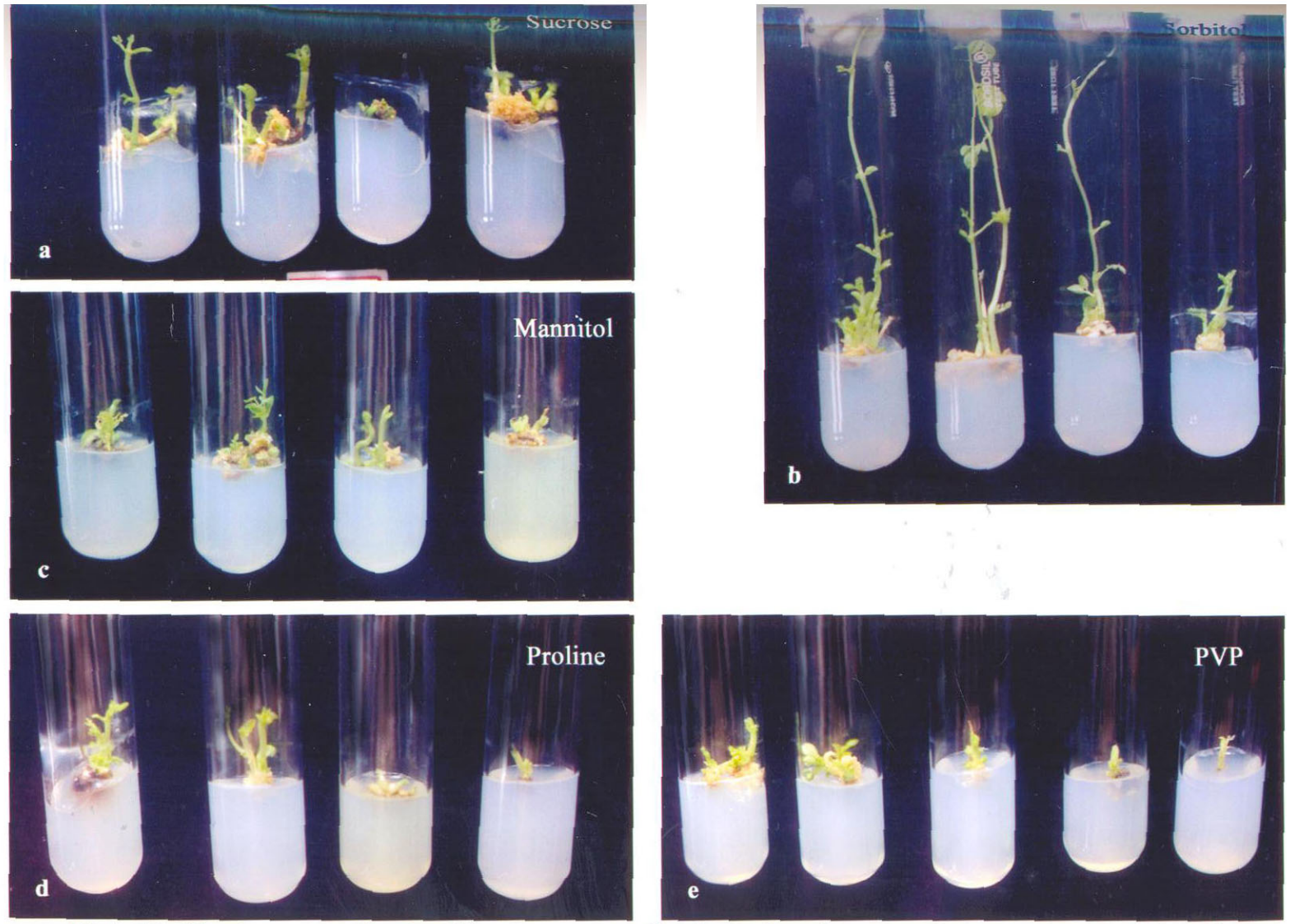
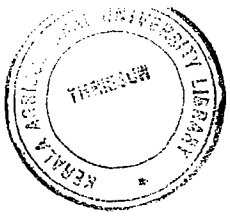


Plate 10. Shoot regeneration in *Clitoria ternatea* after imposing slow growth treatments for 28 weeks

(Shoot proliferation medium: MS + BA 0.5 mg l^{-1} + Kn 0.5 mg l^{-1})



ternatea is illustrated in Plate 9 and shoot regeneration after imposing slow growth treatments for 28 weeks in Plate10.

Among the selected treatments which gave 50 per cent or more regeneration in the slow growth experiment, all cultures except those of SbT4 showed callusing. Rooting was noticed in treatments, SuT1, SuT2, SuT3, SbT1, SbT2, SbT3, SbT4, MnT1, MnT2, PrT1 and control. PVP did not help rooting at any level tried.

Among the selected treatments, maximum plant growth inhibition with reference to plant height (0.61 cm) and node number (1.17) was shown by SuT4, and was on par with PrT4 (Table 31). SuT4 and PrT4 gave 83.33 per cent regeneration at 28 weeks after incubation. Among the various levels of additives tried, maximum plant height (4.66 cm) was recorded by PrT1 and node number (7.17) by SbT1 which was on par with PrT1. Maximum shoot number (1.67) was recorded in SbT1, PrT1, PvpT1 and PvpT2. Among those treatments which gave cent percent survival and regeneration after 28 weeks of incubation, PvpT4 recorded maximum growth inhibition with regard to plant height, shoot number and node number. On storage upto 40 weeks, PvpT4 recorded only 83.33 per cent survival and 40 per cent regeneration .Among the different levels of additives which gave cent per cent survival and regeneration on storage up to 40 weeks, PvpT3 recorded maximum plant growth inhibition.

All the treatments, except PvpT4 and PvpT5, subcultured on to fresh slow growth medium, when transferred to regeneration medium after 40 weeks gave more than 50 per cent regeneration.

Mucuna pruriens

4.2.1.2.1 Addition of Osmotica: Sucrose, Sorbitol and Mannitol

In *M. pruriens*, SuT5 (sucrose at 15 per cent) recorded the lowest plant height and SuT1 (sucrose at 6 per cent) the highest, among the different levels of sucrose tried.

At all stages of growth control recorded maximum plant height compared to various levels of sucrose (Table 32). The rate of plant growth in relation to plant height is also presented in Table 32. In all the sucrose levels rate of growth showed a decreasing trend from eight weeks onwards in *M. pruriens* except control which showed an increasing trend up to eight weeks.

SbT1 (sorbitol 0.50 per cent) recorded maximum plant height (1.88 cm), which is higher than the control, four weeks after incubation. But with later stages SbT1 recorded comparatively lower plant height than the control. SbT5 recorded a plant height of 0.47 cm, which was higher than SbT3 and SbT4, four weeks after incubation. But later stages followed the trend; higher the concentration of sorbitol, lower was the plant height. Moreover after 12 weeks of incubation, SbT4 and SbT5 did not show any increase in plant height; plant height remained constant thereafter. Rate of growth showed a decreasing trend eight week onwards.

MnT1 (0.5 per cent) recorded maximum plant length (3 percent) and MnT5 (3 per cent) the least in all stages of incubation. In MnT1, MnT2 and MnT3, there was no increase in plant height after 16 weeks of incubation, while with MnT4 and MnT5 constant plant height was observed from 12 weeks after incubation. With regard to rate of growth, decreasing trend was noticed from eighth week on wards.

Cent per cent survival rate was obtained with SuT1 (sucrose 6 per cent), SbT1, SbT2, SbT3, SbT4 and all levels of mannitol 28 weeks after inoculation. SuT4 and SuT5 recorded low survival per cent of 33.33 and 8.33 respectively and SbT5, 75 per cent (Table 33). When transferred to regeneration medium, cent per cent regeneration was obtained in cultures SuT1, all levels of sorbitol and mannitol. SuT2 recorded a regeneration percent of 83.33 per cent followed by SuT3 (33.33 per cent). SuT4 and SuT5 did not give any regeneration, while SuT3 recorded low regeneration of 33.33 per cent. All the treatment which showed more than 50 per cent regeneration were further maintained till 40 weeks to study the effect by transferring

the cultures to same slow growth medium and the rest of the cultures were discarded. The control plants did not regenerate on transfer to regeneration medium (Table 33).

After 28 weeks of incubation, there was significant variation with regard to plant height, shoot number and number of nodes among different levels of sucrose, sorbitol and mannitol (which gave 50 per cent regeneration) (Table 34). Among the different levels of various osmoticum tried, SbT1 (sucrose at 6 per cent) gave maximum (3.0 cm) plant height and minimum (0.4 cm) by MnT5 (mannitol at 3 per cent) which is on par with MnT4 (0.43) and SbT5 (0.49 cm). SbT1 recorded highest node number (1.33) and lowest (1.00) by SbT5, MnT4 and MnT5. All treatments recorded lower plant height and node number compared to control. With regard to shoot number maximum shoot number (1.33) was recorded by SbT1. At all levels of sucrose and mannitol, cultures developed only single shoots. SbT4 and SbT5 also produced only single shoots. MnT5 recorded maximum growth inhibition and cent percent survival and regeneration after 28 weeks of incubation. But this did not survive when subcultured on to fresh slow growth medium. After 40 weeks of incubation cent per cent survival and regeneration was obtained with SbT1, SbT2, MnT1 and MnT2. Among these, maximum plant growth inhibition was recorded in MnT2 with a plant height (1.18 cm), shoot number (1) and node number (1.67).

When subcultured on to fresh slow growth medium after 28 weeks, most cultures did not give promising regeneration after 40 weeks of incubation except SbT1, SbT2, MnT1, MnT2 and MnT3. MnT3 gave 83.33 per cent survival after 40 weeks of incubation and 80 per cent regeneration on transfer to regeneration medium (Table 33).

4.2.1.2.2 Addition of Cryoprotectant: Proline

With regard to effect of proline on plant height at various stages of incubation, higher concentration gave lower plant height. PrT1 (proline 5 mM) recorded maximum plant height and minimum by PrT4 (proline 20 mM and PrT5 (proline 30 mM). In fact, PrT4 and PrT5 did not show any increase in plant height after 4 weeks of

Table 32. Effect of slow growth treatments on plant height and rate of growth of *Mucuna pruriens* cultures at different lengths of incubation *in vitro*

Tr. No.	Additives		Mean plant height (cm)							Rate of growth (cm/4 weeks)						
			4 wks	8wks	12wks	16wks	20wks	24wks	28 wks	4wks	8wks	12wks	16wks	20wks	24wks	28wks
SuT1	Sucrose (%)	6.00	0.50	0.80	0.87	0.91	0.95	0.98	1.00	0.50	0.30	0.07	0.04	0.04	0.03	0.02
SuT2		8.00	0.40	0.58	0.72	0.76	0.80	0.82	0.83	0.40	0.18	0.14	0.04	0.04	0.02	0.01
SuT3		10.00	0.38	0.62	0.79	0.86	0.87	0.88	0.89	0.38	0.24	0.19	0.07	0.01	0.01	0.01
SuT4		12.00	0.38	0.47	0.55	0.60	0.60	0.60	0.60	0.38	0.09	0.08	0.05	0.00	0.00	0.00
SuT5		15.00	0.33	0.43	0.50	0.50	0.50	0.50	0.50	0.33	0.10	0.07	0.00	0.00	0.00	0.00
SbT1	Sorbitol (%)	0.50	1.88	2.35	2.70	2.80	2.87	2.94	3.00	1.88	0.47	0.35	0.10	0.07	0.07	0.06
SbT2		1.00	1.41	201	2.41	2.63	2.70	2.76	2.80	1.41	0.60	0.40	0.22	0.07	0.06	0.04
SbT3		1.50	0.40	0.67	0.72	0.75	0.78	0.80	0.82	0.40	0.27	0.05	0.03	0.03	0.02	0.02
SbT4		2.00	0.37	0.62	0.67	0.67	0.67	0.67	0.67	0.37	0.25	0.05	0.00	0.00	0.00	0.00
SbT5		3.00	0.47	0.48	0.49	0.49	0.49	0.49	0.49	0.47	0.01	0.01	0.00	0.00	0.00	0.00
MnT1	Mannitol (%)	0.50	0.65	1.15	1.23	1.25	1.25	1.25	1.25	0.65	0.50	0.08	0.02	0.00	0.00	0.00
MnT2		1.00	0.45	0.85	1.10	1.15	1.15	1.15	1.15	0.45	0.40	0.25	0.05	0.00	0.00	0.00
MnT3		1.50	0.40	0.73	0.92	0.95	0.95	0.95	0.95	0.40	0.33	0.19	0.03	0.00	0.00	0.00
MnT4		2.00	0.37	0.40	0.43	0.43	0.43	0.43	0.43	0.37	0.03	0.03	0.00	0.00	0.00	0.00
MnT5		3.00	0.30	0.37	0.40	0.40	0.40	0.40	0.40	0.30	0.07	0.03	0.00	0.00	0.00	0.00
PrT1	Proline (mM)	5.00	0.92	1.35	1.47	1.55	1.58	1.61	1.63	0.92	0.43	0.12	0.08	0.03	0.03	0.02
PrT2		10.00	0.48	0.90	1.10	1.17	1.21	1.22	1.22	0.48	0.42	0.20	0.07	0.04	0.01	0.00
PrT3		15.00	0.37	0.69	0.89	0.93	0.95	0.96	0.96	0.37	0.32	0.20	0.04	0.02	0.01	0.00
PrT4		20.00	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.00	0.00	0.00	0.00	0.00	0.00
PrT5		30.00	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.00	0.00	0.00	0.00	0.00	0.00
PvpT1	PVP (%)	2.00	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.00	0.00	0.00	0.00	0.00	0.00
PvpT2		4.00	0.30	0.35	0.35	0.35	0.35	0.35	0.35	0.30	0.05	0.00	0.00	0.00	0.00	0.00
PvpT3		6.00	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.00	0.00	0.00	0.00	0.00	0.00
PvpT4		8.00	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.00	0.00	0.00	0.00	0.00	0.00
PvpT5		10.00	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.00	0.00	0.00	0.00	0.00	0.00
Aba T1	ABA (mg l^{-1})	10.00	0.40	0.55	0.60	0.63	0.65	0.67	0.67	0.40	0.05	0.05	0.03	0.02	0.02	0.00
Aba T2		15.00	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.00	0.00	0.00	0.00	0.00	0.00
Aba T3		20.00	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.00	0.00	0.00	0.00	0.00	0.00
Aba T4		25.00	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.00	0.00	0.00	0.00	0.00	0.00
Aba T5		30.00	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.00	0.00	0.00	0.00	0.00	0.00
Control	Sucrose (%)	3.00	1.05	2.62	3.02	3.28	3.49	3.62	3.68	1.05	1.57	0.40	0.26	0.21	0.13	0.06

The data represent mean of only live plants; Culture medium: ½ MS + IBA 0.5 mg l^{-1}

Table 33. Effect of slow growth treatments on survival (SI) and regeneration (R) of Mucuna pruriens cultures at different length of incubation in vitro

Tr. No.	Additives		4 wks	8wks	12wks	16wks	20wks	24wks	28wks		32 wks	36wks	40wks	
			SI (%)	SI (%)	SI (%)	SI(%)	SI (%)	SI (%)	SI (%)	R (%)	SI (%)	SI (%)	SI (%)	R (%)
SuT1	Sucrose (%)	6.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00^{S}	83.33	66.67	50.00	00.00^{D}
SuT2		8.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	83.33^{S}	66.67	50.00	50.00	00.00^{D}
SuT3		10.00	100.00	100.00	100.00	83.33	83.33	83.33	83.33	33.33^{D}	-	-	-	-
SuT4		12.00	50.00	50.00	33.33	33.33	33.33	33.33	33.33	00.00^{D}	-	-	-	-
SuT5		15.00	33.33	33.33	16.67	8.33	8.33	8.33	8.33	00.00^{D}	-	-	-	-
SbT1	Sorbitol (%)	0.50	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00^{S}	100.00	100.00	100.00	100.00
SbT2		1.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00^{S}	100.00	100.00	100.00	100.00
SbT3		1.50	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00^{S}	100.00	83.33	83.33	60.00
SbT4		2.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00^{S}	100.00	50.00	50.00	00.00^{D}
SbT5		3.00	75.00	75.00	75.00	75.00	75.00	75.00	75.00	100.00^{S}	33.33	00.00	00.00	00.00^{D}
MnT1	Mannitol (%)	0.50	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00^{S}	100.00	100.00	100.00	100.00
MnT2		1.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00^{S}	100.00	100.00	100.00	100.00
MnT3		1.50	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00^{S}	100.00	100.00	83.33	80.00
MnT4		2.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00^{S}	83.33	66.67	50.00	33.33^{D}
MnT5		3.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00^{S}	00.00	00.00	00.00	00.00^{D}
PrT1	Proline (mM)	5.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00^{S}	66.67	50.00	50.00	33.33^{D}
PrT2		10.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00^{S}	66.67	50.00	50.00	33.33^{D}
PrT3		15.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00^{S}	50.00	50.00	50.00	33.33^{D}
PrT4		20.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	16.67^{D}	-	-	-	-
PrT5		30.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	00.00^{D}	-	-	-	-
PvpT1	PVP (%)	2.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00^{S}	16.67	16.67	00.00	00.00^{D}
PvpT2		4.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00^{S}	00.00	00.00	00.00	00.00^{D}
PvpT3		6.00	83.33	83.33	83.33	83.33	66.67	66.67	66.67	00.00^{D}	-	-	-	-
PvpT4		8.00	100.00	83.33	83.33	66.67	50.00	50.00	50.00	00.00^{D}	-	-	-	-
PvpT5		10.00	91.67	66.67	66.67	66.67	50.00	50.00	50.00	00.00^{D}	-	-	-	-
Aba T1	ABA (mg l^{-1})	10.00	100.00	100.00	100.00	100.00	83.33	83.33	83.33	100.00^{S}	00.00	00.00	00.00	00.00^{D}
Aba T2		15.00	66.67	66.67	66.67	66.67	50.00	50.00	50.00	100.00^{S}	00.00	00.00	00.00	00.00^{D}
Aba T3		20.00	75.00	66.67	58.33	50.00	50.00	50.00	50.00	16.67^{D}	-	-	-	-
Aba T4		25.00	66.67	66.67	66.67	50.00	50.00	50.00	50.00	16.67^{D}	-	-	-	-
Aba T5		30.00	58.33	50.00	50.00	50.00	50.00	50.00	50.00	00.00^{D}	-	-	-	-
Control	Sucrose (%)	3.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	00.00^{D}	-	-	-	

S: subcultured; D: discarded; Regeneration medium: MS + BA 0.5 mg l^{-1} + Kn 0.5 mg l^{-1}

Table 34. Effect of effective slow growth treatments on morphological parameters in Mucuna pruriens cultures after 28 and 40 weeks of incubation in vitro

Tr. No.	Additives		*28 weeks after incubation				**40 weeks after incubation			
			Plant height (cm)	No.of shoots	No of nodes	Remarks	Plant height (cm)	No.of shoots	No of nodes	Remarks
SuT1	**Sucrose**	6.00	1.00	1.00	1.33	Ca, Rp	D	D	D	--
SuT2	**(%)**	8.00	0.83	1.00	1.17	Ca, Rp	D	D	D	--
SbT1		0.50	3.00	1.33	2.83	Ca, Rp	3.03	1.33	2.83	Ca, Rp
SbT2	**Sorbitol**	1.00	2.80	1.17	2.67	Ca, Rp	2.85	1.17	2.67	Ca, Rp
SbT3	**(%)**	1.50	0.82	1.17	1.33	Ca, Rp	0.88	1.17	1.40	Ca, Rp
SbT4		2.00	0.67	1.00	1.17	Ca, Rp	D	D	D	--
SbT5		3.00	0.49	1.00	1.00	Ca, Rp	D	D	D	--
MnT1		0.50	1.25	1.00	1.67	Ca, Rp	1.35	1.00	1.83	Ca, Rp
MnT2	**Mannitol**	1.00	1.15	1.00	1.50	Ca, Rp	1.18	1.00	1.67	Ca, Rp
MnT3	**(%)**	1.50	0.95	1.00	1.17	Ca, Rp	0.98	1.00	1.17	Ca, Rp
MnT4		2.00	0.43	1.00	1.00	Ca, Rp	D	D	D	--
MnT5		3.00	0.40	1.00	1.00	Ca, Ra	D	D	D	--
PrT1	**Proline**	5.00	1.61	1.00	1.83	Ca, Rp	D	D	D	--
PrT2	**(mM)**	10.00	1.22	1.00	1.33	Ca, Ra	D	D	D	--
PrT3		15.00	0.96	1.00	1.17	Ca, Ra	D	D	D	--
PvpT1	**PVP**	2.00	0.37	1.33	1.00	Ca, Ra	D	D	D	--
PvpT2	**(%)**	4.00	0.35	1.17	1.00	Ca, Ra	D	D	D	--
AbaT1	**ABA**	10.00	0.67	1.00	1.00	Ca, Ra	D	D	D	--
AbaT2	**(mg l^{-1})**	15.00	0.30	1.00	1.00	Ca, Ra	D	D	D	--
Control	**Sucrose (%)**	3.00	3.68	1.50	3.33	Ca, Rp	D	D	D	--
CD			0.52	0.30	0.57	--	--	--	--	--
F value			26.76	1.97	11.48	--	--	--	--	--

*The data represent mean of six replications; ** The data represent mean of live plants in each treatment
Cp: callus present; Ca: callus absent, Rp: root present; Ra: root absent; D: discarded

incubation. Single node inoculated on to the medium remained green and viable which did not increase in length beyond four weeks. Rate of growth showed a decreasing trend from eighth weeks onwards (Table 32)

Proline at all levels recorded cent per cent survival till 28 weeks of incubation (Table 33). At this stage the cultures were transferred to regeneration medium, on which PrT1, PrT2 and PrT3 recorded cent per cent regeneration. PrT4 recorded low regeneration (16.67 per cent) and Pr T5 did not regenerate at all. These two were discarded from storing further by transfer of cultures to fresh slow growth medium.

After 28 weeks, there was significant variation with regard to plant height and node number among three levels of PrT1, PrT2 and PrT3. Maximum plant height (1.61cm) and node number (1.83) were recorded by PrT1 and lowest (0.96 and 1.17 respectively) by PrT3 (Table 34). Only a single shoot was produced at all levels of proline in *M. pruriens.* But when these cultures were transferred to fresh slow growth medium, it gave only very low survival of 50 per cent, after 40 weeks of incubation and very low regeneration (33.33 per cent) when inoculated on to regeneration medium. As such, PrT3 recorded maximum plant growth inhibition and is suited to slow growth storage for 28 weeks and not any further.

