IN VITRO CONSERVATION OF CHETHIKODUVELI (*Plumbago rosea* L.) USING ENCAPSULATION AND VITRIFICATION TECHNIQUES

by

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THESIS

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DECLARATION

I, hereby declare that this thesis entitled **"*IN VITRO* CONSERVATION OF CHETHIKODUVELI (*Plumbago rosea* L.) USING ENCAPSULATION AND VITRIFICATION TECHNIQUES"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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Certified that this thesis entitled **"*IN VITRO* CONSERVATION OF CHETHIKODUVELI (*Plumbago rosea* L.) USING ENCAPSULATION AND VITRIFICATION TECHNIQUES"** is a record of research work done independently by Mrs. Sowmya A. S under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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LIST OF ABBREVIATIONS

A_{260}	absorbance at 260 nm wavelength
A_{280}	absorbance at 280 nm wavelength
Ads	adenine sulphate
B5	Gamborg medium, 1968
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
h	hour
HCL	hydrochloric acid
IAA	indole-3-acetic acid
ISSR	inter-simple sequence repeat
LN	liquid nitrogen
M	molar
mg	milligram
ml	milliliter
min	minute
mM	millimolar
MS	Murashige and Skoog,1962
μM	micromolar
NaCl	sodium chloride
NaOH	sodium hydroxide
nm	nanometer
pH	potential of hydrogen
PCR	polymerase chain reaction
PGR	plant growth regulator
PLBs	protocorm like bodies
PVP	polyvinylpyrrollidone
PVS1	plant vitrification solution 3
PVS2	plant vitrification solution 2
PVS3	plant vitrification 3
RAPD	random amplified polymorphic DNA

rpm	revolutions per minute
s	second
SA	sodium alginate
sp.	species
spp.	species plural
SH	Schenk and Hildebrandt medium,1972
TE	tris-EDTA buffer
Tris HCl	tris(hydroxymethyl)aminomethanehydrochloride
Tdz	thidiazuron
V	Volt
viz.	namely
WHO	world health organization
X	times

LIST OF SYMBOLS

°C	degree celsius
%	per cent
±	plus or minus

APPENDIX

Introduction

1. INTRODUCTION

Plant derived drugs are being widely used not only in developing countries but also in the most advanced countries. According to WHO, eighty per cent of the world's population rely on medicinal plants for their primary health care. Plants are important sources of pharmacologically active compounds which contributes to cure and prevention of diseases (Newman *et al.,* 2000; Raskin *et al.*, 2002). The traditional health care system is mainly dependent on medicinal plants collected from the wild, without being replenished. This has led to the decline in the area of the medicinal plant biodiversity. The indigenous knowledge associated with the conservation and use of medicinal plants is also disappearing at an alarming rate.

Biotechnological strategies for plant germplasm conservation developed as complementary to conventional methods of conservation are based on concepts of *in vitro* plant cell, tissue and organ culture (Vasanth and Vivier, 2011). These strategies have the potential to overcome the inherent limitations of conventional methods of conservation *ex situ,* and to facilitate the exchange of pathogen free germplasm (Ray and Bhattacharya, 2008).

Plumbago rosea (syn. *P. indica*, Family - Plumbaginaceae) is known to be the richest source of plumbagin (Mallavadhani *et al.*, 2002). Plumbagin (2-methoxy- 5hydroxy-1, 4-napthoquinone), a natural napthoquinone, accumulated in the roots of the plant, possesses various pharmacological properties like anticancer (Parimala and Sachdanandam, 1993; Aziz *et al.*, 2002), antimicrobial (Didry *et al.*, 1994), cardiotonic (Itoigawa *et al.*, 1991), antifertility (Bhargava, 1984), antioxidant (Tilak *et al.*, 2004), insecticidal, antidiabetic (Paiva *et al.*, 2005) etc. The plant is of great demand in ayurvedic pharmaceutical industry. This demand cannot be met from the small scale cultivation that is undertaken in Kerala. Hence, the plant is heavily collected from the wild to meet this demand.

Moreover, the plant does not set seeds, thus restricts their easy regeneration in the wild. The establishment of plants in the field via vegetative propagation by cutting is also slow and takes a long time for the roots to reach the harvestable stage (Panichayupakaranant and Tewtrakul, 2002). These had resulted in the decline of the plant population and has become near extinct in the wild (Chetia *et al.*, 1998).

Advances in plant biotechnology, especially those associated with *in vitro* culture and molecular biology, have provided powerful tools to support and improve conservation and management of plant diversity (Withers, 1991). These biotechnological startegies include (a) *in vitro* conservation (b) *in vitro* propagation and re-introduction of plants to their natural habitats, and (c) molecular marker technology. *In vitro* conservation is especially important for vegetatively propagated plant species and those with recalcitrant seeds (Engelmann, 2011).

Short term and long term storage are available for the conservation of plant germplasm *in vitro*. Short term conservation by the manipulation of media, culture conditions, addition of osmoticum etc., enables for germplasm exchange between research institutions and for distribution to end-users. While long term conservation, by cryopreservation in liquid nitrogen, the plant material is conserved for use by future generations and can be maintained as base collections. As the metabolic activities of the plant tissue are interrupted under ultra low temperature of -196˚C in cryopreservation, the material could be stored without any genetic variation for indefinite period.

Synthetic seed technology could be effectively utilized for both short term and long term *in vitro* conservation as it provides for easy handling, storage, distribution and exchange. It offers an efficient means for mass propagation of plant species irrespective of season, space and environmental factors.

Encapsulation of vegetative propagules *viz.*, axillary buds, shoot tips, nodal segments etc. has become a potentially cost effective clonal propagation system (Sarkar and Naik, 1998; Chand and Singh, 2004). These encapsulated vegetative propagules can also be used for germplasm conservation of elite plant species and exchange of axenic plant materials between laboratories and pharmaceutical industries (Hasan and Takagi, 1995; Rai *et al.*, 2008). Alginate encapsulation of shoot tips along with cold preservation offers a strong possibility for germplasm storage and plant regeneration. (Hasan and Takagi, 1995; Singh *et al.*, 2006: West *et al.*, 2006).

Synthetic seeds are amenable for storage using encapsulation and vitrification techniques. Vitrification enables conversion to amorphous glassy state of the intercellular fluid while freezing. This technique exposes the plant tissue to a higher concentration of cryoprotectants, which dehydrates the tissue and makes it tolerant to stresses under ultra low temperature storage. The pretreatments of the plant tissue, concentration of the cryoprotectants and period of expose to cryoprotectants decides for the successful conservation by vitrification technique to cryopreservation.

Hence, the study "*In vitro* conservation of chethikoduveli (*Plumbago rosea* L.) using encapsulation and vitrification techniques" has been proposed with the following objectives

a) Standardization of protocol for short term conservation of *P. rosea* using encapsulation technique,
b) Long term conservation of *P. rosea* using vitrification techniques of cryopreservation *viz.*, simple vitrification and encapsulation vitrification, and
c) Assessment of genetic fidelity of recovered and regenerated plantlets from storage, using molecular markers.

Review of Literature

2. REVIEW OF LITERATURE

Medicinal plants and traditional medicine play an important role in the health care system of most developing countries. The medicinal plants collected from the wild are the main source of medicine in traditional health care practice. In spite of this, due to man-made and natural calamities, the medicinal plant biodiversity is declining. Moreover, the indigenous knowledge associated with the conservation and use of medicinal plants is also disappearing at an alarming rate (Kasagana and Karumuri, 2011).

Plumbago rosea (L.) is a valuable perennial herb of Plumbaginaceae family, which is cultivated throughout India for medicinal and ornamental purposes. *P. rosea* is locally known as 'chethikoduveli' in Kerala. The roots of *P. rosea* is the main source of the alkaloid called 'plumbagin (2-methyl-5-hydroxy-1,4-naphthoquinone), an orange yellow pigment. The roots are pungent, germicidal, vesicant, diuretic and abortifacient (Harikrishnan and Hariharan, 1996). Plumbagin, in small doses has stimulant action on the central nervous system, on muscle pain and on the secretion of sweat, urine and bile (Pillai *et al.*, 1981). It is used in the treatment of leprosy, dyspepsia, diarrhoea, skin diseases etc. Its roots are exploited as the source of plumbagin which possess pharmacological properties like anticancer, antimicrobial, cardiotonic, antifertility etc. (Mallavadhani *et al.*, 2002).

Though cultivated on a small scale, due to the increasing demand, the roots of *P. rosea* are heavily collected from the wild. This has resulted in the near extinction of these species (Komaraiah *et al.*, 2004). Conventional method of field conservation of the plant involves high cost in terms of land and labour; moreover there are risks of deterioration from nature disaster and plant pests during conservation. *In vitro* conservation is an alternative method for plant conservation that eliminates the obstacles of field conservation and acts complementary to field conservation.

In this chapter literature on *in vitro* conservation of various plants including medicinal plants has been reviewed.

2.1 ENHANCEMENT OF MULTIPLICATION RATE

2.1.1 Effect of different medium and additives on multiplication rate

In vitro regeneration of the plant depends on various aspects such as explant type, media composition, PGRs and the culture conditions. A consistent and reproducible protocol looks into the apposite use of basal media, plant growth regulators and various growth promoting additives

2.1.1.1 Medium

The type and concentration of mineral nutrients supplied in different types of media have a profound influence on tissue proliferation and morphogenetic responses (Gantait *et al.,* 2016).

In a study on organogenesis of *Acacia nilotica* Abbas *et al.* (2010) stated that MS media was more apposite than the B5 medium, resulting in higher shoot regeneration frequency.

The differential influence of MS and WPM on morphogenetic responses when axillary buds were used as explants in *Acacia sp.* was reported by Shahinozzaman *et al.* (2012). Between the two media *viz.,* MS and WPM tried, maximum shoot proliferation was observed in MS media, while shoot length was higher in WPM media.

Soni *et al.* (2012) stated that MS medium was found to the best basal medium than B5 medium for the establishment of nodal explants in *Adhatoda vasica.* MS medium supplemented with 1.0 mg L^{-1} BAP gave the best response, with bud break (100 per cent), shoots per explants (2.5), average shoot length (6.2cm) and nodes per shoot (6.0) after 30 days of incubation, while on B5

medium supplemented with BAP, the nodal segments induced bud break (80-100 per cent), shoots per explants (1.0), average shoot length (2.0 cm) and nodes per shoot (3.0) after 30 days of incubation.

In shoot initiation and elongation of *in vitro* nodal explants of *Morus indica,* MS was found to be superior to B5, WPM and SH media (Niratker, 2015).

Upadhyay and Koche (2015) reported that MS medium gave the best shoot bud induction and multiplication in *Clerodendrum serratum* among the different growth media *viz*., MS, SH, WPM and B5. MS medium supplemented with 0.5 mg L^{-1} BAP, gave maximum shoot induction of 90 per cent. Shoot bud induction was observed in 5-8 days of culture. The same medium recorded highest shoot length of 4.52 cm.

Among the different media *viz*., MS, B5, SH, White's, KM, WPM, AR and half MS tried, MS media supplemented with 0.62 µM BA recorded best morphogenetic response and shoot initiation from nodal segment explants of *Phyllanthus stipulatus* after 50 days (Catapan *et al.*, 2001)

2.1.1.2 Additives (Chitosan, adenine sulphate and thidiazuron)

Chitosan, a cationic polymer and N-deacetylated product derivative of chitin, is present in shells of crustaceans and cell wall of fungi (Devlieghere *et al.,* 2004). This component is a carbohydrate polymer and is reported to stimulate growth of plant species (Nge *et al*., 2006). Chitosan being widely available, cheap and safe material for humans, can be used as an environmental friendly plant growth stimulator.

Chitosan stimulated plant growth *in vitro* in the grape vine, *Vitis vinifera* L. (Ait Barka *et al*., 2004). It was also shown to improve the potato micro plant

quality *in vitro* and to increase the yield and seed quality of minitubers (Kowalski *et al.*, 2006).

The effect of chitosan as growth stimulator was studied in *Grammatophyllum speciosum* PLBs by providing in different concentrations *viz.*, 0, 5, 10, 15, 20, 25, 50 or 100 mg L^{-1} to the half strength MS liquid and agar media containing 2 per cent (w/v) sucrose. The results showed that liquid medium supplemented with 15 mg L^{-1} chitosan showed the highest relative growth rate in PLBs (Sopalun *et al.*, 2010).

Nahar *et al.* (2012) stated that by applying chitosan at the rate of 1 mg L^{-1} to the *in vitro* cultures of *Cymbidium dayanum*, the highest PLB induction (93 per cent) and shoots formation (79 per cent) was observed.

Plantlets of *Solanum tubersoum* in *in vitro* culture when treated with soluble chitosan at different concentrations *viz.*, 0, 5, 15, 50, 150, 500, 750 and 1000 mgL^{-1} along with MS medium exhibited different traits. At the concentrations of 750 and 1000 mg L^{-1} of chitosan the culture medium failed to solidify. Lower concentrations (0, 5, 15, 50, 150 mg L^{-1}) did not significantly affect shoot fresh weight, whereas application of 500 mg L^{-1} increased the shoot fresh weight. The root fresh weight was significantly reduced in concentrations of chitosan beyond 500 mg L^{-1} whereas the concentrations (5 and 15 mg L^{-1}) led to the increase in root fresh weight. Improved acclimatization and increase in minituber number was observed in plantlets of greenhouse by applying 500 mg L^{-1} of chitosan in *in vitro* cultures. The tested lower concentrations had no effect on yield parameters (Zakaria *et al.*, 2009).

Chitosan may play a role in enhancing growth and development by some signaling pathway to auxin biosynthesis *via* a tryptophan-independent pathway (Uthairatanakij, 2007). Also, chitosan as a growth promoter and elicitor of plant

defense mechanisms could alleviate stress caused by *in vitro* conditions and acclimatization (Zakaria *et al.*, 2009).

Adenine in the form of adenine sulphate (Ads) stimulate cell growth and enhance shoot formation. The shoot enhancing role of Ads has been demonstrated in various plants (Dhar and Upreti, 1999: Husain *et al.*, 2008). It provides an available source of nitrogen to the cell and can generally be taken up more rapidly than inorganic nitrogen (Vengadesan *et al.*, 2002). It improves growth and reinforces the cytokinin responses of the plants in culture (Gatica *et al.*, 2010).

Khan *et al.* (2014) reported that the best shoot proliferation was observed from the nodal segments in *Stevia rebaudiana* within 7 days of inoculation on MS media supplemented with kinetin (9.3 μM), NAA (0.5 mg L^{-1}) and adenine sulphate (40 mg L^{-1}).

Xiaoping *et al.*, 2006 stated that by supplementing BA (2.0 mg L^{-1}), NAA (0.75 mg L^{-1}), adenine (50 mg L^{-1}) and 10 per cent (v/v) coconut milk in MS basal medium under subdued light at 25±2°C highest frequency of shoot regeneration was achieved in hypocotyl segments of *Plumbago zeylanica*. Each hypocotyl segment produced over 30 shoots arising primarily through direct organogenesis after 3 weeks of culture.

According to Siwach *et al.* (2012) MS medium supplemented with BA 2.5 mg L^{-1} and 50 mg L^{-1} of adenine sulphate as highly effective in enhancing the shoot multiplication rate (4.88 shoots per explant) in *Citrus reticulata.* This effect could be attributed to the low energy utilization in the enhancing mobility of the organic source of nitrogen (Kim and Moon, 2007).

Rout *et al.* (2008) stated that the combination of BA (1.5 mg L^{-1}), IAA (0.05 mg L^{-1}) and adenine sulphate (50 mg L^{-1}) gave efficient shoot regeneration and multiplication in *Acacia arabica*.

According to Ramesh *et al.* (2006), addition of adenine sulphate at 60 mg L^{-1} along with IAA 0.2 mg L^{-1} and BAP 1.5 mg L^{-1}) in MS medium could induce the highest number of shoots (18) in *Bacopa monnieri.*

Thidiazuron (Tdz), a phenylurea, has been reported to have high cytokinin activity at low concentrations (Matsuda and Hirabayashi, 1989). Its cytokinin-like effects on several species include stimulation of *in vitro* shoot proliferation and somatic embryogenesis, promotion of growth in cell and callus cultures, and budbreak of dormant tree (Mok *et al.*, 1987).

Effects of Tdz on *in vitro* proliferation of shoots from axillary buds of grapevine was studied by Gribaudo and Fronda (1991). The regeneration of 96 per cent was reported at Tdz 10 µM while the treatment devoid of Tdz gave only 47 per cent. The highest number of shoots was obtained in Tdz 1 µM (2.15) and the highest shoot length (13.9 mm) in TDZ 0.1 µM.

According to Sudarsono and Goldy (1991) thidiazuron (2.3 to 4.5 µM) alone or in combination with BA (1.0 to 5.0 µM) or KIN (1.0 or 5.0 µM) in MS medium was found to be effective in the establishment of axillary buds and further shoot proliferation . Similar levels were also effective for promoting shoot proliferation in *Vitis rotundifolia.*

Brar *et al.* (1995) stated that Barlo IINora cultivar of carnation cultured on TDZ-containing Gamborg's media produced maximum shoots (8) per explants. But, large amounts of calli were associated with these shoots.

Kim *et al.* (1997) reported that concentration of Tdz 10 µM and BA 40 µm in a combination medium consisting of MS salts with B5 vitamins increased the shoot biomass (total dry weight of axillary shoots) of *Fraxinus pennsylvanica.*

Preece *et al.* (1997) stated that Thidiazuron, stimulated maximum shoot proliferation in *Fraxinus americana* L. but shoots failed to elongate in the same medium and resulted in stunted growth. By transferring the explants to medium supplemented with 1.7 mM gibberellic acid (GA3) elongation of shoots was observed.

Al-Wasel (2000) stated that a combination of PGRs promoted direct organogenesis in *Acacia seyal* compared to single source. BA unaided, produced very few shoots and NAA when employed alone, could not induce shoot development but BA, thidiazuron and NAA in combination, gave efficient organogenesis.

Thidiazuron induced axillary shoot proliferation from nodal explants of *Rauvolfia tetraphylla* and *Psoralea corylifolia* was reported by Faisal *et al.* (2005) and Faisal and Anis (2006) respectively.

Bhattacharyya *et al.* (2007) reported that cytokinins *viz.,* BAP, Kinetin, and Tdz, when applied singly in MS were found to be more effective in shoot bud initiation and multiplication of shoots of *Plumbago indica* than in combination with auxins.

The shoot regeneration using thidiazuron (Tdz) of *Withania somnifera* L. was estalished through *in vitro* culture of nodal segments with axillary buds. MS media supplemented with Tdz 0.5 µM gave effective bud break and also produced maximum regeneration frequency (98 per cent), number of shoots (23.8) with shoot length of (4.83 cm), after 4 weeks of culture (Fathima and Anis, 2011).

A combination *of* of 8.87 µM BAP and 4.54 µM Tdz in WPM gave good proliferation in *Garcinia indica*. After third subculture, 3-4 shoots with average shoot length of 5.92 cm were induced. Shoots elongated with repeated subculture (Deodhar *et al.*, 2014).

In vitro propagation of *Viola pilosa* was carried out in MS basal media supplemented with different plant growth regulators. The best media was found to be MS media supplemented with BA (0.5 mg L^{-1}), Tdz (0.5 mg L^{-1}) and GA3 (0.5 mg L^{-1}) (Soni and Kaur, 2014).

The hypocotyls excised from seedlings of *Solanum melongena* L. (Arka Shirish) were inoculated into basal MS medium with varying concentration of thidiazuron (Mallaya and Ravishankar, 2013). The maximum number of shoot buds was obtained in MS media supplemented with TDZ 0.5 mg L^{-1}.

Triggering of shoots on exposure of explants to an optimum Tdz supplemented medium followed by its withdrawal effectively was observed in many plant species such as *Hibiscus cannabinus* (Srivatanakul *et al.*, 2000), *Medicago truncatula* (Neves *et al.*, 2001), *Saintpaulia ionantha* (Mithila *et al.*, 2003) and *Tamarindus indica* (Mehta *et al.*, 2005). Distorted and fasciated shoots were observed in cultures continuously grown on Tdz containing media. Deleterious effect of continued incubation in Tdz was reported on growth and multiplication of *Cicer arietinum* (Murthy *et al.*, 1996) and *Rauwolfia tetraphylla* (Faisal *et al.*, 2005).

2.2 SHORT TERM CONSERVATION BY ENCAPSULATION TECHNIQUE

The plant materials from short term conservation could be utilized for effective germpalsm exchange and distribution. In short term conservation, the biological material is stored for several months to 2-3 years, by increasing the intervals between subcultures. Growth reduction is generally attained, by modifying the culture medium and/or the environmental conditions. Modifications of the culture medium includes reduction in sugar concentration, dilution of mineral elements, changes in the nature and/or concentration of growth regulators and addition of osmotically active compounds (Engelmann, 2011). The most commonly used combination of physical and chemical factors involves the use of

low light intensity, decrease of temperature, reduction of mineral elements, modifying the carbon source and addition of osmoticum, in the medium (Holobiuc *et al.*, 2007).

Synthetic seed production *via* alginate encapsulation offers an efficient means propagation and short-term to mid-term storage of many plant species (Mohanraj *et al.*, 2009; Singh *et al.*, 2010). This system provides a low-cost, high-volume propagation (Saiprasad and Polisetty, 2003) and easy handling for storage and transportation, restoration of storage potential and upholding the clonal property of the regenerating plantlets (Ghosh and Sen, 1994). Alginate hydrogel given as coating for micropropagules has low toxicity, quick gellation and moderate viscocity. The encapsulation safeguards plant tissues from physical and environmental injuries, reduces dehydration, and offers mechanical support to hold the explants inside gel matrix during storage (Ara *et al.*, 2000). Encapsulation hence, would allow for *in vitro* conservation and storage of rare, endangered and desirable genotypes. (Germana *et al.*, 2011).

Synseeds are demonstrated as good entities for medium-term conservation in various plant species. They retain their viability for a sufficient period required for exchange of germplasm. These are transferred onto fresh medium at the end of a specific storage period. Before entering the next storage cycle, they are generally placed for a short period under optimal conditions, to stimulate regrowth (Devi *et al.*, 1998; Rai *et al.*, 2008).

2.2.1 Additives in encapsulation matrix

The encapsulation matrix, as per the requirement, can be manipulated to include different additives to induce slow growth, to counteract contamination or to stimulate plant growth and morphogenesis.

Hegazi (2011) stated that the best gel composition of encapsulated shoot tips in *Capparis orientalis* was achieved using sodium alginate 3 per cent and calcium chloride ($CaCl_2.2H_2O$) 100 mM.

The *in vitro* buds of *Epidendrum chlorocorymbos* were encapsulated with different additives *viz*., mannitol (0, 1, 2 and 3 per cent), sucrose (0, 1, 2 and 3 per cent) or sorbitol (0, 1, 2 and 3 per cent) and stored for six months with the culture conditions of 16:8 h photoperiod, 23 ± 2°C temperature and 50-80 per cent relative humidity. On inoculation, onto the MS medium at half ionic strength, the stored buds with 1 per cent sorbitol in the matrix gave the best regeneration (Lopez-Puc, 2013).

According to Mohanty *et al.* (2013) the incorporation of sucrose and mannitol (3 and 5 per cent) in the encapsulating matrix exhibited almost similar results with that of control in *Dendrobium nobile*. At these concentrations of sucrose and mannitol were not suitable for storage studies since in all these cases, more than half of PLBs of *Dendrobium nobile* bursted out from the matrix. Outburst was not observed in encapsulated PLBs, with the increased concentrations of 7.5 and 12.5 per cent in the encapsulating matrix, till 60 days of storage. Hence it can be concluded that osmotic concentrations play an important role in determining the storage.

Mohanty *et al.* (2013) also reported that in regeneration medium, the higher survival (78.20, 64.56 per cent respectively) and faster regeneration was obtained in the beads with 7.5 per cent mannitol and sucrose in the matrix compared to that with 12.5 per cent mannitol and sucrose. A decrease in survival (60.00, 54.13 per cent respectively) of stored encapsulated PLBs was observed when the concentration of mannitol and sucrose were increased to 12.5 percent. PLBs of *Dendrobium nobile* encapsulated in the beads containing 15 per cent sucrose and 15 per cent mannitol in the encapsulating matrix did not survive, after 10 days of storage.

Katouzi *et al.* (2011) demonstrated the effect of sucrose (0.08 M and 0.75 M) in the encapsulation matrix on storage of shoot tips of *Helianthus annuus* at 4°C for different duration *viz.*, 15, 30, 60, 90 days. Encapsulated shoot tips containing sucrose 0.08 M proved to be viable (33 per cent) after 90 days of storage while non-encapsulated ones lost their viability completely after 60 days. . With increase in sucrose concentration to 0.75 M, the conversion per cent dropped to 37 per cent and 18 per cent after 60 and 90 days of storage. This indicated the inhibiting effect of higher concentration of sucrose on plant regeneration.

Synthetic seeds, encapsulated microshoots of *Oryza sativa*, with sucrose 3 per cent in encapsulation matrix had significantly higher plantlets survival rate as compared with synthetic seeds with no sucrose in encapsulation matrix in (Taha *et al.*, 2012).

2.2.2 Storage medium

The encapsulated shoot tips of *Capparis orientalis* were immersed in sterilized storage medium *viz.*, distilled water, full-strength MS medium and full-strength MS medium supplemented with 3 per cent sucrose and stored for 0, 15 and 30 days at 4°C in the dark (Hegazi, 2011). Viability was 100 per cent in all the treatments. Full strength MS medium and full strength MS medium augmented with sucrose showed 100 percent recovery whereas only 83.3 per cent recovery was exhibited by encapsulated shoot tips stored in distilled water. With respect to shoot length, the highest shoot length (2.18cm) was observed in full strength MS medium augmented with sucrose followed by full strength MS medium (0.59 cm) and distilled water (0.42cm).

Nodal segments of grapevine rootstock were excised from *in vitro* cultures and were encapsulated in calcium-alginate beads. The encapsulated segments were stored at 4°C in the dark or light in semi-solid MS medium without any hormones. After 9 months of cold storage, the regrowth was 55.6 per cent for the encapsulated nodal segments maintained in darkness (Benelli, 2016).

