CRYOPRESERVATION OF HAIRY ROOT CULTURE OF AMRITHAPALA, *Decalepis arayalpathra* (Joseph & Chandras.) Venter

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by

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DECLARATION

I hereby declare that the thesis entitled **"Cryopreservation of hairy root culture of amrithapala, *Decalepis arayalpathra* (Joseph & Chandras.) Venter"** is a bonafide record of research done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

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We, the undersigned members of the advisory committee of Ms. Dhanya C. S., a candidate for the degree of **Master of Science in (Integrated) Biotechnology** with major in Biotechnology, agree that the thesis entitled **CRYOPRESERVATION OF HAIRY ROOT CULTURE OF AMRITHAPALA, *Decalepis arayalpathra* (Joseph & Chandras.) Venter** may be submitted by Ms. Dhanya C. S., in partial fulfilment of the requirement for the degree.

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DHANYA C.S

Dedicated to my parents

CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF APPENDICES

LIST OF ABBREVIATIONS

%	Percentage
λ	Wavelength
μM	micromolar
µg	microgram
µm	micrometer
ABA	Abscisic acid
cm	centimeter
cps	centipoise
2,4-D	2,4 Dichlorophenoxyacetic acid
DMSO	Dimethyl sulfoxide
DW	Dry weight
g	gram
h	hour
HMSB	Half Strength MS Basal medium
HPLC	High performance liquid chromatography
l	Litre
LN	Liquid nitrogen
-LN	without LN treatment
+LN	with LN treatment
M	Molar
m. c.	moisture content
MBALD	2- hydroxy-4-methoxy Benzaldehyde
mg	milligram
mg/l	milligram per litre
min	minute
ml	millilitre

mm	millimeter
m*M*	millimolar
MS	Murashige and Skoog
MSB	Murashige and Skoog Basal medium
n	no: of observations
$NH_4H_2PO_4$	Ammonium phosphate
NH_4NO_3	Ammonium nitrate
nm	nanometer
^oC	Degree Celsius
PBZ	Paclobutrazol
PVS_2	Plant Vitrification Solution 2
PVS_3	Plant Vitrification solution 3
Rf value	Retardation Factor
RT	Retention Time
s	second
SD	Standard Deviation
sp.	Species
TLC	Thin layer chromatography
UV	ultraviolet
v/v	volume/volume
w/o	without
w/v	weight/volume

INTRODUCTION

1. INTRODUCTION

Decalepis arayalpathra (Joseph & Chandras.) Venter (*Janakia arayalpathra* Joseph and Chandras. Apocynaceae) is a perennial, woody, lactiferous shrub (Joseph and Chandrasekharan, 1978; Venter and Verhoeven, 1997) with slender, spreading branches. The plant is endemic to Southern forests of the Western Ghats in India and is found distributed at an elevation of 800-1,200 m (Nayar, 1996), in the crevices of the rocks in small populations and is enlisted as critically endangered (CAMP-1, 1995; MOEF, 2009). In recent years, the rapid pace of deforestation in the Western Ghats has depleted the wild stands of *D. arayalpathra* and hampered its natural regeneration potential (Gangaprasad *et al.,* 2005). Considering the urgent need for *ex situ* conservation, a protocol for normal root cultures to produce the plant's specific aromatic compound, 2-hydroxy-4-methoxy benzaldehyde has been reported (Sudha and Seeni, 2001). This protocol provides an effective method for *ex situ* conservation and sustainable utilization of this endemic and endangered species.

The monoliform tuberous roots of the plant are highly aromatic, and the native Kani tribes use it as an effective remedy for peptic ulcer, cancer-like afflictions and as rejuvenating tonic (Pushpangadan *et al.,* 1990). Pharmacological and phytochemical investigations of the plant revealed the presence of several bioactive molecules 2- hydroxy-4-methoxy benzaldehyde, naringenin, naringin (Susan *et al.,* 2000) and immunomodulatory (Subramoniam *et al.,* 1996) and antiulcer activites (Shine *et al.,* 2007). Considering the importance of conservation and production of bioactive molecules, recently hairy root culture has been established by Sudha *et al.* (2013).