4.2.1.2.3 Addition of Antioxidant: PVP

Addition of PVP caused considerable reduction in growth of plants in *M. pruriens.* After a slight initial increase in plant height, it remained constant at all stages of incubation. Highest plant height was obtained in PvpT1 (PVP 2 per cent) and lowest at PvpT4 (PVP 8 per cent) and PvpT5 (PVP 10 per cent). Rate of growth also showed a decreasing trend from eighth week on wards (Table 32).

After 28 weeks of culture, cent per cent survival and regeneration was recorded by PvpT1 and PvpT2; while PvpT3, PvpT4 and PvpT5 did not regenerate on transfer to regeneration medium and hence were discarded from further study (Table

33). PvpT1 and PvpT2 did not show any significant variation with regard to plant height, shoot number and node number. But PvpT1 and PvpT2 when subcultured and stored up to 40 weeks, did not survive at all. So as in the case of proline, PVP also is a good preposition for storage up to 28 weeks only.

4.1.2.2.4 Addition of Growth Retardant: ABA

The different levels of ABA inhibited plant growth in *M. pruriens*. All levels of ABA did not cause any considerable increase in plant height. After an initial sprouting to 0.3 cm, the plant did not develop in height. The sprouts remained green.

With AbaT1 (ABA 10 mg l^{-1}), cent per cent survival was recorded only upto 16 weeks; at 28 weeks the survival was 83.3 per cent (Table 33). With all other levels of ABA, survival at 28 weeks was only 50 per cent. Though survival per cent was less, AbaT1 and AbaT2 when inoculated on to regeneration medium after 28 weeks of incubation gave cent per cent regeneration; while higher concentration of ABA gave very low or no regeneration. But when subcultured to fresh slow growth medium, none of the cultures survived. At 28 weeks, AbaT1 and AbaT2 did not show any significant variation with regard to plant height, shoot number and node number (Table 34).

In cultures of *M. pruriens*, all slow growth treatments recorded lower plant height compared to the control at all stages of incubation. Effect of slow growth additives on rate of plant growth of *M. pruriens* cultures at different lengths of incubation *in vitro* is indicated in Fig.11. Cultures could be maintained for 28 weeks without any subculture.

In the treatments selected 28 weeks after incubation, no callusing was noticed in any of the cultures. Rooting was present in all the treatments except MnT5, PrT2, PrT3, all levels of PVP and ABA (Table 34).

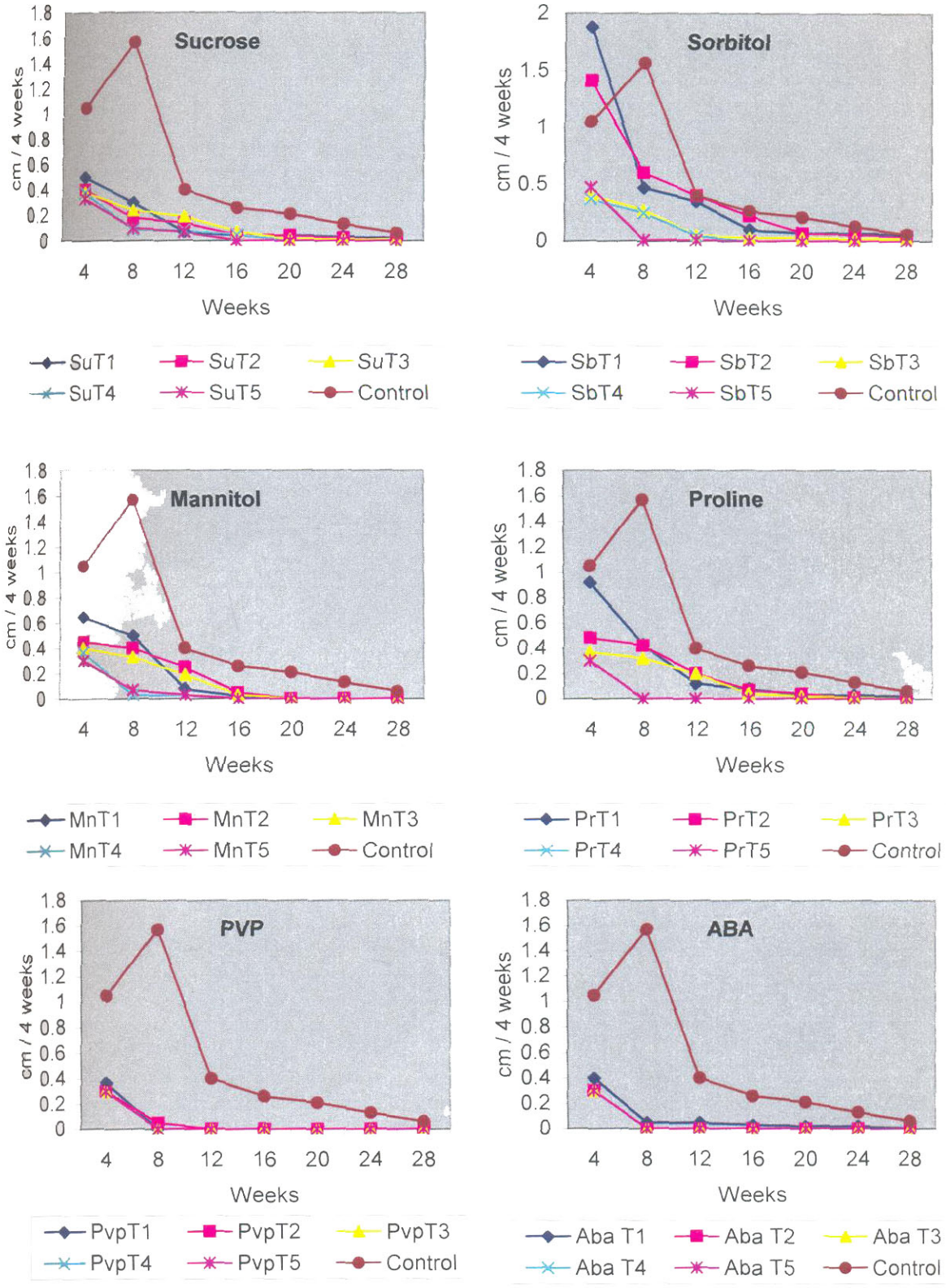


Fig 11. Effect of slow growth additives (osmotica, cryoprotectant, antioxidant and growth retardant) on rate of growth of *Mucuna pruriens* cultures at different lengths of incubation *in vitro*

Plate 11. Effect of different levels of osmotica, cryoprotectant, antioxidant and growth retardant on plant growth of *Mucuna pruriens* cultures after 28 weeks of incubation

a. Plant growth attained by control and different levels of sucrose (6, 8, 10, 12 and 15 %) added to the culture medium (1/2 MS + IBA 0.5 mg l^{-1})

b. Plant growth attained by control and different levels of sorbitol (0.5, 1.0, 1.5, 2.0 and 3.0 %) added to the culture medium (1/2 MS + IBA 0.5 mg l^{-1})

c. Plant growth attained by control and different levels of mannitol (0.5, 1.0, 1.5, 2.0 and 3.0 %) added to the culture medium (1/2 MS + IBA 0.5 mg l^{-1})

d. Plant growth attained by control and different levels of proline (5, 10, 15, 20 and 30 mM) added to the culture medium (1/2 MS + IBA 0.5 mg l^{-1})

e. Plant growth attained by control and different levels of PVP (2, 4, 6, 8 and 10 %) added to the culture medium (1/2 MS + IBA 0.5 mg l^{-1})

f. Plant growth attained by control and different levels of ABA (10, 15, 20, 25 and 30 mg l^{-1}) added to the culture medium (1/2 MS + IBA 0.5 mg l^{-1})

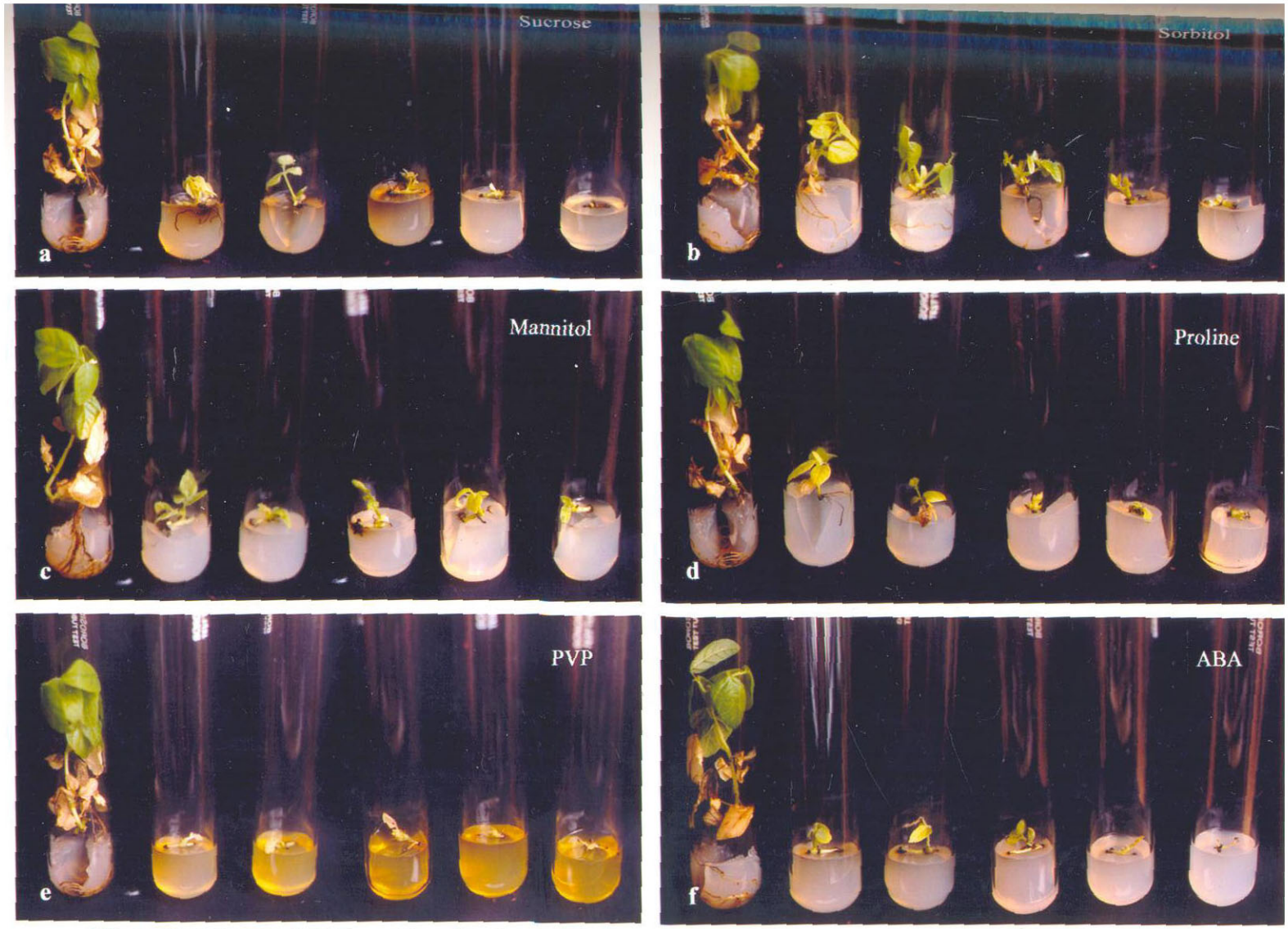


Plate 11. Effect of different levels of osmotica, cryoprotectant, antioxidant and growth retardant on plant growth of *Mucuna pruriens* cultures after 28 weeks of incubation

Plate 12. Shoot regeneration in *Mucuna pruriens* after imposing slow growth treatments for 28 weeks

a. Shoot regeneration in culture medium MS + BA 0.5 mg l^{-1} + Kn 0.5 mg l^{-1} from single nodes excised from slow growth cultures with different levels of sucrose (6, 8 and 10 %) as additives

b. Shoot regeneration in culture medium MS + BA 0.5 mg l^{-1} + Kn 0.5 mg l^{-1} from single nodes excised from slow growth cultures with different levels o f sorbitol (0.5, 1.0, 1.5, 2.0 and 3.0 %) as additives

c. Shoot regeneration in culture medium MS + BA 0.5 mg l^{-1} + Kn 0.5 mg l^{-1} from single nodes excised from slow growth cultures with different levels of mannitol (0.5, 1.0, 1.5, 2.0 and 3.0 %) as additives

d. Shoot regeneration in culture medium MS + BA 0.5 mg l^{-1} + Kn 0.5 mg l^{-1} from single nodes excised from slow growth cultures with different levels of proline (5, 10 and 15 mM) as additives

e. Shoot regeneration in culture medium MS + BA 0.5 mg l^{-1} + Kn 0.5 mg l^{-1} from single nodes excised from slow growth cultures with different levels of PVP (2 and 4 %) as additives

f. Shoot regeneration in culture medium MS + BA 0.5 mg l^{-1} + Kn 0.5 mg l^{-1} from single nodes excised from slow growth cultures with different levels of ABA (10 and 15 mg l^{-1}) as additives

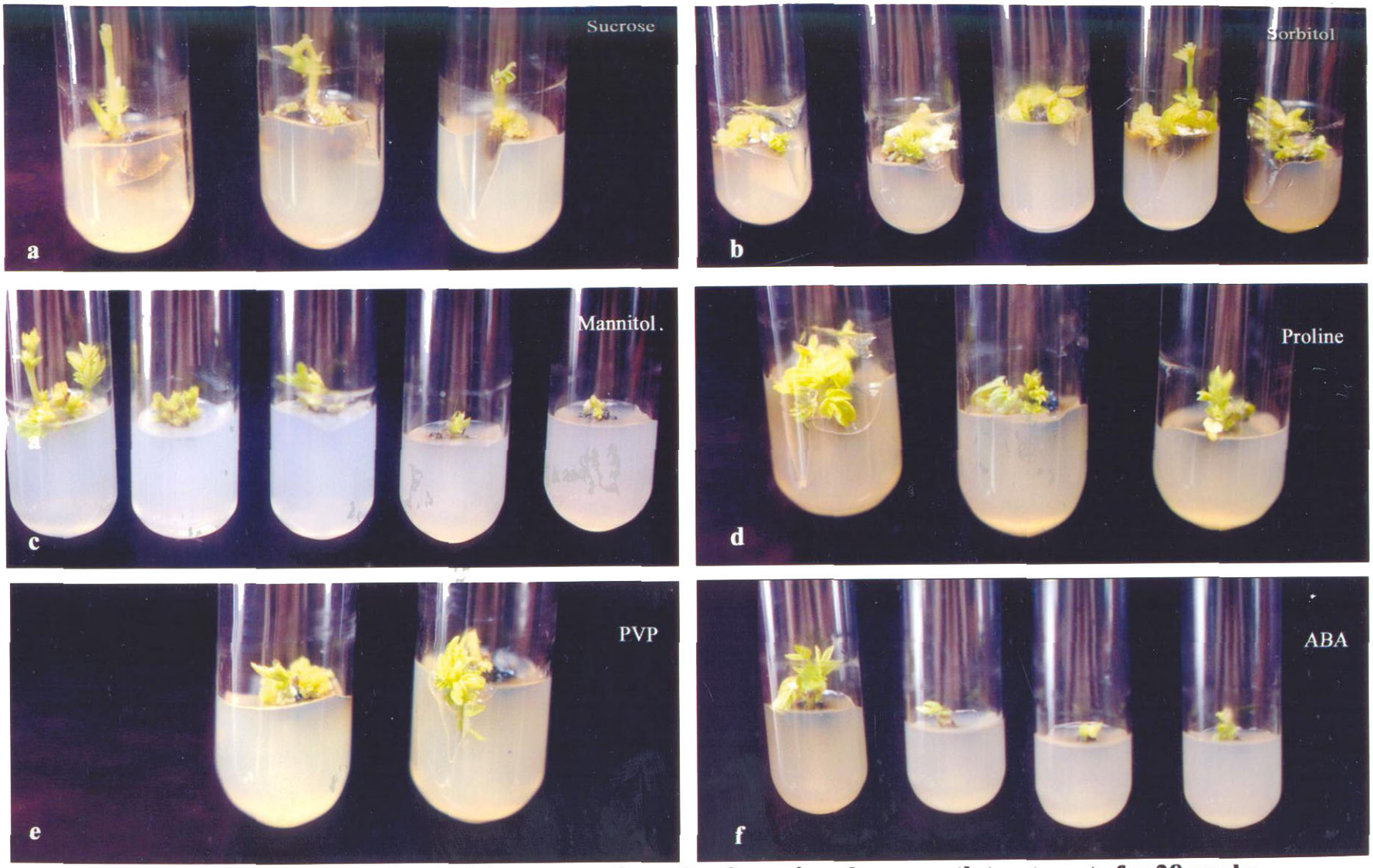


Plate 12. Shoot regeneration in *Mucuna pruriens* after imposing slow growth treatments for 28 weeks

(Shoot proliferation medium: MS + BA 0.5 mg l^{-1} + Kn 0.5 mg l^{-1})

The effect of different levels of osmotica, cryoprotectant, antioxidant and growth retardant on plant growth of *M. pruriens* after 28 weeks of incubation is illustrated in Plate 11 and shoot regeneration after imposing slow growth treatments for 28 weeks in Plate 12. Among the cultures selected for subculture at 28 weeks, there was significant variation with regard to plant height, shoot number and node number. Maximum plant height (3.0 cm) was recorded by SbT1 (sorbitol 0.5 per cent) and the lowest by AbaT2 (ABA 15mg l^{-1}) which was on par with AbaT1, SbT3, SbT4, SbT5, MnT4, MnT5, PvpT1 and PvpT2. With regard to shoot number maximum value (1.33) was recorded by SbT1 and PvpT1 and minimum (1.00) by SuT1, SuT2, SbT4, SbT5, MnT1, MnT2, MnT3, MnT4, MnT5, PrT1, PrT2, PrT3, AbaT1 and AbaT2. Maximum node number (2.83) was recorded by SbT1 and lowest (1.00) by SbT5, MnT4, MnT5, PvpT1, PvpT2, AbaT1 and AbaT2. AbaT2 recorded maximum plant growth inhibition but at 28th week it gave only 50 per cent survival. Considering the maximum plant growth inhibition, survival and regeneration, PvpT2 is identified as the best medium among the various additives tried, which can be stored upto 28 weeks without subculture. But this did not give any regeneration when stored upto 40 weeks. At 40 weeks, SbT3 recorded maximum plant inhibition with regard to plant height but regeneration was only 60 per cent followed by MnT3 which recorded only 80 per cent regeneration. Hence MnT2 with next lower plant height, which gave cent per cent survival and regeneration was selected as the best medium for storage up to 40 weeks. Compared to control, all levels of various additives, caused inhibition in plant growth, with regard to plant height, shoot number and node number.

Indigofera tinctoria

4.2.1.2.1 Addition of Osmotica: Sucrose, Sorbitol and Mannitol

Among the different levels of sucrose tried, SuT4 (sucrose at 12 per cent) and SuT5 (sucrose at 15 per cent) recorded lowest plant height and SuT1 (sucrose at 6 per cent) the highest at all stages of incubation. Su T4 and SuT5 did not increase in

length after an initial sprouting; hence plant height (0.3 cm) remained constant through out the incubation period. At all stages of growth control recorded maximum plant height compared to various levels of sucrose (Table 35). The rate of plant growth in relation to plant height is also depicted in Table 35. In all the sucrose levels rate of growth showed a decreasing trend from 8 weeks on wards including the control.

SbT1 (sorbitol 0.5 per cent) recorded maximum plant height and SbT5 the least. The same trend was followed up to 28 weeks of incubation. Higher the concentration of sorbitol, lower was the plant height (Table 35). Rate of growth showed a decreasing trend from eighth week on wards.

MnT1 (0.5 per cent) recorded highest plant length (3 per cent) and MnT5 (3 per cent) the least in all stages of incubation. With regard to rate of growth, decreasing trend was noticed from eighth week on wards.

Cent per cent survival was obtained with SuT1, SuT2, SuT3, SuT4, all levels of sorbitol and all levels of mannitol, 28 weeks after inoculation. SuT5 recorded low survival per cent of 41.67 per cent. When transferred to regeneration medium, cent per cent regeneration was obtained in cultures SuT1, SuT2, SuT3, SbT3, SbT4, SbT5, MnT1 and MnT2. SuT4 recorded low regeneration of 33.33 per cent; while SuT5 did not give any regeneration. All the treatments which showed more than 50 per cent regeneration were further maintained till 40 weeks to study the effect by transferring the cultures to same slow growth medium and the rest of the cultures were discarded. The control plants recorded a low regeneration of 33.33 per cent on transfer to regeneration medium (Table 36).