Sundararaj *et al.* (2010) reported that synthetic seeds (encapsulated microshoots) of *Zingiber officinale,* dehydrated in 0.25 M sucrose liquid medium for 16h and stored at 25˚C for 8 weeks and 12 weeks, in cryovials, without storage medium gave 53 per cent and 13 per cent plantlet conversion, respectively in the regeneration medium, MS supplemented with BA 2.5 mg L^{-1}.

2.2.3 Storage temperatures

According to Saiprasad and Polisetty (2003) hundred percent germination rates were observed in encapsulated PLBs of Orchids (*Oncidium* and *Dendrobium*) preserved for 45 and 60 days at 4˚C.

Singh *et al.* (2010) observed that encapsulated nodal segments of *Eclipta alba* gave 51.2 per cent survival in the recovery medium after 60 days of storage at low temperature (4°C).

Kikowska and Thiem (2011) reported that successful short term storage for 6 to 12 months of encapsulated plant material could be achieved in a closed sterile container at a temperature of 4°C.

Srivastva *et al.* (2009) observed 82.35 per cent regeneration in *Cineraria maritima,* after 6 months of storage of its alginate encapsulated microshoots, at 25˚C, providing moist environment by spraying with sterile distilled water at 15 days interval.

Thiruvengadam *et al.* (2012) stored encapsulated shoot tips of *Momordica dioica* under dark condition for different duration (0, 2, 4, 6, 8 and 10 weeks) at 4°C in full-strength liquid MS medium. The encapsulated shoot tips could be stored up to 10 weeks at low temperature (4°C) with a survival frequency of 50 per cent.

The encapsulated protocorms of *Cymbidium bicolor*, stored at 25°C gave better germination and regeneration compared to that at 4°C. At both temperatures, the regeneration per cent showed a gradual decline with storage duration. Encapsulated protocorms stored at 4°C up to 30 days retained only 10.23 per cent viability while with 60 days of storage, the synthetic seed turned necrotic, shrunken and brown, resulting in complete death. However, beads stored at 25°C remained green and viable with 52 per cent germination even at 360 days of storage (Mahendran, 2014).

Storage of encapsulated propagules is greatly influenced by the temperature. The response to storage temperature is species specific. There are reports elucidating that the storage life of synthetic seeds is shorter in low temperature of 4°C (Redenbaugh *et al.*, 1987; Gantait *et al.*, 2012).

2.2.4 Storage duration

Hegazi (2011) reported that the encapsulated shoot tips of *Capparis orientalis* gave 100 per cent planlet conversion. However with 15 days of storage at 4°C, it declined to 81.8 per cent and with 30 days of storage, it further declined to 61.9 per cent.

Ali *et al.* (2011) stated that at 4°C, with storage duration, the plantlet conversion of synthetic seeds of *Stevia rebaudiana* decreased. The plantlet conversion frequency after 15, 30, 45 and 60 days of storage are 86, 63, 50 and 23.3 per cent, respectively.

The alginate encapsulated shoot buds of *Centaurium erythraea*, with 3 per cent sucrose as additive in encapsulation matrix, were stored at 4°C for 4, 6 or 14 weeks. The encapsulated buds remained viable for 6 weeks but their developmental parameters significantly decreased. The plantlet recovery of stored

synthetic seeds increased with the addition of nutrient medium and growth regulator to the alginate matrix. The viability and ability to form plantlets retained even after 14 weeks of storage (Piatczak and Wysokinska, 2013).

Akdemir *et al.* (2013) observed a gradual decrease in plantlet conversion of encapsulated shoot apices of both cultivars of *Pistacia vera* Atlı'and 'Siirt' with longer storage periods at 4°C. The maximum plantlet conversion (100 per cent) was obtained in 'Atlı'cultivar with 3 months of storage while encapsulated shoot apices of 'Siirt' cultivar could be stored for 6 months at this temperature. A slight decrease to 88.3 per cent and 90 per cent of plantlet retrieval was achieved in 'Atlı' and 'Siirt' cultivars, respectively on prolonging storage period up to 12 months.

Mahmad *el al.* (2016) stated that the rate of germination of synthetic seeds of *Clitoria ternatea* L. was slightly decreased from 100 per cent to 77 per cent after 60 days of storage at 4°C.

The plantlet conversion efficiency of the encapsulated shoot tips of *Solanum tuberosum* (Nyende *et al.*, 2003) and *Withania somnifera* (Singh *et al.*, 2006) was also reported to decline markedly with extended period of storage at 4°C.

The reason for the decline in plantlet conversion efficiency with storage duration, according to Nyende *et al.* (2003) can be ascribed to the desiccation of the plant tissue and possible accumulation of metabolic wastes in the alginate capsules due to low ventilation storage conditions.

2.3 LONG TERM CONSERVATION USING VITRIFICATION

Cryopreservation is the maintenance of living cells, tissues organs and microorganisms at ultralow temperature (usually that of liquid nitrogen, −196°C). Cryopreservation in liquid nitrogen (–196°C) would serve as 'base collection' and is

so far the only viable procedure for long-term germplasm conservation of vegetatively propagated species (Engelmann, 2004). Cells will not undergo genetic changes under cryogenic storage as all the metabolic activity and cell divisions are arrested. Thus, the biological material can be conserved for extended period.

Engelmann (2004) described seven vitrification-based procedures in use for cryopreservation *viz.,* encapsulation dehydration, simple vitrification, encapsulation-vitrification, desiccation, pregrowth, pregrowth desiccation, and droplet freezing, which have been reported to be successfully used for a number of different plant species.

Vitrification, the most widely used cryopreservation technique, involves treatment of samples with cryoprotective substances, dehydration with highly concentrated vitrification solutions, rapid freezing and thawing, removal of cryoprotectants and recovery (Sakai and Engelmann, 2007). Vitrification solutions *viz.* PVS1, PVS2, PVS3 etc. is a combination of different cryoprotectants, in different ratios.

Vitrification-based procedures involve removal of most or all freezable water by physical or osmotic dehydration of explants, followed by ultra-rapid-freezing which results in vitrification of intra cellular solutes, *i.e.* formation of an amorphous glassy structure without occurrence of ice crystals which are detrimental to cellular structural integrity. As a consequence of these processes, plant tissues are protected from damage and remain viable during their long-term storage at –196°C. These techniques are less complex and do not require a programmable freezer, and are more appropriate for complex organs like embryos and shoot apices. Vitrification technique has been successfully applied to a wide range of plant species due to the easiness and high reproducibility (Panis and Lambardi, 2006).

The same procedure is followed for the two vitrification techniques – simple vitrification and encapsulation vitrification used in the study. The simple

vitrification utilizes non encapsulated explants, while encapsulation-vitrification is a combination of encapsulation and vitrification procedures (Benelli *et al.,* 2013; Engelmann, 2011). The encapsulation-vitrification method was reported first by Matsumoto *et al.* (1995) using shoot apices of *Wasabia japonica*. The regrowth of plant germplasm after cryopreservation is markedly increased by encapsulating plant samples with alginate beads. Encapsulation protects the plant tissue from injury due to dehydration by vitrification procedures.

2.3.1 Pre-conditioning

Jin Baek *et al.* (2003) reported that pre-conditioning of apices at 10°C or 23°C for more than 3 days had a detrimental effect on regeneration of garlic shoot tips. The optimal sucrose concentration in the pre-conditioned medium was 0.3-0.5 M.

Khawnium and Te-chato (2011) stated that in the cryopreservation of oil palm using simple vitrification, the highest (16.67 per cent) somatic embryoids formation in the friable embryogenic tissue (FE) was found by preconditioning the FE in 0.25 M sucrose for 7 days followed by storage in liquid nitrogen.

An average of 61 per cent survival was obtained for *Vanilla planifolia* apices directly preconditioned with sucrose (0.3 M) for seven days before loading and vitrification treatments (Ramirez *et al.*, 2014).

2.3.2 Pre-culture

Pre-culture of the explants under high osmoticum provides desiccation tolerance during cryopreservation. Sucrose is a pregrowth additive, which enhances the desiccation tolerance during cryopreservation. High concentration of sucrose medium might be favourable for osmotic adjustments that protect the

cryopreserved plant tissue from injury on exposure to vitrification solutions (Thinh *et al.*, 1999).

Uragami *et al.* (1990) reported that the asparagus nodal segments precultured in 0.7 M sucrose for 2 days and subsequent desiccation to 20 per cent water content with silica gel, gave 63 per cent survival after cryopreservation.

Studies on cryopreservation of carrot (Dereuddre *et al.*, 1991) tissues revealed that survival rates after cryopreservation could be increased by preculturing the tissues on media containing high concentration of sugar. Eighty percent recovery was seen in *Pyrus sp* by following a preculture treatment in a medium supplemented with 0.75 M sucrose (Scottez *et al.*, 1992).

Dumet *et al.* (1993) states that preculturing of zygotic and somatic embryos in a medium supplemented with cryoprotectants such as sucrose or glucose are needed, before exposing it to the liquid nitrogen for cryopreservation.

Panis *et al.* (1996) reported post thaw recovery rate of 69 per cent, when the meristem clumps of *Musa sp,* precultured for 2 weeks on a proliferating medium supplemented with 0.4 M sucrose were subjected to cryostorage. Martinez and Revilla (1999) revealed that high sucrose concentration (up to 1 M) during preculture was toxic for shoot tip survival.

Among different osmoticum, fructose, glucose, sucrose and sorbitol, tried for preculture in date plam cryopreservaiom, sucrose was found to be the best pre culture additive (Bekheet *et al.*, 2007). Senula *et al.* (2007) reported that the shoot tips of *Mentha sp* were pre-cultured on filter paper in liquid MS medium containing 0.3 M sucrose at 25°C for 20-24 hrs for successful cryopreservsation.

Ray and Bhattacharya (2008) revealed the effect of sucrose preculture duration on cryopreservation of *Rauvolfia serpentina.* When the explants were

precultured for 1-7 days, maximum survival (46 per cent) was obtained after 7 days of preculture. The number of viable explants declined gradually when preculture duration was increased from 7 to 21 days.

Yap *et al.* (2011) stated that increasing the duration of preculture in 0.3 M sucrose supplemented medium, from 0 to 3 days enhanced tolerance to PVS2 solution from 5.6 per cent (no preculture) to 49.2 per cent (3-day preculture) in *Garcinia cowa* shoot tips.

MS medium supplemented with 0.4 M sucrose was observed to be the best concentration for the preculturing shoot tips of *Kaempferia galanga* among other concentrations *viz.*, 0.3 M, 0.4 M, 0.5 M and 0.75 M, tried. A survival of 67 per cent could be obtained after cryostorgae in LN (Preetha *et al.*, 2013).

2.3.3 Loading treatment

Direct exposure of plant tissues to vitrification solutions often leads to detrimental effects. Exposure to loading solutions usually minimizes those adverse effects and leads to increased survival and regrowth after cryopreservation. Loading solution is a diluted version of vitrification solution used for dehydration of plant tissues (Sakai, 2000).

The vitrification method was developed by Uragami *et al.* (1989) using asparagus culture cells. Plant tissues were exposed to a loading solution (LS) consisting of sucrose 0.4 M and glycerol 2.0 M, for 30 min at 25°C, for osmoprotection.

Before exposing the samples of *Asparagus officinalis* to vitrification solution, they are treated with loading solution (2.0 M glycerol and 0.4 M sucrose), to induce tolerance to dehydration by the vitrification solution Nishizawa *et al.* (1993).

With the addition of loading phase prior to immersion in cryoprotective solution and then plunging in liquid nitrogen, the post LN survival was found to higher in *Anigozanthos sp.* The signs of survival could be visible 1-2 days earlier for the loaded samples compared to the samples devoid of loading phase. The initial rate of growth was also more rapid in samples subjected to loading phase (Turner *et al.*, 2001).

Ramirez *et al.*, 2014 stated that loading solution containing 0.4 M sucrose and 2 M glycerol produced highest survival (average 82 per cent) in *Vanilla planifolia.*

According to Jin Baek *et al.* (2003) loading apices of garlic, for 30 or 60 min at 23°C in medium containing glycerol 2 M and sucrose 0.4 M or glycerol 1 M and sucrose 0.8 M had no effect on regeneration after cryopreservation, in comparison with apices cryopreserved without loading treatment.

The effect of treatment with a loading solution (2 M glycerol and 0.4 M sucrose) prior to vitrification was studied in *Garcinia cowa* shoot tips. A decreased tolerance of shoot tips to PVS2 vitrification solution after loading treatment was observed. It had a deleterious effect on the ultrastructure of *G. cowa* meristematic cells (Yap *et al.,* 2011).

2.3.4 Vitrification, Cryopreservation and Recovery

The successful vitrification requires the use of highly concentrated and non-toxic solution of cryoprotectants. The key to success of cryopreservation is to carefully standardise the procedures for dehydration and cryoprotectant permeation and prevent injury by chemical toxicity and or excessive osmotic stress during the treatment. This could be achieved by exposure to vitrification for

varying duration. The different vitrification solutions used in cryopreservation studies include PVS1, PVS2, PVS3 etc

PVS2, the most efficient vitrification solution developed so far, is composed of 30 per cent (w/v) glycerol, 15 per cent (w/v) ethylene glycol, 15 per cent (w/v) dimethyl sulfoxide (DMSO) in MS medium with 0.4 M sucrose (Sakai *et al.*, 1990; Matsumoto and Sakai, 2003; Wang *et al.*, 2003).

Banana shoot tips were found to be very sensitive to PVS2 treatment. Exposure to PVS2 for 5 minutes at 25°C significantly reduced the survival rate of shoot tips Pannis (1995), while orchids (*Cymbidium*) were found to more tolerant to PVS2 treatment as 64.5-70 per cent shoot tips resumed growth even after 40 minutes of PVS2 exposure. (Thinh and Takagi, 2000).

PVS3 is a combination of glycerol 50 per cent (w/v) and sucrose 50 per cent (w/v) in water and was reported to be successful in cryopreserving only in a limited number of species such as asparagus (Nishizawa *et al.*, 1993).

Effective cryopreservation could be achieved in *Anigozanthos viridis* by subjecting the shoot apices in PVS2 solution for for 25 min. The dehydrating and penetrating chemicals of the highly concentrated vitrification solutions prior to the exposure to LN, could promote survival of the cryoexposed cells of shoot tips (Turner *et al.*, 2001).

Ryynanen and Aronen (2005) obtained 71 per cent recovery of birch axillary buds from cryostorage after preculture in 0.7M sucrose for 24 h, loading treatment in solution containing 2M glycerol and 0.4M sucrose for 20 min at room temperature and dehydration in PVS2 for 20 minutes.

The effect of different vitrification solutions *viz.*, PVS1, PVS2 and PVS2 on cryopreservation of *Rauvolfia serpentina* were studied. The best response was

obtained with PVS2 treatment. On exposing the nodal segments to PVS2 solution for 45 min, the highest survival of 66 per cent was obtained after cryopreservation (Ray and Bhattacharya, 2008).

Markovic *et al.* (2013) reported that on comparing the effect of PVS2 and PVS3 on *Vitis vinifera* dehydration, no plant recovery was observed in plantlets exposed to PVS3 vitrification solution for 40 minutes, whereas 40 per cent of survival was achieved with PVS2 dehydration on same time of exposure.

Preetha *et al.* (2013) reported that *Kaempferia galanga* shoot tips were able to resume 36.67 per cent shoot growth after exposure to PVS2 at 0 °C for 20 minutes followed by plunging in LN for one hour. The increased sensitivity of *K. galanga* shoot tips to PVS2 was confirmed when a drastic drop was observed in post-LN recovery after 30 minutes PVS2 exposure at 0 °C.

In encapsulation vitrification of *Populas alba*, toxic effects of PVS2 treatment could not be observed after 120 mins of exposure but none of the cryo-preserved explants survived after 3 weeks in regeneration medium (Tsvetkov *et al.*, 2009).

The protocorm- like bodies (PLBs) of Dendrobium Sonia-28 could be cryopreserved through encapsulation- vitrification method that involved the pre-culture of 3-4 mm PLBs for six days in 0.5 M sucrose, followed by dehydration in PVS2 at 0°C for 150 min. (Ching *et al.*, 2012).

Yamuna *et al.* (2007) opined that regrowth was higher in simple vitrification (80 per cent) than encapsulation vitrification (66 per cent) in *Zingiber officinale* after cryostorage.

Dhital *et al.* (2010) observed encapsulation vitrification gave higher survival and regeneration than simple vitrification, which recorded 36 per cent survival and 28 per cent regeneration in *Solanum tuberosum.*

According to Vianna *et al.* (2012) encapsulation- vitrification was found to be more effective than simple vitrification in *Passiflora sp*. The highest recovery rates for *P. suberosa* (28 per cent) and *P. foetida* (60 per cent) were obtained with the encapsulation-vitrification protocol after pre-treating the encapsulated shoot tips with 0.3 M sucrose, followed by exposure to PVS2 for 60 or 120 min, respectively, and post-freezing incubation on MS medium supplemented with BA 0.44 µM, in the absence of light for 30 days.

Gogoi *et al.* (2012) also reported the better cryopreservation response with encapsulation vitrification (66 per cent regeneration) than simple vitrification (50 per cent regeneration) in *Cymbidium eburneum*. An exposure duration of 20 min. to PVS2 solution led to optimal regeneration after cryopreservation.

Preetha *et al.* (2013) reported that shoot tips of *Kaempferia galanga* were successfully recovered and remained green and resumed growth within 2-3 weeks after the exposure to vitrification and cryopreservation. On transferring the shoots to multiplication medium emergence of shoots was induced directly from apical dome without callus formation.

2.4 GENETIC FIDELITY ASSESSMENT OF PLANTLETS RECOVERED AND REGENERATED FROM *IN VITRO* STORAGE

The assessment of genetic fidelity of plantlets recovered from *in vitro* storage is inevitable in germplasm conservation programme. Two PCR-based techniques, RAPD and ISSR, could be used to test the genetic fidelity of the plants recovered and regenerated from *in vitro* storage because of their simplicity and cost-effectiveness. The two markers, which amplify different regions of the genome, allow better chances for the identification of genetic variations in the plantlets (Martin *et al.*, 2004).

According to Martín and González-Benito (2005) the genetic stability of s *Dendranthema grandiflora* was confirmed when an identical RAPD pattern was obtained in the micro-propagated shoots before and after cryopreservation using vitrification. Yamuna *et al.* (2007) confirmed genetic stability of cryopreserved ginger shoot buds *via* vitrification techniques by RAPD profiling.

The genetic fidelity of the vitrified birch material seems to have remained unchanged according to the morphology and the RAPD profiles of regenerated plants in the greenhouse (Ryynanen and Aronen, 2005).

Genetic fidelity assessment of encapsulated *in vitro* tissues of *Bacopa monnieri* after six months of storage by using ISSR and RAPD markers was reported by Muthiah *et al.* (2013). Results obtained corresponds to little or very low genetic variation pertaining to the clonal nature of the regrown plantlets with that of the mother plant.

Muthiah *et al.* (2013) confirmed the genetic stability of plantlets from encapsulated explants of *Bacopa monnieri* regenerated after 6 months *in vitro* storage at 4°C using RAPD and ISSR markers.

According to Soni and Kaur (2014) genetic stability of the *in vitro* conserved plants of *Viola pilosa* were tested using RAPD and ISSR markers. The plantlets recovered from slow growth (by modifying temperature) and vitrification exhibited no somaclonal variation. All RAPD and ISSR primers produced distinct and scorable bands. All banding profiles of recovered plants were monomorphic and similar to those of the mother plant. Negligible polymorphism was detected during the marker analysis of *in vitro* conserved clones.

Materials and Methods

3. MATERIALS AND METHODS

The present study, '*In vitro* conservation of chethikoduveli (*Plumbago rosea* L.) using encapsulation and vitrification techniques' was carried out in Department of Plant Biotechnology, College of Agriculture, Vellayani during 2014-2016. The study aimed at standardization of protocol for short term and long term conservation of axillary buds of *P. rosea* using encapsulation and vitrification techniques and assessment of genetic fidelity of recovered and regenerated plantlets from storage using molecular markers.

The study was carried out in three phases *viz.,* enhancement of multiplication rate, short term conservation by encapsulation technique and long term conservation using vitrification technique.

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

3.1 PHASE I: ENHANCEMENT OF MULTIPLICATION RATE

In this phase of the investigation, different media and different levels of additives were tried to enhance the shoot multiplication in *P. rosea.*

3.1.1 Explant

Axillary buds from the *in vitro* maintained cultures of *P. rosea* var. Agni were used as the explants in the study. Single node segments of 1-2 cm, containing an axillary bud were excised from the *in vitro* maintained cultures established in MS (Murashige and Skoog, 1962) medium supplemented with BA 1.5 mg L^{-1} and IAA 1 mg L^{-1}. This media has been standardized for axillary shoot proliferation of *P. rosea* in a previous study at the Department of Plant Biotechnology, College of Agriculture, Vellayani.

3.1.2 Media

Four different media *viz.*, White's medium (1963), SH medium (Schenk and Hildebrandt,1972), B5 medium (Gamborg *et al.*, 1968), and MS (control) each supplemented with BA 1.5 mg L^{-1} and IAA 1 mg L^{-1} were tried. The treatments are presented in Table 1. Observations on regeneration per cent, days to bud initiation, shoots initiated per explant, shoot length and nodes per shoot were recorded after six weeks of culture.

Table 1. Media tried for enhancement of multiplication

Treatment	Media	Hormones
ME1	White	BA 1.5 mg L^{-1} and IAA 1 mg L^{-1}
ME2	SH	
ME3	B5	
ME4	MS	

3.1.3 Additives

The effect of different additives *viz.,* chitosan, adenine sulphate and thidiazuron on multiplication rate of axillary buds were assessed. Different levels of each of these additives were added to the standardized control medium (MS medium + BA 1.5 mg L^{-1} + IAA 1 mg L^{-1}).

Stock solutions of different additives were prepared by dissolving required quantities of salts in double distilled water (adenine sulphate) or 1 N NaOH (thidiazuron) or 0.1 M acetic acid (chitosan). The final volume was made up using double distilled water in a volumetric flask. The stock solutions are then kept in refrigerator (4°C) in sterilized bottles. Different concentrations of additives *viz.,* chitosan (0, 5, 10, 15, 20, 25 and 30 mg L^{-1}), adenine sulphate (20, 40, 60 80, 100 and 120 mg L^{-1}) and thidiazuron (0.5, 1, 2, 3, 4 and 5 mg L^{-1}) were added from the stock solutions to the control medium maintaining the pH at 5.7. The treatments for the study are represented in Tables 2, 3 and 4. Each of the treatments were replicated six times.

Observations on regeneration per cent, days to bud initiation, shoots initiated per explant, shoot length and nodes per shoot were recorded after six weeks of culture.

Table 2. Different levels of chitosan tried for shoot multiplication

Treatment	Chitosan Concentration (mg L^{-1})	Hormones
CH1	5	BA 1.5 mg L^{-1} and IAA 1 mg L^{-1}
CH2	10	
CH3	15	
CH4	20	
CH5	25	
CH6	30	
CH7	0	

Table 3. Different levels of Adenine sulphate tried for shoot multiplication

Treatment	Adenine sulphate concentration (mg L^{-1})	Hormones
AD1	20	BA 1.5 mg L^{-1} and IAA 1 mg L^{-1}
AD2	40	
AD3	60	
AD4	80	
AD5	100	
AD6	120	
AD7	0	

Table 4. Different levels of Thidiazuron tried for shoot multiplication

Treatment	Thidiazuron concentration (mg L^{-1})	Hormones
TD1	0.5	BA 1.5 mg L^{-1} and IAA 1 mg L^{-1}
TD2	1	
TD3	2	
TD4	3	
TD5	4	
TD6	5	
TD7	0	

3.2 PHASE II: STANDARDIZATION OF PROTOCOL FOR SHORT TERM CONSERVATION BY ENCAPSULATION TECHNIQUE.

3.2.1 Encapsulation matrix

Single node cuttings of 0.5-1.0 cm with axillary buds were excised from *in vitro* grown cultures for encapsulation. The encapsulation matrix were prepared using 2.5 per cent w/v sodium alginate (Sigma St. Louis, USA) solution in distilled water and 100 mM calcium chloride allowing a complexing time of 30 min. Different osmoticum *viz*., sucrose (10 per cent w/v and 15 per cent w/v) and mannitol (10 per cent w/v and 15 per cent w/v) were supplemented to this encapsulation matrix by mixing it in 2.5 per cent sodium alginate. The Table 5 represents different encapsulation matrices tried for short term conservation studies. Each of the treatments were replicated six times.

Table 5: Different encapsulation matrices tried for short term conservation

Treatment No.	Encapsulation Matrix
A_1	sodium alginate 2.5%+ sucrose 10%+ calcium chloride 100 mM
A_2	sodium alginate 2.5%+ sucrose 15%+ calcium chloride 100 mM
A_3	sodium alginate 2.5%+ mannitol 10%+ calcium chloride 100 mM
A_4	sodium alginate 2.5%+ mannitol 15%+ calcium chloride 100 mM
A_5	sodium alginate 2.5%+ calcium chloride 100 mM

3.2.2 Storage medium

The axillary buds encapsulated in different matrices, were transferred to 2 ml Eppendorf tubes. The beads were suspended in sterile double distilled water and liquid MS medium devoid of calcium chloride. One set of encapsulated beads were kept as such, without any storage medium, as control. The above two media were added afresh to the Eppendorf tubes every 5^{th} day, inside the laminar air flow chamber. The treatments are represented in Table 6. Each of the treatments were replicated six times.

Table 6: Different storage media tried for short term conservation

Treatment No:	Storage media
M_1	Liquid MS
M_2	Sterile distilled water
M_3	No media

3.2.3 Storage temperatures

The encapsulated beads in different storage medium were maintained at two different temperature regimes *viz.,* 4°C and 25°C, *i.e.,* in refrigerated and culture room conditions, respectively. The treatments are represented in Table 7. Each treatments were replicated six times. The beads were maintained in these conditions for different duration *viz.*, 15, 30, 45, 60, and 90 days.

