CRYOPRESERVATION OF CHETHIKODUVELI (*Plumbago rosea* L.) AND ASSESSMENT OF GENETIC FIDELITY OF REGENERATED PLANTLETS USING MOLECULAR MARKERS

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by

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2014

DECLARATION

I hereby declare that this thesis entitled **"Cryopreservation of Chethikoduveli (*Plumbago rosea* L.) and assessment of genetic fidelity of regenerated plantlets using molecular markers"** is a bonafide record of research work done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

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To Lux

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"When you want something, all the universe conspires in helping you to achieve it."

— Paulo Coelho, the Alchemist

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LIST OF ABBREVIATIONS

2, 4-D	2, 4-dichlorophenoxyacetic acid
2 iP	2-isopentenyladenine
A_{260}	absorbance at 260 nm wavelength
A_{280}	absorbance at 280 nm wavelength
BA	N^6-benzyl adenine
$CaCl_2$	calcium chloride
CD (0.05)	critical difference at 5 % level
cm	centimetre
CTAB	cetyltrimethylammonium bromide
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
dNTPS	deoxynucleotides
EDTA	ethylenediaminotetraacetic acid
et al.	and others
Fig.	figure
g	gram
g	standard acceleration due to gravity at the Earth's surface
GA	gibberellic acid
h	hour
HCl	hydrochloric acid
$HgCl_2$	mercuric chloride
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
Kn	kinetin (6-furfurylaminopurine)
LN	liquid nitrogen
mg	milligram
ml	milliliter
min	minute
mM	millimolar

MS	Murashige and Skoog (1962)
NAA	α-naphthaleneacetic acid
NaCl	sodium chloride
Na OH	sodium hydroxide
μM	micromolar
nm	nanometer
PCR	polymerase chain reaction
PLBs	protocorm like bodies
PVP	polyvinylpyrrollidone
RAPD	random amplified polymorphic DNA
rpm	revolution per minute
s	second
SA	sodium alginate
sp.	species
spp.	species (plural)
TE	Tris-EDTA buffer
Tris HCl	tris(hydroxymethyl)aminomethanehydrochloride
V	volt
viz.	namely
X	times

LIST OF SYMBOLS

ºC	degree celsius
%	per cent
±	plus or minus

Introduction

1. INTRODUCTION

Medicinal plants are an important source of traditional as well as modern medicines. Hence they are largely being used for preparing different types of organic compounds having therapeutic properties. The dawn of twentieth century has witnessed increasing realization about the importance of medicinal plants at the global level. Tropical countries, which are a treasure houses of these valuable plant species, are under constant pressure and progressively losing these resources due to several man-made as well as climatic factors. Rural communities in most of these tropical developing economies are greatly dependent on locally produced plant based remedies which are mainly harvested from wild sources. Approximately 80 per cent of the people in developing countries rely on traditional medicines for their primary health care (Vieira and Skorupa 1993). The herbal pharmaceutical industry is largely dependent on the regular supply of medicinal and aromatic plant materials. At present the demand is met mostly from the wild. Consequently, medicinal plant species are disappearing at an alarming rate, from their natural habitat. Rapid agricultural and urban development, deforestation, indiscriminate collection *etc.* all have added to the rapid depletion of this valuable resource. The loss is insurmountable and necessitates action for conservation and sustainable use of medicinal plants.

Conservation of plant genetic resources can be carried out either *in situ* (in the natural habitats of species' population) or *ex situ* (outside the natural habitat). Conventional field conservation of the plant needs high cost of land and labour; moreover there are risks of deterioration from natural disaster and plant pests during conservation. *Ex situ* plant genetic resources conservation can be carried out in an efficient and economical way by seed conservation in seed banks but this is rather difficult for vegetatively propagated species.

Biotechnology has ushered a new era in the domain of conservation of vegetatively propagated 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; Sagare *et al*., 2000; Lakshmi and Mythili, 2003). The rapid strides made in the past few years with regard to development of *in vitro* techniques for conservation have enhanced the value of gene banks and clonal repositories. Cryopreservation, the biotechnological endeavour, holds great potential for long-term conservation of germplasm.

Plumbago rosea L. / Syn. *Plumbago indica* L. (Fa. Plumbaginaceae) containing the alkaloid, plumbagin, is an important ingredient in recipes of many traditional medicines (Jain, 1991). *P. rosea* seldom sets seeds and is conventionally propagated by stem cuttings. The growth of plant is very slow. Though cultivated on a small scale, due to increased demand, the roots of *P. rosea* are heavily collected from the wild. The plant faces near extinction in the wild (Komaraiah *et al.*, 2004). Chetia and Handique (2000) also have reported that the plant has become rare in many parts of India. Conventional *in situ* or *ex situ* conservation of the plant is expensive in terms of land and labour. Moreover, there are risks of deterioration from natural disaster and plant pests and diseases. *In vitro* conservation has been recognised as an alternative method for plant conservation that eliminates the obstacles of field conservation. Charoensub and Phansiri (2004) has standardised the *in vitro* slow growth culture in *P. rosea*. *In vitro* culture has been reported to induce genetic changes (somaclonal variation) in some cases (Scowcroft, 1984). To avoid the genetic alterations that may occur in long tissue culture storage, the storage of germplasm at very low temperatures known as cryopreservation is attempted (Martin *et al.*, 1998).

Cryopreservation (in liquid nitrogen (LN) at -196^{o} C) is, so far, the only viable procedure for long-term germplasm conservation of vegetatively propagated species (Engelmann, 2004). An efficient *in vitro* regeneration system

is a pre-requisite for any cryoconservation study. The encapsulation-dehydration procedure is based on a successive osmotic and evaporation dehydration of plant cells (Swan *et al*., 1999). The conserved propagules could be effectively utilized for subsequent mass regeneration to plantlets as and when required. A major concern with regard to this technique is the genetic stability of conserved material and hence genetic evaluation of the recovered plants is to be strictly followed (Ashmore, 1997). PCR based molecular markers have been used in the genetic fidelity assessment of cryostorage derived plantlets (Zarghami *et al*., 2008; Nair and Reghunath, 2009).

Hence, it becomes relevant to evolve protocols for *in vitro* conservation and regeneration of *P. rosea*, a very valuable and commercially exploited medicinal plant. The present study 'Cryopreservation of Chethikoduveli (*Plumbago rosea* L.) and assessment of genetic fidelity of regenerated plantlets using molecular markers' was undertaken with the following objectives.

1. Optimisation of protocol for *in vitro* regeneration of *Plumbago rosea* var. Agni
2. Standardization of cryoconservation protocol by encapsulation-dehydration technique for long-term conservation
3. Genetic fidelity assessment of plantlets recovered and regenerated after cryostorage using molecular marker, random amplification of polymorphic DNA (RAPD)

Review of Literature

2. REVIEW OF LITERATURE

The World Health Organization has estimated that more than 80 per cent of the world's population in developing countries depends primarily on herbal medicine for basic healthcare needs (Vines, 2004). Approximately two third of the 50,000 different medicinal plant species in use are being collected from the wild. Unscrupulous wild harvesting threatened the existence of many species (Edwards, 2004). Concern about diminishing populations, loss of genetic diversity and local extinctions has made the adoption of conservation strategies inevitable.

Plumbago rosea L. is a valuable medicinal plant belonging to the Plumbaginaceae family. The medicinal properties can be attributed to the presence of an alkaloid present in the root and root bark, plumbagin (2-methyl-5-hydroxy-1, 4-naphthoquinone) that possess anti HIV (Krishnaswamy and Purushothaman, 1980), antimalarial, antimicrobial (Didry *et al.*, 1994) anticancer (Devi *et al.,* 1998), antioxidant (Tilak *et al.,* 2004) anti allergic (Dai *et al.,* 2004) and antiovulatory (Sheeja *et al.,* 2009) properties. *P. rosea* is the best and richest source of plumbagin (Mallavadhani *et al.,* 2002; Kapadia *et al.,* 2005). It is an important ingredient in many recipes of traditional medicine (Jain, 1991). In addition to plumbagin, root also provides plumbaginol, sitosterol and stigmasterol (Dinda *et al.,* 1994) for pharmaceutical uses. Though cultivated on a small scale, due to increased demand, the roots of *P. rosea* are heavily collected from the wild. The plant faces near extinction in the wild (Komaraiah, 2004). Chetia and Handique (2000) also have reported that the plant has become rare in many parts of India. Conventional *in situ* or *ex situ* conservation of the plant is expensive in terms of land and labour. Moreover, there are risks of deterioration from natural disaster and plant pests and diseases. *In vitro* conservation has been recognised as an alternative method for plant conservation that eliminates the obstacles of field conservation.

The present study 'Cryopreservation of Chethikoduveli (*Plumbago rosea* L.) and assessment of genetic fidelity of regenerated plantlets using molecular markers', involved the standardization of *in vitro* conservation protocol for encapsulation-dehydration technique of cryopreservation for long term conservation of *Plumbago rosea.*

In this chapter, literature on *in vitro* 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* REGENERATION

Plant tissue culture is based on the property of totipotency of the plant cell (Butenko, 1999; Griga, 2001). This process of *de novo* reconstruction of an organism from a cell in differentiated stage is highly linked to the process of dedifferentiation when the cell is returning back to its early embryogenic/meristematic stage. In this stage cells undergo division and may form undifferentiated callus tissue or may redifferentiate to form new tissue, organs and an entire organism. Morphogenesis *in vitro* is realized *via* two major pathways: (i) organogenesis when a group of cells is involved for *de novo* formation of organs and (ii) somatic embryogenesis when the new organism is initiated from a single cell (Kalimuthu and Prabakaran, 2013).

In vitro culture may be directed to cater to different applications. The most prominent application being clonal propagation, producing a large number of identical plants. Complex and integrated approaches for cultivation of plant systems may be the basis for future development of new, effective, safe and high quality products. These scientific achievements might be used for the establishment of *ex situ* and *in vitro* collections, multiplication of desired species and to obtain raw material for the pharmaceutical and cosmetic industries (Julsing *et al.,* 2006).

In vitro culture techniques offer a viable tool for the mass multiplication of genetically identical plant material, and the germplasm conservation of rare endangered plants (Arora and Bhojwani 1989; Sudha and Seeni 1994; Baskaran and Jayabalan 2005). Murch *et al.* (2004) consider *in vitro* propagation of medicinal plants a successful strategy that addresses the problems associated with supply and variability in product quality. *In vitro* propagation has been achieved in several medicinal plants using tissue culture techniques (Rout *et al*., 2000; Nalawade *et al*., 2003).

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

Enhanced release of axillary buds involves the stimulation of axillary buds present in the axil of the leaf to develop into a shoot exploiting the normal ontogenetic route of branch development from axillary meristems. The merit of using axillary bud proliferation as a means of regeneration is that incipient shoots are already differentiated *in vivo* and hence, allows recovery of genetically stable and true to type progeny, which is of utmost importance with respect to propagation and conservation of plant species *in vitro* (Hu and Wang, 1983; George and Sherrington, 1984; Chawla, 2009).

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).

Micropropagation of medicinal plants has been achieved through rapid proliferation of shoot-tips and axillary buds in culture. According to Rout *et al.* (2000) numerous factors are reported to influence the success of *in vitro* propagation of different medicinal plants *viz*., age of donor plant, explant type, culture medium, growth regulators and culture conditions. The factors that influence micropropagation of medicinal and aromatic plants have been reviewed by Murashige (1977), Hussey (1980, 1983), Hu and Wang (1983), Bhagyalakshmi and Singh (1988), and Short and Roberts (1991).

2.1.1.1 Age of Donor Plant and Explant Type

The right choice of the explant is important for the success of any *in vitro* propagation system. 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 *viz*., horizontal or vertical, also influences axillary shoot proliferation. Length of explant is a critical factor for *in vitro* micro propagation (Cheong and Pooler, 2003).

Maintaining the donor plants in clean and controlled environmental conditions delivers healthy and sterile explants (Sagare *et al.,* 2001). The physiological age of the explants, and the explant type and size also influence the formation of organs *in vitro* (Rout *et al.,* 2000).

The nature and condition of explants has been shown to have a significant influence on the multiplication rate of *Clerodendrum colebrookianum* by Mao *et al.* (1995). Actively growing materials were more responsive to shoot induction than dormant buds.

Young shoots of *Plumbago zeylanica* L. harvested from two year old, field grown plants were used for the regeneration of shoots *in vitro* by Sahoo and Debata

(1998). The explants used were single node segments (0.8-1.2cm) containing a dormant axillary bud.

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

A rapid and efficient protocol for the large-scale propagation of a potential medicinal plant, *Mucuna pruriens*, through *in vitro* culture of nodal segment explants obtained from 15-day-old aseptic seedlings was described by Faisal *et al.* (2006).

Joshi and Dhawan (2007) observed the formation of shoot clusters in nodal explants derived from four week old seedlings of *Swertia chirayita.*

Karadi *et al.* (2007) reported establishment of *in vitro* callus cultures from leaves and shoot tips of *P. indica* on MS and B5 medium containing varying amounts of auxins and cytokinins.

Induction of direct and indirect organogenesis from leaf and nodal segments of two month old seedlings of *Plumbago indica* L. has been standardized by Bhadra *et al.,* (2009). The nodal segments produced either multiple shoot buds (MSBs) or callus of different nature depending on the combinations of plant growth regulators.

Leaf explants of *Plumbago rosea* L. were used for multiple shoot regeneration by Gopalakrishnan *et al.* (2009). High frequency shoot bud regeneration was seen in tuberous root explants obtained from one year old *Plumbago rosea* L. (Jose and Satheeshkumar, 2010).

2.1.1.2 Explant Surface Sterilization

Explants collected from field grown plants are usually colonised by various microorganisms. Besides surface contaminants, there may be some internal infectants *viz.,* endophytic bacteria or fungi that can be expressed even after years in culture, for which eradication has not been achieved so far (Rout *et al.,* 2000). However surface sterilization is achieved with common sterilizing agents like sodium or calcium hypochlorite (5–10 %), ethyl alcohol (50–95 %), and mercuric chloride (0.01–0.1 %) by washing in the appropriate solution for 10–25 min followed by several rinses in sterile water.

The nodal segments of *Plumbago zeylanica* were given a treatment of 5 per cent (w/v) solution of fungicide (Carbendazim 50 % W.P.) for 30 min on a shaker and further surface-decontaminated by immersing in 80 per cent ethanol containing streptomycin (2 mg ml^{-1}) for 30 s followed by 0.1 per cent $HgCl_2$ for 4 min (Selvakumar *et al.,* 2001).

Arya *et al.* (2003) gave the nodal and apical shoot segments of *Leptadenia reticulate*, a surface sterilization treatment of 0.1 per cent (w/v) mercuric chloride ($HgCl_2$) for 4–5 min and finally dipped in 90 per cent ethyl alcohol for 30–40 s.

Surface sterilization of nodal explants of *Clitoria ternatea* was conducted using a 0.1 per cent (w/v) aqueous mercuric chloride solution for 15 min (Rout, 2005).

Tender leaves and nodal explants of *Justicia gendarussa* were washed thrice with sterile distilled water followed by treatment with 0.03 per cent Carbendazim for 20 min and again washed thrice in sterile distilled water. Explants were further

surface sterilized with 0.1 per cent mercuric chloride for 4 min (Agastian *et al.,* 2006).

Nodal stem segments (4–5 cm) of *Swertia chirata,* were washed thoroughly in tap water and soaked in 5 per cent (v/v) commercial bleach for 30 min followed by surface sterilization with 0.1 per cent $HgCl_2$ (w/v) for 25–30 min (Chaudhuri *et al.,* 2007).

Nodal explants of *Justicia gendarussa* were sterilized by immersion in 50 per cent (v/v) ethanol solution for 30 s, followed by 8 min immersion with agitation in 0.1 per cent $HgCl_2$ (w/v) (Thomas and Yoichiro, 2010).

Surface sterilization of nodal explants of *Sapindus trifoliatus* was carried out with 0.05 per cent (w/v) mercuric chloride for 3–5 min after a brief rinse in 70 per cent (v/v) ethanol under aseptic condition (Asthana *et al.,* 2011).

2.1.1.3 Culture Media

Different media formulations *viz.,* White's medium (White, 1943), MS medium (Murashige and Skoog, 1962), B5 medium (Gamborg *et al*., 1968), SH medium (Schenk and Hildebrant, 1972), Woody Plant Medium (WPM) (Lloyd and Mc Cown, 1980) *etc* were developed for the *in vitro* culture of various plant species. MS medium characterised by high salt concentration is the most commonly used, often with minor alterations. 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).

In vitro plantlet regeneration from nodal explants in MS medium was reported in medicinal plant species *viz*., *Baliospermum montanum* (Johnson and Manickam, 2003), *Wedelia chinensis* (Martin *et al.,* 2003) *Baliospermum axillare* (Singh and Sudarshana, 2003), *Curcuma longa* L. (Islam *et al*., 2004), *Cardiospermum halicacabum* (Thomas and Maseena, 2006), *Swertia chirayita* (Joshi and Dhawan, 2007), *Vernonia amygdalina* (Khalafalla *et al*., 2007), *Balanites aegyptiaca,* (Siddique and Anis, 2009), *Justicia gendarussa,* (Thomas ad Yoichiro, 2010), *Psoralea corylifolia* L. (Baskaran and Jayabalan, 2010), *Sapindus trifoliatus* L. (Asthana *et al.,* 2011), and *Ceropegia* species (Kalimuthu and Prabakaran, 2013).

Kumar *et al.* (1993) utilized B5 basal media for multiple shoot regeneration in *Clitoria ternatea.* Axillary bud release in *Adhatoda beddomi* was induced on SH medium by Sudha and Seeni (1994). Micropropagation of *Wedelia calendulacea* was carried out using nodal explants on MS medium containing B5 vitamins (Emmanuel *et al.*, 2000). Catapan *et al.* (2001) reported that MS medium was the best for shoot initiation followed by B5, SH, and WPM in *Phyllanthus stipulates.* Mechanda *et al*. (2003) obtained plantlet regeneration on WPM medium in *Echinacea purpurea* plants.

MS medium has been found the most suitable medium for shoot proliferation in *Plumbago rosea* L. (Jose *et al.,* 2007). High frequency hairy root induction from stem and leaf explants were obtained in *Plumbago indica* L. and axenic root cultures were established under darkness in hormone free liquid Murashige and Skoog medium containing 3 per cent sucrose (Gangopadhyay *et al.,* 2008).

Leaf and nodal segments of *Plumbago indica* L. were cultured on MS medium to yield either multiple shoot buds (MSBs) or callus of different nature depending on the combinations of plant growth regulators (PGRs) (Bhadra *et al*., 2009).

2.1.1.4 Plant Growth Regulators

The success of any plant tissue culture system is determined largely by different kinds and concentrations of plant growth regulators included in the culture medium. Root and shoot initiation, and the process of differentiation from unorganised callus tissue, are closely regulated by the relative concentrations of auxins and cytokinin in the medium (Skoog and Miller, 1957; Ammirato, 1983; Bajaj *et al.,* 1988; Rout and Das, 1997a). Auxin:cytokinin ratio of approximately 10 yields rapid growth of undifferentiated callus, a ratio of approximately 100 favours root development and a ratio of approximately four favours shoot morphogenesis (Murashige, 1979). The gibberellins in medium enhance shoot elongation but not *in vitro* organogenesis (Gaba, 2005).

Interaction and balance between the growth regulators supplied to the medium and the growth substance produced endogenously by the cultured cell regulates the growth and morphogenesis of the *in vitro* grown plants. Selection and addition of growth regulators at optimum concentration are critical for successful plant tissue culture.

In vitro proliferation and development of shoots from axillary buds is stimulated by cytokinins. BA has been the most favourable cytokinin for multiplication of axillary buds followed by kinetin (Chawla, 2009).

Benjamin *et al.* (1987) has shown that BA (1-5 mg l^{-1}) stimulates the development of the axillary meristems and shoot tips of *Atropa belladonna* when cultured in liquid MS medium. Lal and Ahuja (1996) observed a rapid proliferation rate in *Picrorhiza kurroa* using kinetin at 1.0–5.0 mg l^{-1}.

Tiwari *et al.* (2001) investigated the effect of various cytokinins (BA, TDZ, Kinetin and 2ip) on multiple shoot induction in *Bacopa monnieri* using different explants *viz.,* with node, internode and leaf in MS medium. Among the four cytokinins tested, thidiazuron (1.5 mg l^{-1}) and 6-benzyladenine (2 mg l^{-1}) proved superior and optimum adventitious shoot buds induction was obtained with 1.5 mg l^{-1} thidiazuron.

High frequency shoot formation was induced in nodal segments of *Salvadora persica* in MS medium supplemented with 4 mg l^{-1} BA. But higher concentration of BA resulted in decline in the number of shoot buds produced per explant (Batra *et al.,* 2001).

Rai (2002) reported shoot proliferation in hypocotyl segments of 20–25 days old seedlings of *Nothapodytes foetida* in MS medium supplemented with 0.5 mg l^{-1} thidiazuron (TDZ).

The highest number of regenerated shoots was obtained on MS medium supplemented with 1.5 mg l^{-1} benzyladenine, in *Orthosiphon stamineus.* (Leng and Keng, 2004).

A high frequency direct shoot proliferation was observed in nodal explants cultured on MS medium supplemented with 2.25 mg l^{-1} BAP, by Purkayastha *et al.* (2008) in *Andrographis paniculata.*

Balaraju *et al.* (2008) found that the regeneration frequency of *Vitex agnus-castus* was highest for both apical meristem and nodal explants when explants were cultured on MS medium supplemented with BAP (2.0 mg l^{-1}) and Kin (0.1 mg l^{-1}).

Siddique and Anis (2009) found out that MS medium supplemented with BA (2.8 mg l^{-1}) was the most effective in inducing bud break and growth and also in initiating multiple shoot proliferation, in *Balanites aegyptiaca.*

A rapid shoot proliferation was observed on the nodal explants of *P. zeylanica* in MS medium supplemented with 1.0 mg l^{-1} BA and 1.0 mg l^{-1} GA_3 (Chinnamadasamy *et al.,* 2010).

Thomas and Yoichiro (2010) observed that the nodal cuttings of *Justicia gendarussa* gave maximum shoot proliferation in MS medium supplemented with 4 mg l^{-1} BA and 1.0 mg l^{-1} NAA.

MS medium with BA at 3 mg l^{-1} induced multiple shoot proliferation from nodal explants of *Plumbago indica* L. (Chetia and Handique, 2000), but higher concentrations (3 mgl^{-1} BA and 25 mg l^{-1} AS) gave development of adventitious buds in high frequency, with reduced bud differentiation. The buds differentiated into shoots on transfer to media containing low BA concentrations (0.1-1.5 mg l^{-1}).

Auxin inhibits axillary shoot proliferation but in combination with cytokinin, it promotes shoot proliferation (Rout *et al.,* 1999). Synergistic effect of cytokinins and auxins in shoot proliferation is well documented in medicinal plants.

Wakhlu and Barna (1989) has indicated that the production of multiple shoots is higher in *Plantago ovata* on MS medium having 4.0 mgl^{-1} kinetin along with 0.01 mgl^{-1} NAA.

An optimum number of shoots per nodal explant was obtained in *Adhatoda beddomi* in MS medium using 3.0 mg l^{-1} BAP, 0.5 mg l^{-1} 2-ip and 1.0 mg l^{-1} IAA. Shoot multiplication was a function of cytokinin activity but sustained growth of the

shoots was dependent on the synergistic effect with the auxin, IAA (Sudha and Seeni, 1994).

According to Faria and Illg (1995), the addition of 2.25 mg l^{-1} BA along with 1 mg l^{-1} IAA or 1 mg l^{-1} NAA induced a higher rate of shoot proliferation of *Zingiber spectabile* when rhizome axillary buds were cultured in MS medium. BA was proved superior to 2ip and TDZ for multiple shoot induction.

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*.

2.1.1.5 Culture Conditions

Light, temperature, and relative humidity are important parameters in culture incubation. Photosynthetic activity is not very important during initial phases of culture *in vitro*. Light is essential for morphogenetic processes like shoot and root initiation and somatic embryogenesis (Rout *et al*., 2000).

