CRYOCONSERVATION OF KOOVALAM (*Aegle marmelos* L. Corr.) BY ENCAPSULATION-DEHYDRATION TECHNIQUE

by

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(2015-11-072)

THESIS

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2018

DECLARATION

I, hereby declare that this thesis entitled **"Cryoconservation of Koovalam (*Aegle marmelos* L. Corr.) by encapsulation-dehydration technique"** 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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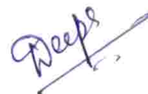

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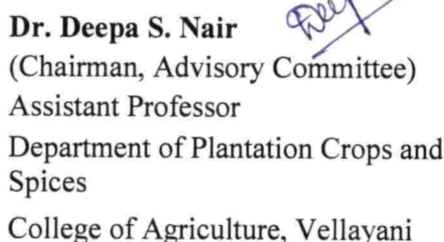

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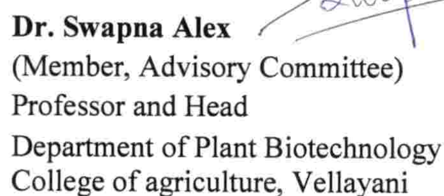

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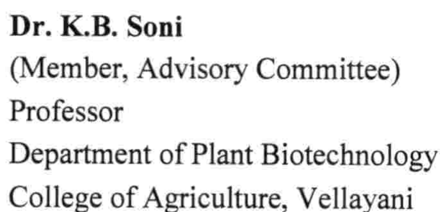

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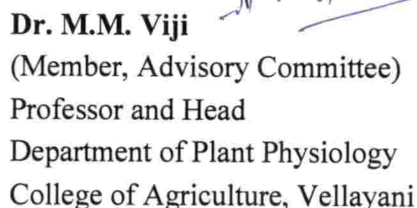

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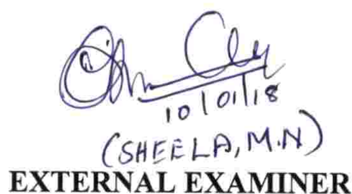

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CONTENTS

Sl. No.	CHAPTER	Page No.
1	INTRODUCTION	1
2	REVIEW OF LITERATURE	5
3	MATERIALS AND METHODS	26
4	RESULTS	42
5	DISCUSSION	67
6	SUMMARY	82
7	REFERENCES	86
8	ABSTRACT	114
9	APPENDICES	113

LIST OF TABLES

Table No.	Title	Page No.
1.	Auxins and cytokinins on multiplication of axillary buds	30
2.	Different levels of chitosan tried for shoot multiplication	30
3.	Different levels of adenine sulphate tried for shoot multiplication	31
4.	Different levels of thidiazuron tried for shoot multiplication	31
5.	Treatments tried for preconditioning	33
6.	Treatments tried for encapsulation	34
7.	Treatments tried for pre-culture	36
8.	Sequence of ISSR primers used for DNA amplification by PCR to evaluate the genetic stability of plantlets regenerated from cryopreserved materials of *Aegle marmelos*	41
9.	Effect of cytokinins and auxins on enhanced release of axillary buds from nodal explants	43
10.	Effect of chitosan on shoot proliferation of *A.marmelos*	45
11.	Effect of adenine sulphate on shoot proliferation of *A.marmelos*	47

12.	Effect of thidiazuron on shoot proliferation of *A.marmelos*	49
13.	Effect of different additives *viz.,* Chitosan (CH), Adenine sulphate (AdS) and Thidiazuron (TDZ) on shoot proliferation of *Aegle marmelos*	51
14.	.Effect of preconditioning duration and sucrose concentration on plant regeneration	53
15.	Effect of different encapsulation matrices on bead formation and survival	55
16.	Effect of different encapsulation matrices on plant regeneration	57
17.	Effect of different pre-culture treatment on plant regeneration	59
18.	Effect of dehydration on survival and plant regeneration before (-LN) and after (+LN) cryopreservation	61
19.	Effect of desiccation on plant regeneration before (-LN) and after (+LN) cryopreservation	63
20.	Survival and regeneration of encapsulated axillary buds after different periods of cryostorage	64
21	ISSR markers screened for the assessment of genetic fidelity of plantlets regenerated from cryostorage	66

LIST OF FIGURES

Fig. No.	Title	Pages Between
1.	Effect of cytokinins and auxins on enhanced release of axillary buds from nodal explants	43-44
2.	Effect of chitosan on shoot proliferation of *A. marmelos*	45-46
3.	Effect of adenine sulphate on shoot proliferation of *A.marmelos*	47-48
4.	Effect of thidiazuron on shoot proliferation of *A.marmelos*	49-50
5.	Effect of preconditioning duration and sucrose concentration on plant regeneration	53-54
6.	Effect of different encapsulation matrices on plant regeneration	57-58
7.	Effect of different pre-culture treatment on plant regeneration	59-60
8.	Effect of dehydration on survival and plant regeneration before (-LN) and after (+LN) cryopreservation	61-62

LIST OF PLATES

Fig. No.	Title	Pages Between
1.	Effect of cytokinins and auxins on enhanced release of axillary buds from nodal explants	43-44
2.	Effect of chitosan on shoot proliferation of *A.marmelos*	45-46
3.	Effect of adenine sulphate on shoot proliferation of *A. marmelos*	47-48
4.	Effect of thidiazuron on shoot proliferation of *A. marmelos*	49-50
5.	Effect of preconditioning duration and sucrose concentration on plant regeneration	53-54
6.	Plant regeneration from encapsulated beads	57-58
7.	Effect of different pre-culture treatment on plant regeneration	59-60
8.	Plant regeneration from dehydrated beads before cryostorage	61-62
9.	Steps in cryopreservation of *A. marmelos* using encapsulation-dehydration technique	64-65
10.	Plant regeneration from dehydrated beads after cryostorage	64-65
11.	Comparison of ISSR banding profile between plants recovered from LN and non cryopreserved control plantlets	66-67

LIST OF APPENDICES

Sl. No:	Title	Appendix No:
1	CTAB Extraction buffer	I
2	TE buffer	II
3	TBE buffer	III

XI.

LIST OF ABBREVIATIONS

2, 4-D	2, 4-Dichlorophenoxyacetic acid
2iP	Isopentenyladenine
A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 280 nm wavelength
AdS	Adenine sulphate
BA	N^6- benzyl adenine
$CaCl_2$	Calcium chloride
CH	Chitosan
CD (0.05)	Critical difference at 5% level
cm	centimetre
CTAB	Cetyl Trimethyl Ammonium Bromide
DMSO	Dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotides
EDTA	Ethylenediaminotetraacetic acid
et al.	and others
Fig.	Figure
g	gram
GA	Gibberellic acid
h	hour
HCL	Hydrochloric acid
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
ISSR	Inter-Simple Sequence Repeat
Kn	Kinetin (6-furfurylaminopurine)
LN	Liquid nitrogen
M	molar
mg	milligram
ml	milliliter
min	minute
mM	millimolar
MS	Murashige and Skoog, 1962
MC	Moisture content
NaCl	Sodium chloride
NaOH	Sodium hydroxide
µM	micromolar

O. D.	Optical Density
pH	potential of hydrogen
PCR	Polymerase chain reaction
PGR	Plant growth regulator
PLBs	Protocorm like bodies
PVP	Polyvinylpyrrollidone
rpm	revolutions per minute
s	second
SA	Sodium alginate
sp.	species
TE	Tris-EDTA buffer
Tris HCl	Tris (hydroxymethyl) aminomethanehydrochloride
TDZ	Thidiazuron
V	Volt
viz.	namely
X	times

LIST OF SYMBOLS

°C	degree celsius
%	per cent
±	plus or minus

Introduction

1. INTRODUCTION

Medicinal plants are the most important source of life saving drugs for the majority of the world's population (Khan *et al.,* 2009). These plants provide biologically active molecules and lead structures for the development of modified derivatives with enhanced activity and reduced toxicity. The World Health Organization (WHO) reported that 80% of people in the developing world use medicinal plants for their primary health care (Vines, 2004). Currently, the huge demand of the medicinal plants is mostly met from the wild. Their biodiversity is being exploited without adequate replacement by the way of organised cultivation. The genetic diversity of medicinal plants is under continuous threat due to over exploitation, unorganized harvesting practices, loss of growth habitat and unmonitored trade of medicinal plants.

Aegle marmelos, commonly known as bael, is widely used in the indigenous system of Indian medicine, Ayurveda. Bael leaves are useful in treating jaundice, conjunctivitis, typhoid etc. (Chakraborty *et al.,* 2012). The leaf and bark extract exhibits antioxidant and antidiabetic activities (Kumar *et al.*, 2015). The roots of the plant are widely used in many ayurvedic formulations and is one of the top ten forest sourced medicinal plants traded from India (Ved and Goraya, 2008; Bhattacharya *et al.*, 2014). The requirement of bael root, an ingredient of *Dasamoola*, is nearly 400 mt per year in Kerala (Swaminathan, 2014). The demand is mostly met from the wild (Ved and Goraya, 2008). Exploitation by pharmaceutical industries, indiscriminate or unregulated harvesting practices with no concern for the sustainability of the resource and lack of organised cultivation, has enlisted the plant as RET (rare, endangered and threatened) species (Venudevan and Srimathi, 2014; Kumar *et al.*, 2015). This plant is redlisted by the Kerala State Medicinal Plant Board.

Conventional *in situ* or *ex situ* conservation of the plant is expensive in terms of land and labour. Moreover, they are liable to risks due to biotic and abiotic factors. Conventional seed conservation is also restricted in this species due to its intermediate seed storage characteristics. *In vitro* conservation has been recognised as an alternative method for plant conservation that eliminates the obstacles of field conservation.

Cryopreservation is a safe, cost-effective *in vitro* strategy for long-term conservation of biological materials (Reed, 2008a). It provides for long-term conservation of plant germplasm without genetic alterations, at ultra-low temperature (-196°C) of liquid nitrogen. It also offers an option for long-term base as well as backup active collections. Low maintenance cost, limited storage space and requirement of less number of replicates makes cryopreservation a viable conservation technology (Shibli and Al-Juboory, 2000). The encapsulation-dehydration procedure of cryopreservation is based on the technology developed for the production of artificial seeds and involves successive osmotic and evaporation-dehydration of plant cells (Gupta, 2014). The water content has to be brought down to an optimum level for successful cryopreservation by encapsulation-dehydration. Desiccated beads on freezing enter into an amorphous glassy state rather than ice crystalline state, which is damaging to the tissues (Dumet *et al.,* 2000). This technique has been applied for cryopreservation of many plant species including woody plant species (Panis and Lambardi, 2005; Shibli *et al.,* 2006)

The major concern with regard to this technique is the genetic stability of conserved material as the plant tissues are exposed to a series of stress inducing steps to enhance its freezing tolerance during cryopreservation. Hence genetic evaluation of the recovered plants is to be strictly followed. PCR based molecular markers have been used in the genetic fidelity assessment of cryostorage derived plantlets (Nair and Reghunath, 2009; Prakash, 2014; Coelho *et al.,* 2014).

Hence, it is pertinent to evolve protocols for *in vitro* conservation and regeneration of *A. marmelos*, a valuable, redlisted and commercially exploited woody medicinal plant. The present study 'Cryoconservation of koovalam (*Aegle marmelos* L. Corr.) by encapsulation-dehydration technique' was undertaken with the following objectives.

1. Standardization of cryopreservation protocol using encapsulation and dehydration technique in *Aegle marmelos*.

2. The genetic fidelity assessment of plantlets recovered and regenerated from cryostorage using Inter Simple Sequence Repeats (ISSR) marker.

Review of Literature

2. REVIEW OF LITERATURE

Aegle marmelos commonly known as bael, is widely used in the indigenous system of Indian medicine, Ayurveda. Marmelosin, skimmianine and umbelliferone are the therapeutically active principles of bael plant (Dhankhar *et al*., 2011). Bael leaves are useful in treating jaundice, conjunctivitis, typhoid etc. (Chakraborty *et al.,* 2012). The leaf and bark extract exhibits antioxidant and antidiabetic activities (Kumar *et al*., 2015). It also yields yellow dye from the fruit rind (Murugan *et al*., 2015). The roots of the plant are widely used in many ayurvedic formulations and is one of the top ten forest sourced medicinal plants traded from India (Ved and Goraya, 2008; Bhattacharya *et al*., 2014).

According to Swaminathan (2014), the average requirement of bael roots, an ingredient of *Dasamoola*, is nearly 400 mt per year. This demand is mostly met from the wild (Ved and Goraya, 2008). Exploitation to meet the demand of pharmaceutical industry, indiscriminate or unregulated harvesting practices with no concern for the sustainability of the resource and lack of organised cultivation, have enlisted the plant as RET (rare, endangered and threatened) species (Venudevan and Srimathi, 2014; Kumar *et al*., 2015). Also, this species is red listed by the State Medicinal Plant Board of Kerala.

In vitro technology has to be resorted for the conservation of *A. marmelos* rather than conventional seed conservation due to its intermediate seed storage characteristics (Malik *et al., 2*012). *In situ* or *ex situ* field conservation of the plant is expensive in terms of land and labour and is liable to risks due to biotic and abiotic factors. Moreover, conventional methods of propagation *via* seed and root suckers are very slow. *In vitro* technology offer viable prospects for mass multiplication and germplasm conservation of this rare and endangered medicinal plant, eliminating the obstacles of field conservation.

The present study 'Cryoconservation of koovalam (*Aegle marmelos* L. Corr.) by encapsulation-dehydration technique' was laid out with the objective of standardization of cryopreservation protocol using encapsulation-dehydration technique for long term conservation of *A. marmelos.*

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

2.1 ENHANCEMENT OF MULTIPLICATION RATE

2.1.1 Effect of different media and additives on multiplication rate

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

2.1.1.1 Enhanced Release of Axillary Buds

Micropropagation by axillary bud proliferation has proved to be the most reliable method for large scale production of many crop plants. The plants regenerated *via* axillary shoot proliferation would be true to type to the mother plant as they develop from the pre-existing meristem (Nair and Reghunath, 2009).

Micropropagation of medicinal plants has been achieved through rapid proliferation of shoot tips and axillary buds in culture. According to Rout *et al.* (2000) numerous factors are reported to influence the success of *in vitro* propagation of different medicinal plants *viz.,* age of donar plant, explant type, culture medium, growth regulators and culture conditions.

2.1.1.2 Explant

In vitro plant regeneration has been reported in *A. marmelos* from different explants, *i.e.,* from root segments (Hossain *et al.*, 1994), single-node segments (Ajithkumar and Seeni, 1998) and cotyledonary explants (Nayak *et al.,* 2007; Hazeena and Sulekha, 2008). Ajithkumar and Seeni (1998) observed that nodal stem segments of *A. marmelos,* were found to be the best explants due to the presence of protected axillary buds, which could withstand surface sterilization procedures. Raghu *et al.* (2007) reported an efficient and rapid *in vitro* clonal propagation by enhanced axillary shoot proliferation from mature single node of *A. marmelos*. In this study, the explants exhibited marked seasonal variation in their response, under *in vitro* conditions.

2.1.1.3 Culture Media

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).

Ajithkumar and Seeni (1998) reported rapid clonal multiplication of *A. marmelos via* axillary bud proliferation using young single-node segments in Murashige and Skoog (MS) nutrient medium. Sandhiya *et al.* (2008) used MS medium supplemented with BA 0.5 mg L^{-1} and IAA 0.1 mg L^{-1} for micropropagation of *A. marmelos* using nodal explants. Hazeena and Sulekha (2008) reported that callus induction and shoot regeneration from cotyledon explants of *A. marmelos* using MS medium supplemented with BA 2.2 μM and 2, 4-D 2.26 μM.

Soni *et al.* (2012) stated that MS medium was found to be the best basal medium than B5 medium for the establishment of nodal explants in *Adhatoda vasica.* 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.

2.1.1.4 Culture Conditions

Ajithkumar and Seeni (1998) obtained good shoot proliferation when *A. marmelos* cultures were incubated at 24±2°C, with a relative humidity of 50-60 per cent and a 12 h photoperiod, at a photon flux density of 50-60 mE m^{-2} s^{-1} using cool white fluorescent tubes.

Das *et al.* (2008) reported multiple shoot proliferation in *A. marmelos* cultures when incubated at 25±2°C with a photoperiod of 16 h at 3000 lux light intensity provided by cool white fluorescent tubes.

According to Hazeena and Sulekha (2008), *A. marmelos* cultures were maintained at 24± 2°C with 60 per cent relative humidity under fluorescent lights of 2000 lux intensity, with 16/8 h light/ dark cycles.

In vitro grown *A. marmelos* cultures were maintained under 14 h light and 10 h dark cycle, at 23 ± 2°C with 55 ± 5 per cent relative humidity and 1000 Lux intensity light using white cool fluorescent lights to induce morphogenesis (Raj and Basavaraju, 2012).

2.1.1.5 Plant Growth Regulators

Plant growth regulators function as chemical messengers for intercellular communication. They work together coordinating the growth and development of cells. A plant growth regulator is an organic compound, either natural or synthetic, that modifies or control one or more specific physiological processes within a plant.

Ajithkumar and Seeni (1998) conducted *in vitro* propagation studies in *A. marmelos.* The studies substantiated the effect of auxin-cytokinin combination on shoot proliferation. The synergistic combination of BA 2.5 mg L^{-1} and IAA 1.0 mg L^{-1} induced best shoot proliferation.

Yadav and Singh (2011) reported multiple shoot regeneration from nodal segments of *A. marmelos*, cultured on MS medium supplemented with various concentrations of auxins and cytokinins individually and in various combinations. BA was found to be more effective than kinetin for shoot multiplication. Nodal explants responded most favourably at BA 2.0 mg L^{-1} producing maximum number of shoots (8.0).

Nayak *et al.* (2007) investigated the effect of BA on shoot proliferation in *A. marmelos* and found optimal response in MS medium supplemented with BA 6.6 μM.

2.1.1.6 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. 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.

Barka *et al.* (2004) reported that chitosan stimulated plant growth *in vitro* in grape vine, *Vitis vinifera* L. Chitosan improved the quality of potato micro plant *in vitro* and increased the yield and seed quality of mini tubers (Kowalski *et al.*, 2006).

The effect of chitosan as growth stimulator was studied in *Grammatophyllum speciosum* Protocorm Like Bodies (PLBs) by providing its

varying concentrations in half strength MS liquid and agar media containing 2 per cent (w/v) sucrose. The liquid medium supplemented with chitosan 15 mg L^{-1} 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 *Cymbidium dayanum*, the highest PLBs induction (93 per cent) and shoots formation (79 per cent) was observed.

Chitosan enhanced growth and development by some signalling pathway to auxin biosynthesis *via* a tryptophan-independent pathway (Uthairatanakij *et al.,* 2007). Also, chitosan as a growth promoter and elicitor of plant defence mechanisms could alleviate stress caused by *in vitro* conditions and acclimatization (Zakaria *et al.,* 2009).

Adenine in the form of adenine sulphate (AdS) stimulates cell growth and enhances shoot formation. The shoot enhancing role of AdS has been demonstrated in various plants (Husain *et al.*, 2008). It provides an available source of nitrogen to the cell initially, and can be assimilated 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).

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

Xiaoping *et al.* (2006) stated that by supplementing BA 2.0 mg L^{-1}, NAA 0.75 mg L^{-1}, adenine sulphate 50 mg L^{-1} and coconut milk 10 per cent in MS basal medium under subdued light at 25±2°C, highest frequency of shoot regeneration (30 shoots) was achieved in hypocotyl segments of *Plumbago zeylanica.*

Rout and Nanda (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 Siwach *et al.* (2012), MS medium supplemented with BA 2.5 mg L^{-1} and adenine sulphate 50 mg L^{-1} could enhance shoot multiplication rate (4.88 shoots per explant) in *Citrus reticulata.*

Imran *et al.* (2012) reported that nodal explant of *Carissa carandas* induced large number of shoots in MS medium supplemented with BAP 1.5 mg L^{-1} + Kn 1.0 mg L^{-1} + TDZ 1.0 mg L^{-1} + AdS 15 mg L^{-1}. Adenine sulphate played an important role for improving the frequency of multiple shoot induction when compared to culture media without adenine sulphate.

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 Kn 2.15 mg L^{-1}, NAA 0.5 mg L^{-1} and adenine sulphate 40 mg L^{-1}.

Thidiazuron (TDZ) is most active cytokinin-like substances for woody plant tissue culture. It facilitates efficient micropropagation of many recalcitrant woody species (Murthy *et al.,* 1998).

Sudarsono and Goldy (1991) reported that in *Vitis rotundifolia,* TDZ (2.3 to 4.5 μM) alone or in combination with BA (1.0 to 5.0 μM) or Kn (1.0 or 5.0 μM) in MS medium was found to be effective in the establishment of axillary buds and also in further shoot proliferation.

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

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

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

Fathima and Anis (2011) established *in vitro* culture using nodal segments of *Withania somnifera* using TDZ. MS media supplemented with TDZ 0.5 μ M gave effective bud break and gave 98 per cent regeneration with 23.8 shoots after 4 weeks of culture.

Pavendan and Rajasekaran (2011) observed that the combined effect of BAP and TDZ on shoot regeneration was found to be the highest and recorded 93 per cent of explants produced shoots and maximum number of shoots per explant was recorded as 21.7 with 5.8 cm shoot length.

Mallaya and Ravishankar (2013) could obtain maximum number of shoot buds in MS media supplemented with TDZ 0.5 mg L^{-1}, when hypocotyls excised from seedlings of *Solanum melongena* L. were used as explant.

A combination of BAP 8.87 μM and TDZ 4.54 μM in WPM (Woody Plant Medium) gave good proliferation in *Garcinia indica.* Shoot elongation was

observed with repeated subculture. After third subculture, 3-4 shoots with average shoot length of 5.92 cm were obtained (Deodhar *et al.,* 2014).

Soni and Kaur (2014) reported that the best response with respect to *in vitro* propagation was obtained in *Viola pilosa,* when cultured in MS media supplemented with BA 0.5 mg L^{-1}, TDZ 0.5 mg L^{-1} and GA_3 0.5 mg L^{-1}.

2.2 *IN VITRO* CONSERVATION

Ex situ conservation *via in vitro* technology involves the maintenance of explants in a pathogen free environment for short to medium or long term period. Conventional field conservation of the plant demands high cost in terms of land and labour. Moreover, the germplasm is prone to risk from biotic and abiotic factors. *In vitro* conservation of plant genetic resources would serve as a complementary approach to the conventional conservation strategies. It helps to overcome the obstacles associated with conventional strategies. In *in vitro* systems, plants are withdrawn into a protected environment, where they are less likely to be affected by pathogens or predators. *In vitro* stored material serves as an entity for international exchange of germplasm, as they are maintained under aseptic conditions (Shibli *et al.,* 2006).

In vitro technology for germplasm conservation could ensure the survival of endangered plant species, and rapid mass propagation for large scale revegetation (Yadav *et al*., 2012). *In vitro* propagation technology is an integral component in a tree improvement or conservation program, especially for high value tropical tree species. This would complement seed banking and *ex situ* measures for long term conservation and clonal propagation of germplasm (Pence, 2010; Sarasan, 2010).

An efficient regeneration system is a prerequisite for any *in vitro* conservation program. *In vitro* slow/minimal growth techniques offer medium-term storage option, avoiding risk of loss of germplasm in field gene bank due to

pest and diseases or natural disasters. This technique is commonly adopted for the conservation of vegetatively propagated species, non-orthodox seeded species and wild species which produce little or no seeds (Ogbu *et al.,* 2010).

In general, active or working collections are held in short/medium-term storage and base collections are held in long-term storage as in cryopreservation (Khanna and Singh, 1998).

2.2.1 Cryopreservation

Cryopreservation is the only technique currently available to ensure the safe and cost-efficient long-term conservation of different types of plant germplasm (Engelmann, 2004). In this technique, cell divisions and metabolic processes are restrained at ultra-low temperature of liquid nitrogen (LN) (-196°C). Plant material, thus would remain unchanged without loss of characteristic properties for unlimited time (Pawłowska, 2008). Cryopreservation is hence considered as the only viable method for safe long-term storage of plant genetic resources (Kaviani, 2010), without causing any alterations in genetic stability.

Cryopreservation offers for the long term conservation of genetic resources of vegetatively propagated plant species and nonorthodox seed species. Cryopreservation involves various steps to minimize desiccation and freezing damage to ensure high propagule recovery. This is based on either dehydration or vitrification of internal solutions (Gonzalez-Benito *et al.*, 2004).

Cryopreservation of organized structures had significantly progressed, especially for species of tropical origin. Cryogenic procedures such as simple freezing, vitrification, encapsulation-dehydration and encapsulation-vitrification had been developed for conserving a number of plant species. Cryopreservation is considered as the most promising choice for the conservation of germplasm owing to safety, repeatability and possibility for long term storage (Reed, 2008; Hazubska *et al.,* 2010). Cryopreservation would involve multiple steps to be

successful with tree species. In most cases, terminal or axillary shoot tips/ meristems obtained from *in vitro* grown shoots are the plant material used for cryopreservation (Gonzalez-Arnao *et al*., 2008). Cryopreservation requires only a minimum of space and low level of maintenance. Hence, cryopreservation protocols had been developed for a large number of plant species (Li and Pritchard 2009; Pritchard *et al.,* 2014) using organised structures.

Many plants were successfully cryopreserved by encapsulation dehydration namely *Melia azadarah* (Scocchi *et al*., 2004), *Vitis vinifera* L. (Miaja *et al*., 2004), *Fragaria x ananassa* (Clavero-Ramirez *et al*., 2005) and *Oncidium bifolium* (Flachsland *et al*., 2006). Mandal and Sharma (2007) successfully cryopreserved *in vitro* shoot tips of *Dioscorea deltoidea* using encapsulation-dehydration technique with subsequent high frequency plant regeneration. The cryopreserved shoot tips maintained their viability and an unaltered level of regeneration capability after up to one year of storage in LN. Kaviani (2010) observed that cryopreservation of germplasm in LN using dehydration and encapsulation technique is the perfect method for embryonic axes of *Melia azedarach, Camellia sinensis* and seeds of *Lilium ledebourii.* Shatnawi (2011) reported successful cryopreservation using *in vitro* shoot tips of *Capparis spiunosa* and Cordeiro *et al.* (2014) using nodal explants of *Mandevilla moricandiana* by encapsulation-dehydration technique.

2.2.1.1.1 Preconditioning

Preconditioning prepares the explants to withstand freezing. It involves manipulations of the culture conditions and culture medium such as subjecting the explants to high sucrose concentration for specific period (Grospietch *et al*., 1999) or culturing at low temperature (Sakai, 2000; Wu *et al*., 2001).

Dumet *et al*. (1993) observed marked improvement in recovery could be obtained by 7 day pre growth period on 0.75 M sucrose when embryos of oil palm (*Elaeis guinensis*) were subjected to cryopreservation.

Kiwi, strawberry, chrysanthemum and wasabi shoot tips were cultured from one to several days on agar based medium with a high sucrose concentration (0.1- 1 M) (Sakai, 2000; Clavero-Ramirez *et al.*, 2005) for cryopreservation and subsequent recovery.

Freshly excised embryonic axes of *Citrus madurensis,* were initially cultured on solidified MS medium supplemented with BA 0.1 mg L^{-1}, NAA 0.1 mg L^{-1} and gibberellic acid (GA_3) 0.1 mg L^{-1} with 0.1 M sucrose, for three days followed by one day culture on same medium with 0.3 M sucrose and 0.5 M glycerol prior to cryopreservation (Cho *et al.*, 2002). Black currant shoot tips were cold acclimatized for 2 weeks or cultured for 7 days on 0.75 M sucrose solid medium before encapsulation (Reed *et al.*, 2005).

Nair and Reghunath (2007) reported cryopreservation of *Clitoria ternatea* somatic embryos after preconditioning them on half-strength MS medium supplemented with 0.5 M sucrose for three weeks.

2.2.1.1.2 Encapsulation

The encapsulation-dehydration procedure is based on the technology developed for the production of artificial seeds. Artificial seeds are encapsulated plant micropropagules, used for sowing as seeds, which have the potential for plant conversion both under *in vitro* and *in vivo* conditions and maintain the conversion potential even after storage (Capuano *et al.*, 1998). They are prepared by suspending the explant in calcium free liquid basal medium with usually 3 per cent (w/v) sodium alginate, 0.1 M calcium chloride (Gonzalez-Arnao and Engelmann, 2006).

Encapsulation of explants in alginate beads provides enhanced protection from mechanical damage and enables easy handling. Encapsulation would allow them to withstand drastic treatments at various steps in cryopreservation, including pre-culture with high sucrose concentration and desiccation to low moisture contents which would be highly damaging or lethal to non-encapsulated

samples. The nutritive nature of the beads will promote survival and regrowth of cryopreserved explants (Paulet *et al.,* 1993; Panis *et al.,* 2005; Engelmann *et al.,* 2008).

In cryopreservation, complementary to exposure to cryoprotectants, encapsulation within alginate beads, before dehydration was shown to be beneficial, by increasing considerably the resistance of plant tissues to desiccation and freezing (Dereuddre *et al*., 1990; Bernard *et al*., 2002).

Encapsulation-dehydration technique being simple and inexpensive, is applied for cryopreservation of many plant species (Shibli *et al*., 2006; Shatnawi, 2011).

Successful cryopreservation of somatic embryos of *Clitoria ternatea* and axillary meristems of *Indigofera tinctoria* using encapsulation-dehydration technique were reported by Nair and Reghunath (2007; 2009).

Protocorm-like bodies (PLBs) of *Brassidium* Shooting Star, a commercial ornamental orchid hybrid, were cryopreserved by an encapsulation-dehydration technique. The highest survival of PLBs was recorded when they were encapsulated using sodium alginate 3.5 per cent with $CaCl_2$ 0.1 mM (Yin *et al.,* 2011).