Hairy roots obtained by transforming plant tissues with *Agrobacterium rhizogenes* are an attractive and relatively new source of important phytochemicals (Carpentier *et al.,* 2007). Stable and extensive production of anthraquinones by hairy

root cultures induced from various *Rubia* species has been reported (Sato *et al.*, 1991). The advantages of hairy roots over undifferentiated cells are their genetic and biosynthetic stability, rapid growth and intensive branching on phytohormone-free medium (Hu and Du, 2006; Georgiev *et al.*, 2007). Rapid biomass accumulation with high production of secondary metabolites makes hairy root cultures suitable for scaling up in bioreactors. The down side of the use of *in vitro* hairy root cultures is that the maintenance is labor intensive and involves culture handling with a high risk of microbial contamination and subsequent loss of original cultures (Grout, 1995). Prolonged subcultures may affect quantitative or qualitative ability for the production of the compound. Cryopreservation is reported as a reliable method for long term storage of the germplasm of endangered species (Bramwell, 1990). Hairy root sample stored in liquid nitrogen through cryopreservation technology will eliminate labor intensive and energy requiring periodic subculture. This technology can be economically used to restore the cryopreserved cultures to the original culture status and to use the same for biochemical, phytochemical, pharmacological and physiological studies in addition to the scale up studies using bioreactor.

Cryopreservation of roots, in general, has received very little attention, and the development of protocols for root cryopreservation is still in its infancy. There is no standard protocol for cryopreservation of hairy roots. Therefore, some empirical studies are required to develop a successful protocol. The situation may change with increasing interest in transformed roots as potential production systems for secondary metabolites, thus leading to the development of cryopreservation protocols useful for a variety of plant species for both germplasm conservation as well as commercial purposes.

There are only a few reports regarding cryopreservation of hairy root cultures and all the works were done on temperate and subtropical plant species. When dealing with species of tropical origin, specific treatments are required to artificially

induce cold tolerance, because tropical plants do not develop cold-tolerance mechanisms, and are thus highly sensitive to low temperatures (Engelmann, 2000).

Different cryopreservation protocols have been developed for isolated root cultures through slow freezing in *Artemisia annua* (Teoh *et al.*,1996); vitrification in *Atropa belladonna* (Touno *et al.*, 2006), *Panax ginseng* (Yoshimatsu *et al.*, 1996) and *Angelica acutiloba* (Yoshimatsu, 2000); encapsulation-dehydration in *Vinca minor* (Hirata *et al.*, 2002) and horseradish hairy roots (Hirata *et al.*, 1998); encapsulation-vitrification in *Eruca sativa* (Xue *et al.*, 2008) and droplet-vitrification in *Rubia* (Kim *et al.*, 2010). However, the protocols are reported to have advantages and disadvantages in terms of post-cryopreservation recovery, applicability/simplicity and possibility of adaptation to diverse materials. As the protocol development phase has a trial and error rather than a systematic approach, such protocol seems to be case specific. Mostly, the protocols are developed by optimizing the conditions of the following steps: explant preparation (physiological state, size and permeability), preconditioning (cold acclimation, pre-culture), loading treatment (composition of the loading solution [LS]), dehydration (composition of the vitrification solution [VS] and treatment duration), cooling and rewarming, and post-cryopreservation (post-cryo) regrowth (Kim, 2011).

Considering the importance of cryopreservation, the present study aims to develop a suitable cryopreservation protocol for hairy root culture of *Decalepis arayalpathra*. As it is a tropical species, extensive studies on preconditioning and pre-culture treatments were undertaken to harden the root tips for dehydration as well as LN treatment. As different methods are reported as effective for different species, both encapsulation-dehydration and vitrification methods are attempted in the present investigation anticipating that at least one method will be useful in *D. arayalpathra*.

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Decalepis arayalpathra (Joseph & Chandras.) Venter (*Janakia arayalpathra* Joseph and Chandras. Apocynaceae) is a perennial, woody, lactiferous shrub (Venter and Verhoeven, 1997; Joseph and Chandrasekharan, 1978) with slender, spreading branches. The plant is endemic to southern forests of the Western Ghats in South India and is found distributed at an elevation of 800-1,200 m (Nayar, 1996), in the crevices of the rocks in small populations and it is enlisted as critically endangered (CAMP-1, 1995; MOEF, 2009).

In recent years the rapid pace of deforestation in the Western Ghats has depleted the wild stands of *D. arayalpathra* and hindered its natural regeneration potential (Gangaprasad *et al.,* 2005). The natural regeneration as well as conventional propagation of this plant is beset with several factors like poor fruit set, seed germination and rooting on stem cuttings (Sudha and Seeni, 2001). Recently, the Government of India prohibited and regulated the collection of this plant, as it is on the verge of extinction (MOEF, 2009).