After storing for different duration under different temperatures, the encapsulated beads were inoculated aseptically to the best multiplication medium standardized in the Phase I investigation. The observations on regeneration per cent, days to bud initiation, shoots initiated per explant, shoot length and nodes per shoot were recorded after six weeks of culture.

Table 7: Different storage temperature tried for short term conservation

Treatment	Storage temperature
T_1	4°C
T_2	25°C

3.3 PHASE III: LONG TERM CONSERVATION USING VITRIFICATION TECHNIQUES

In long term conservation experiments, encapsulated and non-encapsulated axillary buds were subjected to cryostorage, *i.e.* liquid nitrogen at -196°C. Two cryopreservation techniques resorted to in this study were simple vitrification and encapsulation vitrification.

3.3.1 Pre-conditioning

Axillary buds from *in vitro* established *P. rosea* cultures were inoculated MS medium supplemented with sucrose 0.5M and agar 0.57 per cent. Cultures were incubated at 25± 2°C under 14h photoperiod at a photon flux intensity of 30-50 μM m^{-2} s^{-1} for 7 days.

3.3.2 Pre-culture

The pre-conditioned axillary buds were pre-cultured in liquid MS medium supplemented with sucrose 0.5M and DMSO 3 per cent, dispensed into 100 ml Erlenmeyer flasks (50 buds in 25 ml) and incubated at 25± 2°C under 14 h photoperiod at a photon flux intensity of 40 μM m^{-2} s^{-1} for three days without agitation. The medium was drained out after every 24 h compensated with fresh medium of the same composition. In case of encapsulation vitrification technique, precondioned axillary buds were encapsulated in sodium alginate 2.5 per cent and calcium chloride 100 mM, and further subjected to preculture treatment as described.

3.3.3 Loading treatment

The pre-cultured axillary buds or the encapsulated beads, as the case may be, were transferred to 4 ml of autoclaved cryo-tubes (Nalgene Co.) containing sterile loading solution (glycerol 2 M and sucrose 0.4 M in MS medium; pH-5.7) and the left for 30 minutes at room temperature.

3.3.4 Vitrification

After the loading treatment, loading solution was pipetted out from the cryo-tube and replaced with sterile vitrification solution *viz.,* PVS2 (30 per cent (w/v) glycerol, 15 per cent (w/v) ethylene glycol and 15 per cent (w/v) DMSO in

MS liquid medium with 0.4M sucrose; pH 5.7) and PVS3 (50 per cent (w/v) glycerol and 50 per cent (w/v) sucrose in MS liquid medium, pH 5.7). The axillary buds were exposed to vitrification solution for different duration *viz.*, 0, 30, 60, 90, 120, 150, 180 and 210 min at room temperature. The treatments are represented in Table 8 and Table 9.

3.3.5 Cryopreservation and recovery

Axillary buds/beads exposed to vitrification solutions in different durations were directly immersed in liquid nitrogen, after replacing the vitrification solution in the cryovial with fresh solution, where they were stored at least for 2 hours. Cryo-tubes were then removed from liquid nitrogen and transferred to water under constant circulation in a water bath maintained at 40°C for 2 min, for thawing. The re-warmed shoots tips were transferred to recovery medium (standardized in Phase I investigation) and incubated in culture room at 25± 2°C under 14h photoperiod and photon flux intensity of 40 $\mu E\ m^{-2}\ s^{-1}$. The results were expressed in survival percent and regeneration per cent. Survival was estimated as percentage of treated shoots remaining green and showing early symptoms of *in vitro* response (swelling/shoot bud initiation). Regeneration was estimated as percentage of nodal segments differentiated into shoots. The observations on days to bud initiation, shoots initiated per explant, regeneration percentage, shoot length (cm) and nodes per shoot were recorded. The treatments were replicated three times. Each replicate consisted of 30 beads/ axillary buds.

3.4 STATISTICAL ANALYSIS

Completely randomized design was followed for statistical analysis (Panse and sukhatme, 1954), wherever applicable. Data were subjected to analysis of variance (ANOVA) and significant differences between treatments were determined by Duncan multiple range test.

Table 8: Vitrification solution and period of exposure tried for simple vitrification

Treatment No:	Vitrification solution	Period of exposure to vitrification solution (min)
S1	PVS 2	0
S2		30
S3		60
S4		90
S5		120
S6		150
S7		180
S8		210
S9	PVS 3	0
S10		30
S11		60
S12		90
S13		120
S14		150
S15		180
S16		210

Table 9: Vitrification solution and period of exposure tried for encapsulation vitrification

Treatment No:	Vitrification solution	Period of exposure to vitrification solution (min)
E1	PVS 2	0
E2		30
E3		60
E4		90
E5		120
E6		150
E7		180
E8		210
E9	PVS 3	0
E10		30
E11		60
E12		90
E13		120
E14		150
E15		180
E16		210

3.5 MEDIA PREPARATION AND STERILISATION

The chemicals used for the preparation of culture media in the regeneration and conservation experiments were of analytical grade from Merck (Mumbai) and HiMedia Laboratories Pvt. Ltd. (Mumbai).

Stock solutions of major and minor nutrients, organic supplements and plant growth regulators for different media was prepared by following the standard procedures. The stock solutions of different media were prepared by dissolving weighed amounts of salts (using electronic balance; Shimadzu AUX120) of major and minor nutrients and organic supplements in double distilled water.

Stock solutions of growth regulators (GR) were prepared by adding few drops of solvent (ethyl alcohol 50 per cent/ HCl 0.1N / NaOH 0.1N) for the dissolution of required amount of growth regulator. After dissolution, the required volume was made up by the addition of double distilled water in volumetric flask. All the stock solutions were stored in refrigerator (4°C) in sterilized bottles.

The media was prepared by adding calculated amount of aliquots from the stock solutions. Sucrose and inositol required for different media were added afresh and dissolved by constant stirring. The pH of the media was adjusted to 5.6- 5.8 (NaOH 1N or HCl 1N) using electronic pH meter (Susima MP-1 PLUS) after mixing all the constituents except the gelling agent. Agar agar (gelling agent) was added and final volume was made using volumetric flask. Agar was melted using microwave oven. The molten medium was dispersed into pre-sterilized culture bottles (300 ml). The culture bottles were tightly covered using autoclavable plastic lids and media sterilized for 20 min. at 121°C and 15 lbs/(inch)^2 pressure in STERI horizontal autoclave (Yorko, India).

The glasswares, plasticwares and tools used in the regeneration and conservation experiments were initially cleaned by washing with dilute liquid

detergent (Laboline) followed by rinsing with distilled water. All these were dried in hotair oven (160°C -180°C) and wrapped in autoclavable polypropylene covers. These were then sterilized by autoclaving at 121°C and 15 lbs/ $(inch)^2$ pressure for 45 min.

3.6 INOCULATION AND INCUBATION

All the inoculations in the study were carried out inside the laminar air flow chamber. The laminarhood was wiped with 70 per cent v/v ethyl alcohol followed by UV sterilization for 30 min. All the tools used for the inoculations were flame sterilized using absolute ethanol inside the laminar air flow chamber. The explants were aseptically inoculated into the culture bottles and were then incubated in the culture room. Cultures were incubated at 25± 2°C, providing a photoperiod of 14h light/ 8 h dark, at a photon flux intensity of 40 $\mu E\ m^{-2}\ s^{-1}$.

3.7 GENETIC FIDELITY ASSESSMENT OF PLANTLETS RECOVERED AND REGENERATED FROM *IN VITRO* STORAGE

The assessment of genetic fidelity of the plantlets recovered from short and long term conservation was attempted using RAPD and ISSR markers.

3.7.1 Genomic DNA isolation

CTAB method of DNA extraction (Doyle and Doyle, 1990) with slight modification as described by Nathar and Gadge (2013) was used. An extraction buffer consisting of 2 per cent CTAB (w/v), 0.5 M Tris HCl (pH 8.0), 0.5 M EDTA (pH 8.0), 5M NaCl, was prepared.

The leaf samples (0.4 g) were taken from normal *in vitro* cultures and the cultures regenerated from short term and long term conservation treatments. The leaves were washed in distilled water and midrib was removed. They were ground using 1 - 2 ml of extraction buffer in an ice cold mortar and pestle. While grinding 0.1 g of PVP was added. The pulverized leaves were quickly transferred to 3 ml

of freshly prepared prewarmed (65°C) extraction buffer and shaken vigorously by inversion. The tubes were kept for incubation at 65° C for 1 h. After incubation an equal volume (5 ml) of chloroform: isoamyl alcohol (24:1) was added and mixed gently by inversion. The mixture was centrifuged at 10000 rpm for 15 min at 25 to 30 °C. Top aqueous layer was transferred to fresh tube and equal volume of cold isopropanol was added to each tube and again centrifuged at 10000 rpm for 15 min at room temperature. Liquid was decanted leaving the pellet attached to the wall of tube. The pellet was washed with 70 per cent ethanol (4 °C), air dried for 5 min at room temperature. Then it was resuspended in 2ml TE buffer (Appendix I) and 5 µl of RNase was added and incubated overnight at 37° C. The extracted DNA samples were stored at -20°C (Lab-Line Low Temperature Cabinet, India).

3.7.2 Quantification of DNA

Spectrophotometer readings of DNA samples were recorded to determine the quantity and quality of DNA. T60- UV visible spectrophotometer was used for measuring the optical density (OD) of the sample. Spectrophotometer was calibrated to blank (zero absorbance) at 260 nm and 280nm wavelength with 3ml TE buffer and OD reading of 5µl DNA sample dissolved in 3ml of TE buffer at respective wavelengths. An optical density (OD) value of one at 260 nm indicates the presence of 50 ng/µl of double stranded DNA. Hence the quantity of DNA in the extracted sample was estimated by employing the following formula:

$$\text{Concentration of DNA (ng/µl)} = A_{260} \times 50 \times \text{dilution factor}$$

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

Proteins are known to absorb strongly at 280nm, while absorption maximum of DNA/ RNA is at 260 nm. The quality of DNA was determined by ratio of OD 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 proteins.

3.7.3 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 II) was prepared to fill the electrophoresis tank and to cast the gel. Prepared agarose solutions (0.8 per cent) 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 microwave oven until the agarose dissolved. On cooling the molten gel ethidium bromide (EtBr) was added to a final concentration of 0.5µg ml^{-1} and mixed thoroughly. The comb was positioned above the tray and then warm agarose solution was poured into the moulded casting tray. The gel was allowed to set completely (30-45 mins) at room temperature. After the gel was set, the comb was carefully removed and the gel was mounted in electrophoresis tank. Electrophoresis buffer was added just enough to cover the gel to a depth of about 1mm above the gel. DNA sample (5µl) mixed with 1µl 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 was applied and was turned off when the dye migrated three-fourth of the distance through the gel. The gel was removed carefully and visualized under gel documentation system (BIORAD) using 'Quantity One Software'.

3.7.4 RAPD and ISSR Analysis

Seven DNA samples were amplified with five RAPD and four ISSR primers supplied by HiMedia Laboratories Pvt. Ltd. (Mumbai). Primers selected for the analysis with their specified T_m are listed in Table 10.

Sterile water	:	14.4 µl
10 X Taq buffer	:	2.5 µl
$MgCl_2$ (15*mM*)	:	0.4 µl

dNTPs (10*mM* each)	:	1.0 µl
Primer (10 *µM*)	:	1.0 µl
Template DNA(100 ng μl^{-1})	:	0.5 µl
Taq polymerase (5U μl^{-1})	:	0.2 µl
Total volume	:	20 µl

PCR programme was set with initial denaturation at 94°C for 4 min. followed by 35 cycles of denaturation at 94°C for 1 min, annealing ($T_m \pm 5$) for 1 min, and extension at 72°C for 2 min and final extension was done at 72°C for 7 min. The amplified products were separated on agarose gel (1.5 per cent). The gel was viewed under gel documentation system (BIORAD, USA).

Table 10. Sequence of primers used for DNA amplification to evaluate the genetic stability of plantlets regenerated from *in vitro* storage

Sl no:	Primers		Sequence (5'-3')	T_m (°C)
1	RAPD	RPI 2	AACGCGTCGG	27
2		RPI 3	AAGCGACCTG	25
3		RPI 5	AATCGGGCTG	25
4		RPI 4	AATCGCGCTG	25
5		RPI 6	ACACACGCTG	25
6	ISSR	UBC-810	GAGAGAGAGAGAGAGAT	42.9
7		UBC-811	GAGAGAGAGAGAGAGAC	43.3
8		UBC-827	ACACACACACACACACG	54.9
9		HBO-816	CACACACACACACACAT	51.2

Results

4. RESULTS

Investigations were carried out in "*In vitro* conservation of chethikoduveli (*Plumbago rosea* L.) using encapsulation and vitrification techniques" at the Department of Plant Biotechnology, College of Agriculture, Vellayani. The results of the study are presented in this chapter.

4.1 PHASE I: ENHANCEMENT OF MULTIPLICATION RATE

4.1.1 Effect of different medium and additives on multiplication rate

Single nodal segments from *in vitro* raised cultures were used as explants to study the effect of different media and different additives on the multiplication in *P. rosea.* A medium for shoot multiplication from the single nodal explants of *P. rosea*, MS supplemented with BA 1.5 mg L^{-1} and IAA 1 mg L^{-1}, has already been established in a previous study at the Department of Plant Biotechnology.

4.1.1.1 Medium

Four different media *viz.,* White's medium, SH medium, B5 medium and MS (control) each supplemented with BA 1.5 mg L^{-1} and IAA 1 mg L^{-1} were used to assess their effect on shoot multiplication. All the media tried gave 100 per cent regeneration.

Among the different media tried, the control medium (MS + BA 1.5 mg L^{-1} +IAA 1 mg L^{-1}) gave significantly higher response with respect to different parameters *viz*., days to bud initiation, shoots per explant and shoot length (Plate 1).

The earliest shoot bud initiation (7.50 days) was seen in MS media. The bud initiation was late (12 days) in ME3 (B5 + BA 1.5 mg L^{-1}+ 1 mg L^{-1} IAA). This is on par with ME1 (White+ BA 1.5 mg L^{-1}+ 1 mg L^{-1} IAA) and ME2 (SH+ BA 1.5 mg L^{-1}+ 1 mg L^{-1} IAA).

With respect to shoots initiated per explant, the maximum number of shoots (3.75 shoots/ explant) was obtained in ME4 (MS+BA 1.5 mg L^{-1}+ IAA 1 mg L^{-1}). The least (2.50 shoots/explants) number of shoots was obtained in ME3. This value was on par with ME1 and ME2.

Maximum shoot length (4.30cm) was observed in ME4 and had shown a significant variation among other treatments. The least shoot length (3.65cm) was seen in ME3 and its value is on par with ME1 and ME2.

There was no significant variation with respect to the number of nodes per shoots, among the four different media tried.

The MS medium supplemented with BA 1.5 mg $L^{-1}$and IAA 1 mg L^{-1} was selected for the further study on shoot multiplication using different additives. The results of the study are presented in Table 11 (Fig. 1).

4.1.1.2 Additives

Additives *viz.*, chitosan, adenine sulphate and thidiazuron at different levels were tried to study the effect on shoot multiplication. MS medium supplemented with BA 1.5 mg $L^{-1}$and IAA 1 mg $L^{-1}$was selected as the basal medium to which each of these was added.

4.1.1.2.1 Chitosan

Seven treatments with varying concentration of chitosan (0, 5, 10, 15, 20, 25 and 30 mg L^{-1}) were tried to assess the effect on shoot proliferation.

All the different levels of chitosan gave 100 per cent regeneration. Significant variation was observed in the study with respect to days to bud initiation, shoots per explant and shoot length (Plate 2).

Table 11: Effect of different medium on shoot proliferation of *Plumbago rosea*

Treatment no:	Medium	Regeneration percentage	Days to bud initiation	Shoots per explant	Shoot length (cm)	Nodes per shoot
ME1	White+1.5 mg L^{-1} BA+1 mg L^{-1} IAA	100	11.75 ± 0.96^{a}	2.75 ± 0.50^{b}	3.88 ± 0.53^{ab}	2.63 ± 0.14
ME2	SH+ 1.5 mg L^{-1} BA+ 1 mg L^{-1} IAA	100	11.75 ± 0.96^{a}	2.50 ± 0.58^{b}	3.82 ± 0.56^{b}	2.50 ± 0.23
ME3	B5+ 1.5 mg L^{-1} BA+1 mg L^{-1} IAA	100	12.00 ± 0.82^{a}	2.50 ± 0.58^{b}	3.65 ± 0.64^{b}	2.29 ± 0.08
ME4	MS +1.5 mg L^{-1} BA +1 mg L^{-1} IAA	100	**7.50 ± 0.58^{b}**	**3.75 ± 0.50^{a}**	**4.30 ± 0.88^{a}**	2.70 ± 0.31
CD (5%)		-	1.29	0.83	0.44	NS

A. MS + BA 1.5 mg L^{-1} + IAA 1 mg L^{-1}

B. Whites + BA 1.5mg L^{-1} + IAA 1 mg L^{-1}

C. SH + BA 1.5mg L^{-1} + IAA 1 mg L^{-1}

D. B5 + BA 1.5mg L^{-1} + IAA 1 mg L^{-1}

Plate 1: Regeneration of axillary buds of *P. rosea* in different media

The earliest (7.42 days) shoot bud initiation was seen in the control treatment CH7 (MS+ BA 1.5 mg L^{-1}+ IAA 1 mg L^{-1}). It is on par (7.50 days) with CH3 (MS + BA 1.5 mg L^{-1}+ IAA 1 mg L^{-1} + chitosan 15 mg L^{-1}). In CH6 (MS+ BA 1.5 mg L^{-1}+ IAA 1 mg L^{-1} + chitosan 30 mg L^{-1}) delayed bud initiation (13.13 days) was observed.

The maximum shoot per explant of 3.67 cm was seen both in CH7 and CH3. The minimum number of shoots (1.88 cm) was observed in by CH6. This was on par with CH4 (MS + BA 1.5 mg L^{-1}+ IAA 1 mg L^{-1} + chitosan 20 mg L^{-1}) and CH5(MS + BA 1.5 mg L^{-1}+ IAA 1 mg L^{-1} + chitosan 25 mg L^{-1}).

The maximum shoot length (4.29 cm) was seen in CH3 which was on par with CH7 (4.28 cm), the control treatment. The lowest shoot length (3.47 cm) was recorded both in CH5 and CH6. This was found to be on par with CH4.

There was no significant variation with respect to nodes per shoot among the different levels of chitosan tried.

Though, better response with respect to days to bud initiation shoots per explant and shoot length was obtained with MS medium supplemented with BA 1.5 mg L^{-1}, IAA 1 mg L^{-1} and chitosan 15 mg L^{-1}. This was on par with the control treatment devoid of chitosan, *i.e.* MS supplanted with BA 1.5 mg $L^{-1}$and IAA 1 mg L^{-1}. The results of the study are presented in Table 12 (Fig. 2).

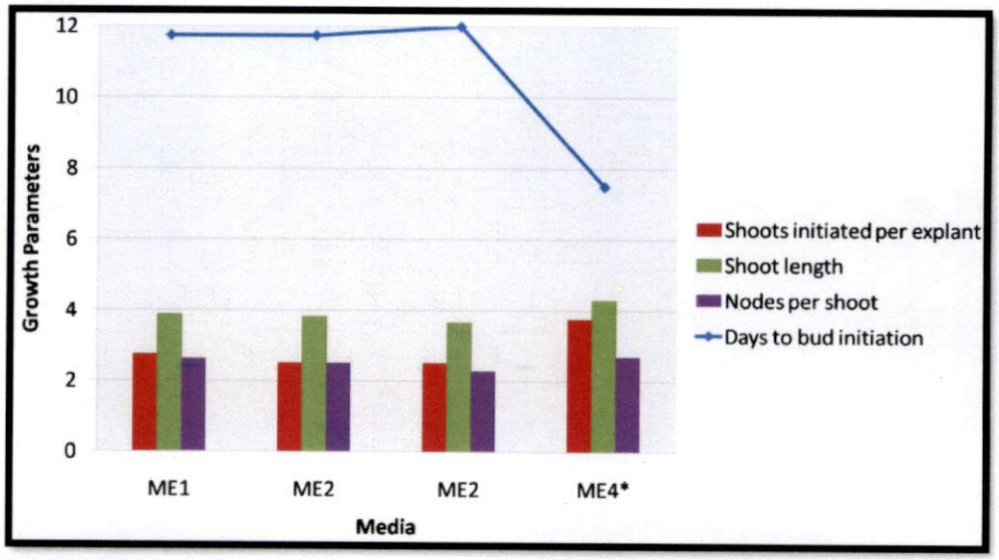
4.1.1.2.2 Adenine sulphate

Seven treatments with different levels of adenine sulphate (20, 40, 60, 80, 100 and 120 mg L^{-1}) were tested to study the effect on shoot proliferation. The results of the study are presented in Table 13 (Fig. 3).

Table 12: Effect of chitosan on shoot proliferation of *P. rosea*

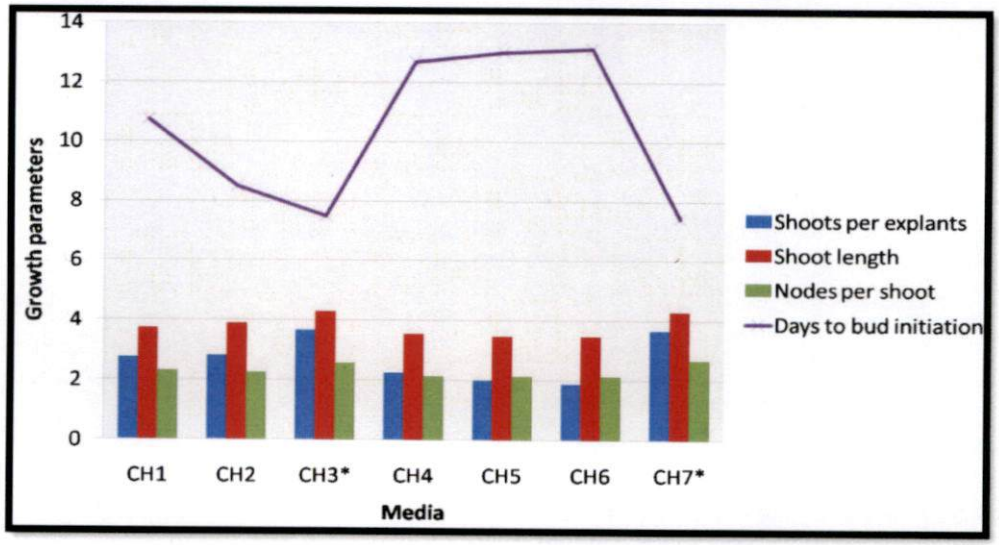
Treatment No:	Chitosan supplemented media	Regeneration percentage	Days to bud initiation	Shoots per explant	Shoot length	Nodes per shoot
CH1	SM + Chitosan 5 mg L^{-1}	100	10.75 ± 0.20^{c}	2.75 ± 0.35^{bc}	3.72 ± 0.10^{c}	2.31 ± 0.24
CH2	SM + Chitosan 10 mg L^{-1}	100	8.50 ± 0.20^{d}	2.81 ± 0.52^{b}	3.88 ± 0.09^{b}	2.25 ± 0.29
CH3	SM + Chitosan 15 mg L^{-1}	100	**7.50 ± 0.20^{e}**	**3.67 ± 0.39^{a}**	**4.29 ± 0.06^{a}**	2.57 ± 0.55
CH4	SM + Chitosan 20 mg L^{-1}	100	12.69 ± 0.24^{b}	2.25 ± 0.35^{cd}	3.54 ± 0.06^{d}	2.13 ± 0.25
CH5	SM + Chitosan 25 mg L^{-1}	100	13.00 ± 0.46^{ab}	2.00 ± 0.20^{d}	3.47 ± 0.06^{d}	2.13 ± 0.32
CH6	SM + Chitosan 30 mg L^{-1}	100	13.13 ± 0.32^{a}	1.88 ± 0.32^{d}	3.47 ± 0.07^{d}	2.13 ± 0.48
CH7	Control (SM*)	100	**7.42 ± 0.17^{e}**	**3.67 ± 0.27^{a}**	**4.28 ± 0.07^{a}**	2.67 ± 0.27
CD (5%)		-	0.40	0.52	0.106	NS

*MS medium + BA 1.5 mg L^{-1} + IAA 1 mg L^{-1}



ME1 (White's+ 1.5mg L^{-1} BA+ 1mg L^{-1} IAA), ME2 (SH + 1.5mg L^{-1} BA+ 1mg L^{-1} IAA), ME3 (B5+ 1.5mg L^{-1} BA+ 1 mg L^{-1} IAA), **ME4- MS+ 1.5mg L^{-1} BA+ 1mg L^{-1} IAA**

Fig. 1: Shoot proliferation of *P. rosea* in different medium



CH1- SM+ chitosan 5mg L^{-1}, CH2- SM+ chitosan 10mg L^{-1}, **CH3- SM+ chitosan 15mg L^{-1}**, CH4- SM+ chitosan 20mg L^{-1}, CH5- SM+ chitosan 25mg L^{-1}, CH6- SM+ chitosan 30mg L^{-1}, **CH7- Control (SM- MS+ 1.5 mg L^{-1} BA+ 1 mg L^{-1} IAA)**

Fig. 2: Effect of chitosan on shoot proliferation of *P. rosea*

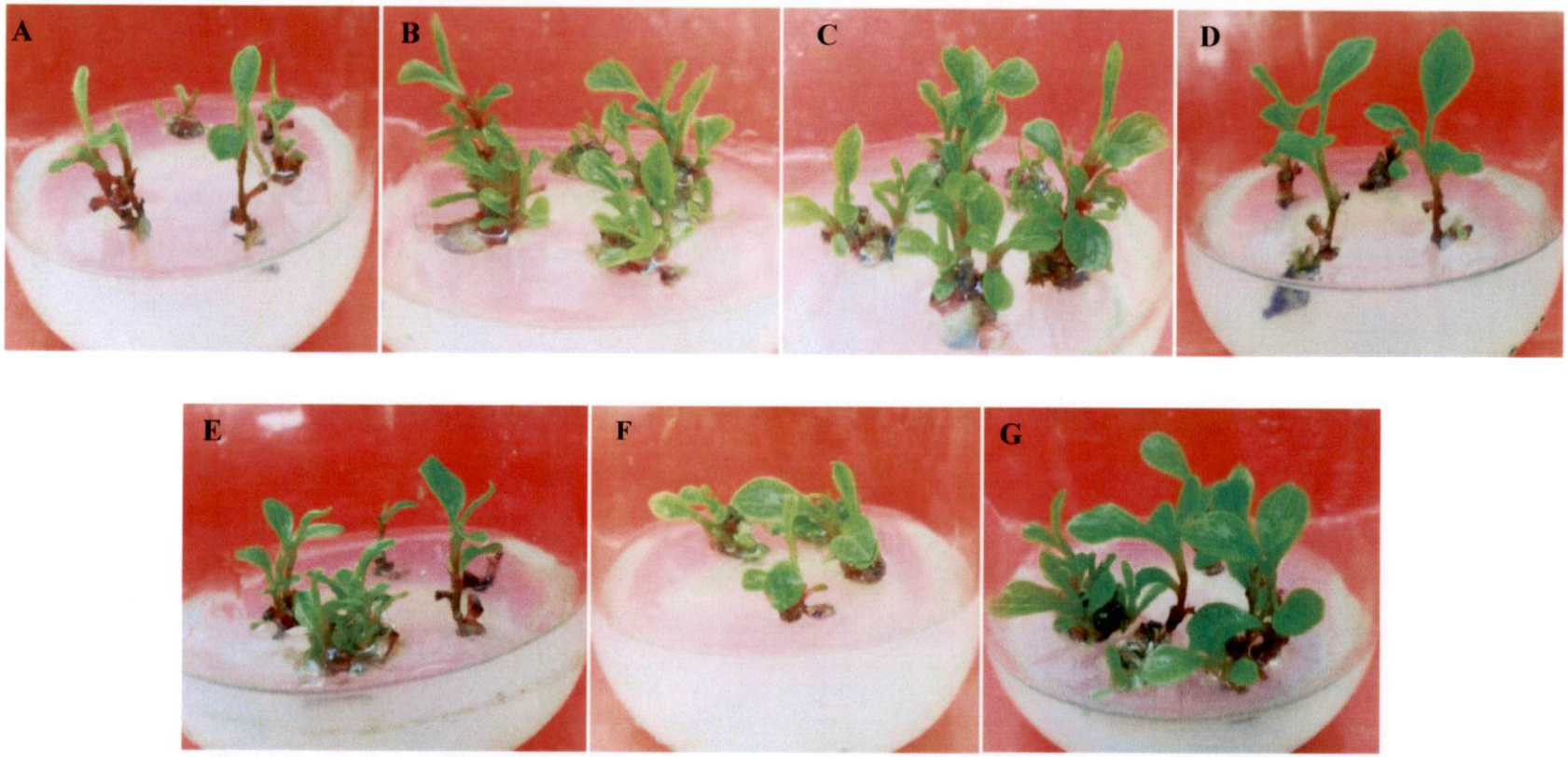


Plate 2. Effect of chitosan on shoot proliferation *P. rosea* : A) SM + chitosan 5 mg L^{-1}, B) SM+ chitosan 10 mg L^{-1}, C) SM + chitosan 15 mg L^{-1}, D) SM + chitosan 20 mg L^{-1}, E) SM + chitosan 25 mg L^{-1}, F) SM + chitosan 30 mg L^{-1}, G) SM (MS+ 1.5 mg L^{-1} BA +1 mg L^{-1} IAA)

All treatments with different concentrations of adenine sulphate recorded 100 per cent regeneration. Significant variation was observed with respect to different parameters *viz.*, days to bud initiation, shoots per explants, shoot length and nodes per shoot (Plate 3).