Hegazi (2011) stated that the best gel composition of encapsulated shoot tips in *Capparis orientalis* was achieved using sodium alginate 3 per cent and $CaCl_2$ 100 mM. Padro *et al.* (2012) reported the use of 3 per cent sodium alginate solution as the best concentration for the formation of beads by complexing with $CaCl_2$ 100mM for cryopreserving *Morus alba.*

Protocom Like Bodies (PLBs) of *Dendrobium chrysanthum* were osmoprotected with a mixture of 0.4 M sucrose and 2 M glycerol, incorporated in the encapsulation matrix comprising sodium alginate 3 per cent (w/v) and calcium

chloride 0.1 M (Mohanty *et al.*, 2013). Hongthongkham and Bunnag (2014) reported that cryopreservation of leaf segment derived PLBs of *Aries odorata* was successful using the encapsulation-dehydration method. The maximum survival percentage of cryopreserved PLBs was achieved with 2 per cent sodium alginate complexed with 2 M glycerol, 0.4 M sucrose and 100mM $CaCl_2$.

2.2.1.1.3 Pre-culture and cryoprotection

Pre-culture of encapsulated shoot apices is a step to induce desiccation tolerance in them during the process of encapsulation-dehydration. Pre-culture is relatively simple, and the concentration of sucrose and the treatment duration are the variables that need to be optimized in this step. Pre-culture duration played an important role in maintaining the viability and survival of cryopreserved plant tissue (Ishikawa *et al.*, 1997).

Optimisation of pre-culture duration enables to reduce cell injuries during encapsulation-dehydration. Sucrose pre-culture of the explants could improve vitrification of plant tissues and cryopreservation tolerance as confirmed in various studies (Gonzalez-Arnao *et al.*, 1996; Fang *et al.*, 2004; Valladares *et al.*, 2004). Sugars may act as an osmoticum and desiccate the tissues. The accumulation of sucrose inside the tissues helps in maintaining cell viability during cryopreservation by preventing freeze injury to cell membranes (Touchell *et al.,* 2002).

Wang *et al.* (2000) resorted to stepwise pre-culture of encapsulated shoot tips of grapevine, on half-strength MS medium supplemented with increasing sucrose concentrations of 0.25, 0.5, 0.75 and 1.0 M (one day each in each concentration) for four days, during encapsulation-dehydration method of cryopreservation.

After pre-culture in MS medium with 5 per cent (v/v) dimethyl sulfoxide for 5 d, the survival rates after cryopreservation of *Artemisia annua* calli were 15-

20 per cent higher than those without pre-culture as reported by Chenshu *et al.* (2003).

Saintpaulia ionantha shoot tips pre-cultured in semi solid MS medium supplemented with 0.3 M sucrose for 48h exhibited high viability, post cryopreservation (Moges *et al.*, 2004). Antony *et al.* (2013) pre-cultured the *Dendrobium* Bobby Messina PLBs in half strength semi-solid MS media supplemented with 0.4 M sucrose at 25°C for 3 days under 16 h photoperiod, when the PLBs could be successfully cryopreserved *via* encapsulation-dehydration.

In vitro shoot tips of *Dendrobium* Walter Oumae were cryopreserved using the encapsulation-dehydration technique. Encapsulated shoot tips were pre-cultured on modified Vacin and Went (1949) agar medium supplemented with 0.3 M sucrose for 2 d. Sucrose could induce cytoplasmic vitrification in order to avoid the formation of intracellular ice crystal during the rapid cooling in LN (Lurswijidjarus and Thammasiri, 2004).

In gentian buds, dehydration tolerance was efficiently increased by two-step pre-culturing with sucrose and each of the two steps was indispensable and interdependent. The first step, where mild osmotic stress was given, involved ABA-mediated cellular changes and the second step incorporation of sucrose into the cells (Suzuki *et al.*, 2006).

Nair and Reghunath (2007) reported successful cryopreservation of encapsulated somatic embryos of *Clitoria ternatea,* pre-cultured in modified half MS liquid medium supplemented with 0.75 M sucrose and 3 per cent dimethylsulphoxide (DMSO) for one day without agitation. In encapsulation-dehydration, Shatnawi (2011) reported pre-culture of calcium-alginate encapsulated shoot tips of *Capparis spiunosa,* from 4 week old hardened plants, on MS medium supplemented with 0.75 M sucrose for one day. Subramaniam *et*

al. (2011) cryopreserved *Dendrobium* sonia-17 by pre-culturing in MS medium supplemented with 0.5 M of sucrose for one day.

For successful cryopreservation of *Rosa hybrida* using encapsulation-dehydration technique, encapsulated beads could be osmoprotected in 0.75 M sucrose for two days (Mubbarakh *et al.*, 2014).

Cordeiro *et al.* (2014) reported that *Mandevilla moricandiana,* a woody crop were encapsulated and pre-cultured in liquid MS basal medium with 0.4 M sucrose for two days on a rotary shaker (100 *rpm*) in the growth room, during cryopreservation.

The exposure to cryoprotectants during pre-culture increases the stability of the plasma membrane to withstand various stresses imposed by freezing and accelerates the recovery process after thawing (Uemura *et al.*, 2009). It is necessary to induce high levels of dehydration tolerance in the plant cells, tissues and organs for preservation, for successful cryopreservation.

2.2.1.1.4 Dehydration

Explants have to be sufficiently dehydrated prior to plunging in LN to avoid lethal intracellular ice formation during rapid ultra-cooling. Hence, after pre-culture, the beads could be subjected to additional physical dehydration by evaporation at room temperature. The reduction of the water content of synthetic seeds to a minimal level is a necessary step for a successful cryopreservation of the encapsulated explants (Bouafia *et al.*, 1996; Gonzalez-Arnao *et al.,* 1996). It is achieved by combining pre-culture of encapsulated beads in sucrose containing medium, with their dehydration in silica gel or under a sterile air flow (Adela and Deliu, 2006).

Clavero-Ramirez *et al.* (2005) reported 3 to 4 h of desiccation could give better recovery after cryopreservation in strawberry (*Fragaria* x *ananassa)* and *Fragaria chiloensis* compared to lower levels of desiccation.

Nair and Reghunath (2007) found that 5 h desiccation of encapsulated somatic embryos of *Clitoria ternatea* gave maximum survival and regeneration after cryopreservation at a moisture content of 18.32 per cent.

Nair and Reghunath (2009) observed that the encapsulated axillary buds of *Indigofera tinctoria* when subjected to 4 h desiccation resulted in 16 per cent moisture content (MC). At this MC, maximum freeze tolerance could be obtained as indicated by higher survival and regeneration rates after cryopreservation.

Encapsulated and non-encapsulated embryonic axes of *Melia azedarach* and *Camellia sinensis* and seeds of *Lilium ledebourii* were dehydrated by exposing them to the sterile airflow of a laminar flow cabinet at 25°C for 1 h for effective cryostorage and further recovery (Kaivani, 2010).

Gupta (2014) reported that when explants encapsulated in alginate beads, pre grown in liquid medium enriched with sucrose are partially desiccated to a water content around 20 per cent (on fresh weight basis) and then frozen rapidly would give successful recovery following cryopreservation.

2.2.1.1.5 Cryopreservation in Liquid Nitrogen

Rapid cooling by direct immersion of plant tissues in liquid nitrogen has been reported in several species (Engelmann, 2011). However in potato (Fabre and Dereuddre, 1990), citrus shoot tips (Gonalez-Arnao *et al*., 1998) and grape (Zhao *et al*., 2001) slow precooling using a programmable freezing apparatus followed by immersion in LN was required to obtain the higher survival rate after cryopreservation.

Chenshu *et al.* (2003) optimised cryopreservation technique for the conservation of callus tissue of *Artemisia annua*, with a high survival rate of 87 per cent, after 2 h of storage in liquid nitrogen.

The tolerance of plants to harsh cryopreservation procedures depends on the ability of that particular plant to withstand cryoinjury. Ice formation and colligative damage cause stress to plants stored in LN. Ice formation causes structural and osmotic damages in cells and leads to mechanical injury. The manipulation of liquid, glassy and solid (ice) states of water is the main goal in order to avoid intracellular ice formation (Day *et al.,* 2008). Hence, the main approach in cryopreservation is to control freezing rate which in turn means the control of extracellular ice crystallization (Day *et al.*, 2008).

After desiccation, encapsulated beads of *Plumbago rosea* were placed in 2 ml sterile polypropylene cryotubes and immersed rapidly in LN where they were kept for 2 h (Prakash, 2014).

2.2.1.1.6 Thawing or Rewarming

Thawing after cryopreservation could be carried out either slowly or rapidly at room temperature. For slow rewarming, beads are taken out of the cryotubes and placed in open petri dishes under the laminar air flow cabinet for about 5 min or they are placed directly on the recovery medium. Rapid warming is performed by stirring the cryotubes in a water bath at +40°C

Rapid thawing in a water bath at 40°C was preferable than slow thawing and higher thawing temperatures, with date palm meristems (Bagniol and Engelmann, 1992). Rapid rewarming would avoid recrystallization phenomena which induce lethal damages of the cells. However, too high thawing rates can also be detrimental to cell survival. They may provoke a too rapid cellular rehydration which creates an important osmotic shock, detrimental to the cells (Jian *et al.*, 1987).

Nair and Reghunath (2007) reported that cryostored somatic embryos of *Clitoria ternatea* were transferred to a water bath maintained at 40°C, for 30-60 s for recovery. The rewarmed shoot tips were transferred to recovery medium consisting of half MS + sucrose 30 g L^{-1} + GA 0.5 mg L^{-1} + BA 0.2 mg L^{-1} + agar 6.0 g L^{-1}.

Subsequent to ultra-cooling of *Crateva nurvala,* cryovials were thawed either by immersing them in water bath at 40°C for 1.5 min (rapid thawing) or by allowing them to reach room temperature by slow thawing (Sanayaima *et al*., 2006). Mandal and Sharma (2007) reported that, following 1 h storage of *Dioscorea deltoidea* in LN, rewarming was performed by placing the cryovials in 40°C water-bath for 2 min. After cryopreservation of *Artemisia herba-alba*, cryogenic vials containing beads were thawed in a water bath at 38°C for 2-3 min (Sharaf *et al*., 2012).

Dehydrated beads of *Rosa hybrida* were placed in cryovials and directly immersed in LN for 24 h. Cryovials were warmed in a water bath at 40°C for 90 seconds. The beads from the cryovial were taken out and transferred aseptically into petri dishes containing growth recovery medium and were put in dark culture room condition for a week followed by semi-light and light condition for another 1 week each (Mubbarakh *et al.,* 2014).

Prakash (2014) reported that axillary buds of *Plumbago rosea* was thawed at 40°C for 30-60 s for better recovery. Then it was transferred to the recovery medium MS+ BA 1.5 mg L^{-1} +IAA 1.0 mg L^{-1} for shoot proliferation.

2.2.1.1.7 Recovery

Regrowth generally takes place on standard semi-solid culture medium. However, the composition of the recovery medium may be transitorily modified either to eliminate the phenolic compounds produced by dead cells by adding activated charcoal to the medium; or by transferring samples on media with

progressively decreasing osmoticum; or the growth regulator content of the medium may also be modified to stimulate proliferation of frozen explants. The culture conditions to which the plant tissues recovered from cryostorage, has to be maintained is also very important. It has been found beneficial for organized structures, such as meristems, to perform the post-thaw recovery in the dark for a short period, to prevent or decrease detrimental photo-oxidation of frozen samples (Benson, 1990).

The rewarmed encapsulated meristems of *Indigofera tinctoria* were transferred to recovery medium, consisting of half-strength MS supplemented with gibberellic acid (GA) 0.5 mg L^{-1}, BA 0.2 mg L^{-1}, and sucrose 0.09 M, gelled with 0.6 per cent agar and the rate of survival was recorded as 56.66 per cent (Nair and Reghunath, 2009).

Maximum regrowth upto 83 per cent was obtained from *Capparis spiunosa* shoot tips recovered from cryopreservation on MS medium supplemented with BA 1.0 mg L^{-1} and IAA 0.1 mg L^{-1} (Shatnawi, 2011).

Encapsulated and cryopreserved (+LN) beads of *Artemisia herba-alba* were inoculated onto a solid MS recovery medium containing isopentenyladenin (2iP) 1.0 mg L^{-1} and sucrose 0.1 M and then kept in the dark for 3 days. After cryopreservation the highest survival (40%) and regrowth (6%) rates were achieved (Sharaf *et al.*, 2012).

Encapsulated nodal explants of *Mandevilla moricandiana* showed 93.30 per cent recovery on semisolid MS basal medium after cryopreservation (Cordeiro *et al.*, 2014).

2.2.2 Genetic stability assessment of cryopreserved materials using IISR

Cryopreservation procedures result in the exposure of tissues to physical, chemical and physiological stresses that cause cryoinjury and therefore

assessment of genetic stability should be performed to validate newly established cryopreservation protocols (Harding, 2004). The assessment of genetic fidelity of plantlets recovered from *in vitro* storage is inevitable in germplasm conservation programme.

According to Harding and Benson (1995), molecular analysis of plant DNA is ideal for genetic stability assessments. PCR based molecular markers have been used in the genetic fidelity assessment of cryostorage derived plantlets (Mikula *et al.,* 2011). 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. These markers, which amplify different regions of the genome, allow better chances for the identification of genetic variations in the plantlets (Martin *et al*., 2002). PCR based molecular markers have been used in the genetic fidelity assessment of cryostorage derived plantlets (Nair and Reghunath, 2009; Prakash, 2014; Coelho *et al.*, 2014).

PCR based markers, ISSR and RAPD confirmed the genetic stability of cryopreserved and regenerated plants of *Zingiber officinale* (Yamuna *et al*., 2007) and *Passiflora pohlii* (Merhy *et al*., 2014)

Prakash (2014) confirmed the genetic stability of *Plumbago rosea* shoot tips from cryopreservation using RAPD marker.

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.

However, the use of different genetic markers has also generated controversial results regarding the genetic fidelity of cryopreserved material. In addition, epigenetic variation in chromatin and DNA methylation of gene sequences has been observed in plants after cryopreservation, which suggests altered patterns of gene expression (Jokipii *et al.*, 2004).

An important prerequisite for any conservation technique is that the regenerants produced from the conserved material should be true-to-type. The practical application of this technology is useful only if it does not lead to the genetic changes in the cryopreserved plant (Zarghami *et al.,* 2008).

Materials and Methods

3. MATERIALS AND METHODS

The present study, 'Cryoconservation of koovalam (*Aegle marmelos* L. Corr.) by encapsulation and dehydration technique' was carried out at the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2015-2017. The study aimed at standardisation of *in vitro* conservation protocol for *A. marmelos* using encapsulation dehydration technique of cryopreservation. The assessment of genetic accuracy of the regenerated plantlets using molecular markers was also attempted.

Investigation was carried out in three phases *viz.,* enhancement of multiplication rate; standardization of *in vitro* conservation using the encapsulation-dehydration technique; and the assessment of genetic fidelity of the regenerated plantlets by ISSR marker.

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

3.1 PHASE I: ENHANCEMENT OF MULTIPLICATION RATE

In this phase of investigation, varying concentrations and combination of auxins and cytokinins, additives (chitosan, thidiazuron and adenine sulphate) were tried to enhance the shoot multiplication in *A. marmelos.*

3.1.1 Source of explant

Axillary buds from the *in vitro* maintained cultures of *A. marmelos* 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 2 mg L^{-1}.

3.1.2 Preparation of Culture Media

The basal medium used for the study was MS (Murashige and Skoog, 1962) medium. The chemicals used in the culture media were of analytical grade from Sisco Research Laboratories (Mumbai), Merck (Mumbai) and HiMedia Laboratories Pvt. Ltd. (Mumbai).

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

The glassware used were washed with dilute liquid detergent (Labolene) and rinsed with single distilled water. Specific quantities of the stock solutions were pipetted out into 1000 ml beaker containing 400 ml of distilled water. Sucrose and inositol were added fresh in required quantity, weighed using an electronic balance (Shimadzu AUX120) and dissolved by constant stirring. The pH of the medium was adjusted to 5.7 using an electronic pH meter (Susima MP-1 PLUS). Agar was added to the medium @ 5.9 g $L^{-1}$and final volume made up to 1000 ml, using a volumetric flask. Agar was melted using microwave oven. The melted medium was dispensed into pre-sterilised culture vessels such as test tubes (25 x 150 mm) and jam bottles (300 ml). Measure of culture medium in test tubes and jam bottles were 15 and 30 ml, respectively. The test tubes containing medium were plugged firmly with non-absorbent cotton wool and the jam bottles were closed tightly with autoclavable plastic lid. They were then autoclaved at 121°C and 1.06 Kg cm^{-2} pressure for 20 minutes using STERI horizontal cylindrical autoclave (Yorko, India). The composition of basal medium was MS salts + sucrose 30 g L^{-1} + agar 5.9 g L^{-1} + inositol 100 mg L^{-1}.

3.1.3 Inoculation

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

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

3.1.4 Incubation

The cultures were incubated in a culture room maintained at light intensity, 40µmol m^{-2} s^{-1} using white fluorescent tubes. The temperature of the room was regulated using an air conditioner at 25±2°C at a relative humidity (RH) of 60 per cent. The photoperiod cycle was maintained at 16h light/8h of darkness.

3.1.5 Effect of cytokinins and auxins on shoot multiplication (Experiment I)

Nodal cuttings (1.5 cm) with a single axillary bud from the *in vitro* established plantlets were inoculated for culture multiplication. The nodal segments were cultured in basal medium supplemented with different concentrations and combinations of auxins (NAA, IAA and IBA) and cytokinins (BA and Kn). Treatments involved different levels of BA (0.5 to 3 mg L^{-1}), Kn (1 to 3 mg L^{-1}), IAA (0.5 mg L^{-1}), IBA (0.5 mg L^{-1}) and NAA (0.5 mg L^{-1}) (Table1). The treatments were replicated three times.

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

3.1.6 Effect of Additives on multiplication of shoots (Experiment II)

The MS medium supplemented with BA 2 mg L^{-1} and IBA 0.5 mg L^{-1} was used as the basal medium to which different additives *viz*., chitosan, adenine

sulphate and thidiazuron were added. These additives were added to two different media, (1) MS medium + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1} (SDM), the best responsive treatment of the experiment 4.1.1 and (2) plain MS medium. The medium, MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1} was taken as the control.

Stock solutions of different additives were prepared by dissolving required quantities of salts in appropriate solvent *viz*., adenine sulphate in double distilled water, thidiazuron in 1 N NaOH and chitosan in 0.1 M acetic acid. The final volume was made up to 200 ml using double distilled water in a volumetric flask. The stock solutions were then kept in refrigerator (4°C) in sterilized bottles. Different concentrations of additives *viz.,* chitosan (10, 20 and 30 mg L^{-1}), adenine sulphate (20, 40 and 60 mg L^{-1}) and thidiazuron (2, 1, 0.5 and 0.02 mg L^{-1}) were added from the stock solutions to the basal media, maintaining the pH at 5.7. The treatments for the study are represented in Tables 2, 3 and 4. Each treatment was replicated three times.

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

3.1.7 Statistical Analysis

Completely randomized design (Panse and Sukhatme, 1985) was followed for statistical analysis, wherever applicable. Data were subjected to analysis of variance (ANOVA) and significant differences between treatments were determined by pairwise comparison. Each treatment was replicated three times.

Table1. Auxins and cytokinins on multiplication of axillary buds

Treatment No.	Plant growth regulators (mg L^{-1})				
	BA	Kn	IAA	IBA	NAA
T1	0.5	-	-	-	-
T2	1	1	-	-	-
T3	3	-	-	-	-
T4	2	-	0.5	-	-
T5	2	-	-	0.5	-
T6	-	2	-	-	-
T7	-	2	-	-	0.5
T8	-	2	-	0.5	-
T9	-	2	0.5	-	-
T10	-	3	-	-	-
T11	2	-	-	-	-
T12	-	-	-	-	-

Table 2. Different levels of chitosan tried for shoot multiplication

Treatment No.	Chitosan (mg L^{-1})	BA (mg L^{-1})	IBA (mg L^{-1})
CH1	10	2	0.5
CH2	20	2	0.5
CH3	30	2	0.5
CH4	10	-	-
CH5	20	-	-
CH6	30	-	-
Control (SDM)	-	2	0.5

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

Treatment No.	Adenine sulphate (mg L^{-1})	BA (mg L^{-1})	IBA (mg L^{-1})
AdS1	20	2	0.5
AdS2	40	2	0.5
AdS3	60	2	0.5
AdS4	20	-	-
AdS5	40	-	-
AdS6	60	-	-
Control (SDM)	-	2	0.5

Table 4. Different levels of thidiazuron tried for shoot multiplication

Treatment No.	Thidiazuron (mg L^{-1})	BA (mg L^{-1})	IBA (mg L^{-1})
TDZ 1	2	2	0.5
TDZ 2	2	-	-
TDZ 3	1	2	0.5
TDZ 4	1	-	-
TDZ 5	0.5	2	0.5
TDZ 6	0.5	-	-
TDZ 7	0.02	2	0.5
TDZ 8	0.02	-	-
Control (SDM)	-	2	0.5

3.2 PHASE II: *IN VITRO* CONSERVATION

3.2.1 Cryopreservation of *Aegle marmelos* using encapsulation-dehydration technique

3.2.1.1 Preconditioning

Single node segments containing an axillary bud from *in vitro* established *A. marmelos* were inoculated on to MS medium supplemented with different concentrations of sucrose (0.1, 0.3, 0.5 M) and keeping the agar concentration 0.59 per cent. Cultures were incubated at 25 ± 2°C under 16h light/8h dark photoperiod at a photon flux intensity of 30-50 µmol $m^{-2}s^{-1}$ for different periods such as 1 day, 1 week, 2 week and 3 weeks. The treatments are represented in Table 5. After weekly intervals of incubation explants were removed from the preconditioning media and cultured on the medium standardised in Phase I (MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1}). The experiment was replicated three times. Observations were recorded on number of days for bud initiation, shoots per explant, shoot length and nodes per shoot after six weeks of incubation. The best preconditioning medium and exposure period was determined based on the above mentioned observations.

3.2.1.2 Encapsulation

Nodal axillary buds excised from *in vitro* grown plantlets were suspended in calcium free MS medium supplemented with different concentration (2.5, 3, 3.5, 4 and 5 per cent) of sodium alginate (Sigma, St. Louis) and 30 g L^{-1} sucrose. This mixture was dropped with a 1 ml micropipette into different concentrations of calcium chloride solution (50, 75, 100 and 200 mM) for encapsulation. The treatments are represented in Table 6. After 30 mins of complexing time in calcium chloride solution, the beads were cultured on the modified inoculation medium (½ MS+ BA 2 mg L^{-1} + IBA 0.5 mg L^{-1}). The treatments were replicated three times. Observations were recorded on number of bud initiation, shoots per explant, shoot length and nodes per shoot after six weeks of culture. Based on the observations, the best encapsulation medium was determined.

Table 5. Treatments tried for preconditioning.

Treatment No.	Sucrose (M)	No. of days
PC1	0.1	1
PC2	0.1	7
PC3	0.1	14
PC4	0.1	21
PC5	0.3	1
PC6	0.3	7
PC7	0.3	14
PC8	0.3	21
PC9	0.5	1
PC10	0.5	7
PC11	0.5	14
PC12	0.5	21

Inoculation medium: MS+ BA 2 mg L^{-1} + IBA 0.5 mg L^{-1}

Table 6. Treatments tried for encapsulation

Treatment No.	SA (%)	$CaCl_2$ (mM)
E1	2.5	50
E2	2.5	75
E3	2.5	100
E4	2.5	200
E5	3	50
E6	3	75
E7	3	100
E8	3	200
E9	3.5	50
E10	3.5	75
E11	3.5	100
E12	3.5	200
E13	4	50
E14	4	75
E15	4	100
E16	4	200
E17	5	50
E18	5	75
E19	5	100
E20	5	200

Modified inoculation medium: ½ MS+ BA 2 mg L^{-1} + IBA 0.5 mg L^{-1}

3.2.1.3 Pre-culture

The preconditioned and encapsulated beads were pre-cultured in liquid MS medium supplemented with different concentrations of sucrose (0.1, 0.5 and 1M) and DMSO 3 per cent dispensed into 100 Erlenmeyer flasks (25 beads in 15 ml medium) and incubated in darkness at 4°C (in a refrigerator) for different periods- 1, 2, 3, 4 and 5 days.

After each day in liquid medium, the beads were transferred to the same medium afresh. The treatments are represented in Table 7. After the respective periods of incubation the beads were cultured on the modified inoculation medium (½ MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1}). The treatments were replicated three times. Observations were recorded on number of days for bud initiation, shoots per explant, shoot length and nodes per shoot after six weeks of culture. Based on the observations best pre-culture condition was determined.

3.2.1.4 Dehydration

To determine the optimum drying time, beads after pre culture were desiccated for 0 to 7 h in a sterile laminar flow cabinet. Moisture content of the beads after every one hour was determined on a fresh weight basis from three replicates of beads dehydrated prior to oven drying at 103°C for two hours using the formula,

$$MC = [(IW - FW)/ IFW]*100$$

IW - Initial weight of the beads after a specific period of drying

FW - Dry weight of the bead after placing in hot oven at 103°C for 2h

IFW- Initial fresh weight of the beads before drying

Liquid nitrogen (LN) tolerance was tested for each drying time at one hour interval by culturing the desiccated beads on modified inoculation medium (½ MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1}). The survival and regeneration percentage were

also recorded for desiccated beads without LN exposure as control for each drying time.

Table 7. Treatments tried for pre-culture

Treatment No.	Sucrose (M)	No. of days
PT1	0.1	1
PT2	0.1	2
PT3	0.1	3
PT4	0.1	4
PT5	0.1	5
PT6	0.5	1
PT7	0.5	2
PT8	0.5	3
PT9	0.5	4
PT10	0.5	5
PT11	1	1
PT12	1	2
PT13	1	3
PT14	1	4
PT15	1	5

3.2.1.5 Cryopreservation and Recovery

Dehydrated beads were transferred to 5 ml cryovial and directly immersed in liquid nitrogen where they were stored for 2 hr, one day and one week. On rewarming, cryotubes were removed from liquid nitrogen and transferred to water under constant circulation in a water bath maintained at 40°C for 30-60 s. The rewarmed shoot tips were transferred to recovery medium (½ MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1} + sucrose 30 g L^{-1} + agar 5.9 g L^{-1}) and incubated in culture room at 25±2°C under 16 h photoperiod at a photon flux intensity of 40 µmol $m^{-2}s^{-1}$. The experiment was replicated three times. The results were expressed as survival per cent and/or regeneration into shoot. Survival (indicated by green colouration of the explant) and regeneration (indicated by emergence of shoot from axillary bud). Regeneration was estimated as percentage of nodal segments differentiated into shoots after six weeks of culture. Observations were recorded on number of days for bud initiation, shoots per explant, shoot length and nodes per shoot after six weeks of culture.

3.2.2 Statistical Analysis

Completely randomized design (Panse and Sukhatme, 1985) was followed for statistical analysis, wherever applicable. Data were subjected to analysis of variance (ANOVA) and significant differences between treatments were determined by pairwise comparison. Each treatment was replicated three times.

3.2.3 Estimation of genetic fidelity of cryopreserved materials using ISSR

3.2.3.1 Genomic DNA Isolation

CTAB (Cetyl Trimethyl Ammonium Bromide) method of DNA extraction (Doyle and Doyle, 1990) with slight modifications was used for genomic DNA isolation.

Young leaves from the cryopreserved plants as well as control plants (*in vitro* cultures maintained at ambient conditions) were used for DNA extraction.

Leaf material (0.5 g) was washed in distilled water and dried by spreading on tissue paper. The samples were chilled and pulverized to a fine powder in liquid nitrogen using a sterile mortar and pestle and transferred about 100 mg of powder into a sterile 2 ml centrifuge tube containing 1 ml of freshly prepared warm extraction buffer (prepared by adding β-mercaptoethanol and polyvinylpyrrolidone (PVP) freshly to the CTAB extraction buffer (Appendix I) to give final concentrations of 0.2 per cent (v/v) and 4 per cent (w/v) respectively, which are then heated to 65°C in water bath (ROTEK, India). The content was homogenized by gentle inversion. The samples were incubated at 60°C in water bath for one hour with intermittent shaking. The homogenate was then extracted with an equal volume of 24:1 (v/v) chloroform/isoamyl alcohol and mixed well by inversion for 5-10 min. The homogenate was centrifuged (Eppendorf centrifuge 5430 R, Germany) at 7500 x g for 10 min at 25°C. The upper phase was transferred to a new tube and the extraction process chloroform/isoamyl alcohol was repeated twice. 0.5 ml of 5 M NaCl was added to the aqueous phase and mixed properly by gentle inversion. This was followed by addition of 0.6 volume of chilled isopropanol to the mixture and mixed by inversion. The mixture was then incubated at 4°C overnight to precipitate the nucleic acid. After incubation, the precipitated DNA was pelleted by centrifugation at 11,000 x g for 10 min at 25°C. The supernatant was decanted and the pellet was washed in 0.5 ml ethanol (80 per cent) twice, each time centrifuging at 5000 x g for 7 min at 25°C and discarding the supernatant. The pellet was air dried for 30-40 min and dissolved in 40 µl of TE buffer (Appendix II). The extracted DNA samples were then stored at -20°C (Lab-Line Low Temperature Cabinet, India).

3.2.3.2 Quantification of DNA

Spectrophotometer absorbance reading of the DNA samples was recorded at 260-280nm to determine the quantity and quality of DNA. UV-Visible Spectrophotometer (ELICO, Model No.SL178) was used for measuring the optical density (O.D.) of the sample. Spectrophotometer was calibrated to blank (zero absorbance) at 260 nm and 280 nm wavelength with 3 ml TE buffer. The

O.D. readings of 5 µl DNA samples dissolved in 3 ml of TE buffer at respective wavelength were also recorded.