There was significant variation with regard to plant height, shoot number and number of nodes among different levels (which gave 50 per cent or more regeneration) of sucrose, sorbitol and mannitol after 28 weeks of incubation (Table 37). Among the different levels of various osmoticum tried, SbT1 (sucrose at 6 per cent) gave maximum (5.5 cm) plant height and minimum (0.63 cm) by SuT3 (sucrose

Table 35. Effect of slow growth treatments on mean plant height and rate of growth of Indigofera tinctoria cultures at different lengths of incubation in vitro

Tr. No.	Additives		Mean plant height (cm)							Rate of growth (cm/4 weeks)						
			4 wks	8wks	12wks	16wks	20wks	24wks	28wks	4 wks	8wks	12wks	16wks	20wks	24wks	28wks
SuT1	Sucrose (%)	6.00	2.00	2.58	3.05	3.28	3.37	3.42	3.46	2.00	0.58	0.47	0.23	0.09	0.05	0.04
SuT2		8.00	1.05	1.45	1.80	2.05	2.12	2.17	2.21	1.05	0.40	0.35	0.25	0.07	0.05	0.04
SuT3		10.00	0.37	0.47	0.53	0.57	0.60	0.62	0.64	0.37	0.10	0.06	0.04	0.03	0.02	0.02
SuT4		12.00	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.00	0.00	0.00	0.00	0.00	0.00
SuT5		15.00	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.00	0.00	0.00	0.00	0.00	0.00
SbT1	Sorbitol (%)	0.50	2.35	4.37	4.90	5.17	5.32	5.42	5.50	2.35	2.02	0.53	0.27	0.15	0.10	0.08
SbT2		1.00	2.05	2.67	3.13	3.45	3.57	3.67	3.74	2.05	0.62	0.46	0.32	0.12	0.10	0.07
SbT3		1.50	0.98	1.62	1.85	2.05	2.17	2.28	2.36	0.98	0.64	0.23	0.20	0.12	0.11	0.08
SbT4		2.00	0.75	1.40	1.50	1.58	1.62	1.66	1.69	0.75	0.65	0.10	0.08	0.04	0.04	0.03
SbT5		3.00	0.30	0.60	0.85	0.95	0.98	0.99	1.00	0.30	0.30	0.25	0.10	0.03	0.01	0.01
MnT1	Mannitol (%)	0.50	1.67	2.25	2.57	2.80	2.90	2.99	3.07	1.67	0.58	0.32	0.23	0.10	0.09	0.08
MnT2		1.00	1.28	1.60	1.72	1.82	1.85	1.87	1.88	1.28	0.32	0.12	0.10	0.03	0.02	0.01
MnT3		1.50	0.77	1.30	1.47	1.63	1.70	1.77	1.82	0.77	0.53	0.17	0.16	0.07	0.07	0.05
MnT4		2.00	0.50	0.75	0.87	0.90	0.93	0.95	0.97	0.50	0.25	0.12	0.03	0.03	0.02	0.02
MnT5		3.00	0.30	0.48	0.53	0.58	0.62	0.64	0.64	0.30	0.18	0.05	0.05	0.04	0.02	0.00
PrT1	Proline (mM)	5.00	2.57	4.62	5.30	5.63	5.85	6.04	6.16	2.57	2.05	0.68	0.33	0.22	0.19	0.12
PrT2		10.00	2.82	3.85	4.65	5.37	5.87	6.30	6.53	2.82	1.03	0.80	0.72	0.50	0.43	0.23
PrT3		15.00	3.07	4.72	5.95	6.23	6.37	6.50	6.59	3.07	1.65	1.23	0.28	0.14	0.13	0.09
PrT4		20.00	2.38	4.06	5.60	6.22	6.35	6.44	6.51	2.38	1.68	1.54	0.62	0.13	0.09	0.07
PrT5		30.00	1.45	3.53	5.43	5.67	5.87	6.04	6.16	1.45	2.08	1.90	0.24	0.20	0.17	0.12
PvpT1	PVP (%)	2.00	0.88	1.73	2.10	2.17	2.22	2.26	2.30	0.88	0.85	0.37	0.07	0.05	0.04	0.04
PvpT2		4.00	0.43	0.73	1.00	1.07	1.08	1.09	1.10	0.43	0.30	0.27	0.07	0.01	0.01	0.01
PvpT3		6.00	0.43	0.73	0.98	1.05	1.07	1.08	1.09	0.43	0.30	0.25	0.07	0.02	0.01	0.01
PvpT4		8.00	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.00	0.00	0.00	0.00	0.00	0.00
PvpT5		10.00	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.00	0.00	0.00	0.00	0.00	0.00
Aba T1	ABA (mg l^{-1})	10.00	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.00	0.00	0.00	0.00	0.00	0.00
Aba T2		15.00	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.00	0.00	0.00	0.00	0.00	0.00
Aba T3		20.00	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.30	0.00	0.00	0.00	0.00	0.00	0.00
Aba T4		25.00	0.30	0.30	0.30	0.30	0.30	0.30	0.00	0.30	0.00	0.00	0.00	0.00	0.00	0.00
Aba T5		30.00	0.30	0.30	0.30	0.30	0.30	0.30	0.00	0.30	0.00	0.00	0.00	0.00	0.00	0.00
Control	Sucrose (%)	3.00	3.08	4.25	4.98	5.51	5.78	5.97	6.10	3.08	1.17	0.73	0.53	0.27	0.19	0.13

The data represent mean of only live plants; Culture medium: ½ MS + IBA 0.5 mg l^{-1}

Table 36. Effect of slow growth treatments on survival (SI) and regeneration (R) of Indigofera tinctoria cultures at different lengths of incubation in vitro

Tr. No.	Additives		4 wks	8wks	12wks	16wks	20wks	24wks	28wks		32wks	36wks	40 wks	
			SI (%)	SI (%)	SI (%)	SI (%)	SI(%)	SI(%)	SI (%)	R (%)	SI (%)	S I(%)	SI (%)	R (%)
SuT1	Sucrose (%)	6.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00 S	100.00	100.00	83.33	80.00
SuT2		8.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00 S	50.00	50.00	50.00	33.33 D
SuT3		10.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00 S	00.00	00.00	00.00	00.00 D
SuT4		12.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	33.33 D	-	-	-	-
SuT5		15.00	100.00	100.00	83.33	58.33	50.00	41.67	41.67	00.00 D	-	-	-	-
SbT1	Sorbitol (%)	0.50	100.00	100.00	100.00	100.00	100.00	100.00	100.00	66.67 S	100.00	100.00	100.00	66.67
SbT2		1.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	66.67 S	100.00	100.00	100.00	66.67
SbT3		1.50	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00 S	100.00	83.33	83.33	60.00
SbT4		2.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00 S	100.00	83.33	83.33	60.00
SbT5		3.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00 S	100.00	83.33	83.33	60.00
MnT1	Mannitol (%)	0.50	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00 S	100.00	100.00	100.00	100.00
MnT2		1.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00 S	100.00	83.33	83.33	80.00
MnT3		1.50	100.00	100.00	100.00	100.00	100.00	100.00	100.00	83.33 S	100.00	83.33	83.33	80.00
MnT4		2.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	83.33 S	83.33	50.00	50.00	66.67
MnT5		3.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	83.33 S	83.33	83.33	50.00	66.67
PrT1	Proline (mM)	5.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	16.67 D	-	-	-	-
PrT2		10.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	33.33 D	-	-	-	-
PrT3		15.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	33.33 D	-	-	-	-
PrT4		20.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	16.67 D	-	-	-	-
PrT5		30.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	16.67 D	-	-	-	-
PvpT1	PVP (%)	2.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00 S	100.00	100.00	100.00	66.67
PvpT2		4.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00 S	100.00	100.00	100.00	66.67
PvpT3		6.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00 S	100.00	100.00	100.00	66.67
PvpT4		8.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00 S	100.00	100.00	100.00	83.33
PvpT5		10.00	100.00	91.66	83.33	66.67	50.00	50.00	41.67	16.67 D	-	-	-	-
Aba T1	ABA (mg l^{-1})	10.00	100.00	100.00	100.00	100.00	100.00	25.00	25.00	00.00 D	-	-	-	-
Aba T2		15.00	100.00	50.00	50.00	41.67	33.33	25.00	25.00	00.00 D	-	-	-	
Aba T3		20.00	100.00	50.00	50.00	50.00	25.00	8.33	25.00	00.00 D	-	-	-	-
Aba T4		25.00	100.00	50.00	50.00	33.33	16.67	8.33	00.00	00.00 D	-	-	-	-
Aba T5		30.00	100.00	50.00	33.33	33.33	8.33	8.33	00.00	00.00 D	-	-	-	-
Control	Sucrose (%)	3.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	33.33 D	-	-	-	

S: subcultured; D: discarded; Regeneration medium: MS + BA 1.0 mg l^{-1} + IAA 0.1 mg l^{-1}

Table 37. Effect of effective slow growth treatments on morphological parameters in Indigofera tinctoria cultures after 28 and 40 weeks of incubation in vitro

Tr. No.	Additives		*28 weeks after incubation				**40 weeks after incubation			
			Plant height (cm)	No.of shoots	No of nodes	Remarks	Plant height (cm)	No.of shoots	No of nodes	Remarks
SuT1	**Sucrose (%)**	6.00	3.45	1.00	3.83	Ca, Rp	3.70	1.00	4.00	Ca, Rp
SuT2		8.00	2.22	1.00	2.50	Ca, Rp	D	D	D	-
SuT3		10.00	0.63	1.00	1.17	Ca, Rp	D	D	D	-
SbT1	**Sorbitol (%)**	0.50	5.50	1.00	5.50	Ca, Rp	5.57	1.00	5.67	Ca, Rp
SbT2		1.00	3.73	1.00	3.67	Ca, Rp	3.82	1.00	3.67	Ca, Rp
SbT3		1.50	2.35	1.00	2.50	Ca, Rp	2.38	1.00	2.50	Ca, Rp
SbT4		2.00	1.68	1.00	2.00	Ca, Rp	1.74	1.00	2.00	Ca, Rp
SbT5		3.00	1.00	1.00	1.67	Ca, Rp	1.14	1.00	1.80	Ca, Rp
MnT1	**Mannitol (%)**	0.50	3.07	1.33	3.17	Ca, Rp	3.12	1.33	3.33	Ca, Rp
MnT2		1.00	1.88	1.17	2.17	Ca, Rp	1.92	1.17	2.17	Ca, Rp
MnT3		1.50	1.82	1.17	2.00	Ca, Rp	1.84	1.17	2.20	Ca, Rp
MnT4		2.00	0.97	1.00	1.00	Ca, Rp	D	D	D	-
MnT5		3.00	0.63	1.00	1.00	Ca, Rp	D	D	D	-
PvpT1	**PVP (%)**	2.00	2.30	1.33	3.33	Ca, Rp	2.38	1.33	3.50	Ca, Rp
PvpT2		4.00	1.12	1.33	1.50	Ca, Rp	1.18	1.33	1.67	Ca, Rp
PvpT3		6.00	1.08	1.00	1.33	Ca, Rp	1.12	1.00	1.33	Ca, Rp
PvpT4		8.00	0.30	1.00	1.00	Ca, Ra	0.30	1.00	1.00	Ca, Ra
Control	**Sucrose (%)**	3.00	6.10	1.50	6.50	Ca, Rp	D	D	D	-
CD (5%)		-	0.88	0.32	0.86	-	-	-	-	-
F value		-	27.27	2.01	26.24	-	-	-	-	-

*The data represent mean of six replications; ** The data represent mean of live plants in each treatment
Cp: callus present; Ca: callus absent, Rp: root present; Ra: root absent; D: discarded

10 per cent) and MnT5 (mannitol at 3 per cent) which was on par with MnT4 (0.97) and SbT5 (1.0 cm). SbT1 recorded highest node number (5.5) and lowest (1.0) by MnT4 and MnT5 which are on par with SuT3 and SbT5. With regard to shoot number, maximum shoot number (1.33) was recorded by MnT1. All levels of sucrose and sorbitol, MnT4 and MnT5 cultures developed only single shoots. All treatments recorded lower plant height, shoot number and node number compared to control.

MnT5 recorded maximum growth inhibition and cent per cent survival but recorded only 83.33 per cent regeneration when transferred to regeneration media after 28 weeks of incubation. Among those cultures which gave cent per cent regeneration 28 weeks after incubation, maximum plant inhibition was recorded by SuT3. But after subculturing and storing up to 40 weeks it did not survive at all. After 40 weeks of storage cent per cent survival and regeneration was obtained with SbT1 and MnT1. Among these, maximum plant growth inhibition was recorded in MnT1 with a plant height (1.14 cm), shoot number (1) and node number (1.80).

4.2.1.2.2 Addition of Cryoprotectant: Proline

In *I. tinctoria*, proline was found to promote plant growth considerably in contradiction to expected plant growth inhibition. Though higher concentration of proline gave lower plant height, in later stages of incubation, 16 weeks onwards even the PrT5 recorded a plant height that is higher than the control. PrT1 (proline 5 mM) recorded maximum plant height and minimum by PrT5 (proline 30 mM). Rate of growth showed a decreasing trend from eighth weeks onwards (Table 36) except for PrT5 where rate of growth showed a diminishing trend from 12^{th} week onwards.

Proline at all levels recoded cent per cent survival till 28 weeks of incubation. The cultures when transferred to regeneration medium, all levels of proline gave very low regeneration per cent (Table 36). PrT1, PrT4 and PrT5 recorded low regeneration (16.67 per cent) than PrT2 and Pr T3 (33.33 per cent). Hence all the levels of proline were discarded from storing further by transfer of cultures to fresh slow growth medium.

4.2.1.2.3 Addition of Antioxidant: PVP

The highest plant height was obtained in PvpT1 (PVP 2 per cent) and lowest at PvpT4 (PVP 8 per cent) and PvpT5 (PVP10 per cent). In PvpT4 and PvpT5, there was no increase in plant height. After an initial sprouting, it remained constant for entire length of incubation. Rate of growth showed a decreasing trend from eighth week on wards at all levels of PVP (Table 35).

After 28 weeks of culture, cent per cent survival and regeneration was recorded by PvpT1, PvpT2, PvpT3 and PvpT4; while PvpT5 recorded a survival of 41.67 per cent and regeneration of 16.67 per cent (Table 36). There was significant variation with regard to plant height, shoot number and node number among the selected levels of PVP (Table 37). PvpT1 recorded maximum plant height (2.3 cm) and node number (3.33); while PvpT4 recorded least plant height (0.3 cm) and node number (1.0). Maximum shoot number (1.33) was recorded by PvpT1 and PvpT2 and least (1.0) by PvpT3 and PvpT4. When subcultured and stored up to 40 weeks, Pvp T4 recorded cent per cent survival upto 40 weeks of storage and thereafter a regeneration of 83.33 per cent when transferred to regeneration medium.

4.2.1.2.4 Addition of Growth Retardant: ABA

All levels of ABA did not cause any considerable increase in plant height in *I. tinctoria*. After an initial sprouting to 0.3cm, the plant did not develop in height. With AbaT1 (ABA 10 mg l^{-1}), cent per cent survival was recorded only up to 20 weeks; at 28 weeks the survival was 25 per cent. AbaT2 and AbaT3 also recorded 25 per cent survival 28 weeks after incubation. At this stage AbaT4 and AbaT5 did not survive. None of the cultures regenerated when inoculated on to regeneration medium. Hence ABA was discarded from further study.

The effect of slow growth additives on rate of plant growth of *I. tinctoria* cultures at different lengths of incubation *in vitro* is illustrated in Fig 12.

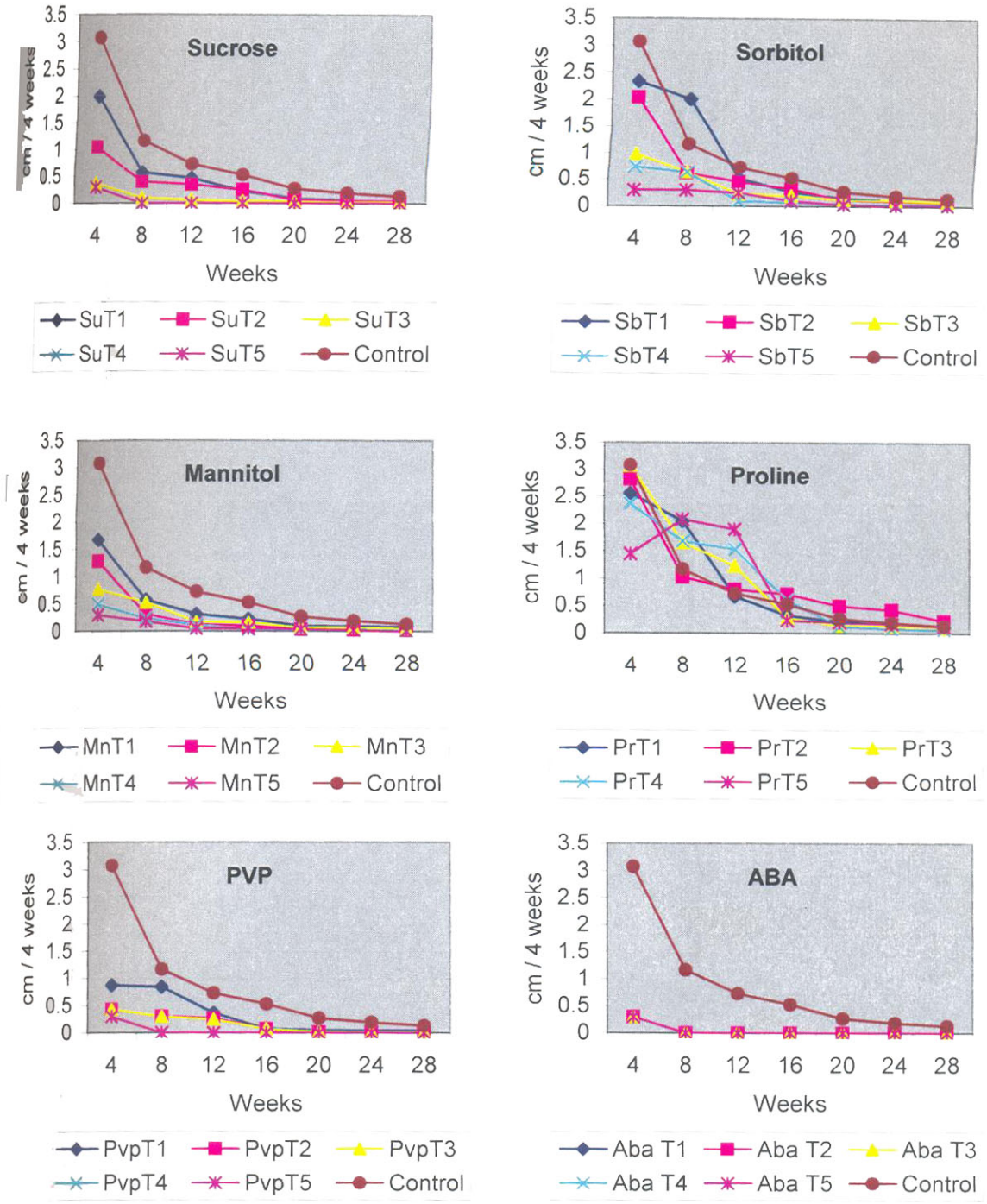


Fig 12. Effect of slow growth additives (osmotica, cryoprotectant, antioxidant and growth retardant) on rate of growth of *Indigofera tinctoria* cultures at different lengths of incubation *in vitro*

Plate 13. Effect of different levels of osmotica, cryoprotectant, antioxidant and growth retardant on plant growth of *Indigofera tinctoria* cultures after 28 weeks of incubation

a. Plant growth attained by control and different levels of sucrose (6, 8, 10, 12 and 15 %) added to the culture medium (1/2 MS + IBA 0.5 mg l^{-1})

b. Plant growth attained by control and different levels of sorbitol (0.5, 1.0, 1.5, 2.0 and 3.0 %) added to the culture medium (1/2 MS + IBA 0.5 mg l^{-1})

c. Plant growth attained by control and different levels of mannitol (0.5, 1.0, 1.5, 2.0 and 3.0 %) added to the culture medium (1/2 MS + IBA 0.5 mg l^{-1})

d. Plant growth attained by control and different levels of proline (5, 10, 15, 20 and 30 mM) added to the culture medium (1/2 MS + IBA 0.5 mg l^{-1})

e. Plant growth attained by control and different levels of PVP (2, 4, 6, 8 and 10 %) added to the culture medium (1/2 MS + IBA 0.5 mg l^{-1})

f. Plant growth attained by control and different levels of ABA (10, 15, 20, 25 and 30 mg l^{-1}) added to the culture medium (1/2 MS + IBA 0.5 mg l^{-1})

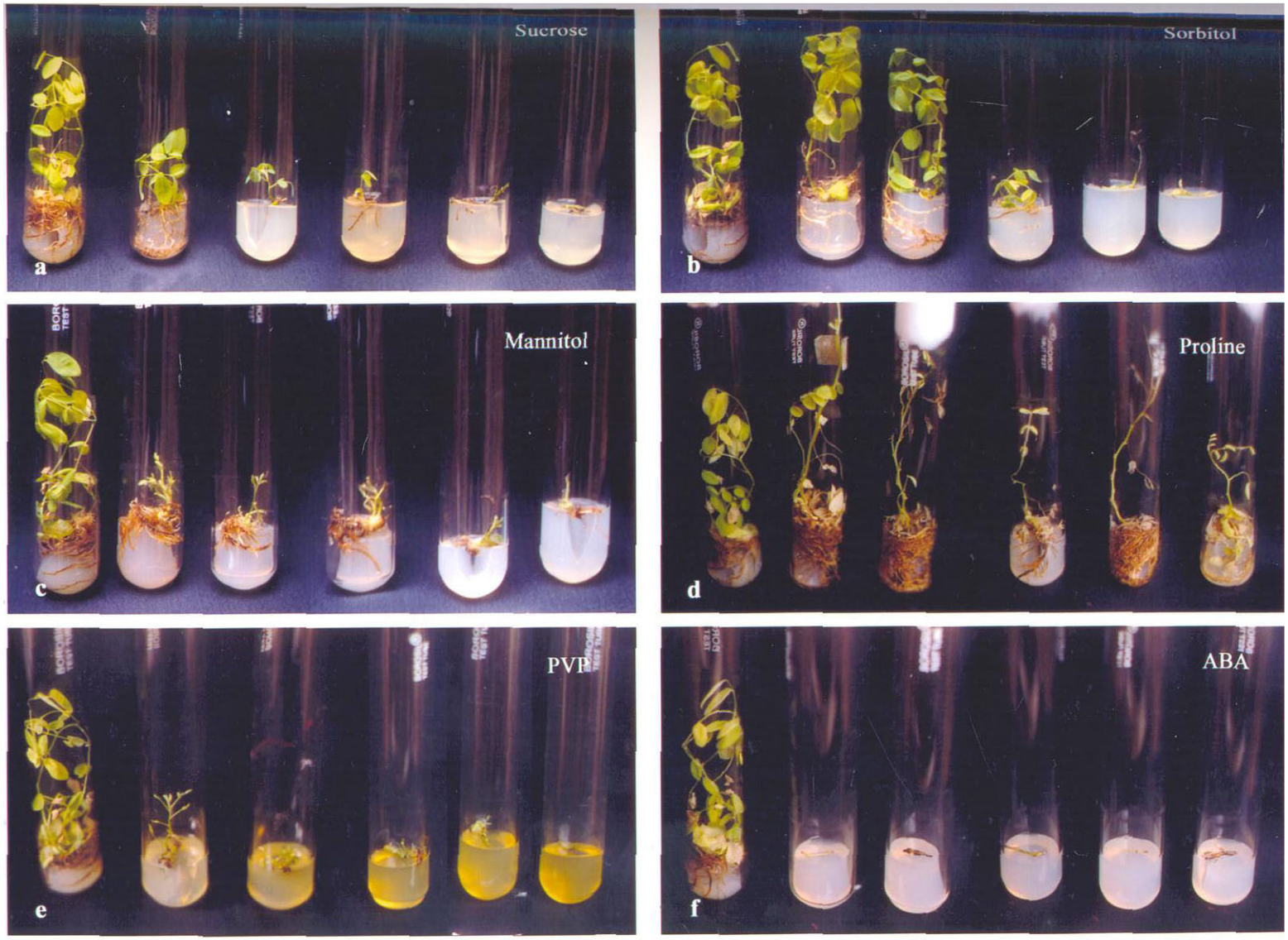


Plate 13. Effect of different levels of osmotica, cryoprotectant, antioxidant and growth retardant on plant growth of *Indigofera tinctoria* cultures after 28 weeks of incubation