Both quality and intensity of light as well as the photoperiod are very critical to the success of certain culture experiments. Exposure to light for 12–16 h per day under 35–112 μmol m^{-2} s^{-1} provided by cool, white fluorescent lamps is usually recommended. Blue light promotes shoot formation while, red light induces rooting in many species (Murashige, 1977).

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). The pH of the medium also affects nutrients uptake and shoot proliferation (Parliman *et al.,* 1982). The optimum pH for shoot proliferation and

elongation was found to be 5.8 in *Mucuna pruriens*, and multiplication was severely inhibited in more acidic medium (Faisal *et al*., 2006). An optimum pH range of 5.80 ± 0.02 was reported for the *in vitro* regeneration of *Sapindus trifoliatus* by Asthana *et al.* (2011).

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 ± 2^{o}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.

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

Explants of *Justicia gendarussa* were grown in culture environment of either 25 ± 2^{o} C or 20^{o} C in continuous darkness or 16 h light/8 h dark cycle with a light intensity of 27 µmol $m^{-2}s^{-1}$ or a continuous low light regime of 0.5 µmol $m^{-2}s^{-1}$ (Agastian *et al.,* 2006).

The cultures of *Cassia angustifolia* were maintained at 24 ± 2^{o}C under 16 h photoperiod with a photosynthetic photon flux density (PPFD) of 50 µmol $m^{-2}s^{-1}$ provided by cool white fluorescent lamps and with 60–65 per cent relative humidity (Siddique and Anis, 2007).

Sapindus trifoliatus cultures which were incubated at 25 ± 2° C with 16/8 (light/dark) photoperiod at a photon flux of 50-70 µmol $m^{-2}s^{-1}$ from cool and white fluorescent tubes gave optimum regeneration (Asthana *et al.* 2011).

2.1.2 *In vitro* Rooting

Adventitious root formation is a key step in micropropagation. The plantlets produced *in vitro* should have a strong and functional root system. *In vitro* induction of roots from growing shoots is achieved in standard medium containing auxin and in medium in the absence of auxin depending on plant genotype (Rout *et al.,* 1989). Roots are mostly induced in presence of a suitable auxin (Razdan, 2003). IAA, IBA and NAA were found to initiate rooting in plants.

IAA is known to control the activity of peroxidases involved in lignification (Barcelo and Munoz, 1992), a process inevitably involved in root formation, through the diffentiation of xylem cells.

Maximum rooting of the excised shoots on MS medium containing 0.5 mg l^{-1} NAA preceded by callus formation was observed in *Rauwolfia micrantha* (Sudha and Seeni, 1996). The promotory effect of reducing salt concentrations of MS medium on *in vitro* rooting of shoots has been described by Upreti and Dhar (1996).

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). Saha *et al.* (2003) found that half strength MS with 1.5 mg l^{-1} IBA was best for inducing maximum number of roots in *Hemidesmus indicus*. NAA was found inferior and favoured callus formation at the base.

According to Martin *et al.* (2003), half strength hormone free MS medium was superior to full strength for root induction in *Wedelia chinensis*. MS medium augmented with 0.2 mg l^{-1} IBA induced rooting in *in vitro* regenerated shoots of *Mucuna pruriens* (Faisal *et al*., 2006). Full strength MS solid medium with 0.75 mg l^{-1} indole-3-butyric acid (IBA) exhibited the best *in vitro* rooting in *Vitex negundo* L. (Vadawale *et al.,* 2006).

Different concentrations and combinations of auxins were tested for the induction of roots from the regenerated shoots in *Swertia chirata* (Chaudhuri *et al.,* 2007). Inclusion of IAA and IBA was found to stimulate the production of adventitious roots from shoots of *Swertia chirata*. However, IBA was found to be significantly more effective when used alone. The highest rate of rhizogenesis was observed on medium supplemented with a combination of IAA (5.71 μM) and IBA (4.9 μM).

IBA was found most effective in inducing roots in *Andrographis paniculata* (Purkayastha *et al*., 2008). The maximum frequency of root formation was achieved within a week when shoots were cultured on MS medium containing 0.5 mg l^{-1} IBA. It is observed that the roots were induced directly from the shoot base without an intervening callus phase on medium supplemented with IBA. In contrast, rooting of shoots occurred through an intervening callus phase on IAA and NAA supplemented medium.

IBA (0.2–1.4 mg l^{-1}) was found to be better than NAA (0.18–1.3 mg l^{-1}) in terms of both the percentage of cultures that responded and the average number of roots per explant in *Sarcostemma brevistigma* (Thomas and Shankar, 2009).

2.1.3 Hardening and Planting Out

Micropropagation system is successful only when plants after transfer from culture to ambient conditions *ex vitro* gives a high survival rate. Transfer involve change from a substrate rich in organic compounds to substrate providing mostly inorganic nutrients. Plant *in vitro* are not photosynthetically active. Hardening and acclimatisation is an important and critical process of micropropagation that enable the plant to withstand the transplanting shock and ensure survival and to grow vigorously *ex vitro.*

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).

Satheeshkumar and Bhavanandan (1988) reported that when micropropagated plants of *Plumbago rosea* were transferred to pots containing a 1:1 soil and sand mixture under greenhouse conditions, about 60 per cent of the plants survived. A high survival (96 %) was recorded when plantlets of *Pinellia ternata* were transplanted into a 1:2:1 mixture of vermiculite:loam soil:peat moss (Tsay *et al*., 1989).

Approximately 60 per cent of the rooted plants of *Centella asiatica* survived in pots containing a 1:1:1 mixture of soil, sand, and well-rotted cow dung (Patra *et al.,* 1998). *Zingiber officinale* plants were supplied with MS inorganic salts twice a week before transfer to the greenhouse (Palai *et al*., 1997).

Saxena *et al*. (1997) reported that 95 per cent of *in vitro* rooted plantlets of *Psoralea corylifolia* were successfully transferred to a 1:1 mixture of soil and sand. Rout *et al*. (1999) reported that about 95 per cent of micropropagated plantlets of *Plumbago zeylanica* were established in the greenhouse within 2–3 weeks of transfer

under 85 per cent relative humidity when planted in earthen pots containing a sterile mixture of sand, soil and cow dung manure in the ratio of 1:1:1 (w/v).

Plantlets of *Andrographis paniculata* with well developed roots were successfully planted out and maintained in the greenhouse in plastic pots containing soil, vermiculite, and vermicompost (1:1:1). Plants were covered with transparent polyethylene bags to maintain adequate moisture for a week and transferred to the greenhouse (Purkayastha *et al.,* 2008).

2.2 *IN VITRO* CONSERVATION

In vitro technology has a specialized and potentially important role to play in the conservation of biological diversity. The methods employed for *in vitro* conservation vary according to the storage duration required (Engelmann, 1991). For short and medium-term conservation, the aim is to reduce growth, thus increasing intervals between subcultures. For long-term conservation, cryopreservation is the only method currently available. Indeed, all metabolic processes as well as cell divisions are stopped at this temperature. Plant material can thus be conserved without modification or alteration for extended periods, under a reduced volume and with reduced maintenance.

In vitro culture is envisaged as a means for germplasm conservation to ensure survival of endangered plant species and rapid mass propagation for large scale domestication (Al-Ababneh *et al.,* 2003; Moges *et al.,* 2003). Storage of shoot tips or meristem derived explants under slow rate of growth has a significant application in germplasm conservation and germplasm exchange programme (Wilkins and Dodds, 1983). Germplasm conservation calls in for germplasm exchange programmes where seeds are not a preferred entity due to risk of seed borne pests and pathogens. To overcome the quarantine problem, *in vitro* grown cultures are preferred for

germplasm exchange. Meristem derived explants such as shoot tips and buds were mostly suggested for their genetic and generative stability (Bajaj, 1986).

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*.

In vitro conservation of plant genetic resources is becoming a complementary approach to the conventional conservation methods and 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. *In vitro* conservation save plant material for short, medium or long-term in small space and under controlled conditions. It's cost-effective and stored material is suitable for international exchange (Shibli *et al.,* 2006).

2.2.1 Cryopreservation

Cryopreservation, *i.e*., the storage of biological material at ultralow temperature, usually that of liquid nitrogen (-196^{o}C), is the only technique currently available to ensure the safe and cost-efficient long-term conservation of different types of germplasm. This technique involves suspension of metabolic activities and cellular divisions of a specimen by subjecting to ultra-low temperature (-150 to -196^{o} C). Conventionally liquid nitrogen (LN) is used as the coolant to achieve the temperature of -196^{o}C in liquid phase and -150^{o}C in vapour phase. The plant material can thus be stored without alteration or modification for a theoretically unlimited period of time (Engelmann, 2004).

The successful cryopreservation protocol was first reported in silver birch twigs by Sakai (1960). Quatrano (1968) could freeze *in vitro* cultured flax cells successfully. The first protocol developed in the 1980s included pre-treatment with cryoprotectants followed by controlled rate of cooling. 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). The cryopreservation protocols have been standardized for several plants including medicinal plants (Bajaj, 1995).

Seeds, as the natural regenerating organs of plants, are the preferred choice of material as used in a range of *Piper spp.* (Chaudhury and Chandel 1994; Decruse and Seeni 2003) and *Rauvolfia micrantha* (Decruse *et al.,* 1999). Those plants which are clonally propagated or producing recalcitrant large seeds or otherwise do not set seeds require the intervention of *in vitro* methods for their successful cryopreservation. Excised zygotic embryo as utilized in other recalcitrant seed crops (Engelman, 2000) has proven useful for the cryopreservation of *Myristica malabarica, Celastrus paniculatus*, and *Nothapodytes nimmoniana* (Radha *et al.,* 2006, 2010b, 2010d).

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. Furthermore, 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).

Cryogenic procedures such as simple freezing, vitrification, encapsulation–dehydration and encapsulation-vitrification have been developed for conserving a number of plant species (Tsai *et al.,* 2009; Hua and Hong 2010). Cryopreservation is the most promising choice for conservation of germplasm owing to safety, repeatability and long-term storage possibility (Hazubska *et al.,* 2010).

2.2.1.1. Cryopreservation by Encapsulation-Dehydration

The encapsulation-dehydration procedure is based on the technology developed for the production of artificial seeds. Explants are encapsulated in alginate beads, pre-grown in liquid medium enriched with sucrose for several days, partially desiccated down to a water content around 20 per cent (fresh weight basis), then frozen rapidly. Survival rates are high and growth recovery of cryopreserved samples is generally rapid and direct, without callus formation. This technique has been developed for apices of various species from temperate origin such as apple, pear, grape, eucalyptus, and of tropical origin such as sugarcane and cassava (Dereuddre, 1992; Engelmann, 1997).

A modified protocol has been proposed by Sakai *et al.* (2000) in which encapsulation and pre-growth in medium with sucrose and glycerol are performed simultaneously. This technique has been applied to apices of numerous species of temperate and tropical origin as well as to cell suspensions

and somatic embryos of several species (Engelmann, 1997b; Engelmann and Takagi, 2000).

The encapsulation-dehydration technique is easy to handle and appears to be a practical method for cryopreservation of meristems and somatic embryos. (Dereuddre *et al.,* 1990; Fabre and Dereuddre, 1990). In the encapsulation-dehydration technique, gradual extraction of water from encapsulated meristems in a bead of alginate is performed during the pre-culture in sucrose-enriched medium (usually around 0.8 *M* sucrose). The sucrose molarity in the beads is further increased by the additional air-desiccation, and reached/exceeded the saturation point of the sucrose solution resulting in a glass transition during cooling to –196°C (Dereuddre *et al.,* 1990). The induction of desiccation tolerance, through the subsequent increase of sucrose concentration during pre-culture of encapsulated shoot apices, is the key for successful cryopreservation. For recovery, samples are usually placed directly under standard culture conditions.

Matsumoto *et al.* (1995) reported that encapsulated lily meristems pre-cultured with a mixture of 0.8 *M* sucrose and 1.0 *M* glycerol produced considerably high post-thaw shoot recovery of 90 per cent while pre-culture only with 0.8 *M* sucrose gave 57 per cent. However, a mixture of 0.8 *M* sucrose in combination with ethylene glycol and DMSO produced toxic effects during the dehydration process. It is considered that glycerol contributes to minimizing the injurious membrane changes resulting from severe dehydration. This revised procedure was also applied to strawberry (Hirai *et al.* 1998).

Matsumoto *et al.* (1995) and Hirai *et al.* (1998) reported low rate of shoot formation and later recovery growth in meristems cryopreserved by

encapsulation-dehydration technique compared to that cryopreserved by vitrification.

Encapsulation-dehydration protocols have been standardized for cryoconservation of medicinal plants like *Holostemma annulare* using shoot tips (Decruse and Seeni, 2002), *Clitoria ternatea* using somatic embryos derived from leaf induced callus (Nair and Reghunath, 2007) and *Indigofera tinctoria* using axillary buds (Nair and Reghunath, 2009).

Studies of Reed *et al.* (2005) on *Cyanodon* revealed that the encapsulation-dehydration cryopreservation protocol was most effective, especially when combined with a 1 to 4 week cold-acclimation period and dehydration to 19 to 23 per cent moisture, before exposure to LN.

Encapsulation within alginate beads was found beneficial in Persian lilac (*Melia azedarach* L.) and tea (*Camellia sinensis* L.) for long term cryostorage in LN (Kaviani, 2010).

Cryopreservation by encapsulation dehydration is a multi-component procedure which involves preconditioning, encapsulation, pre-culture and cryoprotection, desiccation/dehydration, freezing, thawing, recovery and plant regeneration. 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 (Harding and Benson, 1994).

2.2.1.1.1 Preconditioning

Preconditioning of mother plant cultures aims to condition explants to withstand freezing. Preconditioning involves various manipulations of the culture conditions such as culturing mother plants at low temperature, in the case of cold-tolerant species (Wu *et al.,* 2001; Matsumoto and Sakai 1995; Zhao *et al.,* 1999; 2001; Sakai *et al.,* 2000; Dereuddre *et al.,* 1990), or on medium with high sucrose content (Decruse *et al.,* 1999; Grospietsch *et al.,* 1999).

Kiwi, strawberry, chrysanthemum and wasabi shoot tips are cultured from one to several days on agar-based medium with a high sucrose concentration (Clavero-Ramirez *et al.,* 2005; Sakai *et al.,* 2000). *Citrus madurensis* shoot tips are first cultured on solid medium with 0.1 *M* sucrose, then on medium with 0.3 *M* sucrose and 0.5 *M* glycerol (Cho *et al.,* 2002).

Excised shoot tips of *Vitis vinifera* were preconditioned on solidified half MS medium supplemented with different concentrations of sucrose (0.1 to 0.7 *M*) for 1 or 3 days at 25º C and highest recovery (70 %) rate was observed in 0.3 *M* sucrose for 3 days, after plunging into LN (Matsumoto and Sakai, 2003).

Black currant shoot tips were cold acclimated for 2 weeks or cultured for 7 days on 0.75 *M* sucrose solid medium before encapsulation (Reed *et al.,* 2005).

Preconditioning is the culture of the explants after excision for several hours or days, before their encapsulation on standard medium or on medium with high sucrose concentration for one or several days (Engelmann *et al.,* 2008).

2.2.1.1.2 Encapsulation

Encapsulating the explants allows to submit them to very drastic treatments including pre-culture with high sucrose concentrations and desiccation to low moisture contents (MCs) which would be highly damaging or lethal to non-encapsulated samples. For preparing synthetic beads, samples are suspended in calcium-free liquid basal medium with usually 3 per cent (w/v) sodium alginate (low viscosity, 250 centipoises) (Gonzalez-Arnao and Engelmann, 2006). The mixture is dropped with a pipette in liquid culture medium containing a high concentration of calcium chloride (usually 100 mM $CaCl_2$). Alginate polymerizes in presence of the elevated concentration of calcium, thereby producing calcium alginate beads containing the explants. These spherical beads are usually about 4 or 5 mm in diameter, depending on the size of explants, and contain one explant or more. They are held in the calcium solution for 20-30 min after the last bead has been formed in order to guarantee complete polymerization (Engelmann, 2011).

Nair and Reghunath (2007, 2009) reported encapsulation using 2.5 per cent SA and 100 *mM* $CaCl_2$ in somatic embryos of *Clitoria ternatea* and axillary buds of *Indiofera tinctoria*. Padro *et al.* (2012) reported the use of 3 per cent sodium alginate solution as the best concentration for the formation of beads for cryopreservation in *Morus alba.*

Excised shoot tips of *Rabdosia rubescens* were suspended in calcium-free MS inorganic medium supplemented with 3 per cent (w/v) sodium alginate and 0.4 *M* sucrose. The mixture, with shoot tips, was dispensed with a sterile pipette into 0.1 *M* $CaCl_2$ solution containing 0.4 *M* sucrose at room temperature for half an hour, to form beads (4–5 mm in diameter), each bead containing one shoot tip (Ai *et al.*, 2012).

Protocorm-like bodies (PLBs) of *Dendrobium chrysanthum* were osmoprotected with a mixture of 0.4 *M* sucrose and 2 *M* glycerol, incorporated in the encapsulation matrix (comprising of 3 % (w/v) sodium alginate and 0.1 *M* $CaCl_2$) (Mohanty *et al.,* 2013).

Hongthongkham and Bunnag (2014) reported that cryopreservation of leaf segment-derived PLBs of *Aerides odorata* was successful using the encapsulation-dehydration method. The maximum survival percentage of cryopreserved PLBs was achieved by encapsulating PLBs with 2 per cent sodium alginate combined with 2 *M* glycerol and 0.4 *M* sucrose.

2.2.1.1.3 Pre-culture and Cryoprotection

Pre-culture of encapsulated shoot apices is a step to induce desiccation tolerance in the encapsulation-dehydration technique. Pre-culture is relatively simple, and the concentrations of sucrose and treatment duration are the only variables to be optimized for this step.

Pre-culture and cryoprotection involves the treatment of encapsulated explants in medium with cryoprotectants for several hours/days, immediately before their desiccation and freezing. Beads are transferred to Erlenmeyer flasks and agitated in liquid medium with high sucrose concentration and a cryoprotectant. The most common cryoprotective substances are dimethylsulfoxide (DMSO), polyethylene glycol (PEG), sucrose, sorbitol and mannitol. These substances have the osmotic actions; however some of them such as DMSO can enter to cells and protect cellular integrity during cryopreservation (Rajasekharan, 2006). 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. Sucrose treatment durations

may vary between 16-18 h as for sugarcane (Paulet *et al.,* 1993) to 7-10 days in the case of coffee (Mari *et al.,* 1995) and yam (Malaurie *et al.,* 1998).

De Carlo and Lambardi (2005) successfully employed slow-cooling method that involved pretreatment of embryogenic callus of *Citrus* with cryoprotectants, mainly DMSO (3 %) and sucrose before being slowly cooled to -40°C and then immersed in LN.

A progressive increase in sucrose concentration by daily transfers of explants in medium with higher concentration is required to reduce the toxic effect of sucrose. In certain species, a gradual increase of sucrose levels by 0.25 *M* incremental steps ensured over 80 per cent survival for shoot-tips of grape for final sucrose concentrations of 1 *M* and 70 per cent with 1.5 *M*, whereas direct cryoprotection in these media led to very low survival (Plessis *et al.,* 1991).

A progressive increase in sucrose concentration during the pre-culture was used for coffee (Mari *et al.,* 1995) and sugarbeet (Vandenbussche and de Proft 1996). Higher concentration of sucrose (0.4 *M* sucrose) in beads was applied to wasabi (Matsumoto *et al.,* 1995), strawberry (Hirai *et al.,* 1998) and Japanese pink lily (Matsumoto and Sakai 1995). It is an approach to simplify the procedure but results benefited from a progressive increase of sucrose concentration.

To increase the tolerance to liquid nitrogen (LN), the excised shoot tips of *Crateva nurvala,* were pre-cultured in sucrose (0.09 *M*, 0.4 *M* and 0.7 *M*) enriched liquid MS medium for 16, 24 or 48 h. When the sucrose in the pre-culture medium was increased to 0.4 *M*, post-thaw survival and regeneration also improved as compared to 0.09 *M* (Sanayaima *et al.,* 2006).

Sucrose is an important pre-culture additive for most cryopreservation method, which enhance desiccation tolerance during cryopreservation. Among different types of sugars (fructose, glucose, sorbitol, and sucrose) used as somatic agents in pre-culture medium, sucrose was the best for the survival of cryopreserved date palm tissue culture (Bekheet *et al.,* 2007).

In case of *Dendrobium candidum,* survival of cryopreserved PLBs increased to 87.6 per cent when these were pre-cultured in the presence of 0.75 *M* sucrose for 5 days (Yin and Hong 2009).

Sharaf *et al.* (2012) reported that significant variations in survival and regrowth of noncryopreserved shoot tips were obtained along with different concentrations of sucrose in the pre-culture medium and the duration of pre-culture for *Artemisia herba-alba*. High survival (96–100 %) rates were also obtained after pre-culture with 0.0, 0.1 or 0.3 *M* sucrose at 2 or 4 days.

Sucrose concentration in pre-culture medium and duration of pre-culture with 0.5 *M* sucrose supplemented MS medium for 2 days significantly improved the regrowth rates of embryogenic calli of *Anemarrhena asphodeloides* cooled to -196º C by vitrification (Yin and Hong, 2012).

Encapsulated protocorm-like bodies (PLBs) of *Dendrobium chrysanthum* were pre-cultured on MS liquid medium supplemented with different concentrations of sucrose (0.06, 0.3, 0.5, 0.7 *M*) for 2 days keeping on a rotary shaker at 98 rpm (25º C). The best result (survival 63.2 %) was obtained when the pre-culture of PLBs was carried out in 0.3 *M* sucrose whereas negative impact on survival percentage of cryopreserved PLBs were obtained with further increase in sucrose concentration from 0.5 *M* to 0.7 *M* (Mohanty *et al.,* 2013).

2.2.1.1.4 Dehydration

After pre-culture and cryoprotection, beads are submitted to an additional physical dehydration by evaporation at room temperature. To avoid lethal intracellular ice crystallization during rapid ultra-cooling, explants have to be sufficiently dehydrated prior to a plunge into LN. *In vitro* materials are not inherently tolerant to dehydration as they contain large amount of water. Hitmi *et al*. (1999) demonstrated that sucrose decreased the water content of *Chrysanthemum cinerarifolium* shoot tips and thus enhanced their freezing tolerance. The dehydration after encapsulation is also important for the maximum survival of plants in cryo. Gonzalez-Arnao and Engelmann (2006) described two desiccation levels *viz.,* dehydration under the air current of a laminar flow cabinet or dehydration in sealed containers with dry silica gel.

The moisture content varies depending on the species and the type of samples as it was 27 per cent for pear apical shoot-tips encapsulated in larger alginate beads (Scottez *et al.,* 1992) while microspore embryos of oilseed rape survived best at 18-20 per cent MC (Uragami, 1993). In general, the bead water content that ensures the highest survival after cooling in liquid nitrogen is around 20 per cent, which corresponds to the amount of unfreezable water in biological samples. At such water contents, only glass transitions are recorded upon freezing using differential scanning calorimetry (Sherlock *et al.,* 2005).

The embryonic axes of *Nothapodytes nimmoniana* lost MC rapidly in the laminar air flow cabinet, showing reduction of 43.7, 31.3, 24.4, 19.6 and 15.4 per cent on a fresh weight basis after ½, 1, 1 ½, 2 and 2 ½ h desiccation, respectively. Reduction in the MC of axes was accompanied by reduction in the survival rate. Cryopreservation was only efficient for the axes desiccated up to

19.6 per cent MC, whereas embryonic axes having moisture content below this lost the viability gradually when exposed to LN (Radha *et al.,* 2010d)

Damiano *et al.* (2011) reported that after cryopreservation of *Prunus persica* by encapsulation dehydration, no regrowth was obtained with 4 h physical desiccation treatment in silica gel (bead moisture content of 28 %) while initial viability and regrowth increased with silica treatments reducing bead moisture to 20.1 per cent.

The control of water content of plant samples before freezing is the key factor in developing successful cryoprotection protocols (Zhang *et al.* 2001). If the cells are not sufficiently dehydrated, intracellular ice will be formed resulting in cryoinjury during cold storage in liquid nitrogen and if over-dehydrated, the osmotic stress can be damaging (Bian *et al.* 2002). Maximum survival of PLBs of *Dendrobium nobile* (53.3 %) was achieved after cryopreservation when pre-cultured beads were dehydrated for 5 h with reduced water content of 28.3 per cent and gradually decreased (37.4 %) with 6 h of dehydration with bead water content of 16.7 per cent (Mohanty *et al.,* 2012).