The days to bud initiation was the earliest (6.42 days) in the treatment AD2 (MS+BA 1.5 mg L^{-1}+ IAA 1 mg L^{-1} + Ads 40 mg L^{-1}) which was significantly different from all other treatments. Bud initiation was significantly late (12.42 days) in AD6 (MS + BA 1.5 mg L^{-1} + IAA 1 mg L^{-1} + Ads 120 mg L^{-1}).

Regarding the number of shoots per explant, the treatment AD2 gave the best response with 4.25 shoots/ explants, which was significantly higher compared to all other treatments. The least number of shoots (1.42 shoots/ explant) were obtained in AD4 (MS+ BA 1.5 mg L^{-1} + IAA 1 mg L^{-1}+ Ads 80 mg L^{-1}) AD5 (MS+ BA 1.5 mg L^{-1} + IAA 1 mg L^{-1}+ Ads 100 mg L^{-1}) and AD6. This is on par with AD1 (MS+ BA 1.5 mg L^{-1} + IAA 1 mg L^{-1}+ Ads 20 mg L^{-1})

Shoot length was maximum (4.69 cm) in the treatment AD2, which was significantly higher compared to all other treatments. The least shoot length (3.79 cm) was obtained in AD6. This is on par with AD1, AD5 and AD6.

Maximum number of nodes per shoot (3.25) was also obtained in AD2. The minimum number of nodes per shoot (1.69) was recorded in AD6, which is on par with AD5.

4.1.1.2.3 Thidiazuron

Seven treatments involving different concentrations of thidiazuron (0.5, 1, 2, 3, 4 and 5 mg L^{-1}) were tried to study their effect on shoot multiplication. The results of the study are presented in Table 14 (Fig. 4).

The different levels of thidiazuron gave 100 per cent regeneration. Significant variation was observed with respect to days to bud initiation, shoots per explant, shoot length and nodes per shoot (Plate 4).

The bud initiation was the earliest (7.41 days) in the control treatment TD7 (MS + BA 1.5 mg L^{-1} + IAA 1 mg L^{-1}). Among the treatments, the bud initiated late (10.00 days) in TD5 (MS+ BA 1.5 mg L^{-1} + IAA 1 mg L^{-1}+ Tdz 4 mg L^{-1}) and TD6 (MS+ BA 1.5 mg L^{-1} + IAA 1 mg L^{-1}+ Tdz 5 mg L^{-1}). This was on par with TD4 (MS+ BA 1.5 mg L^{-1} + IAA 1 mg L^{-1}+ Tdz3 mg L^{-1}).

The treatment TD5 and TD6 gave the maximum number of shoots per explant (11.50) which was on par with TD3 (MS+ BA 1.5 mg L^{-1} + IAA 1 mg L^{-1} + Tdz 2mg L^{-1}) and TD4. The least number of shoots per explants (3.67) was recorded in the control treatment, TD7.

Shoot length was maximum (4.28 cm) in the control treatment TD7 (MS+ BA 1.5 mg L^{-1} + IAA 1 mg L^{-1}), which was significantly different from other treatments. The minimum shoot length of 1.36 cm to 1.43 cm was observed in all the treatments supplemented with thidiazuraon. The shoot length recorded in all these treatments were on par.

Maximum number of nodes per shoot (2.67) was registered in the control treatment TD7. The minimum number of nodes per shoot (1.5) was recorded in TD6. This was on par with all the treatments supplemented with 1 to 4 mg L^{-1} of thidiazuron.

In the study, it is observed that the control treatment, devoid of thidiazuron *i. e.*, (MS+ BA 1.5 mg L^{-1} + IAA 1 mg L^{-1}) gave significantly better response with respect to days to bud initiation, shoot length and nodes per shoot. However, shoots per explant was significantly higher in TD5 and TD6 supplemented with thiadiazuron at 4 mg L^{-1} and 5 mg L^{-1}. Though the shoot proliferation was higher

Table 13. Effect of adenine sulphate on shoot proliferation of *P. rosea*

Treatment No:	Adenine sulphate supplemented media	Regeneration percentage	Days of bud initiation	Shoots per explant	Shoot length	Nodes per shoot
AD1	SM + adenine sulphate 20 mg L^{-1}	100	9.34 ±0.27^{d}	1.67 ± 0.00^{d}	3.79 ±0.10^{d}	2.31 ±0.12^{c}
AD2	SM + adenine sulphate 40 mg L^{-1}	100	**6.42 ±0.17^{f}**	**4.25 ±0.17^{a}**	**4.69 ±0.13^{a}**	**3.25 ±0.20^{a}**
AD3	SM + adenine sulphate 60 mg L^{-1}	100	7.42 ±0.42^{e}	2.99 ±0.47^{c}	4.20 ±0.03^{b}	2.25 ±0.20^{c}
AD4	SM + adenine sulphate 80 mg L^{-1}	100	10.42 ±0.42^{c}	1.42 ±0.17^{d}	3.92 ±0.05^{c}	2.06 ±0.24^{cd}
AD5	SM + adenine sulphate 100 mg L^{-1}	100	11.67 ±0.27^{b}	1.42 ±0.32^{d}	3.82 ±0.08^{cd}	1.88 ±0.32^{de}
AD6	SM + adenine sulphate 120 mg L^{-1}	100	12.42 ±0.17^{a}	1.42 ±0.17^{d}	3.79 ±0.04^{d}	1.69 ±0.31^{e}
AD7	Control (SM*)	100	7.42 ±0.32^{e}	3.67 ±0.27^{b}	4.27 ±0.06^{b}	2.69 ±0.12^{b}
CD (5%)		-	0.457	0.382	0.113	0.344

*MS medium + BA 1.5 mg L^{-1} + IAA 1 mg L^{-1}

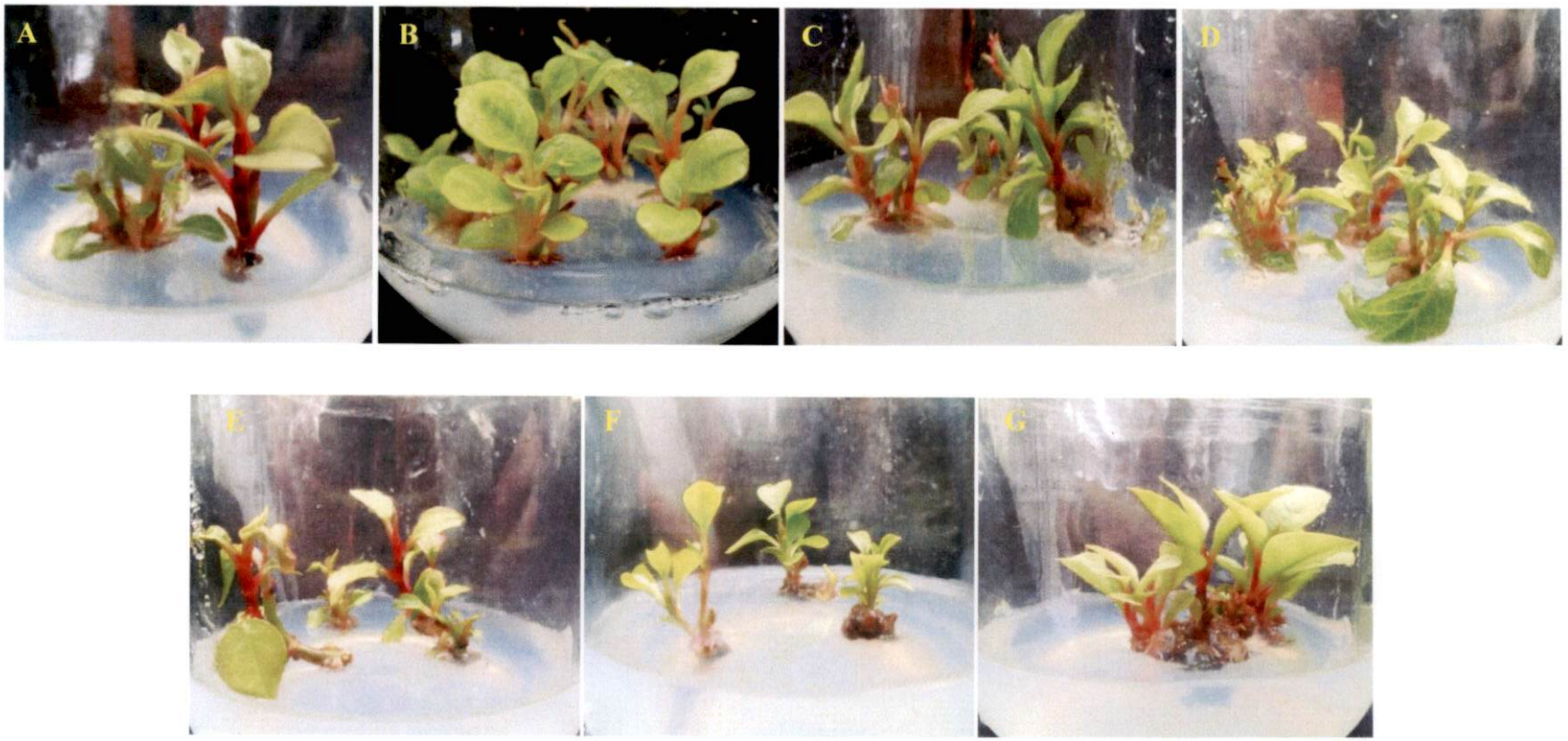
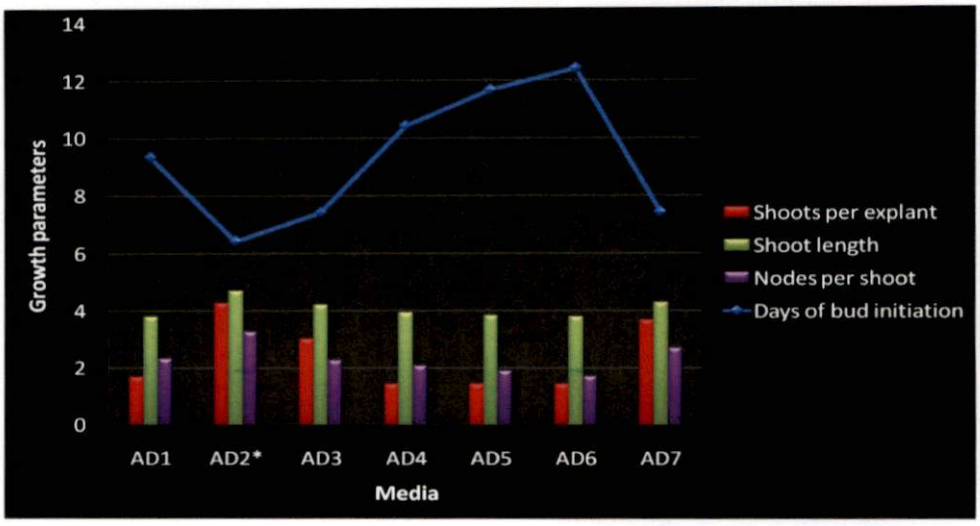


Plate 3: Effect of adenine sulphate on shoot proliferation *P. rosea* :A) SM + adenine sulphate 20 mg L^{-1} B) SM + adenine sulphate 40 mg L^{-1}, C) SM + adenine sulphate 60 mg L^{-1} D) SM + adenine sulphate 80 mg L^{-1} E) SM + adenine sulphate 100 mg L^{-1} F) SM + adenine sulphate 120 mg L^{-1} G) SM (MS +1.5 mg L^{-1} BA +1 mg L^{-1} IAA)

Table 14. Effect of thidiazuron on shoot proliferation of *P. rosea*

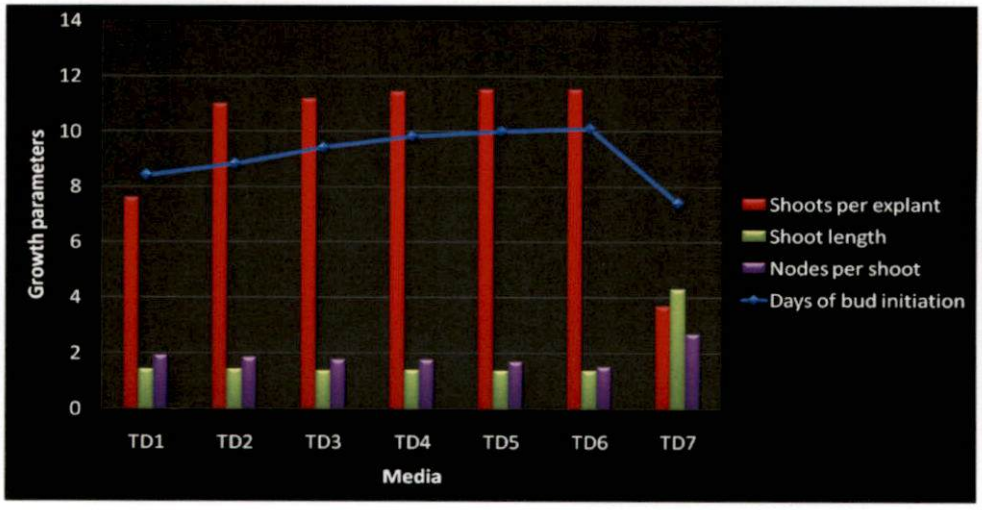
Treatment No:	Thidiazuron supplemented media	Regeneration percentage	Days of bud initiation	Shoots per explant	Shoot length	Nodes per shoot
TD1	SM + thidiazuron 0.5 mg L^{-1}	100	8.42 ± 0.03^{c}	7.58 ± 0.32^{c}	1.42 ± 0.02^{b}	1.92 ± 0.17^{b}
TD2	SM + thidiazuron 1 mg L^{-1}	100	8.83 ± 0.18^{c}	11.00 ± 0.38^{b}	1.43 ± 0.01^{b}	1.84 ± 0.19^{bc}
TD3	SM + thidiazuron 2 mg L^{-1}	100	9.42 ± 0.25^{b}	11.17 ± 0.34^{ab}	1.36 ± 0.02^{b}	1.75 ± 0.17^{bc}
TD4	SM + thidiazuron 3 mg L^{-1}	100	9.83 ± 0.11^{ab}	11.42 ± 0.17^{ab}	1.38 ± 0.01^{b}	1.75 ± 0.32^{bc}
TD5	SM + thidiazuron 4 mg L^{-1}	100	10.00 ± 0.07^{a}	**11.50 ± 0.43^{a}**	1.36 ± 0.01^{b}	1.67 ± 0.27^{bc}
TD6	SM + thidiazuron 5 mg L^{-1}	100	10.10 ± 0.18^{a}	**11.50 ± 0.20^{a}**	1.36 ± 0.01^{b}	1.50 ± 0.20^{c}
TD7	Control (SM*)	100	**7.41 ± 0.03^{d}**	3.67 ± 0.27^{d}	**4.28 ± 0.04^{a}**	**2.67 ± 0.27^{a}**
CD (5%)		-	0.566	0.458	0.144	0.343

*MS medium + BA 1.5 mg L^{-1} + IAA 1 mg L^{-1}



AD1- SM + Ads 20mg L^{-1}, **AD2- SM + Ads 40 mg L^{-1}**, AD3- SM + Ads 60 mg L^{-1}, AD4- SM + Ads 80mg L^{-1}, AD5- SM + Ads 100 mg L^{-1}, AD6- SM + Ads 120 mg L^{-1}, AD7- Control (SM- MS + 1.5mg L^{-1} BA + 1 mg L^{-1} IAA)

Fig. 3: Effect of adenine sulphate on shoot proliferation of *P. rosea*



TD1- SM+ 0.5 mg L^{-1} thidiazuron,TD2- SM + 1 mg L^{-1} thidiazuron ,TD3- SM + 2 mg L^{-1} thidiazuron , TD4- SM + 3 mg L^{-1} thidiazuron , TD5- SM + 4 mg L^{-1} thidiazuron , TD6- SM + 5 mg L^{-1} thidiazuron, TD7- Control (SM- MS + 1.5mg L^{-1} BA+ 1mg L^{-1} IAA)

Fig. 4: Effect of thidiazuron on shoot proliferation of *P. rosea*

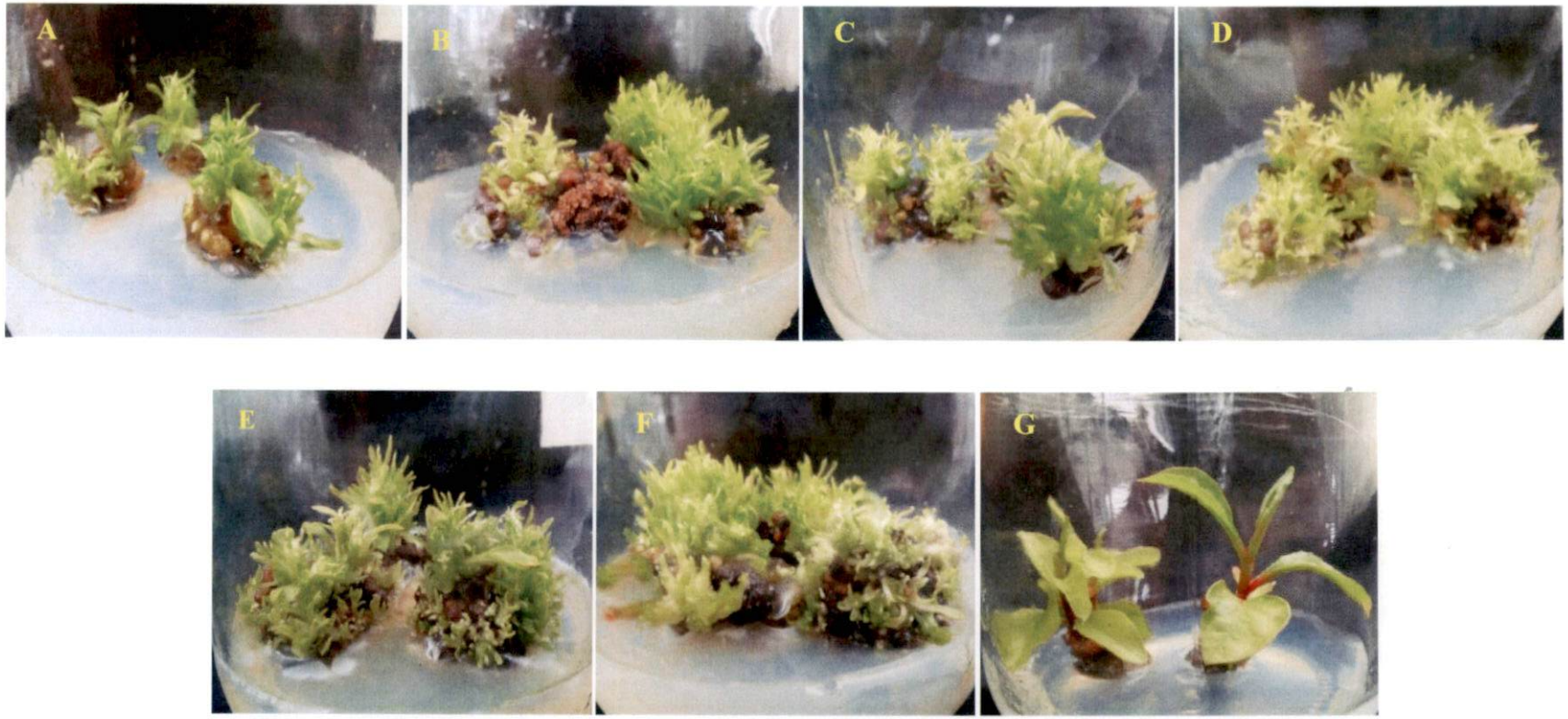


Plate 4: Effect of thidiazuron on shoot proliferation *P. rosea* : A) SM + thidiazuron 0.5 mg L^{-1}B) SM + thidiazuron 1 mg L^{-1}, C) SM + thidiazuron 2 mg L^{-1} D) SM + thidiazuron 3 mg L^{-1} E) SM + thidiazuron 4 mg L^{-1}F) SM + thidiazuron 5 mg L^{-1} G) SM (MS +1.5 mg L^{-1} BA +1 mg L^{-1} IAA)

in these treatments, the shoots gave a stunted and vitrified appearance. The leaf area was also appeared to be much less compared to that of the control treatment. The cultures also showed callusing at the base. Hence, inspite of profuse proliferation recorded by TD5 and TD6, these were not selected as the proliferation medium for the further experiments.

Among the different levels of additives tried, AD2 (MS + BA 1.5 mg L^{-1} + IAA 1 mg L^{-1} + Ads 40 mg L^{-1}) and CH3 (MS + BA 1.5 mg L^{-1}+ IAA 1 mg L^{-1} + chitosan 15 mg L^{-1}) gave better response compared to all other treatments, with respect to shoot multiplication. But the CH3 was on par with the control treatment. But AD2 gave significantly higher response compared to the control treatment. Hence, AD2 was selected as the regeneration/ multiplication medium for short term and long term conservation studies.

4.2 PHASE II: SHORT TERM CONSERVATION BY ENCAPSULATION TECHNIQUE

The standardization of protocol for short term conservation was attempted by optimizing the additives of the encapsulation matrix, storage medium and storage temperature. The encapsulated axillary buds were stored for different duration ranging from 15 to 90 days at 15 days interval.