Plate 14. Shoot regeneration in *Indigofera tinctoria* after imposing slow growth treatments for 28 weeks

a. Shoot regeneration in culture medium MS + BA 1.0 mg l^{-1} + IAA 0.1 mg l^{-1} from single nodes excised from slow growth cultures with different levels of sucrose (6, 8 and 10 %) as additives

b. Shoot regeneration in culture medium MS + BA 1.0 mg l^{-1} + IAA 0.1 mg l^{-1} from single nodes excised from slow growth cultures with different levels of sorbitol (0.5, 1.0, 1.5, 2.0 and 3.0 %) as additives

c. Shoot regeneration in culture medium MS + BA 1.0 mg l^{-1} + IAA 0.1 mg l^{-1} from single nodes excised from slow growth cultures with different levels of mannitol (0.5, 1.0, 1.5, 2.0 and 3.0 %) as additives

d. Shoot regeneration in culture medium MS + BA 1.0 mg l^{-1} + IAA 0.1 mg l^{-1} from single nodes excised from slow growth cultures with different levels of PVP (2 , 4, 6 and 8 %) as additives

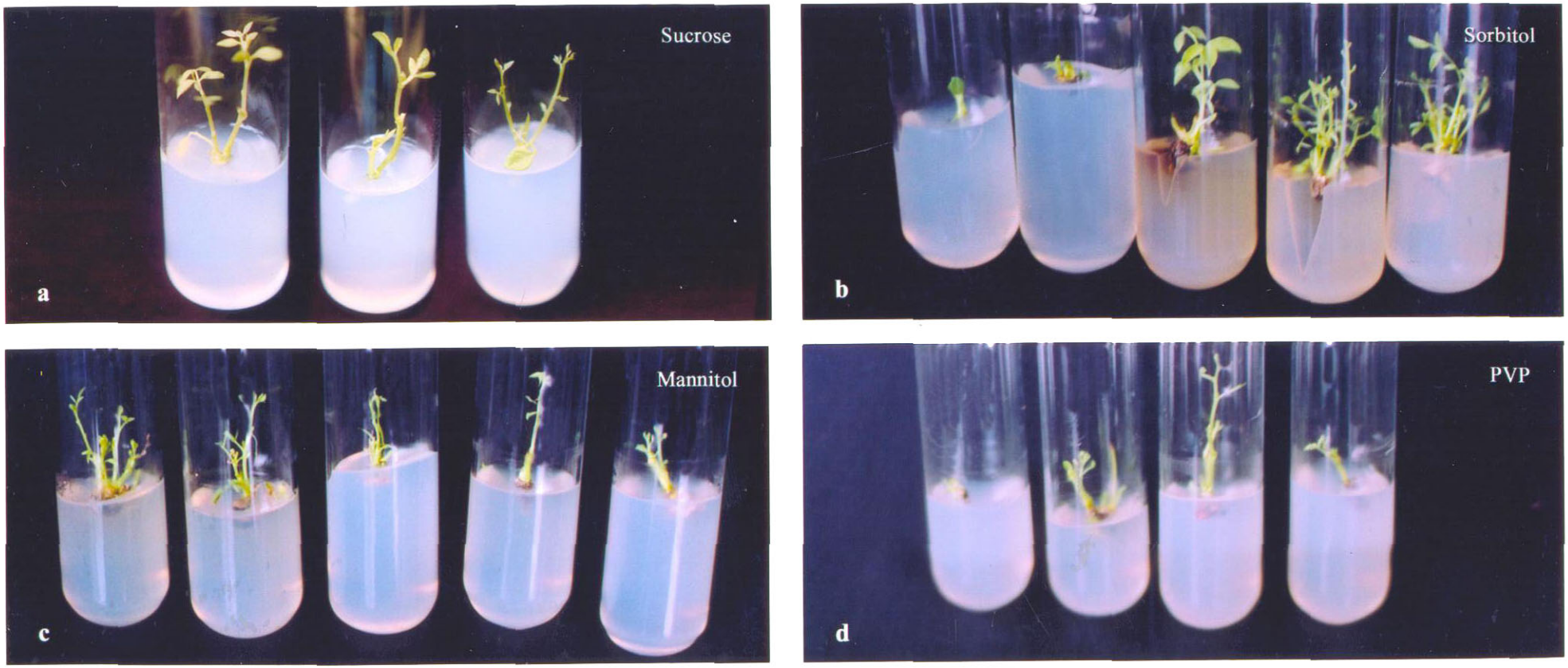


Plate 14. Shoot regeneration in *Indigofera tinctoria* after imposing slow growth treatments for 28 weeks

(Shoot proliferation medium: MS + BA 1.0 mg l^{-1} + IAA 0.1 mg l^{-1})

Cultures of *I. tinctoria* were maintained for 28 weeks without any subculture. The effect of different additives on plant growth after 28 weeks of incubation *in vitro* is illustrated in Plate 13 and shoot regeneration after imposing slow growth treatments for 28 weeks in Plate 14. The cultures selected at 28 weeks after incubation did not show callusing in any of the treatments. Moreover, rooting was observed in all treatments except PvpT4.

Among the cultures selected for subculture at 28 weeks, there was significant variation with regard to plant height, shoot number and node number (Table 37). Maximum plant height (5.5 cm) was recorded by SbT1 (sorbitol 0.5 per cent) and lowest (0.3 cm) by PvpT4 (ABA 15 mg l^{-1}) which was on par with SuT3, SbT5, MnT4, MnT5, PvpT2 and PvpT3. With regard to shoot number maximum value (1.33) was recorded by MnT1, PvpT1 and PvpT2 and minimum (1.0) by SuT1, SuT2, SuT3, all levels of sorbitol, MnT4, MnT5, PvpT3 and PvpT4. Maximum node number (5.5) was recorded by SbT1 and lowest (1.0) by MnT4, MnT5 and PvpT4. PvpT4 recorded maximum plant growth inhibition with cent per cent survival and regeneration 28 weeks after incubation. This gave a regeneration of 83.33 per cent when inoculated onto regeneration medium after 40 weeks of storage. Cent per cent regeneration was recorded by MnT1 after 40 weeks of storage with a plant height of 3.12 cm, shoot number of 1.33 and node number of 3.33.

4.2.2. Cryopreservation

4.2.2.1 Seeds

Clitoria ternatea

Seeds of *C. ternatea* extracted from dried pods contain 12.65 per cent moisture. Their germination rate was 96 per cent in the open. At this moisture status, when stored in LN and subjected to germination, only 56 per cent of the seeds germinated (Table 38).

Seeds were subjected to two different moisture levels MC1-9.37 per cent and MC2 - 7.03 per cent and six different levels of storage in liquid nitrogen (1 day, 1 week, 3 weeks, six weeks, 9 weeks and control (not stored in LN)) to find their effect on germination and survival.

In control treatment (not stored in LN) germination was 96.22 per cent and 94.63 per cent for moisture content levels 9.37 per cent and 7.03 per cent, respectively. Germination occurred normally in 15-20 days after incubation. However, after mechanical scarification by means of breaking and removal of a small portion of seeds coat from the micropylar end, the seeds started germinating within ten days.

Factorial completely randomized block design was used for the statistical analysis of the data following square root transformation. The results are presented in Table 39. Significant variation in germination per cent between treatments and control was observed. Control recorded a higher germination per cent compared to the treatments. However at each moisture levels, there was no significant variation in germination per cent at different periods of storage. But there was significant difference between the germination per cent at two different moisture levels. As such, at low moisture level of 7.03 per cent (MC2), *C. ternatea* recorded higher germination at all periods of storage in LN. But control recorded low germination at MC2 than at MC 1 (9.37 per cent moisture).

On storage in LN, breakage of seed coat was noticed in few seeds of *C. ternatea* which facilitated germination and restricted the necessity of mechanical scarification. Breakage of seed coat occurred in 20- 30 per cent seeds, irrespective of duration of storage in LN.

Mucuna pruriens

Seeds of *M. pruriens* extracted from dried pods contained 11 per cent moisture. On incubation, cent per cent germination was observed under ambient conditions. When these seeds were stored in LN for one day, only of 53.33 per cent of them germinated (Table 38).

Seeds were subjected to two different moisture levels MC1- 8.63 per cent and MC2 - 5.24 per cent and six different levels of storage in liquid nitrogen (1 day, 1 week, 3 weeks, six weeks, 9 weeks and control (not stored in LN)) to find their effect on germination and survival.

Cent per cent germination was recorded in control at 8.63 per cent moisture level. While at moisture level 5.24 per cent, it recorded 93.27 per cent germination. Germination occurred normally within a week after incubation. No mechanical scarification was needed in *M. pruriens.* When sprayed with water, seeds imbibed water and seed coat splitted longitudinally in 2-3 days.

Factorial completely randomized block design was used for the statistical analysis of the data following square root transformation. The results are presented in Table 39. There was significant variation in germination per cent between treatments and control. Control recorded a higher germination per cent compared to the treatments. At a particular moisture level, no significant variation in germination per cent was observed at different periods of storage. But there was significant difference between the germination percent at two different moisture levels. At the low moisture level of 5.24 per cent (MC 2), *M. pruriens* recorded higher germination at all periods of storage in LN. But control recorded low germination at MC2 than at MC1 (8.63 per cent moisture).

Indigofera tinctoria

Seeds of *I. tinctoria* were derived from dried pods. Their moisture content was 10.64 per cent. Seeds gave a germination of 95 per cent under ambient conditions. When the seeds were stored in LN for one day, they gave a germination of 57.33 per cent (Table 38).

Seeds were subjected to two different moisture levels MC1-9.79 per cent and MC2 - 5.35 per cent and six different levels of storage in liquid nitrogen (1 day, 1 week, 3 weeks, six weeks, 9 weeks and control (not stored in LN)) to find their effect on germination and survival.

At MC1 (9.79 per cent moisture), 88.64 per cent germination was recorded in control. While at MC2 (5.35 per cent moisture), it recorded 91.31 per cent germination. Germination occurred normally between 30-40 days after incubation. Mechanical scarification of seed is essential to facilitate germination in *I. tinctoria.*, which enabled seeds to germinate within 15-20days.

Factorial completely randomized block design was used for the statistical analysis of the data following square root transformation. The results are presented in Table 39. There was significant variation in germination per cent between treatments and control. The effect of two MC levels on germination of seeds was very significant. Control recorded a higher germination percent compared to the treatments. At a particular MC level, no significant variation in germination per cent was observed at different periods of storage. However, the highest germination was recorded at 9 weeks after storage in LN at moisture levels, 9.79 per cent and 5.35 respectively. There was significant difference between the germination percent at two different moisture levels. At the low moisture level of 5.35 per cent (MC2), *I. tinctoria* recorded higher germination at all periods of storage in LN. But control recorded low germination at MC 2 than at MC1 (9.79 per cent moisture).

Table 38. Moisture content and seed germination as influenced by immersion in LN (24h) of *Clitoria ternatea, Mucuna pruriens* and *Indigofera tinctoria*

	Clitoria ternatea	*Mucuna pruriens*	*Indigofera tinctoria*
No. of seeds	50.00	10.00	100.00
Moisture content (%)	12.65	11.00	10.64
Germination (%) control	96.00	100.00	95.00
Germination (%) after 24h immersion in LN	56.00	53.33	57.33

Table 39. Effect of cryostorage for different periods on seed germination at two different moisture levels in *Clitoria ternatea, Mucuna pruriens and Indigofera tinctoria*
CD (5%) for treatments :*C. ternatea:* 0.54; *M. pruriens*: 0.77; *I. tinctoria* : 0.63

Storage in LN	*Clitoria ternatea*		*Mucuna pruriens*		*Indigofera tinctoria*	
	MC1=9.37 %	MC2= 7.03 %	MC1=8.63 %	MC2= 5.24 %	MC1=9.79 %	MC2=5.35%
1day	69.27 (8.32)	90.62(9.51)	69.76(8.35)	79.79(8.93)	72.33(8.50)	82.59(9.09)
1 wk	68.64(8.29)	91.91(9.59)	76.39(8.74)	86.60(9.31)	82.41(9.08)	87.64(9.36)
3 wk	69.76(8.35)	91.30(9.56)	73.26(8.56)	79.79(8.93)	65.18(8.07)	81.20(9.01)
6 wk	68.57(8.28)	88.54(9.41)	73.26(8.56)	86.60(9.31)	78.21(8.84)	75.54(8.69)
9 wk	68.99(8.31)	89.95(9.48)	76.39(8.74)	86.60(9.31)	80.32(8.96)	90.55(9.52)
Control	96.22(9.81)	94.63(9.72)	100.00(10.00)	93.27(9.66)	88.64(9.42)	91.33(9.56)

Mean table: Period of storage in LN

Storage in LN	*Clitoria ternatea*	*Mucuna pruriens*	*Indigofera tinctoria*
1day	79.57(8.92)	74.65(8.64)	77.44(8.80)
1 wk	79.92(8.94)	81.54(9.03)	85.01(9.22)
3 wk	80.28(8.96)	76.56(8.75)	72.93(8.54)
6 wk	78.32(8.85)	79.92(8.94)	76.91(8.77)
9 wk	79.21(8.90)	81.54(9.03)	85.38(9.24)
CD (1%)	NS	NS	NS

Mean table: Moisture levels

Moisture content	*Clitoria ternatea*	*Mucuna pruriens*	*Indigofera tinctoria*
MC1	69.06(8.31)	73.79(8.59)	75.52(8.69)
MC2	90.44(9.51)	83.91(9.16)	83.36(9.13)
CD (5%)	0.28	0.35	0.28

Transformed values are presented in the parenthesis

The germinated plants were field planted and cent per cent survival was obtained in seeds, cryopreserved at different moisture levels and different periods of storage in liquid nitrogen in all the three plant species.

The effect of preservation of *C. ternatea*, *M. pruriens* and *I. tinctoria* seeds in LN for nine weeks on germination and establishment is illustrated in Plate 15.

4.2.2.2 Zygotic Embryo

4.2.2.2.1 Simple Desiccation

Freshly excised embryo of *M. pruriens* showed 100 per cent survival in basal MS medium without any hormones. It recorded an average moisture content of 90.54 per cent. However, these explants did not produce shoots after immersion in LN.

The data was analysed statistically using completely randomized block design following arc sine transformation. The results are presented in Table 40 and Fig 13. There was significant variation with regard to moisture content of embryos at different periods of desiccation. Moisture content of zygotic embryo decreased from 60.94 per cent to 8.14 per cent, during the desiccation period. Survival of control embryos (-LN), decreased over the desiccation period tested, from 100 per cent without desiccation to 63.40 per cent after 240 min desiccation. After freezing in LN for 24 hours, no survival was noted without desiccation, survival increased progressively to 90.76 per cent at 150 min desiccation. From 180 min desiccation on wards, survival showed a decreasing trend; decreased to 46.65 per cent after 240 min desiccation. Plant regeneration also followed the same pattern. It progressively decreased from 100 per cent without desiccation to 51.82 per cent after 240 min desiccation in control embryos. After freezing in LN, plant regeneration was nil without desiccation, and increased up 80.05 per cent at 150 min desiccation and then decreased to 52.39 per cent at 240 min desiccation.

Table 40. Effect of desiccation of zygotic embryos of *Mucuna pruriens* on moisture content (%), survival (%) and plant regeneration (%) with and without cryopreservation

DD(min)	MC(%)	Survival (%)		Plant regeneration (%)	
		-LN	+LN	-LN	+LN
0	60.94(51.30)	100.00(90.00)	00.00(00.00)	100.00(90.00)	00.00(00.00)
30	45.64(42.48)	98.86(83.85)	26.52(30.98)	95.22(77.34)	50.00(44.98)
60	30.59(33.56)	95.48(77.69)	36.60(37.21)	93.01(77.63)	53.05(46.74)
90	22.82(28.51)	90.00(71.54)	60.14(50.83)	85.53(67.62)	72.11(58.10)
120	17.74(24.90)	86.99(68.83)	73.48(58.98)	81.26(64.32)	63.69(58.43)
150	14.17(22.10)	83.64(66.12)	90.76(72.27)	75.94(60.60)	80.05(63.45)
180	12.09(20.34)	77.55(61.69)	73.48(58.98)	69.09(56.20)	68.08(55.58)
210	10.16(18.58)	70.33(56.97)	63.40(52.75)	53.35(46.90)	58.68(49.98)
240	8.14(16.57)	63.40(52.75)	46.65(43.06)	51.82(46.03)	52.39(46.35)
CD(5%)	1.54	11.38	11.19	12.01	12.21

Transformed values are presented in the parenthesis
-LN: without cryopreservation; +LN: after cryopreservation in LN for one day;
MC : moisture content; DD: desiccation duration

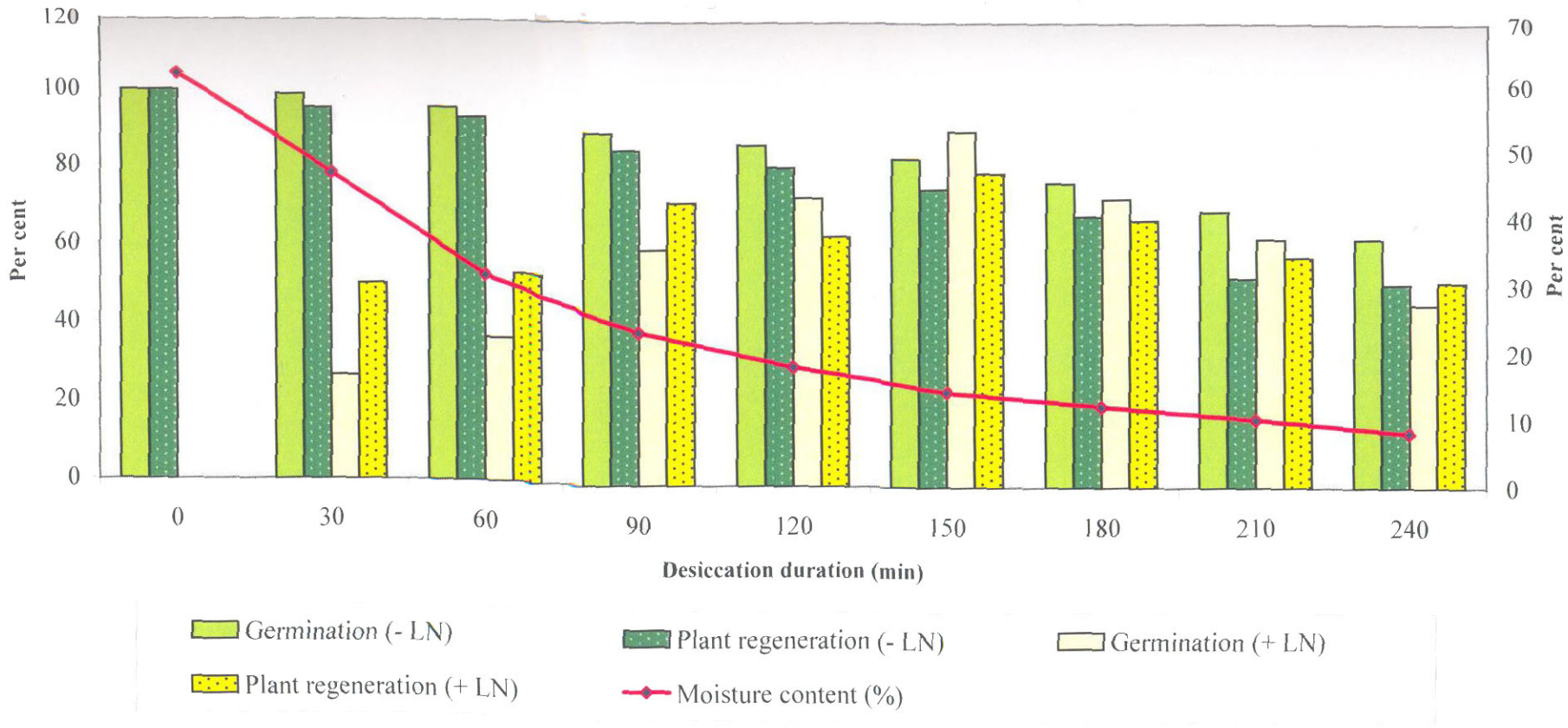


Fig. 13 Effect of desiccation of zygotic embryos of *Mucuna pruriens* on moisutre content, survival and plant regeneration with and without cryopreservation

Plate 15. Effect of preservation of *Clitoria ternatea*, *Mucuna pruriens* and *Indigofera tinctoria* seeds in LN (-196°C) for 9 weeks on germination and establishment

a. Germination of *C. ternatea* seeds (moisture content 7.03 %)

a1. Establishment of seedling from cryopreserved seeds of *C. ternatea*

b. Germination of *M. pruriens* seeds (moisture content 5.24 %)

b1. Establishment of seedling from cryopreserved seeds of *M. pruriens*

c. Germination of *I. tinctoria* seeds (moisture content 5.35 %)

c1. Establishment of seedling from cryopreserved seeds of *I. tinctoria*

Plate16. Different stages of development of cryopreserved zygotic embryos (pre air-desiccated for 150 min) of *M. pruriens*

a. Excised zygotic embryo(before desiccation and cryopreservation)

b. *In vitro* establishment after cryopreservation

c. Root initiation

d. Shoot initiation

e. Whole plant regeneration

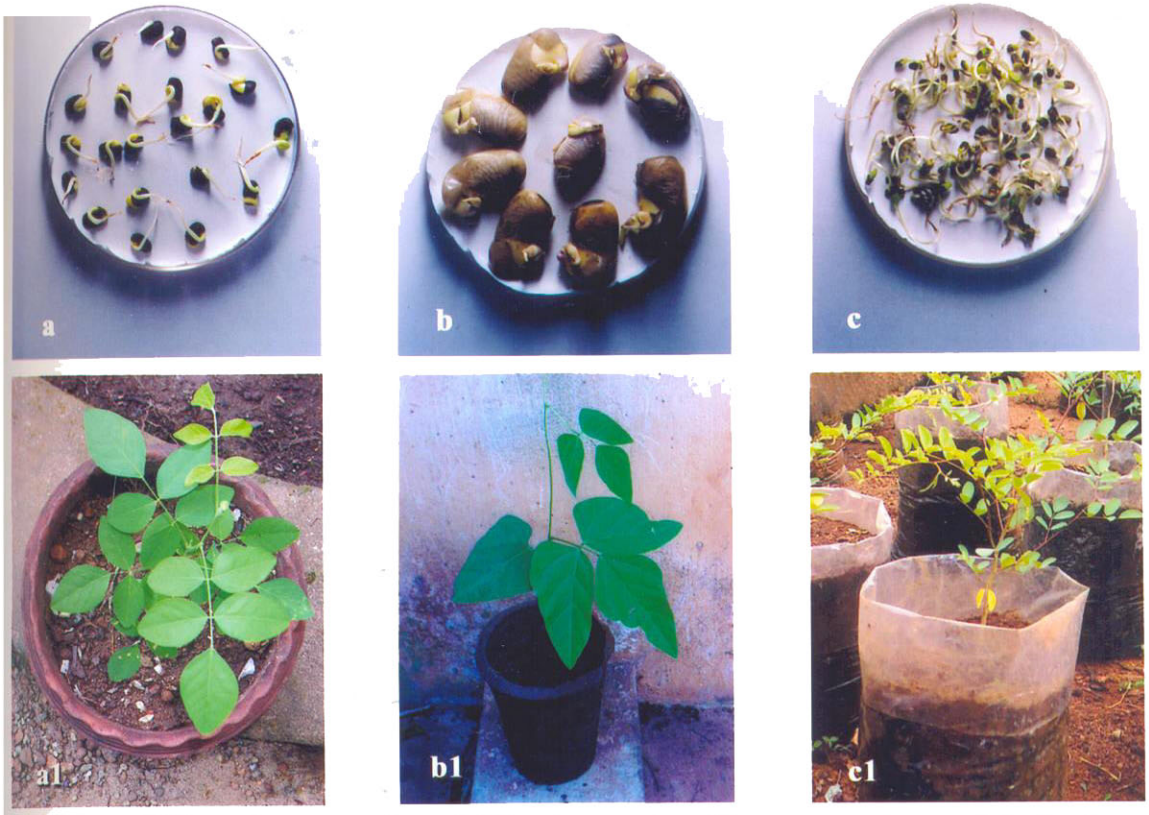


Plate 15. Effect of preservation of *Clitoria ternatea*, *Mucuna pruriens* and *Indigofera tinctoria* seeds in LN (-196°C) for 9 weeks on germination and establishment