Optimal desiccation time is related to the species and to the type of explant; for instance 32 per cent of final moisture content was suitable for protocorm like bodies of *Phalaenopsis bellina* (Khoddamzadeh *et al.,* 2011) while 18-28 per cent was optimal for *Sabal embryos* (Wen and Wang, 2010). However, water content of about 20 per cent is reported to induce best recovery in several species including *Picea* (Hazubska-Przybyl *et al*., 2010), *Pyrus* (Condello *et al.,* 2009) and *Morus bombicys* (Niino *et al.,* 1992b). Padro *et al.* (2012) reported highest re-growth after cryopreservation (67 %) with an osmoprotection of shoot apices in 0.75 *M* sucrose for 3 days, followed by a physical desiccation in silica gel for 9 h (19 % MC) in *Morus alba*.

Le Bras *et al.* (2014) reported that, the level of dehydration significantly influenced shoot regrowth after cryopreservation of axillary buds of *Rosa chinensis* by encapsulation dehydration technique. The best regrowth rate (60 %) was obtained at a bead water content of 0.35 g water per g dry weight.

Encapsulated PLBs of *Aerides odorata* were dehydrated on a sterile filter paper laid down on an open Petri dish (40 beads/Petri dish) and exposed to sterile air flow in a laminar air flow cabinet for 0-8 h (Hongthongkham and Bunnag, 2014).

2.2.1.1.5 Cryopreservation in Liquid Nitrogen

After dehydration, beads are placed in 1 or 2 ml sterile polypropylene cryotubes for freezing. Rapid cooling by direct immersion of the cryotubes in liquid nitrogen has been reported in many plant species. However in potato (Fabre and Dereuddre, 1990), grape (Plessis *et al.,* 1991; Zhao *et al.,* 2001) and *Citrus* (Gonzalez-Arnao *et al.,* 1998) shoot tips, slow precooling using a programmable freezing apparatus followed by immersion of samples in liquid nitrogen was required to obtain higher survival after cryopreservation (Engelmann, 2011).

Sharaf *et al.* (2012) observed the greatest survival (40 %) for cryopreserved (+LN) shoot tips of *Artemisia herba-alba* when shoot tips were pre-treated with 1.0 *M* sucrose for 3 days followed by 0 h dehydration where the beads attained 32.45 per cent moisture content, while the highest regrowth (6 %) was obtained with 0.5 *M* sucrose after 4 h of dehydration for *Artemisia herba-alba* shoot tips or with 1.0 *M* sucrose after 0 or 2 h of dehydration.

After desiccation, encapsulated beads of *Prunus persica* were placed in 2 ml sterile polypropylene cryotubes (10 beads/tube) and immersed rapidly in LN where they were kept for 24 h (Damiano *et al.,* 2011).

Osmoprotected and encapsulated seeds of *Lilium iedebourii* were placed in 1.8 ml cryotubes (3 or 4 seeds per cryotube) and directly plunged and maintained into LN for 24 h (Kaviani, 2011).

2.2.1.1.6 Thawing or Rewarming

In encapsulation-dehydration technique, rewarming is carried out at room temperature, since samples are sufficiently dehydrated before freezing and there is no risk of ice recrystallization upon warming (Gonzalez-Arnao and Engelmann, 2006). In cases where rapid warming is performed, cryotubes are plunged for 2-3 min in a water bath at +40°C (Gupta and Reed, 2006).

Subsequent to ultra-cooling of *Crateva nurvala*, cryovials were thawed either by immersing them in a water bath at 40° C for 1.5 min (rapid thawing) or by allowing them to reach room temperature slowly (slow thawing) (Sanayaima *et al.,* 2006).

Mandal and Sharma (2007) reported that, following 1 h storage of *Dioscorea deltoidea* in LN, rewarming was performed by placing cryovials in a 40° C water-bath for 2 min.

Damiano *et al.* (2011) reported that re-warming was performed by placing the cryotubes in the air current of the laminar flow cabinet.

For thawing and rewarming of cryopreserved seeds of *Lilium iedebourii* cryotubes were thawed in a water-bath at 37-38° C for 3 min (Kaviani, 2011).

The cryopreserved embryos of *Byrsonima intermedia* were thawed by plunging the cryotubes into a water bath for 5 minutes at the temperature of 37±2°C (Nogueira *et al.,* 2011).

After cryopreservation of *Artemisia herba-alba,* cryogenic vials containing beads were thawed in a water bath at 38° C for 2–3 min (Sharaf *et al.,* 2012).

2.2.1.1.7 Recovery

Regrowth generally takes place on standard semi-solid culture medium. However, the composition of the recovery medium may be transitorily altered to eliminate phenolic compounds produced by dead cells by adding activated charcoal to the medium as with sugarcane apices (Paulet *et al.,* 1993; Gonzalez-Arnao, 1996) or to stimulate regrowth of frozen explants by modifying the growth regulator content of the medium as with sugarcane and yam apices (Paulet *et al.,* 1993; Malaurie *et al.,* 1998). Environmental conditions also influence the recovery of plant tissues after cryopreservation. Organized structures such as meristems, are subjected to post-thaw recovery in the dark for a short period (around one week) (Gonzalez-Arnao and Engelmann, 2006). Exposure to dark period during post-thaw recovery stage helps to prevent or decrease detrimental photo oxidation of frozen samples (Benson, 1990).

Gonzalez-Arnao, (1996) reported that sugarcane apices of 15 different commercial varieties representing a broad genetic diversity were successfully cryopreserved with recovery ranging between 24 per cent and 91 per cent.

The effect of glycine and leucine on the recovery of frozen-thawed *Lavandula vera* cells was studied by Kuriyama *et al.* (2000). Glycine was found to be inhibiting the recovery of frozen-thawed cells and promoted the lethal process of freezing injury.

Regrowth of *Glehnia littoralis* after cryopreservation varied with respect to both desiccation time and moisture content of beads. No regrowth was obtained following 2 h of desiccation, however, the regrowth percentage subsequently increased at 6 h desiccation (MC <19 %) and regrowth reached the maximum (43.3 ± 3.3 %) (Otokita, *et al.,* 2009).

The cryopreserved encapsulated shoot tips of *Cacanska bestrna* were transferred to Petri dishes containing standard medium (MS medium supplemented with BA 1 mg l^{-1}, IBA 0.1 mg l^{-1} and GA3 0.1 mg l^{-1}, pH 5.7, 0.06 M sucrose and 7.2 g l^{-1} agar) and kept in growth room in darkness for 7 d and after that transferred to standard growth conditions (Ruzic and Vujovic, 2012).

After recovery, explants from the optimal cryopreservation treatment were multiplied on the standard culture medium and rooting was performed on an MS basal medium supplied with IBA 1.0 mg l^{-1} (Padro *et al.,* 2012).

Cryostorage periods of 1–360 days were tested in *Anemarrhena asphodeloides* and no significant differences were found among the regrowth rates of embryogenic calli in all treatments (Yin and Hong, 2012).

Encapsulated non-cryopreserved (-LN) and encapsulated cryopreserved (+LN) beads of *Artemisia herba-alba* were inoculated onto a solid MS recovery medium containing 2iP 1.0 mg l^{-1} and sucrose 0.1 *M* and then kept in the dark for 3 days (Sharaf *et al.,* 2012).

2.2.2 Genetic Stability Assessment of Cryopreserved Materials Using RAPD

The condition for successful cryopreservation is not only that the stored culture can start regrowth after thawing, but also that the culture has retained the same characteristics as it had before cryopreservation. Genetically stable true to type plants are expected to be recovered from cryopreservation as metabolic activities at temperature of LN are reduced to zero (Panis *et al.,* 2001). Cryopreserved tissue should be genetically identical to non-treated phenotype and can directly produce normal plants (Dumet and Benson, 2000). A large number of reports showing no evidence of morphological, cytological, biochemical, or molecular alterations in plants from storage at -196°C (Harding, 2004). However, genetic variation in cryopreserved materials has occasionally been detected at morphological, biochemical, chromosomal and molecular levels (Muller *et al.,* 2007; Kaity *et al.,* 2008; Sanchez *et al.,* 2008; Zeng *et al*., 2010; Martin *et al.,* 2011).

A series of stresses (e.g. dehydration, osmotic pressure, low temperature) throughout the cryopreservation process may result in genetic variation (Engelmann, 2004; Hazubska-Przbyl *et al.,* 2010). 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). Genetic instability and somaclonal variations may be caused some differences in genotype and phenotype profiles of cryopreserved plants (Harding, 2004). So, viability and genetic stability are two important factors after cryopreservation (Anand, 2006). The identification of genetic variation is important for effective management and use of genetic resources.

The practical application of this technology is useful only if it does not lead to the genetic changes in the cryopreserved plant. According to Harding and Benson (1995) molecular analysis of plant DNA is ideal for genetic stability assessments. PCR based molecular markers have been used in the genetic fidelity assessment of cryostorage derived plantlets (Zhai *et al.,* 2003). 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).

Characteristics such as growth rate, regeneration capability and ploidy levels were retained after cryogenic storage in many studies (Kobayashi *et al.,* 1990; Meijer *et al.,* 1991; Ward *et al.,* 1993; Wang *et al.,* 1994; Ribeiro *et al.*, 1996). In contrast, Wan and Vasil (1996) reported that efficiency of regeneration of napier grass (*Pennisetum purpureum* Schum.) plants from cryopreserved suspension cells was reduced in comparison with non cryopreserved controls.

RAPD technique has been used to study the genetic stability of cryopreserved tissue cultures of date palm (Bekheet *et al.,* 2007). According to RADP analysis, plantlets derived from cryopreserved cultures were identical to that derived from non-treated cultures and both were similar with the field growth plants (Bekheet *et al.,* 2007). Assessment of genetic fidelity by Random Amplified Polymorphic DNA analysis (RAPD) in *Pistacia vera* revealed out high levels of genetic stability between donor plant and cryopreserved plants (Akdemir *et al.,* 2013).

RAPD based assessment of the genetic stability of *in vitro* grown micropropagated plants has been reported for many plant species (Rani *et al.,* 1995;

Rout and Das, 2002; Martins *et al.,* 2004; Venkatachalam *et al.,* 2007). Yamuna *et al*. (2007) used ISSR and RAPD profiling to confirm the genetic stability of cryopreserved ginger shoot buds.

Mohanty *et al.,* (2008) evaluated the genetic stability of micropropagated *Zingiber officinale* clones using RAPD markers. RAPD analysis revealed monomorphic bands showing the absence of polymorphism thus confirming the genetic uniformity among *in vitro* grown somaclones of *Z. officinale.* Similar findings are reported in *Centaurea ultreiae* (Mallon *et al.,* 2010) and in *Sapindus trifoliatus* (Asthana *et al.,* 2011). The amplification products were found to be monomorphic in micropropagated plants and similar to those of mother plant.

Materials & Methods

3. MATERIALS AND METHODS

The present study, 'Cryopreservation of Chethikoduveli (*Plumbago rosea* L.) and assessment of genetic fidelity of regenerated plantlets using molecular markers' was carried out at the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2011-2013. The study aimed at optimization of protocol for *in vitro* regeneration of *P.rosea* var. Agni and standardization of its *in vitro* conservation procedure by cryopreservation. The assessment of genetic accuracy of the regenerated plantlets using molecular markers was also attempted.

Investigations were carried out in two phases *viz*., *in vitro* regeneration and *in vitro* conservation. *In vitro* regeneration studies were carried out by enhanced release of axillary buds method and *in vitro* conservation was done using the encapsulation-dehydration technique of cryopreservation. The assessment of genetic fidelity of the regenerated plantlets was carried out by RAPD technique.

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

3.1 PHASE I: *IN VITRO* REGENERATION

3.1.1 Culture Media

The basal medium used for the study was MS medium (Murashige and Skoog, 1962). The chemicals used in the culture media were of analytical grade from Sisco Research Laboratories (Mumbai), Merck (Mumbai) and HiMedia Laboratories Pvt. Ltd. (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 glassware 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 containing 400 ml of distilled water. Sucrose and inositol were added fresh in required quantity, weighed using an electronic balance (Shimadzu AUX120) and dissolved by constant stirring. The pH of the medium was adjusted to 5.7 using an electronic pH meter (Susima MP-1 PLUS). Agar was added to the medium and final volume made up to 1000 ml, using a volumetric flask. Agar was melted using a microwave oven. The melted medium was dispensed into pre-sterilised culture vessels such as test tubes (25 x 150 mm) and jam bottles (300 ml). Measure of culture medium in test tubes and jam bottles were 15 and 30 ml, respectively. The test tubes containing medium were plugged firmly with non-absorbent cotton wool and the jam bottles were closed tightly with plastic lid. They were then autoclaved at 121° C and 1.06 kg cm^{-2} pressure for 20 minutes using STERI horizontal cylindrical autoclave (Yorko, India). The composition of basal medium was MS + 30 g l^{-1} sucrose + 5.7 g l^{-1} agar + 100 mg l^{-1} inositol.

3.1.2 Inoculation

The glassware and tools required for inoculation were washed thoroughly in tap water, rinsed with distilled water, covered with polypropylene cover and autoclaved at 121° C and 1.06 kg cm^{-2} pressure for 45 minutes. Horizontal type autoclave (Yorco, India) was used for autoclaving.

Inoculation operations for enhanced release of axillary buds and *in vitro* rooting were carried out in a laminar air flow chamber (Klenzaids, India). The closure of the culture vessels was removed before inoculation, the rim of the culture vessels was flamed before and after inoculation and then the closure is replaced.

3.1.3 Incubation

The cultures were incubated in a culture room maintained at light intensity, 40 μmol m^{-2} s^{-1} using white fluorescent tubes for 14 h. The temperature of the room was regulated using an air conditioner at 25 ± 2° C at a relative humidity (RH) of 60 per cent.

3.1.4 Enhanced Release of Axillary Buds

Plumbago rosea var. Agni (Plate 1) was collected from the Aromatic and Medicinal Plants Research Station (AMPRS), Odakkali, Ernakulam district and was maintained at the Department of Plant Biotechnology, College of Agriculture, Vellayani as source of explants during the course of the study.

3.1.4.1 Surface Sterilization

Single node cuttings of 3-4 cm long were excised from actively growing shoots of one year old plants of *Plumbago rosea* var. Agni. The leaves were removed leaving a small portion of petiole on the cuttings. Nodal segments were washed thoroughly under running tap water for 15 min and subsequently immersed in water with a few drops of detergent, (Labolene) for 30 min, followed by washing 4-5 times with distilled water. The nodal cuttings were then immersed in a systemic fungicide solution, 0.1 per cent carbendazim 50 per cent W.P., for 30 min followed by washing several times with distilled water. Further, the explants were aseptically sterilised with a dip for 2-3 seconds in absolute alcohol followed by sterilisation with disinfectant, mercuric chloride. Different levels of mercuric

Plate 1. *Plumbago rosea* var. Agni

chloride (0.05 %, 0.08 % 0.1 %, 0.15 %, 0.2 %) and two exposure period (5 min and 10 min) were tried to obtain contamination free cultures. After treating with mercuric chloride, nodal segments were washed 4-5 times with sterile water to remove any trace of mercuric chloride. The exposed ends of the nodal cuttings were further cut to remove the dead tissues due to mercuric chloride exposure. Single node cuttings of 2-2.5 cm were inoculated in a vertical position on hormone free MS medium. Contamination due to microbial growth was recorded per cent after six weeks of culture.

3.1.4.2 Culture Initiation and Multiplication

Nodal cuttings (1.5 cm) with a single axillary bud from the *in vitro* established plantlets were inoculated for culture initiation and multiplication. The nodal segments were cultured in basal medium supplemented with different concentrations and combinations of auxins (NAA, IAA and IBA) and cytokinins (BA and Kn). Treatments involved different levels of BA (0.5 to 1.5 mg l^{-1}), Kn (0.5 to 1.0 mg l^{-1}), IAA (0.5 to 1.0 mg l^{-1}), IBA (0.5 to 1.0 mg l^{-1}) and NAA (0.5 to 1.0 mg l^{-1}) (Table 1). The treatments were replicated six times.

Observations were recorded on number of days for bud initiation, shoots/culture, shoot length and nodes/culture after six weeks of culture.

3.1.5 *In vitro* Rooting

Well developed shoots having 3-4 cm length were separated and subjected to different rooting treatments. The shoots were inoculated into MS medium with varying levels of IBA (0.1 to 1.5 mg l^{-1}), IAA (0.1 to 1.5 mg l^{-1}) and NAA (0.1 to 1.5 mg l^{-1}) (Table 2). Each treatment was replicated six times.

Observations were taken on per cent cultures initiating roots, number of days for root initiation, roots/culture and length of root after six weeks of culture.

Table 1. Plant growth regulators (Cytokinin and Auxin) tried for i*n vitro* regeneration

Treatment No.	Plant growth regulators (mg l^{-1})				
	BA	IAA	IBA	NAA	Kn
T1	0.5	0.5	-	-	-
T2	0.5	1	-	-	-
T3	1	0.5	-	-	-
T4	1	1	-	-	-
T5	1.5	0.5	-	-	-
T6	1.5	1	-	-	-
T7	0.5	-	0.5	-	-
T8	0.5	-	1	-	-
T9	1	-	0.5	-	-
T10	1	-	1	-	-
T11	1.5	-	0.5	-	-
T12	1.5	-	1	-	-
T13	0.5	-	-	0.5	-
T14	0.5	-	-	1	-
T15	1	-	-	0.5	-
T16	1	-	-	1	-
T17	1.5	-	-	0.5	-
T18	1.5	-	-	1	-
T19	0.5	-	-	-	0.5
T20	0.5	-	-	-	1
T21	1	-	-	-	0.5
T22	1	-	-	-	1
T23	1.5	-	-	-	0.5
T24	1.5	-	-	-	1
T25	0.5	-	-	-	-
T26	1	-	-	-	-
T27	1.5	-	-	-	-
T28	2	-	-	-	-
Control	-	-	-	-	-

Table 2. Plant growth regulators tried for *in vitro* rooting

Treatment No.	Plant growth regulators (mg l^{-1})		
	NAA	IBA	IAA
R1	0.1	-	-
R2	0.5	-	-
R3	1.0	-	-
R4	1.5	-	-
R5	-	0.1	-
R6	-	0.5	-
R7	-	1.0	-
R8	-	1.5	-
R9	-	-	0.1
R10	-	-	0.5
R11	-	-	1.0
R12	-	-	1.5
Control	-	-	-

3.1.6 Planting Out and Acclimatization

Plantlets of 4-7 cm length each with 9-12 roots were removed from the bottles with the help of forceps. The agar adhering to the roots was completely removed by thorough washing with running tap water. The plants were treated with 0.1 per cent carbendazim 50 per cent W.P. for 1 h before planting out. The plants were then transferred to polythene bags (10x15 cm) filled with potting medium consisting of red soil and coir pith (3:1) and red soil and coir pith (2:1) supplemented with VAM (Vesicular Arbuscular Mycorrhiza). Twenty five plants each were planted in the two potting media. The plants were kept under 25 per cent shade net house and irrigated twice a day with rose can. Observations were recorded on number of plants survived and survival percentage after six weeks of planting out.

3.1.7 Statistical Analysis

Completely randomized design (Panse and Sukhatme, 1985) was followed for statistical analysis, wherever applicable. Data were subjected to analysis of variance (ANOVA) and significant difference between treatments were determined by Duncan Multiple Range Test (DMRT).

3.2 PHASE II: *IN VITRO* CONSERVATION

3.2.1 Cryopreservation of *Plumbago rosea* Using Encapsulation Dehydration Technique

3.2.1.1 Preconditioning

Axillary buds of *in vitro* established *P. rosea* were inoculated on to MS medium (devoid of glycine) (Kuriyama *et al.,* 2000) supplemented with different concentrations of

sucrose (0.1, 0.3, 0.5, 0.75 and 1.0 *M*) and 0.57 per cent agar. Cultures were incubated at 25 ± 2° C under 14 h photoperiod at a photon flux intensity of 30-50 µmol m^{-2} s^{-1} for different periods such as 1 day, 1 week and 2 weeks. The treatments are represented in Table 3. The incubated axillary buds were then removed from the treatment media and cultured on the medium which gave the best response to axillary shoot proliferation. The experiment was replicated six times. Observations were recorded on number of days for bud initiation, shoots/culture, shoot length and nodes/culture after six weeks of culture. The best preconditioning medium and exposure period was determined based on the above mentioned observations.

3.2.1.2 Encapsulation

Nodal axillary buds excised from *in vitro* grown plantlets were suspended in calcium free MS medium (devoid of glycine) supplemented with different concentrations (2.5, 3. 4, and 5 %) of sodium alginate (Sigma, St. Louis, USA) and 0.5 M sucrose. This mixture was dispensed with a 1 ml micropipette into different concentrations of calcium chloride solution (50, 75, 100 and 200 *mM*) for encapsulation. The treatments are represented in Table 4. After 30 min, the beads were cultured on the medium which gave the best response to axillary shoot proliferation. The treatments were replicated six times. Observations were recorded on number of days for bud initiation, shoots/culture, shoot length and nodes/culture after six weeks of culture. Based on the observations best encapsulation medium was determined.

3.2.1.3 Pre-culture

The preconditioned and encapsulated beads were pre-cultured in MS liquid medium (devoid of glycine) supplemented with different concentrations of sucrose (0.1, 0.3, 0.5, 0.75, and 1.0 *M*) and 3 per cent DMSO dispensed into 100 ml Erlenmeyer flasks (50 beads in 25 ml medium) and incubated at 25 ± 2° C under 14 h photoperiod at a photon

Table 3. Treatments tried for preconditioning

Treatment No.	Sucrose (*M*)	No. of days
PC1	0.1	1
PC2	0.1	7
PC3	0.1	14
PC4	0.3	1
PC5	0.3	7
PC6	0.3	14
PC7	0.5	1
PC8	0.5	7
PC9	0.5	14
PC10	1.0	1
PC11	1.0	7
PC12	1.0	14

Table 4. Treatments tried for encapsulation

Treatment No.	SA (%)	$CaCl_2$ (*mM*)
E1	2.5	50
E2	2.5	75
E3	2.5	100
E4	2.5	200
E5	3.0	50
E6	3.0	75
E7	3.0	100
E8	3.0	200
E9	4.0	50
E10	4.0	75
E11	4.0	100
E12	4.0	200
E13	5.0	50
E14	5.0	75
E15	5.0	100
E16	5.0	200

flux intensity of 40 µmol m^{-2} s^{-1} 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 different periods- 1, 3, 5 and 7 days. The treatments are represented in Table 5. After the respective periods of incubation the beads were cultured on the medium which gave the best response to axillary shoot proliferation. The treatments were replicated six times. Observations were recorded on number of days for bud initiation, shoots/culture, shoot length and nodes/culture after six weeks of culture. Based on the observations best pre-culture condition was determined.

3.2.1.4 Dehydration

To determine the optimum drying time, beads after pre culture were desiccated for 0 to 7 h in a sterile air laminar flow cabinet. Liquid nitrogen (LN) tolerance was tested for each drying time at one hour interval. The survival and regeneration percentage were also recorded for desiccated beads without LN exposure as control for each drying treatments. 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 using the formula,

$$MC = [(\text{Fresh weight} - \text{Final weight}) / \text{Fresh weight}] * 100$$

3.2.1.5 Cryopreservation and Recovery

Dehydrated beads were transferred to 4 ml cryovial and directly immersed in liquid nitrogen where they were stored at least for two hours. 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 (MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1} + sucrose 3 per cent + agar 0.57 per cent) and incubated in culture room at 25 ± 2° C under 14 h photoperiod at a photon flux

Table 5. Treatments tried for pre-culture

Treatment No.	Sucrose (*M*)	No. of days
PT1	0.1	1
PT2	0.1	3
PT3	0.1	5
PT4	0.1	7
PT5	0.3	1
PT6	0.3	3
PT7	0.3	5
PT8	0.3	7
PT9	0.5	1
PT10	0.5	3
PT11	0.5	5
PT12	0.5	7
PT13	0.75	1
PT14	0.75	3
PT15	0.75	5
PT16	0.75	7
PT17	1.0	1
PT18	1.0	3
PT19	1.0	5
PT20	1.0	7

intensity of 40 µmol m^{-2} s^{-1}. Six replications were given with each having four beads. The results were expressed as survival per cent 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 six weeks of post-culture. Observations were recorded on number of days for bud initiation, shoots/culture, shoot length and nodes/culture after six weeks of culture.