Single node cuttings of 0.5-1.0 cm with axillary buds were excised from *in vitro* grown cultures for encapsulation. Different osmoticum *viz*., sucrose (10 per cent w/v and 15 per cent w/v) and mannitol (10 per cent w/v and 15 per cent w/v) were supplemented to the encapsulation matrix. The differentially encapsulated beads (A_1, A_2, A_3, A_4, A_5) were stored in liquid MS (M_1), sterile distilled water (M_2) and without any medium (M_3) at two temperature regimes of 4°C (T_1) and 25°C (T_2) for different duration. The short term storage of encapsulated axillary buds of *P. rosea* are depicted in Plate 5

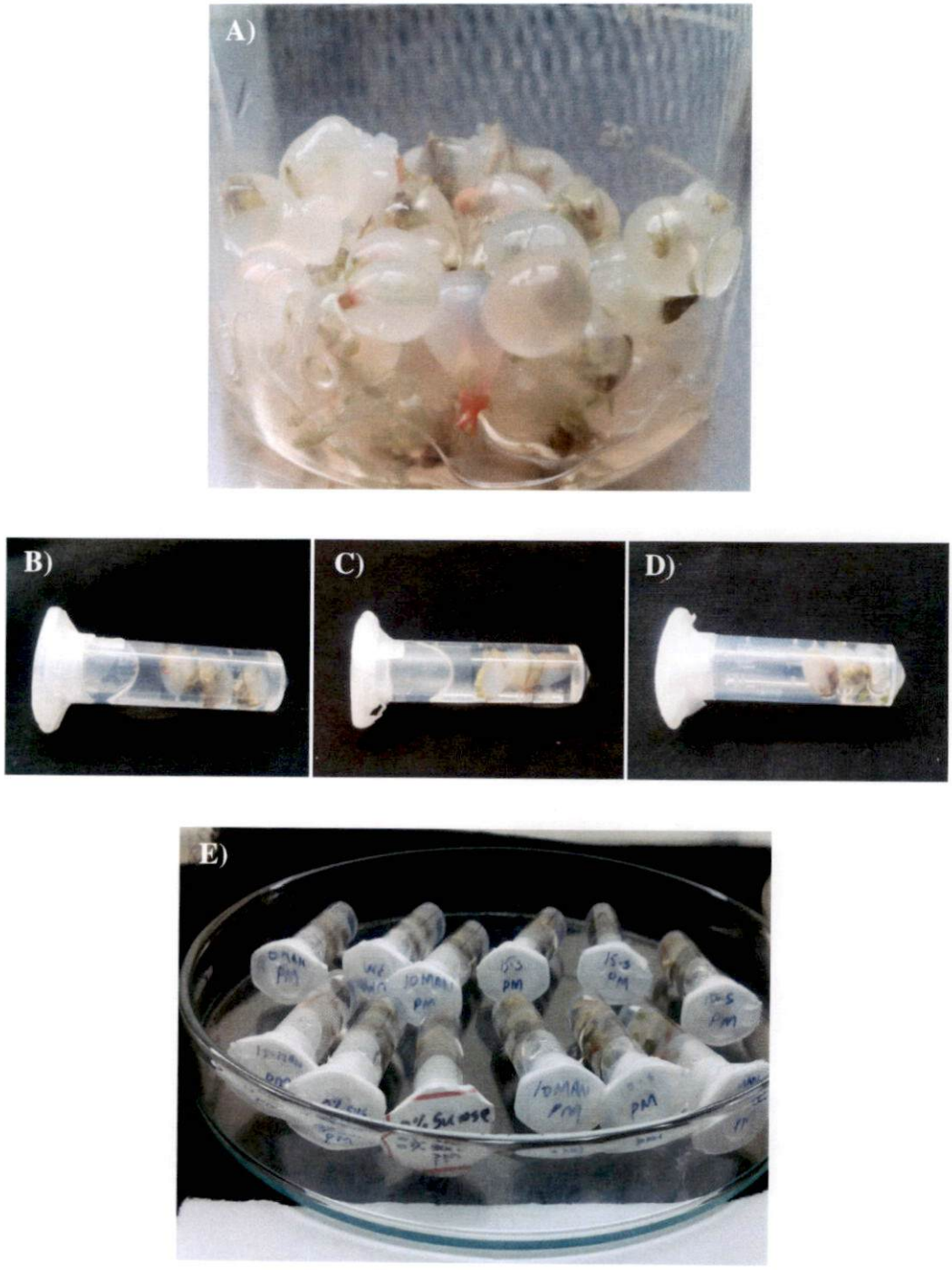


Plate 5: Short term storage of encapsulated axillary buds of *P. rosea* : A) Encapsulated buds, Storage media: B) Liquid MS C) Sterile distilled water D) No media, E) Storage at 25°C.

4.2.1 Regeneration from short term storage at 4°C

The maximum regeneration of 91.67 per cent was obtained with mannitol 10 per cent supplemented encapsulation matrix, stored in liquid MS at 4°C after 15 days of storage. The regeneration per cent showed a gradual decline with extended period of storage. However, 79.17 per cent regeneration could be obtained after 30 days of storage under the same conditions and declined to 50 per cent with 45 days of storage. With 60 days and 75 days of storage the regeneration per cent declined drastically to 37.50 per cent and 12.50 per cent, respectively.

Sucrose 10 per cent supplemented encapsulation matrix stored in liquid MS gave next highest regeneration per cent of 83.33 per cent. This treatment also showed a similar trend with respect to decline in regeneration per cent with extended period of storage. It gave 75 per cent, 45.83 per cent, 33.33 per cent and 8.33 per cent with 30, 45, 60, 75 days of storage. At 90 days, no regeneration could be obtained under both the situations.

It is observed in the study that, when the encapsulated beads showed greater regeneration per cent when stored in liquid MS followed by storage without any medium. With all the differentially prepared encapsulated beads, same trend could be observed. Sterile distilled water gave comparatively lower regeneration per cent in all the treatments.

At 15 days of storage, sucrose 10 per cent supplemented matrix (A_1) gave 67 to 83 per cent regeneration under different storage situations (M_1, M_2, M_3), Sucrose 15 per cent (A_2) gave 63 to 75 per cent; mannitol 10 per cent (A_3) gave 75 to 92 per cent; mannitol 15 per cent (A_4) gave 63 to 75 per cent; without any additive (A_5) gave 67 to 80 per cent.

At 30 days of storage, sucrose 10 per cent supplemented matrix (A_1) gave 50 to 75 per cent regeneration under different storage situations (M_1, M_2, M_3), Sucrose 15 per cent (A_2) gave 38 to 63 per cent; mannitol 10 per cent (A_3) gave 58 to 79 per cent; mannitol 15 per cent (A_4) gave 38 to 63 per cent; without any additive (A_5) gave 41 to 71 per cent.

The regeneration was observed to be less than 50 per cent at 45, 60 and 75 days of storage in all the different treatments tried. No regeneration was obtained with any of these short-term conservation treatments at 90 days of storage. The results of the study are presented in Table 15 (Fig. 5).

4.2.2 Regeneration from short term storage at 25°C

The maximum regeneration of 95.83 per cent was obtained with mannitol 10 per cent supplemented encapsulation matrix, stored in liquid MS at 25°C after 15 days of storage. At 25°C of storage also, the regeneration per cent showed a gradual decline with extended period of storage. At 30 days of storage, 83.33 per cent regeneration could be obtained and with 45 days of storage, it declined to 58.33 per cent. With 60 days, 75 days and 90 days of storage, drastic decline in regeneration per cent *viz.*, 37.50 per cent, 16.67 per cent and 4.16 per cent respectively, was observed.

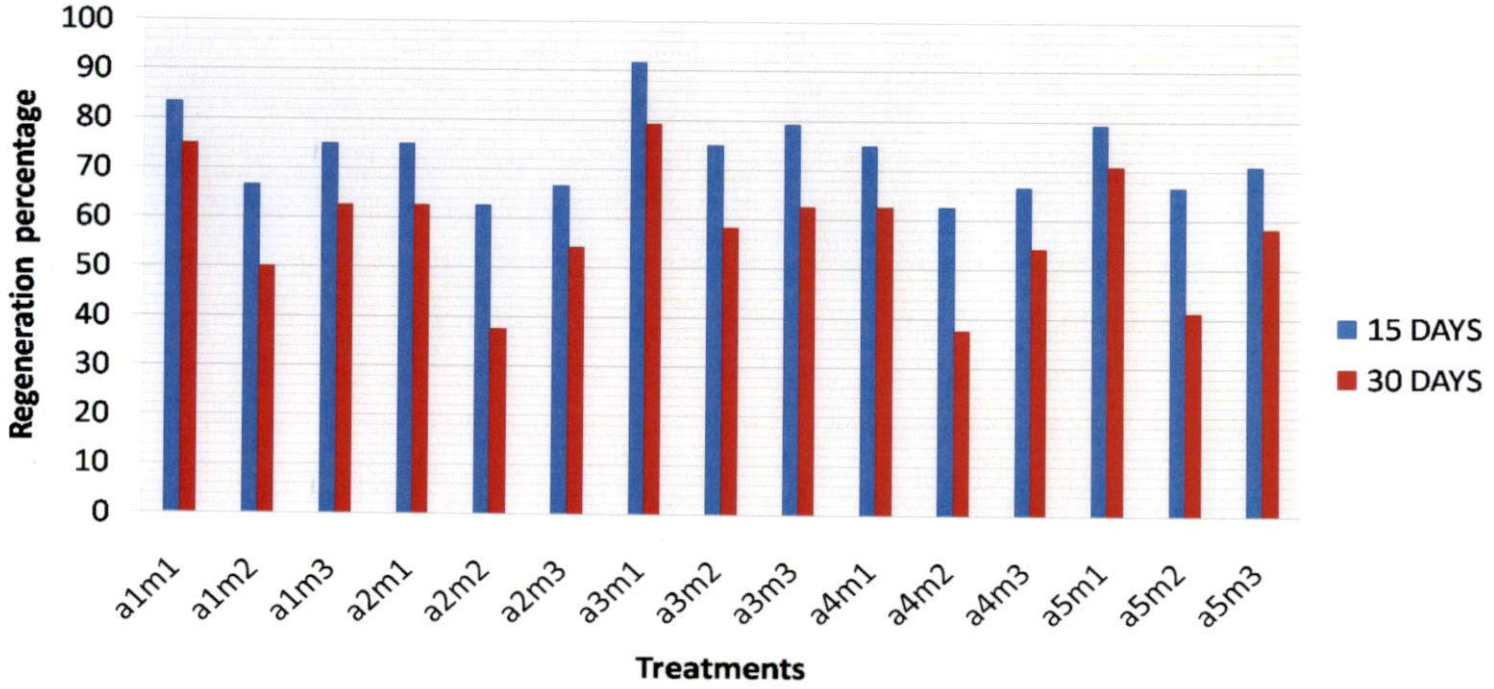
At this temperature regime also, sucrose 10 per cent supplemented encapsulation matrix stored in liquid MS gave the next highest regeneration per cent of 87.50 per cent. This treatment also showed a similar trend with respect to decline in regeneration per cent with extended period of storage, as observed at 4°C. It gave 79.17 per cent, 50 per cent, 29.17 per cent and 12.50 per cent with 30, 45, 60, 75 days of storage. At 90 days, no regeneration could be obtained.

It is observed in the study that, the encapsulated beads showed greater regeneration per cent when stored in liquid MS followed by storage without any

Table 15. Regeneration percentage of encapsulated axillary buds of *P. rosea* after short term conservation (storage temperature: 4˚C)

Storage medium	Storage duration	Additives in encapsulation matrix (2.5 % SA+100 *mM* $CaCl_2$)				
		A1 (sucrose 10%)	A2 (sucrose 15%)	A3 (mannitol 10 %)	A4 (mannitol 15 %)	A5 (no additive)
M1 (MS)	15 days	83.33	75.00	**91.67**	75.00	79.16
M2 (SDW*)		66.67	62.50	75.00	62.50	66.67
M3 (No media)		75.00	66.67	79.17	66.67	70.83
M1 (MS)	30 days	75.00	62.50	**79.17**	62.50	70.83
M2 (SDW)		50.00	37.50	58.33	37.50	41.16
M3 (No media)		62.50	54.16	62.50	54.16	58.33
M1 (MS)	45 days	45.83	29.17	50.00	37.50	41.67
M2 (SDW)		20.83	-	25.00	-	-
M3 (No media)		25.00	-	29.17	16.67	20.83
M1 (MS)	60 days	33.33	-	37.50	-	25.00
M2 (SDW)		-	-	-	-	-
M3 (No media)		-	-	16.67	-	-
M1 (MS)	75 days	8.33	-	12.50	-	-
M2 (SDW)		-	-	-	-	-
M3 (No media)		-	-	-	-	-
M1 (MS)	90 days	-	-	-	-	-
M2 (SDW)		-	-	-	-	-
M3 (No media)		-	-	-	-	-

*sterile distilled water, Regeneration medium-MS+BA 1.5 mg L^{-1}+IAA1.5 mg L^{-1}+Adenine sulphate 40 mg L^{-1}



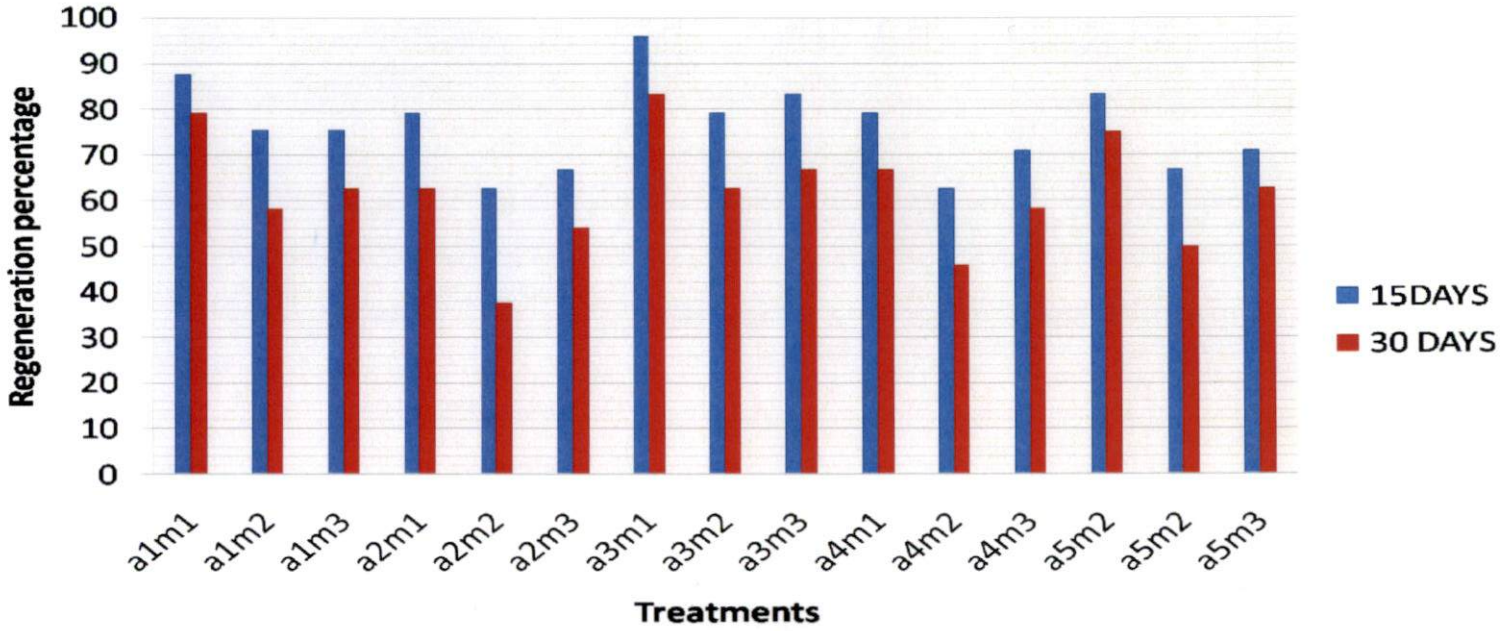
a1m1- sucrose 10 %+ MS, a1m2- sucrose 10%+ sterile distilled water+a1m3- sucrose 10% + no media, a2m1- sucrose 15%+Ms media, a2m2- sucrose 15%+ sterile distilled water, a2m3- sucrose 15%+ no media, **a3m1- mannitol 10%+ MS**, a3m2- mannitol 10%+ sterile distilled water, a3m3- mannitol 10%+ no media, a4m1- mannitol 15 %+ MS, a4m2- mannitol 15%+ sterile distilled water, a4m3- mannitol 15%+ no media,, a5m1- no additive+ no media, a5m2- no additive+sterile distilled water, a5m3- no additive+ no media

Fig. 5- Regeneration percentage of encapsulated axillary buds of *P. rosea* after short term conservation (4° C)

Table 16: Regeneration percentage of encapsulated axillary buds of *P. rosea* after short term conservation (storage temperature:25°C)

Storage medium	Storage duration	Additives in encapsulation matrix (2.5 % SA+100 *mM* $CaCl_2$)				
		A1 (sucrose 10%)	A2 (sucrose 15%)	A3 (mannitol 10 %)	A4 (mannitol 15 %)	A5 (no additive)
M1 (MS)	15 days	87.50	79.17	**95.83**	79.17	83.33
M2 (SDW*)		75.50	62.50	79.17	62.50	66.67
M3 (No media)		75.50	66.67	83.33	70.83	70.83
M1 (MS)	30 days	79.17	62.50	**83.33**	66.67	75.00
M2 (SDW)		58.33	37.50	62.50	45.83	50.00
M3 (No media)		62.50	54.16	66.67	58.33	62.50
M1 (MS)	45 days	50.00	29.17	58.33	37.50	41.66
M2 (SDW)		29.17	-	25.00	-	-
M3 (No media)		25.00	16.67	29.17	20.83	25.00
M1 (MS)	60 days	29.17	-	37.50	-	-
M2 (SDW)		-	-	-	-	-
M3 (No media)		16.67	-	25.00	-	-
M1 (MS)	75 days	12.50	-	16.67	-	-
M2 (SDW)		-	-	-	-	-
M3 (No media)		8.33	-	-	-	-
M1 (MS)	90 days	-	-	4.16	-	-
M2 (SDW)		-	-	-	-	-
M3 (No media)		-	-	-	-	-

*sterile distilled water, Regeneration medium-MS+BA 1.5 mg L^{-1}+IAA1.5 mg L^{-1}+Adenine sulphate 40 mg L^{-1}



a1m1- sucrose 10 %+ MS, a1m2- sucrose 10%+ sterile distilled water+a1m3- sucrose 10% + no media, a2m1- sucrose 15%+Ms media, a2m2- sucrose 15%+ sterile distilled water, a2m3- sucrose 15%+ no media, **a3m1- mannitol 10%+ MS**, a3m2- mannitol 10%+ sterile distilled water, a3m3- mannitol 10%+ no media, a4m1- mannitol 15 %+ MS, a4m2- mannitol 15%+ sterile distilled water, a4m3- mannitol 15%+ no media,, a5m1- no additive+ no media, a5m2- no additive+sterile distilled water, a5m3- no additive+ no media

Fig. 6- Regeneration percentage of encapsulated axillary buds of *P. rosea* after short term conservation (25° C)

medium. With all the differentially prepared encapsulated beads same trend could be observed. Sterile distilled water gave comparatively lower regeneration per cent in all the treatments.

At 15 days of storage, sucrose 10 per cent supplemented matrix (A_1) gave 76 to 88 per cent regeneration under different storage situations (M1, M2, M3), Sucrose 15 per cent (A_2) gave 62 to 79 per cent; mannitol 10 per cent (A_3) gave 79 to 96 per cent; mannitol 15 per cent (A4) gave 63 to 79 per cent and without any additive (A5) gave 67 to 83 per cent regeneration.

At 30 days of storage, sucrose 10 per cent supplemented matrix (A_1) gave 58 to 79 per cent regeneration under different storage situations (M_1, M_2, M_3), Sucrose 15 per cent (A_2) gave 38 to 63 per cent; mannitol 10 per cent (A_3) gave 63 to 83 per cent; mannitol 15 per cent (A_4) gave 46 to 67 per cent; without any additive (A_5) gave 50 to 75 per cent.

The regeneration was less than 50 per cent in all the treatments from 45 days of storage except in the treatment with mannitol 10 per cent supplemented encapsulation matrix in liquid MS medium, where the regeneration was observed to be 58.33 per cent. The results of the study are presented in Table 16 (Fig. 6).

4.2.3 Plantlet conversion potential of encapsulated axillary buds after short-term storage

The plantlet conversion potential of encapsulated axillary buds was assessed at 15 days and 30 days of storage. The regeneration per cent being less than 50 per cent on extended periods of storage *i.e.*, from 45 days to 90 days, these were discarded from the study.

4.2.3.1 Storage for 15 days

4.2.3.1.1 Encapsualtion matrices (supplemented with additives)

Among the five treatments with different concentration of additives *viz.*, sucrose and mannitol (A_1, A_2, A_3, A_4, A_5) in encapsulation matrix tried, the earliest (13.63 days) to bud initiation was observed in the treatment A_3 (mannitol 10 per cent supplemented encapsulation matrix). This was significantly different from all the other treatments. Bud initiation was significantly late (16 days) in A_2 (sucrose 15 per cent supplemented encapsulation matrix).

Significant variation was observed with respect to shoot per explants among the different treatments tried. The treatment A_3 recorded maximum shoot per explants (4.7) followed by A1 (Sucrose 10 per cent supplemented encapsulation matrix) with 4.4 shoots per explant. The least number of shoots (3.00) was produced in treatment A_2.

Shoot length was significantly higher (1.54 cm) in A_3 and the least shoot length (1.10 cm) was observed in the treatment A_2.

The number of nodes per shoot also exhibited significant variation among the treatments tried. Maximum number of nodes per shoot (1.93) was recorded in treatment A_3 and the lowest number (1.24) was recorded in A_2.

Among the different encapsulation matrices tried, mannitol 10 per cent supplemented matrix gave the best response with respect to plantlet conversion of the encapsulated axillary buds, after 15 days of storage.

4.2.3.1.2 Storage media

The plantlet conversion potential of differentially encapsulated axillary buds under three different storage conditions *viz.*, in liquid MS (M_1), sterile distilled water (M_2) and without any media (M_3) were assessed.

Significant variation was found among three treatments, with all the four parameters *viz.*, days to bud initiation, shoots per explant, shoot length and nodes per shoot.

The days taken for bud initiation were the earliest (13.88 days) in the treatment M_1 and the bud initiated late (15.42 days) in M_2.

The treatment M1 was found to produce maximum number (4.6) of shoots formed per explants. The least number (3.38) of shoots per explant was observed in M_2.

The maximum shoot length (1.55 cm) was recorded in M_1 and the minimum (1.16 cm) in M_2.

The number of nodes per shoot was maximum (1.88) in treatment M_1. The minimum number of nodes per shoot (1.32) was recorded in the treatment M_2.

Among the three different storage media (M_1, M_2, M_3) tried, M_1 (liquid MS) was found to give best response with respect to plantlet conversion after short-term storage at 15 days of storage.

4.2.3.1.3 Storage temperature

The effect of two different temperature regimes *viz.*, 4°C and 25°C on plantlet conversion potential of encapsulated axillary buds of *P. rosea* were studied.

All the parameters *viz.*, days to bud initiation, shoots per explant, shoot length and nodes per shoot were found to have significant variation, between the two treatments tried.

The days taken for bud initiation were the earliest (11.52 days) at 25°C (T_2). The bud initiation was late (17.96 days) at 4°C (T_1).

The number of shoots per explant was the highest (4.65) in the treatment T_2. The least number (3.08) of shoots per explant was observed in T_1.

The maximum shoot length (1.30 cm) was recorded in T_2. The minimum shoot length of 1.28 cm was observed in the treatment T_1.

The maximum nodes per shoot (1.60) were observed in T_2 and the minimum (1.49) in T_1.

The best response with respect to plantlet conversion potential of encapsulated beads was obtained when recovered from the storage temperature of 25°C compared to 4°C.

4.2.3.1.4 Interaction effect of storage conditions

Thirty different combinations of treatments were analysed to study the interaction effect of storage conditions *viz.*, encapsulation matrix supplement with additives, storage medium and storage temperature on plantlet conversion potential (Plate 6). Among the thirty treatments tried, axillary buds encapsulated

Table 17. Effect of different encapsulation matrix, storage medium and temperature on the plantlet conversion potential of synthetic seeds (duration of storage: 15 days)

Treatments	Days to bud initiation	Shoots per explant	Shoot length	Nodes per shoot
Encapsulation matrix* supplemented with additives				
A_1 (sucrose 10%)	14.17^{d}	4.4^{b}	1.40^{b}	1.69^{b}
A_2 (sucrose 15%)	16.00^{a}	3.00^{e}	1.10^{e}	1.24^{e}
A_3 (mannitol 10%)	**13.63^{e}**	**4.7^{a}**	**1.54^{a}**	**1.93^{a}**
A_4 (mannitol 15%)	15.36^{b}	3.33^{d}	1.14^{d}	1.32^{d}
A_5 (no additive)	14.53^{c}	3.9^{c}	1.28^{c}	1.53^{c}
SEm (±)	0.09	0.09	0.004	0.02
CD (5%)	0.17	0.17	0.007	0.04
Medium				
M_1 (MS medium)	**13.88^{c}**	**4.60^{a}**	**1.55^{a}**	**1.88^{a}**
M_2 (sterile distilledwater)	15.42^{a}	3.38^{c}	1.16^{c}	1.32^{c}
M_3 (no media)	14.92^{b}	3.62^{b}	1.17^{b}	1.46^{b}
SEm (±)	0.07	0.07	0.003	0.015
CD (5%)	0.13	0.13	0.006	0.03
Temperature				
T_1(4˚C)	17.96^{b}	3.08^{b}	1.28^{b}	1.49^{b}
T_2 (25˚C)	**11.52^{a}**	**4.65^{a}**	**1.30^{a}**	**1.60^{a}**
SEm (±)	0.06	0.05	0.002	0.01
CD (5%)	0.11	0.10	0.005	0.02
Interaction (additive, medium and temperature)				
$a_1m_1t_1$(sucrose 10 %+ MS + 4˚C)	16.6^{j}	4.2^{cd}	1.66^{d}	1.96^{e}
$a_1m_1t_2$ (sucrose 10 %+ MS + 25˚C)	9.4^{u}	**6.2^{a}**	1.70^{c}	2.32^{c}
$a_1m_2t_1$ (sucrose 10 %+ SDW** + 4˚C)	18.4^{d}	3.2^{d}	1.23^{m}	1.32^{h}
$a_1m_2t_2$ (sucrose 10 %+ SDW+ 25˚C)	11.8^{o}	4.6^{c}	1.25^{l}	1.48^{g}
$a_1m_3t_1$ (sucrose 10 %+ no media+ 4˚C)	17.6^{g}	3.4^{d}	1.25^{l}	1.48^{g}

table 17 continued.