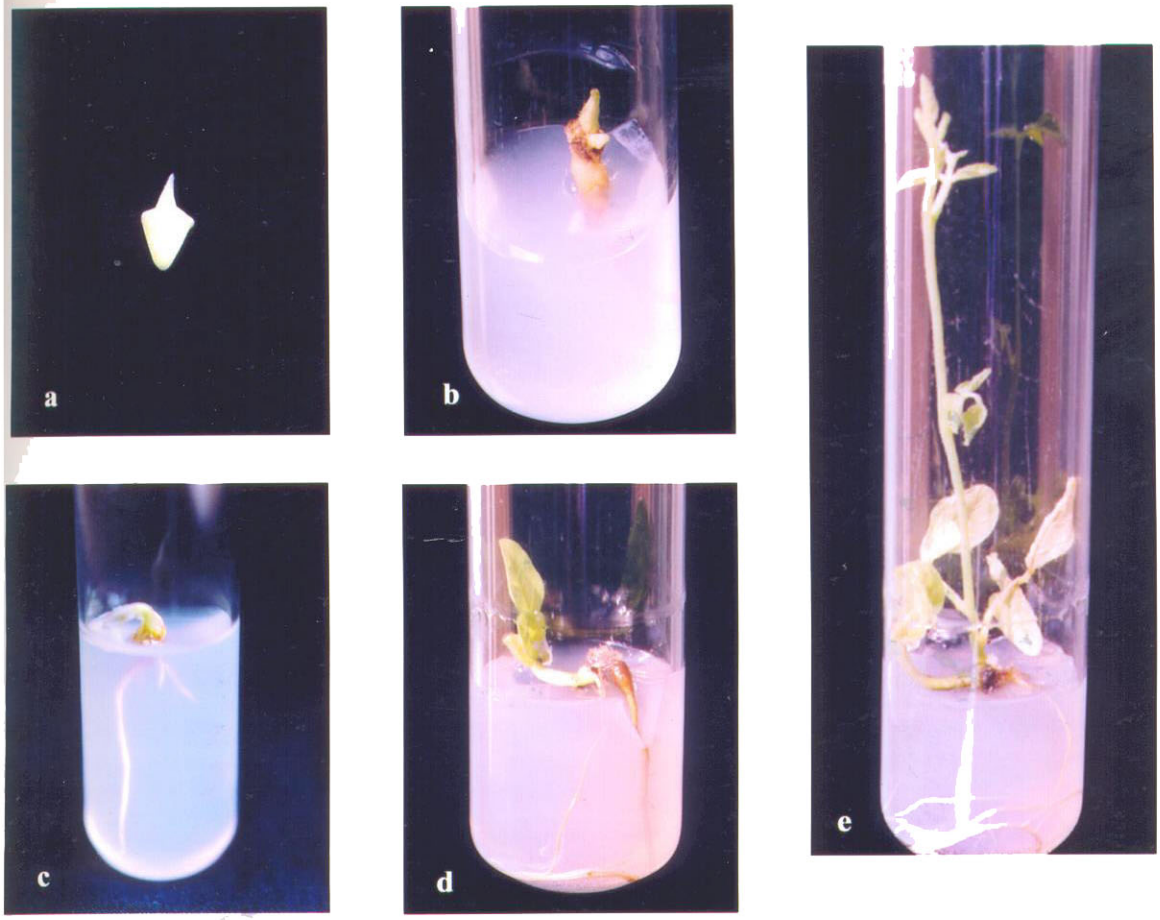


Plate 16. Different stages of development of cryopreserved zygotic embryos (pre air desiccated for 150 min) of *Mucuna pruriens*

After freezing in LN, the highest survival (90.76 per cent) and plant regeneration (80.05 per cent) was obtained at a moisture level of 14.17 per cent, 150 min after desiccation (Plate 16).

4.2.2.2.2 Rapid Freezing with Addition of Cryoprotectant

Zygotic embryos of *M. pruriens* pretreated with cryoprotectant solutions did not survive after one hour of freezing in any of the treatments tried. The embryos became black in 2-3 days and did not germinate even after a month of incubation. non- treated embryos when cultured in normal incubation conditions gave 90 per cent germination.

4.2.2.3 Shoot Meristem

4.2.2.3.1 Encapsulation Dehydration

The results are presented in Table 41.

Clitoria ternatea

After pre conditioning, encapsulation and pre-culture of the excised axillary buds of *C. ternatea*, they were subjected to different hours of dehydration (0h, 1h, 2h, 3h and 4h). After cryopreservation for 1h in LN, the samples were rewarmed and placed in regeneration medium to study the rate of survival and regeneration. Moisture content was determined after different periods of dehydration tried.

Alginate beads encapsulating the axillary buds possessed 62.63 per cent moisture. Upon dehydration under laminar air flow for 4 h, it reduced to 20.09 per cent. Encapsulated axillary buds desiccated to moisture content of 20.09 per cent (4h) and 17.79 per cent (5h) exhibited survival of 60 and 40 per cent respectively. With higher moisture content, the axillary buds did not survive at all.

With no desiccation, encapsulated buds gave a survival of 84.85 per cent and regeneration 82.22 per cent. At 4h desiccation, control plants (-LN) gave a survival of 70 per cent and regeneration of 66.27 per cent; while after freezing in LN, survival was 60 per cent and regeneration 65 per cent. Survival recorded was 56.67 per cent and regeneration 52.38 per cent in control plants at 5 h of desiccation, while after freezing in LN, survival was 40 per cent and regeneration 50 per cent.

Hence 4 h of desiccation with a moisture content of 20.09 per cent is found most effective for cryopreservation of encapsulated axillary buds of C. *ternatea* (Plates 17a and 17b).

Mucuna pruriens

Encapsulated axillary buds of *M. pruriens*, by subjecting to desiccation (0- 5 h), moisture content was reduced gradually from 68.99 per cent to 18.70 per cent at 5 h desiccation. With no desiccation (0h) and freezing, the rate of survival was 86.67 percent and regeneration 84.72 per cent; while at 5 h desiccation, survival and regeneration was 30 and 44.44 per cent, respectively. None of the desiccated buds survived after freezing in liquid nitrogen (Plate 17c).

Indigofera tinctoria

Encapsulated axillary buds of *I. tinctoria,* by subjecting to desiccation (0 – 5h), moisture content was reduced gradually from 65.98 per cent to 9.42 per cent on 5h desiccation. With no desiccation (0h), survival of control plants were 93.33 per cent and regeneration 78.33 per cent. On freezing the buds did not survive at all. Maximum survival after freezing in LN was obtained in treatment with 4 h desiccation, 56.66 per cent and a regeneration of 62.22 per cent, but at the same desiccation duration control plants (-LN) gave 50 per cent survival and 58.89 per cent regeneration. At 5 h desiccation, survival and regeneration per cent recorded on freezing was very low, 20 and 33.33 per cent respectively (Plate 17d); while control plants recorded 33.33 per cent survival and 47.22 per cent regeneration.

4.2.2.3.2 Rapid Freezing with Addition of Cryoprotectant

Clitoria ternatea

Cryoprotectants *viz.,* glycerol, DMSO, mannitol, ethylene glycol, proline, glucose, sucrose and their combinations were tried to study their effect on cryopreservation of axillary meristem of *C. ternatea.* Control plants gave survival and regeneration of 83.33 and 80.09 per cent, respectively. But on rapid immersion in LN after treating with 1 ml of cryoprotectant, axillary buds did not survive in any of the cryoprotectant treatments tried. Moreover, the explants turned white on inoculation to the recovery medium, particularly in DMSO (10 per cent) treated meristems (Plate 18a)

Mucuna pruriens

In *M. pruriens,* among the different cryoprotectants tried, only Cp10 (Sucrose 0.7 M + DMSO 5 %) gave successful results. This treatment gave 60 per cent survival and 66.67 per cent regeneration (Plate 18c). In all other treatments with cryoprotectants, axillary buds did not survive after freezing in LN. It turned black in three to four days (Plate 18 b). The control plants gave survival and regeneration per cent of 70 and 66.07 per cent, respectively.

Indigofera tinctoria

In *I. tinctoria,* treatments with cryoprotectants did not give satisfactory results. However, Cp10 (Sucrose 0.7 M + DMSO 5 per cent) gave a survival and regeneration of 33.33 per cent and 38.89 per cent respectively. Axillary buds did not survive in any of the other treatments after freezing in LN. Control plants gave a survival and regeneration of 70 and 65.47 percent, respectively. In treatments with DMSO, the axillary buds turned white (Plate 18d) but in all other treatments, where there was no survival, plants turned black in 2-3 days.

Table 41. Effect of desiccation of encapsulated meristems on the moisture content (%), survival (%) and plant regeneration (%) in *Clitoria ternatea, Mucuna pruriens and Indigofera tinctoria* with or without cryopreservation

DD (h)	*Clitoria terantea*					*Mucuna pruriens*					*Indigofera tinctoria*				
	MC (%)	Survival (%)		Plant regeneration (%)		MC (%)	Survival (%)		Plant regeneration (%)		MC (%)	Survival (%)		Plant regeneration (%)	
		-LN	+LN	-LN	+LN		-LN	+LN	-LN	+LN		-LN	+LN	-LN	+LN
0	62.63	84.85	00.00	82.22	00.00	68.99	86.67	00.00	84.72	00.00	65.98	93.33	00.00	78.33	00.00
1	46.82	80.00	00.00	78.90	00.00	58.99	86.67	00.00	80.56	00.00	52.34	80.00	00.00	75.20	00.00
2	34.68	80.00	00.00	74.73	00.00	49.23	73.33	00.00	76.79	00.00	33.65	73.33	00.00	73.21	00.00
3	24.98	75.00	00.00	72.23	00.00	37.50	66.67	00.00	75.40	00.00	25.00	63.33	00.00	63.58	00.00
4	20.09	70.00	60.00	66.27	65.00	29.55	33.33	00.00	66.67	00.00	16.01	50.00	56.66	58.89	62.22
5	17.79	56.67	40.00	52.38	50.00	18.70	30.00	00.00	44.44	00.00	9.42	33.33	20.00	47.22	33.33

-LN: without cryopreservation; +LN: after cryopreservation in LN for one day; MC: moisture content; DD: Desiccation duration

Table 42. Effect of desiccation of encapsulated somatic embryos of *Clitoria ternatea* on moisture content (%), survival (%) and plant regeneration (%) with or without cryopreservation

DD (h)	MC (%)	Survival (%)		Regeneration (%)	
		-LN	+LN	-LN	+LN
0	63.67	73.33	00.00	67.85	00.00
1	51.18	63.33	00.00	53.17	00.00
2	33.91	53.33	00.00	42.06	00.00
3	25.79	50.00	00.00	41.11	00.00
4	21.38	33.33	26.66	38.89	38.89
5	18.32	26.66	43.33	38.89	55.00

-LN: without cryopreservation; +LN: after cryopreservation in LN for one day; MC: moisture content; DD: Desiccation duration

Plate 17. ***In vitro*** **regeneration of encapsulated, desiccated and cryopreserved shoot meristems of** ***Clitoria ternatea, Mucuna pruriens*** **and** ***Indigofera tinctoria***

a. Sodium alginate encapsulated shoot meristems of *C. ternatea* before desiccation and cryopreservation

b. Establishment and regeneration of *C. ternatea* plants in recovery medium (1/2 MS + GA 0.5 mg l^{-1} + BA 0.2 mg l^{-1})

c. Dried up shoot meristem of *M. pruriens* (after desiccation and cryopreservation) in recovery medium (1/2 MS + GA 0.5 mg l^{-1} + BA 0.2 mg l^{-1})

d. Establishment and regeneration of *I. tinctoria* plants in recovery medium (1/2 MS + GA 0.5 mg l^{-1} + BA 0.2 mg l^{-1})

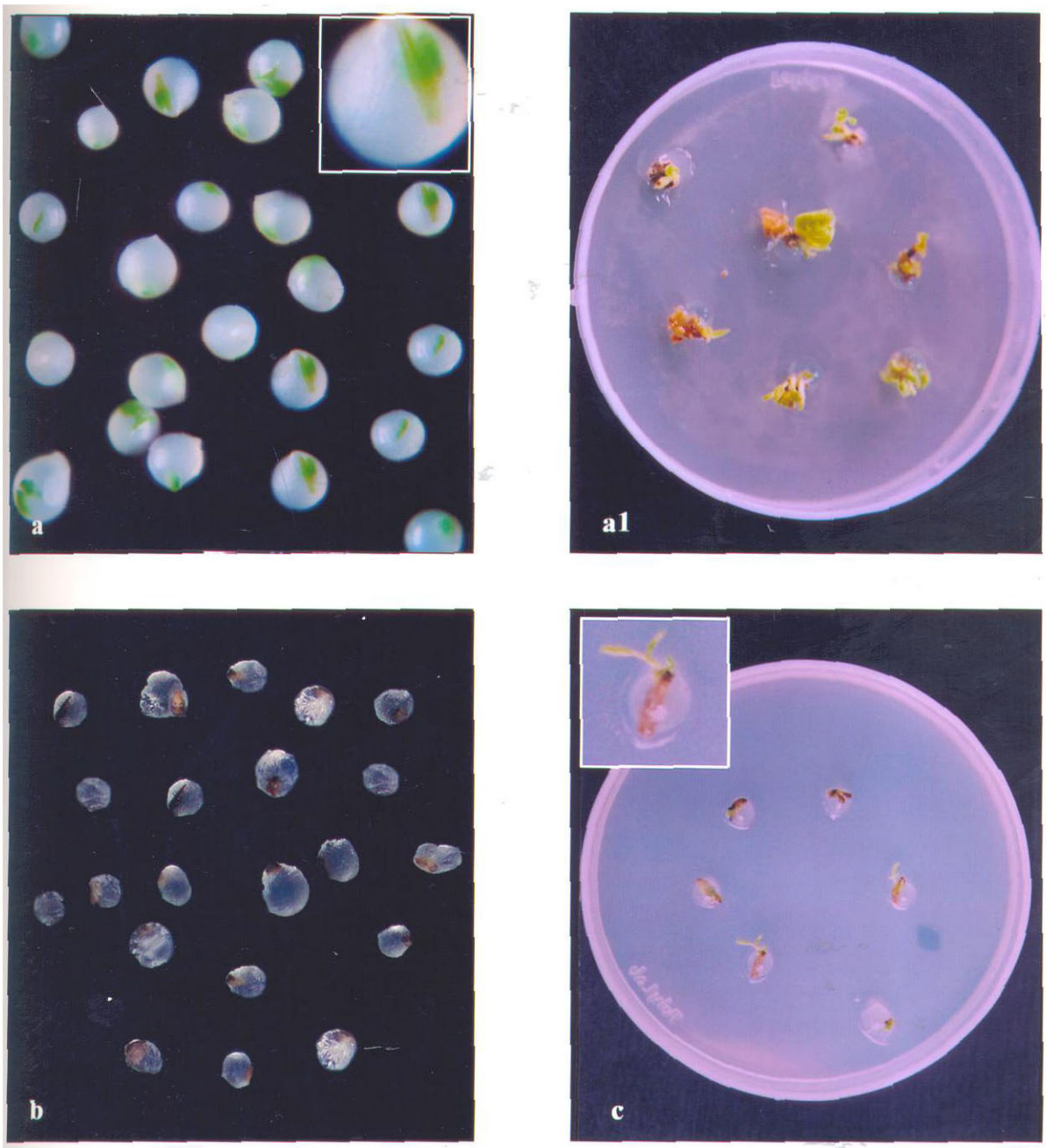


Plate 17. ***In vitro*** **regeneration of encapsulated, desiccated and cryopreserved shoot meristems of** ***Clitoria ternatea*****,** ***Mucuna pruriens*** **and** ***Indigofera tinctoria***

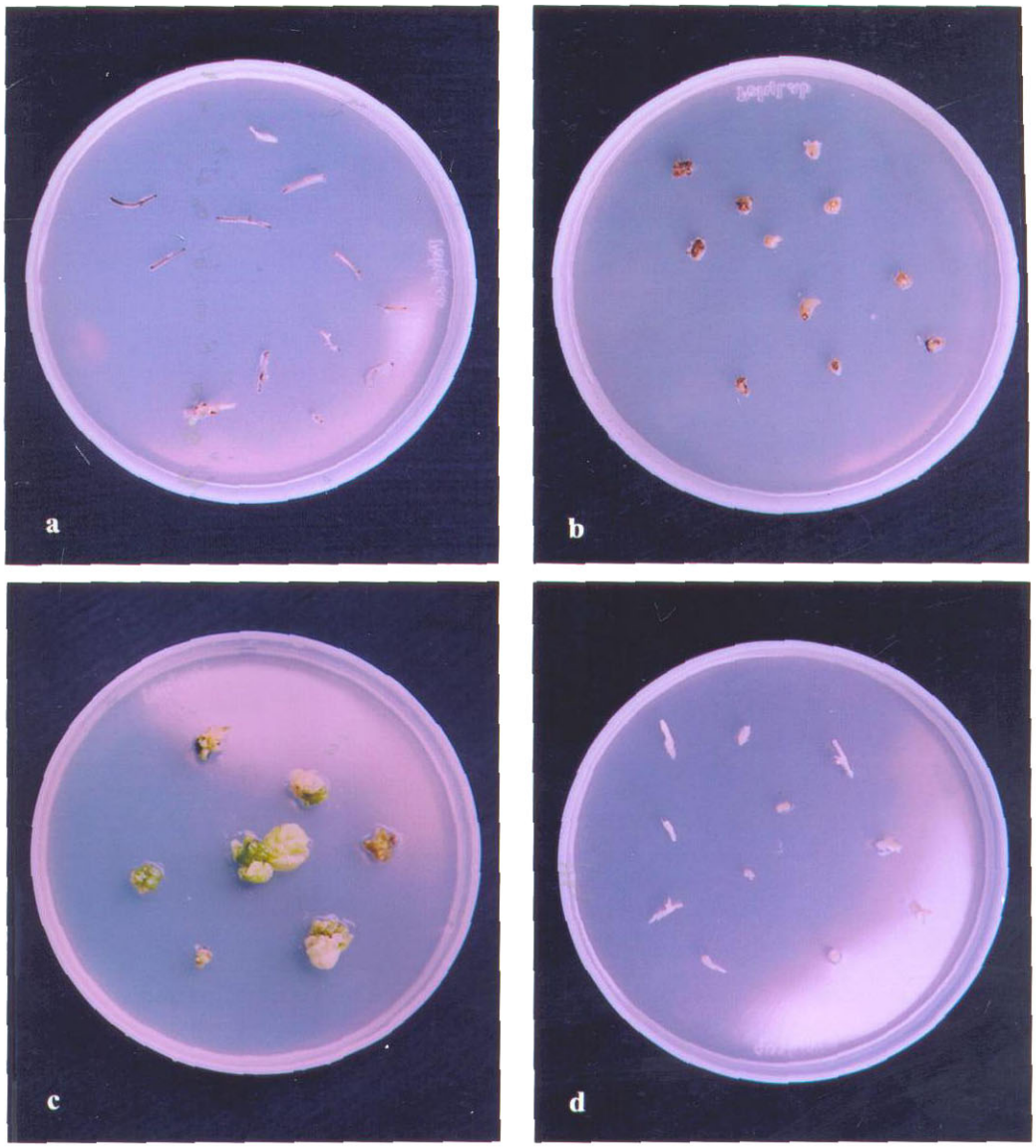


Plate 18. Effect of cryoprotectants on survival and growth of shoot meristems of *Clitoria ternatea, Mucuna pruriens* and *Indigofera tinctoria* after rapid freezing in LN

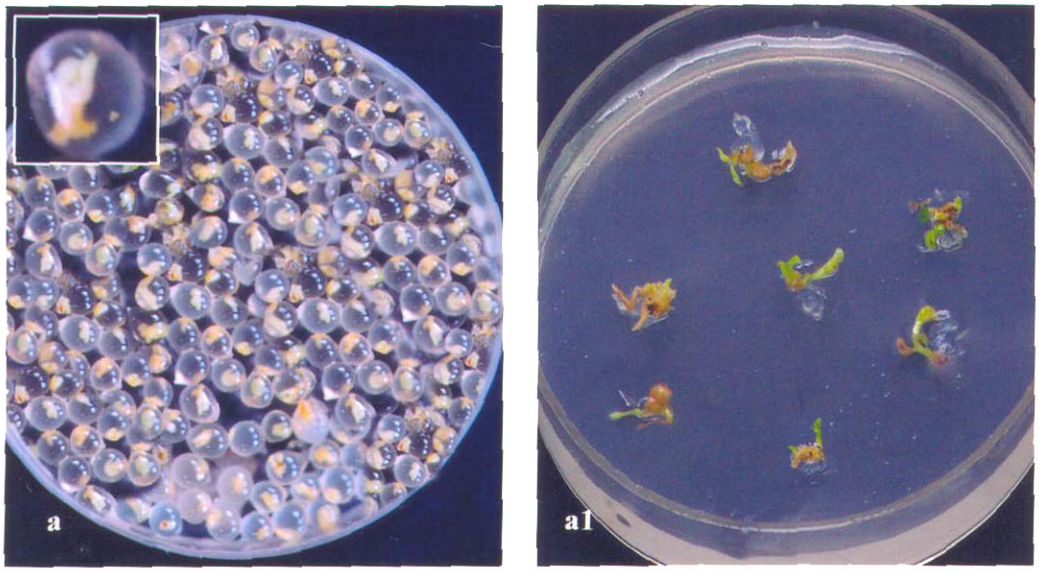


Plate 19. *In vitro* regeneration of encapsulated, desiccated and cryopreserved somatic embryos of *Clitoria ternatea*

Plate 18. Effect of cryoprotectants on survival and growth of shoot meristems of *Clitoria ternatea*, *Mucuna pruriens* and *Indigofera tinctoria* after rapid freezing in LN

a. Shoot meristem of *C. ternatea* treated with cryoprotectant,.DMSO 10 %, turning white when inoculated on to recovery medium (1/2 MS + GA 0.5 mg l^{-1} + BA 0.2 mg l^{-1}) after freezing in LN

b. Shoot meristem of *M. pruriens* treated with cryoprotectant, DMSO 10 %, turning black when inoculated on to recovery medium (1/2 MS + GA 0.5 mg l^{-1} + BA 0.2 mg l^{-1}) after freezing in LN

c. Regenerated shoot meristem of *M. pruriens* treated with cryoprotectant, sucrose 0.7 M + DMSO 5 % when inoculated on to recovery medium (1/2 MS + GA 0.5 mg l^{-1} + BA 0.2 mg l^{-1}) after freezing in LN

d. Shoot meristem of *I .tinctoria* treated with cryoprotectant, DMSO 10 % turning white when inoculated on to recovery medium (1/2 MS + GA 0.5 mg l^{-1} + BA 0.2 mg l^{-1}) after freezing in LN

Plate 19. *In vitro* regeneration of cryopreserved, desiccated, encapsulated somatic embryos of *Clitoria ternatea*

a. Sodium alginate encapsulated somatic embryos before desiccation

a1. Establishment and regeneration of *C. ternatea* plants in recovery medium (1/2 MS + GA 0.5 mg l^{-1} + BA 0.2 mg l^{-1}) after freezing in LN

4.2.2.4 Somatic Embryos

Encapsulated somatic embryos of *Clitoria ternatea* were subjected to different duration of desiccation; their survival and regeneration per cent were recorded. On 5h desiccation, moisture content reduced gradually from 63.67 per cent to 18.32 per cent (Table 42). Without desiccation, encapsulated embryos showed a survival and regeneration per cent of 73.33 percent and 67.85 per cent, respectively; but after freezing in LN, embryos did not survive at all. At 4 h desiccation, when embryos attained a moisture content of 21.38 per cent, it gave a survival rate of 26.66 per cent and regeneration of 38.89 per cent, after freezing in LN. Maximum survival after freezing in LN was obtained at 5h desiccation when the moisture content was reduced to 18.32 per cent. Survival and regeneration was 43.33 and 55 per cent respectively (Plate 19).