3.2.2 Statistical Analysis

Completely randomized design (Panse and Sukhatme, 1985) was followed for statistical analysis, wherever applicable. Data were subjected to analysis of variance (ANOVA) and significant difference between treatments were determined by Duncan Multiple Range Test (DMRT).

3.2.3 Estimation of Genetic Fidelity of Cryopreserved Materials Using RAPD

3.2.3.1 Genomic DNA Isolation

C-TAB method of DNA extraction (Doyle and Doyle, 1990) with slight modifications was used for genomic DNA isolation.

Young leaves from the cryopreserved and regenerated plants as well as control plants (*in vitro* seedlings maintained at ambient conditions) were used for DNA extraction. Leaf material (500 mg) was washed in distilled water and dried by spreading on tissue paper. The samples were chilled and pulverized to a fine powder in liquid nitrogen using a sterile mortar and pestle and transferred about 100 mg of powder into a sterile 2 ml centrifuge tube containing 1 ml of freshly prepared warm extraction buffer (prepared by adding β-mercaptoethanol and polyvinylpyrrolidone

(PVP) freshly to the CTAB extraction buffer (Appendix I) to give a final concentration of 0.2 per cent (v/v) and 4 per cent (w/v) respectively, which was then heated to 65^{o} C in water bath (ROTEK, India)). The content was homogenized by gentle inversion. The samples were incubated at 60^{o} C in water bath for 1 h with intermittent shaking. The homogenate was then extracted with an equal volume of 24:1 (v/v) chloroform/isoamyl alcohol and mixed well by inversion for 5-10 min. The homogenate was centrifuged (Eppendorf centrifuge 5430 R, Germany) at 7500 x *g* for 10 min at 25^{o}C. The upper phase was transferred to a new centrifuge tube and the extraction process with chloroform/isoamyl alcohol was repeated twice. 0.5 ml of 5 *M* NaCl was added to the aqueous phase and mixed properly by gentle inversion. After that, 0.6 volume of chilled isopropanol was added to the mixture and mixed by inversion. The mixture was then incubated at 4^{o} C overnight to precipitate the nucleic acid. After incubation, the precipitated DNA was pelletized by centrifugation at 11,000 x *g* for 10 min at 25^{o} C. The supernatant was decanted and the pellet was washed in 0.5 ml ethanol (80 %) twice, each time centrifuging at 5000 x *g* for 7 min at 25^{o} C and discarding the supernatant. The pellet was air dried for 30-40 min and dissolved in 40 µl of TE buffer (Appendix II). The extracted DNA samples were then stored at -20^{o} C (Lab-Line Low Temperature Cabinet, India).

3.2.3.2 Quantification of DNA

Spectrophotometer reading of the DNA samples was recorded to determine the quantity and quality of DNA. T60 UV- Visible Spectrophotometer was used for measuring the optical density (O.D.) of the sample. Spectrophotometer was calibrated to blank (zero absorbance) at 260 nm and 280 nm wavelength with 3 ml TE buffer and O.D. reading of 5 µl DNA sample dissolved in 3 ml of TE buffer at respective wavelengths.

An optical density (O.D.) value of 1.0 at 260 nm indicates the presence of 50 ng/µl of double stranded DNA. Hence, the quantity of DNA present in the extracted sample was estimated by employing the following formula:

$$\text{Concentration of DNA (ng } \mu l^{-1}) = A_{260} \times 50 \times \text{dilution factor}$$

(Where, A_{260} is absorbance reading at 260 nm)

Proteins are known to absorb strongly at 280 nm while absorption maximum of DNA/RNA is at 260 nm. The quality of DNA was determined by the ratio of O.D. value of the sample at 260 nm and 280 nm. A value ranging from 1.70 to 1.90 is an indicator of reduced contamination from protein.

3.2.3.3 Agarose Gel Electrophoresis

The presence of genomic DNA and PCR products were confirmed and analysed by using horizontal gel electrophoresis unit (Genei, Bangalore). Electrophoresis buffer (0.5 x TBE) (Appendix III) was prepared to fill the electrophoresis tank and to cast the gel. Prepared agarose solution (0.8 %) in electrophoresis buffer (0.24 g in 30 ml of buffer solution) in a conical flask while 1.5 per cent of agarose solution was used for separation of PCR products. The slurry was heated in a microwave oven until the agarose dissolved. On cooling the molten gel, added ethidium bromide (EtBr) to a final concentration of 0.5 µg ml^{-1} and mixed thoroughly. The comb was positioned above the tray, and then the warm agarose solution was poured into the moulded casting tray. The gel was allowed to set completely (30-45 min) at room temperature. On gel setting, carefully removed the comb and the tape and the gel was mounted in the electrophoresis tank. Electrophoresis buffer was added just enough to cover the gel to a depth of about 1 mm above the gel. DNA sample (5 µl) mixed with 1 µl of 6x gel loading buffer (Genei) and the sample mixtures were slowly loaded into the wells of the submerged

gel using a micropipette. A voltage of 80 V (5V cm^{-1} - measured as the distance between the positive and negative electrodes) was applied and it was turned off when the dye migrated 3/4th of the distance through the gel. The gel was removed carefully and visualized under the gel documentation system (BIORAD) using 'Quantity One Software'.

3.2.3.4 RAPD Analysis

Two DNA samples were amplified with six RPI decamer primers supplied by Genei, Bangalore. Primers selected for the analysis are listed in Table 6. The components of the reaction mixture were optimized as listed below.

Water	:	12.9 µl
10x Taq buffer A (Tris with 15 *mM* $MgCl_2$)	:	2 µl
dNTPS (2.5 *mM* each)	:	1.6 µl
Primer (10 *µM*)	:	2 µl
Template DNA (50 ng μl^{-1})	:	1 µl
Taq polymerase (3U μl^{-1})	:	0.5 µl
Total volume	:	20 µl

PCR was carried out in Eppendorf Mastercycler (Germany). PCR programme was set with initial denaturation at 94^o C for 4 min followed by 35 cycles of denaturation at 94^o C for 45 s, annealing at 50^o C for 30 s and extension at 72^o C for 45 s. Final extension was done at 72^o C for 5 min. Control reactions were carried out to distinguish the target products from non-target products and primer dimer. The amplified products along with Quantum PCR Marker (low range) from 'Genei, Bangalore' were separated on agarose gel (1.5 %). The gel was viewed under gel documentation system (BIORAD, USA).

Table 6. Sequence of selected RAPD primers used for DNA amplification by PCR to evaluate the genetic stability of plantlets regenerated from cryopreserved materials of *Plumbago rosea*

Sl. No.	Primers	Sequence (5'-3')
1	RPI 2	AACGCGTCGG
2	RPI 3	AAGCGACCTG
3	RPI 5	AATCGGGCTG
4	RPI 4	AATCGCGCTG
5	RPI 6	ACACACGCTG
6	RPI 8	ACCACCCACC

The genetic fidelity assessment of cryopreserved axillary buds of *P. rosea* was done by comparing the RAPD banding pattern of the cryo-regenerated plantlets with the RAPD bands of control non-cryopreserved *in vitro* grown plantlets.

Results

4. RESULTS

Investigations were carried out on 'Cryopreservation of Chethikoduveli (*Plumbago rosea* L.) and assessment of genetic fidelity of regenerated plantlets using molecular markers' at the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2011-2013. The results of the study are presented here.

4.1 PHASE I: *IN VITRO* REGENERATION

4.1.1 Enhanced Release of Axillary Buds

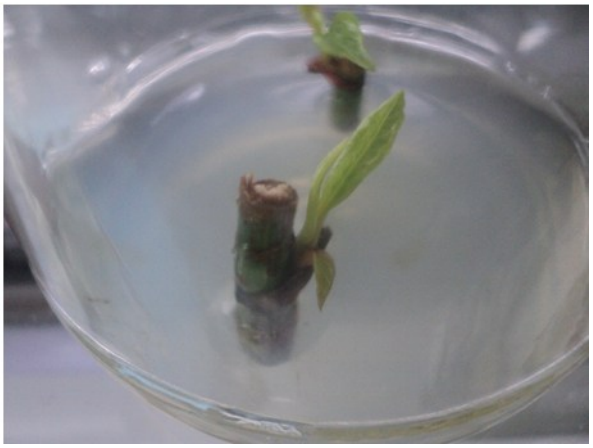
4.1.1.1 Surface Sterilization

Single nodal segments excised from actively growing shoots of one year old field grown plants were used as explants. They were subjected to different surface sterilization treatments. Among the different treatments tried, treatment T5 (0.2 % $HgCl_2$ for 5 min) gave 100 per cent survival without any contamination. All the treatments gave bud initiation within a week of culture. No explants survived the treatments T1 (0.05 % $HgCl_2$ for 10 min) and T2 (0.08 % $HgCl_2$ for 10 min), while T3 (0.1 % $HgCl_2$ for 5 min) and T4 (0.15 % $HgCl_2$ for 5 min) resulted in 4.17 per cent and 8.33 per cent survival respectively (Plate 2). The contamination was predominantly fungal (Plate 3).

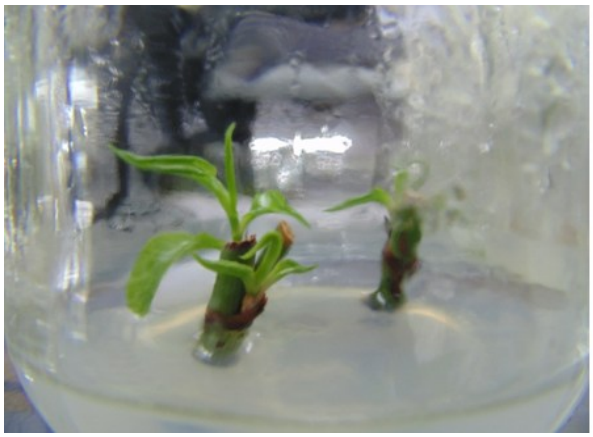
4.1.1.2 Culture Initiation and Multiplication

Nodal segments of 1.5 cm with a single axillary buds from *in vitro* established plantlets were used as explants for culture initiation and multiplication studies.

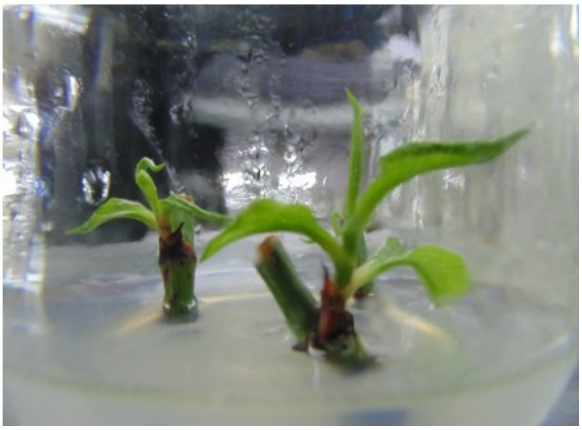
A. After 2 weeks of culture



B. After 3 weeks of culture

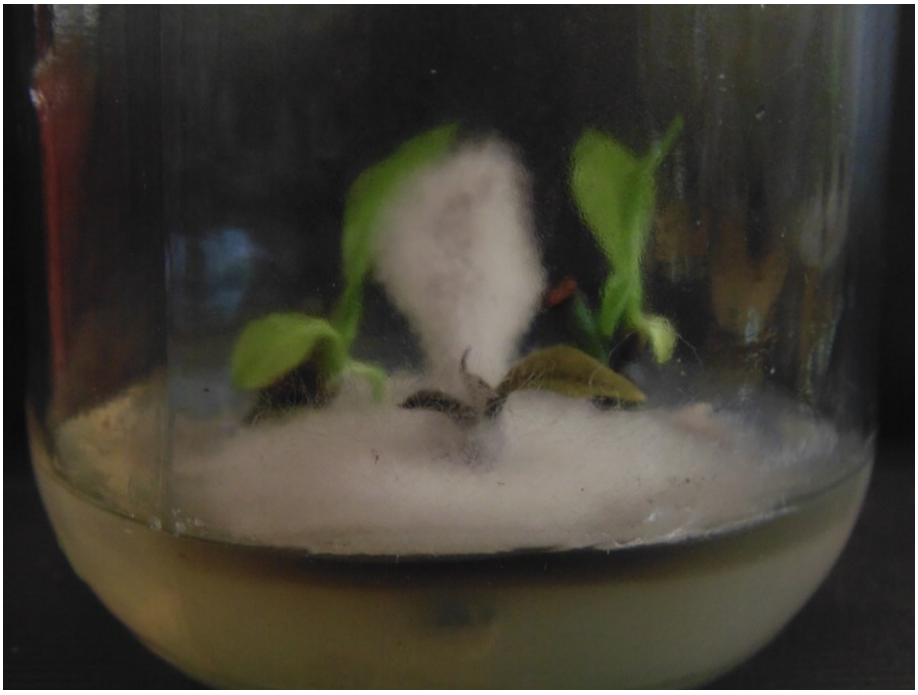


C. After 4 weeks of culture



D. After 4 weeks of culture

Plate 2. Establishment of culture in MS medium from single nodal stem cuttings



A

B

Plate 3. (A&B) Fungal contamination in the establishment medium (hormone free MS)

Thirty treatments involving different combinations of plant growth regulators (BA, IAA, IBA, NAA and Kn) in MS medium were tried to study their effect on shoot regeneration from the explants. Results obtained from the study are furnished in Table 7 (Fig 1).

Cent per cent bud initiation was obtained with all the treatments used. The bud initiation was indicated as swelling of axillary buds and shoot emergence in the explant (Plate 4).

The different treatments showed considerable variation with regard to the number of days taken for bud initiation, shoots/culture, shoot length and nodes/explant. The earliest bud initiation (7.94 days) was obtained with T6 (MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1}) which was on par with T1, T2, T3, T4, T25 and T29. The bud initiation was late (20.33 days) in T18 (MS + BA 1.5 mg l^{-1} + NAA 1.0 mg l^{-1}). This was on par with T17 (MS + BA 1.5 mg l^{-1} + NAA 0.5 mg l^{-1}) (20.05 days) and the control (MS medium free of growth hormones) (19.61 days).

With respect to the number of shoots/culture, the maximum number of shoots (5.28 shoots/culture) per culture was obtained in T6 (MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1}). This treatment showed significant variation with respect to all other treatments. The least number of shoots/culture (1.28 shoots/culture) was obtained in the control (MS medium free of growth hormones). This value was on par with the treatments T1 and T2, T7 to T23 and T25.

Maximum shoot length (5.33 cm) was observed in T6 (MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1}) which was on par with T29 (half MS + BA 1.0 mg l^{-1} + IAA 1.0 mg l^{-1}). The minimum value (2.14 cm) was seen in T24 (MS + BA 1.5 mg l^{-1} + Kn 1.0 mg l^{-1}) which was on par with T7, T12, T14, T15, T17, T18, T20 to T25, T27, T28 and the control (MS medium free of growth hormones).

Table 7. Effect of cytokinins and auxins on enhanced release of axillary buds from nodal explants

Treatment No.	Plant growth regulators (mg l^{-1})									
	BA	IAA	IBA	NAA	Kn	Survival (%)	Days for bud initiation	Shoots/culture	Shoot length	Nodes/shoot
T1	0.5	0.5	-	-	-	100	9.39 ± 0.54[a]	1.89 ± 0.19[b]	4.64 ± 0.22	5.22 ± 0.37
T2	0.5	1	-	-	-	100	10.00 ± 0.77[a]	1.56 ± 0.11[b]	4.31 ± 0.13	4.50 ± 0.22
T3	1	0.5	-	-	-	100	10.16 ± 0.73[a]	2.11 ± 0.29	3.92 ± 0.30	5.11 ± 0.20
T4	1	1	-	-	-	100	9.78 ± 0.54[a]	3.00 ± 0.17	3.89 ± 0.47	4.72 ± 0.26
T5	1.5	0.5	-	-	-	100	11.05 ± 0.92	2.22 ± 0.11	3.28 ± 0.32	4.22 ± 0.29
T6	**1.5**	**1**	**-**	**-**	**-**	**100**	**7.94 ± 0.22[a]**	**5.28 ± 0.26[a]**	**5.33 ± 0.19[a]**	**5.83 ± 0.14[a]**
T7	0.5	-	0.5	-	-	100	11.28 ± 0.78	1.39 ± 0.10[b]	2.81 ± 0.17[b]	3.06 ± 0.10[b]
T8	0.5	-	1	-	-	100	11.72 ± 0.42	1.33 ± 0.09[b]	2.92 ± 0.18	2.94 ± 0.28[b]
T9	1	-	0.5	-	-	100	12.83 ± 1.04	1.39 ± 0.16[b]	3.11 ± 0.29	3.39 ± 0.16
T10	1	-	1	-	-	100	11.50 ± 1.28	1.44 ± 0.19[b]	3.08 ± 0.31	3.22 ± 0.24
T11	1.5	-	0.5	-	-	100	10.83 ± 0.83	1.56 ± 0.17[b]	3.22 ± 0.28	2.72 ± 0.20[b]
T12	1.5	-	1	-	-	100	10.83 ± 0.59	1.67 ± 0.12[b]	2.69 ± 0.12[b]	2.83 ± 0.14[b]
T13	0.5	-	-	0.5	-	100	14.66 ± 1.21	1.45 ± 0.11[b]	3.19 ± 0.12	3.78 ± 0.19
T14	0.5	-	-	1	-	100	16.55 ± 1.23	1.50 ± 0.14[b]	2.31 ± 0.21[b]	3.11 ± 0.19
T15	1	-	-	0.5	-	100	16.16 ± 0.67	1.33 ± 0.15[b]	2.22 ± 0.18[b]	2.94 ± 0.13[b]

Table 7. Continued...

Treatment No.	Plant growth regulators (mg l^{-1})									
	BA	IAA	IBA	NAA	Kn	Survival (%)	Days for bud initiation	Shoots/culture	Shoot length	Nodes/shoot
T16	1	-	-	1	-	100	17.28 ± 0.62	1.83 ± 0.14[b]	3.14 ± 0.16	3.83 ± 0.19
T17	1.5	-	-	0.5	-	100	20.05 ± 0.64[b]	1.56 ± 0.17[b]	2.86 ± 0.10	3.67 ± 0.23
T18	1.5	-	-	1	-	100	20.33 ± 0.58[b]	1.72 ± 0.20[b]	2.83 ± 0.10[b]	3.44 ± 0.20
T19	0.5	-	-	-	0.5	100	14.16 ± 0.59	1.33 ± 0.15[b]	2.92 ± 0.20	3.61 ± 0.13
T20	0.5	-	-	-	1	100	15.33 ± 0.53	1.39 ± 0.13[b]	2.36 ± 0.11[b]	3.39 ± 0.22
T21	1	-	-	-	0.5	100	15.66 ± 0.40	1.78 ± 0.22[b]	2.64 ± 0.15[b]	3.17 ± 0.25
T22	1	-	-	-	1	100	16.50 ± 0.85	1.83 ± 0.19[b]	2.39 ± 0.24[b]	3.28 ± 0.18
T23	1.5	-	-	-	0.5	100	14.72 ± 0.49	2.00 ± 0.12[b]	2.36 ± 0.07[b]	3.33 ± 0.12
T24	1.5	-	-	-	1	100	15.50 ± 0.31	2.11 ± 0.25	2.14 ± 0.13[b]	3.00 ± 0.21
T25	0.5	-	-	-	-	100	9.94 ± 0.22[a]	1.86 ± 0.32[b]	2.17 ± 0.23[b]	2.83 ± 0.21[b]
T26	1	-	-	-	-	100	11.17 ± 0.67	2.75 ± 0.17	2.92 ± 0.20	3.20 ± 0.18
T27	1.5	-	-	-	-	100	10.72 ± 0.57	2.72 ± 0.57	2.64 ± 0.15[b]	3.16 ± 0.11
T28	2	-	-	-	-	100	10.56 ± 0.56	3.47 ± 0.37	2.81 ± 0.17[b]	2.95 ± 0.10[b]
T29*	1	1	-	-	-	100	9.50 ± 0.24[a]	4.50 ± 0.27	4.81 ± 0.12	5.08 ± 0.12
Control	-	-	-	-	-	100	19.61 ± 0.90[b]	1.28 ± 0.16[b]	2.17 ± 0.22[b]	2.44 ± 0.16[b]
CD (5%)	-	-	-	-	-	-	2.02	0.6	0.59	0.56
FV (5%)	-	-	-	-	-	-	23.06	19.31	15.33	17.96

*½ MS

Means denoted by the same letters are not significantly different (P = 0.05) using Duncan's Multiple Range Test.