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

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

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

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

3.2.3.3 Agarose gel electrophoresis

The presence of genomic DNA and PCR products were confirmed and analysed by using horizontal gel electrophoresis unit (Genei, Bangalore). Agarose solution (0.9 per cent) in electrophoresis buffer (0.24 g agar in 30 ml of buffer solution) was prepared in a conical flask for the identification of genomic DNA. Electrophoresis buffer (0.5 x TBE) (Appendix III) was prepared to fill the electrophoresis tank and to cast the gel. Agarose 1.5 per cent solution was used for casting the gel to separate out the PCR products. The slurry was heated in a microwave oven until the agarose was dissolved. On cooling to the molten gel, added ethidium bromide (EtBr) to a final concentration of 0.5 µg ml^{-1} and mixed thoroughly. The comb was positioned above the tray, and then the warm agarose solution was poured into the moulded casting tray. The gel was allowed to set completely (30-50 min) at room temperature. On gel setting, the comb was carefully removed. The gel was then, taken out from the casting tray and mounted in the electrophoresis tank. Electrophoresis buffer was added just enough to cover the gel to a depth of about 1 mm above the gel. DNA sample (5 µl) mixed with 1 µl of 6X gel loading buffer (Genei) and the sample mixtures were slowly loaded

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

3.2.3.4 ISSR Analysis

DNA samples were amplified with nine different ISSR primers supplied by Genei, Bangalore. Primers selected for the analysis are listed in Table 8. The components of the reaction mixture were optimized as listed below.

Water	:	12.1 µl
10X Taq buffer A (Tris with 15 mM $MgCl_2$)	:	2.0 µl
dNTPs (10 mM each)	:	1.5 µl
Primer (10 µM)	:	2.0 µl
Template DNA (50 ng μl^{-1})	:	2.0 µl
Taq polymerase (3U μl^{-1})	:	0.4 µl
Total volume	:	20 µl

PCR was carried out in a Eppendorf Master cycler (Germany). PCR programme was set with initial denaturation at 94°C for 4 min followed by 35 cycles of denaturation at 94°C for 45 s, annealing at 50°C for 1min and extension at 72°C for 2 min. Final extension was done at 72°C for 8 mins. Control reactions were carried out to distinguish the target products from non-target products and primer dimer. The amplified products along with quantum PCR Marker (low range) from 'Genei, Bangalore' were separated on agarose gel (1.5 per cent). The gel was viewed under gel documentation system (BIORAD, USA).

The genetic fidelity assessment of cryopreserved axillary buds of *A. marmelos* was done by comparing the ISSR banding pattern of the cryo-

regenerated plantlets with that of control non-cryopreserved *in vitro* grown plantlets.

Table 8: Sequence of ISSR primers used for DNA amplification by PCR to evaluate the genetic stability of plantlets regenerated from cryopreserved materials of *Aegle marmelos*

SI. No.	Primers	Sequence (5'-3')	T_m (°C)
1	UBC-810	GAGAGAGAGAGAGAGAT	42.9
2	UBC-811	GAGAGAGAGAGAGAGAC	43.3
3	UBC-827	ACACACACACACACACG	54.9
4	HBO-816	CACACACACACACACAT	51.1
5	UBC 807	AGAGAGAGAGAGAGAGT	42.5
6	UBC 840	GAG AGA GAG AGA GAG AYT	52.9
7	UBC 811	GAGAGAGAGAGAGAGAC	43.3
8	UBC 847	CACACACACACACACART	53.7
9	UBC 826	ACACACACACACACACC	53.3

Results

4. RESULTS

Investigations were carried out on "Cryoconservation of koovalam (*Aegle marmelos* L. Corr.) by encapsulation-dehydration technique," at the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2015-2017. The results of the study are presented here.

4.1 PHASE I: ENHANCEMENT OF MULTIPLICATION RATE

Single node segments of 1-2 cm, containing an axillary bud excised from *in vitro* cultures maintained in MS medium supplemented with BA 2 mg L^{-1}, were used as explants.

4.1.1 Effect of cytokinins and auxins on shoot multiplication

The different concentrations and combinations of cytokinins (BA and Kn) and auxins (NAA, IAA and IBA) were tried to study their effect on shoot multiplication. The results of the study are presented in Table 9.

Among the twelve treatments (Table 1) tried, four treatments with less than 50 per cent regeneration were discarded from statistical analysis. The discarded treatments include T1 (MS + BA 0.5 mg L^{-1}), T7 (MS + Kn 2.0 mg L^{-1} and NAA 0.5 mg L^{-1}), T9 (MS + Kn 2.0 mg L^{-1} and IAA 0.5 mg L^{-1}) and T12 (hormone free MS medium). The observations were recorded on various plant growth parameters *viz.,* days to bud initiation, shoots per explant, shoot length and nodes per shoot after six weeks of incubation.

There was no significant variation among the different treatments with respect to days to bud initiation. The bud initiation took place in 6 to 8.33 days. In the treatment, T5 (MS+BA 2 mg L^{-1}+ IBA 0.5 mg L^{-1}) giving maximum number (9.33) of shoots per explant recorded significant variation among the treatments. This treatment was found to be significantly superior to all other treatments. The least number (1.00) of shoots per explant was obtained in T8 (MS

Table 9. Effect of cytokinins and auxins on enhanced release of axillary buds from nodal explants

Treatment No.	Plant growth regulators (mg L^{-1})					RG (%)	DBI	SpE	SL (cm)	NpS
	BA	Kn	IAA	IBA	NAA					
T1*	0.5	-	-	-	-	16.67	8.00	1.00	0.33	1.00
T2	1	1	-	-	-	83.33	6.67±1.79	6.33±1.53^{b}	1.13±0.27^{c}	7.00± 1.69^{a}
T3	3	-	-	-	-	100.00	6.83±1.74	2.67±0.68^{c}	2.63±0.67^{b}	1.00± 0.24^{c}
T4	2	-	0.5	-	-	66.67	8.33±0.20	1.83±0.04^{c}	1.33±0.03^{c}	2.67± 0.06^{b}
T5	2	-	-	0.5	-	100.00	7.00±0.45	9.33±1.63^{a}	3.63±0.63^{a}	8.33± 1.45^{a}
T6	-	2	-	-	-	83.33	7.17±0.45	4.33±0.27^{b}	2.37±0.15^{b}	3.50±0.22^{b}
T7*	-	2	-	-	0.5	16.67	8.68	1.00	0.57	1.00
T8	-	2	-	0.5	-	100.00	6.67±1.22	1.50±0.27^{c}	1.67±0.30^{b}	1.50±0.27^{b}
T9*	-	2	0.5	-	-	0.00	0.00	0.00	0.00	0.00
T10	-	3	-	-	-	66.67	6.00±1.04	4.30±0.74^{b}	2.00±0.34^{b}	2.17±0.37^{bc}
T11	2	-	-	-	-	50.00	6.33±0.08	4.83±1.55^{b}	4.00±0.05^{a}	3.67±0.04^{b}
T12*	-	-	-	-	-	16.67	8.33	1.00	0.17	1.00
CD (5%)	-	-	-	-	-	-	NS	2.824	1.215	2.636

RG – Regeneration; DBI – Days to Bud Initiation; SpE – Shoots per Explant; SL- Shoot length; NpS- Nodes per shoot; * Treatments have been excluded from statistical analysis

Basal culture medium: MS

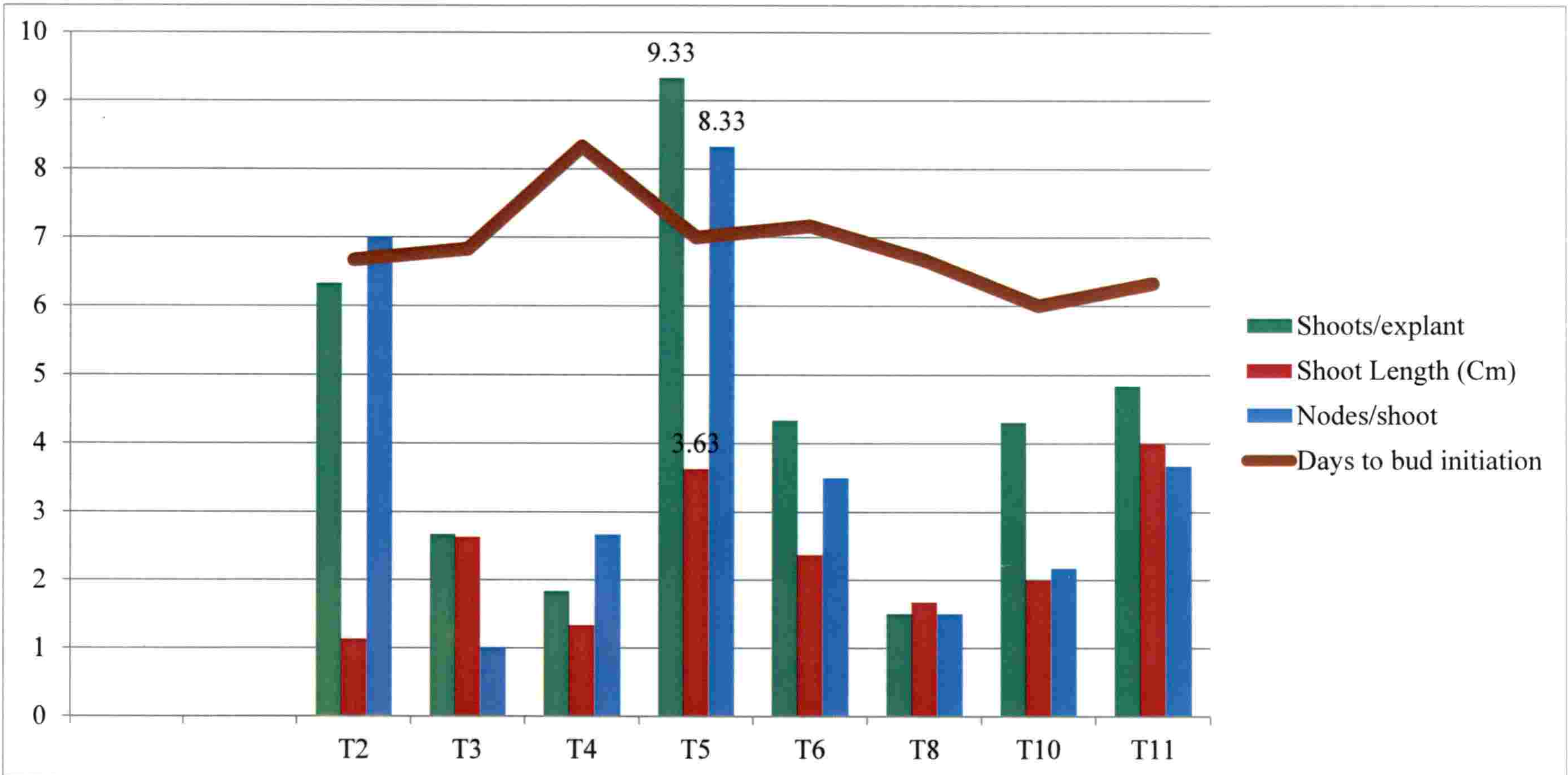
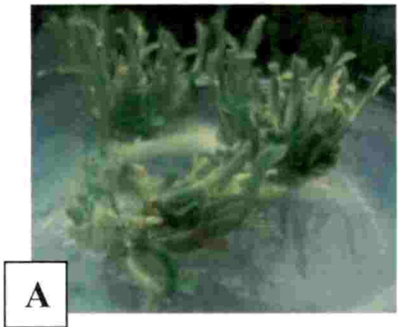


Fig 1. Effect of cytokinins and auxins on enhanced release of axillary buds from nodal explants



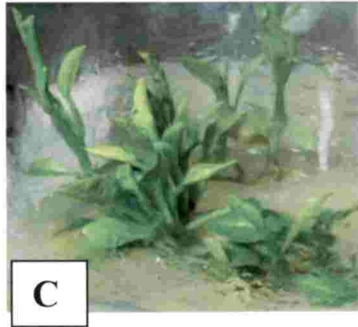


Plate 1. Effect of cytokinins and auxins on enhanced release of axillary buds from nodal explants. A) MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1} , B)MS + BA 1 mg L^{-1} + Kn 1 mg L^{-1} , C) MS + Kn 3 mg L^{-1} D) MS + BA 2 mg L^{-1} , E) Control (MS).

+ Kn 2 mg L^{-1}+ IBA 0.5 mg L^{-1}). This value was on par with the treatment T3 (MS + BA 3 mg L^{-1}) and T4 (MS + BA 2 mg L^{-1} +IAA 0.5 mg L^{-1}).

Shoot length and nodes per shoot exhibited significant variation with the different treatments. Maximum shoot length (4.00 cm) was observed in T11 (MS +BA 2 mg L^{-1}) which was on par with T5 (MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1}). The least shoot length (1.13 cm) was seen in T2 (MS + BA 1 mg L^{-1}+ Kn 1 mg L^{-1}) and the value is on par with T4 and T8. Nodes per shoot (8.33) were the highest in T5 (MS + BA 2 mg L^{-1}+ IBA 0.5 mg L^{-1}). This was on par with the treatment T2. The least number (1.00) was found in T3 (MS + BA 3 mg L^{-1}), which was on par with T4, T6, T8 and T10.

Among the twelve treatments tried, the medium MS + BA 2 mg L^{-1} +IBA 0.5 mg L^{-1} yielded significantly higher response with respect to different parameters *viz*., shoots per explant, shoot length and nodes per shoot (Plate 1). The results are graphically represented in Fig. 1.

4.1.1.1 Additives

Chitosan, adenine sulphate and thidiazuron were added to the medium at different levels to study their effect on shoot multiplication. These additives were added to two different media, (1) MS medium + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1} (SDM), the best responsive treatment of the experiment 4.1.1 and (2) plain MS medium.

4.1.1.1.1 Chitosan

Six treatments (Table 2) with varying concentration of chitosan (10, 20 and 30 mg L^{-1}) were tried to assess the effect on shoot proliferation. The results are depicted in Table 10 and Fig. 2.

Table 10. Effect of chitosan on shoot proliferation of *Aegle marmelos*

Treatment No.	Chitosan (mg L^{-1})	BA (mgL^{-1})	IBA (mg L^{-1})	RG (%)	DBI	SpE	SL	NpS
CH1	10	2	0.5	100	7.00±0.78	35.67 (5.97) ±0.03^{a}	5.23±0.58^{a}	8.50±0.95^{a}
CH2	20	2	0.5	100	8.00±1.94	7.00 (2.63) ±0.14^{c}	1.65±0.40^{c}	4.16±1.01^{b}
CH3	30	2	0.5	100	9.00±2.29	2.33 (1.50) ±0.18^{fg}	1.08±0.27^{c}	1.50±0.38^{c}
CH4	10	-	-	100	7.00±0.17	6.00 (2.40) ±0.32^{d}	3.48±0.08^{b}	5.33±0.13^{b}
CH5	20	-	-	100	7.17±1.25	2.17 (1.47) ±0.01^{g}	1.00±0.17^{c}	1.66±0.29^{c}
CH6	30	-	-	100	8.17±0.51	2.83 (1.66) ±0.14^{ef}	1.77±0.11^{c}	1.83±0.11^{c}
Control (SDM)	-	2	0.5	100	7.00±0.45	9.33 (3.03) ±0.27^{b}	3.63±0.63^{b}	8.33±1.45^{a}
CD (5%)	-	-	-	-	NS	0.639	0.994	1.867

RG – Regeneration; DBI – Days to Bud Initiation; SpE – Shoots per Explant; SL- Shoot Length; NpS- Nodes per shoot, Transformed (square root) values are presented in the parenthesis.

Basal culture medium: MS

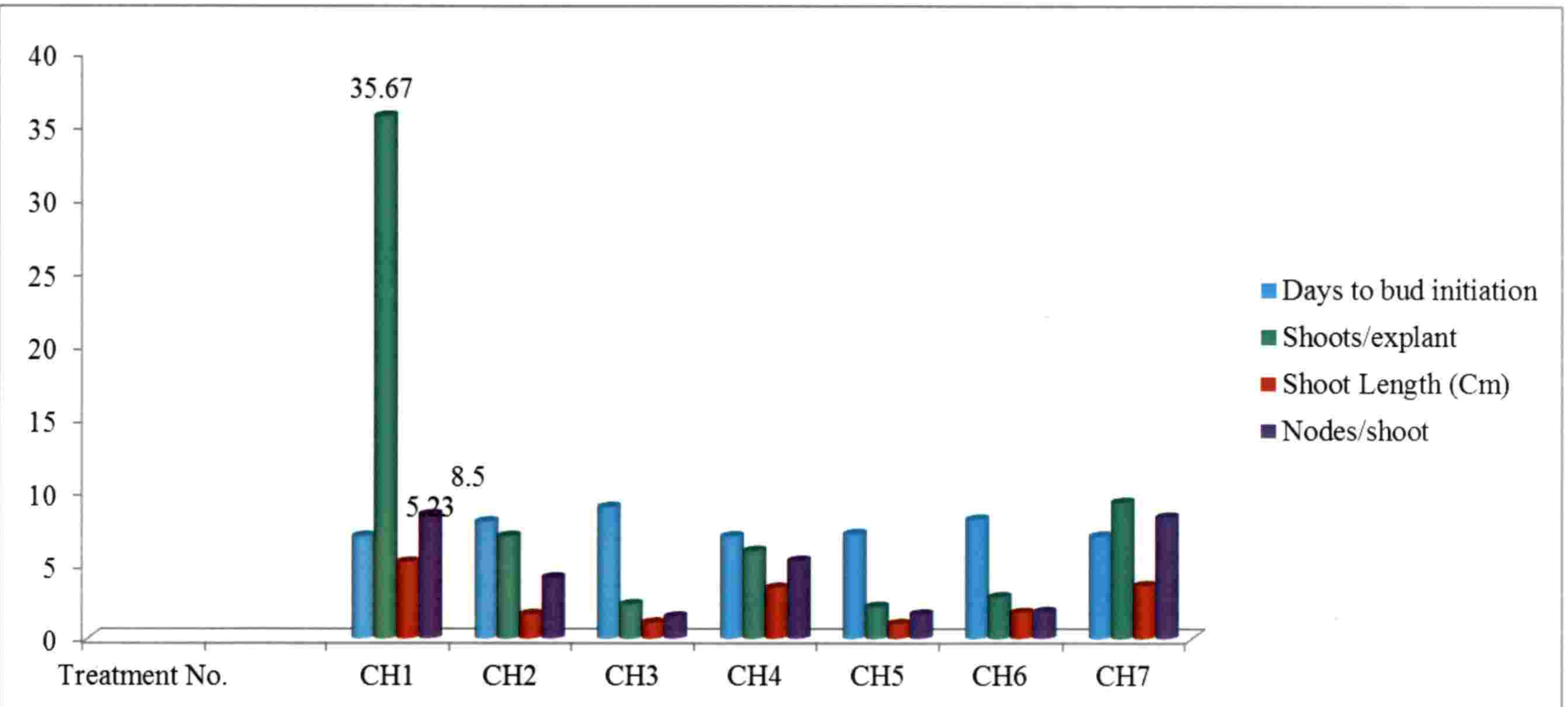


Fig 2. Effect of chitosan on shoot proliferation of *Aegle marmelos*

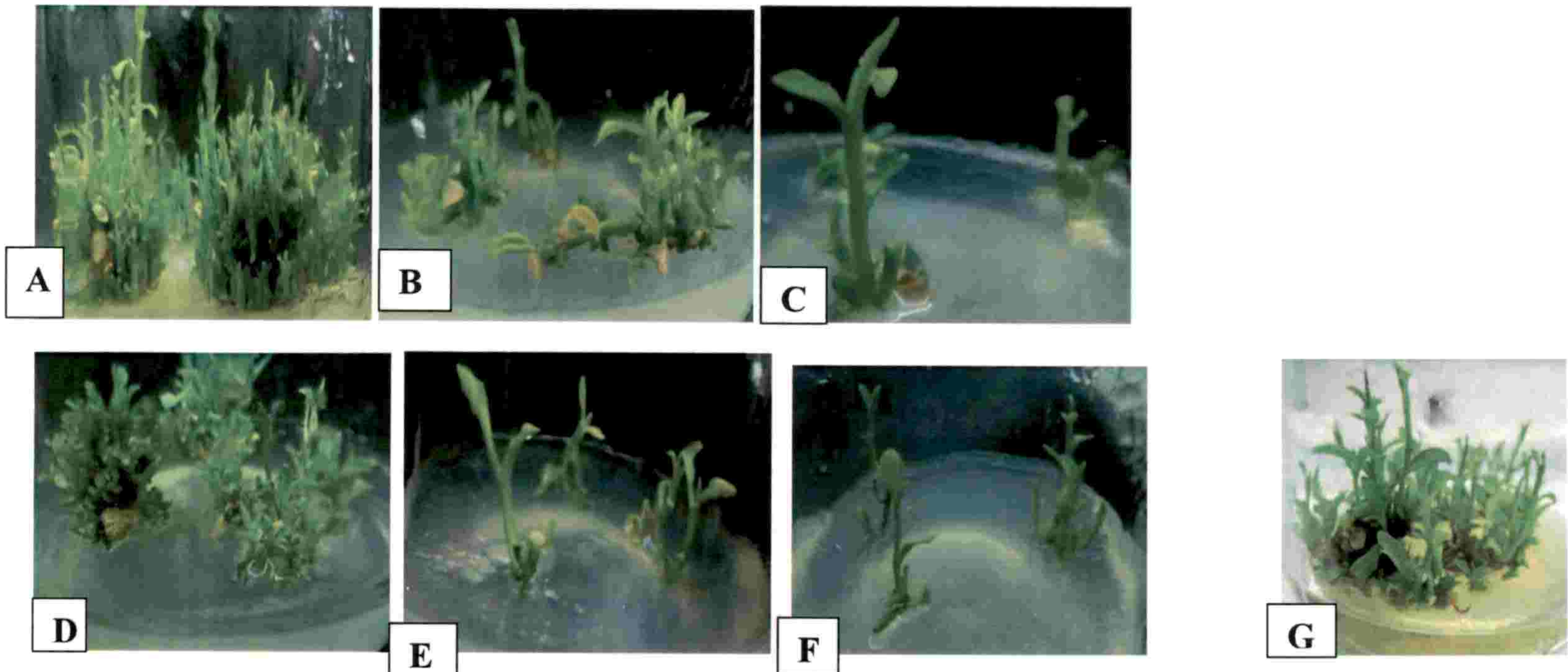


Plate 2. Effect of chitosan on shoot proliferation of *A. marmelos* : A) SDM + chitosan 10 mg L^{-1}, B) SDM+ chitosan 20 mg L^{-1}, C) SDM + chitosan 30 mg L^{-1}, D) MS + chitosan 10 mg L^{-1}, E) MS + chitosan 20 mg L^{-1}, F) MS + chitosan 30 mg L^{-1}, G) SDM (MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1})

Basal culture medium: MS

All treatments including the control, provided 100 per cent regeneration. Significant variation with respect to shoots per explant, shoot length and nodes per shoot was observed in the study. Days to bud initiation did not show any significant difference among the various treatments. The number of days to bud initiation ranged from 7 to 9.

The treatment, CH1 (SDM + chitosan 10 mg L^{-1}) recorded maximum number of shoots (35.67) per explant and shoot length (5.23 cm). These two parameters were significantly higher in CH1 compared to other treatments. The shoots per explant were found to be the lowest (2.17) in CH5 (MS + chitosan 20 mg L^{-1}). This was on par with the treatments CH3. The same treatment CH5 recorded the lowest shoot length (1.00 cm). The treatments CH2, CH3 and CH6 were found to be on par with CH5.

The nodes per shoot were also observed to be maximum (8.50) in the treatment CH1 (SDM + chitosan 10 mg L^{-1}). This was on par with the treatment control. The minimum number of nodes (1.50) per shoot was seen in the treatment CH3 (SDM+ chitosan 30 mg L^{-1}) which was found to be on par with CH5 and CH6.

The shoot proliferation with respect to different chitosan treatments and the control are represented in Plate 2.

4.1.1.1.2 Adenine sulphate

Six treatments involving different levels of adenine sulphate (20, 40 and 60 mg L^{-1}) were tested to study the effect on shoot proliferation. The results of the study are presented in Table 11 and Fig 3.

All treatments with different concentrations of adenine sulphate recorded 100 per cent regeneration. Significant variation was observed among the shoots

Table 11. Effect of adenine sulphate on shoot proliferation of *Aegle marmelos*

Treatment No.	Adenine sulphate (mg L^{-1})	BA (mg L^{-1})	IBA (mg L^{-1})	RG (%)	DBI	SpE	SL	NpS
AdS1	20	2	0.5	100	7.00±0.78	1.00±0.11^{d}	1.00±0.11^{c}	1.17±0.01^{g}
AdS2	40	2	0.5	100	8.33±0.73	1.33±0.32^{d}	1.07±0.25^{c}	2.17±0.02^{e}
AdS3	60	2	0.5	100	8.17±0.31	3.00±0.76^{c}	2.00±0.51^{c}	1.50±0.02^{f}
AdS4	20	-	-	100	8.67±0.87	1.00±0.02^{d}	2.07±0.05^{c}	4.67±0.06^{d}
AdS5	40	-	-	100	8.83±0.39	2.50±0.43^{c}	3.83±0.67^{b}	5.33±0.06^{c}
AdS6	60	-	-	100	8.50±1.02	5.33±0.33^{b}	7.17±0.45^{a}	9.33±0.23^{a}
Control (SDM)	-	2	0.5	100	7.00±0.45	9.33±1.63^{a}	3.63±0.63^{b}	8.33±1.45^{b}
CD (5%)	-	-	-	-	NS	1.275	1.234	0.277

RG – Regeneration; DBI – Days to Bud Initiation; SpE – Shoots per Explant; SL- Shoot length; NpS - Nodes per shoot

Basal culture medium: MS

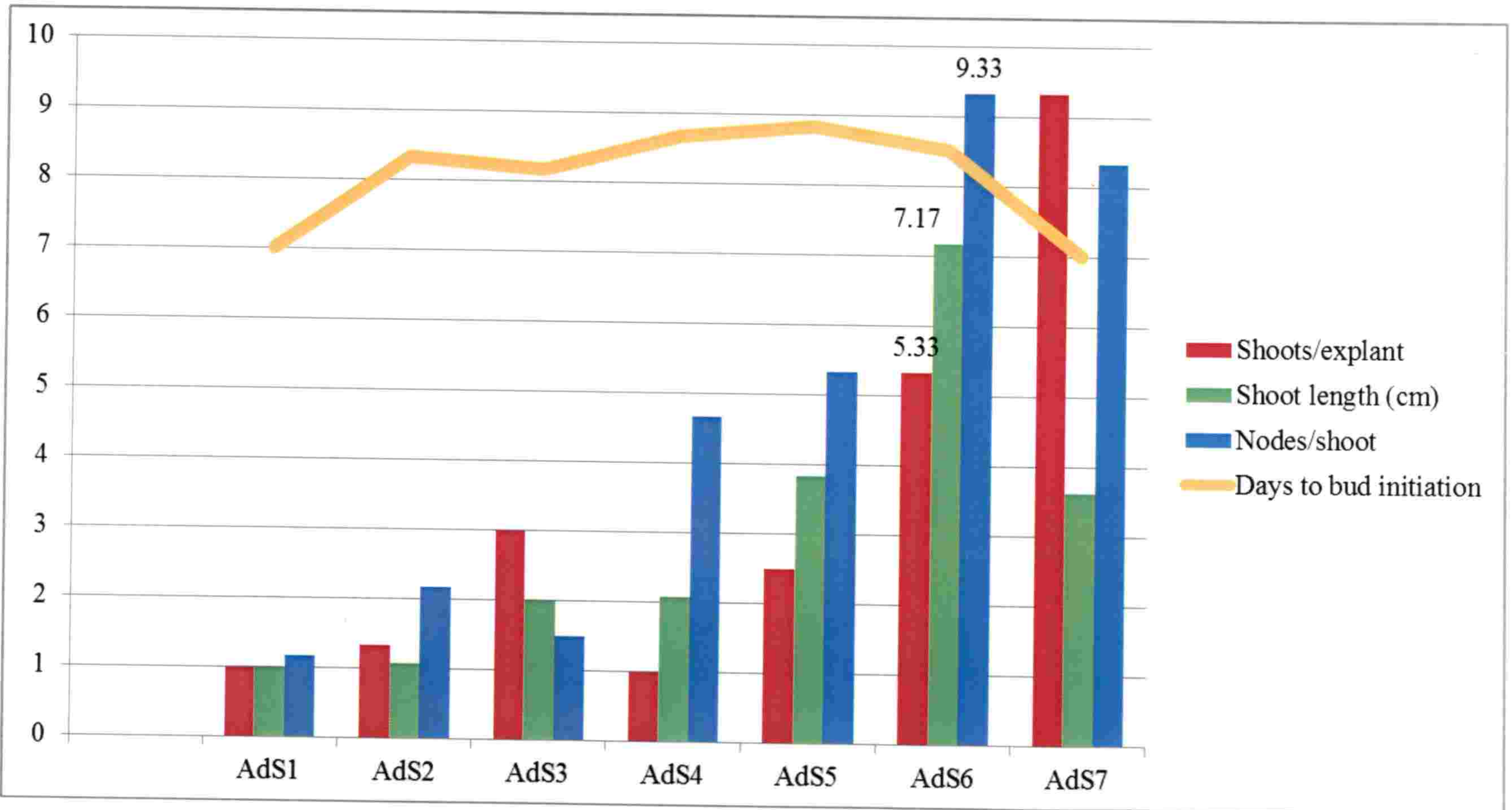


Fig 3. Effect of adenine sulphate on shoot proliferation of *Aegle marmelos*

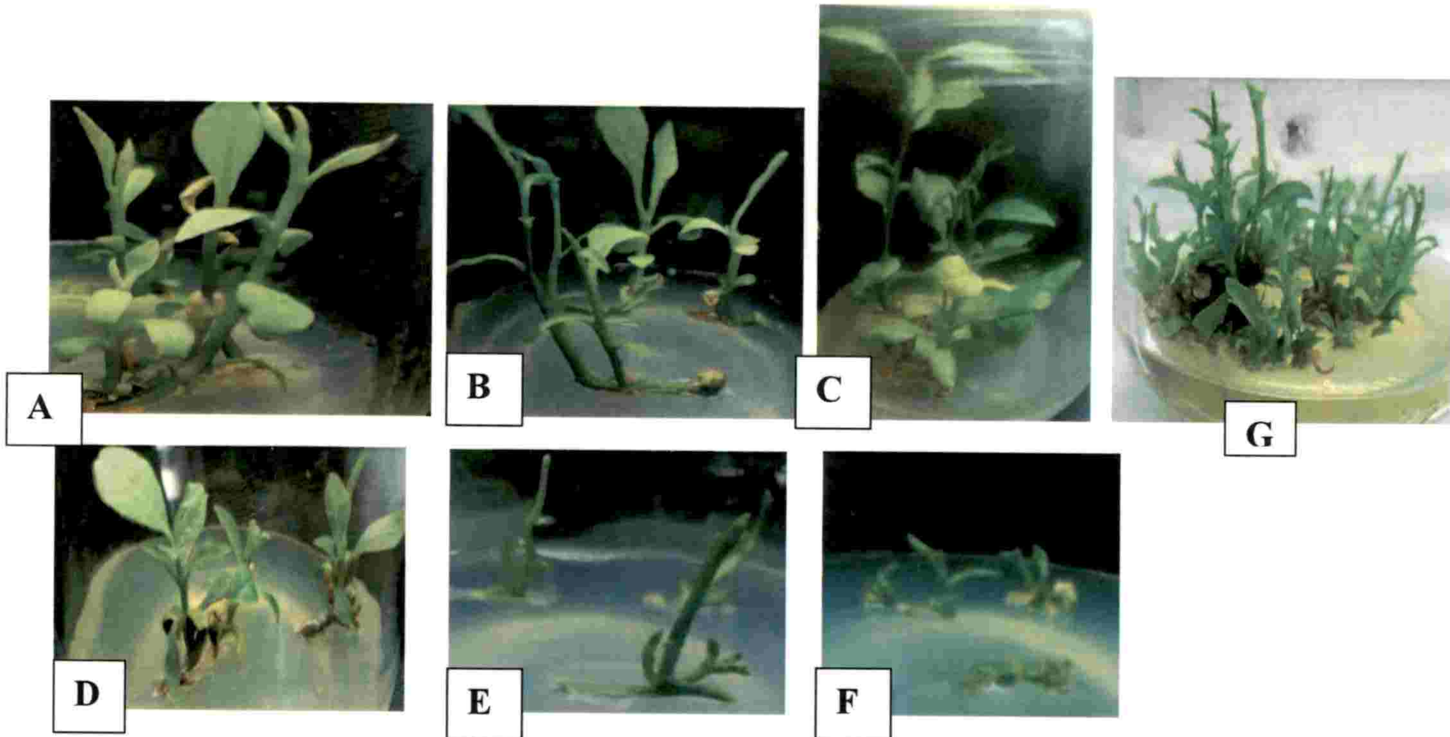


Plate 3: Effect of adenine sulphate on shoot proliferation of *A. marmelos*: A) SDM + adenine sulphate 20 mg L^{-1} B) SDM + adenine sulphate 40 mg L^{-1}, C) SDM + adenine sulphate 60 mg L^{-1} D) MS + adenine sulphate 20 mg L^{-1} E) MS + adenine sulphate 40 mg L^{-1} F) MS + adenine sulphate 60 mg L^{-1} G) SDM (MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1})

Basal culture medium: MS

per explant, shoot length and nodes per shoot (Plate 3). Days to bud initiation did not show significant variation among the different treatments. It ranged from 7 to 8.83 days.