$a_1m_3t_2$ (sucrose 10 %+ no media+ 25˚C)	11.2^{r}	4.8^{bc}	1.28^{k}	1.56^{fg}
$a_2m_1t_1$ (sucrose 15 %+ MS+ 4˚C)	18.6^{c}	2.6^{e}	1.26^{k}	1.32^{h}
$a_2m_1t_2$ (sucrose 15 %+ MS+ 25˚C)	12.4^{n}	4.4^{c}	1.27^{k}	1.32^{h}
$a_2m_2t_1$ (sucrose 15 %+ SDW+ 4˚C)	19.0^{a}	1.8^{e}	1.02^{q}	1.12^{i}
$a_2m_2t_2$ (sucrose 15 %+ SDW+ 25˚C)	13.8^{k}	3.4^{d}	1.02^{q}	1.16^{i}
$a_2m_3t_1$ (sucrose 15 %+no media+ 4˚C)	18.8^{b}	2.2^{e}	1.03^{q}	1.24^{h}
$a_2m_3t_2$ (sucrose 15 %+no media+ 25˚C)	13.4^{l}	3.6^{cd}	1.03^{q}	1.28^{h}
$a_3m_1t_1$ (mannitol 10 %+ MS+ 4˚C)	16.2^{j}	4.6^{c}	1.90^{b}	2.48^{b}
$a_3m_1t_2$ (mannitol 10 %+ MS+ 25˚C)	**8.4^{v}**	**6.6^{a}**	**1.94^{a}**	**2.68^{a}**
$a_3m_2t_1$ (mannitol 10 %+ SDW+ 4˚C)	17.8^{f}	3.4^{d}	1.34^{i}	1.52^{g}
$a_3m_2t_2$ (mannitol 10 %+ SDW+ 25˚C)	11.4^{q}	4.8^{bc}	1.35^{i}	1.64^{f}
$a_3m_3t_1$ (mannitol 10 %+no media+ 4˚C)	17.4^{h}	3.6^{d}	1.37^{h}	1.64^{f}
$a_3m_3t_2$ (mannitol 10 %+ no media+ 25˚C)	10.6^{s}	5.2^{b}	1.38^{g}	1.64^{f}
$a_4m_1t_1$ (mannitol 15 %+ MS+ 4˚C)	18.2^{e}	3.4^{d}	1.33^{ij}	1.44^{g}
$a_4m_1t_2$ (mannitol 15 %+ MS+ 25˚C)	11.6^{p}	4.6^{c}	1.37^{h}	1.48^{g}
$a_4m_2t_1$ (mannitol 15 %+ SDW+ 4˚C)	18.8^{b}	2.2^{e}	1.03^{q}	1.16^{i}
$a_4m_2t_2$ (mannitol 15 %+ SDW+ 25˚C)	12.8^{m}	3.6^{d}	1.05^{p}	1.24^{h}
$a_4m_3t_1$ (mannitol 15 %+ no media+ 4˚C)	18.4^{d}	2.4^{e}	1.05^{p}	1.28^{h}
$a_4m_3t_2$ (mannitol 15 %+ no media+ 25˚C)	12.4^{n}	3.8^{cd}	1.05^{p}	1.32^{h}
$a_5m_1t_1$ (no additive+ MS+ 4˚C)	17.2^{i}	3.8^{cd}	1.55^{f}	1.72^{f}
$a_5m_1t_2$ (no additive+ MS+ 25˚C)	10.2^{t}	5.6^{b}	1.58^{e}	2.08^{d}
$a_5m_2t_1$ (no additive+ SDW+ 4˚C)	18.6^{c}	2.6^{e}	1.12^{o}	1.28^{h}
$a_5m_2t_2$ (no additive+ SDW+ 25˚C)	11.8^{o}	4.2^{cd}	1.13^{o}	1.28^{h}
$a_5m_3t_1$ (no additive+ no media+ 4˚C)	17.8^{f}	2.8^{de}	1.13^{o}	1.32^{h}
$a_5m_3t_2$(no additive+ no media+ 25˚C)	11.6^{p}	4.4^{c}	1.15^{n}	1.48^{g}
SEm (±)	0.08	0.21	0.009	0.05
CD (5%)	0.17	0.42	0.018	0.09

*sodium alginate 2.5 per cent and calcium chloride 100 mM

**sterile distilled water,Regeneration media- MS+ BA 1.5 mg L^{-1}+ IAA1.5 mg L^{-1}+ Adenine sulphate 40 mg L^{-1}

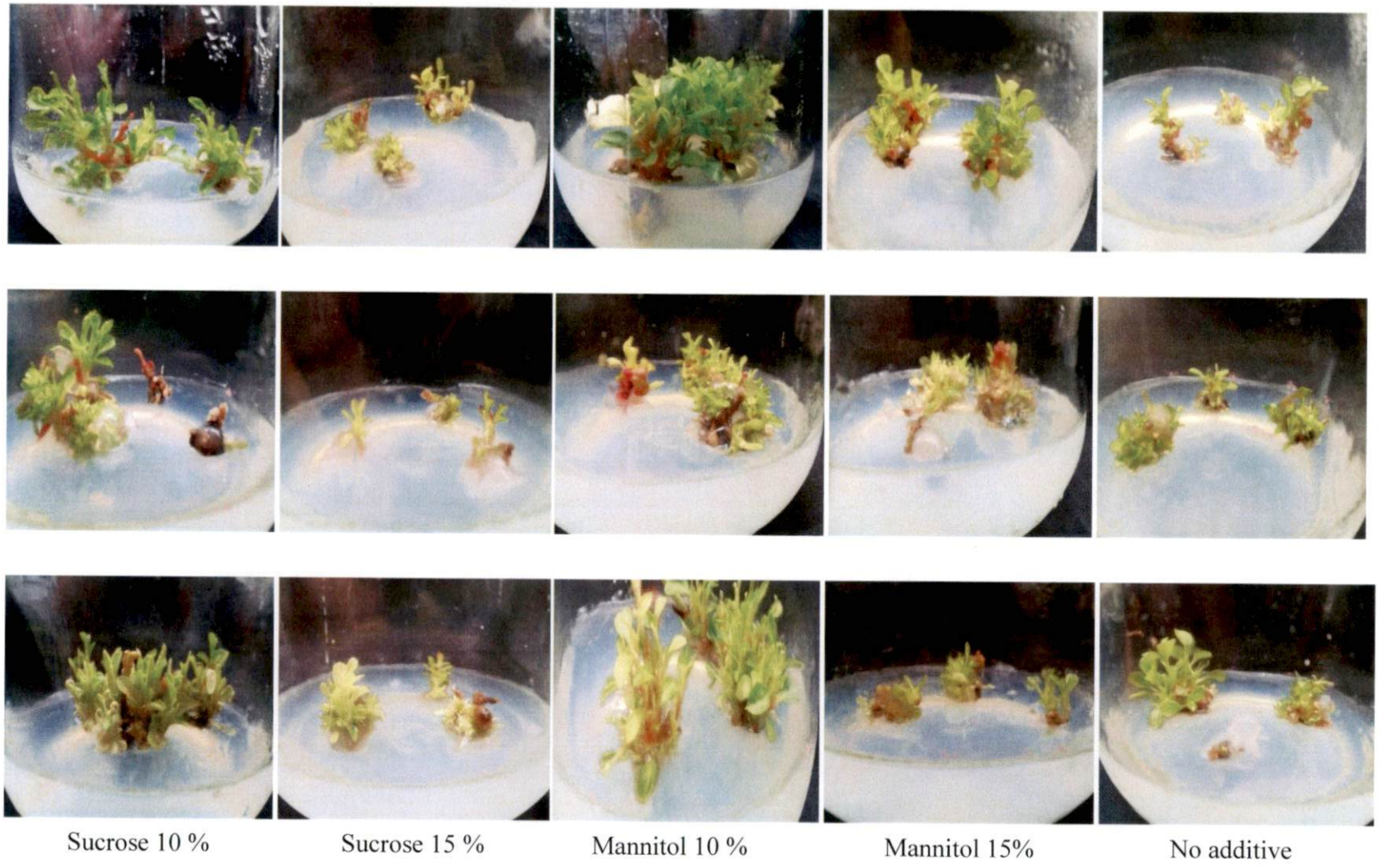


Plate 6: Response of encapsulated buds of *P. rosea* after 30 days of storage at 4˚C (6 weeks of culture)

*sterile distilled water

in mannitol 10 per cent supplemented encapsulation matrix, stored in liquid MS medium at 25˚C ($a_3m_1t_2$) was found to give best plantlet conversion potential with respect to days to bud initiation (8.4 days), shoots per explant (6.6), shoot length (1.94 cm) and nodes per shoot (2.68). The variation among the thirty treatments was significant with respect to all these parameters tried. Days to bud initiation shoot length and nodes per shoot were significantly superior to all other treatment combinations. However, shoots per explant (6.2) was found to be on par with $a_1m_1t_2$ (sucrose 10 per cent supplemented encapsulation matrix, liquid MS as the storage medium; 25˚C storage temperature). The data is presented in Table 17.

4.2.3.2 Storage for 30 days

4.2.3.2.1 Encapsualtion matrices (supplemented with additives)

Among the five treatments with different concentration of additives *viz.,* sucrose and mannitol (A_1, A_2, A_3, A_4, A_5) in encapsulation matrix tried, the earliest days (16.23) to bud initiation was observed in the treatment A_3 (mannitol 10 per cent supplemented encapsulation matrix). This was significantly different from all the other treatments. Bud initiation was significantly late (18.20 days) in A_2 (sucrose 15 per cent supplemented encapsulation matrix).

Significant variation was observed with respect to shoot per explant, shoot length and nodes per shoot among the different treatments tried. The treatment A_3 recorded maximum shoots per explant (4.33). The least number of shoots (2.67) was obtained in treatment A_2. Shoot length was significantly higher (1.46 cm) in A_3 and the least shoot length (1.03 cm) was observed in the treatment A_2. Maximum number of nodes per shoot (1.76) was recorded in treatment A_3 and the lowest number (1.21) was recorded in A_2.

Among the different encapsulation matrices tried, mannitol 10 per cent supplemented matrix gave the best response with respect to the plantlet

conversion of the encapsulated axillary buds after 30 days of storage, as with 15 days of storage.

4.2.3.2.2 Storage media

The plantlet conversion potential of differentially encapsulated axillary buds under three different storage conditions *viz.*, in liquid MS (M_1), sterile distilled water (M_2) and without any media (M_3) were assessed.

Significant variation was found among three treatments, with all the four parameters *viz.*, days to bud initiation, shoots per explant, shoot length and nodes per shoot.

The days taken for bud initiation were the earliest (16.16 days) in the treatment M_1 and the bud initiated late (18.10 days) in M_2.

The treatment M1 was found to produce maximum number (4.38) of shoots per explant. The least number (2.92) of shoots per explant was observed in M_2.

The maximum shoot length (1.43 cm) was recorded in M_1 and the minimum (1.09 cm) in M_2.

The number of nodes per shoot was maximum (1.70) in treatment M_1. The minimum number of nodes per shoot (1.25) was recorded in the treatment M_2.

Among the three different storage media (M_1, M_2, M_3) tried, M_1 (liquid MS was found to give the best response with respect to plantlet conversion after short-term storage at 30 days of storage, as with 15 days of storage.

4.2.3.2.3 Storage temperature

The effect of two different temperature regimes *viz*., 4°C and 25°C on plantlet conversion potential of encapsulated axillary buds of *P. rosea* were studied.

All the parameters *viz*., days to bud initiation, shoots per explant, shoot length and nodes per shoot were found to have significant variation, between the two treatments tried.

The days taken for bud initiation were the earliest (14.27 days) at 25°C (T_2). The bud initiation was late (20.35 days) at 4°C (T_1).

The number of shoots per explant was the highest (4.37) in the treatment T_2. The least number (2.67) of shoots per explant was observed in T_1.

The maximum shoot length (1.22 cm) was recorded in T2. The minimum shoot length of 1.20 cm was observed in the treatment T_2.

The maximum nodes per shoot (1.46) were observed in T_2 and the minimum (1.42) in T_2.

The best response with respect to plantlet conversion potential of encapsulated beads was obtained when recovered from the storage temperature of 25°C as compared to 4°C.

4.2.3.2.4 Interaction effect of storage conditions

Thirty different combinations of treatments were analysed to study the interaction effect of storage conditions *viz*., encapsulation matrix supplement with additives, storage medium and storage temperature on plantlet conversion

Table 18. Effect of different encapsulation matrix, storage medium and temperature on the plantlet conversion potential of synthetic seeds (duration of storage: 30 days)

Treatments	Days to bud initiation	Shoots per explant	Shoot length	Nodes per shoot
Encapsulation matrix* supplemented with additives				
A_1 (sucrose 10%)	16.77^{d}	3.93^{b}	1.32^{b}	1.55^{b}
A_2 (sucrose 15%)	18.20^{a}	2.67^{e}	1.03^{e}	1.21^{d}
A_3 (mannitol 10%)	**16.23^{e}**	**4.33^{a}**	**1.46^{a}**	**1.76^{a}**
A_4 (mannitol 15%)	17.93^{b}	3.00^{d}	1.07^{d}	1.25^{d}
A_5 (no additive)	17.40^{c}	3.60^{c}	1.19^{c}	1.42^{c}
SEm (±)	0.086	0.08^{5}	0.002	0.02
CD (5%)	0.17	0.17	0.004	0.04
Medium				
M_1 (MS medium)	**16.16^{c}**	**4.38^{a}**	**1.43^{a}**	**1.70^{a}**
M_2 (sterile distilledwater)	18.10^{a}	2.92^{c}	1.09^{c}	1.25^{c}
M_3 (no media)	17.66^{b}	3.22^{b}	1.10^{b}	1.36^{b}
SEm (±)	0.067	0.066	0.002	0.01
CD (5%)	0.13	0.13	0.003	0.03
Temperature				
T_1(4°C)	20.35^{a}	2.67^{b}	1.20^{b}	1.42^{b}
T_2 (25°C)	**14.27^{b}**	**4.37^{a}**	**1.22^{a}**	**1.46^{a}**
SEm (±)	0.05	0.05	0.001	0.01
CD (5%)	0.11	0.10	0.002	0.02
Interaction (additive medium and temperature)				
$a_1m_1t_1$(sucrose 10 %+ MS + 4°C)	18.8^{e}	4.2^{cd}	1.63^{c}	1.76^{c}
$a_1m_1t_2$ (sucrose 10 %+ MS + 25°C)	12.4^{j}	5.8^{b}	1.68^{b}	1.88^{b}
$a_1m_2t_1$ (sucrose 10 %+ SDW** + 4°C)	20.8^{c}	2.4^{e}	1.12^{ij}	1.28^{e}
$a_1m_2t_2$ (sucrose 10 %+ SDW+ 25°C)	14.6^{h}	4.2^{cd}	1.13^{i}	1.44^{d}
$a_1m_3t_1$ (sucrose 10 %+ no media+ 4°C)	19.8^{d}	2.6^{e}	1.16^{h}	1.48^{d}

table 18 continued.

$a_1m_3t_2$ (sucrose 10 %+ no media+ 25°C)	14.2^{h}	4.4^{c}	1.17^{h}	1.48^{d}
$a_2m_1t_1$ (sucrose 15 %+ MS+ 4°C)	19.8^{d}	2.2^{ef}	1.06^{k}	1.28^{e}
$a_2m_1t_2$ (sucrose 15 %+ MS+ 25°C)	14.4^{h}	4.4^{c}	1.07^{k}	1.24^{ef}
$a_2m_2t_1$ (sucrose 15 %+ SDW+ 4°C)	21.6^{a}	1.6^{f}	1.02^{l}	1.12^{g}
$a_2m_2t_2$ (sucrose 15 %+ SDW+ 25°C)	16.2^{f}	2.8^{e}	1.02^{l}	1.12^{g}
$a_2m_3t_1$ (sucrose 15 %+no media+ 4°C)	21.8^{a}	1.8^{f}	1.02^{l}	1.24^{ef}
$a_2m_3t_2$ (sucrose 15 %+no media+ 25°C)	15.4^{g}	3.2^{de}	1.02^{l}	1.24^{ef}
$a_3m_1t_1$ (mannitol 10 %+ MS+ 4°C)	18.6^{e}	4.4^{c}	**1.85^{a}**	**2.32^{a}**
$a_3m_1t_2$ (mannitol 10 %+ MS+ 25°C)	**11.6^{k}**	**6.4^{a}**	**1.87^{a}**	**2.48^{a}**
$a_3m_2t_1$ (mannitol 10 %+ SDW+ 4°C)	20.4^{c}	2.6^{e}	1.24^{g}	1.36^{de}
$a_3m_2t_2$ (mannitol 10 %+ SDW+ 25°C)	13.8^{hi}	4.6^{c}	1.25^{g}	1.44^{d}
$a_3m_3t_1$ (mannitol 10 %+no media+ 4°C)	19.6^{d}	3.2^{de}	1.25^{g}	1.48^{d}
$a_3m_3t_2$ (mannitol 10 %+ no media+ 25°C)	13.4^{i}	4.8^{c}	1.27^{f}	1.48^{d}
$a_4m_1t_1$ (mannitol 15 %+ MS+ 4°C)	19.6^{d}	2.8^{e}	1.14^{i}	1.36^{de}
$a_4m_1t_2$ (mannitol 15 %+ MS+ 25°C)	14.2^{h}	4.6^{c}	1.16^{h}	1.32^{e}
$a_4m_2t_1$ (mannitol 15 %+ SDW+ 4°C)	21.4^{a}	1.8^{f}	1.03^{l}	1.12^{g}
$a_4m_2t_2$ (mannitol 15 %+ SDW+ 25°C)	15.6^{g}	3.2^{de}	1.03^{l}	1.16^{g}
$a_4m_3t_1$ (mannitol 15 %+ no media+ 4°C)	21.6^{a}	2.2^{ef}	1.03^{l}	1.28^{e}
$a_4m_3t_2$ (mannitol 15 %+ no media+ 25°C)	15.2^{g}	3.4^{d}	1.03^{l}	1.28^{e}
$a_5m_1t_1$ (no additive+ MS+ 4°C)	19.4^{d}	3.6^{d}	1.42^{e}	1.68^{c}
$a_5m_1t_2$ (no additive+ MS+ 25°C)	12.8^{j}	5.4^{b}	1.47^{d}	1.72^{c}
$a_5m_2t_1$ (no additive+ SDW+ 4°C)	21.2^{ab}	2.2^{ef}	1.05^{kl}	1.24^{ef}
$a_5m_2t_2$ (no additive+ SDW+ 25°C)	15.4^{g}	3.8^{d}	1.06^{k}	1.24^{ef}
$a_5m_3t_1$ (no additive+ no media+ 4°C)	20.8^{c}	2.4^{e}	1.06^{k}	1.32^{e}
$a_5m_3t_2$(no additive+ no media+ 25°C)	14.8^{gh}	4.2^{cd}	1.07^{k}	1.32^{e}
SEM (±)	0.21	0.21	0.005	0.04
CD	0.41	0.41	0.01	0.09

*sodium alginate 2.5 per cent and calcium chloride 100 mM

**sterile distilled water, Regeneration medium-MS+ BA 1.5 mg L^{-1}+ IAA1.5 mg L^{-1}+ Adenine sulphate 40 mg L^{-1}

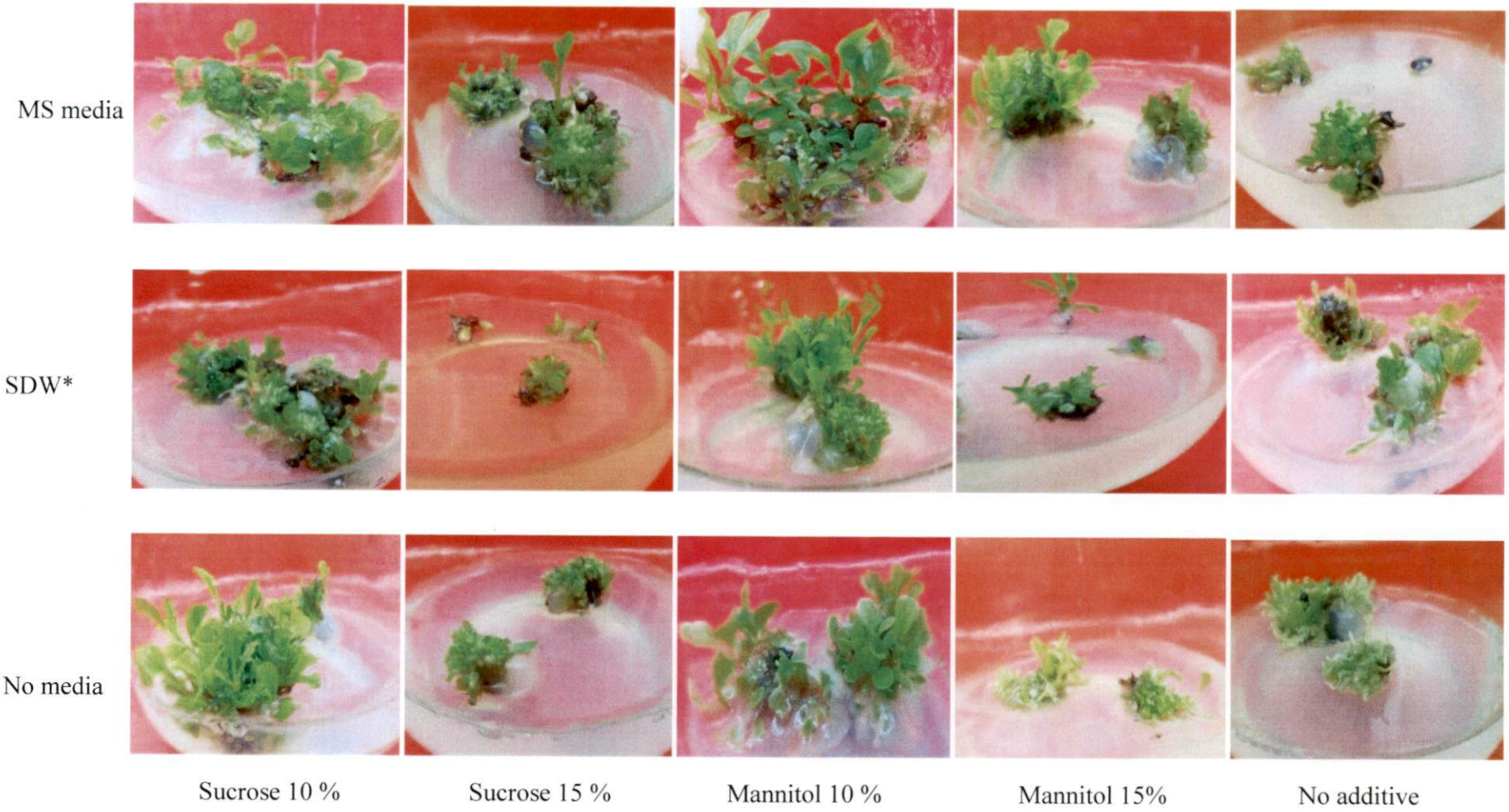


Plate 7: Response of encapsulated buds *P. rosea* after 30 days of storage at 25˚C (6 weeks of culture)

*sterile distilled water

potential (Plate 7). Among the thirty treatments tried, axillary buds encapsulated in mannitol 10 per cent supplemented encapsulation matrix, stored in liquid MS medium at 25°C ($a_3m_1t_2$) was found to give best plantlet conversion potential with respect to days to bud initiation (11.6 days), shoots per explants (6.4), shoot length (1.87 cm) and nodes per shoot (2.48). The variation among the thirty treatments was significant with respect to all these parameters. Days to bud initiation and shoots initiated per explant were significantly superior to all other treatment combinations. However, shoot length (1.87 cm) and nodes per shoot (2.32) was found to be on par with $a_3m_1t_1$ (mannitol 10 per cent supplemented encapsulation matrix, liquid MS as the storage medium; 4°C storage temperature). The data is presented in Table 18.

4.3 PHASE III: LONG TERM CONSERVATION USING VITRIFICATION

The long term conservation of axillary buds of *P. rosea* was attempted using two vitrification techniques of cryopreservation *viz*., simple vitrification using non-encapsulated axillary buds and encapsulation vitrifiction using encapsulated axillary buds. The axillary buds from *in vitro* cultures were preconditioned 0.5 M sucrose for 7 days, encapsulated (in case of encapsulation vitrifcation technique) and precultured in 0.5 M sucrose containing liquid MS for 3 days. The precultured materials were further subjected to loading treatment for 20 minutes in 2 M glycerol and 0.4 M sucrose in MS medium followed by exposure to different periods in vitrification solutions *viz*., PVS2 and PVS3. After the specified periods of exposure, they were frozen in liquid nitrogen for 2 hours. They were then rewarmed/ thawed and placed in recovery medium to study the survival and regeneration. Steps in simple and encapsulated vitrification are depicted in Plate 8.