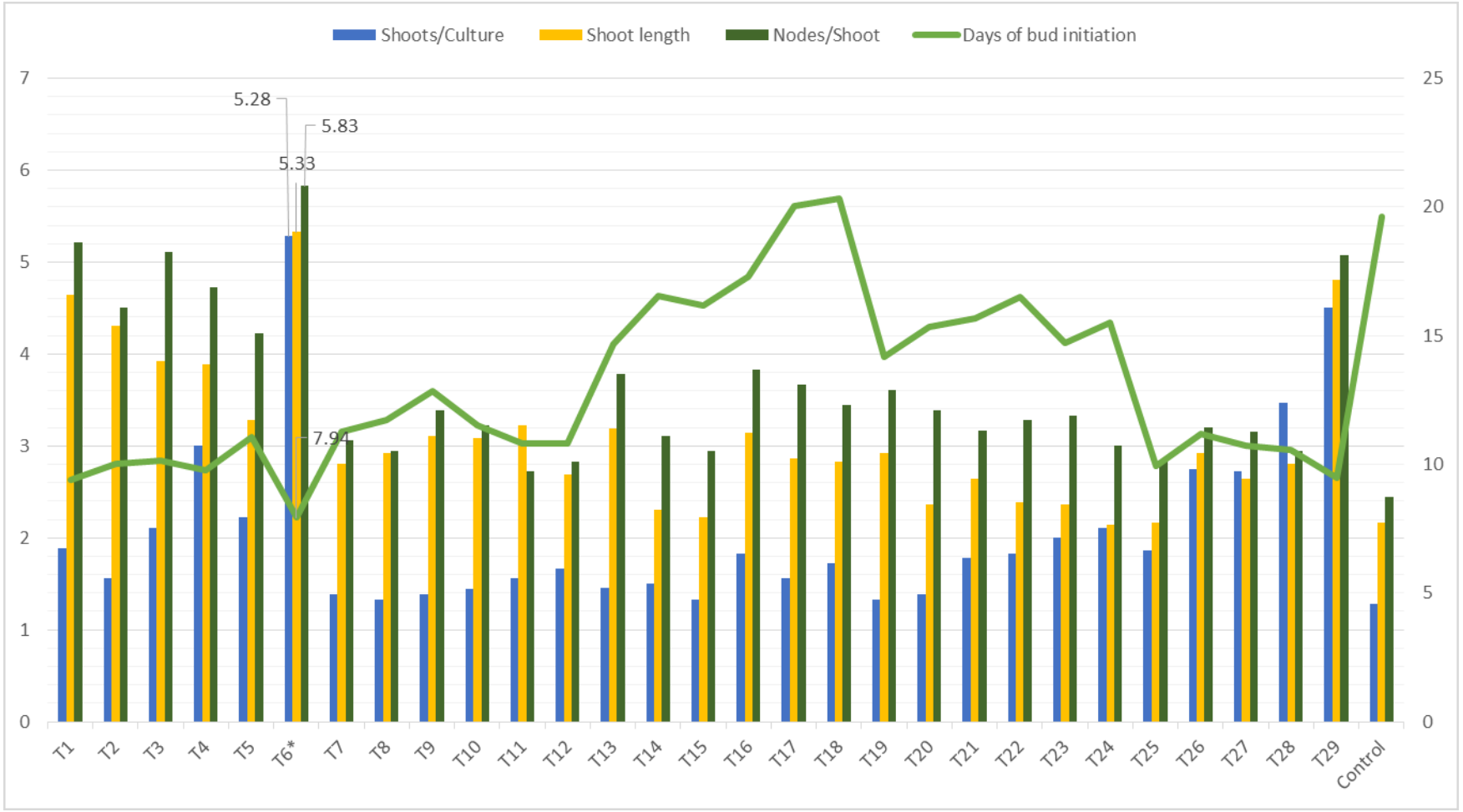
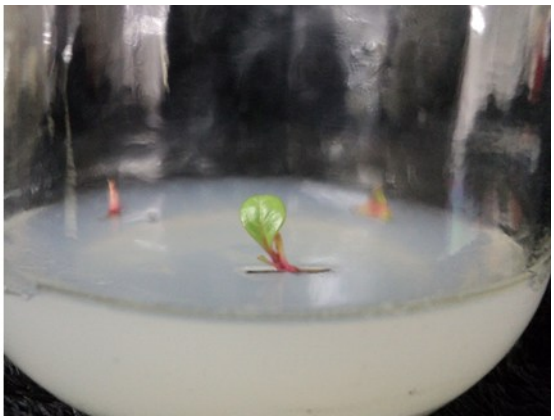


Fig 1. Effect of cytokinins and auxins on enhanced release of auxillary buds from nodal explants

A. After one week of culture

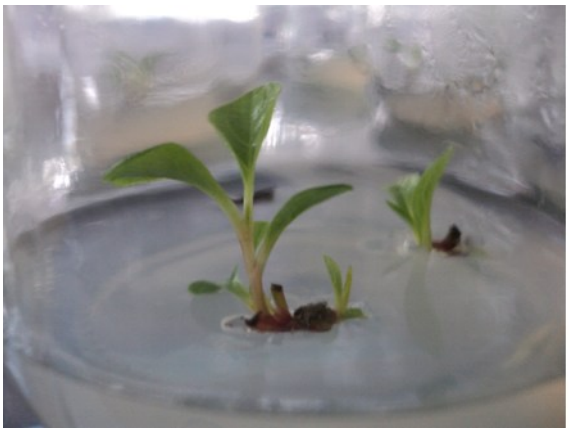


B. After 10 days of culture

C. After 2 weeks of culture

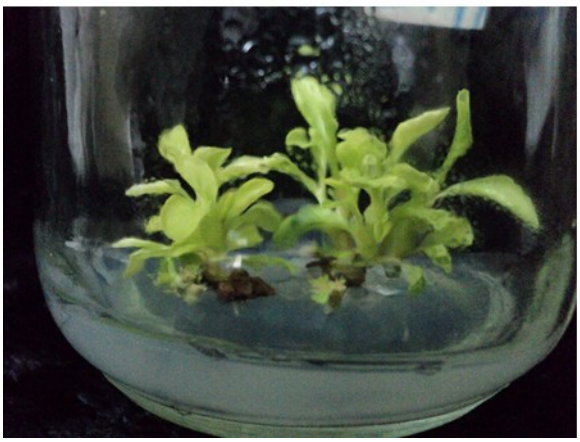
D. After three weeks of culture

Plate 4. Axillary shoot proliferation from single nodal explants in MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1}



A. After four weeks of culture

B. After four weeks of culture



C. After five weeks of culture

D. After seven weeks of culture

Plate 4. Axillary shoot proliferation from single nodal explants in MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1}, continued…

A

B

Plate 5. (A&B) Basal callusing observed in the treatment MS + BA 1.5 mg l^{-1} + NAA 1.0 mg l^{-1}

With respect to the number of nodes per shoots, treatment T6 (MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1}) gave maximum number of nodes (5.83 nodes/shoot) which was significantly different from all other treatments. The control (MS medium free of growth hormones) gave the least (2.45 nodes/shoot) number of nodes per shoot which was on par with the treatments T7, T8, T11, T12, T14, T15, T24, T25 and T28.

Earliest bud initiation, maximum shoot/culture, shoot length and nodes per shoot were obtained in T6 (MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1}). The treatments from T15 to T18 involving higher concentrations of BA and NAA showed excessive callusing (Plate 5).

4.1.2 *In vitro* Rooting

Twelve treatments with various levels of auxins (IAA, IBA and NAA) were tried for *in vitro* rooting. Result of the study are presented in Table 8 (Fig 2).

All treatments with different growth hormones resulted in cent per cent root initiation (Plate 6). No rooting was observed in control treatments with no growth regulators.

Earliest (7.28 days) root initiation was observed in R3 (NAA 1.0 mg l^{-1}) followed by R2 (NAA 0.5 mg l^{-1}). Root initiation was late (11.39 days) in R5 (IBA 0.1 mg l^{-1}) which was on par with R7, R8, R9, R11 and R12. The maximum number of roots (10.67 roots/culture) was recorded in R3 (NAA 1.0 mg l^{-1}) which was on par with R4, R5, R6, R7 and R8. The least number of roots (6.56 roots/culture) was given by R2 (NAA 0.5 mg l^{-1}) which was on par with R1, R9, R10, R11 and R12. The maximum root length (5.42 cm) was seen in R3 (NAA 1.0 mg l^{-1}) which was significantly different from all other treatments. The minimum root length (2.94 cm)

Table 8. Effect of different plant growth regulators on *in vitro* rooting

Treatment No.	NAA	IBA	IAA	Rooting (%)	Days for root initiation	Roots/culture	Root length
R1	0.1	-	-	100	9.22 ± 0.34	7.17 ± 0.45^{b}	3.69 ± 0.16
R2	0.5	-	-	100	7.78 ± 0.27^{a}	6.56 ± 0.79^{b}	3.36 ± 0.21^{b}
R3	**1.0**	**-**	**-**	**100**	**7.28 ± 0.26^{a}**	**10.67 ± 0.94^{a}**	**5.42 ± 0.29^{a}**
R4	1.5	-	-	100	8.83 ± 0.32	9.33 ± 0.33^{a}	3.58 ± 0.22^{b}
R5	-	0.1	-	100	11.39 ± 0.25^{b}	10.39 ± 0.61^{a}	3.53 ± 0.19^{b}
R6	-	0.5	-	100	9.22 ± 0.39	9.06 ± 0.44^{a}	3.53 ± 0.17^{b}
R7	-	1.0	-	100	10.94 ± 0.65^{b}	9.06 ± 0.56^{a}	3.72 ± 0.35
R8	-	1.5	-	100	10.83 ± 0.49^{b}	9.78 ± 0.70^{a}	2.94 ± 0.14^{b}
R9	-	-	0.1	100	10.22 ± 0.62^{b}	8.06 ± 0.54^{b}	3.17 ± 0.12^{b}
R10	-	-	0.5	100	10.00 ± 0.38	8.33 ± 0.46^{b}	3.92 ± 0.10
R11	-	-	1.0	100	10.42 ± 0.35^{b}	8.11 ± 0.48^{b}	4.08 ± 0.33
R12	-	-	1.5	100	10.36 ± 0.54^{b}	8.17 ± 0.41^{b}	3.50 ± 0.11^{b}
Control	-	-	-	0.00	0.00	0.00	0.00
CD (5%)	-	-	-	-	1.21	1.75	0.61
FV (5%)	-	-	-	-	8.86	4.46	8.21

Means denoted by the same letters are not significantly different (P = 0.05) using Duncan's Multiple Range Test.

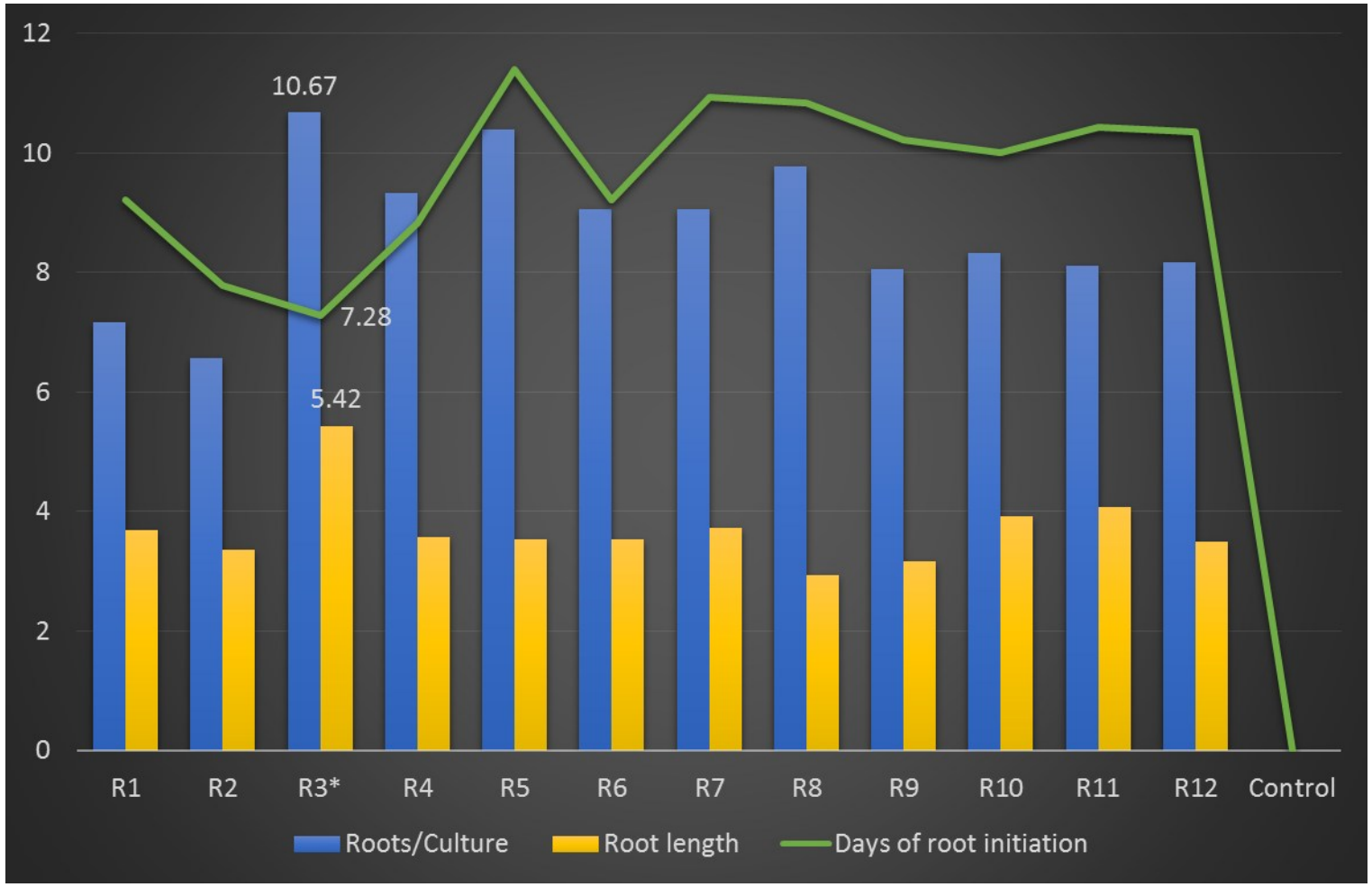


Fig 2. Effect of different plant growth regulators on *in vitro* rooting

A. After two weeks of culture

B. After three weeks of culture

C. After five weeks of culture

D. After six weeks of culture

Plate 6. *In vitro* rooting of regenerated shoots in MS + NAA 1.0 mg l^{-1}

was observed in R8 (IBA 1.5 mg l^{-1}) which was on par with R2, R4, R5, R6, R9 and R12.

4.1.3 Planting Out and Acclimatization

The *in vitro* rooted plants were carefully removed from the culture vessels and planted out in two different potting media, red soil and coir pith (3:1) and red soil and coir pith (2:1) supplemented with VAM to study the effect on *ex vitro* establishment. The survival rate of plants was estimated after six weeks of culture. There was no significant difference between the effects shown by two potting media. The plants showed a survival rate of 76 per cent and 72 per cent in the treatments PM1 (red soil:coir pith (3:1)) and PM2 (red soil:coir pith (2:1) supplemented with VAM), respectively. Plate 7 illustrates the *ex vitro* establishment of the plants.

4.2 PHASE II: *IN VITRO* CONSERVATION

4.2.1 Cryopreservation of *Plumbago rosea* Using Encapsulation Dehydration Technique

Different steps of cryopreservation using encapsulation dehydration *viz.,* preconditioning, encapsulation, pre-culture, dehydration and thawing and recovery were standardized to optimize the protocol for cryopreservation of *P. rosea*. Explants used were axillary buds.

4.2.1.1 Preconditioning

Axillary buds of *P. rosea* excised from the *in vitro* raised plantlets were preconditioned on hormone free MS medium with different concentrations of sucrose for varying time periods. The preconditioned nodes were cultured on the medium


A. Red soil: coir pith (3:1)


B. Red soil: coir pith (2:1) supplemented with VAM

Plate 7. *Ex vitro* establishment

which gave the best response for enhanced release of axillary buds. (T6 - MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1}). The results obtained are illustrated in Table 9 (Fig 3).

All the treatments except PC10 (sucrose 1 *M* for 1 day) PC11 (sucrose 1.0 *M* for 7 days) and PC12 (sucrose 1.0 *M* for 14 days) showed cent per cent survival (Plate 8). The treatments PC10, PC11 and PC12 gave a survival rate of 38.89 per cent, 16.67 per cent and zero survival respectively.

The days for bud initiation was the earliest (8.56 days) in the treatment PC8 (sucrose 0.5 *M* for 7 days) which was on par with PC2, PC3, PC7 and PC9. Bud initiation was late (13.72 days) in PC4 (sucrose 0.3 *M* for 1 day) followed by PC5 (sucrose 0.3 *M* for 14 days).

Regarding the number of shoots produced, the treatment PC8 (sucrose 0.5 *M* for 7 days) gave the best result (2.67 shoots/culture) which was significantly different from all other treatments. The least number of shoots (1.33 shoots/culture) was produced in PC5 (sucrose 0.3 *M* for 14 days) which was on par with treatments PC1 to PC4, PC6, PC7 and PC9.

Shoot length was maximum (4.89 cm) in the treatment PC8 (sucrose 0.5 *M* for 7 days) which was followed by PC3 (sucrose 0.1 *M* for 14 days). Minimum length of shoot (3.45 cm) was recorded in the treatment PC2 (sucrose 0.1 *M* for 7 days) which was on par with PC1, PC4 to PC7 and PC9.

Maximum number of nodes per shoot (4.39 nodes/shoot) was recorded in the treatment PC8 (sucrose 0.5 *M* for 7 days) which was significantly different from all the other treatments. The minimum (2.83 nodes/shoot) number of nodes was recorded in PC3 (sucrose 0.1 *M* for 14 days) which was on par with PC1, PC2, PC4, PC5, PC6 and PC9.

Table 9. Effect of preconditioning duration and sucrose concentration on axillary bud proliferation

Treatment No.	Sucrose (*M*)	No. of days	Survival (%)	Regeneration (%)	Days for bud initiation	Shoots/culture	Shoot length	Nodes/shoot
PC1	0.1	1	100	100	10.78 ± 0.22	1.67 ± 0.12^{b}	3.55 ± 0.19^{b}	3.06 ± 0.22^{b}
PC2	0.1	7	100	100	9.39 ± 0.71^{a}	1.39 ± 0.13^{b}	3.45 ± 0.40^{b}	3.22 ± 0.14^{b}
PC3	0.1	14	100	100	8.94 ± 0.40^{a}	1.56 ± 0.14^{b}	4.33 ± 0.24^{a}	2.83 ± 0.17^{b}
PC4	0.3	1	100	100	13.72 ± 0.47^{b}	1.45 ± 0.14^{b}	3.86 ± 0.26^{b}	3.00 ± 0.17^{b}
PC5	0.3	7	100	100	12.67 ± 0.37^{b}	1.33 ± 0.12^{b}	3.64 ± 0.22^{b}	2.94 ± 0.26^{b}
PC6	0.3	14	100	100	10.94 ± 0.93	1.39 ± 0.10^{b}	3.72 ± 0.16^{b}	3.22 ± 0.11^{b}
PC7	0.5	1	100	100	9.33 ± 0.26^{a}	1.72 ± 0.20^{b}	4.14 ± 0.15^{b}	3.50 ± 0.14
PC8	**0.5**	**7**	**100**	**100**	**8.56 ± 0.31^{a}**	**2.67 ± 0.19^{a}**	**4.89 ± 0.13^{a}**	**4.39 ± 0.32^{a}**
PC9	0.5	14	100	100	8.67 ± 0.49^{a}	1.55 ± 0.20^{b}	4.11 ± 0.29^{b}	3.44 ± 0.14^{b}
PC10*	1.0	1	38.89	27.7	-	-	-	-
PC11*	1.0	7	16.67	11.11	-	-	-	-
PC12*	1.0	14	0.00		-	-	-	-
CD (5%)	-	-	-		1.45	0.44	0.68	0.56
FV (5%)	-	-	-		13.14	6.93	3.62	5.69

*Treatments which failed to give >40% survival

Means denoted by the same letters are not significantly different (P = 0.05) using Duncan's Multiple Range Test.

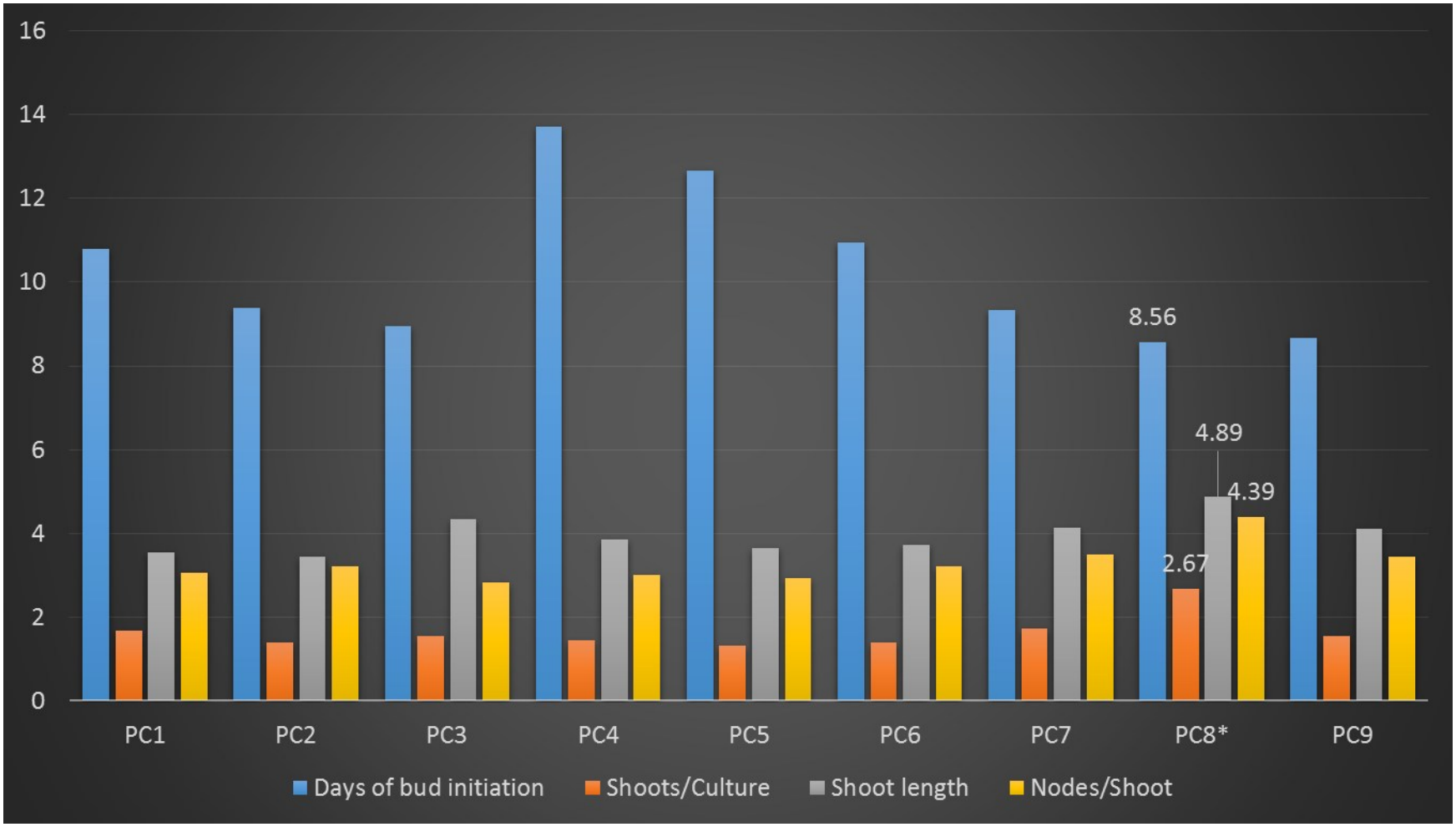
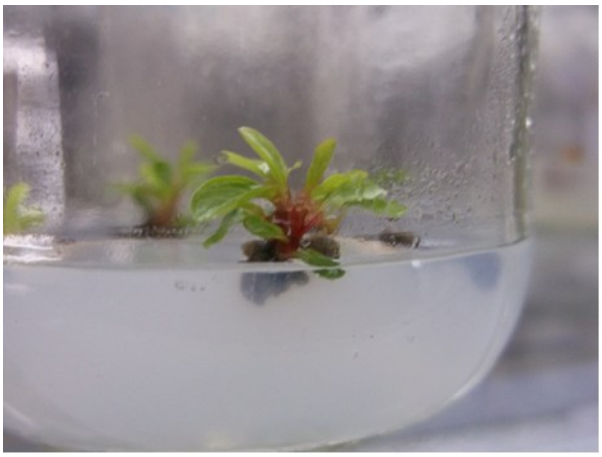


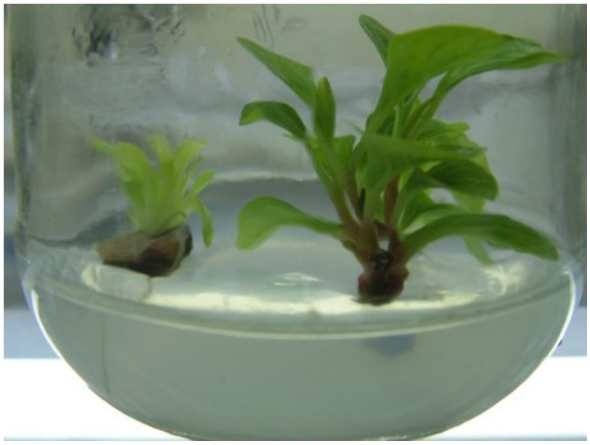
Fig 3. Effect of preconditioning duration and sucrose concentration on axillary bud proliferation



A. After two weeks of culture

B. After three weeks of culture

C. After four weeks of culture



D. After four weeks

Plate 8. Shoot proliferation in preconditioned (sucrose 0.5 *M* in MS for 7 days) axillary buds in MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1}

Analysing the result, the best preconditioning treatment was found to be PC8 (sucrose 0.5 *M* for 7 days)

4.2.1.2 Encapsulation

Single nodal segments were encapsulated using different concentrations of sodium alginate (2.5, 3. 4 and 5 % m/v) and calcium chloride (50, 75, 100 and 200 *mM*). Sixteen treatments were tried. The sodium alginate beads formed after encapsulation were cultured on the medium which gave the best response for enhanced release of axillary buds. (T6 - MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1}). The results are given in Table 10 (Fig 4).

All the sixteen treatments gave cent per cent survival. Compared to the regeneration after preconditioning, the days taken for bud initiation after encapsulation was late. Also the values obtained for shoots per culture, shoot length and nodes per shoot were less, after six weeks of observation. The regeneration of the plants from encapsulated beads are illustrated in Plate 9.

The days for bud initiation was earliest (9.19 days) in the treatment E3 (MS + SA 2.5 % + $CaCl_2$ 100 *mM*) which was significantly different from all the other treatments. Bud initiation was late (17.53 days) in E12 (MS + SA 4.0 % + $CaCl_2$ 200 *mM*), which was on par with the treatments E9, E10, E11 and E16.