In this experiment, the control (SDM) treatment gave maximum shoot proliferation with 9.33 shoots per explant. This was significantly superior to all other treatments, which were supplemented with adenine sulphate. Among the adenine sulphate supplemented medium, AdS6 (MS + adenine sulphate 60 mg L^{-1}) gave 5.33 shoots per explant. The least number of shoots (1.00) per explant were obtained in AdS1 (SDM + AdS 20 mg L^{-1}) and AdS4 (MS + AdS 20 mg L^{-1}). These were on par with AdS2 (SDM + AdS 40 mg L^{-1}).

However, shoot length was found to be maximum (7.17 cm) in the treatment AdS6, which was significantly higher compared to all other treatments. The least shoot length (1.00 cm) was obtained in AdS1. This was on par with AdS2, AdS3 and AdS4.

Also, AdS6 recorded highest number of nodes (9.33) per shoot and was found to be superior to all other treatments. The minimum number of nodes (1.17) per shoot was recorded in AdS1.

4.1.1.1.3 Thidiazuron

Six treatments (TDZ1 to TDZ6) with different concentrations of thidiazuron (0.5, 1 and 2 mg L^{-1}) were tried initially to study their effect on shoot multiplication. But these treatments did not give any morphogenesis. The explant turned yellow and later dried off. Hence a lower concentration (0.02 mg L^{-1}) of thidiazuron was tried. The results of the study are presented in Table 12 (Fig 4).

The two media supplemented with thidiazuron 0.02 mg L^{-1} (TDZ7 & 8) yielded 100 per cent regeneration. Buds were initiated early (7.00 days) in the

Table 12. Effect of Thidiazuron on shoot proliferation of *Aegle marmelos*

Treatment No.	TDZ (mg L^{-1})	BA (mg L^{-1})	IBA (mg L^{-1})	RG (%)	DBI	SpE	SL	NpS
TDZ 7	0.02	2	0.5	100	8.00±0.89	20.00±0.32	5.50±0.61	15.56±0.43
TDZ 8	0.02	-	-	100	9.13±1.21	3.15±0.76	4.35±0.15	6.23±0.95
Control (SDM)	-	2	0.5	100	7.00±0.45	9.33±1.63	3.63±0.63	8.33±1.45

RG – Regeneration; DBI – Days to Bud Initiation; SpE – Shoots per Explant; SL- Shoot length; NpS- Nodes per shoot

Basal culture medium: MS

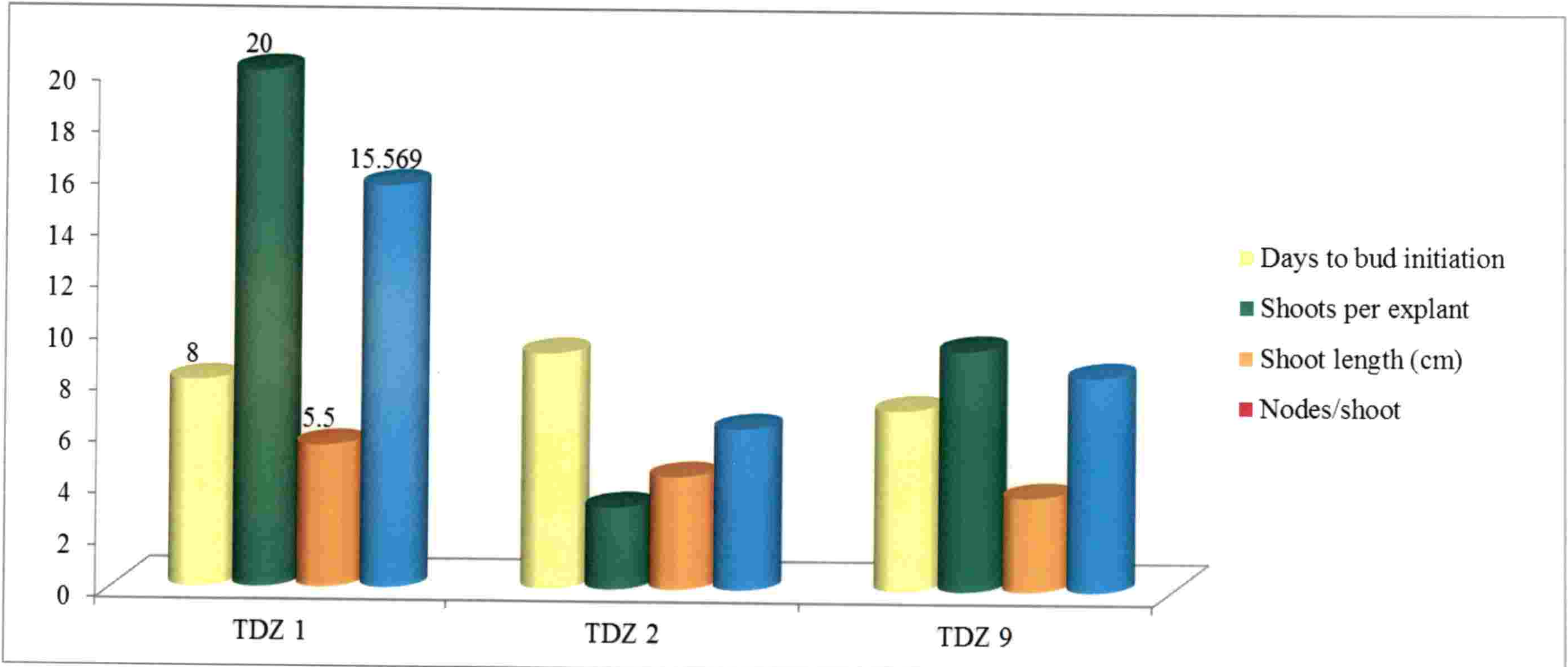
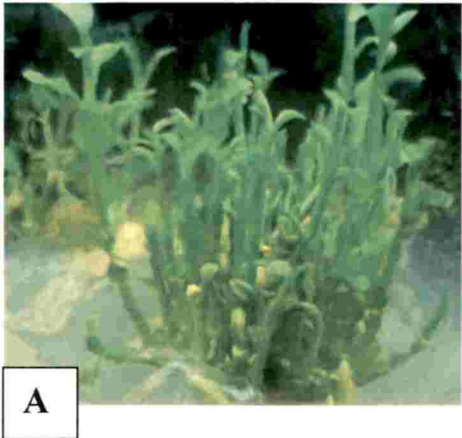


Fig 4. Effect of thidiazuron on shoot proliferation of *Aegle marmelos*



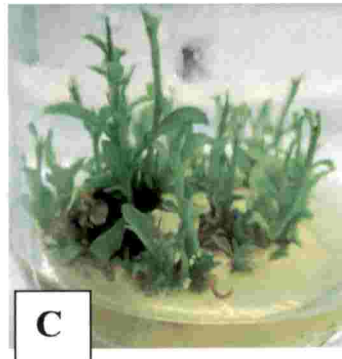


Plate 4: Effect of thidiazuron on shoot proliferation *A. marmelos* : A) SDM + TDZ 0.02 mg L^{-1} , B) MS + TDZ 0.02 mg L^{-1}, C) SDM (MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1})

Basal culture medium: MS

control compared to thidiazuron supplemented medium. The bud initiation started in 8 to 9 days in thidiazuron supplemented medium (Plate 4). TDZ7 (SDM + thidiazuron 0.02 mg L^{-1}) gave better shoot proliferation (20 shoots per explant) compared to control (9.33 shoots per explant) and TDZ 8 (MS + thidiazuron 0.02 mg L^{-1}) (3.15 shoots per explant). Shoot length (5.50 cm, 4.35cm and 3.63 cm) and nodes (15.56, 6.23 and 8.33) per shoot were obtained in TDZ7, TDZ8 and control, respectively.

The additives chitosan (CH), thidiazuron (TDZ) and adenine sulphate (AdS) at different concentrations were supplemented in two different media *i.e.* 1) hormone free MS medium and 2) MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1} to study their effect on shoot proliferation. The best shoot proliferation response obtained for each additives were MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1} + chitosan 10 mg L^{-1} (35.67 shoots per explant), MS + AdS 60mg L^{-1} (5.33 shoots per explant) and MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1} + TDZ 0.02 mg L^{-1} (20.00 shoots per explant). Even though higher shoot proliferation was observed in CH and TDZ supplemented media, they exhibited morphological abnormalities. Normal shoots were obtained with AdS supplemented medium, but the shoot proliferation was less compared to MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1}. Hence MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1} was identified as the best shoot proliferation medium and this medium was used as basal medium in the cryopreservation studies.

4.2 PHASE II: *IN VITRO CONSERVATION*

4.2.1 Cryopreservation of *Aegle marmelos* using encapsulation-dehydration technique

In the study, a protocol has been developed for long term conservation of *A. marmelos via* encapsulation-dehydration technique of cryopreservation. The different steps of encapsulation-dehydration technique *viz.,* preconditioning, encapsulation, pre-culture, dehydration and recovery were standardized.

Table 13. Effect of different additives *viz.*, Chitosan (CH), Adenine sulphate (AdS) and Thidiazuron (TDZ) on shoot proliferation of *Aegle marmelos*

Treatment No.	Additives (mg L^{-1})	BA (mg L^{-1})	IBA (mg L^{-1})	RG (%)	DBI	SpE	SL	NpS
CH1	10	2	0.5	100	7.00±1.35	35.67 (5.97) ±0.03[a]	5.23±0.58[b]	8.50±0.95[bc]
CH2	20	2	0.5	100	8.00±3.36	7.00 (2.63) ±0.14[c]	1.65±0.40[e]	4.16±1.01[e]
CH3	30	2	0.5	100	9.00±3.98	2.33 (1.50) ±0.18[e]	1.08±0.27[g]	1.50±0.38[f]
CH4	10	-	-	100	7.00±0.29	6.00 (2.40) ±0.32[d]	3.48±0.08[d]	5.33±0.13[d]
CH5	20	-	-	100	7.17±2.17	2.17 (1.47) ±0.01[e]	1.00±0.17[f]	1.66±0.29[f]
CH6	30	-	-	100	8.17±0.89	2.83 (1.66) ±0.14[e]	1.77±0.11[e]	1.83±0.11[f]
AdS1	20	2	0.5	100	7.00±0.78	1.00 (0.99) ±0.05[f]	1.00±0.11[f]	1.17±0.01[g]
AdS2	40	2	0.5	100	8.33±0.73	1.33 (1.13) ±0.14[f]	1.07±0.25[f]	2.17±0.02[f]
AdS3	60	2	0.5	100	8.17±0.31	3.00 (1.70) ±0.22[e]	2.00±0.51[e]	1.50±0.02[f]
AdS4	20	-	-	100	8.67±0.87	1.00 (0.99) ±0.01[f]	2.07±0.05[e]	4.67±0.06[e]
AdS5	40	-	-	100	8.83±0.39	2.50 (1.56) ±0.14[e]	3.83±0.67[c]	5.33±0.06[d]
AdS6	60	-	-	100	8.50±1.02	5.33 (2.30) ±0.07[d]	7.17±0.45[a]	9.33±0.23[b]
TDZ 7	0.02	2	0.5	100	8.00±0.20	20.00 (4.47) ±0.02[b]	5.50±0.13[b]	15.57±0.39[a]
TDZ 8	0.02	-	-	100	9.10±0.19	3.15 (1.77) ±0.08[e]	4.36±0.09[c]	6.23±0.13[d]
SDM	-	2	0.5	100	7.00±0.45	9.33 (3.03) ±0.27[c]	3.63±0.63[d]	8.33± 1.45[c]
CD (5%)	-	-	-	-	NS	0.465	0.685	0.995

RG – Regeneration; DBI – Days to Bud Initiation; SpE – Shoots per Explant; SL- Shoot length; NpS- Nodes per shoot, Transformed (square root) values are presented in the parenthesis.

Basal culture medium: MS

4.2.1.1 Preconditioning

Single node segments containing an axillary bud excised from the *in vitro* maintained cultures of *A. marmelos* were used as explant for preconditioning experiments. The preconditioning treatments included exposure of the explants to different concentrations of sucrose in hormone free semisolid MS medium for varying periods (Table 5). The explants from various preconditioning treatments were inoculated on to MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1} (standardised as best shoot proliferation medium in Phase I studies), to study the plant regeneration/proliferation responses of preconditioned explants.

The explants from all the preconditioning treatments showed 100 per cent survival in the inoculation medium. Significant variation was observed with respect to the different parameters like days to bud initiation, shoots per explant, shoot length and nodes per shoot. The results of the study are represented in the Table 14 and Fig 5.

The bud initiated early (8.00 days) in the explants from the treatment PC1 and PC2 (sucrose 0.1 M for 1 day and 7 days respectively). These were found to be on par with PC5, PC6, PC7, PC9 and PC10. Bud initiation was late (15.33 days) in PC12 (sucrose 0.5 M for 21 days) followed by PC3, PC4, PC8 and PC11, which were on par.

Regarding the number of shoots produced, explant from PC2 (sucrose 0.1 M for 7 days) gave the best result (5.50 shoots per explant) which was on par with those from PC5 (sucrose 0.3 M for 1 day). The least number of shoots (1.00) per explant was produced from explants from PC6 (sucrose 0.3 M for 7 days) and PC12 (sucrose 0.5 M for 21 days) which was on par with those from PC3 and PC4.

Table 14. Effect of preconditioning duration and sucrose concentration on plant regeneration

Treat ment No.	Semi-solid MS + Sucrose (M)	No. of days	RG (%)	DBI	SpE	SL	NpS
PC1	0.1	1	100	8.00±0.89[b]	3.50±0.39[b]	7.50±0.89[a]	6.50±0.72[b]
PC2	0.1	7	100	8.00±1.94[b]	5.50±1.33[a]	6.50±1.57[a]	9.83±2.38[a]
PC3	0.1	14	100	11.00±2.80[a]	1.33±0.34[c]	5.40±1.37[a]	7.00±1.78[a]
PC4	0.1	21	100	13.00±0.31[a]	1.23±0.03[c]	1.67±0.04[c]	4.17±0.10[b]
PC5	0.3	1	100	8.67±1.51[b]	5.43±0.95[a]	7.00±1.22[a]	6.00±1.05[b]
PC6	0.3	7	100	9.00±0.57[b]	1.00±0.06[c]	6.00±0.38[a]	4.17±0.26[b]
PC7	0.3	14	100	10.00±0.17[b]	1.83±0.03[c]	2.78±0.04[b]	2.17±0.03[c]
PC8	0.3	21	100	14.67±2.70[a]	3.00±0.31[b]	5.17± 0.95[a]	2.00±0.36[c]
PC9	0.5	1	100	8.33±1.44[b]	2.67±0.55[b]	5.00±0.86[a]	7.17±1.24[a]
PC10	0.5	7	100	10.00±0.12[b]	1.73±0.02[c]	2.83±0.03[b]	6.33±0.08[b]
PC11	0.5	14	100	12.67±0.12[a]	1.67±0.01[c]	4.58±0.04[b]	5.03±0.05[b]
PC12	0.5	21	100	15.33±0.12[a]	1.00±0.06[c]	3.17±0.02[b]	2.67±0.02[c]
CD (5%)	-	-	-	4.239	1.630	2.518	3.007

RG – Regeneration; DBI – Days to Bud Initiation; SpE – Shoots per Explant; SL- Shoot length; NpS- Nodes per shoot

Inoculation medium: MS+ BA 2 mg L^{-1} +IBA 0.5 mg L^{-1}

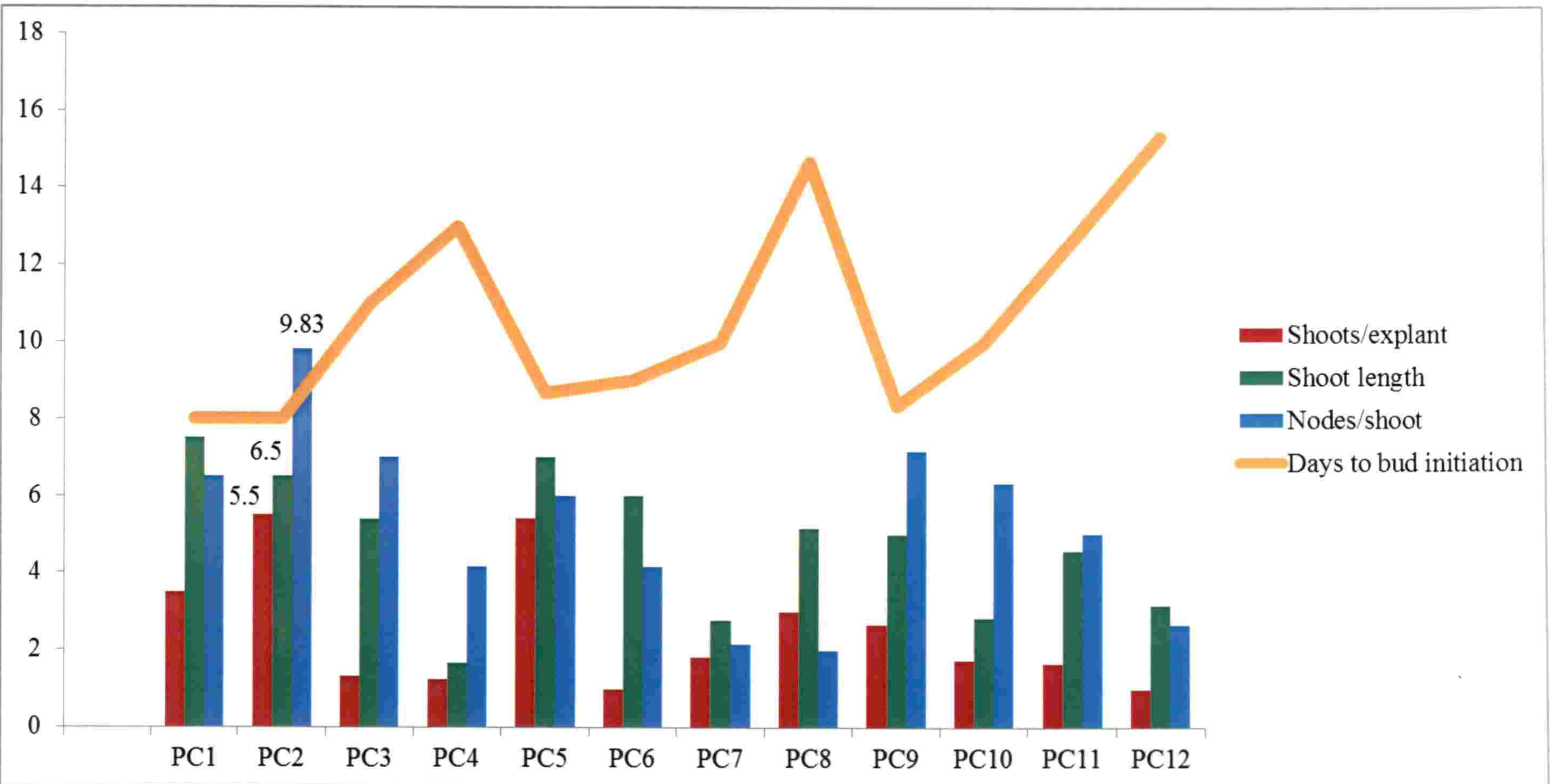


Fig 5. Effect of preconditioning duration and sucrose concentration on plant regeneration

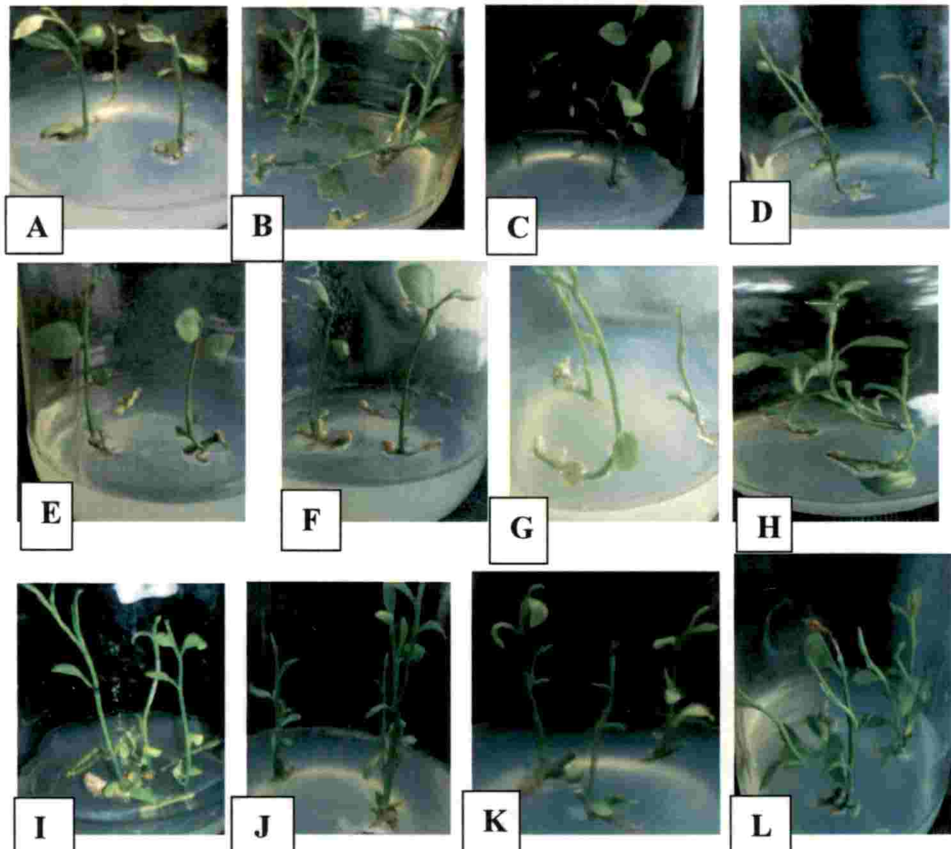


Plate 5. Effect of preconditioning duration and sucrose concentration on plant regeneration (A) 0.1 M for 1 day, (B) 0.1 M for 7 days, (C) 0.1M for 14 days, (D) 0.1M for 21 days, (E) 0.3 M for 1day, (F) 0.3 M for 7 days, (G) 0.3 M for 14 days, (H) 0.3 M for 21 days, (I) 0.5 M for 1 day, (J) 0.5 M for 7 days, (K)0.5 M for 14 days (L) 0.5 M for 21 days

Inoculation medium: MS + BA 2mg L^{-1} + IBA 0.5 mg L^{-1}

The explants from PC1 (sucrose 0.1 M for 1 day) recorded maximum shoot length (7.50 cm) which was on par with those from PC 2, PC3, PC5, PC6, PC8 and PC9. The lowest shoot length (1.67 cm) was recorded in the explants from PC4 (sucrose 0.1 M for 21 days), which was on par with those from PC7, PC10 and PC12.

Maximum number of nodes (9.83) per shoot was recorded in the explant from PC2 (sucrose 0.1 M for 7 days) which was on par with those from PC3 and PC9. The minimum number of nodes (2.00) per shoot was recorded in explants from PC8 (sucrose 0.3 M for 21 days) which was on par with those from PC7 and PC12.

Analysing the result, the best preconditioning treatment was found to be PC2 (sucrose 0.1 M for 7 days) in which the explant showed maximum shoot proliferation, shoot length and nodes per shoot. The plant regeneration response of the explants from various preconditioning treatments is presented in Plate 5.

4.2.1.2 Encapsulation

Single nodal segments containing an axillary bud were encapsulated using different concentrations of sodium alginate (2.5, 3, 3.5, 4 and 5% m/v) prepared in modified liquid MS (calcium free) and calcium chloride (50, 75, 100 and 200 mM) solution. Twenty encapsulation matrices formed by various combinations of sodium alginate and calcium chloride (Table 6) were tried to study their effect on plant regeneration. There was no bead formation in the treatments E1 to E5, E9, E13. The calcium alginate beads formed after encapsulation were cultured on the modified inoculation medium (½ MS + BA 2 mg L^{-1}+ IBA 0.5 mg L^{-1}). The beads cultured on inoculation medium (MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1}), they did not show any regeneration even after three months of incubation.

Table 15. Effect of different encapsulation matrices on bead formation and survival

Treatment No.	Encapsulation matrix		Beads formation	Survival (%)
	Liquid MS** + SA (%)	$CaCl_2$ (mM)		
E1*	2.5	50	No beads formation	-
E2*	2.5	75	No beads formation	-
E3*	2.5	100	No beads formation	-
E4*	2.5	200	No beads formation	-
E5*	3.0	50	No beads formation	-
E6	3.0	75	Formation of beads	100.00
E7	3.0	100	Formation of beads	100.00
E8*	3.0	200	Formation of beads	33.33
E9	3.5	50	No beads formation	-
E10	3.5	75	Formation of beads	100.00
E11	3.5	100	Formation of beads	100.00
E12*	3.5	200	Formation of beads	33.33
E13	4.0	50	No beads formation	-
E14	4.0	75	Formation of beads	100.00
E15	4.0	100	Formation of beads	100.00
E16*	4.0	200	Formation of beads	16.67
E17	5.0	50	Formation of beads	100.00
E18	5.0	75	Formation of beads	100.00
E19	5.0	100	Formation of beads	100.00
E20*	5.0	200	Formation of beads	16.67

SA - Sodium alginate; * Treatments were excluded from further experiments
** Calcium free liquid MS medium

Modified Inoculation medium: ½ MS + BA 2 mg L^{-1} +IBA 0.5 mg L^{-1}

Treatments *viz.,* E8, E12, E16 and E20 that gave less than 50 per cent survival were also excluded from statistical analysis. The encapsulated beads formed in the treatments E6, E7, E10, E11, E14, E15, E17, E18 and E19 gave 100 per cent survival (Table 15) when cultured on modified inoculation medium.

A low level of significance was observed among the beads developed in different encapsulation matrices, with respect to days to bud initiation. The earliest bud initiation was found in beads formed in E6 and E11. These were found to be on par with all other encapsulation matrices except the beads formed in E19, which took 17.33 days, the last to initiate buds.

The parameters, shoots per explant, shoot length and nodes per shoot showed significant variation among beads developed in different encapsulation matrices.

The beads from E11 (SA 3.5% in modified liquid MS + $CaCl_2$ 100 mM) gave the maximum number of shoots (6.67) per explant, shoot length (4.67 cm) and nodes (9.00) per shoot. This treatment was significantly superior to all other treatments with respect to shoots per explant and nodes per shoot. The shoot length was found to be on par with the beads formed in E6, E7, E14, E15 and E17. The lowest value with respect to shoots (1.33) per explant and shoot length (1.00) was recorded in E19 (SA 5% in modified liquid MS + $CaCl_2$ 100 mM). The minimum number of nodes (1.17) per shoot was observed in E18 (SA 5 % in modified liquid MS + $CaCl_2$ 75 mM) which was observed to be on par with E17 and E19.