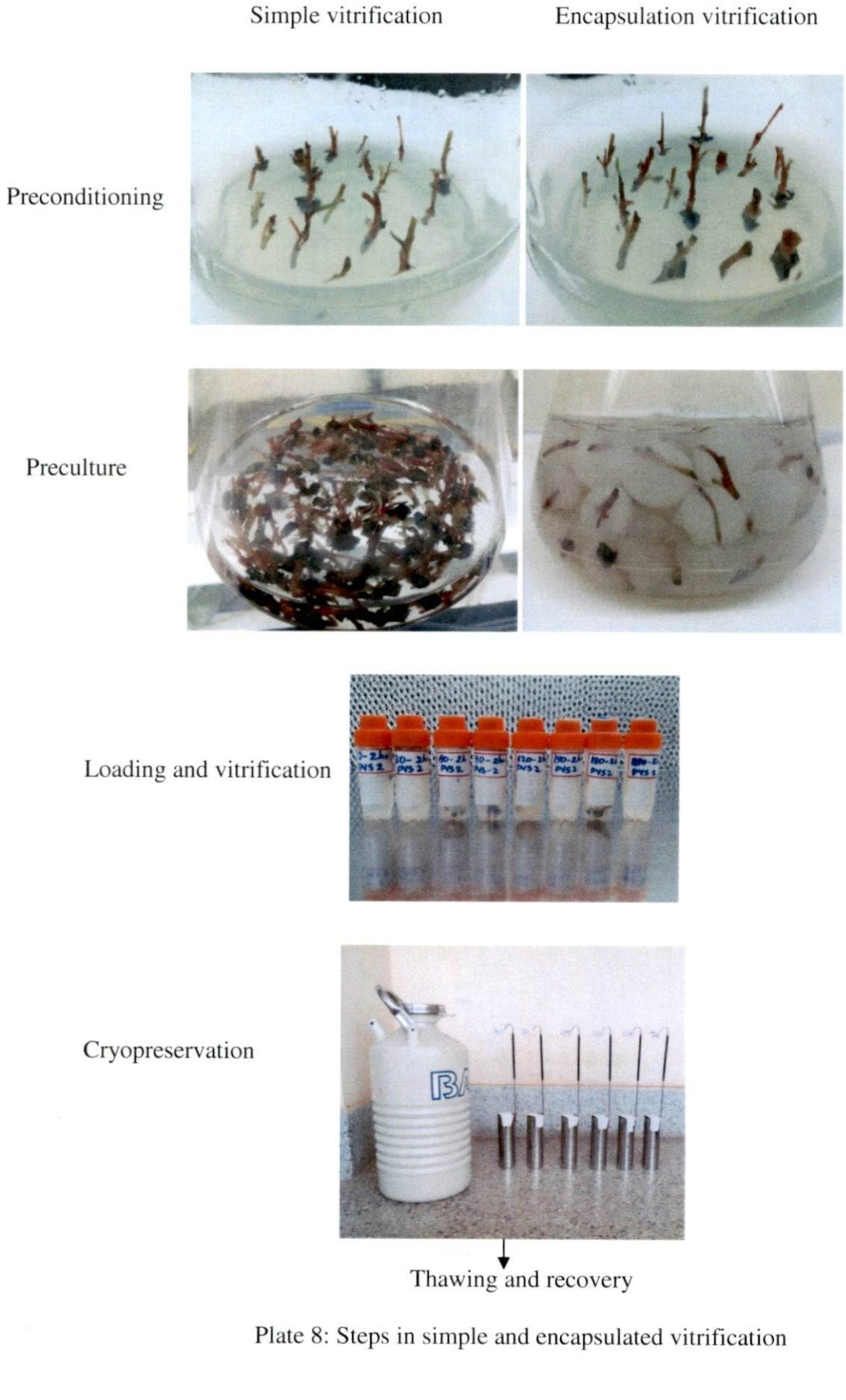


Plate 8: Steps in simple and encapsulated vitrification

4.3.1 Recovery of cryostored axillary buds

The survival was indicated by, the explants remaining green, in the recovery medium and regeneration was indicated by the emergence of shoot from the explants in the same medium.

The effect of simple and encapsulation vitrification techniques on survival and regeneration of cryoderived axillary buds are depicted in Tables 19 and 20.

In simple vitrification, after 2 h of cryostorage, survival and regeneration could be observed only in four treatments, among the sixteen tried. The recovery in terms of survival and regeneration could be obtained on exposure of preconditioned and precultured axillary buds to vitrification solutions PVS2 and PVS3 for 30 and 60 min. PVS2 exposure for 30 min (S2) gave maximum survival (62.22 per cent) and regeneration (47.78 per cent) among the four responded treatments. This was followed by PVS3 exposure for 30 min (S10) with a survival of 54.44 per cent and regeneration 37.67 per cent. PVS2 exposure for 60 min (S3) and PVS3 exposure for 60 min (S11) gave very low survival (24.44 and 18.89 per cent, respectively) and regeneration (16.67 and 11.11 per cent, respectively).

In encapsulation vitrification, recovery could in terms of survival and regeneration could be obtained in five treatments *i. e.*, the exposure to vitrification solutions, PVS2 for 30, 60 and 90 min and PVS3 for 30 and 60 min, among the sixteen treatments attempted. Maximum survival (78.89 per cent) and regeneration (70.00 per cent) was observed on PVS2 exposure for 30 min. This was followed by PVS3 exposure for 30 min (E10) which gave a survival of 67.78 per cent and regeneration of 55.52 per cent. PVS3 for 60 min (E11) gave very low survival (27.78 per cent and regeneration (17.78 per cent), among the five responded treatments.

Table 19: Effect of simple vitrification of axillary buds of *P. rosea* on survival and regeneration percentage

Treatment No:	Period of exposure to vitrification solution (min)	Vitrification solution	2 h Cryostorage	
			SP*	RP**
S1	0	PVS 2	-	-
S2	30		62.22	47.78
S3	60		24.44	16.67
S4	90		-	-
S5	120		-	-
S6	150		-	-
S7	180		-	-
S8	210		-	-
S9	0	PVS 3	-	-
S10	30		54.44	37.67
S11	60		18.89	11.11
S12	90		-	-
S13	120		-	-
S14	150		-	-
S15	180		-	-
S16	210		-	-

*survival percentage

**regeneration percentage

Regeneration medium-MS+ BA 1.5 mg L^{-1}+ IAA1.5mg L^{-1}+ Adenine sulphate 40 mg L^{-1}

Table 20: Effect of encapsulation vitrification of axillary buds of *P. rosea* on survival and regeneration percentage

Treatment No:	Period of exposure to vitrification solution (min)	Vitrification solution	2 hr cryostorage	
			SP*	RP**
S1	0	PVS 2	-	-
S2	30		78.89	70.00
S3	60		65.56	52.22
S4	90		37.78	28.89
S5	120		-	-
S6	150		-	-
S7	180		-	-
S8	210		-	-
S9	0	PVS 3	-	-
S10	30		67.78	55.52
S11	60		27.78	17.78
S12	90		-	-
S13	120		-	-
S14	150		-	-
S15	180		-	-
S16	210		-	-

*survival percentage

**regeneration percentage

Regeneration medium-MS+ BA 1.5 mg L^{-1}+ IAA1.5 mg L^{-1}+ Adenine sulphate 40 mg L^{-1}

The explants were cryostored for different duration *viz*., 2 h, 1 day, 1 week, 1 month and 2 months to study the effect on recovery. It was observed that there is no significant variation in terms of survival and regeneration per cent with respect to different periods of storage in LN (Table 21). However, significant variation was observed with different vitrification treatments with varying periods of exposure in LN. Only those treatments which gave more than 50 per cent survival after 2 h of exposure were carried over to extended periods of storage in LN. Hence, explants from five vitrification treatments *viz*, Simple vitrification with PVS2 exposure for 30 min (S2), PVS3 exposure for 60 min (S10), Encapsulation vitrification with PVS2 exposure for 30 min (E2), 60 min (E3) and PVS3 exposure for 30 min (E10) were carried forward for extended periods of storage in LN. In all the different period of LN storage, E2 gave maximum survival (76 to 79 percent) and regeneration (63 to 70 per cent). Effect of the five different cryopreservation treatments on shoot proliferation of axillary buds recovered from 2 h of cryostorage is presented in Fig. 7.

This was followed by E10, which was found to be on par with E3 with respect both survival and regeneration. The lowest survival and regeneration was recorded in S10 with survival of 49-56 per cent and regeneration of 36-38 per cent. However among the simple vitrification treatments, S2 gave better survival (59- 62 per cent) and regeneration (43-48 per cent).

In the study, it was observed that encapsulation vitrification treatments gave better recovery compared to simple vitrification treatments.

4.3.2 Shoot proliferation of axillary buds recovered from cryostorge.

The data on shoot proliferation of axillary buds, recovered from cryostorage following different vitrification treatments is presented in Table 22.

Table 21: Survival and regeneration percentage of axillary buds exposed to different vitrification solutions after cryostorage

Treatment No:	Vitrification solutions										
	2 hour		1 day		1 week		1 month		2 month		
	SP* (%)	RG** (%)	SP (%)	RG (%)	SP (%)	RG (%)	SP (%)	RG (%)	SP (%)	RG (%)	CD
S2	62.22^{c}	47.78^{c}	58.89^{d}	45.56^{c}	60.00^{b}	50.00^{b}	58.89^{c}	44.44^{c}	60.00^{cd}	43.33^{c}	NS
S10	54.44^{d}	36.67^{d}	55.56^{d}	37.78^{d}	51.11^{c}	35.55^{c}	48.89^{d}	35.56^{d}	55.56^{d}	35.55^{d}	NS
E2	**78.89^{a}**	**70.00^{a}**	**76.67^{a}**	**66.67^{a}**	**73.33^{a}**	**63.33^{a}**	**76.67^{a}**	**66.67^{a}**	**75.59^{a}**	**65.56^{a}**	NS
E3	65.56^{bc}	52.22^{bc}	64.44^{c}	48.89^{c}	62.22^{b}	50.00^{b}	63.33^{bc}	47.78^{c}	64.44^{bc}	50.22^{b}	NS
E10	67.78^{b}	55.52^{b}	68.89^{b}	55.55^{b}	65.56^{b}	55.55^{b}	64.44^{b}	54.44^{b}	67.78^{b}	55.55^{b}	NS
CD	3.51	4.69	4.14	4.96	6.06	5.85	5.43	4.14	4.96	5.65	

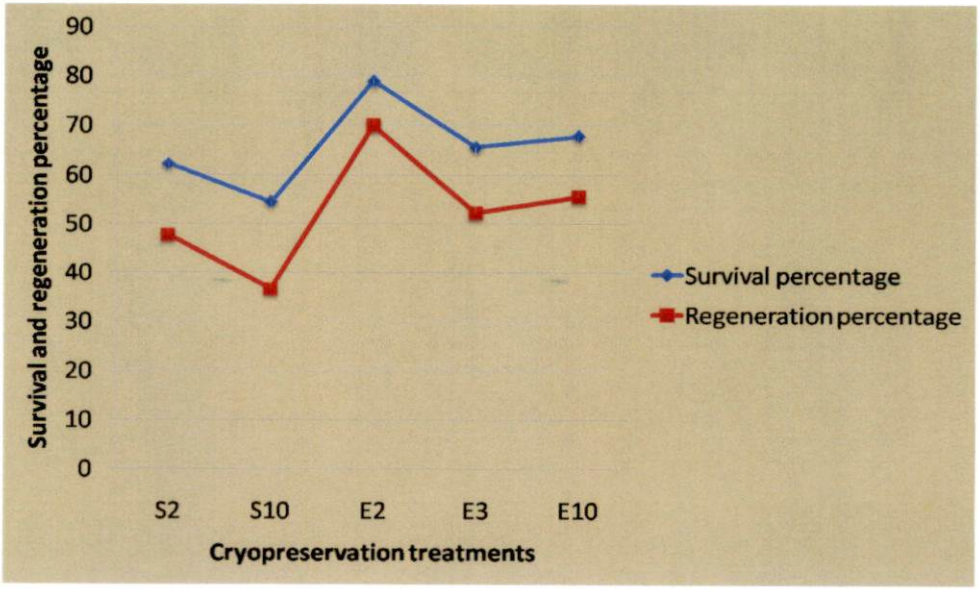
S2 - Simple vitrification (PVS2- 30 min exposure)
S10 - Simple vitrification (PVS3-30 min exposure)
E2 - Encapsulation vitrification (PVS2-30 min exposure)
E3 - Encapsulation vitrification (PVS2- 60 min exposure)
E10 - Encapsulation vitrification (PVS3- 30 min exposure)

* survival percentage
**regeneration percentage

Table 22. Effect of different cryopreservation treatments on shoot proliferation of axillary buds, recovered from cryostorage

Treatment no:	Regeneration percentage	Days to bud initiation	Shoots per explant	Shoot length	Nodes per shoot
S2	100	34.13 ± 0.83^{c}	1.63 ± 0.52^{d}	2.25 ± 0.08^{d}	2.00 ± 0.76
S10	100	38.58 ± 0.79^{a}	1.15 ± 0.37^{e}	1.98 ± 0.07^{e}	1.70 ± 0.75
E2	**100**	$\mathbf{28.89 \pm 0.80^{e}}$	$\mathbf{2.56 \pm 0.52^{a}}$	$\mathbf{2.88 \pm 0.12^{a}}$	2.11 ± 0.79
E3	100	36.43 ± 0.53^{b}	1.85 ± 0.69^{c}	2.75 ± 0.05^{b}	2.15 ± 0.69
E10	100	31.75 ± 1.28^{d}	1.88 ± 0.65^{b}	2.49 ± 0.09^{c}	1.88 ± 0.65
CD		0.90	0.57	0.12	NS

S2 - Simple vitrification (PVS2- 30 min exposure)
S10 - Simple vitrification (PVS3-30 min exposure)
E2 - Encapsulation vitrification (PVS2-30 min exposure)
E3 - Encapsulation vitrification (PVS2- 60 min exposure)
E10 - Encapsulation vitrification (PVS3- 30 min exposure)
Regeneration medium- MS+BA 1.5 mg L^{-1}+IAA1.5 mg L^{-1}+Adenine sulphate 40 mg L^{-1}



S2- Simple vitrification (PVS2- 30 min.), S10 - Simple vitrification (PVS3- 30 min), E2- Encapsulation vitrification (PVS2-30 min), E3- Encapsulation vitrification (PVS2- 60 min), E10 - Encapsulation vitrification (PVS3-30 min).

Fig. 7: Effect of different cryopreservation treatments on shoot proliferation of axillary buds of *P. rosea*, recovered from cryostorage (2 h storage in liqiuid nitrogen)

The parameters, days to bud initiation, shoots per explants and shoot length showed significant variations among the different vitrification treatments. The earliest bud initiation was observed in E2 (28.89 days). The same treatment recorded maximum shoots per explant (2.56) and shoot length (2.88 cm). There was no significant variation with respect to number of nodes per shoot among the different treatments. Bud initiated late (38.58 days) in S10. The least number of shoots (1.15) and shoot length (1.98 cm) were also recorded in the same treatment.

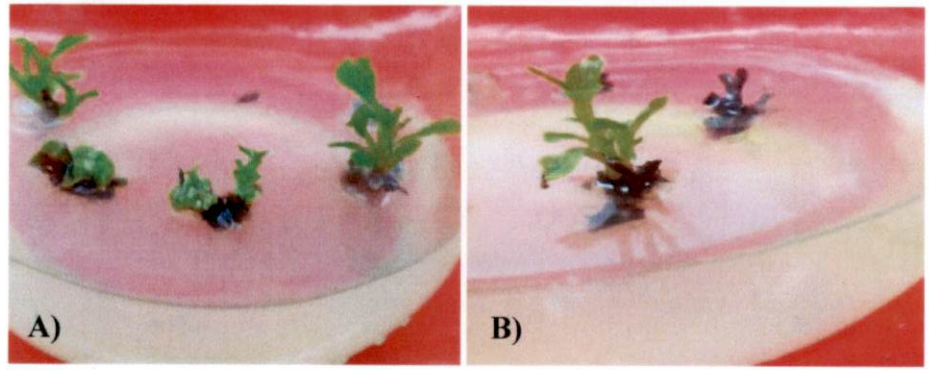
Hence, encapsulation vitrification with PVS2 exposure for 30 min (Plate 9) gave best response with respect to recovery and further shoot regeneration and proliferation among the different vitrification treatments tried.

4.3.3 Estimation of genetic stability of cryopreserved materials using RAPD and ISSR markers

Five RAPD and four ISSR markers were tried for the assessment of genetic fidelity in the plants regenerated after *in vitro* conservation. Of the different primers used, the ISSR primers UBC-810 (GAGAGAGAGAGAGAGAT) and UBC-811 (GAGAGAGAGAGAGAGAC) had produced clear and distinguished bands. The number of bands for each primer varied from 6 to 7. The five RAPD primers tested did not give any bands.

ISSR profiles of the plantlets regenerated from both short and long term storage, were identical to those of control plants with both the two primes. Plate 10 illustrates amplified banding patterns produced by the two primers in plantlets regenerated after *in vitro* storage and control plants. No difference was observed in the banding pattern in any of the plantlets.

Simple vitrification



ncapsulation vitrification

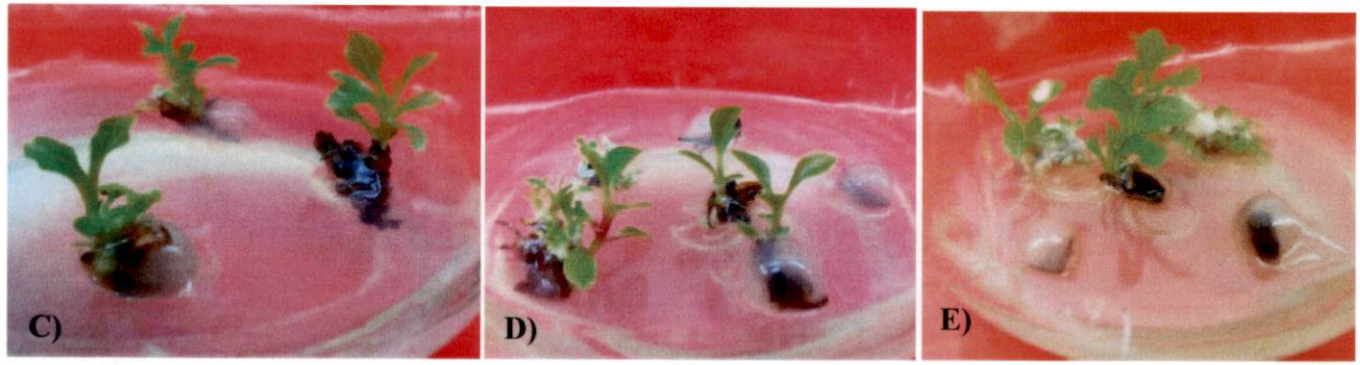


Plate 9: Regenerated axillary buds *P. rosea* after cryopreservation-

Simple vitrification : A) 30 mins exposure to PVS2 vitrification solution, B) 30 min exposure to PVS3 vitrification solution

Encapsulation vitrification : C) 30 mins exposure to PVS2 vitrification solution, D) 60 mins exposure to PVS2 vitrification solution, E) 30 mins exposure to PVS3 vitrification solution

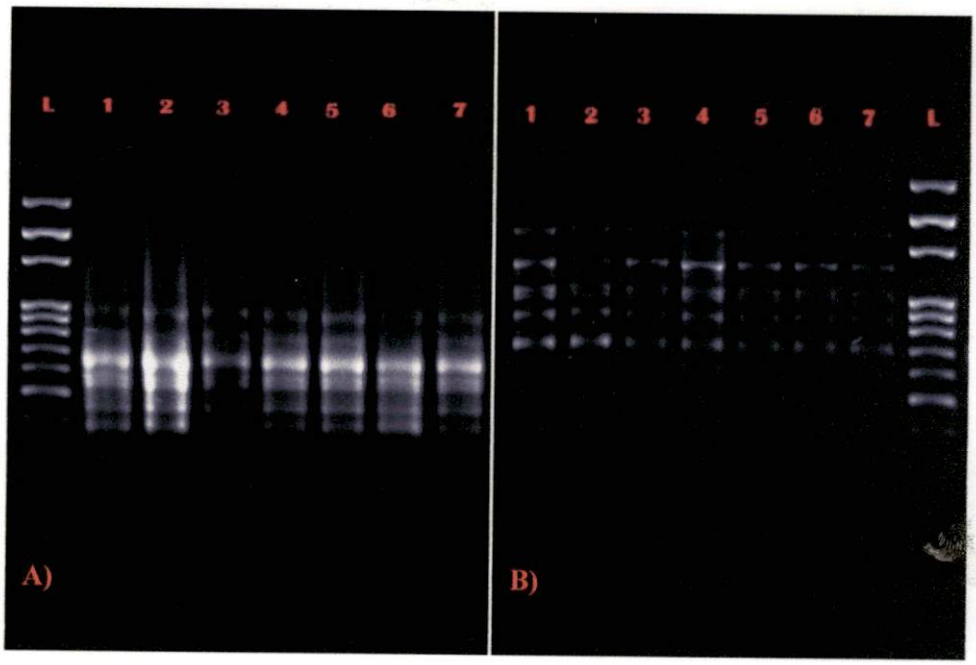


Plate 10: ISSR banding profile of plantlets regenerated from *in vitro* storage and control plantlets of *P. rosea*: A) UBC- 810, B) UBC- 811

1- Control
2- Short term conservation(4˚C)
3- Short term conservation (25˚C)
4- Simple vitrification PVS2
5- Simple vitrification PVS3
6- Encapsulation vitrification PVS2
7- Encapsulation vitrification PVS3
L- Ladder (100 bp)

Discussion

5. DISCUSSION

The present study, '*In vitro* conservation of Chethikoduveli (*Plumbago rosea* L.) using encapsulation and vitrification techniques' was carried out at Department of Plant Biotechnology, College of Agriculture, Vellayani. The results obtained in the study are discussed in this chapter.

5.1 ENHANCEMENT OF MULTIPLICATION RATE

5.1.1 Effect of different medium and additives on multiplication rate

5.1.1.1 Medium

The type and concentration of mineral nutrients supplied in different types of media have a profound influence on tissue proliferation and morphogenetic responses (Gantait *et al.,* 2016). A shoot multiplication medium, MS medium supplemented with BA 1.5mg L^{-1} and IAA 1mg L^{-1}, using nodal plants of *P. rosea* has been standardized in the Department of Plant Biotechnology, College of Agriculture, Vellayani (Prakash, 2014).

In the present study, effect of different media on enhancement of shoot multiplication was attempted. Among the different media MS, White's, SH and B5 supplemented with the same hormonal combination BA 1.5mg L^{-1} and IAA 1mg L^{-1} tried, the days to bud intiation, shoots per explant (3.75 shoots/explant), shoot length and nodes per shoots were found to be the best in MS medium supplemented with BA 1.5mg L^{-1} and IAA 1mg L^{-1}). Soni *et al.* (2012) reported that MS medium gave better shoot proliferation compared to B5 medium from the nodal explants of *Adhatoda vasica.* The shoots per plant, average shoot length and nodes per shoot were higher in MS medium supplemented with BAP 1.0 mg L^{-1}. The findings are also in agreement with the study on the effect of different media on *in vitro* regeneration of *Morus indica* by Niratker *et al.* (2015). MS medium was found to be superior to Gamborg and SH media with respect to shoot initiation as well as elongation. The probable reason for the better shoot

proliferation response of MS medium compared to B5, SH and Whites media may be due to its higher ammonium content (Catapan *et al.*, 2001).

5.1.1.2 Additives

The MS medium supplemented with BA 1.5 mg L^{-1} and IAA 1 mg L^{-1} was used as the basal medium to which different additives *viz.*, chitosan, adenine sulphate and thidiazuron were added. This medium was used as the control treatment in the studies on enhancement of shoot multiplication with different additives.

5.1.1.2.1 Chitosan

Seven treatments with different concentrations of chitosan, supplemented to the basal medium were tried for the assessment of shoot proliferation. The least days to bud initiation (7.50) and highest shoot proliferation (3.67 shoots per explant) and maximum shoot length (4.28 cm) was obtained in MS media supplemented with BA 1.5 mg L^{-1}, IAA 1 mg L^{-1} and chitosan 15 mg L^{-1}. The results were on par with that of control. In agreement with our finding, Sopalun *et al.* (2010) reported that chitosan 15 mg L^{-1} supplemented MS medium gave the better shoot proliferation from the PLBs of *Grammatophyllum speciosum.*

In the study, chitosan upto 15 mg L^{-1} exhibited an increasing trend in shoot proliferation. Beyond this level of chitosan, the shoot growth declined. This confirms that an optimum concentration of additive is needed for shoot proliferation, beyond which it may lead to toxicity. Also at higher concentration, 30 mg L^{-1} of chitosan, the media did not solidify properly. Zakaria *et al.* (2009) has reported non solidification of media at higher concentration of chitosan at the rate of 750 mg L^{-1}.

The chitosan enhances growth and development by some signaling pathway to auxin biosynthesis *via* a tryptophan-independent pathway (Uthairatanakij, 2007). It acts as a growth promoter and elicitor of plant defense mechanisms that could alleviate stress caused by *in vitro* conditions and acclimatization (Zakaria *et al.,* 2009).

5.1.1.2.2 Adenine sulphate

Adenine sulphate at seven different concentrations in the basal medium were used to study the effect on shoot multiplication. Ads 40 mg L^{-1} supplemented medium gave best response with respect to days to bud initiation (6.42), maximum shoot proliferation (4.25 shoots per explant), shoot length (4.69 cm) and nodes per shoot (3.25). A declining trend in shoot proliferation was observed with higher levels of Ads.

The best shoot proliferation from nodal explants was observed in *Stevia rebaudiana* by supplementing adenine sulphate (40 mg L^{-1}), (Khan *et al.*, 2014). This is consistent with the results obtained in our study. Ads can stimulate cell growth and shoot multiplication probably by acting as organic nitrogen source and/or acting as pre-cursor for natural cytokinin synthesis.

The nitrogen utilization of *in vitro* grown culture can be enhanced by the use of reduced nitrogen forms particularly adenins and amino acids. Ads being an organic source of nitrogen could be taken up more rapidly than inorganic nitrogen (Vengadesan *et al.*, 2002). It regulates primary nitrogen assimilation and improves cell proliferation as well as regeneration in many species (Shrivastava and Banerjee, 2008).

The natural and synthetic purine cytokinins are degraded to adenine sulphate and related nucleotides. Van Staden *et al.* (2008) stated the addition of Ads to the medium may retard the degradation of cytokinins by feed-back

inhibition or by competing for the enzyme systems involved in cytokinin metabolism. The benefits of Ads supplementation are often only noticed when it is associated with ammonium nitrate or with cytokinins such as BAP or kinetin. Gatica *et al.* (2010) reported that adenine sulphate show cytokinin activity and therefore, augmented to different culture medium to reinforce the the cytokinin responses and improve growth of plants in culture.