The treatment E3 (SA 2.5 % + $CaCl_2$ 100 *mM*) gave the maximum number of shoots per culture (2.31 shoots/culture) which was on par with E1, E2, E4, E6, E7, E13, E15 and E16. The least number of shoots per culture (1.39 shoots/culture) was recorded in the treatment E12 (MS + SA 4.0 % + $CaCl_2$ 200 *mM*), which was on par with all the treatments except E3, E4, E6, E12 and E15.

Table 10. Effect of encapsulation using different concentration of sodium alginate and calcium chloride on axillary bud proliferation

Treatment No.	SA (%)	$CaCl_2$ (*mM*)	Survival (%)	Days for bud initiation	Shoots/culture	Shoot length	Nodes/shoot
E1	2.5	50	100	12.08 ± 0.38^{b}	1.83 ± 0.19^{ab}	2.51 ± 0.20^{ab}	2.94 ± 0.26^{b}
E2	2.5	75	100	11.44 ± 0.38^{b}	1.78 ± 0.10^{ab}	2.40 ± 0.26^{b}	3.00 ± 0.22^{ab}
E3	**2.5**	**100**	**100**	**9.19 ± 0.39^{a}**	**2.31 ± 0.16^{a}**	**3.35 ± 0.21^{a}**	**3.75 ± 0.08^{a}**
E4	2.5	200	100	11.83 ± 0.50^{b}	2.08 ± 0.25^{a}	1.89 ± 0.20^{b}	2.53 ± 0.14^{b}
E5	3.0	50	100	12.75 ± 0.60^{b}	1.56 ± 0.19^{b}	1.93 ± 0.30^{b}	2.47 ± 0.25^{b}
E6	3.0	75	100	11.67 ± 0.59^{b}	2.06 ± 0.27^{a}	2.56 ± 0.11^{ab}	3.00 ± 0.17^{ab}
E7	3.0	100	100	11.72 ± 0.39^{b}	1.89 ± 0.31^{ab}	2.83 ± 0.43^{a}	3.36 ± 0.29^{a}
E8	3.0	200	100	13.47 ± 0.42^{b}	1.61 ± 0.16^{b}	2.04 ± 0.23^{b}	2.78 ± 0.28^{b}
E9	4.0	50	100	16.36 ± 0.70^{b}	1.44 ± 0.13^{b}	2.36 ± 0.15^{b}	2.83 ± 0.17^{b}
E10	4.0	75	100	15.83 ± 0.85^{b}	1.47 ± 0.18^{b}	1.90 ± 0.15^{b}	2.47 ± 0.10^{b}
E11	4.0	100	100	16.42 ± 0.72^{b}	1.56 ± 0.19^{b}	2.67 ± 0.08^{ab}	3.08 ± 0.08^{ab}
E12	4.0	200	100	17.53 ± 1.28^{b}	1.39 ± 0.18^{b}	1.81 ± 0.23^{b}	2.53 ± 0.18^{b}
E13	5.0	50	100	12.28 ± 0.69^{b}	1.86 ± 0.21^{ab}	2.79 ± 0.41^{a}	3.31 ± 0.33^{a}
E14	5.0	75	100	11.25 ± 0.51	1.44 ± 0.11^{ab}	3.29 ± 0.33^{a}	3.72 ± 0.28^{a}
E15	5.0	100	100	13.61 ± 0.74	2.06 ± 0.13^{a}	3.10 ± 0.34^{a}	3.72 ± 0.39^{a}
E16	5.0	200	100	16.08 ± 1.16^{b}	1.72 ± 0.13^{ab}	3.01 ± 0.22^{a}	3.44 ± 0.16^{a}
CD (5%)	-	-	-	1.96	0.53	0.73	0.65
FV (5%)	-	-	-	11.79	2.11	3.90	3.86

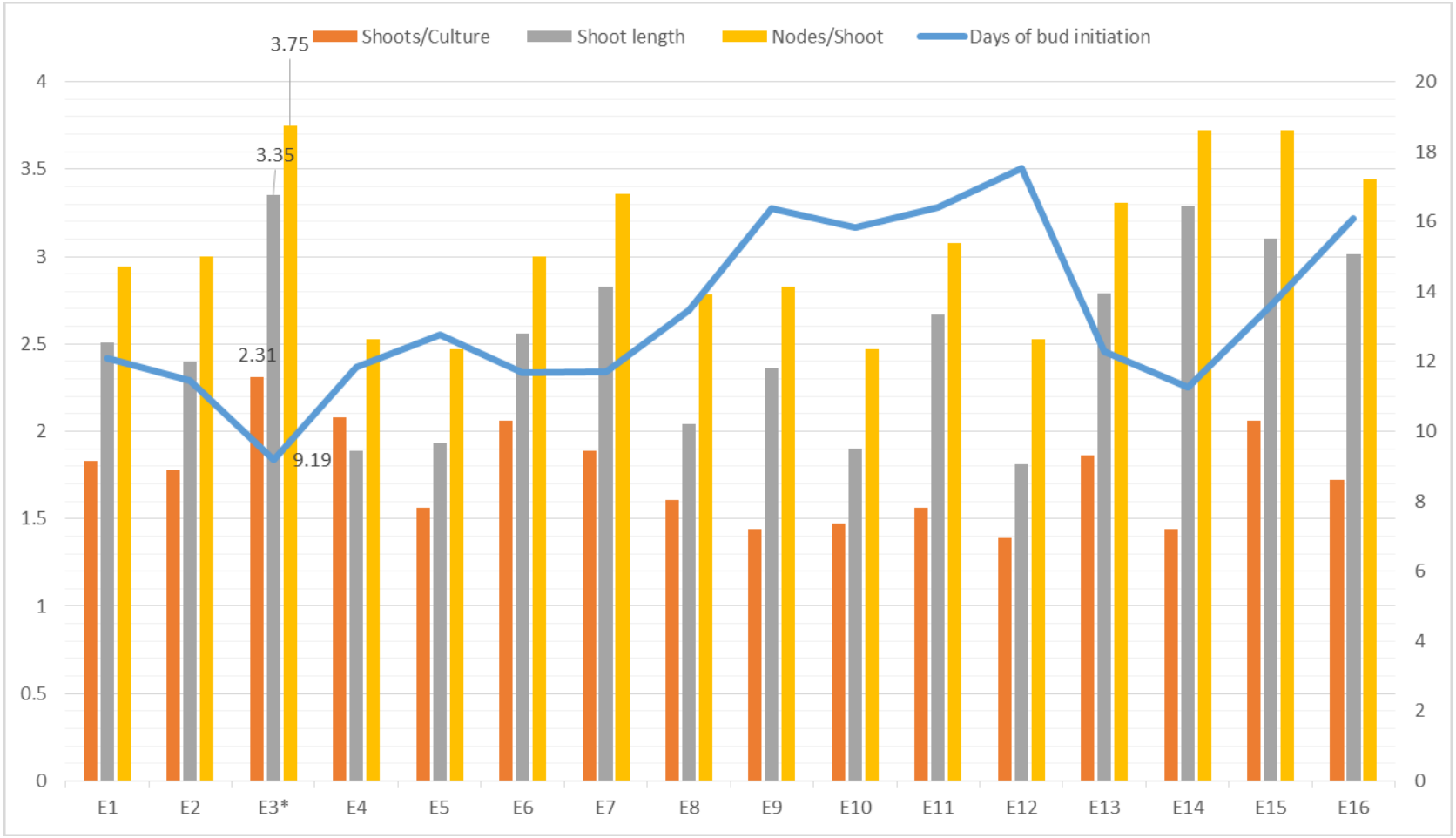
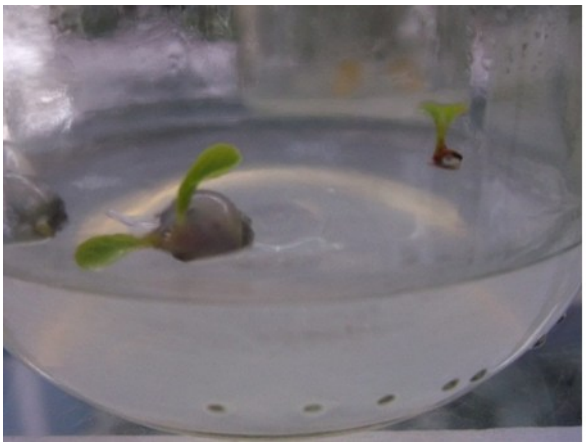


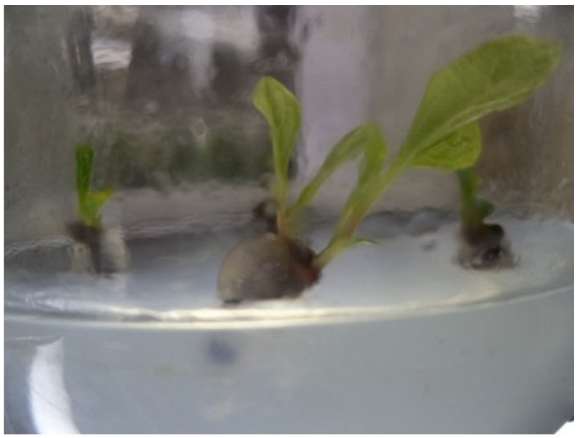
Fig 4. Effect of encapsulation using different concentration of sodium alginate and calcium chloride on axillary bud proliferation

A. Encapsulated axillary buds

B. Encapsulated axillary buds



C. After two weeks of culture



D. After four weeks of culture

Plate 9. Shoot proliferation of encapsulated (MS - glycine + SA 2.5% + $CaCl_2$ 100 *mM*) axillary buds in MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1}

Shoot length was maximum (3.35 cm) in the treatment E3 (SA 2.5 % + $CaCl_2$ 100 *mM*), which was on par with E1, E6, E7, E11 and E13 to E16. The minimum (1.81 cm) shoot length was observed in the treatment E12 (MS + SA 4.0 % + $CaCl_2$ 200 *mM*), which was on par with E1, E2, E4, E5, E6 and E8 to E12.

Maximum number of nodes per shoot (3.75 nodes/shoot) was registered in the treatment E3 (MS + SA 2.5 % + $CaCl_2$ 100 *mM*), which was on par with E2, E6, E7, E11 and E13 to E16. The minimum number of nodes per shoot (2.47 nodes/shoot) was observed in E5 (MS + SA 3.0 % + $CaCl_2$ 50 *mM*) which was on par with E1, E2, E4, E5, E6 and E8 to E12.

With respect to the results obtained, the best encapsulation treatment was found to be E3 (MS + SA 2.5 % + $CaCl_2$ 100 *mM*).

4.2.1.3 Pre-culture

Single nodal segments, those gave the best preconditioning treatment (PC8 - sucrose 0.5 *M* for 7 days) were encapsulated using the best treatment (E3 - SA 2.5 % + $CaCl_2$ 100 *mM*) obtained after encapsulation studies and the beads were then given twenty pre-culture treatments involving different concentrations of sucrose (0.1, 0.3, 0.5, 0.75, and 1.0 *M*) for different time periods (1, 3, 5 and 7 days) in hormone free MS liquid medium with 3 per cent DMSO in every treatments. The beads were taken out from the medium after the respective periods of culturing and were cultured on the medium which gave the best response for enhanced release of axillary buds (T6 - MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1}). The results obtained are illustrated in Table 11 (Fig 5).

A cent per cent survival rate was recorded in all the treatments except treatments from PT11 to PT20. Evidently higher concentrations of sucrose (0.75 *M* and 1.0 *M*) did not support the growth and regeneration of the plants irrespective of

the different time periods tried. However the treatments PT11 (sucrose 0.5 *M* for 5 days), PT12 (sucrose 0.5 *M* for 7 days), PT 13 (sucrose 0.75 *M* for 1 day) and PT14 (sucrose 0.75 *M* for 3 days) were given a survival per cent of 88.89, 77.78, 16.67 and 11.11 respectively while all the other treatments with higher concentrations of sucrose yielded zero per cent survival. Also, all the survived treatments showed a lag for bud initiation compared to the normal *in vitro* regeneration. The regeneration of the plants after pre-culture is illustrated in Plate 10.

The days taken for bud initiation was the earliest (18.94 days) in the treatment PT10 (sucrose 0.5 *M* for 3 days) which was on par with the treatments PT1, PT2, PT3 and PT5. Bud initiation was late (22.81 days) in the treatment PT12 (sucrose 0.5 *M* for 7 days) which was on par with PT3, PT4 and PT6 to PT9 and PT11.

Number of shoots formed per culture was the most (3.44 shoots/culture) in the treatment PT10 (sucrose 0.5 *M* for 3 days) which was significantly different from all the other treatments. The least number of shoots per culture (1.5 shoots/culture) was observed in PT1 (sucrose 0.1 *M* for 1 day) which was on par with PT2 to PT6 and PT7.

The maximum length of shoot (4.36 cm) was recorded in PT10 (sucrose 0.5 *M* for 3 days) followed by PT11 (sucrose 0.5 *M* for 5 days). The minimum value of shoot length (2.72 cm) was seen in the treatment PT2 (sucrose 0.1 *M* for 3 days), which was on par with PT1 to PT8 and PT12.

The number of nodes formed per shoot was the maximum (4.33 nodes/shoot) in PT10 (sucrose 0.5 *M* for 3 days) followed by PT11 (sucrose 0.5 *M* for 5 days). The minimum number of nodes per shoot (3.00 nodes/shoot) was seen in the treatment PT9 (sucrose 0.5 *M* for 1 day), which was on par with PT1 to PT8 and PT12.

Table 11. Effect of pre-culture duration and sucrose concentration on axillary bud proliferation

Treatment No.	Sucrose (*M*)	No. of days	Survival (%)	Regeneration (%)	Days for bud initiation	Shoots/culture	Shoot length	Nodes/shoot
PT1	0.1	1	100	100	20.22 ± 0.56^{a}	1.50 ± 0.14^{b}	3.25 ± 0.20^{b}	3.33 ± 0.12^{b}
PT2	0.1	3	100	100	19.72 ± 0.39^{a}	1.83 ± 0.19^{b}	2.72 ± 0.16^{b}	3.28 ± 0.18^{b}
PT3	0.1	5	100	100	20.89 ± 0.7^{ab}	1.67 ± 0.15^{b}	2.97 ± 0.16^{b}	3.28 ± 0.18^{b}
PT4	0.1	7	100	100	22.00 ± 0.58^{b}	1.61 ± 0.16^{b}	2.78 ± 0.19^{b}	3.56 ± 0.14^{b}
PT5	0.3	1	100	100	19.83 ± 0.56^{a}	1.83 ± 0.19^{b}	2.89 ± 0.22^{b}	3.22 ± 0.19^{b}
PT6	0.3	3	100	100	20.94 ± 0.61^{b}	1.89 ± 0.22^{b}	3.25 ± 0.18^{b}	3.11 ± 0.22^{b}
PT7	0.3	5	100	100	21.06 ± 0.83^{b}	1.78 ± 0.20^{b}	2.89 ± 0.10^{b}	3.28 ± 0.16^{b}
PT8	0.3	7	100	100	21.61 ± 0.42^{b}	2.39 ± 0.16	3.31 ± 0.31^{b}	3.22 ± 0.20^{b}
PT9	0.5	1	100	100	22.33 ± 0.50^{b}	2.22 ± 0.14	3.67 ± 0.32	3.00 ± 0.15^{b}
PT10	**0.5**	**3**	**100**	**100**	**18.94 ± 1.02^{a}**	**3.44 ± 0.22^{a}**	**4.36 ± 0.08^{a}**	**4.33 ± 0.19^{a}**
PT11	0.5	5	88.89	83.33	21.39 ± 0.43^{b}	2.72 ± 0.29	3.81 ± 0.41^{a}	3.94 ± 0.26^{a}
PT12	0.5	7	77.78	61.11	22.81 ± 0.57^{b}	2.28 ± 0.20	3.22 ± 0.23^{b}	3.36 ± 0.12^{b}
PT13*	0.75	1	16.67	11.11				
PT14*	0.75	3	11.11	5.56	-	-	-	-
PT15*	0.75	5	-		-	-	-	-
PT16*	0.75	7	-		-	-	-	-
PT17*	1.0	1	-		-	-	-	-
PT18*	1.0	3	-		-	-	-	-
PT19*	1.0	5	-		-	-	-	-
PT20*	1.0	7	-		-	-	-	-
CD (5%)	-	-	-		1.76	0.55	0.65	0.51
FV (5%)	-	-	-		3.39	8.27	4.36	4.27

*Treatments which failed to give >40 % survival

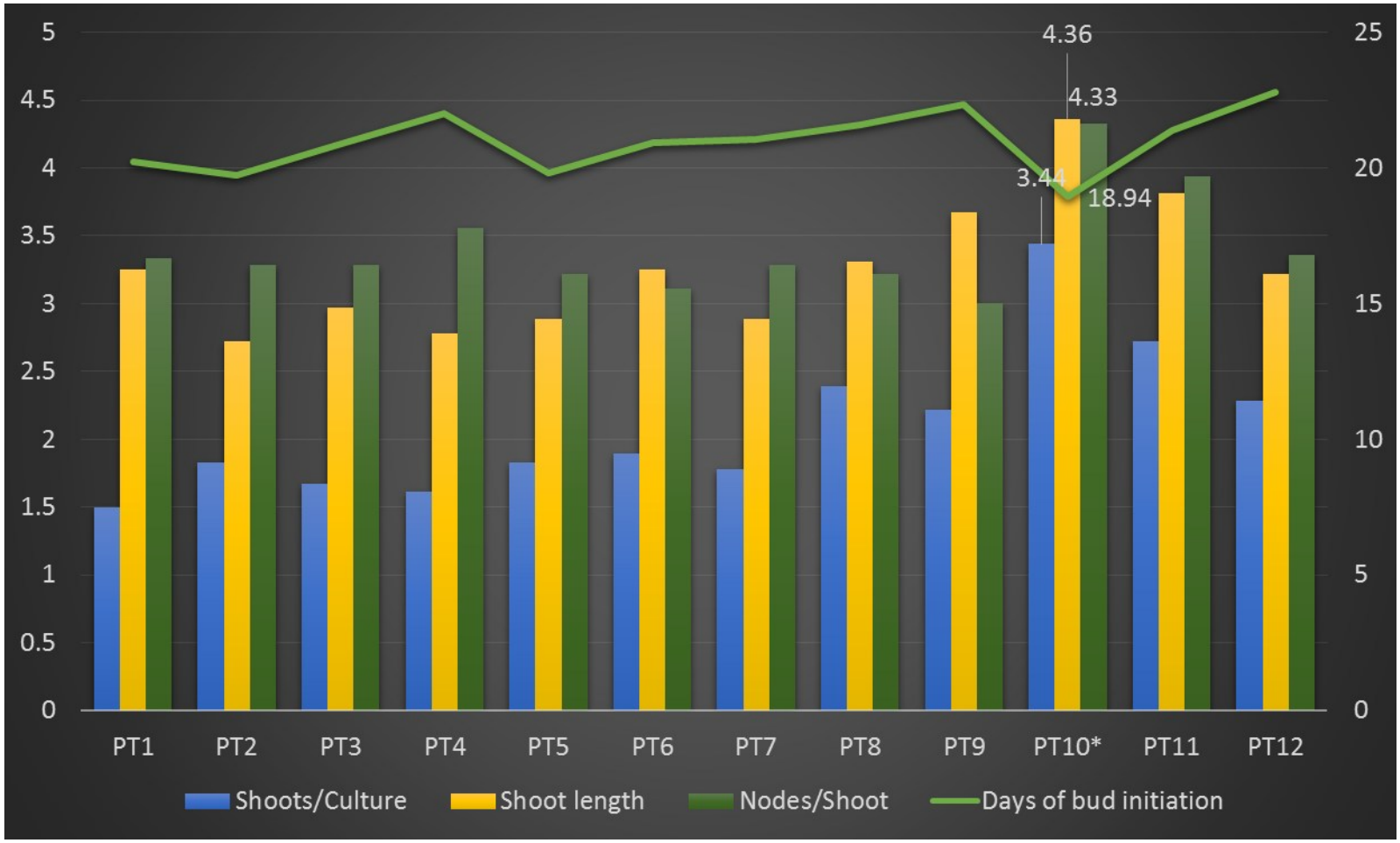
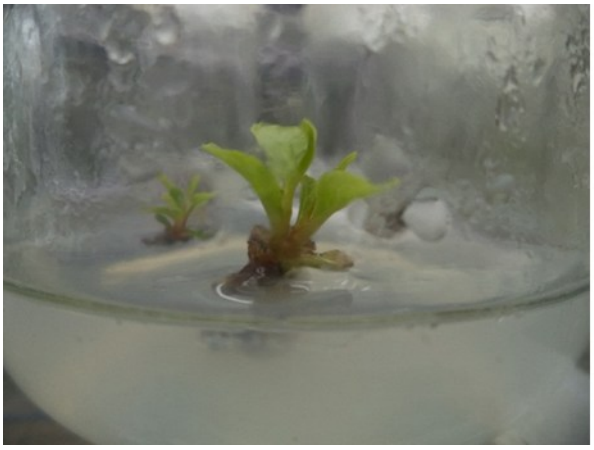
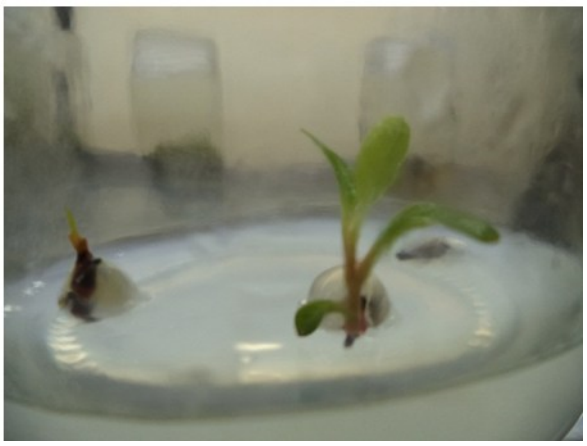


Fig 5. Effect of pre-culture duration and sucrose concentration on axillary bud proliferation



A. After 25 days of culture



B. After 30 days of culture

C. After five weeks

D. After six weeks

Plate 10. Regeneration of preconditioned, encapsulated and pre-cultured (Liquid MS + 3 % DMSO + sucrose 0.5 *M* for 3 days) axillary buds in MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1}

From the results obtained, the treatment PT10 (sucrose 0.5 *M* for 3 days) was selected as the best treatment.

4.2.1.4 Dehydration, Freezing in LN and Recovery

After preconditioning, encapsulation and pre-culture treatments with the best results obtained in the respective fields, the encapsulated axillary buds were subjected to different hours of dehydration (0 h, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h and 7 h). After cryopreservation of the dehydrated samples for at least 2 h in LN (Plate 11), they were rewarmed and placed in the recovery medium to study the rate of survival and regeneration. Moisture content was determined after different periods of dehydration on a fresh weight basis. The results are illustrated in the Table 12 (Fig 6).

Alginate beads encapsulating the axillary buds possessed 71.82 per cent moisture before desiccation. With no desiccation (0 h), the control plants (-LN) gave cent per cent survival and regeneration. After dehydration under laminar air flow for 6h, the moisture content of the beads was reduced to 15.43 per cent. Encapsulated axillary buds of control plants (-LN) dehydrated to 18.13 per cent (5 h) and 15.43 per cent (6 h) resulted in 72.22 per cent and 61.11 per cent survival, respectively.

Survival (indicated by green colouration of the explant) and regeneration (indicated by emergence of shoot from axillary bud) of cryopreserved axillary buds were observed for those undergone 5 h and 6 h desiccation. At 5 h desiccation, the plants immersed in LN gave 66.67 per cent survival and 62.50 per cent regeneration. Survival recorded for cryopreserved plants at 6 h desiccation was 45.83 per cent and regeneration was 41.67 per cent. All other desiccation hours yielded no survival and regeneration after recovery from liquid nitrogen. Plate 12 illustrates the regeneration after immersion in liquid nitrogen.