From the encapsulation experiment, the best encapsulation matrix was identified as E11 (SA 3.5 % in modified liquid MS + $CaCl_2$ 100 mM) based on its response to plant regeneration, in the modified inoculation medium.

The results of the study are presented in Table 16 and Fig. 6.

Table 16. Effect of different encapsulation matrices on plant regeneration

Treatment No.	Encapsulation matrix		RG (%)	DBI	SpE	SL (cm)	NpS
	Liquid MS* + SA (%)	$CaCl_2$ (mM)					
E6	3.0	75	100	9.00±1.00[b]	2.50±0.28[b]	4.00±0.46[a]	4.17±0.46[b]
E7	3.0	100	100	11.00±2.66[b]	2.83±0.68[b]	4.33±1.05[a]	5.33±1.29[b]
E10	3.5	75	100	9.33±2.38[b]	2.07±0.52[b]	2.03±0.51[b]	5.50±0.42[b]
E11	3.5	100	100	9.00±0.22[b]	6.67±0.16[a]	4.67±0.11[a]	9.00±0.22[a]
E14	4.0	75	100	10.00±1.74[b]	3.17±0.55[b]	4.50±0.78[a]	7.50±0.46[a]
E15	4.0	100	100	13.67±0.86[a]	1.83±0.11[c]	3.83±0.24[a]	7.40±0.42[a]
E17	5.0	50	100	10.33±0.17[b]	2.00±0.03[b]	3.67±0.06[a]	1.33±0.02[c]
E18	5.0	75	100	14.67±2.70[a]	2.10±0.38[b]	1.50±0.27[b]	1.17±0.21[c]
E19	5.0	100	100	17.33±3.00[a]	1.33±0.23[c]	1.00±0.17[b]	1.50±0.26[c]
CD (5%)	-	-	-	5.82	1.226	1.636	2.579

RG – Regeneration; DBI – Days to Bud Initiation; SpE- Shoots per Explant; SL- Shoot length; NpS- Nodes per shoot; * Calcium free liquid MS medium

Modified inoculation medium: ½ MS+ BA 2 mg L^{-1} +IBA 0.5 mg L^{-1}

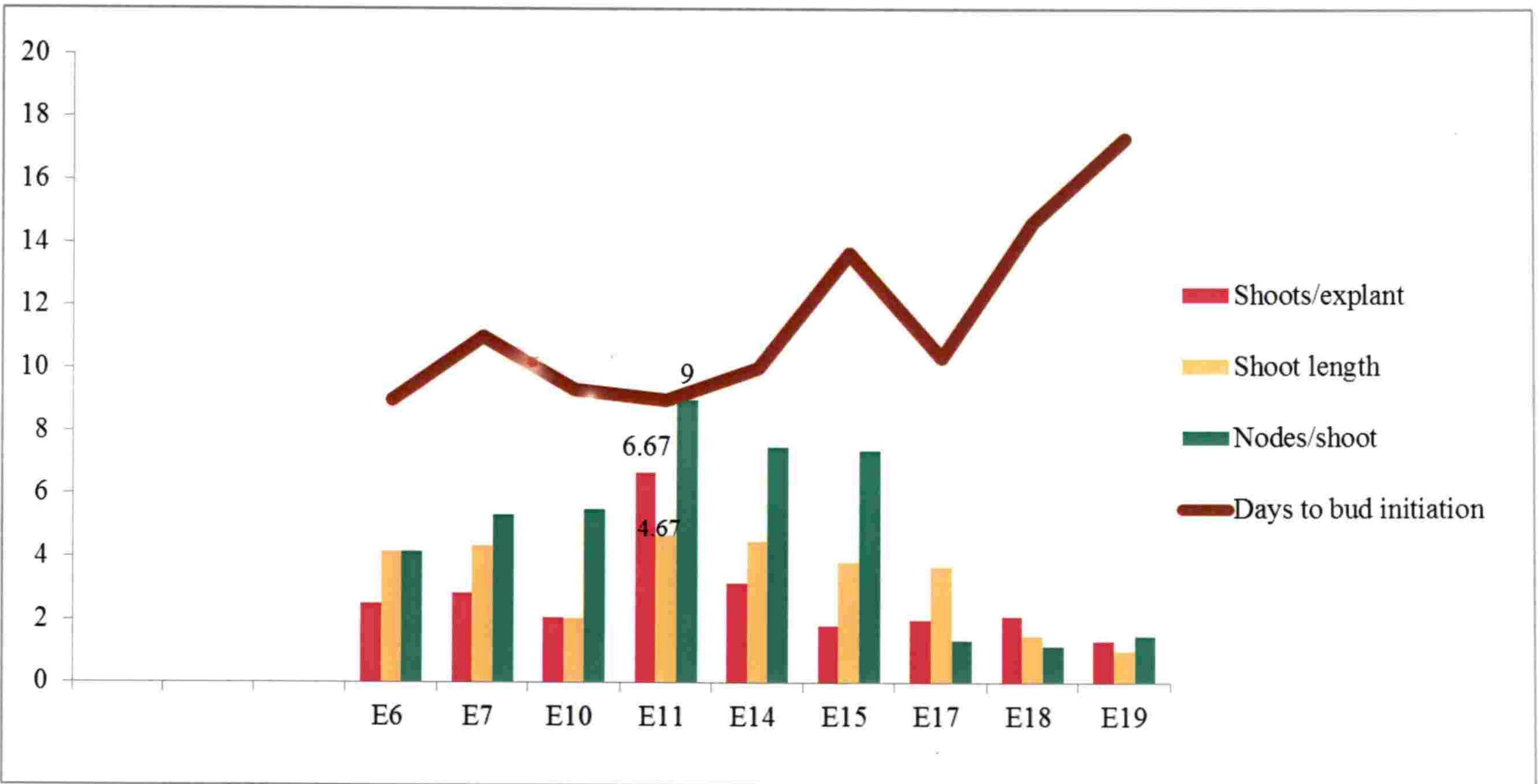


Fig 6. Effect of different encapsulation matrices on plant regeneration

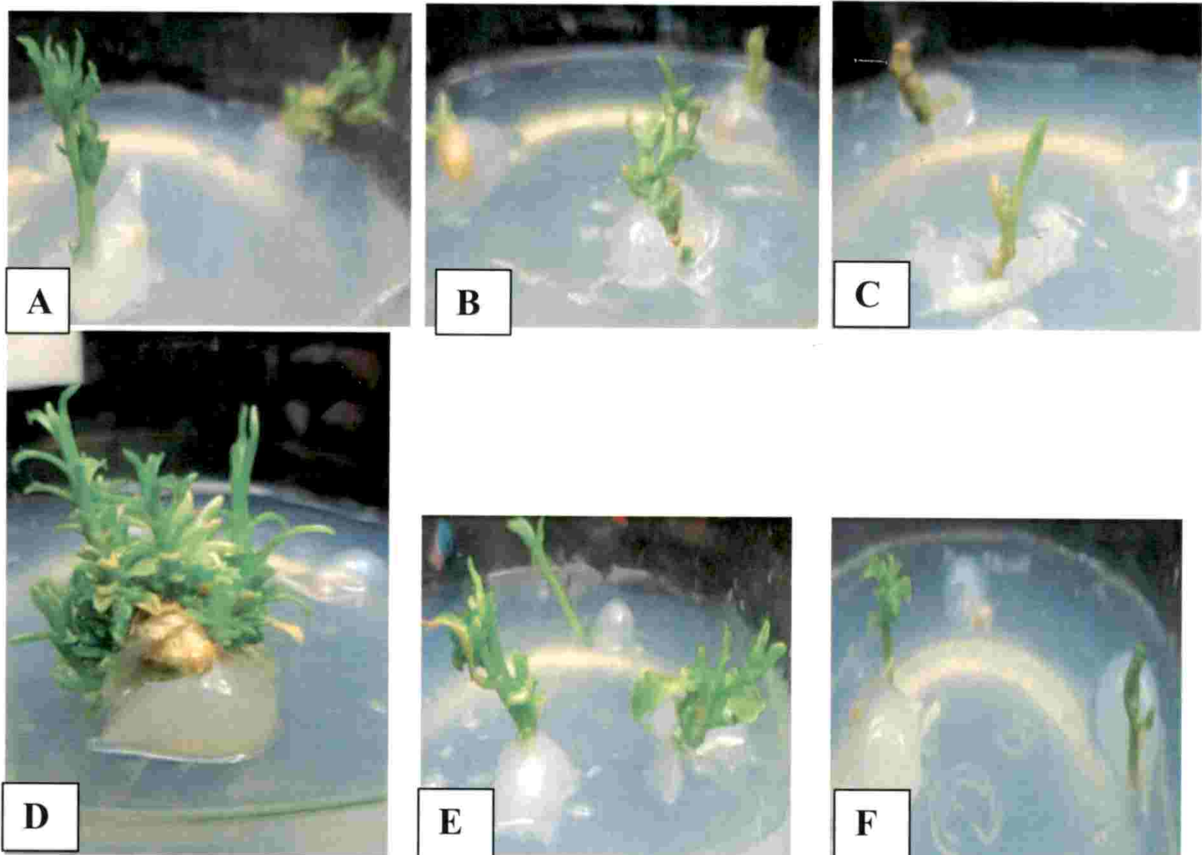


Plate 6. Plant regeneration from encapsulated beads A) 3.5% Sodium alginate + 75mM $CaCl_2$,B) 4% Sodium alginate + 75mM $CaCl_2$,C) 5% Sodium alginate + 75mM $CaCl_2$, D) 3.5% Sodium alginate + 100 mM $CaCl_2$,E) 4% Sodium alginate + 100 mM $CaCl_2$, F) 5% Sodium alginate + 100 mM $CaCl_2$

Modified Inoculation medium: ½ MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1}

4.2.1.3 Pre-culture

Preconditioned (sucrose 0.1 M in semisolid MS medium for 7 days) and encapsulated (SA 3.5 % in modified liquid MS + $CaCl_2$ 100 mM) axillary buds were used as explants in pre-culture studies. The beads thus formed were subjected to fifteen pre-culture treatments involving different concentrations of sucrose (0.1, 0.5 and 1 M) in hormone free MS liquid medium supplemented with 3 per cent DMSO for different time periods (1-5 days). The beads were taken out from the medium after the respective periods of culturing and were inoculated on to the modified inoculation medium (½ MS+BA 2 mg L^{-1}+ IBA 0.5 mg L^{-1}). The results obtained are illustrated in Table 17 (Fig. 7).

A cent per regeneration was recorded in pre-culture treatments, PT1 to PT3 (liquid MS + 3 % DMSO + sucrose 0.1 M for 1-3 days) and PT6 to PT8 (liquid MS + 3 % DMSO + sucrose 0.5 M for 1-3 days). The higher concentration of sucrose (1 M) did not support the growth and regeneration of the plants irrespective of the different periods tried. The treatments PT11 to PT15 (liquid MS + 3 % DMSO + sucrose 1.0 M for 1-5days) did not give any regeneration when the beads from them were cultured on to the inoculation medium. Hence, these five treatments were excluded from statistical analysis. The treatments PT4 (sucrose 0.1 M for 4 days), PT5 (sucrose 0.1 M for 5 days), PT 9 (sucrose 0.5 M for 4 days) and PT 10 (sucrose 0.5 M for 5 days) recorded a regeneration per cent of 83.33, 66.67, 66.67 and 50.00 respectively. The regeneration of the plants after pre-culture is illustrated in Plate 7.

Among the ten treatments, which gave plant regeneration, no significant variation was observed with respect to days to bud initiation. However, all these treatments exhibited a delay in bud initiation compared to the normal *in vitro* regeneration. The days to bud initiation ranged from 19 -25 days (Table 17) after pre-culturing the beads as against 9 days (Table 16) in normal *in vitro* regeneration.

Table 17. Effect of different pre-culture treatment on plant regeneration

Treatment No.	Pre-culture treatment		RG (%)	DBI	SpE	SL (cm)	NpS
	Liquid MS+ 3% DMSO + Sucrose (M)	Period of exposure					
PT1	0.1	1	100.00	21.67±0.14	1.00±0.11[c]	1.57±0.01[b]	1.33±0.02[e]
PT2	0.1	2	100.00	22.67±0.22	2.33±0.34[b]	1.30±0.02[c]	1.63±0.02[d]
PT3	0.1	3	100.00	23.66±0.23	1.33±0.45[c]	1.83±0.02[a]	1.63±0.02[d]
PT4	0.1	4	83.33	23.00±0.43	1.56±0.03[c]	1.07±0.03[d]	1.35±0.03[e]
PT5	0.1	5	66.67	23.33±0.03	1.00±0.20[c]	1.00±0.02[d]	1.24±0.02[f]
PT6	0.5	1	100.00	24.73±0.41	2.00±0.11[b]	1.27±0.02[c]	2.97±0.03[b]
PT7	0.5	2	100.00	22.67±0.34	2.67±0.03[b]	1.40±0.02[b]	2.83±0.02[c]
PT8	0.5	3	100.00	19.00±0.03	3.67±0.17[a]	2.00±0.02[a]	3.33±0.01[a]
PT9	0.5	4	66.67	24.67±0.20	1.23±0.17[c]	1.00±0.11[d]	1.00±0.02[g]
PT10	0.5	5	50.00	25.33±0.22	1.00±0.21[c]	1.00±0.18[d]	1.00±0.03[g]
PT11*	1	1	-	-	-	-	-
PT12*	1	2	-	-	-	-	-
PT13*	1	3	-	-	-	-	-
PT14*	1	4	-	-	-	-	-
PT15*	1	5	-	-	-	-	-
CD (5%)	-	-	-	NS	0.938	0.177	0.078

RG – Regeneration; DBI – Days to Bud Initiation; SpE – Shoots per Explant; SL- Shoot length; NpS- Nodes per shoot, * Treatments were excluded from statistical analysis.

Modified inoculation medium: ½ MS + BA 2 mg L^{-1} +IBA 0.5 mg L^{-1}.

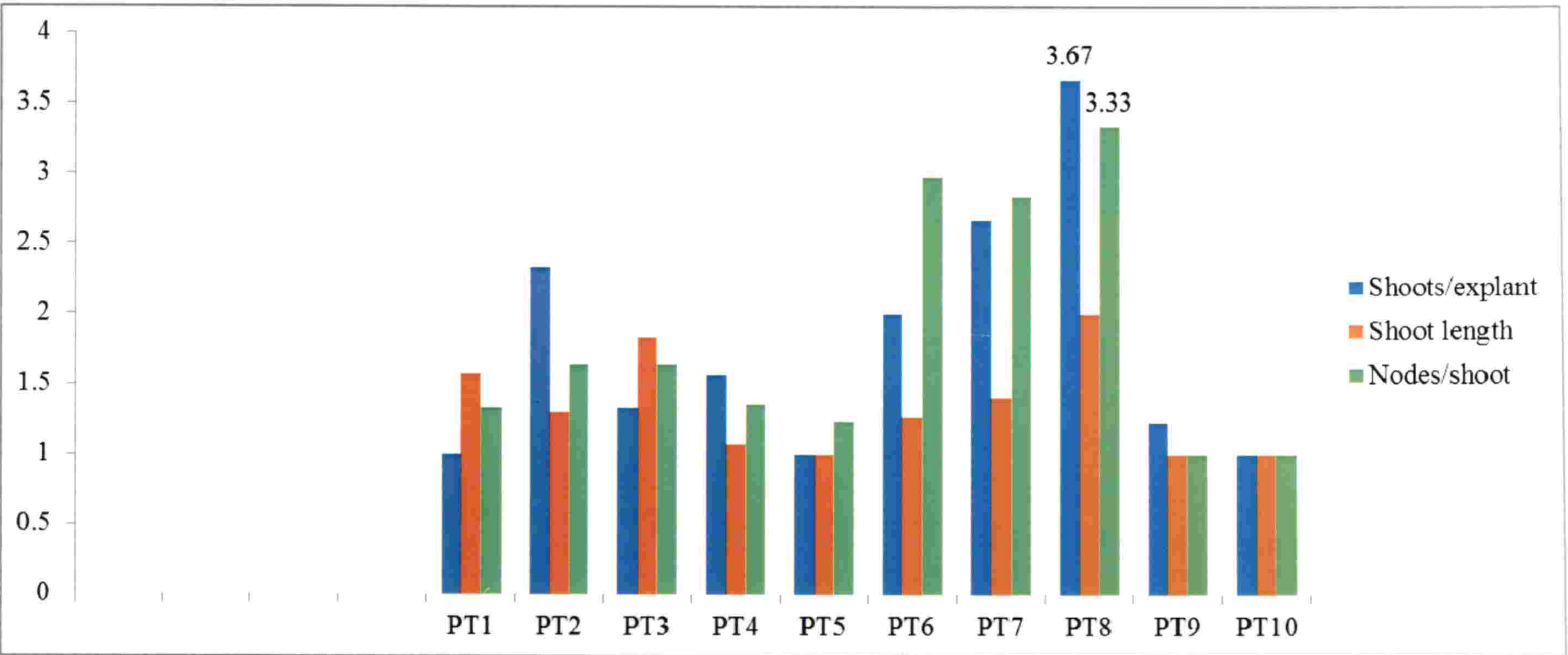


Fig 7. Effect of different pre-culture treatment on plant regeneration

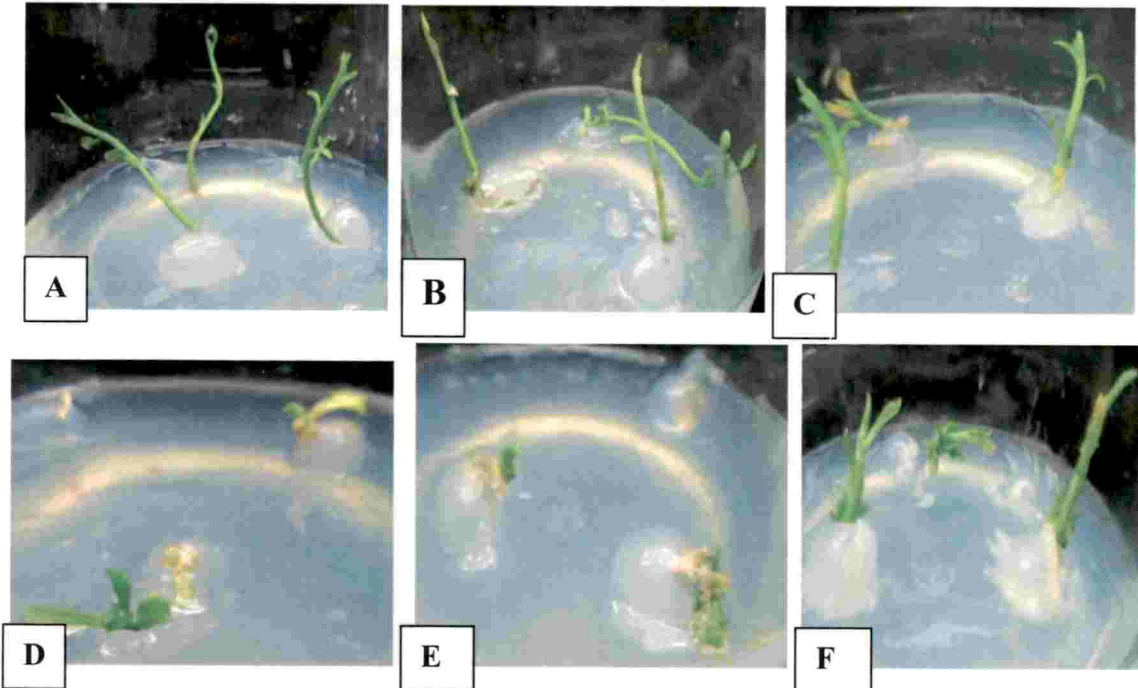


Plate 7. Effect of different pre-culture treatment on plant regeneration (A) 0.1 M sucrose + 3% DMSO for 1 day (B) 0.1 M sucrose + 3% DMSO for 2 day (C) 0.1 M sucrose + 3% DMSO for 3 days (D) 0.5 M sucrose + 3% DMSO for 1 day (E) 0.5 M sucrose + 3% DMSO for 2 day (F) 0.5 M sucrose + 3% DMSO for3 days.

Inoculation medium: ½ MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1}

The treatment PT8 (liquid MS + DMSO 3% + sucrose 0.5 M for 3 days) registered the highest value in terms of shoots (3.67) per explants, shoot length (2 cm) and nodes (3.33) per shoot. This treatment was found to be significantly superior to all other treatments with regard to the said parameters.

The least number of shoots (1.00) per explant was observed in PT1 (liquid MS + DMSO 3% + sucrose 0.1 M for one day), PT5 (liquid MS + DMSO 3% + sucrose 0.1 M for 5 days) and PT10 (liquid MS + DMSO 3% + sucrose 0.5 M for 5 days) which were on par with PT3, PT4 and PT9. The minimum value in terms of shoot length (1.00 cm) was seen in the treatment PT5 (liquid MS + DMSO 3% + sucrose 0.1 M for 5 days), PT9 (liquid MS + DMSO 3% + sucrose 0.5 M for 4 days) and PT10 (liquid MS + DMSO 3% + sucrose 0.5 M for 5 days) which were on par with PT4. The minimum number of nodes (1.00) per shoot was seen in the treatment PT9 (liquid MS + DMSO 3% + sucrose 0.5 M for 4 days) and PT10 (liquid MS + DMSO 3% +sucrose 0.5 M for 5 days).

The preconditioned and encapsulated beads subjected to PT8 (liquid MS + DMSO 3% + sucrose 0.5 M for 3 days) gave maximum shoot multiplication in the inoculation medium and hence, this was selected as the best pre-culture medium.

4.2.1.4 Dehydration, Freezing in LN and Recovery

After preconditioning, encapsulation and pre-culture treatments with the best results obtained in the respective steps, the axillary buds were subjected to different hours of dehydration (0 h, 1 h, 2 h, 3 h, 4 h, 5 h, 6 h and 7 h). Moisture content was determined after different periods of dehydration on a fresh weight basis and it ranged from 82 per cent to 12.6 per cent with 7 h of dehydration. The results are illustrated in Table 18 (Fig 8). At 0 h & 1h of desiccation, when the moisture content was 82 and 78 per cent respectively, the control plants gave 100 per cent regeneration and survival. At 2h of desiccation with 64.70 per cent

Table 18. Effect of dehydration on survival and plant regeneration before (-LN) and after (+LN) cryopreservation

Sl. No	Dehydration duration (h)	MC (%)	Survival (%)		Regeneration (%)	
			-LN	+LN	-LN	+LN
D0	0	82.00	100.00	0.00	100.00	0.00
D1	1	78.00	100.00	0.00	100.00	0.00
D2	2	64.70	100.00	0.00	83.33	0.00
D3	3	43.50	83.33	0.00	83.33	0.00
D4	4	38.10	83.33	0.00	66.67	0.00
D5	5	26.50	83.33	0.00	66.67	0.00
D6	6	19.50	83.33	66.67	66.67	50.00
D7	7	12.60	0.00	0.00	0.00	0.00

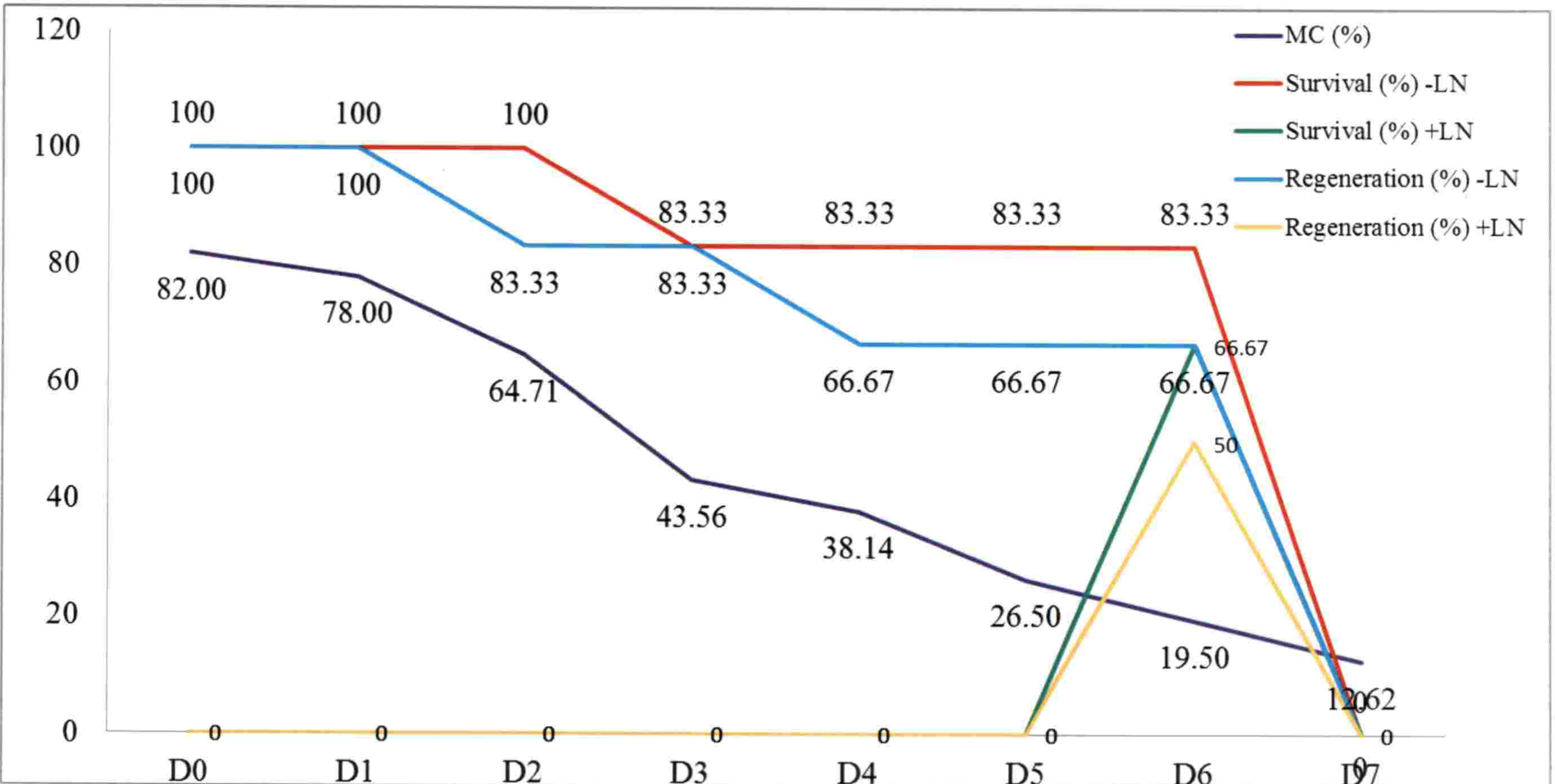


Fig 8. Effect of dehydration on survival and plant regeneration before (-LN) and after (+LN) cryopreservation

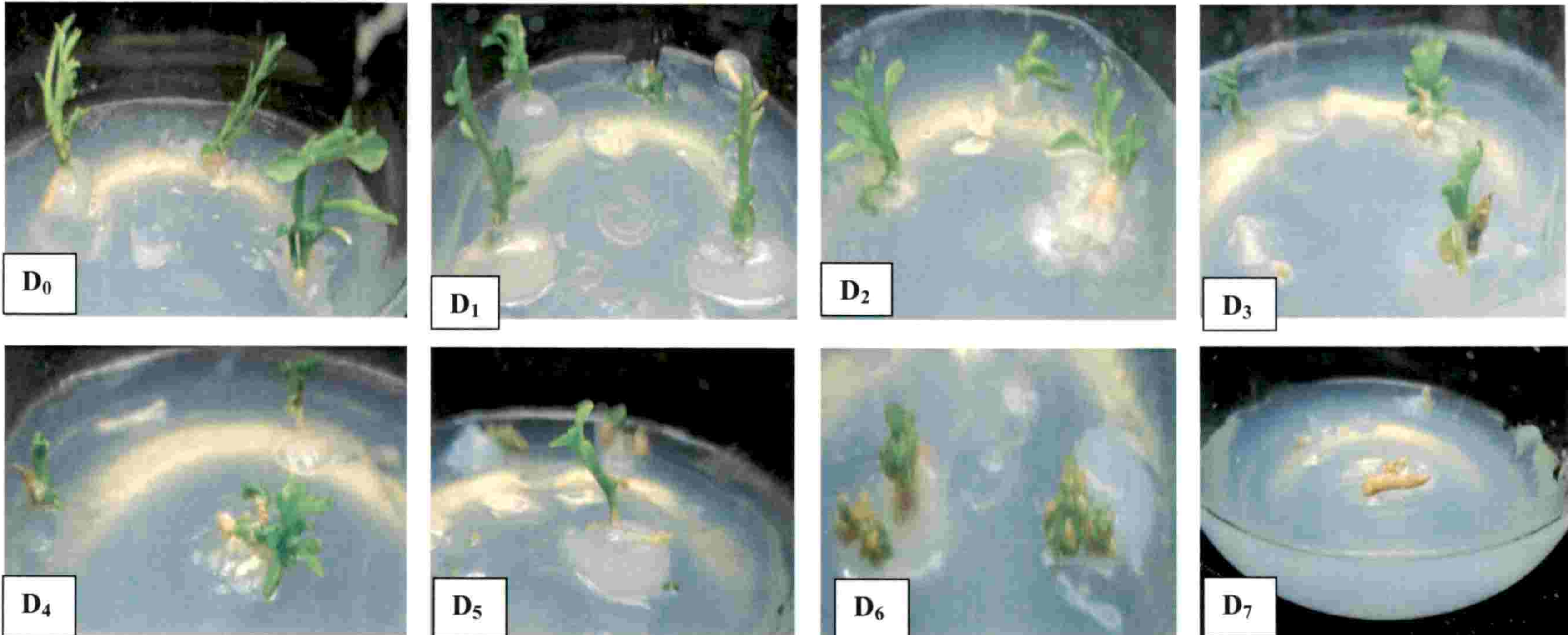


Plate8. Plant regeneration from dehydrated beads before cryostorage (D_0) Without dehydration (0 h), (D_1) Dehydration after 1h, (D_2) Dehydration after 2h, (D_3) Dehydration after 3h, (D_4) Dehydration after 4h, (D_5) Dehydration after 5h, (D_6) Dehydration after 6 h, (D_7) Dehydration after 7h.