4.2.3 Estimation of Genetic Stability of Cryopreserved Materials Using RAPD Technique

To assess the genetic fidelity of plantlets regenerated from cryopreserved seeds, meristem, zygotic and somatic embryos, the RAPD patterns were compared with those of field grown plants in case of seeds and *in vitro* grown plantlets in case of meristems, zygotic and somatic embryos in *C. ternatea*, *M. pruriens* and *I. tinctoria*. All the six primers produced clear and reproducible bands. Primers selected for each plant species and their nucleotide sequence is presented in Table 43.The number of bands of each primer varied from three to six.

RAPD fragment pattern for plantlets from cryopreserved materials were identical to those of control plants for all the six primers tested. Plate 20 illustrates amplified band patterns produced by six primers in plantlets regenerated after cryopreservation and control plants. No differences were observed in the banding pattern of control and cryopreserved samples in all the three plant species.

Table 43. Nucleotide sequence of decamer primers employed for the evaluation of genetic stability of plantlets regenerated from control and cryopreserved materials of *Clitoria ternatea*, *Mucuna pruriens* and *Indigofera tinctoria*

Sl. No.	*Clitoria ternatea*		*Mucuna pruriens*		*Indigofera tinctoria*	
	Primers	Sequence (5'–3')	Primers	Sequence (5'–3')	Primers	Sequence (5'–3')
1.	OperonA1	CAGGCCCTTC	OperonA1	CAGGCCCTTC	OperonA4	AATCGGGCTG
2.	OperonA10	GTGATCGCAG	OperonA3	AGTCAGCCAC	OperonA10	GTGATCGCAG
3.	OperonA17	GACCGCTTGT	OperonA10	GTGATCGCAG	OperonB1	GTTTCGCTCC
4.	OperonA19	CAAACGTCGG	OperonA13	CAGCACCCAC	OperonB4	GGACTGGAGT
5.	OperonA20	GTTGCGATCC	OperonB12	CCTTGACGCA	OperonB5	TGCGCCCTTC
6.	OperonB5	TGCGCCCTTC	OperonB18	CCACAGCAGT	OperonB18	CCACAGCAGT

Plate 20. Comparison of RAPD banding pattern of cryopreserved plant specimens regenerated from various propagules of *Clitoria ternatea*, *Mucuna pruriens* and *Indigofera tinctoria* with those from non cryopreserved propagules

a. Pairwise comparison of RAPD banding pattern (six primers) of plants regenerated *ex vitro* from cryopreserved and non cryopreserved seeds of *C. ternatea*

a1. Pairwise comparison of RAPD banding pattern (six primers) of plants regenerated *in vitro* from cryopreserved and non cryopreserved shoot meristems of *C. ternatea*

a2. Pairwise comparison of RAPD banding pattern (six primers) of plants regenerated *in vitro* from cryopreserved and non cryopreserved somatic embryos of *C. ternatea*

b. Pairwise comparison of RAPD banding pattern (six primers) of plants regenerated *ex vitro* from cryopreserved and non cryopreserved seeds of *M. pruriens*

b1. Pairwise comparison of RAPD banding pattern (six primers) of plants regenerated *in vitro* from cryopreserved and non cryopreserved of zygotic embryos *M. pruriens*

b2. Pairwise comparison of RAPD banding pattern (six primers) of plants regenerated *in vitro* from cryopreserved and non cryopreserved shoot meristems of *M. pruriens*

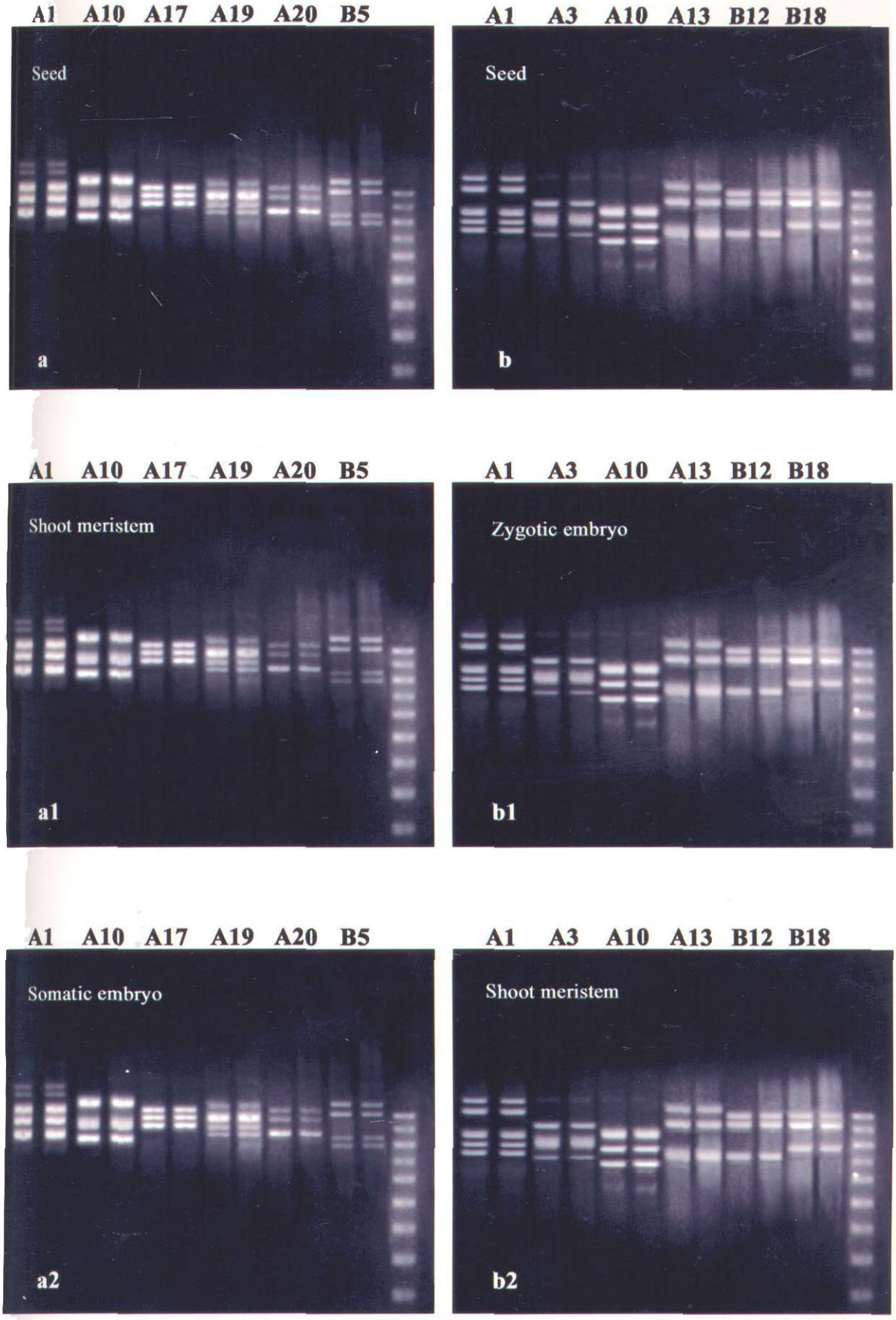
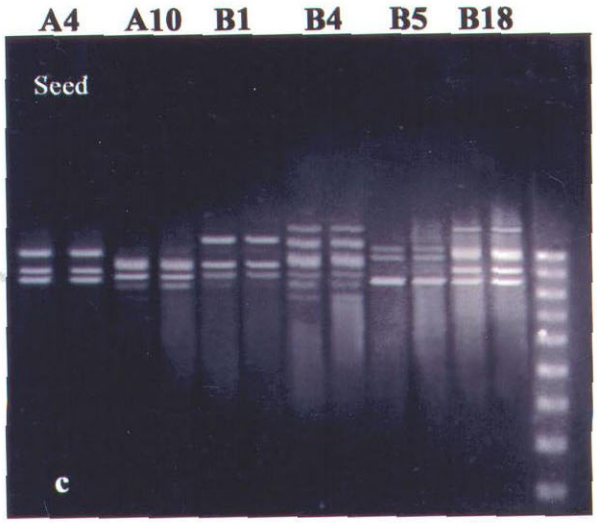


Plate 20. Comparison of RAPD banding pattern of cryopreserved plant specimens regenerated from various propagules of *Clitoria ternatea*, *Mucuna pruriens* and *Indigofera tinctoria* with those from non cryopreserved propagules (Contd..)

c. Pair wise comparison of RAPD banding pattern (six primers) of plants regenerated *ex vitro* from cryopreserved and non cryopreserved seeds of *I. tinctoria*

c1. Pair wise comparison of RAPD banding pattern (six primers) of plants regenerated *in vitro* from cryopreserved and non cryopreserved shoot meristems of *I. tinctoria*



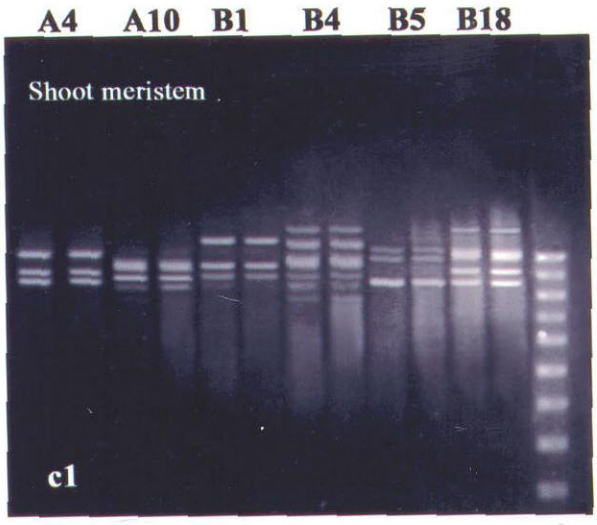


Plate 20. Comparison of RAPD banding pattern of cryopreserved plant specimens regenerated from various propagules of *Clitoria ternatea*, *Mucuna pruriens* and *Indigofera tinctoria* with those from non cryopreserved propagules

Discussion

5. DISCUSSION

The present project on 'Rapid propagation and conservation of selected leguminous plants using *in vitro* techniques' conducted during 2001-2004 at the Department of Plantation Crops and Spices and at the Department of Plant Biotechnology, College of Agriculture , Vellayani has resulted in standardization of *in vitro* techniques for not only rapid multiplication but also for short term as well as long term conservation of valuable germplasm of commercially important plants *viz.*, *Clitoria ternatea*, *Mucuna pruriens* and *Indigofera tinctoria*. The results are discussed in this chapter.

5.1 *IN VITRO* PROPAGATION

Clitoria ternatea, *Mucuna pruriens* and *Indigofera tinctoria* are propagated usually by seed. One of the constraints in this conventional propagation is the very short span of seed viability. No alternative mode of multiplication is available to propagate and conserve genetic stock of these valuable plants. *In vitro* culture technique provides a tool for rapid and large scale multiplication of these crop species. It also permits production of pathogen free planting materials.

5.1.1 Enhanced Release of Axillary Buds

In vitro propagation by axillary shoot proliferation has proved to be the most promising method of clonal propagation in many medicinal plants (Dias *et al.*, 2002). In the present study, enhanced release of axillary buds was attempted using nodal segments, leaf and cotyledons. Only the nodal explants exhibited organogenesis. Explant source is one of the critical parameters for successful regeneration of legume species, which is other wise recalcitrant to *in vitro* regeneration (Parrott *et al.*, 1992).

Growth and morphogenesis *in vitro* are regulated by the interaction and balance between the growth regulators supplied to the medium and growth substances endogenously produced by the cultured cell. BA, Kn and IAA at various concentrations and combinations were tried in the present study to standardize their optimum levels for shoot proliferation in the three plant species. In the case of *C. ternatea* and *M. pruriens*, the best hormone combination for shoot proliferation was BA 0.5 mg l^{-1} + Kn 0.5 mg l^{-1} with regard to shoot number and shoot length, while for *Indigofera tinctoria* BA 1.0 mg l^{-1}+ IAA 0.1 mg l^{-1} produced maximum number of multiple shoots and shoot elongation. Multiple shoot induction in media with Kn alone or in combination with IAA was very less in all the three plant species. Compared to this, multiple shoot induction (shoot number) was high in treatments with BA alone. The potentiality of BA over Kn in shoot induction from nodal explants was also reported in *Houttuynia cordata* (Handique and Bora, 1999), while promising response due to combined effect of BA and Kn was reported in *Withania somnifera* (Deka *et al.*, 1999) and *Rauvolfia tetraphylla* (Sarma *et al.*, 1999). Jagatram *et al.* (2003) also observed that conjoint addition of BA and Kn promoted shoot bud proliferation in nodal explants of *Madhuca latifolia*. In consistence with the findings in *I. tinctoria*, synergistic effect of BA and IAA in multiplication and growth of shoots was reported in *Bacopa monnieri* (Yelne *et al.*, 1997) and *Cardiospermum halicacabum* (Jaysheelan and Rao, 1998). Nunes Ed *et al.* (2002) reported inhibitory effect on addition of NAA to BA in *Cedrela fissilis*.

In *M. pruriens* and *I. tinctoria* bud break was early in treatments with BA in combination with Kn, while in *C. ternatea*, bud initiated early in treatment with BA, Kn and IAA.

In the present study there was no significant variation in number of shoots per culture with the addition of GA into the medium. Except for *C. ternatea*, no variation was observed with regard to shoot length also. In *C. ternatea*, GA (1.0 mg l^{-1}) recorded lower shoot length compared to control. But all the three plant species showed a progressive increase in shoot length with increase in GA level. Addition of

GA along with BA was found to be beneficial for the elongation of shoots in *Murraya koenigii* (Bhuyan *et al.*, 1997) and in *Vitex negundo* (Sahoo and Chand, 1998).

The basal medium requirement depends upon the plant species and the purpose of cell, tissue and organ culture. In the present study full MS was found to be significantly superior to quarter MS, half MS, MS+AC, SH, B5 and WPM in the three plant species. This is in agreement with the findings of Catapan *et al.* (2001) that MS medium was the best for shoot initiation followed by B5, SH and WPM in *Phyllanthus stipulatus.* Komalavalli and Rao (2000) also found that, among the different media tested, MS was the best for shoot sprouting, number of shoots and length followed by B5, SH and WPM in *Gymnema sylvestre.* MS media has been found the most suitable medium for shoot proliferation in grain legumes, soybean (Saka *et al.,* 1980) and chickpea (Arockiasamy *et al.,* 2000).

Effect of two carbon sources, sucrose and glucose at various levels on shoot proliferation of *C. ternatea*, *M. pruriens* and *I. tinctoria* were investigated. According to George and Sherrington (1984), sucrose is the most utilizable carbon form and energy source for *in vitro* cultures. Among the sucrose levels studied, sucrose at 30 g l^{-1} produced maximum number of shoots in *C. ternatea* and *M. pruriens* (6.33 and 5.17 respectively). While in *I. tinctoria*, though there was no significant variation with regard to number of shoots, maximum number of shoots was obtained with sucrose at 50 g l^{-1}. Kumar *et al.* (1993) found that sucrose at 20 g l^{-1} is effective for shoot multiplication in *C. ternatea.* Chattopadhyay *et al.* (1995) supplemented 30 g l^{-1} of sucrose to the medium for multiple shoot regeneration in *M. pruriens.* Sucrose at 50 g l^{-1} evoked best response in studies on direct shoot regeneration from cotyledons of *Citrus sinensis* (Daming *et al.,* 2000). Glucose at different levels were found to be significantly inferior to sucrose in *C. ternatea* and *M. pruriens* with regard to proliferating shoots but in *Indigofera*, there was no significant variation. Still sucrose produced maximum number of shoots (4.67) compared to glucose at different levels. Hazeena (2001) opined that sucrose was a better carbon source compared to glucose in *Aegle marmelos.*

Effect of cobaltous chloride, as an ethylene inhibitor was studied to assess its effect on shoot proliferation. No significant difference was obtained with different levels of it when tried in the three plant species. In contrast to this, Lai *et al.* (2000) observed enhancement in shoot proliferation with the addition of 5 µM cobaltous chloride in *Carica papaya.* Martin (2002) found a reduction in number of shoots formed in *Holostemma adakodien* with the addition of cobaltous chloride in the medium.

The optimum agar concentration creates an osmotic potential favourable for uptake of nutrients. In the present study, there was significant variation among the different levels of agar tried with regard to number of shoots in *C. ternatea* and *M. pruriens,* while in *I. tinctoria* there was no significant variation. However, a decreasing trend in shoot number was observed with increased levels of agar. Maximum number of shoots per culture was observed in agar at 5 g l^{-1} in *C. ternatea, M. pruriens* and *I. tinctoria* (6.67, 5.50 and 5.33 respectively). Inhibitory action of agar on shoot proliferation at higher concentrations has been related to decreased uptake of medium constituents (Debergh, 1983) and decreased availability of water (Stoltz, 1971).

5.1.2 Somatic (Indirect) Organogenesis

Indirect somatic organogenesis is characterized by redetermination of differentiated cells leading to callus formation and subsequent dedifferentiation to form separate shoot and root initials from it. The standardization of *in vitro* plant regeneration protocols with intervening callus phase provides for genetic variation developing in many of its component cells (Bhalsing, 1999). In addition, the protocol can be utilised for generating new genetic variability by somatic hybridization through protoplast fusion (Arcioni *et al.,* 2001)

Stem, leaf and cotyledons were used as explants in *C. ternatea* and *M. pruriens.* In *I. tinctoria*, stem and leaf were used, but due to small size of seed,

cotyledons could not be used as explant. Different combinations of plant growth substances (2, 4-D, NAA and BA) were tried to assess its effect on callus initiation, in the three plant species. Best treatment combination for callus initiation varied with the species and explant type. The manipulation of the appropriate levels of auxins and cytokinins is crucial to define the balance of growth regulators so that there is induction of callus formation in the different types of explant (Franklin and Dixon, 1994).

C28 (NAA 2.0 mg l^{-1} + BA 0.5 mg l^{-1}), C27 (NAA 1.0 mg l^{-1} + BA 0.5 mg l^{-1}) and C17 (2,4-D 2.0 mg l^{-1} + BA 0.5 mg l^{-1}) gave maximum callus induction in stem, leaf and cotyledon explants respectively of *C. ternatea*. In *M. pruriens*, C13 (2,4-D 2.0 mg l^{-1} + BA 0.1 mg l^{-1}), C28 (NAA 2.0 mg l^{-1} + BA 0.5 mg l^{-1}) and C17 (2,4-D 2.0 mg l^{-1} + BA 0.5 mg l^{-1}) recorded best callus induction in stem, leaf and cotyledon explants, respectively. While in *I. tinctoria*, best callus induction was obtained in C17 and C16 (2, 4-D 1.0 mg l^{-1} + BA 0.5 mg l^{-1}) respectively in stem and leaf explants. With the three species and with different explants of each, best callusing was obtained at high ratio of auxin to cytokinin. Mercier *et al.* (1992) obtained profuse callus regeneration from stem explants of *Gomphrena officinalis* in BA + NAA hormone combination. In contrast to this, BA in combination with NAA did not induce any significant callus growth in stem and leaf explants of *Centella asiatica* (Patra *et al.*, 1998). With cotyledon explants of the legume, *Glycine wightti*, Da Silva *et al.* (2003) observed maximum callus induction in medium containing the combination of 2,4-D (1 mg l^{-1}) and Kn (0.1 mg l^{-1}).

In *C. ternatea,* all the three explants failed to produce callus in media lacking growth regulators. Similar was the response with BA alone. This is in agreement with the findings of Saritha *et al.* (2003) in leaf explants of *Spilanthes acmella.* In *M. pruriens* stem explant alone responded to BA, whereas both stem and leaf explants responded in *I. tinctoria.* Rout *et al.* (1999) observed callus induction in leaf and stem explants of *Plumbago zeylanica* in BA supplemented MS medium, though with a low callus induction per cent.

NAA (0.5 – 2.0 mg l^{-1}) in culture medium showed root initiation from both leaf and cotyledonary explants with limited or no callusing in *C. ternatea.* This is in consistent with the findings of Rout *et al* (1999), with stem and leaf explants of *Plumbago zeylanica.* Even with *M. pruriens* and *I. tinctoria,* NAA gave either low or no response with different explants.

The medium containing 2, 4-D in combination with NAA induced callus in all the explants tried in the three plant species. But within 4 weeks of culture, it turned dark brown and did not give regeneration. Shoot regeneration was observed with callus induced in media with BA in combination with 2, 4-D or NAA. Callus from induction media were subcultured on to various regeneration media containing different combinations of BA, Kn, NAA and adenine sulphate.