Table 12. Effect of desiccation of encapsulated axillary buds on moisture content, survival and plant regeneration with or without cryopreservation

Dehydration duration (h)	MC (%)	Survival (%)		Regeneration (%)	
		-LN	+LN	-LN	+LN
D0	71.82	100	0.00	100	0.00
D1	56.11	100	0.00	95.83	0.00
D2	43.07	91.67	0.00	83.33	0.00
D3	32.68	83.33	0.00	79.17	0.00
D4	24.87	75.00	0.00	70.83	0.00
D5	18.13	72.22	66.67	66.67	62.50
D6	15.43	61.11	45.83	55.56	41.67
D7	13.71	33.33	0.00	0.00	0.00

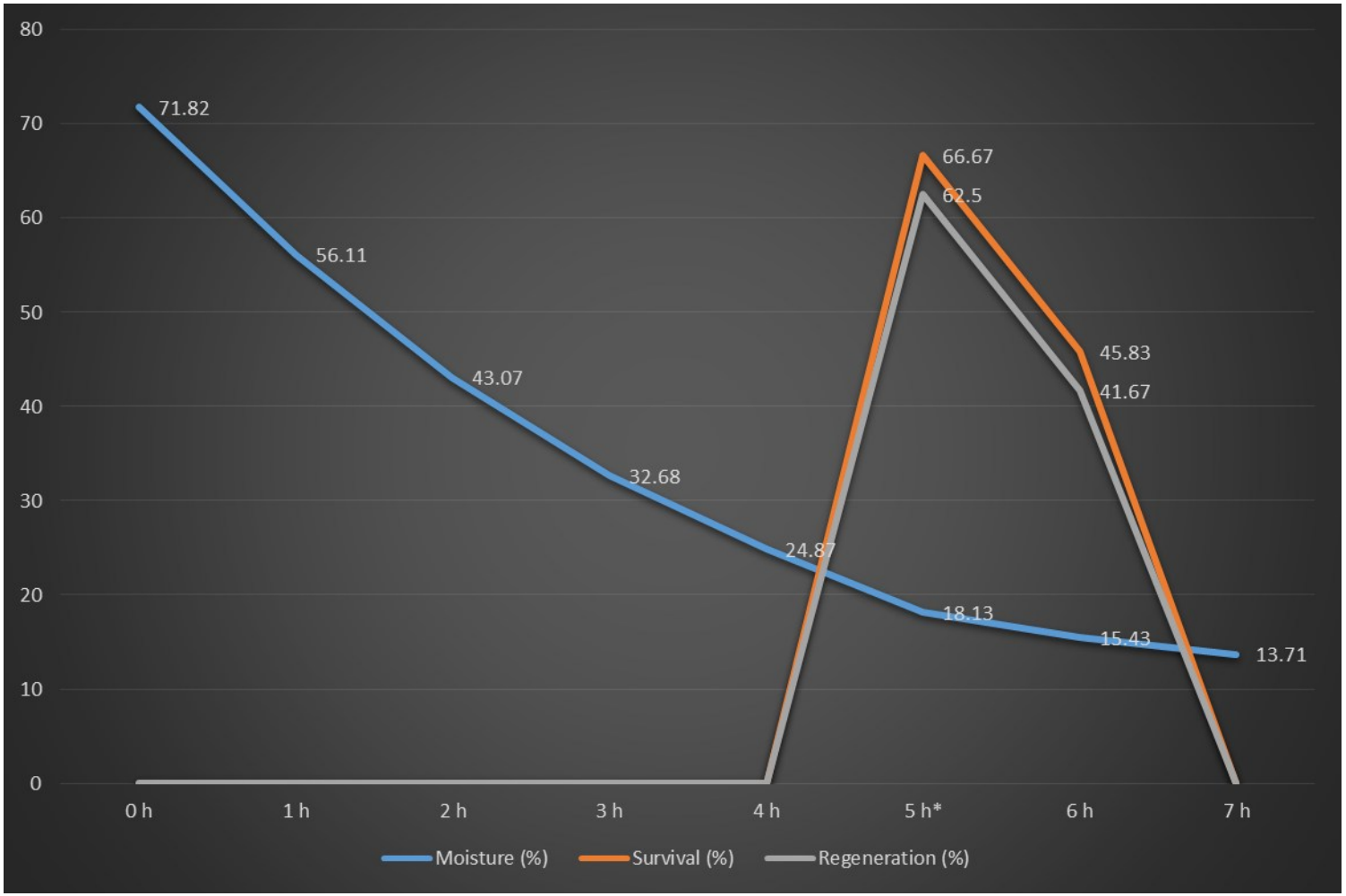


Fig 6. Effect of dehydration duration on moisture content, survival and regeneration in cryo recovered beads

The days of bud initiation when cultured on recovery medium after cryopreservation with 5 h and 6 h desiccation was 25.39 days and 26.28 days respectively. Shoots per culture obtained was 4.06 for 5 h desiccation and 3.33 for 6 h desiccation. Shoot length was maximum (3.56 cm) for 5 h desiccation and 6 h desiccation yielded a shoot length of 2.89 cm. Maximum number of nodes per shoot (3.58 nodes/shoot) was observed for 5 h desiccation while 6 h desiccation gave 2.86 nodes per shoot. The results are given in Table 13.

Root initiation was 100 per cent for both 5 h and 6 h desiccation periods. Days taken for root initiation was earliest (9.22 days) in 5 h while 6 h desiccation took 11.22 days for root initiation. Roots per culture was maximum (8.39 roots/culture) in 6 h desiccation, however it was on par with the value given by 5 h desiccation (8.28 roots/culture). Root length was maximum (4.17 cm) for 6 h desiccation period while 5 h desiccation gave a root length of 3.81cm. The results are presented in Table 14.

The beads when stored in liquid nitrogen for different duration and cultured on recovery medium did not show any significant variation with respect to survival and regeneration per cent (Table 15).

4.3 Estimation of Genetic Stability of Cryopreserved Materials Using RAPD

RAPD profiles of plantlets regenerated from cryopreserved axillary buds were compared with those of the *in vitro* grown plantlets to assess the genetic fidelity. All the six primers produced clear and reproducible bands. The number of bands of each primer varied from 2 to 9.

RAPD profiles of the plantlets regenerated from cryopreserved materials were identical to those of control plants for all the six primers tested. Plate 13 illustrates amplified band patterns produced by six primers in plantlets regenerated after

Table 13. Effect of desiccation on proliferation of axillary buds with or without cryopreservation

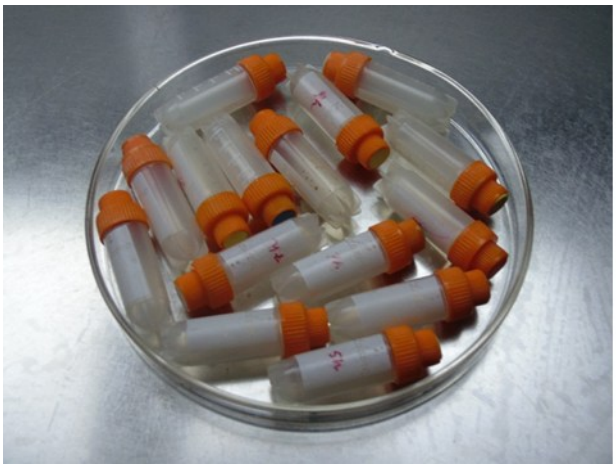
Dehydration duration (h)	Days for bud initiation		Shoots/culture		Shoot length		Nodes/shoot	
	-LN	+LN	-LN	+LN	-LN	+LN	-LN	+LN
D0	20.28 ± 0.71	-	3.56 ± 0.24	-	3.97 ± 0.18	-	3.97 ± 0.22	-
D1	21.00 ± 0.70	-	2.44 ± 0.20	-	3.94 ± 0.25	-	3.64 ± 0.42	-
D2	22.11 ± 0.59	-	2.72 ± 0.23	-	4.03 ± 0.37	-	4.17 ± 0.33	-
D3	20.33 ± 0.58	-	2.78 ± 0.47	-	3.56 ± 0.33	-	3.72 ± 0.45	-
D4	21.00 ± 0.64	-	2.33 ± 0.34	-	3.42 ± 0.37	-	3.67 ± 0.27	-
D5	21.00 ± 0.91	25.39 ± 0.45	2.61 ± 0.34	4.06 ± 0.23	3.56 ± 0.25	3.56 ± 0.35	4.00 ± 0.34	3.58 ± 0.16
D6	22.17 ± 0.65	26.28 ± 0.42	2.83 ± 0.25	3.33 ± 0.12	2.31 ± 0.25	2.89 ± 0.10	3.22 ± 0.25	2.86 ± 0.28
D7	-	-	-	-	-	-	-	-
CD (5%)	1.99	1.37	0.89	0.59	0.84	0.81	0.96	0.72
FV (5%)	1.2	3.97	1.64	7.48	4.13	3.36	0.86	5.01

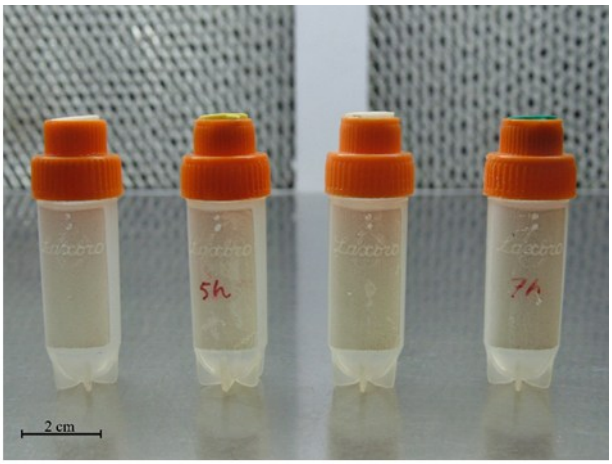
Table 14. Effect of desiccation on *in vitro* rooting with or without cryopreservation

Dehydration duration (h)	Rooting (%)		Days for root initiation		Roots/culture		Root length	
	-LN	+LN	-LN	+LN	-LN	+LN	-LN	+LN
D0	100	-	9.50 ± 0.48	-	9.72 ± 0.45	-	3.78 ± 0.18	-
D1	100	-	11.39 ± 0.50	-	8.33 ± 0.66	-	3.44 ± 0.17	-
D2	100	-	11.00 ± 0.35	-	8.89 ± 0.73	-	3.86 ± 0.11	-
D3	100	-	10.39 ± 0.58	-	9.22 ± 0.70	-	3.53 ± 0.24	-
D4	100	-	10.44 ± 0.35	-	9.06 ± 0.54	-	3.64 ± 0.18	-
D5	100	100	11.25 ± 0.28	9.22 ± 0.29	9.39 ± 0.77	8.28 ± 0.60	3.58 ± 0.35	3.81 ± 0.24
D6	100	100	10.42 ± 0.47	11.22 ± 0.48	8.17 ± 0.49	8.39 ± 0.53	3.36 ± 0.27	4.17 ± 0.29
D7	-	-	-	-	-	-	-	-
CD (5%)	-	-	1.27	1.25	1.81	1.78	0.65	0.84
FV (5%)	-	-	2.14	12.68	0.78	0.02	0.62	0.92

Table 15. Effect of cryopreservation duration on survival (%) and regeneration (%) of axillary buds

	Cryopreservation duration							
Dehydration duration (h)	2 h		1 day		1 week		1 month	
	SP (%)	RG (%)	SP (%)	RG (%)	SP (%)	RG (%)	SP (%)	RG (%)
D5	65.63	59.38	66.67	62.50	63.33	63.33	63.89	61.11
D6	43.75	40.63	45.83	41.67	46.67	40.00	44.44	41.67





A. Cryovials

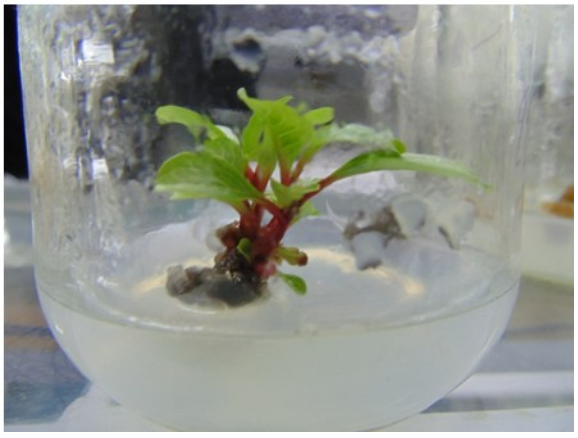
B. Rapid plunging in LN

Plate 11. Freezing in LN

A. After four weeks of culture

B. After five weeks of culture

C. After six weeks of culture



D. After seven weeks of culture

Plate 12. Regeneration of encapsulated, pre-cultured, dehydrated (5 h) and cryopreserved beads in MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1}

cryopreservation and control plants. No differences were observed in the banding pattern of control and cryopreserved samples.

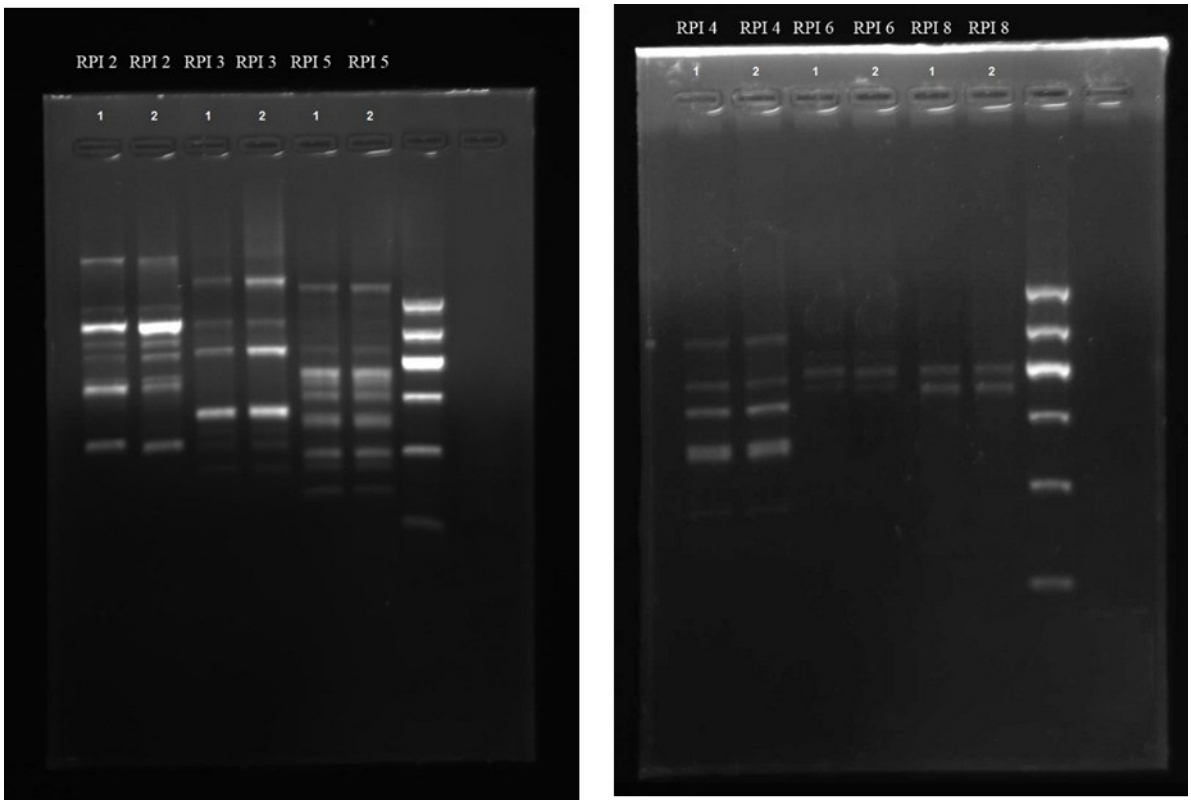


Plate 13. Comparison of RAPD banding profile between plants recovered from LN and non cryopreserved control plantlets

Discussion

5. DISCUSSION

The present study, 'Cryopreservation of Chethikoduveli (*Plumbago rosea* L.) and assessment of genetic fidelity of regenerated plantlets using molecular markers', was carried out during 2011-2013 at the Department of Plant Biotechnology, College of Agriculture, Vellayani. The results obtained are discussed in this chapter.

5.1 *IN VITRO* REGENERATION

P. rosea rarely sets seed and hence conventionally propagated by stem cuttings. Though cultivated on a small scale, due to increased demand, the roots of *P. rosea* are heavily collected from the wild. *In vitro* culture technique enables rapid and large scale multiplication of this plant. Also, pathogen free plantlets can be produced by this method. *In vitro* propagation is used widely for the commercial propagation of a large number of plant species, including medicinal plants (Rout *et al*., 2000).

5.1.1 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). In the present study, enhanced release of axillary buds was attempted using nodal segments having single axillary bud. The right choice of the explant is important for the success of any *in vitro* propagation system. It depends on the kind of culture to be initiated, the purpose of the proposed culture and the plant species to be used. Maintaining the donor plants in clean and controlled environmental conditions delivers healthy and sterile explants (Sagare *et al.,* 2001).

5.1.1.1 Surface Sterilization

Explants collected from field grown plants are usually contaminated by various microorganisms. Hence the surface sterilization of the explant is a critical procedure to establish healthy and disease free cultures (Rout *et al.,* 2000).

In the present study, single nodal segments excised from actively growing shoots of one year old field grown plants were used as explants. Different concentrations of $HgCl_2$ for different duration were tried for surface sterilization. Among which, the treatment with 0.2 % $HgCl_2$ for 5 min followed by a dip in absolute alcohol for 2-3 s resulted in 100 per cent survival of the plants. The contamination found in other treatments were predominantly fungal, which might be due to endophytic associations (Chetia and Handique, 2000).

According to Jose *et al.* (2007), approximately 65 per cent of the young nodal explants of *P. rosea* remained contamination free during culture initiation when surface sterilized with 0.1 % $HgCl_2$ for 4-7 min. Efforts to reduce the contamination percentage without affecting the viability and morphogenetic response of the explants by changing the surface sterilization procedures did not yield encouraging results. In contrast to this observation, in the present study, it was found that it is possible to get 100 per cent contamination free plantlets by surface sterilizing with 0.2 per cent $HgCl_2$ for 5 min and a dip in absolute alcohol for 2-3 s, in *Plumbago rosea* var. Agni.

5.1.1.2 Culture Initiation and Multiplication

The levels and kinds of plant growth regulators included in the culture medium largely determine the success rate of culture initiation and multiplication. Auxin : cytokinin ratios of approximately 10 yield rapid growth of undifferentiated callus, a ratio of approximately 100 favours root development and a ratio of

approximately 4 favours the development of shoots (Murashige, 1979). The inclusion of cytokinins and auxins to the culture medium stimulated *in vitro* multiplication and growth of shoots in several plant species (George, 1993).

In the present study, different combinations of cytokinins and auxins were tried for inducing shoot proliferation in the nodal explants. Among the thirty treatments tried all the treatments gave 100 per cent survival. Maximum shoot proliferation (5.28 shoots/culture) was observed in the treatment, MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1}. Satheeshkumar and Bhavanandan (1988) reported that callus induced stem segment of *P. rosea* gave multiple shoot formation on MS medium containing auxin and cytokinin. MS medium supplemented with 2, 4-D 2.5 mg l^{-1} and Kn 1.5 mg l^{-1} gave maximum callus induction while BAP 2 mgl^{-1} and NAA 1 mgl^{-1} induced shoot formation from the callus. Asthana *et al.* (2011) observed a maximum shoot regeneration frequency (97.22 %) of *Sapindus trifoliatus* on MS medium supplemented with BAP 1.0 mg l^{-1}.

In the present study, when cytokinins were solely supplemented in the MS medium (BA alone or BA and Kn together) they did not give shoot proliferation as with BA-IAA combination. This indicates the synergistic effect of BA and IAA on shoot proliferation as reported in *Bacopa monnieri* (Yelne *et al*., 1997), *Cardiospermum helicacabum* (Jaysheelan and Rao, 1998), *Gardenia jasminoides* (Duhoky and Rasheed, 2010) and *Lysimachia vulgaris* (Turker and Guner, 2013).

Earliest bud initiation (7.94 days), maximum shoot length (5.33 cm) and maximum nodes per shoot (5.83 nodes/shoot) were obtained in the treatment MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1}. The control treatment which lacked the plant growth regulators produced the least number of shoots/culture. These finding were in accordance with the report of Asthana *et al.* (2011) in *Sapindus trifoliatus.*

Multiple shoot induction in MS medium containing combinations of BA and NAA/Kn was very less. MS medium supplemented with BA alone gave better shoot proliferation. Higher concentrations of BA (1.0 to 1.5 mg l^{-1}) along with NAA (0.5 to 1.0 mg l^{-1}) resulted in excessive callusing at the base of the nodal explants. This observation was in contrast with the report on *Justicia gendarussa* by Agastian *et al.* (2006), that when nodal explants were grown in MS medium supplemented with low concentrations of BAP (0.1 mg l^{-1}) and NAA (1.0 mg l^{-1}) produced compact chlorophyllous calli with shoots. Vengadesan *et al.* (2002) also reported that when cotyledonary nodes of *Acacia sinuata* were cultured on MS medium containing a combination of BAP and auxins (NAA, IBA or IAA), gave very low shoot proliferation but in turn, produced basal callus. None of the combinations of BAP along with NAA was effective in enhancing shoot proliferation, but supported prolific callus growth at the basal end of the explants in *Sapindus trifoliatus* (Asthana *et al.,* 2011).

5.1.2 *In vitro* Rooting

In vitro induction of roots from growing shoots has been achieved in standard medium containing auxin or absence of auxin depending on plant genotype (Rout *et al.,* 1989).

In the present study twelve treatments with different concentrations of IBA (0.1 to 1.5 mg l^{-1}), IAA (0.1 to 1.5 mg l^{-1}) and NAA (0.1 to 1.5 mg l^{-1}) were tried for *in vitro* root formation. Earliest (7.28 days) root initiation, maximum number of roots (10.67 roots/culture) and maximum root length (5.42 cm) were recorded in MS medium supplemented with NAA 1.0 mg l^{-1}. No rooting was observed in control treatment with no growth regulators. Agastian *et al.* (2006) reported in *Justicia gendarussa* the formation of long chlorophyllous roots in medium supplemented with NAA (0.2-2.0 mg l^{-1}). However, root induction in *P. rosea* with higher

concentrations of IBA was reported by Satheeshkumar and Bhavanandan (1988), Rout *et al.* (2002) and Satheeshkumar and Seeni (2003). Gopalakrishnan *et al.* (2009) reported the root formation of *P. rosea* in hormone free medium.

5.1.3 Planting Out and Acclimatization

Micropropagation system can be marked successful only if a successful planting out protocol is embedded with it. The survival percentage of *ex vitro* established plants is crucial for any micropropagation protocol.

In the present study *in vitro* rooted plants were planted out in two different potting media, red soil and coir pith (3:1) and red soil and coir pith (2:1) supplemented with VAM. The plants showed a survival rate of 76 % and 72 % respectively in both the treatments and there was no significant difference between them.

Gopalakrishnan *et al.* (2009) observed that more than 90 per cent of plantlets survived on hardening of *P. rosea* in red soil, vermiculite and farmyard manure (1:1:1) for one week. They also observed that gradual acclimatization of *in vitro* grown plants to external environment is most essential for *P. rosea.* More than 80 per cent of the plants transferred to pots survived and resumed growth.

Jose and Satheeshkumar, (2010) reported that 96 per cent of the rooted plants of *P. rosea* were established in soil and sand (1:1) mixture in the shade net house with regular watering.

5.2 *IN VITRO* CONSERVATION

5.2.1 Cryopreservation of *Plumbago rosea* Using Encapsulation Dehydration Technique

The successful cryopreservation protocol was first reported in silver birch twigs by Sakai (1960). Quatrano (1968) could freeze in vitro cultured flax cells successfully. Encapsulating the explants allows exposure to extreme treatments including pre-culture with high sucrose concentrations and desiccation to low moisture content that would be highly damaging or lethal to non-encapsulated samples. In cryopreservation protocols using desiccation, survival is often increased by encapsulation as reported in rapeseed microspore embryos (Niino and Sakai, 1992) apple shoot tips (Uragami *et al.,* 1993). Due to the extreme desiccation of explants, most or all freezable water is removed from cells, and vitrification of internal solutes takes place during rapid exposure to LN, thus avoiding lethal intracellular ice crystallization (Engelmann, 1997). As a consequence, the whole or a large part of the frozen explant is kept intact after rewarming, which results in high survival, rapid and direct regrowth and reproducible results after cryopreservation (Engelmann, 2000).