Inoculation medium: ½ MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1}

moisture content 100 per cent survival and 83.33 per cent regeneration was obtained. With 3h to 6h of desiccation, the control plants showed a declining trend with respect to survival and regeneration reaching a level of 83.33 and 66.67 per cent respectively by the 6th h. After 7 h of desiccation the control did not survive at all.

After dehydration, the beads were cryopreserved in LN for 2 h (Plate 10) and then they were rewarmed and placed in the recovery medium to study the rate of survival and regeneration. Those beads desiccated at 0 to 5h after 2h of cryostorage, did not give any sign of survival, on culturing in the recovery medium. The beads that were subjected to 6h desiccation, resulting in a moisture content 19.50 per cent, gave 66.67 per cent survival and 50 per cent regeneration. Also, when the beads at 7 h of desiccation at a moisture content of 12.60 per cent, were cryopreserved for 2h, no survival could be observed when cultured on to the recovery medium.

The various plant regeneration parameters *viz,* days to bud initiation, shoots per explant, shoot length and nodes per shoot were recorded, when the desiccated beads were cultured on to the recovery medium, before and after cryopreservation. Cryopreserved beads gave regeneration only after 6h of desiccation. The days to bud initiation was higher in cryopreserved beads (32.96 days) compared to non cryopreserved ones (23.07 days). Cryopreserved beads recorded lower number of shoots (1.89) per explant, lower shoot length (1.5 cm) and lower number of nodes (2.00) per shoot compared to non cryopreserved beads. The value recorded for non cryopreserved beads were shoots (2.50) per explant, shoot length (2.35 cm) and nodes (2.47) per shoot was produced. The results are given in the Table 19. Among the control (non cryopreserved) treatments, maximum shoots per explant, shoot length and nodes per shoot was obtained at 0h of desiccation.

Table 19. Effect of desiccation on plant regeneration before (-LN) and after (+LN) cryopreservation

Dehydration duration (h)	DBI		SpE		SL (cm)		NpS	
	-LN	+LN	-LN	+LN	-LN	+LN	-LN	+LN
0	21.84±1.03	-	3.26±0.36	-	4.78±0.53[a]	-	4.95±0.55[a]	-
1	22.43±1.21	-	2.72±0.65	-	3.47±0.84[b]	-	3.55±0.86[ab]	-
2	23.75±0.53	-	2.42±0.61	-	3.94±1.00[ab]	-	2.83±0.07[b]	-
3	21.28±0.30	-	2.00±0.04	-	2.12±0.05[b]	-	2.86±0.51[b]	-
4	22.00±1.76	-	2.60±0.45	-	2.23±0.39[b]	-	2.96±0.18[b]	-
5	22.11±1.21	-	2.23±0.14	-	2.63±0.16[b]	-	2.90±0.01[b]	-
6	23.07±0.34	32.96±0.24	2.50±0.03	1.89±0.21	2.35±0.01[b]	1.50 ±0.37	2.47±0.18[b]	2.00±0.51
7	-	-	-	-	-	-	-	-
CD (5%)	NS		NS		1.711		1.672	-

DBI – Days to Bud Initiation; SpE – Shoots per Explant; SL- Shoot length; NpS- Nodes per shoot

Recovery medium: ½ MS + BA 2 mg L^{-1} +IBA 0.5 mg L^{-}

Table 20. Survival and regeneration of encapsulated axillary buds after different periods of cryostorage

Periods of cryostorage	Survival (%)	Regeneration (%)
2 h	66.67	50.00
1 Day	50.00	50.00
1 week	66.67	50.00

Recovery medium: ½ MS + BA 2 mg L^{-1} +IBA 0.5 mg L^{-}

Preconditioning

Encapsulation

Pre-culture

Dehydration

Cryopreservation

Thawing and Recovery

Plate 9: Steps in cryopreservation of *A. marmelos* using encapsulation-dehydration technique

2 h cryostored plants

1 day cryostored plants

1 week cryostored plants

Plate 10. Plant regeneration from dehydrated beads after cryostorage

Recovery medium: **½ MS + BA 2** mg L^{-1} + **IBA 0.5** mg L^{-1}

The beads when stored in liquid nitrogen for different duration and cultured on recovery medium did not show any significant variation with respect to survival and regeneration per cent (Table 20).

4.3 Estimation of genetic stability of cryopreserved materials using ISSR markers

ISSR profiles of plantlets regenerated from cryopreserved axillary buds were compared with those of the *in vitro* grown plantlets to assess the genetic stability. Among the nine different ISSR primers used, four primers *viz.,* UBC 807 (AGAGAGAGAGAGAGAGT), UBC 840 (GAG AGA GAG AGA GAG AYT), UBC 847 (CACACACACACACACART) and UBC 826 (ACACACACACACACACC) produced clear and distinguishable bands. The number of bands for each primer varied from 5 to 7.

ISSR profiles of the plantlets regenerated from cryopreserved materials were identical to those of control plants for all the four primers tested. Plate 11 illustrates amplified banding patterns produced by the four primers in plantlets regenerated after cryo storage and control plants. No difference was observed in the banding pattern of control and cryopreserved samples.

Table 21. ISSR markers screened for the assessment of genetic fidelity of plantlets regenerated from cryostorage

Sl. No.	Primers	Sequence (5'-3')	T_m (°C)	Remarks
1	UBC-810	GAGAGAGAGAGAGAGAT	42.9	No amplification
2	UBC-811	GAGAGAGAGAGAGAGAC	43.3	No amplification
3	UBC-827	ACACACACACACACACG	54.9	No amplification
4	HBO-816	CACACACACACACACAT	51.1	No amplification
5	UBC 807*	AGAGAGAGAGAGAGAGT	42.5	6 bands
6	UBC 840*	GAG AGA GAG AGA GAG AYT	52.9	6 bands
7	UBC 811	GAGAGAGAGAGAGAGAC	43.3	No amplification
8	UBC 847*	CACACACACACACACART	53.7	7 bands
9	UBC 826*	ACACACACACACACACC	53.3	5 bands

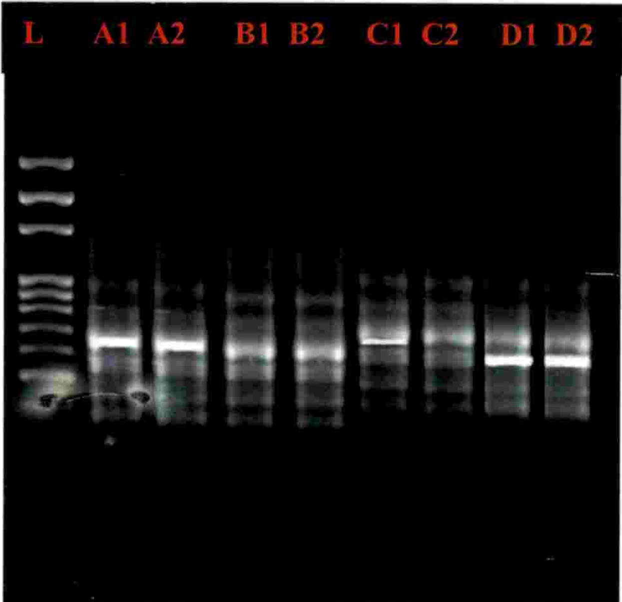


Plate 11. Comparison of ISSR banding profile between plants recovered from LN and non cryopreserved control plantlets

L- Ladder-100 bp
A_1 - UBC 807- Control
A_2 -UBC 807- Cryopreserved plants

B_1 - UBC 840- Control
B_2 - UBC 840- Cryopreserved plants

C_1 -UBC 847- Control
C_2 -UBC 847- Cryopreserved plants

D_1 -UBC 826- Control
D_2 -UBC 826- Cryopreserved plants

Discussion

5. DISCUSSION

The present study, 'Cryoconservation of koovalam (*Aegle marmelos* L. Corr.) by encapsulation-dehydration technique', was carried out during 2015-2017 at the Department of Plant Biotechnology, College of Agriculture, Vellayani. The results obtained are discussed in this chapter.

5.1 ENHANCEMENT OF MULTIPLICATION RATE

5.1.1 Effect of cytokinins and auxins on shoot multiplication

5.1.1.1 Medium

The basal culture medium used in the study was MS (Murashige and Skoog) medium, the mostly widely used medium in plant tissue culture. The types and concentration of media have a significant influence on proliferation and morphogenetic responses of plant tissues (Gantait *et al.,* 2016). The type and concentration of plant growth regulators incorporated in the culture medium have a profound effect on culture initiation, morphogenesis and multiplication. The ratio of auxins to cytokinins plays an important role in determining the morphogenetic responses in plant tissue culture systems. The cytokinins - auxins combinations in the culture medium stimulated *in vitro* multiplication and growth of shoots in several plant species (George, 1993). The auxin-cytokinin ratio is kept low for axillary shoot proliferation.

In the present study, different combinations of cytokinins and auxins were tried for inducing shoot proliferation in the nodal segments. Among the twelve treatments tried three treatments gave 100 per cent regeneration. Maximum shoot proliferation (9.33 shoots per explant) was observed in the treatment, MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1}. In consensus with this, Beena *et al.* (2003) reported that BA - IBA combination in MS medium gave good axillary bud proliferation in *Ceropegia candelabrum* L.

Bindhu (2015) reported that a combination of BAP 0.5 mg L^{-1} and IAA 0.1 mg L^{-1} induced the formation of maximum number (4.5) of shoots (2.5 cm). Similar combination of BA and IAA was also reported by Sandhiya *et al.* (2008) in *A. marmelos*. Das (2008) reported better shoot proliferation with BA (2 mg L^{-1}) + NAA (0.2 mg L^{-1}) combination. However, in our study BA- IAA and BA-NAA combinations gave very low shoot proliferation of 1.83 and 1.00 shoots respectively, per explant.

Jawahar *et al.* (2008) reported shoot formation in the woody medicinal plant *Vitex negundo,* when auxins along with cytokinins were supplemented in the medium.

It was observed in our study that BA 2 mg L^{-1} in the medium, gave a shoot proliferation of 4.83 shoots, while Yadav and Singh (2011) reported a higher shoot proliferation producing eight shoots in the same medium. According to him auxin in combination with cytokinin gave lower shoot proliferation in *A. marmelos.*

BA and Kn when used individually in MS medium, shoot proliferation was observed to be on par but shoot length and nodes per shoot were comparatively less in Kn supplemented medium. However, Bindhu (2015) reported that BA 2 mg L^{-1} was found to be better than Kn, where a 3-fold increase in the number of shoots from the nodal explants of *A. marmelos*.

Yadav and Singh (2011) reported that no bud break was obtained on nodal explants of *A. marmelos* in MS medium devoid of growth regulators. In the present study, bud initiated in 8.33 days but there was no multiple shoot formation.

5.1.1.2 Additives (Chitosan, Adenine sulphate, Thidiazuron)

Chitosan, adenine sulphate and thidiazuron were added to MS medium supplemented with BA 2 mg L^{-1} and IBA 0.5 mg L^{-1} and plain MS medium to study their effect on shoot proliferation.

Six treatments with different concentrations of chitosan, supplemented to the basal medium were tried for the assessment of shoot proliferation. Bud initiation was early in MS medium augmented with BA 2 mg L^{-1}, IBA 0.5 mg L^{-1} and chitosan 10 mg L^{-1} (7.00). The same medium recorded the highest shoot proliferation (35.67 shoots per explant) and shoot length (5.23 cm). Barka *et al.* (2004) reported that chitosan stimulated plant growth *in vitro* in grape vine, *Vitis vinifera* L. The positive influence of chitosan on plant growth was also confirmed by Sopalun *et al.* (2010). They observed that chitosan 25 mg L^{-1} in MS (half strength) medium recorded the highest relative growth rate in PLBs of *Grammatophyllum speciosum*. Nahar *et al.* (2012) stated that by applying chitosan at the rate of 1 mg L^{-1} to the *in vitro* cultures *Cymbidium dayanum*, the highest PLBs induction (93 per cent) and shoots formation (79 per cent) could be observed. According to Mihiretu *et al.* (2016) chitosan improved shoot multiplication rate in chrysanthemum, strawberry and sweet basil.

The chitosan a deacetylated form of chitin, is an environment friendly biopolymer, that enhances growth and development in plants. This effect can be attributed to some signalling pathway to auxin biosynthesis *via* a tryptophan-independent pathway (Uthairatanakij *et al.,* 2007). Zakaria *et al.* (2009) opined that it acts as a growth promoter and elicitor of plant defense mechanisms that could alleviate stress caused by *in vitro* conditions and acclimatization.

Six different media supplemented with Adenine sulphate were used to study the effect on shoot multiplication. MS medium supplemented with AdS 60 mg L^{-1} gave best response with respect to days to bud initiation (8.50 days),

maximum shoot proliferation (5.33 shoots per explant), shoot length (7.17 cm) and nodes per shoot (9.33). The plant growth stimulating responses of adenine sulphate has been reported in many plants. 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 was found to be most effective in inducing maximum number of shoots (18) from nodal explants in *Bacopa monnieri* (Ramesh *et al.,* 2006). For multiple shoot proliferation of *Anthurium andreanum* MS with BAP 0.5 mg L^{-1} and adenine sulphate (AdS) 60 mg L^{-1} proved to be the best resulting ten multiple shoots per inoculated shoot bud within 50 days (Gantait *et al*., 2008). Siwach *et al.* (2012) explained that adenine sulphate stimulated cell growth and shoot multiplication probably by acting as organic nitrogen source and/or acting as precursor for natural cytokinin synthesis. According to Nitsch (1968) adenine acts more as a synergist of cytokinins in *in vitro* cultures. In our study, adenine suphate supplemented medium gave lesser shoot proliferation compared to control (MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1}). Moreover, AdS when added to BA and IBA supplemented medium, shoot proliferation was found to be less (3 shoots per explant) against 5.33 shoots per explant in medium supplemented with AdS alone. This observation explained that adenine suphate did not reinforce the effect of cytokinins and auxins in the medium. In contrast to this, Naaz *et al.* (2014) reported that basal medium with AdS alone did not stimulate shoot formation, while AdS added with BA promoted shoot proliferation in *Syzygium cumini.*

Adenine sulphate show cytokinin activity and could be added to *in vitro* culture systems to promote growth (Gatica *et al.,* 2010). The nitrogen utilization of *in vitro* grown culture could be improved by the incorporation of reduced nitrogen forms particularly adenines and amino acids in the culture media. AdS being an organic source of nitrogen could be assimilated more rapidly than inorganic nitrogen (Vengadesan *et al.*, 2002).

The effect of thidiazuron on shoot proliferation was assessed by supplementing different levels of the additive in basal media. MS+ BA 2 mg L^{-1} +

IBA 0.5 mg L^{-1} + TDZ 0.02 mg L^{-1} recorded the highest number shoots (20.00) per explant. The stimulating effect of TDZ on multiple shoot formation has been reported in several medicinal and aromatic plant species including *Arachis correntina* (Mroginski *et al*., 2004), *Curcuma longa* (Prathanturarug *et al*., 2005), *Curculigo orchioides* (Thomas, 2007), *Ocimum basilicum* (Bacila *et al*., 2010), and *Mentha arvensis* (Faisal *et al.,* 2014). Thidiazuron has been used successfully *in vitro* to induce adventitious shoot formation and to promote axillary shoot proliferation. Thidizuron (TDZ) is one of the best active cytokinin-like substances for woody plant tissue culture. It enables efficient micropropagation of several recalcitrant woody species. TDZ at low concentrations (<1 μM) induce better axillary proliferation but inhibit shoot elongation. The most objectionable effect of TDZ is the formation of fasciated shoots in culture with some species. The intense shoot proliferation due to TDZ, indicative of high cytokinin acivity have recognized it as one of the most active cytokinins for *in vitro* manipulation of woody species (Huetteman and Preece, 1993).

In the study, thidiazuron though there was significant shoot proliferation, abnormal shoots with reduced shoot length and more number of nodes per shoots were produced, resulting in very low internodal length. TDZ at very low concentration of 0.02 mg L^{-1} only gave higher shoot proliferation (20 shoots per explant). At higher concentrations (0.5, 1 and 2 mg L^{-1}) there was no morphogenetic response. The nodal explant became yellow and dried out within 2 weeks of inoculation. Chalupa (1987) also reported high cytokinin activity of thidiazuron at very low concentrations (0.002-0.05 mg L^{-1}). Aggarwal *et al.* (2012) observed that combination of TDZ 0.024 mg L^{-1} and adenine 79.7 mg L^{-1} in MS medium resulted in highly proficient shoot regeneration (80%) from leaf explants of Himalayan poplar (*Populus ciliata* Wall.).

In the present study it was found that TDZ in combination with BA and IBA in MS medium gave high shoot proliferation, while when used alone, the shoot proliferation was much less (3.15 shoots per explant) even less than the

control. Fathima and Anis, (2011) reported the micropropagation of *Withania somnifera* L. through *in vitro* culture of nodal segments with axillary buds. MS medium containing TDZ (0.0 to 10.0 μM) was effective in inducing shoot buds and maintaining high rates of shoot multiplication.

According to Lu (1993), number of shoots produced on medium having thidiazuron were equivalent to or greater than shoots initiated on medium with purine-type cytokinins. Low concentrations of thidiazuron (0.0022 to 0.088 mg L^{-1}) were found to be effective for organogenesis. He also opined that sustained exposure to thidiazuron may induce hyperhydricity and abnormal shoot and root morphology.

TDZ is an urea derivative and does not contain the purine ring common to adenine type cytokinins such as benzyl aminopurine, kinetin or zeatin. This activity of TDZ may be due to the fact that it promotes the conversion of cytokinin ribonucloides to biologically more active ribonucleotides (Capelle *et al.*, 1983). It also encourages the synthesis of endogenous purine cytokinins or inhibits their degradation (Thomas and Katterman, 1986).

Guo *et al.* (2011) reported that TDZ might alter endogenous plant growth regulators, either directly or indirectly and produce reactions in cell or tissue, resulting in the enhancement of its partition/regeneration. The morpho-regulatory potential of TDZ has led to its application in *in vitro* plant culture system for the development of viable morphogenetic systems.

In the study, among the three different additives tested, chitosan and thidiazuron gave very high shoot proliferation. But the shoots were found to be morphologically abnormal with very low internodal elongation and thin needle like leaves. Adenine sulphate gave normal shoots but shoot proliferation was less compared to control. Hence, the control medium (MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1}) was selected as the basal medium for cryopreservation studies.

5.2 *IN VITRO* CONSERVATION

5.2.1 Cryopreservation of *A. marmelos* using encapsulation-dehydration technique

The cryopreservation is an *in vitro* conservation technique that involves the storage of plant tissue in ultra-low temperature of liquid nitrogen (-196°C), where the cell metabolic and biochemical activities are restrained. These materials can be stored for unlimited time without any genetic variation. The encapsulation-dehydration technique of cryopreservation is widely adopted in many plant species as it enables easy handling of tissues and high rates of recovery after cryostorage. This involves the encapsulation of micropropagules within alginate beads and further reduction in moisture level by elevated concentration of osmotic agents or mechanical dehydration under laminar airflow cabinet (Reed, 2008b). The beads surrounding the explant allows it to withstand extreme treatment conditions including exposure to high sucrose concentrations during pre-culture, desiccation to low moisture content and exposure to liquid nitrogen during freezing, which would be highly damaging or lethal to non-encapsulated samples. When the plant tissues are subjected to desiccation, the internal solutes are converted to a vitrified state removing most freezable water. Hence during rapid exposure to LN, intracellular ice crystal formation is eluded, which otherwise would have been lethal to plant tissues (Engelmann, 1997). This enables high rate of recovery after cryopreservation as a large part of the frozen plant tissue is kept intact after rewarming (Engelmann, 2000). Many plant species could be successfully cryopreserved using encapsulation-dehydration, a few of these being *Vinca minor* (Hirata *et al.*, 2002), shoot-tips of *Syzygium francisii* (Shatnawi *et al.*, 2004), *Ceratopetalum gummiferum* shoot-tips (Shatnawi and Johnson 2004), embryogenic tissues of *Pinus nigra* (Salaj *et al.*, 2011), *Artemisia herba-alba* shoot-tips (Sharaf *et al.*, 2012) and medicinal orchid *Dendrobium nobile* (Mohanty *et al.*, 2012).

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

5.2.1.1 Preconditioning

The plant tissue has to be dehydrated to a moisture level of 20-30% to avoid crystallization during cooling. Most hydrated tissues do not tolerate dehydration to this moisture content level. The key for effective cryopreservation is to develop dehydration tolerance rather than freezing tolerance (Panis and Lambardi, 2005). Exogenous sucrose at low concentrations has been reported to serve as a metabolic substrate for low-temperature induced metabolic alterations, while at higher concentrations, it is held to have a cryoprotective effect on cellular membranes (Uemura and Steponkus, 2003)

In the present study, the role of preconditioning treatments in terms of both sucrose concentration in the medium and exposure duration has been investigated using axillary buds of *A. marmelos* excised from the *in vitro* raised plantlets. The preconditioning treatment selected was exposure to sucrose 0.1 M supplemented semisolid MS medium for 7 days. This treatment gave 100 per cent regeneration when cultured on the basal medium MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1} with maximum shoots per explant (5.50), shoot length (6.50 cm) and nodes (9.83) per shoot. Maximum growth of shoots was obtained in sucrose concentrations in the range of 0.15 M to 0.29 M for 8 weeks when *in vitro*-grown shoot tips of *Troyer citrange* were cryopreserved by encapsulation-dehydration (Wang *et al.,* 2002). The sucrose concentration in the preconditioning medium significantly influenced the growth of shoots as well as survival of shoot tips after cryostorage. Cho *et al.* (2002) observed that 3-day treatment with 0.1M sucrose was the optimal preconditioning treatment for the cryopreservation embryonic axes of *Citrus madurensis*.

A sucrose pre-culturing treatment, could induce a high level of resistance to dehydration and subsequent freezing. However, in many cases this treatment alone was not sufficient and the preconditioning of stock shoots before pre-culturing was an essential step (Niino and Sakai 1992; Niino *et al.,* 1997).

5.2.1.2 Encapsulation

Encapsulation involves the ion exchange between sodium alginate and calcium chloride, resulting in calcium alginate bead formation, which helps to protect explant during exposure to ultra low temperature of liquid nitrogen. This allows the explant to withstand exposure to high sucrose concentration during pre-culture, which is an essential step to enhance freezing tolerance during cryopreservation.

In our investigation, axillary buds were encapsulated using different concentrations of sodium alginate and calcium chloride. Encapsulated beads with twenty different combinations of sodium alginate and calcium chloride were cultured on to the inoculation medium (MS + BA 2 mg L^{-1} +IBA 0.5 mg L^{-1}). But the beads did not give regeneration even after three months in culture. Hence modified inoculation medium was tried (½ MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1}). Among the twenty treatments tried, nine treatments showed 100 per cent survival. Bud initiated within 9 days when the medium was modified with half strength MS. Encapsulated buds initiated late (9-17 days) compared to non encapsulated beads (6.00 days). Among the different encapsulation treatments tried, axillary buds encapsulated with SA 3.5% in modified liquid MS and $CaCl_2$ 100mM gave maximum shoot proliferation in the modified inoculation medium. Yin *et al.* (2011) reported that same combination of SA and $CaCl_2$ for artificial seed formation by encapsulation of PLBs in Brassidium Shooting Star.

Shoots per explant, shoot length and nodes per shoot were found to be low in encapsulated beads compared to non encapsulated beads. Firmer the beads, late (17.33 days) was the bud initiation as found with the treatment E19 (SA 5 per cent in modified liquid MS + $CaCl_2$ 100mM). In *Pinus patula* the germination percentage of encapsulated somatic embryos was affected considerably by the concentration of sodium alginate and the duration of exposure to calcium chloride (Malabadi and Staden, 2005). In agreement to our finding, beads formed in nodal segments of *Tylophora indica* using SA 5 percent formed very hard and tailed beads and the regeneration declined drastically to 23.30 per cent. The firmness of the bead depends mainly on the number of sodium ions exchanged with calcium ions during complex formation (Gantait *et al.,* 2017).

5.2.1.3 Pre-culture

In encapsulation dehydration procedure, pre-culture with high sucrose concentration would increase desiccation and freezing tolerance, which are very critical for the recovery of cryopreserved tissues (Takagi, 2000). As like preconditioning, the concentration of sucrose and period of exposure are the two factors that need to be optimised during pre-culture to enhance the survival post cryostorage. Survival of cryopreserved explants increased with increase in sucrose concentration to certain level, as exposure to sucrose increased total soluble protein and sugar content in the treated tissues. The sugars are competitive protein and membrane stabilizers besides having osmotic and colligative effects (Hitmi *et al.,* 1999).

In the present study, preconditioned and encapsulated single nodal segments were subjected to fifteen pre-culture treatments involving different concentrations of sucrose (0.1, 0.5 and 1 M) for different periods (1-5 days) in hormone free MS liquid medium with 3 per cent DMSO. The beads pre-cultured in liquid MS medium supplemented with sucrose 0.5 M for 3 days were selected as the best pre-culture treatment, as it gave the earliest bud initiation (19.00 days),

maximum shoots per explant (3.67), shoot length (2.00 cm) and nodes per shoot (3.33) in the medium ½ MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1}. Higher concentrations of sucrose (1.0 M) did not support the growth and regeneration of the plants irrespective of the different periods tried.

Bekheet *et al.* (2007) opined that sucrose is an important pre-growth additives, as it would enhance desiccation tolerance during cryopreservation. In date palm, it was found that sucrose was the best pre growth additive among different types of sugar tried (fructose, glucose, sorbitol and sucrose) as indicated by the enhanced survival after cryopreservation.

Encapsulated protocorm like bodies (PLBs) of *Dendrobium chrysanthum* pre-cultured on MS liquid medium supplemented with 0.3 M sucrose for 2 days gave 53.3 per cent survival. The survival percentage was found to decline on pre-culturing with higher concentrations of sucrose (0.5 M -0.7 M) (Mohanty *et al.*, 2012). Seed initiated embryonic callus of *Citrus inodora* was cryopreserved using encapsulation-dehydration technique. Alginate encapsulated embryogenic callus was pre-cultured for 3 days in liquid basal medium with sucrose (0.5M).

The plant tissue will undergo vitrification on sucrose pre-culture thus preventing lethal intercellular ice crystal formation during cryopreservation (Koster, 1991). Sucrose would enable maintaining plasma membrane integrity by replacing water on the membrane surface and thus stabilizing the protein under freezing conditions (Crowe *et al.*, 1987).

5.2.1.4 Dehydration

After preconditioning, encapsulation and pre-culture, the beads are subjected to physical dehydration by evaporation prior to freezing in LN. In rapid freezing as in our study, explant dehydration should occur before exposure to liquid nitrogen. This dehydration was carried out by physical methods such as

exposing the encapsulated axillary bud to a laminar flow cabinet. This procedure would enable direct cryostorage after dehydration avoiding the use of cryoprotectants that may induce cytotoxicity (Engelmann, 2004; Gonzalez-Arnao and Engelmann, 2006).

In our investigation it was observed that maximum survival of 66.67 per cent and regeneration of 50 per cent was obtained only when the explants were dehydrated for 6h reaching a moisture content of 19.25 per cent. With lower or higher level of dehydration duration, the encapsulated beads did not show any sign of regeneration after cryostorage. However, the dehydrated beads (not exposed to LN) gave a regeneration ranging from 100 to 66.67 upto 6 h of dehydration.

Similar period of dehydration (6h) resulting in a moisture level of 18- 20 per cent for successful cryopreservation has been reported in various plant species. Priadi *et al.* (2000) optimised the cryopreservation of sengon (*Paraserianthes falcataria* L.) using encapsulation-dehydration method and the highest viability of sengon (27.77 percent) was obtained from shoot tips air-dried for 6 hours in a laminar airflow cabinet.

Al-Ababneh *et al.* (2002) established a cryopreservation protocol for sour orange (*Citrus aurantium* L.), wherein encapsulated shoot tips were dehydrated for 6h reaching a moisture content of 18% resulting in 83 per cent survival and 47 per cent regrowth. Gupta (2009) reported that when encapsulated shoot tips of *Morus* spp. were dehydrated for 6h to a moisture content of ~20 per cent, gave a reasonable recovery of 30-35 per cent. Al- Baba *et al.* (2015) observed 40 per cent survival in cryopreserved *Ziziphora tenuior* shoot tip using encapsulation-dehydration technique when subjected to chemical dehydration in 0.75 M sucrose for one day followed by 6h of air dehydration.

Dehydration would result in increased solute concentration within the plant tissues. On rapid freezing, increased solute concentration would lead to a glassy amorphous state. In this vitrified state, intra cellular ice crystal formation, which is damaging to the tissues, is prevented (Gonzalez-Arnao *et al.,* 2008; Wowk, 2010).

In the present study, preconditioned, encapsulated, pre-cultured and dehydrated axillary buds after cryostorage and thawing at 40°C for 30-60s, when cultured on to recovery medium, initiated buds after 32.96 days of inoculation After two months of culture, 1.89 shoots per explant with a shoot length of 1.50 cm and 2.00 nodes per shoot were obtained.

The different periods of storage in liquid nitrogen did not have any profound influence on survival and regeneration. Three different duration *viz.,* 2h, 1 day and 1 week did not have any significant difference with respect to survival and regeneration.

The encapsulation dehydration has shown to increase the regrowth of many cryopreserved plant species. It increases survivability and decrease cell destruction due to dehydration (Yamamoto *et al.,* 2011; Sherlock *et al.,* 2005). Encapsulation-dehydration being a simple, efficient, safe and cost-effective cryopreservation technique, has been successfully employed in large number of plant species. This also gives a high post-thaw recovery. Encapsulation-dehydration technique, thus could easily be employed for long-term conservation of plant genetic resources.