In case of *C. ternatea,* shoot regeneration was best in stem derived callus with respect to number of shoots (2.33) and length of shoots (5.17 cm). Highest regeneration was obtained in SR 24 (BA 2.0 mg l^{-1} + Kn 2.0 mg l^{-1} + NAA 1.0 mg l^{-1} + adenine sulphate 20 mg l^{-1}). Light brown callus with green nodular structures developed into shoots when transferred to SR 24 medium after 4 weeks of culture. Skoog and Tsui (1948) opined that addition of adenine sulphate to the medium enhances growth and shoot formation. Shoot regeneration per cent was much less or nil with BA (1-2 mg l^{-1}). Similar results have been reported in leaf and stem explants of *Centella asiatica* (Patra *et al.*, 1998).

In *C. ternatea* SR11 (BA 2.0 mg l^{-1} + NAA 0.5 mg l^{-1}) and SR 19 (BA 2.0 mg l^{-1} + Kn 1.0 mg l^{-1} + NAA 0.5 mg l^{-1}) recorded maximum shoot regeneration with regard to shoot number and shoot length in leaf and cotyledon segment respectively. In the present study, low shooting response was observed in all treatments with Kn in combination with NAA in all explants tried in *C. ternatea.*

In *M. pruriens* best shoot regeneration was obtained from leaf derived callus. SR 11 (BA 2.0 mg l^{-1} + NAA 0.5 mg l^{-1}) recorded maximum shoot regeneration in

terms of shoot number and shoot length from leaf derived callus as in *C. ternatea.* Same treatment recorded maximum shoot regeneration with cotyledon explant also. These findings confirm that the presence of auxin at lower concentration favours shoot regeneration and multiplication. Sharma and Wakhlu (2003) observed maximum shoot differentiation from petiole derived callus on MS medium containing BA (1.0 mg l^{-1}) and NAA (0.2 mg l^{-1}) in *Heracleum candicans*. In contrast to this, maximum shoot induction was obtained in MS medium supplemented with higher concentration of NAA (3.5 mg l^{-1}) in combination with BA (1.5 mg l^{-1}) from leaf derived callus of *Albizzia amara* (Ramamurthy and Savithramma, 2003). In case of stem explant of *Mucuna pruriens*, best regeneration was obtained with SR13 (Kn 1.0 mg l^{-1} + NAA 0.5 mg l^{-1}), whereas the same medium recorded poor regeneration with regard to leaf and no regeneration in cotyledon explant. With respect to cotyledon derived callus, the regeneration was obtained only in media containing BA in combination with NAA. In *Vigna unguiculata;* plant regeneration from cotyledon-derived callus was recorded in a medium containing 0.1 mg l^{-1} zeatin (Anand *et al.*, 2001).

In *I. tinctoria*, only stem derived callus gave shoot regeneration, while leaf derived callus did not give any shooting response with the treatments tried. SR 11 (BA 2.0 mg l^{-1} + NAA 0.5 mg l^{-1}) gave maximum shoot regeneration in stem derived callus. However, Howell *et al.* (1987) could regenerate shoot from leaf derived callus in *Indigofera potaninii* in MS medium supplemented with BA and NAA.

5.1.3 Somatic Embryogenesis

Plant regeneration through somatic embryogenesis was achieved in *C. ternatea.* Callus obtained by culturing leaf discs on MS medium supplemented with 2,4-D 0.5 mg l^{-1} + BA 0.1 mg l^{-1}, when subjected to higher cytokinin concentration, BA 1.0 mg l^{-1}, produced numerous somatic embryos. Somatic embryos developed into complete plantlets on medium devoid of growth regulators and in GA supplemented medium. There was no significant variation with regard to different

germination media. Similar results were reported in *Thevetia peruviana* (Kumar, 1992). Embryogenic calli were obtained from leaf explants of *Eryngium foetidum* in LS medium supplemented with 2,4-D 1.0 mg l^{-1} and BA 1.0 mg l^{-1} (Igancimuthu *et al.*, 1999).

Lakshmisita *et al.* (1979) reported the somatic embryogenesis enhancing effect of GA in *Santalum album*. Davey and Power (1988) emphasized the requirement of GA for the development of protoplast derived embryos of *Trifolium pratense* beyond the globular stage. But in the present study, when callus with globular and heart shaped embryo was transferred to GA supplemented medium no further development was obtained. Torpedo shaped embryos when separated and transferred to the germination medium produced root and shoot initials.

5.1.4 Rooting

5.1.4.1 In vitro Rooting

Efficient rooting of *in vitro* regenerated plants and subsequent field establishment is the last and crucial stage of rapid clonal propagation. Auxins frequently used for rooting are IAA, IBA and NAA (George and Sherrington, 1984).

The three plant species gave different rooting response with the three types of auxins tried. Medium supplemented with NAA (1 mg l^{-1}) proved best for *C. ternatea*. Kumar *et al.* (1993) observed best root induction in B5 medium supplemented with NAA 3 mg l^{-1} in *C. ternatea*. IBA (2.5 mg l^{-1}) favoured best rooting in *M. pruriens* with regard to early rooting, number of roots and root length. Profuse rooting was obtained in IBA (1 mg l^{-1}) in *Spilanthes acmella* (Saritha *et al.*, 2003) whereas IBA (3 mg l^{-1}) was required in *Salvadora persica* (Batra *et al.*, 2001). IAA (1.5 mg l^{-1}) is the best rooting media observed for *I. tinctoria* in the present investigation. In *Plumbago zeylanica* best rooting was obtained in media supplemented with 0.57 µM IAA.

In the present study, it is observed that in *C. ternatea*, auxins at higher concentration inhibit root elongation. In *Aegle marmelos*, Hossain *et al.* (1994) observed that at higher concentration of IBA, there was reduction in root length.

When IAA was supplemented to the medium, callusing suppressed root formation in *C. ternatea* and *M. pruriens* in contrast to the observation in *I. tinctoria* wherein it produced profuse root induction. Rooting suppression in the presence of IAA was reported by Batra *et al.* (2001) in *Salvadora persica.*

In *I. tinctoria*, root initiation was observed in medium devoid of growth regulators. Similar findings were reported in *Acorus calamus* (Anu *et al.*, 2001). In the present study, very low root initiation was observed in medium containing NAA in *I. tinctoria*. In contrast to this, Howell *et al.* (1987) observed rooting in *Indigofera potaninii*, at higher levels of NAA (5 mg l^{-1}).

5.1.4.2 Ex vitro Rooting

The major cost involved in the production of *in vitro* plants is for rooting and hardening. *Ex vitro* rooting is preferred in many crops with a view to save time and resources. In the present study, pretreatment with IBA 1000 mg l^{-1} for 20 s recorded high survival percent (83.33 and 75 per cent respectively after four weeks of planting in *C. ternatea* and *I. tinctoria*, while in *M. pruriens* 500 mg l^{-1} gave maximum survival per cent. John (1996) reported similar finding in *Holostemma annulare* where IBA 1000 mg l^{-1} was found to be best for *ex vitro* rooting. *Ex vitro* root induction was achieved with IBA (260 µM) in *Vitex negundo* (Kannan and Jasrai, 1998)

The benefit of any micropropagation system can only be fully realized by the successful transfer of plantlets from tissue culture vessels to the ambient conditions found *ex vitro;* hence acclimatization of *in vitro* raised plants is inevitable. Various potting media were tried in the present investigations to study the effect on the

survival of *in vitro* raised plants. Sand was the ideal potting media registering maximum survival rate (83.33, 75.00 and 91.67 per cent respectively) after four weeks in *C. ternatea*, *M. pruriens* and *I. tinctoria*. Successful *ex vitro* establishment of *in vitro* raised plantlets in sand was previously reported in *Holostemma annulare* (John, 1996) and in *Aegle marmelos* (Hazeena, 2001).

In this study, different *in vitro* propagation methods have been standardized for the three plant species. This has made possible the availability of pathogen free and quality planting materials for year round planting for mass multiplication. Balance between plant growth regulators and other additives *viz.*, casein hydrolysate, coconut water, amino acids etc. is a pre requisite for organogenesis. In the case of enhanced release of axillary buds and callus mediated organogenesis, high ratio of cytokinins to auxins promoted shoot formation and proliferation. Auxins promoted *in vitro* rooting in the three plant species. Though the plant species belong to the same family, auxin which effected maximum rooting in each species is different; NAA in *C. ternatea*, IBA in *M. pruriens* and IAA in *I. tinctoria*. This confirmed the differential response of these plants to auxins in rhizogenesis. A simple and reproducible protocol for the rapid propagation of *Clitoria ternatea* was developed through somatic embryogenesis from the leaf explants. From one gram of embryogenic callus, an average of 60 to 70 plantlets could be produced. This indicates that somatic embryogenesis offers the fastest method of *in vitro* multiplication. The different *in vitro propagation* methods standardized in this study can be effectively made use for germplasm conservation, genetic transformation and biotechnological methods of crop improvement.

5.2 *IN VITRO* CONSERVATION

Seeds of the three legumes selected for the study fall in orthodox category. Orthodox seed storage is carried out using methodologies that offer either 'active' or 'base' conservation conditions for the medium term or long term respectively. *In vitro* active conservation is achieved by maintenance of cultures in the growing state, usually in slow growth medium. *In vitro* base conservation is achieved by

cryopreservation of seeds, *i.e.,* storage in liquid nitrogen (-196°C). Seed is not a preferred entity for international exchange of germplasm as there is risk of seed borne pest and diseases. Generally, the cultures used for distribution are shoots, embryos or plantlets derived from them, due to the greater genetic stability of such cultures, which calls in for cryopreservation of these plant parts.

5.2.1 Slow Growth

Slow growth provides for short to medium term storage by manipulating the culture conditions or culture media to allow the cultures to remain viable but with a slow growth rate. As the germplasm is conserved in a viable but slow growing state, it could be grown into plants on immediate demand (Kartha, 1985).

5.2.1.1 Culture Medium and Explant

In all the three plant species under study, single node cuttings were used as explant. In slow growth studies, type of explant and the size of the explant played a very important role. Westcott *et al.*, (1977) used nodal cuttings to store potato *in vitro.*

In the study, half MS supplemented with IBA (0.5 mg l^{-1}) was slow growth base medium used, which was further supplemented with additives to achieve growth inhibition. In slow growth studies Hansen and Kristiansen (1997) had established positive effect of rooting medium containing auxins on survival rate. Ng and Ng (1991) confirmed that reducing the concentration of mineral salt mixture in the medium retarded the growth of cultures. In slow growth studies with *Janakia arayalpathra* and *Geophila reniformis*, half MS medium gave better survival with or without the addition of osmotica (TBGRI, 1997).

5.2.1.2 Addition of Osmotica: Sucrose, Sorbitol and Mannitol

In the three plant species under study, sucrose at higher levels caused an inhibition in the linear growth of the plants. Schenk and Hildebrandt (1972) explained

this effect of sucrose; high levels of sucrose can be used to maintain cultures in dormant conditions for longer periods as sucrose concentration above 4-5 per cent had an inhibitory but non-toxic effect on plant cell growth. In contradiction to this, survival rate of *M. pruriens* and *Indigofera tinctoria* were less than 50 per cent at SuT5 (sucrose 15 per cent). In the present study, among the different osmotica tried, which gave cent per cent survival and regeneration after 28 weeks of incubation, SuT3 (sucrose 10 per cent) gave maximum plant growth inhibition in *I. tinctoria.* MS medium with 7 per cent sucrose was better for reduced growth and high survival rate of *Holostemma adakodien* (TBGRI, 1997).

Sorbitol also resulted in reduction of growth in all the three plant species. Among the different osmoticum tried, SbT1 (sorbitol 0.5 per cent) gave maximum plant growth in *M. pruriens* and *I. tinctoria*, though it was lower than the control. This is in conformity with by Lizarraga *et al.* (1992) who observed growth reduction with 3 per cent sorbitol in sweet potato cultures. Some enhancement in growth was seen with sweet potato at lower concentration of sorbitol. Same effect was reported in cassava cultures by Roca *et al.* (1984).

Root initiation was observed at all levels of sorbitol in all the three plant species studied. This may be due to the presence of IBA (0.5 mg l^{-1}) in the medium. Also, sorbitol at high concentration was found to enhance root growth in sweet potato (Lizarraga *et al.*, 1992).

Among the different osmotica which gave cent per cent survival and regeneration after 28 weeks of incubation, MnT3 (mannitol 1.5 per cent) and MnT4 (mannitol 3 per cent) gave maximum plant growth inhibition in *C. ternatea* and *M. pruriens,* respectively. This in agreement with the observation of Zandavoort *et al.*, (1994) that mannitol (3 per cent) could effectively bring down the rate of growth of cultures of *Xanthosoma sagittifolium* and could be stored for 3 years without losses.

All levels of mannitol gave 100 per cent survival in *M. pruriens* and *I. tinctoria* up to 28 weeks of storage. In *C. ternatea* also mannitol gave a good survival per cent ranging from 90 to 100. Bessembinder *et al.* (1993) showed that the addition

of mannitol to the storage medium had a positive effect on survival and growth when the temperature regime was 28°C in *Colocasia esculenta*. In this study, mannitol significantly suppressed growth under light conditions but shoots retained a healthy green colour indicating that mannitol had a positive effect on the survival. In contrast to this, Roca *et al.* (1982) observed the deterioration of cassava shoots in presence of even 0.1 per cent mannitol with a storage temperature lower than 20°C.

Osmotica such as mannitol and sorbitol reduced mineral uptake by cells through differences in osmotic pressure thereby retarding plant growth (Dodds and Roberts, 1985; Thompson *et al.*, 1986).

In the present study, three plant species could be stored for 28 weeks without subculture in presence of mannitol (0.5 to 3 per cent) and sorbitol (0.5 to 3 per cent). This is in agreement with the findings of Acedo (1993) and Mandel and Chandel (1990) that in *Ipomoea batatas* subculture interval could be extended to 6-12 months when supplemented with 1-4 per cent sorbitol and 2-4 per cent mannitol, respectively.

5.2.1.3 Addition of Cryoprotectant: Proline

Though there are reports on growth retardation of cultures on addition of cryoprotectant (Engelmann, 1991), no slow growth experiment has yet been done utilizing the cryoprotectant, proline. Proline is usually accumulated in response to water deficits, and salinity stress (Taylor, 1996; Rhodes, 1987). When supplied exogenously, proline has osmoprotective (Handa *et al.*, 1986; Lone *et al.*, 1987) and cryoprotective (Songstad *et al.*, 1990; Santarius, 1992) function. As such it is expected to have a growth promoting effect.

But, in this study, proline was very effective in slowing down the growth rate of *C. ternatea* and *M. pruriens* cultures, but in *I. tinctoria* cultures, proline promoted plant growth and it attained a height higher than that of the control plants. In *C. ternatea*, maximum plant height was obtained with Pr T1 (proline 5 mM) among the additives tried. But at higher levels of proline there was substantial reduction in plant height.

5.2.1.4 Addition of Antioxidant: PVP

Addition of PVP caused plant growth inhibition in all the three plant species. But in *M. pruriens* in particular, it caused a considerable reduction in plant height. Except for a slight increase in plant height during early stages (up to 4 weeks) of incubation, the plant height remained constant. In garlic, by addition of PVP (6-12 per cent) to the culture medium, an initial establishment of 75 to 100 per cent was obtained. But there was no significant effect on plant growth with increasing concentrations of PVP (Wasswa, 1991).

5.2.1.5 Addition of Growth Retardant: ABA

ABA produced significant reduction in plant growth in *M. pruriens* and *I. tinctoria;* while *C. ternatea* cultures did not survive at any levels of ABA tried. Detrimental effect of ABA was reported by Roca *et al.* (1982) in potato. In *M. pruriens*, survival was 83.33 per cent at ABA (10 mg l^{-1}) and 50 per cent at higher levels, 28 weeks after incubation. But with *I. tinctoria*, ABA at 10-30 mg l^{-1} gave 0 - 25 per cent survival but did not regenerate on transfer to regeneration medium, while in *M. pruriens* ABA at 10 and 15 mg l^{-1} gave 100 per cent regeneration after 28 weeks of incubation. This is in agreement with the observation of Negash (1990) on the effect of ABA on garlic. He found that a higher concentration of 15 mg l^{-1} gave better results in terms of survival and reduced growth. Jarret and Gawel (1991) reported a survival rate of 70 to 85 per cent in sweet potato plants in medium containing ABA 5-20 mg l^{-1}.

At different concentrations of ABA, explants of *M. pruriens* showed a little or no extension or development. But explants remained green and viable. Similar observation is reported by Desamero (1990) in sweet potato.

5.2.2 Cryopreservation

The aim of this study is to develop a protocol for the long term conservation of *Clitoria ternatea*, *Mucuna pruriens* and *Indigofera tinctoria* using cryopreservation technique.

Though seed storage is the most convenient and economic method for medium to long-term *ex situ* germplasm conservation, it has inherent limitations such as loss of seed viability, particularly in humid tropical conditions (Roberts, 1991). Traditionally, temperature for storing orthodox seeds range from 10°C to – 20°C (Cromothy *et al.,* 1982). When dried, many seeds are stable for long periods of time under these conditions; but seed deterioration in storage does occur, depending on species, temperature and moisture level (Roberts and Ellis, 1984). But at cryostorage temperature (-196°C), biological process are brought to a halt and it is presumed that deterioration that can occur in seeds at higher temperature will not take place, making very long term storage possible (IBPGR, 1982).

Cryostorage offers a secure method of seed storage, as seed metabolism is suspended at ultra-low temperature of liquid nitrogen (–196°C). The use of liquid nitrogen for long-term storage of seed has been demonstrated for a large number of economically important species. Successful cryostorage of seed is highly dependent on moisture content, which must be maintained below a critical point at which freezing injury occurs. The critical moisture content varies between species, for instance, bean and pea have 28 and 33 per cent critical moisture contents respectively (Vertucci, 1989).

In all the three leguminous plant species, no significant variation was observed on germination per cent at different periods of storage in LN. However, moisture level had significant effect on cryostorage and germination. At lower moisture level, after storage in LN, seeds recorded higher germination per cent compared to higher levels of moisture. Moisture content is the single most important deciding factor affecting the ability of germplasm to be stored in LN (Stanwood and Bass, 1981). Orthodox seeds generally tolerate desiccation below 10 per cent moisture and do not exhibit much reduction in germination after storage in LN. A seed moisture content of 4 to 10 per cent has been proved to be better for a safe storage of several medicinal plants which show orthodox seed behaviour. Chaudhury and Chandel (1994) were of view that seeds that are shed with high moisture content

show a considerable decline in viability below a certain level of moisture and increased seed moisture content lead to a decline in survival after LN storage. This view has been confirmed in our study. At initial seed moisture content, seeds of all the three plants gave only 40-50 per cent germination after a day of storage in LN.

In *I. tinctoria*, control and cryopreserved seeds recorded higher germination at MC2 compared to MC1. While in *C. ternatea* and *M. pruriens*, control recorded high germination rate at MC1 and cryopreserved seeds at MC2 level.

Breakage of seed coat, which was observed only in the seeds of *C. ternatea* on LN storage, can be considered as a sensitivity reaction of the seeds. Though exposure to cryotemperature of -196°C brought about the breakage, it did not affect the germination. Those seeds with broken seed coat germinated faster compared to those with intact seed coat. This may be due to faster imbibition of water and subsequent germination. Moreover, the injury on seed coat might be only peripheral in nature. Physiological and morphological entities related to viability and germination remained intact and could be retrieved on thawing as in normal germination. In contrast to this, mechanical injury to seed by direct immersion into LN causing loss in viability has also been reported in certain species (Wang *et al.,* 1994).

Amenability of seeds to cryopreservation depends on seed size, moisture per cent and viability. Cryopreservation of seeds of many small seeded crop species is important, as it requires very little space and seeds can be conserved for indefinite periods. But, *M. pruriens*, which has large seeds, cannot be identified as good preposition for cryopreservation. Though cryopreservation of seeds was found successful in *M. pruriens*, considering the large size of seeds, zygotic embryos were utilized in cryopreservation experiments. Zygotic embryos are highly organized, small-tissue systems that can be used to produce a whole plant from the meristematic tissues contained therein. Therefore, they are likely to carry lower risk of somaclonal variation in culture. Moreover, zygotic embryo culture has been used with success to cryopreserve the germplasm of many plant species (Berjak *et al.,* 2000).

Simple desiccation gave good results, though treatments with cryoprotectant did not give any positive results with zygotic embryos. Maximum survival and plantlet production was at a MC of 14.17 per cent obtained on 150 min of desiccation. Hor *et al.* (1990) reported that excised embryos must survive desiccation below the threshold freezable moisture content of 18 to 33 per cent for successful cryopreservation.

Dehydration increases the osmotic pressure of the intracellular solution (the cytoplasm) which depresses its freezing temperature and promotes vitrification and inhibits intracellular ice formation. In many species of plants, their organs and cells, survive freezing temperatures by achieving very low intracellular water content. Negative impact of cryoprotectants on cryopreservation of zygotic embryos can be attributed to their toxic effect.

Cultured plantlets germinated from zygotic embryos could be subjected to slow growth storage but the only advantage that this would offer over the field gene bank would be potential protection from infection with pathogens. Without extensive investigation to optimize slow growth conditions for a sufficiently wide range of genotypes, it would be an extremely risky conservation mode since it lacks the opportunity to replicate valuable genotypes in culture.

Meristems of all the three plant species, *C. ternatea, M. pruriens* and *I. tinctoria* were cryopreserved using encapsulation-dehydration method and by rapid freezing in cryoprotectant.

The encapsulation-dehydration method uses sucrose as a cryoprotectant coupled with partial desiccation prior to exposure to liquid nitrogen, thus avoiding the use of chemical cryoprotectants. In this study, pre culturing of encapsulated axillary buds and somatic embryos to be cryopreserved were pre cultured in a medium supplemented with 0.75 M sucrose to provide a stress conditioning effect. Initially, it was assumed that the protective role of sugars was due to an osmotic effect only, but

more recently it has been observed, both directly and indirectly, that sugars enter cells in large quantities (Dumet *et al.,* 1993; Suzuki *et al.,* 1997). It has been reported that, as a result of sucrose uptake, the freezing point is lowered and the amount of freezable water in the tissues decreases and with that the formation of ice crystals upon exposure to subzero temperatures is reduced, improving the post-thaw recovery rates (Panis *et al.*, 1996). Sugars, like sucrose, when added before dehydration and low temperature stresses, are also known to stabilize membranes and proteins during desiccation (Crowe *et al.,* 1990), which would improve recovery following freezing. The experiment was done to study the effect of dehydration duration on encapsulation-dehydration method. But successful storage was recorded only in *C. ternatea* and *I. tinctoria*, with maximum survival rate of 60 and 56.66 per cent respectively at 4h storage with a MC of 17.79 and 16.01 per cent respectively. But among the desiccation duration tried, encapsulated axillary buds of *M. pruriens* did not survive.