Cryopreservation, theoretically conserving materials for an unlimited period at ultra-low temperature, is the most advisable method to preserve plant resources. Encapsulation-dehydration method, which can protect plant materials from crystallization related injury through dehydration with silica-gel or by sterile air, instead of expensive freezer and toxic cryoprotectants (Engelmann 2004; Popova *et al.* 2010), has been frequently used to preserve different tissue and cell types, especially complex organs such as shoot tips and axillary buds (Hao *et al.* 2001; Uchendu and Reed 2008; Nair and Reghunath, 2007, 2009; Martin *et al.* 2011).

In the present study nodal segments with single axillary bud taken from *in vitro* raised *P. rosea* were cryopreserved using the encapsulation-dehydration technique. This technique proceeds through a series of sequential steps *viz.,* preconditioning, encapsulation, pre-culture, dehydration and thawing and recovery.

5.2.1.1 Preconditioning

Most hydrated tissues do not withstand dehydration to the moisture content (20 – 30 %) that is needed to prevent crystallization during cooling. The key to successful cryopreservation is in the induction of dehydration tolerance rather than freezing tolerance (Panis and Lambardi, 2005). Sugar treatment is one of the strategies followed to prepare the plant tissues for dehydration stress to which they will be exposed during cryopreservation process.

Sugar act as osmolyte and stabilise membranes and proteins. They induce the production of certain compounds like proteins, other sugars, glycer, proline, glycine betaine and polyamines. The sucrose treatments prepare the plant tissues for cryopreservation. Sucrose concentration ranges from 0.3 - 1.0 *M* and treatment ranges from 1 to 4 weeks (Panis, 2008).

In the present study axillary buds of *P. rosea* excised from the *in vitro* raised plantlets were preconditioned on hormone free MS medium with different concentrations of sucrose for varying time periods. The preconditioned nodes were then cultured on the medium MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1} and the preconditioning treatment which gave maximum shoot proliferation was selected for the standardisation of cryopreservation protocol.

All the preconditioning treatments gave 100 per cent shoot proliferation except the one with sucrose 1.0 *M* (highest level of sucrose among the treatments tried), irrespective

of duration of treatment. The regeneration was very low (< 40 %) at this concentration of sucrose. When cultured on medium MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1}, the earliest bud initiation (8.56 days), maximum shoots/culture (2.67 shoots/culture), shoot length (4.89 cm) and nodes/culture (4.39 nodes/culture) were obtained in the buds preconditioned in MS medium supplemented with sucrose 0.5 *M* for 7 days.

Suzuki *et al.* (2005) reported that the axillary buds of *Gentiana scabra,* pre-conditioned with 0.1 *M* sucrose for 3–20 days before transferring them to medium containing 0.4 *M* and 0.7 *M* sucrose showed high survival rates (74–94 %).

5.2.1.2 Encapsulation

Encapsulating the explants allows to submit them to very drastic treatments including pre-culture with high sucrose concentrations and desiccation to low moisture contents (MCs) which would be highly damaging or lethal to non-encapsulated samples.

Axillary buds were encapsulated using different concentrations of sodium alginate and calcium chloride. Among the sixteen treatments tried all the treatments showed 100 per cent survival and regeneration. Encapsulated buds were cultured for shoot proliferation in the medium MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1}.

Encapsulated buds initiated (9.19 days) late compared to non encapsulated buds (7.94 days). Shoots per culture, shoot length and nodes per shoot were found to be low in encapsulated buds compared to non encapsulated ones. Among the different encapsulation treatments tried, axillary buds encapsulated with SA 2.5 % and $CaCl_2$ 100 *mM* gave maximum shoots/culture (2.31 shoots/culture), shoot length (3.35 cm) and nodes/culture (3.75 nodes/culture) in the medium, MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1}.

Padro *et al.* (2012) reported the use of 3 per cent sodium alginate solution as the best concentration for the formation of beads for cryopreservation in *Morus alba.* Ai *et al.*, (2012) encapsulated the shoot tips of *Rabdosia rubescens* in MS medium supplemented with SA 3 % and sucrose 0.4 M. The beads were formed by complexing shoot tips in 3 per cent SA with $CaCl_2$ 0.1 *M* solution containing sucrose 0.4 *M*.

5.2.1.3 Pre-culture

Sucrose is an important pre-growth additive for most cryopreservation methods, which enhances desiccation tolerance during cryopreservation. Among different types of sugars (fructose, glucose, sorbitol, and sucrose) used as somatic agents in pre-culture medium, sucrose was the best for the survival of cryopreserved date palm tissue culture (Bekheet *et al.,* 2007).

According to Wang *et al.* (2004), survival of cryopreserved explants or cells increased with increase in sucrose concentration to certain level, as high sucrose concentration increases total soluble protein and sugar content in the treated tissues.

In the present study, single nodal segments preconditioned (in MS medium supplemented with sucrose 0.5 *M* for 7 days) and encapsulated (SA 2.5 % + $CaCl_2$ 100 *mM*) were subjected to twenty pre-culture treatments involving different concentrations of sucrose (0.1, 0.3, 0.5, 0.75, and 1.0 *M*) for different time periods (1, 3, 5 and 7 days) in hormone free MS liquid medium with 3 per cent DMSO in every treatments. The pre-cultured beads were then cultured on the medium (MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1}) to study the effect on shoot proliferation.

The beads pre-cultured in liquid MS medium supplemented with sucrose 0.5 *M* for 3 days gave earliest bud initiation (18.94 days), maximum shoots/culture (3.45 shoots/culture), shoot length (4.36 cm) and nodes/culture (4.33 nodes/culture) in the

medium MS + BA 1.5 mg l^{-1} + IAA 1.00 mg l^{-1}. Higher concentrations of sucrose (0.75 *M* and 1.0 *M*) did not support the growth and regeneration of the plants irrespective of the different periods tried. The blackening of buds observed at these levels may be attributed to the toxicity induced by high sucrose concentration.

Hitmi *et al.* (1999) demonstrated that sucrose decreased the water content of *Chrysanthemum cinerarifolium* shoot tips and thus enhanced their freezing tolerance.

Sanayaima *et al.* (2006) pre-cultured the excised shoot tips of *Crateva nurvala* in sucrose 0.4 *M* for 48 h to increase the LN tolerance as indicated by better post-thaw survival and regeneration compared to sucrose 0.09 *M*. Yin and Hong (2009) reported 87.6 % survival of cryopreserved *Dendrobium candidum* PLBs, when pre-cultured in MS medium containing sucrose 0.75 *M* for 5 days.

Encapsulated protocorm like bodies (PLBs) of *Dendrobium chrysanthum* pre-cultured on MS liquid medium supplemented with 0.3 *M* sucrose gave 63.2 per cent survival. The survival percentage was found to decline on pre-culture with higher concentrations of sucrose (0.5 *M* – 0.7 *M*) (Mohanty *et al.,* 2013).

5.2.1.4 Dehydration

Reduction of water content to a critical level before freezing is the key factor in developing successful cryoprotection protocols (Zhang *et al.* 2001). If the cells are not sufficiently dehydrated, intracellular ice will be formed resulting in cryoinjury during cold storage in LN and if over-dehydrated, the osmotic stress can be damaging (Bian *et al.* 2002). Hence, dehydration must be long enough to ensure sufficient cell dehydration, without cytotoxic effects.

In the present study the preconditioned, encapsulated and pre-cultured axillary buds of *P. rosea* were subjected to different hours of dehydration from 0 to 7 h. Moisture content of encapsulated axillary buds prior to dehydration was 71.82 per cent. This when cultured on medium, MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1} gave cent per cent survival and regeneration but did not give any regeneration when cultured after subjecting to rapid plunging in LN for 2 h. This may be attributed to the cell damage by crystallisation of water in the plant tissue as reported by Meryman and Williams (1985). This was in confirmation with findings of Nair and Reghunath (2009) in encapsulated axillary buds of *Indigofera tinctoria.*

The dehydration after encapsulation is important for the maximum survival of plants recovered from LN in cryostorage. This can be achieved by air dehydration of the encapsulated axillary buds in laminar air flow. In the present study, after dehydration under laminar air flow for 6 h, MC reduced to 15.43 per cent. Encapsulated axillary buds dehydrated to 18.13 per cent (5 h) and 15.43 per cent (6 h) resulted in 72.22 and 61.11 per cent survival, respectively and the same when recovered from LN storage gave 66.67 and 45.83 per cent survival respectively. According to Reed *et al*. (2006), the optimum MC for germplasm of most plants before exposure to LN is normally about 19 to 23 per cent. Alginate beads dried to 20 per cent moisture vitrified on exposure to LN and formed stable glasses that do not form ice crystals on rewarming (Dumet *et al.,* 2000). Thus, a prerequisite for successful application of encapsulation dehydration technique is the avoidance of irreversible cell membrane damage caused by the formation of intracellular ice crystals. Ice crystallization can only be prevented through a reduction of the cellular water content (dehydration) to the strict minimum (Vertommeu *et al.,* 2008).

Khoddamzadeh *et al.* (2011) opined that optimal desiccation time is related to the species and to the type of explants. Moisture content of 32 per cent was suitable for protocorm like bodies (PLBs) of *Phalaenopsis bellina* while 18-28 per cent was

optimal for *Sabal embryos* (Wen and Wang, 2010). However, water content of about 20 per cent is reported to induce best recovery in several species including *Picea* (Hazubska-Przybyl *et al*. 2010), *Pyrus* (Condello *et al.* 2009) and *Morus bombicys* (Niino *et al.* 1992b). Padro *et al.* (2012) reported highest re-growth after cryopreservation (67 %) with an osmoprotection of shoot apices in 0.75 *M* sucrose for 3 days, followed by a physical desiccation in silica gel for 9 h (19 % MC) in *Morus alba*.

Maximum survival (53.3 %) of PLBs of *Dendrobium nobile* was achieved after cryopreservation when pre-cultured beads were dehydrated for 5 h with reduced water content of 28.3 % and survival gradually decreased (37.4 %) with 6 h of dehydration with bead water content of 16.7 per cent (Mohanty *et al.,* 2012).

Many researchers have used a combination of encapsulation, high sucrose pretreatment and dehydration before exposure of gemplasm to LN (Dereuddre *et al.,* 1990; Blakesley *et al.,* 1995; Bernard *et al.,* 2002; Reed *et al.,* 2006) to escape freezing injury.

In the present study the recovered shoots were easily multiplied and rooted on the recovery medium. The plants desiccated to 5 h gave 4.06 shoots/culture after cryopreservation and recovery while those desiccated to 6 h gave a maximum shoot proliferation of 3.33 shoots/culture. On *in vitro* rooting, the 5 h desiccated plants gave 8.28 roots/culture while 6 h desiccated plants gave 8.39 roots/culture.

5.3 Estimation of Genetic Stability of Cryopreserved Materials Using RAPD

Genetic fidelity and stability of cryo-derived regenerants is one of the most important concerns in cryopreservation. Plants recovered following cryogenic treatments should be genetically identical to the starting materials and genetic fidelity

needs to be considered in the cryopreservation process (Ashmore, 1997). The long term genetic consequences of dehydration and freezing injury for *in vitro* conservation need to be evaluated (Harding, 1996; Gagliardi *et al.,* 2007). Random amplification of polymorphic DNA (RAPD) method is fast, simple, and efficient method for evaluating the genetic stability of plant materials (Nair and Reghunath, 2009).

RAPDs were used for assessing the genetic stability of plant species *viz., Dioscorea bulbifera* (Dixit *et al.,* 2003), *Melia azedarach* (Scocchi *et al.,* 2004) and *Dendranthema grandiflora* (Martin and Gonzalez–Benito, 2005) following cryopreservation. Most of the genetic stability analysis using RAPD techniques did not show significant differences between regenerants derived from non cryopreserved and cryopreserved tissues (Turner *et al.,* 2001, Scocchi *et al.,* 2004, Helliot *et al.,* 2002, Zarghami *et al.,* 2008, Zhai *et al,* 2003).

As cryopreservation is a technique wherein all metabolic activities of plant tissues are arrested, the materials are expected to be maintained without any genetic alterations as confirmed from the studies by Moukadiri *et al.* (1999), Hao *et al.* (2001), Condello *et al.* (2009) and Baranek *et al.* (2010). However, RAPD markers revealed 0.34 per cent of variation in the cryopreserved material *Thymus moroderi* (Marco-Medina and Casas, 2013).

In the present study, RAPD profiles of cryopreserved and non cryopreserved regenerants were compared to assess the genetic fidelity of plantlets regenerated from cryopreservation. Six primers were screened and they produced clear and reproducible bands. RAPD profile of regenerants from cryopreserved materials were identical to those of control plants for all the six primers tested. No differences were observed in the banding pattern of control and cryopreserved samples.

Encapsulated axillary buds at two different moisture levels 18.13 per cent (at 5 h desiccation) and 15.43 per cent (at 6 h desiccation) were stored for different duration in LN. Moisture content significantly influenced survival and regeneration per cent per cent while the effect of duration of storage in LN did not have any significant influence on survival and germination per cent. This confirmed the fact that moisture content is a critical factor in cryopreservation.

In vitro regeneration and *in vitro* conservation of *P. rosea* by encapsulation dehydration has been standardized in the present study. The encapsulation dehydration method established in the study appears to be simple and effective for the long term conservation of *P. rosea* by cryopreservation.

Future Lines of Work

- Optimisation of different steps in cryopreservation to get better recovery per cent.
- Cryopreservation techniques like encapsulation-vitrification, droplet immersion can be attempted to enhance the survival rate after long term storage.
- Cryopreservation can be tried with other explants such as roots of *P. rosea.*
- More sophisticated molecular techniques can be employed for accurate interpretation of genetic fidelity assessment.

Summary

6. SUMMARY

The present study on "Cryopreservation of Chethikoduveli (*Plumbago rosea* L.) and assessment of genetic fidelity of regenerated plantlets using molecular markers" was carried out at the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2011-2013. *Plumbago rosea* var. Agni plants were collected from AMPRS, Odakkali, Ernakulam and maintained at the Department of Plant Biotechnology, College of Agriculture, Vellayani as source of explant during the course of the study. The project was carried out in two phases *viz*., optimization of protocol for *in vitro* regeneration of *Plumbago rosea* var. Agni and standardization of cryopreservation protocol by encapsulation-dehydration technique for long-term conservation of *Plumbago rosea.*

6.1 PHASE I: *IN VITRO* REGENERATION

6.1.1 Enhanced Release of Axillary Buds

For surface sterilizing, single nodal explants (3-4 cm long) were subjected to fungicide treatment with 0.1 per cent carbendazim 50 per cent W. P. (for 30 min) followed by aseptic sterilisation dip with absolute alcohol. Further, the explants were surface sterilised with 0.2 per cent $HgCl_2$ (for 5 min) which gave 100 per cent survival without any contamination.

Nodal segments of 1.5 cm with a single axillary buds from in vitro established plantlets were used as explants for culture initiation and multiplication studies. Enhanced release of axillary buds from single nodal explants, with earliest bud initiation (7.94 days), maximum shoot proliferation (5.28 shoots/culture), maximum

shoot length (5.33 cm) and maximum number of nodes per culture (5.83 nodes/culture) was obtained in the medium, MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1}.

6.1.2 *In vitro* Rooting

Well developed shoots having 3-4 cm length were used for *in vitro* rooting. The best response of *in vitro* rooting of plantlets, with earliest root initiation (7.28 days), maximum number of roots (10.67 roots/culture) and maximum root length (5.42 cm) was obtained in the medium, MS + NAA 1.0 mg l^{-1}.

In vitro rooted plants gave a maximum survival rate of 76 per cent and 72 per cent, when planted out in potting media consisting of red soil and coir pith (3:1) and red soil and coir pith (2:1) supplemented with VAM respectively at 25 per cent shade.

6.2 PHASE II: *IN VITRO* CONSERVATION

6.2.1 Cryopreservation of *Plumbago rosea* Using Encapsulation Dehydration Technique

Different steps in encapsulation-dehydration technique of cryopreservation *viz.,* preconditioning, encapsulation, pre-culture, dehydration and thawing and recovery were standardised to evolve a long term conservation protocol for *P. rosea*. Axillary buds of *in vitro* established plantlets were used for the cryopreservation.

Among the different preconditioning treatments tried, earliest bud initiation (8.56 days), maximum shoot proliferation (2.67 shoots/culture), maximum shoot length (4.89 cm) and maximum number of nodes per shoot (4.39 nodes/shoot) was obtained in MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1} medium when nodal segments with single axillary bud were subjected to preconditioning with sucrose 0.5 *M* for 7 days.

Among different encapsulation treatments with varying concentration of sodium alginate and calcium chloride tried, earliest bud initiation (9.19 days), maximum shoot proliferation (2.31 shoots/culture), maximum shoot length (3.35 cm) and maximum number of nodes per shoot (3.75 nodes/shoot) was obtained in beads formed with sodium alginate 2.5 per cent and calcium chloride 100 *mM*, when cultured on the medium, MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1}.

The preconditioned encapsulated axillary buds were subjected to different pre-culture media for different periods. Pre-culture media supplemented with sucrose 0.5 *M* for 3days gave earliest bud initiation (18.94 days), maximum shoot proliferation (3.45 shoots/culture), maximum shoot length (4.36 cm) and maximum number of nodes per shoot (4.33 nodes/shoot), when cultured on the medium, MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1}.

The preconditioned, encapsulated and pre-cultured axillary buds were subjected to different periods of desiccation. A desiccation duration of 5h at 18.13 per cent moisture level was found to be most effective giving 66.67 per cent survival and 62.50 per cent regeneration on thawing and culturing on the recovery medium MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1}.

The beads when stored in liquid nitrogen for different duration and cultured on recovery medium did not show any significant variation with respect to survival per cent.

6.2.2 Estimation of Genetic Stability of Cryopreserved Materials Using RAPD

RAPD markers were tried to study the genetic fidelity of the regenerated plantlets from encapsulated and cryopreserved axillary buds. Six primers were screened and RAPD banding patterns of the cryoregenerated plantlets and control

plants were compared. Polymorphism was not found with any of the primers tested. RAPD profiles of cryoregenerated plantlets were identical to those of the control.

The *in vitro* regeneration protocol optimized included surface sterilization of single node cuttings with 0.2 per cent $HgCl_2$ for 5 min, axillary shoot proliferation in MS medium supplemented with BA 1.5 mg l^{-1} and IAA 1.0 mg l^{-1} , *in vitro* rooting in MS medium supplemented with NAA 1.0 mg l^{-1} and planting out in potting medium, red soil and coir pith (3:1).

The protocol for encapsulation dehydration technique of cryopreservation was standardised for the axillary buds of *P. rosea* with preconditioning in semi solid MS medium supplemented with sucrose 0.5 *M* for 7 days, encapsulation using sodium alginate 2.5 per cent and calcium chloride 100 *mM* followed by pre-culture in liquid MS supplemented with sucrose 0.5 *M* for 3 days and 5 h dehydration (MC 18.13 %), rapid freezing in LN for at least 2 h and recovery in the medium MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1}.

The cryopreservation protocol using encapsulation-dehydration technique standardised in the present study could be utilised for long-term conservation of *P. rosea*.

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* Original not seen

APPENDIX I

CTAB Extraction Buffer

C-TAB	2.5 %	
Tris- HCl (pH 8.0)	100 *mM*	
EDTA	25 *mM*	
NaCl	1.5 *M*	
β-mercaptoethanol	0.2 % (v/v)	freshly added prior to DNA extraction
PVP	4 % (w/v)	

APPENDIX II

TE buffer

Tris- HCl (pH 8.0)	10 *mM*
EDTA	1 *mM*

APPENDIX III

TBE Buffer (5x) for 1 liter solution

Tris base	54 g	(0.445 *M*)
Boric acid	27.5 g	(0.445 *M*)
0.5 M EDTA (pH 8.0)	20 ml	(0.01 *M*)

Abstract

CRYOPRESERVATION OF CHETHIKODUVELI (*Plumbago rosea* L.) AND ASSESSMENT OF GENETIC FIDELITY OF REGENERATED PLANTLETS USING MOLECULAR MARKERS

by

ANAND VISHNU PRAKASH

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8. ABSTRACT

Investigations on "Cryopreservation of Chethikoduveli (*Plumbago rosea* L.) and assessment of genetic fidelity of regenerated plantlets using molecular markers" were carried out at the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2011-2013. *Plumbago rosea* var. Agni plants were collected from AMPRS, Odakkali, Ernakulam and maintained at the Department of Plant Biotechnology, College of Agriculture, Vellayani as source of explant during the course of the study.

The objectives of the present study was to standardise cryopreservation protocol by encapsulation dehydration technique for long term conservation of *P. rosea* and genetic fidelity assessment of plantlets recovered and regenerated from cryostorage using molecular markers. The project was carried out in two phases *viz*., *in vitro* regeneration and *in vitro* conservation by cryopreservation of *P. rosea.*

In vitro regeneration protocol was optimised for *P. rosea* var. Agni. Various steps of *in vitro* regeneration *viz.,* surface sterilization, axillary shoot proliferation, *in vitro* rooting and acclimatization and planting out has been standardised.

For surface sterilizing, single nodal explants (3-4 cm long) were subjected to fungicide treatment with 0.1 per cent carbendazim 50 per cent W. P. (for 30 min) followed by aseptic sterilisation dip with absolute alcohol. Further, the explants were surface sterilised with 0.2 per cent mercuric chloride (for 5 min) which gave 100 per cent survival without any contamination.

Enhanced release of axillary buds from single nodal explants, with maximum shoot proliferation (5.28 shoots/culture) was obtained in the medium, MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1}. The best response (10.67 roots/culture) of *in vitro* rooting of

plantlets was obtained in the medium, MS + NAA 1.0 mg l^{-1}. *In vitro* rooted plants gave a maximum survival rate of 76 per cent and 72 per cent, when planted out in potting media consisting of red soil and coir pith (3:1) and red soil and coir pith (2:1) supplemented with VAM respectively at 25 per cent shade.

In cryopreservation studies, preconditioning treatment (sucrose 0.5 *M* for 7 days) recorded maximum shoot proliferation (2.67 shoots/culture) when nodal segments with single axillary bud were cultured on MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1} medium. Among different encapsulation treatments, maximum shoot proliferation of (2.31 shoots/culture) was obtained in beads formed with sodium alginate 2.5 per cent and calcium chloride 100 *mM*, when cultured on the medium, MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1}. Pre-culture medium supplemented with sucrose 0.5 *M* for 3days gave maximum shoot proliferation (3.44 shoots/culture) when cultured on the medium, MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1}.

A desiccation duration of 5 h at 18.13 per cent moisture level was found to be most effective giving 66.67 per cent survival and 62.50 per cent regeneration on thawing and culturing on the recovery medium MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1}. The beads when stored in liquid nitrogen for different duration and cultured on recovery medium did not show any significant variation with respect to survival per cent.

RAPD markers were tried to study the genetic fidelity of the regenerated plantlets from encapsulated and cryopreserved axillary buds. Six primers were screened and RAPD banding patterns of the cryoregenerated plantlets and control plants were compared. Polymorphism was not found with any of the primers tested. RAPD profiles of cryoregenerated plantlets were identical to those of the control.

The *in vitro* regeneration protocol optimized included surface sterilization of single node cuttings with 0.2 per cent $HgCl_2$ for 5 min, axillary shoot proliferation in MS medium supplemented with BA 1.5 mg l^{-1} and IAA 1.0 mg l^{-1}, *in vitro* rooting in MS medium supplemented with NAA 1.0 mg l^{-1} and planting out in potting medium, red soil and coir pith (3:1).

The protocol for encapsulation dehydration technique of cryopreservation was standardised for the axillary buds of *P. rosea* with preconditioning in semi solid MS medium supplemented with sucrose 0.5 *M* for 7 days, encapsulation using sodium alginate 2.5 per cent and calcium chloride 100 *mM* followed by pre-culture in liquid MS supplemented with sucrose 0.5 *M* for 3 days and 5 h dehydration (MC 18.13 %), rapid freezing in LN for at least 2 h and recovery in the medium MS + BA 1.5 mg l^{-1} + IAA 1.0 mg l^{-1}.

The cryopreservation protocol using encapsulation-dehydration technique standardised could be utilised for long-term conservation of *P. rosea*.