5.1 Estimation of genetic stability of cryopreserved materials using ISSR markers

Genetic stability of cryo-derived regenerants is one of the most important concerns in cryopreservation. Assessment of true-to-type regenerants is important

as cryopreservation goes through a series of stress situations that could induce variations. The plant material is hence liable to epigenetic alterations on cryopreservation (Harding, 2004; Mikula *et al.,* 2011). Plants recovered following cryogenic treatments should be genetically identical to the starting materials (Ashmore, 1997). Hence, the genetic uniformity of the cryopreserved plant material needs to be validated.

In the present study, ISSR markers were used to assess the genetic stability of cryopreserved regenerants. Nine ISSR primers were screened of which four gave amplification of 5-7 distinguishable bands. The banding pattern obtained with respect to cryopreserved regenerants and non-cryopreserved *in vitro* raised control plants were found to be similar. According to Harding and Benson, (1995) molecular analysis of plant DNA is ideal for genetic stability assessments. PCR based molecular markers have been used in the genetic fidelity assessment of cryostorage derived plantlets (Zhai *et al.,* 2003). Molecular marker, ISSR are easy to use, cheap and requiring no previous sequence information, hence could be used effectively for genetic stability assessment (Butiuc-Keul *et al.,* 2016).

The application of ISSR for the characterization of genetic stability has been well documented in *Morus* spp (Rao *et al.,* 2009), *Zingiber offinalis* (Yamuna *et al.,* 2007), *Clivia miniata* (Wang *et al.,* 2011), *Bacopa monnieri* (Muthiah *et al.*, 2013), *Phalaenopsis gigantea* (Samarfard *et al.,* 2013), *Scutellaria altissima* (Grzegorczyk-Karolak *et al.,* 2013) and synthetic seeds of cucumber (Adhikari *et al.,* 2014).

ISSR, a molecular technique developed by Zietkiewicz *et al.* (1973), can screen 100 to 3,000 bp DNA fragments. ISSR is amplified by PCR using microsatellite core sequences as primers with a few selective nucleotides as anchors into the non-repeat adjacent regions (16-18bp). The main advantage of ISSR is that it is randomly distributed throughout the genome and results are reproducible (Walvekar and Kaimal, 2014).

A protocol for cryopreservation of *A. marmelos* by encapsulation-dehydration technique with a reasonable recovery of 50 per cent has been standardized in the present study. The encapsulation-dehydration method established in the study appears to be simple and effective for the long term conservation of *A. marmelos* by cryopreservation.

Future Lines of Work

- Cryopreservation steps *viz.,* preconditioning, pre-culturing and recovery could be further improved to enhance the recovery per cent.
- Vitrification based cryopreservation could also be tried to enhance the recovery per cent.

Summary

6. SUMMARY

The present study on "Cryoconservation of Koovalam (*Aegle marmelos* L. Corr.) by encapsulation-dehydration technique", was carried out during 2015-2017 at the Department of Plant Biotechnology, College of Agriculture, Vellayani. *In vitro* maintained cultures in the Department of Plant Biotechnology were used as source of explant during the course of the study. The proposed research programme was carried out in three phases *viz.,* enhancement of multiplication rate, standardization of cryopreservation protocol using encapsulation and dehydration technique and assessment of genetic fidelity of plantlets recovered and regenerated from cryostorage using molecular markers.

Single node segments with axillary buds from *in vitro* maintained cultures were used as the explants in all the experiments. Among the twelve combinations of auxins and cytokinins tried, MS (Murashige and Skoog, 1962) medium supplemented with BA 2 mg L^{-1} + IBA 0.5 mg L^{-1} was found to be the best treatment with respect to shoot multiplication (9.33 shoots per explant).

Effect of additives *viz.,* chitosan, adenine sulphate and thidiazuron on shoot multiplication was studied by adding them in two different media, (1) MS medium + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1} and (2) plain MS medium. MS medium + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1} without any additive was taken as the control. Hundred per cent regeneration was observed in all the six chitosan supplemented treatments with varying concentration (chitosan at 10, 20 and 30 mg L^{-1}). The best response with respect to days to bud initiation (7.00), shoots initiated per explant (35.67), shoot length (5.23 cm) and nodes per shoot (8.50) was observed in MS medium supplemented with BA 2 mg L^{-1}, IBA 0.5 mg L^{-1} and chitosan 10 mg L^{-1}.

All the six adenosine supplemented media with varying concentration of adenine sulphate (20, 40 and 60 mg L^{-1}) gave 100 percent regeneration. Among

the treatments, the best response was observed in MS media supplemented with adenine sulphate 60 mg L^{-1} with respect to days to bud initiation (8.50), shoots initiated per explant (5.33), shoot length (7.17 cm) and nodes per shoot (9.33). However, shoot proliferation was significantly lower compared to the control which gave a better multiplication rate of 9.33 shoots per explant.

Eight different thidiazuron supplemented media with varying concentrations (thidiazuron at 2, 1, 0.5 and 0.02 mg L^{-1}) were tried to study its effect on shoot proliferation. All treatments except those supplemented with thidiazuron 0.02 mg L^{-1} did not give any regeneration. The inoculated explants initially turned yellow and were completely dried out within two weeks of culture.

The treatments MS+ TDZ 0.02 mg L^{-1} and MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1} + TDZ 0.02 mg L^{-1} recorded 100 per cent regeneration. Maximum shoot proliferation (20.00 shoots per explant) was observed in thidiazuron 0.02 mg L^{-1} supplemented MS medium with BA 2 mg L^{-1} and IBA 0.5 mg L^{-1}. In this medium the bud initiated in 8 days and within six weeks of culture, it recorded a shoot length of 5.50 cm and 15.56 nodes per shoot.

Even though higher shoot proliferation was observed in CH and TDZ supplemented media, they exhibited morphological abnormalities. Normal shoots were obtained with AdS supplemented medium, but shoot proliferation was less compared to control (MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1}). Hence MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1} was used as basal medium for cryopreservation studies.

A protocol for cryopreservation of *A. marmelos* was developed by standardising different sequential steps in encapsulation-dehydration procedure *viz.,* preconditioning, encapsulation, pre-culture, dehydration, thawing and recovery. Axillary buds of *in vitro* established plantlets were used as explant for cryopreservation studies.

The preconditioning medium, sucrose 0.1M in semisolid MS medium for 7 days was selected as the best for preconditioning treatment. The nodal segments with single axillary buds, thus preconditioned when inoculated in MS+BA 2 mg

L^{-1}+ IBA 0.5 mg L^{-1} medium, gave best shoot growth responses *viz.,* the earliest bud initiation (8.00 days), maximum shoot proliferation (5.50 shoots per explant), maximum shoot length (6.50 cm) and maximum number of nodes per shoot (9.83 nodes/shoot).

In encapsulations, the beads formed with sodium alginate 3.5 per cent and calcium chloride 100 mM gave 100 per cent regeneration and it recorded the earliest bud initiation (9.00 days), maximum shoots (6.67) per explant, maximum shoot length (4.67 cm) and maximum number of nodes (9.00) per shoot was obtained when cultured on the modified inoculation medium, ½ MS+BA 2 mg L^{-1} + IBA 0.5 mg L^{-1}. This encapsulation treatment has been selected as the most appropriate considering its regeneration percentage and plant conversion efficiency.

The preconditioned and encapsulated axillary buds were subjected to different pre-culture treatments, involving various sucrose concentration in liquid MS media for varying periods of exposure. The explants from pre-culture medium, liquid MS supplemented with DMSO 3 per cent and sucrose 0.5 M for 3 days gave earliest bud initiation (19.00 days), maximum shoot proliferation (3.67 shoots per explant), maximum shoot length (2.00 cm) and maximum number of nodes per shoot (3.33 nodes per shoot), when cultured on the inoculation medium, ½ MS+BA 2 mg L^{-1}+ IBA 0.5 mg L^{-1}.

The preconditioned, encapsulated and pre-cultured axillary buds were subjected to different periods of desiccation. The survival and regeneration of post cryostorage was obtained only at 6h of desiccation. At 6h, with a moisture content of 19.5 per cent, the encapsulated buds gave 66.67 per cent survival and 50.00 per cent regeneration in the recovery medium ½ MS+BA 2 mg L^{-1}+ IBA 0.5 mg L^{-1}. The buds after cryostorage initiated after 32 days of inoculation with 1.89 shoots per explant.

The beads stored in liquid nitrogen for different duration *viz.,* 2h, 1 day and 1 week when cultured on recovery medium, did not show any significant variation with respect to survival and regeneration per cent.

The genetic fidelity of plantlets regenerated from encapsulated buds after cryostorage were assessed using ISSR markers. Nine ISSR primers were screened. Amplification as indicated by distinguishable bands could be obtained only with four ISSR primers. The number of bands with each primer varied from 5 to 7. Polymorphism was not found with any of these primers. ISSR profiles of cryoregenerated plantlets were identical to those of the control.

The protocol for encapsulation dehydration technique of cryopreservation was standardised using axillary buds of *A. marmelos* with preconditioning in semi solid MS medium supplemented with sucrose 0.1 M for 7 days, encapsulation using sodium alginate 3.5 per cent in modified liquid MS medium and calcium chloride 100mM followed by pre-culture in liquid MS medium supplemented with DMSO 3 per cent and sucrose 0.5 M for 3 days and dehydration 6 h MC (19.5%), rapid freezing in liquid nitrogen for at least 2 h and recovery in the medium ½ MS+BA 2 mg L^{-1}+ IBA 0.5 mg L^{-1}.

The cryopreservation using encapsulation-dehydration technique standardised in the present study could be utilized for long term conservation of *A.marmelos.*

References

7. REFERENCES

Adela, H. and Deliu, C. 2006. Cryopreservation of strawberry shoot tips by encapsulation-dehydration. *Natl. Bot. Hortic. Agrobot*. 34: 28-33.

Aggarwal, G., Sharma, C., and Srivastava, D. K. 2012. Thidiazuron: A potent cytokinin for efficient plant regeneration in Himalayan poplar (*Populus ciliate* Wall.) using leaf explants. *Ann. For. Res*. 55(2): 179-188.

Adhikari, S., Bandyopadhyay, T. K., and Ghosh, P. 2014. Assessment of genetic stability of *Cucumis sativus* L. regenerated from encapsulated shoot tips. *Scientia Hortic.* 170: 115-122.

Ajithkumar, D. and Seeni, S. 1998. Rapid clonal multiplications through *in vitro* axillary shoot proliferation of *Aegle marmelos* (L) Corr., a medicinal tree. *Plant Cell Rep.* 17(5): 422-426.

Al- Baba, H., Shibli, R. A., Akash, M. P. P., Al-Qudah, T., Tahtamouni, R. W., and Al- Ruwaiei, H. 2015. Cryopreservation and genetic stability assessment of threatened medicinal plant (*Ziziphora tenuior* L.) grown wild in Jordan. *Jordon J. Bio. Sci.* 8: 247-256.

Al-Ababneh, S. S., Karam, N. S., and Shibli, R. A. 2002. Cryopreservation of sour orange (*Citrus aurantium* L.) shoot tips. *In vitro Cell. Dev. Biol. Plant* 38: 602-607.

Al-Wasel, A. S. 2000. Micropropagation of *Acacia seyal* Del. *In vitro J. Arid Environ.* 46: 425-431.

Antony, J. J. J., Mubbarakh, S. A., and Subramaniam, S. 2013. Effect of plasmolysis on Protocorm-Like Bodies of *Dendrobium* Bobby Messina orchid following cryopreservation with encapsulation dehydration method. *Appl. Biochem. Biotechnol.* 1-12.

Ashmore, S. E. 1997. Status report on the development and application of *in vitro* techniques for the conservation and use of plant genetic resources. IPGRI, Rome, pp. 67.

Bacila, I., Coste, A., Halmagyi, A., and Deliu, C. 2010, Micropropagation of *Hypericum maculatum* Cranz an important medicinal plant. *Rom. Biotechnol. Lett.* 15: 86-99.

Baginol, S. and Engelmann, F. 1992. Effects of pregrowth and freezing conditions on the resistance of meristems of date palm (*Phoenix dactylifera* L. var. Bou Sthammi Noir) to freezing in liquid nitrogen. *Cryo Lett.* 12: 279-286.

Barka, A., Eullaffroy, E., Clement, P. C., and Vernet, G. 2004. Chitosan improves development and protects *Vitis vinifera* L. against *Botrytis cinerea. Plant Cell Rep.* 22: 608-614.

Beena, M. R., Martin, K. P., Kirt, P. B., and Hariharan, M. 2003. Rapid *in vitro* propagation of medicinally important *Ceropegia candelabrum. Plant Cell. Tissue Org. Cult.* 72: 285-289.

Bekheet, S. A., Taha, H. S., Saker, M. M., and Solliman, M. E. 2007. Application of cryopreservation technique for *in vitro* grown date palm (*Phoenix dactylifera* L.) cultures. *J. Appl. Sci. Res.* 3(9): 859-866.

Benson, E. E. 1990. *Free radical damage in stored plant germplasm.* International Board for Plant Genetic Resources, Rome, pp.121-125.

Bernard, F., Shaker-Bazarnov, H., and Kaviani, B. 2002. Effect of salicylic acid on cold preservation and cryopreservation of encapsulated embryonic axes of Persian lilac (*Melia azedarach* L.). *Euphytica.* 123: 85-88.

Bhattacharya, R., Reddy, K. R. C., and Mishra, A. K. 2014. Export Strategy of Ayurvedic products from India. *Int. J. Ayurvedic Med.* 5(1): 125-128.

Bhattacharyya, R., Ray, A., Gangopadhyay, M., and Bhattacharya, S. 2007. *In vitro* conservation of *Plumbago indica* -a rare medicinal plant. *Plant Cell Biotechnol. Mol. Biol.* 8 (2): 39-46.

Bindhu, K. B. 2015. *In vitro* propagation of *Aegle marmelos* through nodal explants. *Int. J. Sci. Res.* 4 (4): 2319-7064.

Bouafia, S., Jelti, N., Lairy, G., Blanc, A., Bonnel, E., and Dereuddre, J. 1996. Cryopreservation of potato shoot tips by encapsulation-dehydration. *Potato Res.* 39: 69-78.

Butiuc- Keul, A., Farkas, A., and Cristea, V. 2016. Genetic stability assessment of *in vitro* plants by molecular markers. *Studia Universitatis Babeş-Bolyai Biologia.* 1: 107-114.

Brar, M. S., Al-Khayri, J. M., and Klingaman, G. L. 1995. Effect of Thidiazuron and Benzylaminopurine on *in vitro* shoot proliferation of carnation (*Dianthus caryophyllus* L.). *J. Ark. Acad. Sci.* 49(7): 30-33.

Capelle, S. C., Mok, D. W. S., and Kirchner, S. C. 1983. Effects of thidiazuron on cytokinin autonomy and the metabolism of Nt-(/x 2-isopentenyl) [8-

14C] adenosine in callus tissues of *Phaseolus lunatus* L. *Plant Physiol.* 73: 796-802.

Capuano, G., Piccioni, E. and Standardi, A. 1998, Effect of different treatments on the conversion of M-26 apple root stock synthetic seeds obtained from encapsulated apical and axillary micropropagated buds. *J. Hortic. Sci. Biotechnol.* 73(3): 299-305.

Chakraborty, M., Patel, A., Garach, D., and Kamath, J. 2012. *Aegle marmelos* L.: A therapeutic boon for human health. *Int. J. Res. Ayurveda Pharma.* 3(2): 159-162.

Chalupa, V. 1987. Effect of Benzylaminopurine and Thidiazuron on *in vitro* shoot proliferation of *Tilia cordata* Mill., *Sorbus aucuparia* L. and *Robinia pseudoacacia* L. *Biol. Plant* 29 (6): 425-429.

Chenshu, A., Wang, X., Yuan, X., Zhao, B., and Wang, Y. 2003. Optimization of cryopreservation of *Artemisia annua* L. callus. *Biotechnol. Lett.* 25: 35-38.

Cho, E. G., Hor, Y. L., Kim, H.H., Rao, V. R., and Engelmann, F. 2002. Cryopreservation of *Citrus madurensis* embryonic axes by vitrification: Importance of loading and treatment with vitrification solution. *Cryo Lett.* 23: 317-324.

Clavero-Ramirez, I., Galvez-Farfan, J., Lopez- Aranda, J. M., and Gonzalez-Benito, M. E. 2005. Apex cryopreservation of several strawberry genotypes by two encapsulation-dehydration methods. *Cryo Lett.* 26(1): 17-24.

Coelho, N., Benito, M. E. G., Martin, C., and Romano, A. 2014. Cryopreservation of *Thymus lotocephalus* shoot tips and assessment of genetic stability. *Cryo Lett.* 35 (2): 119-128.

Cordeiro, S. Z., Simas, N. K., Henriques, A. B., and Sato, A. 2014. *In vitro* conservation of *Mandevilla moricandiana* (Apocynaceae): short-term storage and encapsulation–dehydration of nodal segments. *In vitro Cell. Dev. Biol. Plant* 50: 326-336.

Crowe, J. H., Crowe, L.M., Carpenter, J. F., and Wistrom, C. A. 1987. Stabilization of dry phospholipid bilayers and proteins by sugars. *J. Biochem.* 242: 1-10.

Das, R., Hasan, M. F., Rahaman, M. S., Rashid, M. H., and Rahman, M. 2008. Study on *in vitro* propagation through multiple shoot proliferation in wood apple (*Aegle marmelos* L.). *Int. J. Sustain. Crop Prod.* 3(6): 16-20.

Day, J. G., Harding, K. C., Nadarajan, J., and Benson, E. E. 2008. Cryopreservation Molecular Biomethods Handbook, Springer, pp. 917-947.

Deodhar, S., Pawar, K., Singh, N., Thengane, R. J., and Thengane, S. R. 2014. Clonal propagation of female plants of *Garcinia indica* Choiss: A tree species of high medicinal value, *J. Appl. Biol. Biotechnol.* 2 (6): 18-25.

Dereuddre, J., Scottez, C., Arnaud, Y., and Duron, M. 1990. Resistance of alginate-coated axillary shoot tips of pear tree *(Pyrus communis* L., Beurre Hardy) *in vitro* plantlets to dehydration and subsequent freezing in liquid nitrogen: effect of previous cold hardening. *CR Acad. Sci. Paris III.* 310: 317-323.

Dhankhar, S., Ruhil, S., Balhara, M., and Chhillar, A. K. 2011. *Aegle marmelos* (Linn.) Correa: A potential source of phytomedicine *J. Med. Plants Res.* 5(9): 1497-1507.

Doyle, J. J. and Doyle, J. L. 1990. Isolation of plant DNA from fresh tissue. *Focus* 12: 13-15.

Dumet, D., Block, W., Worland, R., Reed, B. M. J., and Benson, E. E. 2000. Profiling cryopreservation protocols for *Ribes ciliatum* using differential scanning calorimetry. *Cryo Lett.* 21. 367-378.

Dumet, D., Engelmann, F., Chabrillange, N., and Duva, Y. 1993. Cryopreservation of oil palm *(Elaeis guineensis* Jacq.) somatic embryos involving a desiccation step. *Plant Cell Rep.* 12: 352-355.

Engelmann, F. 1997. *In vitro* conservation methods. Biotechnology and Plant Genetic Resources: Conservation and Use, (eds) BV Ford-Lloyd, JH Newburry & JA Callow, CABI, Wellingford, pp.119-162.

Engelmann, F. 2000. Importance of cryopreservation for the conservation of plant genetic resources. In: Engelmann F, Takagi H (eds) Cryopreservation of tropical plant germplasm: current research progress and application. JIRCAS, Tsukuba/IPGRI, Rome, pp 8-20.

Engelmann, F. 2004. Plant Cryopreservation: Progress and Prospects. *In vitro Cell. Dev. Biol. Plant.* 40: 427-433.

Engelmann, F. 2011. Use of biotechnologies for the conservation of plant biodiversity. *In vitro Cell. Dev. Biol. Plant* 47: 5-16.

Engelmann, F., Gonzalez-Arnao, M. T., Yongjie, W., and Escobar, R. 2008. Development of encapsulation-dehydration plant cryopreservation: A practical guide. pp. 59-76.

Fabre, J. and Dereuddre, J. 1990. Encapsulation-dehydration: a new approach to cryopreservation of *Solanum* shoot tips. *Cryo Lett.* 11: 413-426.

Faisal, M. and Anis, M. 2006. Thidiazuron induced high frequency axillary shoot multiplication in *Psoralea corylifolia. Biol. Plant.* 50: 437-440.

Faisal, M., Ahmad, N., and Anis, M. 2005. Shoot multiplication in *Rauvolfia tetraphylla* L, using thidiazuron. *Plant Cell Tissue Org. Cult.* 80: 187-190.

Faisal, M., Alatar, A. A., Hegazy, A. K., Alharbi, S. A., El-Sheikh, M., Okla, M. K. 2014. Thidiazuron induced *in vitro* multiplication of *Mentha arvensis* and evaluation of genetic stability by flow cytometry and molecular markers. *Ind. Crops Prod.* 62: 100-106.

Fang, J. Y., Wetten, A., and Hadley, P. 2004. Cryopreservation of cocoa (*Theobroma cacao* L.) somatic embryos for long-term germplasm storage. *Plant Sci.* 166: 669-675.

Fathima, N. and Anis, M. 2011. Thidiazuron induced high frequency axillary shoot multiplication in *Withania somnifera* L. Dunal. *J. Med. Plants Res.* 5(30): 6681-6687.

Flachsland, E., Terada, G., Scocchi, A., Rey, H., Mroginski, L., and Engelmann, F. 2006. Cryopreservation of seeds and *in vitro* cultured protocorms of *Oncidium bifolium* Sims. (Orchidaceae) by encapsulation dehydration. *Cryo Lett.* 27: 235-242.

Gantait, S., Kundu, S., and Das, P. K. 2016. Acacia: An exclusive survey on *in vitro* propagation. *J. Saudi Soc. Agric. Sci.* 1-15.

Gantait, S., Mandal, N., Bhattacharyyal, S., and Das, P. K. 2008. *In vitro* mass multiplication with pure genetic identity in *Anthurium andreanum* Lind. *Plant Tissue Cult. Biotechnol.* 18(2): 113-122.

Gantait, S., Kundub, S., Yeasminb, C. L., and Alib, M. N. 2017. Impact of differential levels of sodium alginate, calcium chloride and basal media on germination frequency of genetically true artificial seeds of *Rauvolfia serpentina* (L.) Benth. ex Kurz. *J. Appl. Res. Med. Aromat. Plants* 4: 75-81.

Gatica, A. A. M., Munoz, V. J., Ramirez, F. P., and Valdez, M. M. 2010. *In vitro* plant regeneration system for common bean (*Phaseolus vulgaris*): effect of N6-benzylaminopurine and adenine sulphate. *Electr. J. Biotechnol.* 13(1): 0717-3458.

George, E. F. 1993. Plant propagation by tissue culture. Part I: published by the technology Edington Exegetics Ltd., England. 520p.

Gonzalez-Arnao, M. T., Engelmann, F., Urra, C., Morenza, M., and Rios, M. 1998. Cryopreservation of *Citrus* apices using the encapsulation-dehydration technique. *Cryo Lett.* 19: 177-182.

Gonzalez-Arnao, M. T., Panta , A., Roca, W. M., Escobar, R. H., and Engelmann, F. 2008. Development and large scale application of cryopreservation techniques for shoot and somatic embryo cultures of tropical crops. *Plant Cell Tissue Org. Cult.* 92: 1-13.

Gonzalez-Arnao, M. T., Moreira, T., and Urra, C. 1996. Importance of pre-growth with sucrose and vitrification for the cryopreservation of sugarcane apices using encapsulation-dehydration. *Cryo Lett.* 17: 141-148.

Gonzalez-Arnao, M. T. and Engelmann, F. 2006. Cryopreservation of plant germplasm using the encapsulation-dehydration technique: Review and case study on sugarcane. *Cryo Lett.* 27(3): 155-168.

Gonzalez-Benito, M. E., Clavero-Ramirez, I., and Lopez-Aranda, J. M. 2004. Review: The use of cryopreservation for germplasm conservation of vegetatively propagated crops. *Spanish J. Agric. Res.* 2 (3): 341-351.

Grospietch, M., Stodulkova, E., and Zamecnik, J. 1999. Effect of osmotic stress on the dehydration tolerance and cryopreservation of *Solanum tuberosum* shoot tips. *Cryo Lett.* 20: 339-346.

Grzegorczyk-Karolak, I., Kuzma, L., and Wysokinska, H. 2013. The use of long-term *Scutellaria altissima* callus cultures for shoot regeneration, production of bioactive metabolites and micropropagation. *J. Med. Plants Res.* 7(45): 3303-3313.

Guo, B., Abbasi, B. H., Zeb, A., Xu, L. L., and Wei, Y. H. 2011. Thidiazuron: A multi-dimensional plant growth regulator. *Afr. J. Biotechnol.* 10(45): 8984-9000.

Gupta, S. 2014. Cryopreservation of germplasm through encapsulation-dehydration technique. *Acta Hortic.* 1039:147-153.

Gupta, S. 2009. Cryopreservation of *in vitro*-Grown Shoot Tips of *Morus spp.* and *Pyrus cossonii* Rehder by encapsulation-dehydration. *Acta Hortic.* 8: 309-318.

Harding, K. 2004. Genetic Integrity of Cryopreserved Plant Cells: A Review. *Cryo Lett.* 25: 3-22.

Harding, K. and Benson, E. E. 1995. Biochemical and molecular methods for assessing damage, recovery and stability in cryopreserved plant germplasm. *Genetic preservation of plant cells in vitro* (ed. Grout, B.W.W) Springer Verlag, Heidelberg, pp. 103-151.

Hazeena, M. S. and Sulekha, G. R. 2008. Callus induction and plantlet regeneration in *Aegle marmelos* (L.) Corr. using cotyledon explants. *J. Trop. Agric.* 46 (2): 79-84.

Hazubska-Przbyl, T., Chmielarz, P., Michalak, M. and Bojaraczuk, K. 2010. Cryopreservation of embryonic tissues of *Picea omorika* (Serbian spruce). *Plant Cell Tissue Organ Cult.* 102: 35-44.

Hegazi, M. 2011. Viability of encapsulated shoot tips of *Capparis orientalis* Duh. *Nat. Sci.* 9(8): 223-228.

Hirata, K., Mukai, M., Goda, S., Ishio-Kinugasa, M., Yoshida, K., Sakai, A., and Miyamoto, K. 2002. Cryopreservation of hairy root cultures of *Vinca minor* (L.) by encapsulation-dehydration. *Biotechnol. Lett.* 24: 371-376.

Hitmi, A., Barthomeuf, C., and Sallanon, H. 1999. Cryopreservation of *Chrysanthemum cinerariaefolium* shoot tips and effects of pre-treatment conditions and retention of biosynthetic capacity. *Cryo Lett.* 20: 109-120.

Hongthongkham, J. and Bunnag, S. 2014. *In vitro* propagation and cryopreservation of *Aerides odorata* Lour. (Orchidaceae). *Pakist. J. Biol. Sci.* 17(5): 608-618.

Hossain, M., Islam, R., Karim, M. R., Rahman, S. M., and Joarder, O. I. 1994. Production of plantlets from *Aegle marmelos* nucellar callus. *Plant Cell Rep.* 13: 570-573.

Huetteman, C. A. and Preece, J. E. 1993. Thidiazuron: a potent cytokinin for woody plant tissue culture. *Plant Cell Tissue Org. Cult.* 33: 105-119.

Husain, M. K., Anis, M., and Shahzad, A. 2008. *In vitro* propagation of a multipurpose leguminous tree (*Pterocarpus marsupium* Roxb.) using nodal explants. *Acta. Physiol. Plant*. 30(3): 124-130.

Imran, M. A., Begum, G., Sujatha, K., and Mallaiah, B. 2012. Effect of adenine sulphate (AdS) with cytokinins on multiple shoot production in *Carissa carandas* (L.). *Int. J. Pharma. Bio. sci.* 3(1): 473-480.

Ishikawa, K., Harata, K., Mii, M., Sakai, A., Yoshimatsu, K., and Shimomura, K. 1997. Cryopreservation of zygotic embryos of a Japanese terrestrial orchid (*Bletilla striata*) by vitrification. *Plant Cell Rep.* 16: 754-757.

Jawahar, M., Ravipaul, S., and Jeyaseelan, M. 2008. *In vitro* regeneration of *Vitex negundo* L. A Multipurpose woody aromatic medicinal shrub. *Plant Tissue Cult. Biotechnol.* 18: 37-42.

Jian, L. C., Sun, D. L., and Sun, L. H. 1987. Sugarcane callus cryopreservation. In: P.H. Li (eds), Plant cold hardiness, Alan R. Liss, Inc., New York, pp. 323-337.

Jokipii, S., Ryynanen, L., Kallio, P. T., Aronen, T., and Haggman, H. 2004. A cryopreservation method maintaining the genetic fidelity of a model forest

tree, *Populus Tremula* L. × *Populus Tremuloides* Mich. *Plant Sci.* 166: 799-806.

Kaviani, B. 2010. Cryopreservation by encapsulation-dehydration for long-term storage of some important germplasm: seed of lily [*Lilium ledebourii* (Baker) Bioss.], embryonic axe of persian lilac (*Melia azedarach* L.), and tea (*Camellia sinensis* L.). *Plant Omics J.* 3(6): 177-182.

Khan, M. K., Misra, P., Sharma, T., Shukla, P. K., and Ramteke, P. W. 2014. Effect of adenine sulphate on *in vitro* mass propagation of *Stevia rebaudiana* Bertoni. *J. Med. Plant Res.* 8(13): 543-549.

Khan, M. Y., Aliabbas, S., Kumar, V., and Rajkumar, S. 2009. Recent advances in medicinal plant biotechnology. *Indian J. Biotechnol.* 8: 9-22.

Khanna, P. P. and Singh, N. 1998. Conservation of Plant Genetic Resources. [online]Available:http://www.bioversityinternational.org/fileadmin/bioversity/publications/Web_version/174/ch13.htm. [n.d]

Koster, K. L. 1991. Glass formation and desiccation tolerance in seeds. *Plant Physiol.* 96: 302-304.