In order to study the effect of cryoprotectant, the axillary buds were treated with different cryoprotectant solutions but survival was recorded only in *M. pruriens* and *I. tinctoria*. Only a single treatment *viz.*, Cp10 (sucrose 0.7 M + DMSO 5 per cent) gave positive result with regard to survival in both the plant species. *M. pruriens* recorded a survival of 50.00 per cent and *I. tinctoria* only 33.33 per cent. No survival was recorded in any of the treatments with *C. ternatea.*

Cryoprotection is the phase of minutes to hours during which single protectant compounds or several component mixtures are applied. The most commonly used cryoprotectant is DMSO which can also act as an effective pre growth additive. DMSO can be used alone for organized cultures (Withers *et al.,* 1988), but in the study, DMSO when added alone did not promote survival. In *C. ternatea* and *I. tinctoria*, albino plants were produced and in *M. pruriens* buds turned brown. But when it was added along with sucrose, axillary buds survived in *M. pruriens* and *I. tinctoria*. This effect is confirmed by Withers and King (1980). Sucrose is a naturally occurring compound in plants. One of the attractions of using more natural compounds is that it reduces the risk of cryoprotectant toxicity, a problem that can be

encountered with high concentrations of DMSO and with old or impure stocks of the chemical (Withers, 1985). Hence, there has been a tendency to develop cryopreservation protocols avoiding these chemical cryoprotectants for explant survival, as they are toxic to plant cells, complicate the freezing procedure and in many cases do not significantly improve plant recovery (Arakawa *et al.*, 1990).

In addition to encapsulated meristems, somatic embryo also offers an aid for year-round multiplication and distant distribution of propagules in the form of artificial seeds. Somatic embryos of *C. ternatea* were subjected to encapsulation and artificial seeds thus formed when dehydrated at different length of time gave maximum survival of 43.33 per cent at 5 h desiccation and moisture content of 18.32 per cent.

Somatic embryos and synthetic seeds, if orthodox, could be used for germplasm conservation of clonal lines because manipulation of storage and regeneration would be easier when compared to the tedious excision, pretreatments and cryopreservation of shoot buds (Towill, 1991).

Plants derived from somatic embryos may show a lower frequency of variation than those produced by organogenesis *via* other tissue culture systems. To be suitable for storage, somatic embryos should exhibit desiccation tolerance and show longevity similar to those of sexually derived seeds. Viability after drying is often low but this may be due to the occurrence in the population of somatic embryos in different developmental stages, insufficient drying rates or lack of optimization in any of the several steps leading to a mature somatic embryo (Gray, 1987).

Successful cryopreservation involves subjecting plants to a series of successive stressing events; dehydration, freezing and thawing. Each aspect of the cryopreservation procedure may affect recovery of the tissues and predispose cells to genetic change. Harding and Benson (1995) opined that molecular analysis of plant DNA is ideal for genetic stability assessments. The DNA sequence is essentially the same in all cells in all tissues of the plant, and DNA can be extracted and stored for long periods of time by freezing. Moreover, this technique provides results of greater

resolution. PCR and RAPD technology are highly relevant to stability assessments of plants derived from *in vitro* conservation. This technique has been successfully used to study the genetic stability of cryopreserved materials of *Arachis* species (Gagliardi *et al.*, 2003), *Diascorea bulbifera* (Dixit *et al.*, 2003), *Vitis vinefera* (Zhai *et al.*, 2003). These authors showed that, through careful selection of primers and strict standardisation of protocols, RAPD technique is perfectly adapted to genetic stability assessment. The present study has also confirmed this observation. No variation was detected in the RAPD banding pattern of plants derived from cryopreserved explants compared to control in the three plant species *viz.*, *C. ternatea*, *M. pruriens* and *I. tinctoria.*

In this study, protocol has been standardized for short to medium term as well as long term conservation. For short to medium term conservation, slow growth technique was standardized by which, cultures could be maintained without subculture for 28 weeks as against the normal subculture interval of 4-6 weeks. Though control cultures could be maintained alive for 28 weeks, it did not regenerate after 28 weeks when transferred to shoot proliferation medium. Among various osmotica tried, mannitol was found to be the most suitable additive to induce slow growth. Nevertheless, all the additives brought about an inhibition in plant growth, except proline, in *I. tinctoria.* The higher the concentration of additives, lesser was the plant growth. So far, there are no reports on cryoprotectant (proline) and antioxidant (PVP) on inducing slow growth in *in vitro* cultures. But in this study, PVP was found to inhibit plant growth in cultures of all the three plant species under study. Proline gave similar effect in *C. ternatea* and *M. pruriens.*

For long term conservation, cryopreservation of various propagules was attempted in the three plant species. Seed germination behaviour at two different moisture levels and different lengths of time in LN was studied. Moisture content significantly influenced germination per cent while the effect of duration of storage in LN did not influence germination per cent. This confirmed the traditional hypothesis that moisture content is a critical factor in cryopreservation of seeds and that once the liquid nitrogen temperature is reached, there is practically no variation in germination

with respect to any length of storage in LN. Desiccation duration and moisture level for cryopreservation of zygotic embryo and cryoprotectants for rapid freezing technique for shoot meristem of *M. pruriens* were standardized. Desiccation duration and moisture levels were standardized for encapsulated meristems and somatic embryos of *C. ternatea* and encapsulated meristems of *I. tinctoria.* These techniques can be used effectively in germplasm conservation of these valuable medicinal plants.

Future Lines of Work

The standardization of *in vitro* plant regeneration protocols, with or without intervening callus phase can be exploited for generating new genetic variability in these medicinal plants by somatic hybridization through protoplast fusion and can be utilized for *Agrobacterium* mediated genetic transformation.

More effective slow growth protocols can be standardized by combining low temperature storage with different additives. Moreover, different light regimes can also be tried for inhibition of growth in cultures.

Various steps in cryopreservation *viz.*, pre culturing and use of cryoprotectants need be standardized to obtain a higher per cent of survival and regeneration. Cryoprotectants and their concentration, period of maintaining culture in pre culturing media etc. need be standardized. Vitrification technique of cryopreservation can also be resorted to for long term conservation.

Protocols are to be standardized for cryopreservation of other economically important agri-horticultural crops of our state as well as our country.

Summary

6. SUMMARY

The study, 'Rapid propagation and conservation of selected leguminous medicinal plants using *in vitro* techniques' was carried out at the Department of Plantation Crops and Spices and at the Department of Plant Biotechnology, College of Agriculture, Vellayani during the period from 2001-2004. The experiments on cryopreservation were done at the Division of Biotechnology, Tropical Botanical Garden and Research Institute, Palode. Investigations were carried out in two phases *viz.*, *in vitro* propagation and *in vitro* conservation on three important leguminous medicinal plants *viz.*, *Clitoria ternatea* L., *Mucuna pruriens* Baker non DC. and *Indigofera tinctoria* L. Propagation studies were carried out by enhanced release of axillary buds, somatic organogenesis and somatic embryogenesis. *In vitro* conservation was attempted using slow growth and cryopreservation techniques.

6.1 *IN VITRO* PROPAGATION

6.1.1 Enhanced Release of Axillary Buds

Among the cytokinin – auxin combinations tried, BA 0.5 mg l^{-1} + Kn 0.5 mg l^{-1} in MS medium recorded maximum shoot proliferation in *C. ternatea* and *M. pruriens* and BA 1.0 mg l^{-1} + IAA 0.1 mg l^{-1} in MS medium in *I. tinctoria* from nodal explants. Leaves and cotyledons, when used as explants did not give any shooting response. GA did not influence shoot proliferation in the three plant species studied.

Among the different culture media tried, full MS was found to be significantly superior to all other media with regard to shoot proliferation in all the three plant species.

The best carbon source in the culture medium was found to be sucrose at 30 g l^{-1} in all the three plant species, compared to glucose. Cobaltous chloride,

ethylene inhibitor did not evoke any significant influence on shoot proliferation in any plant species. With regard to number of shoots per culture among various levels of agar tried, maximum shoot proliferation was obtained with agar (5 g l^{-1}), which was on par with the control agar (7 g l^{-1}) in *C. ternatea* and *M. pruriens*. In *I. tinctoria* agar levels did not show much influence on shoot proliferation.

6.1.2 Somatic (Indirect) Organogenesis

In somatic (indirect) organogenesis, NAA 2.0 mg l^{-1} + BA 0.5 mg l^{-1}, NAA 1.0 mg l^{-1} + BA 0.5 mg l^{-1}, 2,4-D 2.0 mg l^{-1} + BA 0.5 mg l^{-1} in MS medium registered maximum callusing rate in stem, leaf and cotyledon explants respectively in *C. ternatea*. 2,4-D 2.0 mg l^{-1} + BA 0.1 mg l^{-1}, NAA 2.0 mg l^{-1} + BA 0.5 mg l^{-1} and 2,4-D 2.0 mg l^{-1} + BA 0.5 mg l^{-1} in MS medium recorded maximum callusing rate in stem, leaf and cotyledon explants respectively in *M. pruriens*. 2,4-D 2.0 mg l^{-1} + BA 0.5 mg l^{-1} and 2,4-D 2.0 mg l^{-1} + BA 0.1 mg l^{-1} in MS medium recorded highest callusing rate from stem and leaf explants respectively in *I. tinctoria*.

Shoot regeneration from callus was achieved in *C. ternatea* with BA 2.0 mg l^{-1} + Kn 1.0 mg l^{-1} + NAA 1.0 mg l^{-1} + adenine sulphate 20 mg l^{-1}, BA 2.0 mg l^{-1} + NAA 0.5 mg l^{-1} and BA 2.0 mg l^{-1} + Kn 1.0 mg l^{-1} + NAA 0.5 mg l^{-1} in MS medium from stem, leaf and cotyledon derived callus respectively. In *M. pruriens*, Kn 1.0 mg l^{-1} + NAA 0.5 mg l^{-1} in MS medium was identified as the best medium for shoot regeneration from stem derived callus and BA 2.0 mg l^{-1} + NAA 0.5 mg l^{-1} in MS medium for shoot regeneration from leaf and cotyledon derived callus. BA 2.0 mg l^{-1} + NAA 0.5 mg l^{-1} in MS medium was identified as the best media for shoot regeneration from stem derived callus in *I. tinctoria*.

6.1.3 Somatic Embryogenesis

For *in vitro* propagation of *C. ternatea via* somatic embryogenesis, first yellow, granular and friable callus was initiated from *in vitro* leaf explants in callus

induction medium containing 2,4-D 0.5 mg l^{-1} + BA 0.1 mgl^{-1}in MS medium. Callus after proliferation when subcultured on to MS medium supplemented with BA 1.0 mg l^{-1}, initiated globular, heart and torpedo shaped embryos. Embryos, when separated and cultured on GA supplemented medium or hormone free MS medium, torpedo shaped embryos developed into plantlets with root and shoot initials. Elongation of plantlets was achieved in hormone free MS medium. The fully developed plantlets, when transferred to pots and acclimatized, 40 per cent survival rate was obtained.

In *M. pruriens* and *I. tinctoria* somatic embryogenesis could not be initiated from various explants in spite of different treatments tried.

6.1.4 Rooting

When *in vitro* raised shoots were cultured in MS medium with NAA 1.0 mg l^{-1}, IBA 2.5 mg l^{-1} and IAA 1.5 mg l^{-1} good rooting *in vitro* was obtained in *C. ternatea, M. pruriens* as well as *I. tinctoria.*

On various pre treatments with IBA, for *ex vitro* rooting and subsequent survival, IBA 1000 mg l^{-1} for 20 s gave maximum survival in *C. ternatea* and *I. tinctoria*, while IBA 500 mg l^{-1} for 20 s in *M. pruriens.*

In all the three plant species, sand was identified to be the best potting medium for planting out and acclimatization.

6.2 *IN VITRO* CONSERVATION

6.2.1 Slow Growth

Half MS with IBA 0.5 mg l^{-1} was identified as the suitable basal culture medium for slow growth in all the three plant species studied. Cultures could be

maintained without subculture for more than 28 weeks. Those cultures which gave more than 50 per cent survival and regeneration were again subcultured on to fresh slow growth medium to reassess the efficacy of the medium to induce slow growth.

In *C. ternatea* cultures, PVP 8 per cent was identified as the best additive to induce slow growth up to first subculture (28 weeks) based on cent per cent survival and growth inhibition and further regeneration, when needed. On storage up to 40 weeks, PVP 8 per cent recorded only 83.33 per cent survival and 40 per cent regeneration. Among the different levels of additives which gave cent per cent survival and regeneration on storage up to 40 weeks, PVP 6 per cent recorded maximum plant growth inhibition.

In the case of *M. pruriens*, PVP 4 per cent was identified as the best medium in which cultures could be conserved up to 28 weeks without subculture. But this did not give any regeneration when the period was extended up to 40 weeks. Among those treatments which gave cent per cent survival and regeneration up to 28 weeks and on subculturing to fresh slow growth medium for conservation for further 12 weeks (total of 40 weeks), mannitol 1 per cent was selected as the best additive to induce slow growth.

In *I. tinctoria*, PVP 8 per cent recorded maximum plant growth inhibition with cent per cent survival and regeneration after 28 weeks of incubation. This gave a regeneration of 83.33 per cent when inoculated on to regeneration medium after 40 weeks of storage. Among those treatments which gave cent per cent survival and regeneration up to 28 weeks and on subculturing to fresh growth medium for conservation for further 12 weeks (total of 40 weeks), mannitol 0.5 per cent was selected as the best additive to obtain maximum growth inhibition.

PVP 6 per cent, mannitol 1.0 and 0.5 per cent were identified as the best among the various additives tried to induce slow growth in *C. ternatea*, *M. pruriens*

and *I. tinctoria* respectively, on conservation up to 40 weeks in terms of cent per cent survival, regeneration and plant growth inhibition.

6.2.2 Cryopreservation

6.2.2.1 Seed

On cryopreservation, germination of seeds is greatly influenced by the moisture content particularly when they are conserved by cryopreservation. At lower moisture level of 7.03, 5.24 and 5.35 per cent higher germination was obtained compared to higher moisture level of 9.37, 8.63 and 9.79 per cent in *C. ternatea*, *M. pruriens* and *I. tinctoria* seeds respectively, after freezing in LN. In the present study, varying periods of storage in LN did not influence the germination of seeds in all the three plant species studied.

6.2.2.2 Zygotic embryo

Due to large size of seeds in *M. pruriens*, zygotic embryos were tried for cryopreservation by simple desiccation for different periods. The highest survival (90.76 per cent) and plant regeneration (80.05 per cent) were recorded at 150 min after desiccation. After freezing in LN for 24 h, no survival was noted without desiccation; survival increased progressively up to 150 min desiccation.

Zygotic embryos of *M. pruriens* pretreated with cryoprotectant solutions did not survive after one hour of freezing in any of the treatments tried. The embryos became black in 2-3 days and did not germinate even after a month of incubation.

6.2.2.3 Shoot Meristem

In *C. ternatea* and *I. tinctoria*, 4 h desiccation was found to be most effective for cryopreservation of encapsulated axillary buds by encapsulation-dehydration

technique, while in *M. pruriens* none of the desiccated buds survived freezing in LN. On rapid freezing in LN with the addition of cryoprotectant, only meristems of *M. pruriens* survived in a cryoprotectant combination sucrose 0.7 M + DMSO 5 per cent. Meristems of none of the three plant species survived in other cryoprotectant treatments.

6.2.2.4 Somatic Embryo

Germinated somatic embryos on encapsulation-dehydration gave maximum survival of 43.33 per cent and plantlet regeneration of 55 per cent at 5 h desiccation and 18.32 per cent moisture content. Without desiccation, encapsulated embryos showed a survival and regeneration of 73.33 per cent and 67.85 per cent, respectively; but after freezing in LN, embryos did not survive at all.

6.2.3 Genetic Stability Assessment of Cryopreserved Materials Using RAPD Technique

To assess the genetic fidelity of plantlets regenerated from cryopreserved materials, the RAPD patterns were compared with those of non-cryopreserved plants. Six primers were selected for each plant species. No difference was observed in the banding pattern of cryopreserved and non-cryopreserved samples in all the three plant species under study, which indicated genetic stability of cryopreserved materials.

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* Original not seen

RAPID PROPAGATION AND CONSERVATION OF SELECTED LEGUMINOUS MEDICINAL PLANTS USING *IN VITRO* TECHNIQUES

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ABSTRACT

Investigations on "Rapid propagation and conservation of selected leguminous medicinal plants using *in vitro* techniques" were carried out at the Department of Plantation Crops and Spices and at the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2001-2003. The cryopreservation experiments were conducted at the Division of Biotechnology, Tropical Botanical Garden and Research Institute, Palode during 2003-2004. Leguminous medicinal plants *viz., Clitoria ternatea* L., *Mucuna pruriens* Baker non DC. and *Indigofera tinctoria* L. having immense medicinal value and of which superior accessions were available in the Department of Plantation Crops and Spices were selected for the study. Standardisation of i*n vitro* techniques for rapid propagation of these medicinal plants and their conservation *via* slow growth and cryopreservation were attempted in the present study.

Enhanced release of axillary buds from nodal explants, with maximum shoot proliferation of 6.33 and 5.17 was obtained in MS medium supplemented with BA 0.5 mg l^{-1} + Kn 0.5 mg l^{-1} in *C. ternatea* and *M. pruriens* respectively. Nodal explants of *I. tinctoria* recorded maximum shoot proliferation of 4.67 in MS medium with BA 1.0 mgl^{-1} + IAA 0.1 mg l^{-1}. Leaves and cotyledons, when used as explants did not give rise to any shoots.

In order to initiate somatic (indirect) organogenesis, maximum callus index (267 to 367) could be produced in MS medium with NAA 2.0 mg l^{-1} + BA 0.5 mg l^{-1}, NAA 1.0 mg l^{-1} + BA 0.5 m gl^{-1} and 2,4-D 2.0 mg l^{-1} + BA 0.5 mg l^{-} in stem, leaf and cotyledon explants respectively in *C. ternatea.* 2,4-D 2.0 m gl^{-1} + BA 0.1 mg l^{-1}, NAA 2.0 mg l^{-1} + BA 0.5 mg l^{-1} and 2,4-D 2.0 mg l^{-1} + BA 0.5 mg l^{-1} in MS medium recorded maximum callus index (199.99 to 283.00) in stem, leaf and cotyledon explants respectively in *M. pruriens.* 2,4-D 2.0 mg l^{-1} + BA 0.5 mg l^{-1} and 2,4-D 2.0 mg l^{-1}+ BA 0.1 mg l^{-1} in MS medium recorded highest callus index (155.34 to 250.00) from stem and leaf explants respectively in *I. tinctoria.*

In *C. ternatea*, BA 2.0 mg l^{-1} + Kn 1.0 mg l^{-1} + NAA 1.0 mg l^{-1} + adenine sulphate 20 mg l^{-1}, BA 2.0 mg l^{-1} + NAA 0.5 mg l^{-1} and BA 2.0 mg l^{-1} + Kn 1.0 mg l^{-1} + NAA 0.5 mg l^{-1} in MS medium were identified as the best media for shoot regeneration from stem, leaf and cotyledon derived callus respectively. In *M. pruriens*, Kn 1.0 mg l^{-1} + NAA 0.5 mg l^{-1} in MS medium was identified as the best medium for shoot regeneration from stem derived callus and BA 2.0 mg l^{-1} + NAA 0.5 mg l^{-1} in MS medium for shoot regeneration from leaf and cotyledon derived callus. BA 2.0 mg l^{-1} + NAA 0.5 mg l^{-1} in MS medium was identified as the best media for shoot regeneration from stem derived callus.

Somatic embryogenesis could be induced in *Clitoria ternatea* using yellow granular and friable leaf derived callus (from callus induction medium 2,4-D 0.5 mg l^{-1} + BA 0.1 mgl^{-1}in MS medium) on culturing callus on to MS medium supplemented with BA 1.0 mg l^{-1}, initiated globular, heart and torpedo shaped embryos. Embryos, when separated and cultured on GA supplemented medium or hormone free MS medium, torpedo shaped embryos developed into plantlets with root and shoot initials. Elongation of plantlets was achieved in hormone free MS medium. The fully developed plantlets could be acclimatized and transplanted to pots under ambient conditions with 40 per cent survival rate.

Rooting of *in vitro* raised shoots was achieved by culturing them on to NAA 1.0 mg l^{-1}, IBA 2.5 mg l^{-1} and IAA 1.5 mg l^{-1} in MS medium in *C. ternatea*, *M. pruriens* and *I. tinctoria*, respectively.

In *in vitro* conservation, half MS with IBA 0.5 mg l^{-1} was identified as the suitable basal culture media for slow growth in all the three plant species under study. Cultures could be maintained without subculture for 28 weeks. Those cultures which gave more than 50 per cent survival and regeneration were again subcultured on to fresh slow growth medium to reassess the efficacy of the medium to induce slow growth. PVP 6.0 per cent, mannitol 1.0 and 0.5 per cent were identified as the best among the various additives tried to induce slow growth in *C. ternatea*, *M. pruriens*

and *I. tinctoria* respectively, on storage up to 40 weeks in terms of cent per cent survival and regeneration and plant growth inhibition.

For cryopreservation, germination of seeds was found significantly influenced by its moisture content. Lower moisture level of 7.03, 5.24 and 5.35 per cent recorded a higher germination per cent of 90.44, 83.91 and 83.36 per cent respectively in *C. ternatea, M. pruriens* and *I. tinctoria*. Varying periods of storage in liquid nitrogen did not affect the germination of seeds in *C. ternatea, M. pruriens and I. tinctoria.*

Due to the large size of seeds in *M. pruriens*, zygotic embryos were found more suitable for cryopreservation. The highest survival (90.76 per cent) and plant regeneration (80.05 per cent) were recorded at a moisture level of 14.17 per cent, 150 min after desiccation.

In *C. ternatea* and *I. tinctoria,* 4 h desiccation was found to be most effective for cryopreservation of encapsulated axillary buds by encapsulation- dehydration method, while in *M. pruriens* none of the desiccated buds survived freezing in liquid nitrogen. On rapid freezing in liquid nitrogen with the addition of cryoprotectant, only meristems of *M. pruriens* survived. Cryoprotectant combination of sucrose 0.7 M + DMSO 5 per cent was found effective and none of the three species survived in other cryoprotectant treatments.

Germinated somatic embryos on encapsulation-dehydration gave maximum survival of 43.33 per cent at 5 h desiccation.

To assess the genetic fidelity of plantlets regenerated from cryopreserved materials, the RAPD patterns were compared with those of the non-cryopreserved plants. Six primers were selected for each plant species. No difference was observed in the banding pattern of cryopreserved and non-cryopreserved samples in all the three plant species under study, which confirmed the genetic stability of cryopreserved materials.