5.1.1.2. Thidiazuron

The effect of thidiazuron on shoot proliferation was assessed by supplementing different levels of the additive in basal medium. Thidiazuron 4 mg L^{-1} and 5 mg L^{-1} recorded the highest shoots per explant (11.50). The stimulating effect of Tdz on multiple shoot formation has been reported in several medicinal and aromatic plant species including *Ocimum basilicum* (Bacila *et al.*, 2010) *Arachis correntina*, (Mroginski *et al.*, 2004) *Curcuma longa*, (Prathanturarug *et al.*, 2005) *Curculigo orchioides* (Thomas, 2007) and *Mentha arvensis* (Faisal *et al.,* 2014). This effect could be attributed to the ability of Tdz in stimulating biosynthesis of endogenous cytokinin or in altering cytokinin metabolism (Li *et al.,* 2000; Ahmad *et al.*, 2006; Guo *et al.*, 2011)

However, in our study, the best response with respect to days to bud initiation, shoot length and number of nodes per shoot was obtained in the control medium devoid of thidiazuron. Though number of shoots initiated were higher in thidiazuron supplemented treatments, the shoots obtained were vitrified, stunted with clustered axillary buds and reduced leaf expansion. Callus formation was also observed at the base. Similar abnormalities were reported in *Vitis vinifera* on supplementing thidiazuron 2- 4 mg L^{-1} in MS media. (Ibanez *et al.*, 2003). Formation of fasciated or distorted shoots are observed in medicinal plant cultures, continuously grown on Tdz supplemnted media (Nawala and Karmakar, 1997, Wala and Jasrai 2003).

5.2 SHORT TERM CONSERVATION BY ENCAPSULATION TECHNIQUE

In the study, the highest regeneration of 95.83 per cent and 83.33 per cent and shoot proliferation (6.6 and 6.4) were obtained in the regeneration medium, MS supplemented with BA 1.5 mg L^{-1}, IAA 1 mg L^{-1} and Ads 40 mg L^{-1}, from the axillary buds encapsulated with mannitol 10 per cent in the matrix, stored in liquid MS medium for 15 and 30 days respectively, at 25°C. The same treatment had shown the highest regeneration (91.67, 79.17 per cent, respectively) at 4°C after 15 and 30 days of storage. The regeneration and plant conversion efficiency of axillary buds in sucrose 10 per cent supplemented encapsulation matrix followed the above treatment, at both temperatures, in the same storage medium. Similar trend of osmotic response was reported in *Dendrobium nobile*, where mannitol 7.5 per cent followed by sucrose 7.5 per cent gave maximum survival and plant conversion from encapsulated PLBs. (Mohanty *et al.*, 2013)

An optimum concentration of osmoticum could inhibit the growth of cultures by inducing osmotic stress without being toxic, thus enabling its storage. Increase in concentration of sucrose and mannitol to 15 per cent had reduced the regeneration and shoot proliferation, in the study. Sucrose and mannitol at optimal concentration would serve both as growth retardants by causing osmotic stress to plant material under conservation and as carbon source when, the stored material need to be recovered and regenerated after storage (Shibli *et al.*, 2006).

Mohanty *et al.*, (2013) opined that osmotic concentrations minimizes the growth of explants during storage condition. This may be due to osmotic stress imposed on the explants by higher concentration of sucrose and mannitol. Increased osmotic stress would cause cell plasmolysis resulting in slower cell division (Loveys *et al.* 1975), cell growth inhibition (Wong and Sussex, 1980) and prolong the storage time (Yun-peng *et al.*, 2012).

In a study by Mohanty *et al.* (2013) in orchid, Dendrobium nobile, it was observed PLBs encapsulated in the beads containing 15 per cent sucrose and mannitol in the encapsulating matrix died after 10 days which may be due to increased rate of cell dehydration resulting in cell death. Though, 15 per cent sucrose and mannitol in the matrix did not cause cell death, it diminished the regeneration and subsequent plantlet conversion, in our study.

The study also confirmed the role of osmoticum in the matrix on shoot regeneration resposes. The plant conversion efficiency of axillary buds encapsulated without any osmotic additives was found to be less compared to 10 per cent osmoticum (mannitol and sucrose) supplemented matrix. Mannitol induces osmotic stress by reducing the hydric potential and restricting the water availability to the explants (Silva and Scherwinski-Pereira, 2011). As sucrose concentration increases, water content of the plant cell would reduce causing a reduction in cell volume. The decrease in cell volume would result in lower turgor pressure in the cells. The cell expansion being a turgor-driven process, loss of turgor would result in growth reduction (Taiz and Zeiger, 2002).

The differentially encapsulated buds were stored in different media, *viz.*, liquid MS, sterile distilled water and without any media as control. The highest regeneration and shoot proliferation was observed in the regeneration medium, from the treatment with liquid MS as storage media. This was in agreement with a study in *Capparis orientalis*, where MS nutrients had significant superiority over the distilled water with respect to plantlet conversion and shoot length (Hegazi, 2011).

This effect may due to the absorbtion of nutrients from the liquid MS storage media that enhanced plant conversion from the encapsulated axillary buds. The lowest regeneration was obtained by storing encapsulated axillary buds in sterile distilled water. This may due to movement of water into the encapsulated matrix from the storage media (sterile distilled water) due to the high

concentration of osmoticum in the encapsulated matrix. This may lead to the rotting of the plant tissue inside the encapsulation matrix.

Next to liquid MS as the storage media, better response with respect to survival and plantlet conversion in encapsulated buds was obtained in the storage treatment without any media. The may be due to the tolerance to desiccation that the buds have obtained due to encapsulation, which otherwise would have resulted in dehydration and reduction in plant conversion. Dereuddre *et al.* (1990) stated that alginate encapsulation would increase considerably the resistance of plant tissues to desiccation.

By storing the encapsulated axillary buds in two different storage temperatures *viz.*, 25˚C than 4˚C the highest regeneration and shoot proliferation was obtained at 25˚C, which exhibited a gradual decline with extended period of storage. Similarly, the encapsulated protocorms of *Cymbidium bicolor*, stored at 25˚C gave better germination and regeneration compared to that at 4˚C. At both temperatures, the regeneration per cent showed a gradual decline with storage duration (Mahendran, 2014).

Silva and Scherwinski- Pereira (2011) explained the influence of different temperatures on survival, shoot length and number of buds per shoot in *P. aduncum* after six months of *in vitro* storage. At 25˚C, 100 per cent survival could be obtained but this was drastically reduced to 2.4 per cent at 10˚C.

Regeneration percentage deteriorated beyond 30 days in all the thirty treatment combination. This may be attributed to the inhibition in respiration of plant tissues, perhaps due to alginate cover during prolonged days of storage, at different temperatures. The decline in plant recovery from stored encapsulated vegetative propagules may be due to both oxygen deficiency in the calcium alginate bead (Hegazi, 2011).

5.3 LONG TERM CONSERVATION USING VITRIFICATION

In the study, long term conservation of axillary buds of *P. rosea* was done using vitrification technique of cryopreservation. Survival above 50 per cent was obtained in five treatments *viz*., 30 min exposure to PVS 2 and 30 min exposure to PVS3 in case of simple vitrification and in case of encapsulation vitrification 30 and 60 min exposure to PVS2 vitrification solution and 30 min exposure to PVS3 vitrification solution, after cryostorage in the regeneration medium. Prolong exposure to vitrification solution may be detrimental to the axillary buds due to the excessive osmotic stress or chemical toxicity exerted by concentrated PVS2 (Matsumoto *et al*.,1995; Thinh *et al*., 1999). The toxicity studies have shown that glycerol-induced damage is aggravated by dimethyl sulphoxide and ethylene glycol (Cho *et al*., 2002).

Among vitrification solutions tried *viz*., PVS2 and PVS3, higher regeneration was attained in PVS2, in the study. This is in agreement with the study on cryopreservation of *Raufolia serpentina* by Ray and Bhattacharya (2008). Volk and Walters (2006) explained that PVS2 acts in cryopreservation by replacing cellular water and changing freezing behaviour of the water remaining in cells. As DMSO is a penetrating component in PVS2, PVS2 treatment broadens the permissible range of water contents in cryopreserved plant tissue, reduces the damage from excessive cell shrinkage and limits the risk of ice formation. Successful cryopreservation with PVS3 entails a longer soaking period than is necessary with PVS2. As penetrating cryoprotectants *viz*., ethylene glycol and dimethyl sulfoxide are not present in PVS3, a longer time needed to accumulate intracellular concentrations of glycerol sufficiently high for cryoprotection. (Volk *et al*., 2006)

Among the five different treatments of vitrification, the highest survival and shoot proliferation was observed in encapsulation vitrification with exposure to PVS2 vitrification solution for 30 min. This is in agreement with the results

obtained in the cryopreservation of gentian shoot apices (Tanaka *et al.*, 2004). Higher survival in encapsulation vitrification may be due to the alginate coating that reduces cell dehydration rate and relatively lower extent of direct contact to toxic chemical components of PVS2 (such as DMSO). In simple vitrification, severe and rapid dehydration of tissues occur due to the direct exposure to vitrification solution. In contradiction to our observation, Yamuna *et al.* (2007) reported that simple vitrification had produced higher regrowth than encapsulation vitrification in *Zingiber officinale.*

5.4 Genetic fidelity assessment of plantlets recovered and regenerated from in vitro storage

The genetic uniformity of the *in vitro* conserved plants needs to be evaluated to assess the feasibility of the technique in germplasm conservation. The genetic stability of *P. rosea* plants recovered from short term as well as long term (cryopreservation) conservation was assessed using five RAPD and four ISSR markers. Of the different primers used, only two ISSR primers produced distinguishable bands. Banding profile was monomorphic and no difference was observed in the banding pattern between the plants regenerated from *in vitro* storage and control plants from *in vitro* culture.

Plant materials could be conserved without genetic change for unlimited periods in cryostorage. Cryopreservation, being stored at low temperature as that of LN (-196˚C), all the metabolic activities are arrested resulting in the restoration of genetic make-up of the stored material for any duration. Al-Ababneh *et al.*, (2002) stated that regeneration and genetic stability of cryopreserved materials could be maintained for indefinite period.

Two PCR-based techniques, RAPD and ISSR, were used to test clonal fidelity because of their simplicity and cost-effectiveness. The use of two markers,

which amplify different regions of the genome, allows better chances for the identification of genetic variations in the clones (Martin *et al*., 2004).

Genetic stability of long term micropropagated shoots of banana, *Vanilla planifolia*, almonds and tea clones were assessed using RAPD and ISSR markers by Lakshmanan *et al*. (2007) Sreedhar *et al*. (2007), Martin *et al*. (2004) and Devarumath *et al*. (2002) respectively tested the genetic nature of *in vitro* grown with the help of RAPD, ISSR, techniques.

The genetic stability assessment studies did not show any significant polymorphism between the cryopreserved plants and their non cryopreserved control.

In vitro short term conservation using encapsulation technique and long term convertion by vitrification technique of cryopreservation has been standardized in the study. Short term conversation, where in encapsulated axillary buds were stored for 30 days could be used as a means for germplasm exchange and distribution. Encapsulation –vitrification which gave 70 per cent regeneration after cryopreservation is an effective long term conservation technique for conserving *P. rosea*. The genetic uniformity could be maintained both in short-term and long term conversation techniques standardized.

Future Lines of Work

- Optimisation of various steps in vitrification method of cryopreservation.
- Optimisation of conservation using synthetic seeds technology to enhance the period of storage.
- Cryopreservation can be tried with other explants such as roots of *P. rosea*.
- The morphological, biochemical and molecular evaluation of cryogenerated plantlets and its comparision with the plantlets.

Summary

6. SUMMARY

The present study on "*In vitro* conservation of chethikoduveli (*Plumbago rosea L.*) using encapsulation and vitrification techniques" was carried out in the Department of Plant Biotechnology, College of Agriculture, Vellayani. The explants for the study were sourced from the cultures of *Plumbago rosea var* Agni maintained at the Department of Plant Biotechnology. The study was carried out in three phases *viz.*, enhancement of multiplication rate, short term conservation by encapsulation technique and long term conservation using vitrification technique.

The objective of the study was to standardise the protocol for short term and long term conservation of axillary buds using encapsulation and vitrification techniques and to assess the genetic fidelity of recovered and regenerated plantlets using molecular markers.

Single nodal segments from *in vitro* raised cultures were used as explants to study the effect of different media and different additives on the multiplication in *P. rosea*. A medium for shoot multiplication from the single nodal explants of *P. rosea*, MS supplemented with BA 1.5 mg L^{-1} and IAA 1 mg L^{-1}, has already been established in a previous study at the Department of Plant Biotechnology.

Four different media *viz.,* B5 medium, White's medium and SH medium and MS (control) each supplemented with BA 1.5 mg L^{-1} and IAA 1 mg L^{-1} were used to assess their effect on shoot multiplication. Among the different media tried, MS medium was observed to give best response in earliest days (7.50) to bud initiation, maximum shoot proliferation (3.75 shoots) and maximum shoot length (4.30 cm).

Different levels of additives (chitosan, adenine sulphate and thidiazuron) were tried for the enhancement of multiplication. Standardized medium, MS supplemented with BA 1.5mg L^{-1} and IAA 1mg L^{-1}, for shoot proliferation was used as the control treatment in the study.

Hundred per cent regeneration was observed in all the seven treatments supplemented BA 1.5 mg L^{-1} and IAA 1mg L^{-1}in MS media along with varying concentration of chitosan (0, 5, 10, 15, 20, 25 and 30 mg L^{-1}). The best reponse with respect to days to bud initiation (7.42), shoots initiated per explant (3.67) and shoot length (4.29 cm) was observed in MS medium supplemented with BA 1.5 mg L^{-1}, IAA 1 mg L^{-1} and chitosan 15 mg L^{-1}. But these results were on par with the control treatment.

Regeneration percentage was observed to be hundred per cent in MS media supplemented with BA 1.5 mg L^{-1} and IAA 1mg L^{-1} and varying levels of adenine sulphate (20, 40, 60, 80, 100 and 120 mg L^{-1}). Among the treatments,the best response was observed in MS media supplemented with BA 1.5 mg L^{-1}, IAA 1mg L^{-1} and 40 mg L^{-1} adenine sulphate with respect to days to bud initiation (6.42), shoots initiated per explant (4.25), shoot length (4.69cm) and nodes per shoot (3.25).

All treatments with different concentrations (0.5, 1, 2, 3, 4 and 5 mg L^{-1}) of thidiazuronin MS media supplemented with BA 1.5 mg L^{-1} and IAA 1mg L^{-1}recorded 100 per cent regeneration. Maximum shoot proliferation (11.50 shoots per explant) was observed in thidiazuron 4 and 5 mg L^{-1}supplemented MS medium with BA 1.5 mg L^{-1} and IAA 1mg L^{-1}. However, the earliest days to bud initiation (7.41), highest shoot length (4.28) and maximum nodes per shoot (2.67) were observed in the control treatment.

Thidiazuron supplemented media produced shoots with stunted and vitrified appearancewith callusing at the base. The leaf expansion was much lower compared to that of the control treatment. Though chitosan 15mg L^{-1} gave maximum shoots per explants (3.67) among different levels of chitosan tried, it was found to be on par with the control. Among the different additives tried, adenine sulphate 40 mg L^{-1}gave the best shoot multiplication response. Hence, MS medium supplemented with BA 1.5mg L^{-1}, IAA 1 mg L^{-1} and adenine

sulphate 40 mg $L^{-1}$was selected as the regeneration / multiplication medium for further conservation studies.

Short term conservation by encapsulation technique was carried out by differentially encapsulating the axillary buds in an encapsulation matrix (sodium alginate 2.5 per cent and calcium chloride 100 mM) with different additives *viz.*, sucrose 10 percent, sucrose 15 percent, mannitol 10 per cent, mannitol 15 per cent and holding them in storage media *viz.*, liquid MS and sterile distilled water at two temperature regimes, 4°C and 25°C.

Maximum regeneration of 95.83 and 83.33 per cent was obtained from encapsulated axillary buds with the additive mannitol 10 per cent in the matrix, stored in MS medium at 25°C for 15 and 30 days respectively. Similar trend was obtained at 4°C for the same treatment combination, stored for 15 and 30 days, giving a regeneration of 91.67 and 79.17 per cent respectively. This treatment recorded the highest regeneration among all the other treatments, but a declining trend in regeneration per cent was observed with extended period of storage (from 45 to 90 days with 15 days interval) at both the temperatures. Encapsulated axillary buds could be stored upto 30 days in all combinations, beyond which the regeneration per cent started to decline.

Among all the treatment combinations after 15 days of storage, the best response with respect to earliest days to bud initiation (8.4), shoots initiated per explant (6.6) shoot length (1.94 cm) and nodes per shoot (2.68) was obtained in encapsulated axillary buds supplemented with mannitol 10 per cent, stored in MS media at 25°C.

After 30 days of storage the same treatment combination had shown the highest plantlet conversion potential of encapsulated axillary buds with earliest days to bud initiation (11.6), maximum number of shoots per explant (6.4), maximum shoot length (1.87 cm) and maximum nodes per shoot (2.48).

Two cryopreservation techniques *viz.*, simple vitrification using non-encapsulated axillary buds and encapsulation vitrification using encapsulated axillary buds were attempted. The axillary buds from *in vitro* cultures were preconditioned 0.5 M sucrose for 7 days, precultured for 3 days in 0.5 M sucrose containing MS media. In case of encapsulation vitrification preconditioned axillary buds were encapsulated using 2.5 per cent sodium alginate and 100 mM $Ca\ Cl_2$, prior to preculture.

The precultured materials were further subjected to loading treatment for 20 minutes in 2 M glycerol and 0.4 M sucrose in MS medium, followed by exposure to different periods in vitrification solutions *viz.*, PVS2 and PVS3. After the specified periods of exposure, they were frozen in liquid nitrogen for 2 hours. They were then rewarmed/ thawed and placed in recovery medium to study the survival, regeneration and plantlet conversion potential.

Among the sixteen treatments tried in simple vitrification, survival could be obtained only in four treatments *viz.*, exposure to PVS2 for 30 and 60 min and exposure to PVS3 for 30 and 60 min. The survival was above 50 per cent, in 30 min exposure for both PVS2 and PVS3 treatments. Only five treatments showed survival, among sixteen treatments tried in encapsulation vitrification. The five treatments were 30, 60 and 90 min exposure to PVS2 vitrification solution and 30 and 60 min exposure to PVS3 vitrification solution. Of the different treatments, in encapsulation vitrification, the survival above 50 per cent was observed in the treatments *viz.*, 30 and 60 min exposure to PVS2 vitrification solution and 30 min exposure to PVS3 vitrification solution.

Among the five vitrification treatments which gave more than 50 per cent survival after 2 h of cryostorge, no significant variation was observed in survival and regeneration per cent among different storage periods *viz.,* 2 h, one day, one week, one month and two months.

After crystorage, the maximum survival as well as regeneration was obtained in encapsulation vitrification treatment with 30 min exposure to PVS2 vitrification solution. The same treatment recorded maximum plantlet conversion potentialwith earliest days to bud initiation (28 days), maximum shoots per explant (2.56) and maximum shoot length (2.88 cm).

The genetic stability of plantlets recovered and regenerated from *in vitro* storage were assessed by using RAPD and ISSR markers. Five RAPD and four ISSR markers were used for the genetic stability assessment. Distinguishable band pattern could be obtained only with two ISSR primers. The similar banding profile of the plantlets derived from different *in vitro* storage treatments indicated that no polymorphism existed among the plantlets. The plantlets recovered from short-term conservation and long term conservation (by cryopreservation) were found to be genetically stable and true to type.

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IN VITRO CONSERVATION OF CHETHIKODUVELI (*Plumbago rosea* L.) USING ENCAPSULATION AND VITRIFICATION TECHNIQUES

by

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ABSTRACT

The present study entitled "*In vitro* conservation of chethikoduveli (*Plumbago rosea L.*) using encapsulation and vitrification techniques" was carried out in the Department of Plant Biotechnology, College of Agriculture, Vellayani. The objective of the study was to standardise the protocol for short term and long term conservation of axillary buds using encapsulation and vitrification techniques and to assess the genetic fidelity of recovered and regenerated plantlets using molecular markers.

The study was carried out in three phases *viz.*, enhancement of multiplication rate, short term conservation by encapsulation technique and long term conservation using vitrification technique.

Axillary buds from *in vitro* raised cultures were used as the explants in all the experiments. Different media (Gamborg, White, SH and MS) and different levels of additives (chitosan, adenine sulphate and thidiazuron) were tried for the enhancement of multiplication. Standardized medium, MS supplemented with BA 1.5mg L^{-1} and IAA 1mg L^{-1}, for shoot proliferation was used as the control treatment in the study.Among the different media tried, MS medium was observed to give the best response with respect to shoot multiplication (3.75 shoots/explant).

Among the different levels of each additive tested, best response was obtained with chitosan 15 mg L^{-1}, adenine sulphate 40 mg L^{-1} and thidiazuron 4 mg L^{-1} and 5mg L^{-1} in MS supplemented with BA 1.5 mg L^{-1} and IAA 1 mg L^{-1}. Thidiazuron at both levels gave maximum shoot proliferation (11.5 shoots/explant). But shoots obtained were stunted, and vitrified with callusing at the base. Adenine sulphate 40mg L^{-1}, gave significantly better response with 4.25 shoots/explant. Though chitosan 15mg L^{-1}gave maximum shoots/explants (3.67) among different levels of chitosan tried, it was found to be on par with the control. Hence, MS medium supplemented with BA 1.5mg L^{-1}, IAA 1mg L^{-1} and adenine sulphate 40 mg L^{-1} was selected as the medium for further conservation studies.

In short term conservation studies, encapsulated axillary buds were used as explants. Effect of different additives (sucrose 10 per cent, sucrose 15 per cent, mannitol 10 per cent, mannitol 15 per cent) in encapsulation matrix (sodium alginate 2.5 per cent and calcium chloride 100 mM), different storage media (liquid MS, sterile distilled water) on shoot regeneration and proliferation were tried at two temperature regimes of 4°C and 25°C.

Encapsulated axillary buds could be stored upto 30 days in all combinations, beyond which the regeneration percentage deteriorated. Maximum regeneration of 83.33 and 79.17 per cent was obtained with mannitol 10 per cent in encapsulation matrix and liquid MS as storage medium at 25°C and 4°C respectively after 30 days of storage. In this treatment at 25°C, the days to bud initiation was the least (11.60 days); Shoots/explant (6.4), shoot length (1.87cm) and nodes per shoot (2.48) was the highest.

Two cryopreservation techniques of simple vitrification and encapsulation vitrification were attempted for long term conservation studies. In simple vitrification, preconditioned (0.5 M sucrose for 7 days), precultured (0.5 M sucrose for 3days) axillary buds were exposed to vitrification solutions, PVS2 (glycerol 30 per cent, ethylene glycol 15 per cent and DMSO 15 per cent in MS with sucrose 0.4 M, pH 5.7) and PVS3 (glycerol 50 per cent and sucrose 50 per cent in MS, pH 5.7) for 0 to 210 minutes at 30 minutes interval. The best response in terms of survival (62.22 per cent) and regeneration (47.78 per cent) was obtained in PVS2 exposure for 30 min after 2 h of cryopreservation *ie.*, storage in liquid nitrogen. In encapsulation vitrification, preconditioned, encapsulated, precultured axillary buds were given exposure to vitrification solutions as above, maximum survival (78.89 per cent) and regeneration (70.00 per cent) was also obtained in the same treatment. The survival and regeneration percentage was found to be on par with different periods of cryopreservation. Among the cryopreservation treatments, encapsulation vitrification was found to be the best.

The cryoregenerated explants were then inoculated on to the best proliferation medium to study the regeneration and multiplication. Encapsulation vitrification in PVS2 for 30 minutes gave best response with respect to bud initiation (28.89 days), shoots/ explant (2.56) and shoot length (2.88 cm).

The genetic fidelity of the plantlets subjected to short and long term conservation was assessed using 5 RAPD and 4 ISSR markers. Among these, two ISSR primers produced 5-7 bands. The banding patterns of the conservation regenerated plantlets and the control were compared. The profiles generated did not show any polymorphism and was identical to those of control, which indicated the genetic stability.

In the study, maximum multiplication rate (4.25 shoots/explant) was obtained in MS media supplemented with BA 1.5mg L^{-1}, IAA 1mg L^{-1} and adenine sulphate 40mg L^{-1}. With respect to short term conservation, the encapsulated axillary buds with the additive in encapsulation matrix (mannitol 10 per cent), storage media (liquid MS) and storage temperature (25°C) gave maximum regeneration (83.33 per cent). Encapsulated axillary buds could be stored upto 30 days in all combinations, beyond which the regeneration percentage deteriorated. In long term conservation the maximum survival (78.89 per cent) and regeneration (70.00 per cent) was obtained in encapsulation vitrification. The genetic stability was maintained in plantlets regenerated from short and long term conservation.

Appendices

APPENDIX I

Chemical composition of various nutrient media

Composition (mg L^{-1})	Nutrient media			
	MS	**B5**	**SH**	**White**
Major nutrients				
NH_4NO_3	1650	-	-	-
KNO_3	1900	2500	2500	80
$CaCl_2.2H_2O$	440	150	200	-
$MgSO_4.7H_2O$	370	250	400	720
KH_3PO_4	170	-	-	-
$(NH_4)_2SO_4$	-	134	-	-
$NaH_2PO_4.H_2O$	-	150	-	16.50
$Ca(NO_3)_2.4H_2O$	-	241	-	300
KCl	-	-	-	65
Na_2SO_4	-	-	-	200
$NH_4H_2PO_4$	-	-	300	-
Minor nutrients				
KI	0.83	0.75	1.0	0.75
H_3BO_3	6.2	3.0	5.0	1.5
$MnSO_4.4H_2O$	22.3	-	-	7.0
$MnSO_4.H_2O$	-	1.12	10.0	-
$ZnSO_4.7H_2O$	8.6	2.0	1.0	3.2
$Na_2MoO_4.2H_2O$	0.25	0.25	0.1	-
$CuSO_4.5H_2O$	0.025	0.025	0.2	-
$CoCl_2.6H_2O$	0.025	0.025	0.1	200
$FeSO_4.7H_2O$	27.8	27.8	27.8	-
$Fe_2(SO_4)_3$	-	-	-	2.5
Na_2EDTA	37.3	37.3	20	-
$FePO_4.4H_2O$	-	-	-	7.0
Organic supplements				
Glycine	2	-	-	3
Myo-inositol	100	100	1000	-
Thiamine HCl	0.1	10.0	5.0	-
Pyridoxine HCl	0.5	1.0	0.5	-
Nicotinic acid	0.5	1.0	5.0	-

APPENDIX II

TE buffer

Tris- HCL (pH 8.0)	10 mM
EDTA	1 mM

APPENDIX III

TBE (5X) FOR 1 litre 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	(0.01M)