Kowalski, B., Terry, J., Herrera, L. F., and Penalver, D. A. 2006. Application of soluble chitosan *in vitro* and in the greenhouse to increase yield and seed quality of potato minitubers. *Potato Res.* 49: 167-176.

Kumar, K. S., Asish, G. R., Mony, R. S., Sundaresan, A., and Venugopalan, V. V. 2015. Comparative evaluation of the antioxidant and antidiabetic activity of different parts of *Aegle marmelos* and its chemical profiling using HPLC and HPTLC technique. *World J. Pharmacy Pharma. Sci.* 4(9):1107-1121.

Li, D. Z. and Pritchard, H. W. 2009. The science and economics of ex situ plant conservation. *Trends Plant Sci.* 14(11): 614-21.

Lu, C. Y. 1993. The use of thidiazuron in tissue culture. *In vitro Cell. Dev. Biol.* 29: 92-96.

Lurswijidjarus, W. and Thammasiri, K. 2004. Cryopreservation of shoot tips of *Dendrobium* Walter Oumae by encapsulation-dehydration. *Sci. Asia* 30: 293-299.

Malabadi, R. B. and Staden, J. V. 2005. Storability and germination of sodium alginate encapsulated somatic embryos derived from the vegetative shoot apices of mature *Pinus patula* trees. *Plant Cell Tissue Org. Cult.* 82 (3): 259-265.

Malik, S. K., Chaudhury, R., and Pritchard, H. W. 2012. Long term, large scale banking of *Citrus* species embryos: Comparisons between cryopreservation and other seed banking temperatures. *Cryo Lett.* 33(6): 453-464.

Mallaya, N. P. and Ravishankar, G. A. 2013. *In vitro* propagation and genetic fidelity study of plant regenerated from inverted hypocotyl explants of eggplant (*Solanum melongena* L.) cv. Arka Shirish. *Biotechnol.* 3: 45–52.

Mandal, B. B. and Sharma, D. S. 2007. Cryopreservation of *in vitro* shoot tips of *Dioscorea deltoidea* Wall., an endangered medicinal plant: Effect of cryogenic procedure and storage duration. *Cryo Lett.* 28: 461-470.

Martin, C., Uberhuaga, E., and Perez, C. 2002. Application of RAPD markers in the characterisation of *Chrysanthemum* varieties and the assessment of somaclonal variation, *Euphytica.* 127: 247-253.

Merhy, T. S. M., Vianna, M. G., Garcia, R. O., Pacheco, G., and Mansur, E. 2014. Cryopreservation of *Passiflora pohlii* nodal segments and assessment of genetic stability of regenerated plants. *Cryo Lett.* 35(3): 204-215.

Miaja, M. L., Gambino, G., Vallania, R., and Gribaudo, I. 2004. Cryopreservation of *Vitis vinifera* L. somatic embryos by vitrification or encapsulation-dehydration. *Acta Hortic.* 663: 599-603.

Mihiretu, H. C., Du Toita, E., Steyna, J. M., and Laurieb, S. M. 2016. Chitosan improved *in vitro* growth, leaf ultrastructure and acclimatization of micropropagated sweet potato. *World Congress on Root and Tuber Crops* Nanning, Guangxi, China, pp. 18-22.

Mikula, A., Tomiczak, K., and Rybczynski, J. J. 2011. Cryopreservation enhances embryogenic capacity of *Gentiana cruciata* (L.) suspension culture and maintains (epi) genetic uniformity of regenerants. *Plant cell Rep.* 30(4): 565-574.

Moges, A. D., Shibli, R. A., and Karam, N. S. 2004. Cryopreservation of African violet (*Saintpaulia ionantha* Wendl.) shoot tips. *In vitro Cell. Dev. Bio. Plant* 40(4): 389-395.

Mohanty, P., Das, M. C., Kumaria, S., and Tardon, P. 2012. High-efficiency cryopreservation of the medicinal orchid *Denrobium nobile* Lindl. *Plant Cell Tissue Org. Cult.* 109: 297-305.

Mohanty, P., Das, M. C., Kumaria, S., and Tandon, P. 2013. Cryopreservation of pharmaceutically important orchid *Dendrobium chrysanthum* Wall. Ex Lindl. Using vitrification based method. *Acta Physiol. Plant.* 35: 1373-1379.

Mroginski, E., Rey, H. Y, Gomzalez, A. M., and Mroginski, L. A. 2004. Thidiazuron promotes *in vitro* plant regeneration of *Arachis correntina* (Leguminosae) via organogenesis. *Plant Growth Reg.* 23: 129-134.

Mubbarakh, S. A., Izhar, N. A., Rajasegar, A., and Subramaniam, S. 2014. Establishment of encapsulation-dehydration technique for *in vitro* fragmented explants of *Rosa hybrida* L. *J. Food Agric.* 26 (6): 565-576.

Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures, *Physiol. Plant.* 15: 473-497.

Murthy, N. S., Murch, S. J., and Saxena, P. K. 1998. Thidiazuron: A potent regulator of *in vitro* plant morphogenesis. *In vitro Cell. Dev. Bio. Plant* 34: 267.

Murugan, K., Aswathy, J. M., Murukan, G., Manoj, G. S., and Babu, D. K. V. 2015. Dye yielding plants of Malabar Region, Kerala: a case study. *Int. J. Biol. Pharmacy Allied Sci.* 4(10): 6191-6199.

Muthiah, J. V. L., Shunmugiah, K. P., and Manikandan, R. 2013. Genetic fidelity assessment of encapsulated *in vitro* tissues of *Bacopa monnieri* after six months of storage by using ISSR and RAPD markers. *Turkish J. Bot.* 37: 1008-1017.

Naaz, A., Shahzad, A., and Mohammad, A. 2014. Effect of Adenine Sulphate interaction on growth and development of shoot regeneration and

174352

inhibition of shoot tip necrosis under *in vitro* condition in adult *Syzygium cumini* L.-a multipurpose tree. *Appl. Biochem. Biotechnol.* 173: 90-102.

Nahar, S., J, Kazuhiko, S., Chieh Li, H., and Kaewjampa, N. 2012. Effect of plant growth regulators on organogenesis in protocorm-like body (PLBS) of *Cymbidium dayanum in vitro.* ARPN. *J. Agric. and Biol. Sci.* 6 (6): 28-33.

Nair, D. S. and Reghunath, B. R. 2007. Effective use of encapsulation-dehydration technique in cryopreserving somatic embryos of Butterfly pea (*Clitoria ternatea* L.). *J. Herbs Spices Med. Plant.* 13 (3): 83-95.

Nair, D. S. and Reghunath, B. R. 2009. Cryoconservation and regeneration of axillary shoot meristems of *Indigofera tinctoria* (L.) by encpasulation-dehydration technique. *In vitro Cell. Dev. Biol. Plant* 45: 565-573.

Nayak, P., Behera, P.R., and Manikkannan, T. 2007. High frequency plantlet regeneration from cotyledonary node cultures of *Aegle marmelos* (L.) Corr. *In vitro Cell. Dev. Biol. Plant* 43: 231-236.

Nge, K. L., New, N., Chandrkrachang, S., and Stevens, W. F. 2006. Chitosan as a growth stimulator in orchid tissue culture. *Plant Sci.* 170(6): 1185-1190.

Niino, T. and Sakai, A. 1992. Cryopreservation of alginate-coated *in vitro*-grown shoot tips of apple, pear and mulberry. *Plant Sci.* 87: 199-206.

Niino, T., Tashiro, K., Suzuki, M., Ohuchi, S., Magoshi, J., and Akihama, T. 1997. Cryopreservation of *in vitro* grown shoot tips of cherry and sweet cherry by one-step vitrification. *Sci. Hortic.* 70: 155-163.

Nitsch, C. 1968. Induction *in vitro de la floraison chez une plante de jours courts: Plumbago indica* L. *Ann. Sci. Nat. Bot.* 9: 1-91.

Ogbu, J. U., Essien, B. A., Essien, J. B., and Anaele, M. U. 2010. Conservation and management of genetic resources of horticultural crops in Nigeria: Issues and biotechnological strategies. *J. Hortic. For.* 2(9): 214-222.

Padro, M. D. A., Frattarelli, A., Sgueglia, A., Condello, E., Damiano, C., and Caboni, E. 2012. Cryopreservation of white mulberry (*Morus alba* L.) by encapsulation-dehydration and vitrification. *Plant Cell Tissue Org. Cult.* 108: 167-172.

Panis, B. and Lambardi, M. 2005. Status of cryopreservation technologies in plants (crops and forest trees). In: Ruane, J. and Sonnino, A. (eds) The role of biotechnology for the characterization and conservation of crop, forest, animal and fishery genetic resources in developing countries. Turin, FAO, pp. 61-78.

Panis, B., Piette, B., and Swennen, R. 2005. Droplet vitrification of apical meristems: A cryopreservation protocol applicable to all Musaceae. *Plant Sci.* 68(1): 45-55.

Panse, V. G. and Sukhatme, P. V. 1985. *Statistical Methods for Agricultural workers.* ICAR, New Delhi, 197p.

Paulet, F., Engelmann, F., and Glazmann, J. C. 1993. Cryopreservation of apices of *in vitro* plantlets of sugarcane (*Saccharum* sp. hybrids) using encapsulation-dehydration. *Plant Cell Rep.* 12: 525-529.

Pavendan, P. and Rajasekaran, C. S. 2011. Effect of different concentrations of plant growth regulators for the micropropagation of *Eugenia singampattiana* Bedd., endangered tree species. *Res. J. Bot.* 6(3): 122-127.

Pawlowska, B. 2008. Employment of encapsulation-dehydration method for liquid nitrogen cryopreservation of ornamental plant explants propagated *in vitro*. *Folia Hortic.* 20(1): 61-71.

Pence, V. C. 2010. The possibilities and challenges of *in vitro* methods for plant conservation. *Kew Bull.* 65: 539-547.

Prakash, A. V. 2014. Cryoconservation of Chethikoduveli (*Plumbago rosea* L.) and assessment of genetic fidelity of regenerated plantlets using molecular markers. M.Sc. (Ag.) thesis, College of Agriculture, Vellayani, 40p.

Prathanturarug, S., Soonthornchareonon, N., Chauakul, W., Phaidee, Y., and Saralamp, P. 2005. Rapid micropropagation of *Curcuma longa* L. using bud explants pre-cultured in thidiazuron- supplemented liquid medium. *Plant Cell Tissue Org. Cult.* 80: 347-351.

Priadi, D., Sudarmonowati, E., Arifani, R., and Parisy, S. 2000. Cryopreservation of sengon (*Paraserianthes falcataria* (L.) Nielsen) and mangium (*Acacia mangium* (Wild) using encapsulation-dehydration method. Conference In Agris science: 2005, Publisher: Puslitbang Bioteknologi.

Pritchard, H. W., Justin, F. M., Ferraz, J. B. S., Marks, T. R., Camargo, L.C., Nadarajan, J., and Ferraz, I. D. K. 2014. Innovative approaches to the preservation of forest trees. *For. Ecol. Manag.* 333: 88-98.

Raghu, A. V., Geetha, S. P., Gerald, M., Indira, B., Ravindran, P. N., and Mohanan, K. V. 2007. An improved micropropagation for Bael- A vulnerable medicinal tree. *Res. J. Bot.* 2: 186-194.

Raj, J. V. and Basavaraju, R. 2012. *In vitro* nodal explants propagation of *Aegle marmelos* (L.) Correa. *Indian J. Innovations Dev.* 8: 576-587.

Ramesh, M., Saravanakumar, R. M., and Pandain, S. K. 2006. Benzyl amino purine and adenine sulphate induced multiple shoot and root induction from nodal explants of Brahmi (*Bacopa monnieri* L). *Penn. Nat. Prod. Rad.* 5: 44-51.

Rao, A. A., Chaudhury, R., Malik, S., Kumar, S., Ramachandran, R., and Qadri, S. M. H. 2009. Mulberry biodiversity conservation through cryopreservation. *In vitro Cell. Dev. Biol. Plant* 45:639-649.

Reed, B. M. 2008a. Plant Cryopreservation: a practical guide. USDAARS National Clonal Germplasm Repository, USA, pp. 3-11, 33-4.

Reed, B. M. 2008b. Plant cryopreservation: a practical guide. In: B. M. Reed (ed.), Springer Science + Business Media LLc, New York, 513p.

Reed, B. M., Schumacher, L., Dumet, D., and Benson, E. E. 2005. Evaluation of a modified encapsulation –dehydration procedure incorporating sucrose pre-treatments for the cryopreservation of *Ribes* germplasm. *In vitro Cell. Dev. Biol. Plant* 41: 431-436.

Rout, G. R. and Nanda, R. M. 2008. Protocol of somatic embryogenesis in *Acacia Arabica* (Lamk.) Wild. In: Protocol for Somatic Embryogenesis in Woody Plants, pp. 401-411.

Rout, G. R., Samantaray, S., and Das, P. 2000. *In vitro* manipulation and propagation of medicinal plants. *Biotech. Adv.* 18: 91-120.

Sakai, A. 2000. Development of cryopreservation techniques. Cryopreservation of Tropical Plant Germplasm, Japan. pp. 1-7.

Salaj, T., Matusikova, I., Fraterova, L., Pirselova, B., and Salaj, J. 2011. Regrowth of embryogenic tissues of *Pinus nigra* following cryopreservation. *Plant Cell Tissue Org. Cult.* 106: 55-61.

Samarfard, S., Kadir, M. A., Kadzimin, S. B., Ravanfar, S., and Saud, H. M. 2013. Genetic stability of *in vitro* multiplied *Phalaenopsis gigantea* protocorm-like bodies as affected by chitosan. *Notulae Bot. Hortic. Agrobot.* 41(1): 177-183.

Sanayaima, R. K. Kaur, A., Agarwal, A., and Babbar, S. B. 2006. Cryopreservation of *in vitro* grown shoot tips of *Crateva murvala* Buch. Ham, an important medicinal tree. *Cryo Lett.* 27(6): 375-386.

Sandhiya, G., Devendra, C., and Manju, B. 2008. Micropropagation of Bael (*Aegle marmelos* L. Corr.)-an indigenous medicinal fruit tree of India. *Indian J. Plant Genet. Resour.* 21(3): 213-216.

Sarasan, V. 2010. Importance of *in vitro* technology to future conservation programmes worldwide. *Kew Bull.* 65: 549-554.

Scocchi, A., Faloci, M., Medina, R., Olmos, S., and Mroginski, L. 2004. Plant recovery of cryopreserved apical meristem-tips of *Melia azedarach* L. using encapsulation/dehydration and assessment of their genetic stability. *Euphytica.* 135: 29-38.

Sharaf, S. A., Shibli, R. A., Kasrawi, M. A., and Baghdadi, S. H. 2012. Cryopreservation of wild Shih (*Artemisia herba-alba* Asso.) shoot-tips by

encapsulation-dehydration and encapsulation-vitrification. *Plant Cell Tissue Org. Cult.* 108: 437-444.

Shatnawi, M. A. 2011. Cryopreservation of *Capparis spinosa* shoot tips *via* vitrification, encapsulation-dehydration and encapsulation-vitrification. *World Appl. Sci. J.* 15 (3): 318-325.

Shatnawi, M. A. and Johnson, K. A. 2004. Cryopreservation by encapsulation-dehydration of Chritsmas bush' *(Ceratopetalum gummiferum)* shoot tips. *In vitro Cell. Dev. Biol. Plant* 40(2): 239-244.

Shatnawi, M. A., Johnson, K. A., and Torpy, F. 2004. *In vitro* propagation and cryostorage of *Syzygium francisii* (Myrtaceae) by encapsulation-dehydration method. *In vitro Cell. Dev. Biol. Plant* 40(4): 403-407.

Sherlock, G., Block, W., and Benson, E. 2005. Thermal analysis of the plant encapsulation-dehydration cryopreservation protocol using silica gel as the desiccant). *Cryo Lett.* 26: 45-54.

Shibli, R. A., Shatnawi, M. A., Subaih, W. S., and Ajlouni, M. M. 2006. *In vitro* conservation and cryopreservation of plant genetic resources: A review. *World J. Agric. Sci.* 2: 372-382.

Shibli, R. A. and Al-Juboory, K. 2000. Cryopreservation of 'Nabali' olive (*Olea europea* L.) somatic embryos by encapsulation-dehydration and encapsulation-vitrification. *Cryo Lett.* 21: 357-366.

Siwach, P., Chanana, S., Gill, A. R., Dhanda, P., Rani, J., Sharma, K., Rani, H., and Kumari, D. 2012. Effects of adenine sulphate, glutamine and casein hydrolysate on *in vitro* shoot multiplication and rooting of Kinnow

mandarin (*Citrus reticulata* Blanco). *Afr. J. Biotechnol.* 11(92): 15852-15862.

Soni, M. and Kaur, R. 2014. Rapid *in vitro* propagation, conservation and analysis of genetic stability of *Viola pilosa*. *Physiol. Mol. Biol. Plants* 20(1): 95-101.

Soni, P., Bahadur, A. N., and Kanungo, V. K. 2012. Study of *in vitro* micropropagation of a medicinally important plant *Adhatoda Vasica* Nees by auxillary bud culture. *Indian J. Innovations Dev.* 1(4): 199-202.

Sopalun, K., Thammasiri, K., and Ishikawa, K. 2010. Effects of chitosan as the growth stimulator for *Grammatophyllum speciosum in vitro* culture. *World Acad. Sci. Eng. Technol. J.* 4: 11-29.

Subramaniam, S., Sinniah, U. R., Khoddamzadeh, A. A., Periasamy, S. and Antony, J. J. J. 2011. Fundamental concept of cryopreservation using *Dendrobium* sonia-17 protocorm-like bodies by encapsulation-dehydration technique. *Afr. J. Biotechnol.* 10(19): 3902-3907.

Sudarsono and Goldy, R. G. 1991. Growth regulator and axillary bud position effects on *in vitro* establishment of *Vitis rotundifolia*. *Hortic. sci.* 26 (3): 304-307.

Suzuki, M., Ishikawa, M., Okuda, H., Noda, K., Kishimoto, T., Nakamura, T., Ogiwara, I., Shimura, I., and Akihama, T. 2006. Physiological changes in gentian axillary buds during two step pre-culture with sucrose that conferred high levels of tolerance to desiccation and cryopreservation. *Ann. Bot.* 97: 1073-1081.

Swaminathan, M. S. 2014. Plantation crops -Challenges ahead in an Era of Climate Change. *Proceedings and Recommendations of International Symposium on Plantation Crops (PLACROSYM XXI).* 10-12 December 2014; Kozhikode. Kerala, ICAR-Indian Institute of Spices Research, pp. 12-25.

Takagi, H. 2000. *In Cryopreservation of Tropical Plant Germplasm*: Current Research Progress and Application (eds) Engelmann, F. and Takagi, H. IPGRI, Rome, Italy, pp. 178-193.

Thomas, D. T. 2007. Pre-treatment in thidiazuron improves the *in vitro* shoot induction from leaves in *Curculigo orchioides* Gaertn., an endangered medicinal plant. *Acta Physiol. Plant*. 29: 455- 461.

Thomas, J. C. and Katterman, F. R. 1986. Cytokinin activity induced by thidiazuron. *Plant Physiol.* 81: 681-683.

Thorpe, A. 1980. *Prespective in plant cell and tissue culture*. International Review of cytology supplement.11A, (ed. Vasil, I. K). Acad Press, New York, pp. 71-111.

Touchell, D., Chiang, V., and Tsai, C. J. 2002. Cryopreservation of embryogenic cultures of *Picea mariana* (black spruce) using vitrification. *Plant Cell Rep.* 21(2): 118-124.

Uemura, M. and Steponkus, P. L. 2003. Modification of the intracellular sugar content alters the incidence of freeze-induced membrane lesions of protoplasts isolated from *Arabidopsis thaliana* leaves. *Plant Cell Environ.* 26: 1083-1096.

Uemura, M., Minami, A., and Kawamura, Y. 2009. Effect of low temperatures and cryoprotectants on plant plasma membranes. 1st International symposium on cryopreservation in horticultural species, Leuven, Belgium, 15p.

Upadhyay, S. and Koche, V. 2015. Comparison of different medium and establishment of an efficient micropropagation technique of *Clerodendrum Serratum* L. an endangered medicinal plant. *IOSR J. Environ. Sci. Toxicol. Food Technol.* 1(2): 27-35.

Uthairatanakij, A., Teixeira da Silva, J. A., and Obi Wan, K. 2007. Chitosan for improving orchid production and quality. *Orchid Sci. Biotechnol.* 1: 9-15.

Vacin, E. F. and Went, F. W. 1949. Some pH changes in nutrient solutions. *Int. J. Plant Sci.* 110: 605-13.

Valladares, S., Toribio, M., Celestino, C. and Vieitez, A. 2004. Cryopreservation of embryogenic cultures from mature *Quercus Suber* trees using vitrification. *Cryo Lett.* 25: 177-186.

Ved, D. K. and Goraya, G. S. 2008. *Demand and supply of medicinal plants in India.* National Medicinal Plant Board, New Delhi, 211p.

Vengadesan, G., Ganapathi, A., Amutha, S., and Selvaraj, N. 2002. *In vitro* propagation of *Acacia* species/a review. *Plant Sci.* 163: 663-671.

Venudevan, B. and Srimathi, P. 2014. Seed technological approaches for restoration of endangered medicinal tree, Bael (*Aegle marmelos* L. *Corr.).* *Life Sci. Leafl.* 54: 61-67.

Vines, G. 2004. Herbal Harvest with future: towards sustainable source for medicinal plants. Plant life International, Available: [www.plantlife.co.uk].

Walvekar, S. and Kaimal, P. 2014. Determination of genetic mutations induced by chemical and radiation treatment in *Aegle marmelos,* using Inter Simple Sequence Repeat (ISSR) primers. *Int. J. Pharma Bio. Sci.* 5 (2): 339 -345.

Wang, Q. M., Gao, F. Z., Gao, X., Zou, F. Y., Sui, X., Wang, M., Hui, Y. J., and Wang, L. 2011. Regeneration of *Clivia miniata* and assessment of clonal fidelity of plantlets. *Plant Cell. Tissue Org. Cult.* 109(2): 191-200.

Wang, G., Yuan, M., and Hong, Y. 2002. *In vitro* flower induction in roses. *In vitro Cell. Dev. Biol. Plant* 38: 513-518.

Wang, Q., Tanne, E., Arav, A., and Gafny, R. 2000. Cryopreservation of *in vitro*-grown shoot tips of grapevine by encapsulation-dehydration. *Plant Cell. Tissue and Organ Cult.* 3: 41-46.

Wowk, B. 2010. Thermodynamic aspects of vitrification. *Cryo biol.* 60: 11-22.

Wu, Y., Zhao, Y., Engelmann, F., and Zhou, M. 2001. Cryopreservation of apple dormant buds and shoot tips. *Cryo Lett.* 22: 227-284.

Xiaoping, W., Xiaoping, G., Yuan, T., and Scott, D. 2006. Russell a highly efficient *in vitro* plant regeneration system and Agrobacterium-mediated transformation in *Plumbago zeylanica. Plant Cell Rep.* 25: 513-521.

Yadav, K. and Singh, N. 2011. *In vitro* propagation and biochemical analysis of field established wood apple (*Aegle marmelos*). *Analele Univ. din Oradea - Fascicula Biol.* 1: 23-28.

Yadav, K., Singh, N., and Verma, S. 2012. Plant tissue culture: A biotechnological tool for solving the problem of propagation of multipurpose endangered medicinal plants in India. *J. Agric. Technol.* 8(1): 305-318.

Yamamoto, S., Rafique, T., Priyantha, W., Fukui, K., Matsumoto, T., and Niino, T. 2011. Development of cryopreservation procedure using aluminium cryo-plates. *Cryo Lett.* 32: 256-265.

Yamuna, G., Sumathi, V., Geetha, S. P., Praveen, K., Swapna, N., and Babu, N. K. 2007. Cryopreservation of *in vitro* grown shoot of ginger (*Zingiber officinale* Rosc). *Cryo Lett.* 28(4): 241-252.

Yin, L. L., Poobathy, R., James, J., Julkifle, A. L., and Subramaniam, S. 2011. Preliminary investigation of cryopreservation by encapsulation-dehydration technique on *Brassidium* Shooting Star orchid hybrid. *Afr. J. Biotechnol.* 10(22): 4665-4672.

Zakaria, R. A., Maleki-Zanjani, B., and Sedghi, E. 2009. Effect of *in vitro* chitosan application on growth and minituber yield of *Solanum tuberosum* L. *Plant Soil Environ.* 55 (6): 252-256.

Zarghami, R., Pirseyedi, M., Hasrak, S., and Sardrood, B. P. 2008. Evaluation of genetic stability in cryopreserved *Solanum tuberosum. Afr. J. Biotechnol.* 7 (16): 2798-2802.

Zhai, Z., Wu, Y., Engelmann, F., Chen, R. and Zhao, Y. 2003. Genetic stability assessments of plantlets regenerated from cryopreserved *in vitro* cultured grapes and kiwi shoot-tips using RAPD. *Cryo Lett.* 24: 315-322.

Zhao, Y., Wu, Y., Engelmann, F., and Zhou, M. 2001. Cryopreservation of axillary buds of grapes (*Vitis vinifera) in vitro* plantlets. *Cryo Lett.* 22: 321-328.

Zietkiewicz, E., Rafalski, A., and Labuda, D. 1973. Genome fingerprinting by Simple Sequence Repeat (SSR)-anchored polymerase chain reaction amplification. *Genomics* 20(2): 176-183.

Appendices

APPENDIX I

CTAB Extraction buffer

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

APPENDIX II

TE buffer

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

APPENDIX III

TBE (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)

CRYOCONSERVATION OF KOOVALAM (*Aegle marmelos* L. Corr.) BY ENCAPSULATION-DEHYDRATION TECHNIQUE

by

DEEPA E.

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Abstract of the thesis

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8. ABSTRACT

The study entitled "Cryoconservation of Koovalam (*Aegle marmelos* L. Corr.) by encapsulation-dehydration technique," was carried out in the Department of Plant Biotechnology, College of Agriculture, Vellayani during 2015-2017. The objectives of the present study were to standardize a cryopreservation protocol for *Aegle marmelos* using encapsulation and dehydration technique and to assess the genetic fidelity of plantlets regenerated from encapsulated axillary buds after cryostorage, using molecular markers.

Investigation was carried out in three phases *viz.,* enhancement of multiplication rate, standardization of *in vitro* conservation using the encapsulation-dehydration technique and assessment of genetic fidelity of the regenerated plantlets using ISSR markers.

Single node segments with axillary buds from *in vitro* maintained cultures were used as the explants in all the experiments. Among the twelve combinations of auxins and cytokinins were tried, MS (Murashige and Skoog, 1962) medium supplemented with BA 2 mg L^{-1} + IBA 0.5 mg L^{-1} was found to be the best treatment with respect to shoot multiplication (9.33 shoots per explant).

The additives chitosan (CH), thidiazuron (TDZ) and adenine sulphate (AdS) at different concentrations were supplemented in two different media *i.e.* 1) hormone free MS medium and 2) MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1} to study their effect on shoot proliferation. The best shoot proliferation response obtained for each additives were MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1} + chitosan 10 mg L^{-1} (35.66 shoots per explant), MS + AdS 60mg L^{-1} (5.33 shoots per explant) and MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1} + TDZ 0.02 mg L^{-1} (20.00 shoots per explant). Even though higher shoot proliferation was observed in CH and TDZ supplemented media, they exhibited morphological abnormalities. Normal shoots were obtained with AdS supplemented medium, but shoot proliferation was less

compared to MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1}. Hence MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1} was used as basal medium for cryopreservation studies.

Encapsulation-dehydration technique of cryopreservation involved various steps like preconditioning, encapsulation, pre-culture, dehydration (desiccation), thawing and recovery. Nodal segments with single axillary buds were used as the explant. In preconditioning experiment, PC2 (sucrose 0.1M in semi solid MS for 7 days) was selected as the best preconditioning treatment, which produced maximum shoot proliferation (5.50 shoots per culture) when cultured on basal medium. Among different encapsulation treatments, maximum shoot proliferation of (6.66 shoots per culture) was obtained in the beads formed with sodium alginate 3.5 per cent in modified MS medium and calcium chloride 100 mM, when cultured on modified basal medium (½ MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1}). Pre-culture experiments were conducted using preconditioned and encapsulated explants. The pre-culture treatment selected was liquid MS medium supplemented with sucrose 0.5 M and DMSO 3 per cent for 3 days, which gave maximum shoot proliferation (3.66 shoots per explant) in modified basal medium.

The preconditioned, encapsulated and pre-cultured beads were subjected to 0 to 7 h of desiccation under laminar airflow. The moisture content declined from 82 per cent to 12.60 percent on 7 h of desiccation. The desiccated beads were then cryopreserved in liquid nitrogen for 2h, followed by thawing for 30-60s at 40°C and inoculated on to recovery medium ½ MS + BA 2 mg L^{-1} + IBA 0.5 mg L^{-1}. Survival (66.67 per cent) and regeneration (50 per cent) could be obtained only at 6h of desiccation when the moisture content was 19.50 per cent. The beads when stored in liquid nitrogen for different duration did not show any significant variation with respect to survival and regeneration.

The genetic fidelity of plantlets regenerated from encapsulated axillary buds subjected to cryostorage were analysed using ISSR markers. Among the nine primers tried, four primers UBC 807 (AGAGAGAGAGAGAGAGT), UBC 840 (GAG AGA GAG AGA GAG AYT), UBC 847 (CACACACACACACACART) and

UBC 826 (ACACACACACACACACC) that gave scorable (5-7) bands were selected for the study. The ISSR banding patterns of the cryoregenerated plantlets and control plants were identical, which indicated the genetic stability.

This study was successful in developing a protocol for cryopreservation using encapsulation-dehydration technique in *A. marmelos* with 50 per cent regeneration efficiency.

174352