The monoliform tuberous roots of the plant are highly aromatic, and the native Kani tribes use it as an effective remedy for peptic ulcer, cancer-like afflictions and as a rejuvenating tonic (Pushpangadan *et al*., 1990). Pharmacological investigation of the root extract of the plant has revealed gastric antisecretory and antiulcer activities (Shine *et al*., 2007) as well as immunomodulatory properties (Subramoniam *et al*., 1996). Roots of the plant are closely similar to that of Indian Sarasaparilla, *Hemidesmus indicus* R. Br, and *D. hamiltonii* Wight & Arn, which are widely used in the traditional system of medicine as a blood purifier. The phytochemical profile of the root tissues revealed secondary metabolites, such as 2-hydroxy-4-methoxy benzaldehyde (MBALD), ά-amyrinacetate, 4-methoxy salicelaldehyde, magnificol, 3-hydroxy p-anisaldehyde, naringenin, kaempferol, and

aroma dendrin (Susan *et al*., 2000). The plant's specific aromatic compound, MBALD possesses antimicrobial and antioxidant activities (Wang *et al.,* 2010). The compound also showed significant antifungal activity against seed-borne fungal pathogen (Mohana *et al*., 2009). The MBALD has been analyzed in normal root cultures of the plant (Sudha and Seeni, 2001) and the allied species, *D. hamiltonii* (Giridhar *et al.,* 2005). Plant biotechnological research is very limited on *Decalepis arayalpathra*. Micropropagation protocol was standardized by Sudha *et al.* (2005) with the objective of *ex situ* conservation. However, the normal root culture system was slow-growing with a short life-span, as noticed in an extensive review (Giri and Narasu, 2000). Further improvement of this plant in culture requires genetic transformation, which is an efficient technique to enhance secondary metabolites. Considering the importance of conservation and production of bioactive molecules, recently hairy root culture has been established by Sudha *et al.* (2013).

The present study, "Cryopreservation of hairy root culture of amrithapala, *Decalepis arayalpathra* (Joseph & Chandras.), Venter" involved the standardization of cryopreservation protocol for long term conservation of hairy root culture of *Decalepis arayalpathra*.

This chapter reviews on the literature of the cryopreservation of hairy root cultures of medicinal plants.

2.1 HAIRY ROOT CULTURE

In vitro culture is envisaged as a means for germplasm conservation to enhance the survival of endangered plant species and its rapid mass propagation (Al-Ababneh *et al.,* 2003; Moges *et al.,* 2003). The development of biotechnology has led to the production of a new sort of germplasm including clones obtained from elite genotypes, cell lines with unique attributes, and genetically transformed material (Engelmann, 1992). The development of *Agrobacterium rhizogenes* mediated hairy

root culture offers a remarkable potential for commercial production of a number of low-volume and high value secondary metabolites (Mehrotra *et al.*, 2010). Besides, hairy root cultures provide an excellent experimental system to study the various aspects of biosynthesis of useful phytochemicals, such as enzymatic pathways, key intermediates, and critical regulation points (Fu *et al.*, 2006) and the production of transgenic herbals (Piatczak *et al.*, 2006). The hairy root culture systems revealed the pharmacological activities in addition to the enhanced production of bioactive molecules (Syklowska-Baranek *et al.*, 2012) and the possibilities of utilizing artificial polyploidization for improving germplasm (He-Ping *et al.*, 2011).

Hairy root cultures are also an attractive experimental system, as they are long-term aseptic root clones, genetically stable with growth rates comparable to those of the fastest-growing cell suspension cultures (Lorence *et al.*, 2004). Other advantages of hairy roots include rapid biomass accumulation, typically accompanied with a high production of secondary metabolites and the possibility for up scaling in specialized bioreactors (Kim *et al.*, 2002; Georgiev *et al.*, 2007).

The down side of the use of *in vitro* hairy root cultures is that the maintenance is labor intensive and involves culture handling with a high risk of microbial contamination and subsequent loss of original cultures (Grout, 1995). Prolonged subcultures may affect quantitative or qualitative ability for the production of the compound. Conservation of original cultures using cryopreservation technology can avoid these problems. Storing samples in liquid nitrogen eliminates the need for periodic sub culturing and reduces the risk of accumulation of somaclonal variation (Teoh *et al.*, 1996).

2.2 CRYOPRESERVATION

Cryopreservation is a safe and cost-effective approach for long-term preservation. Cryogenic storage in liquid nitrogen (LN) at -196^{o}C ceases cell division and the metabolic and biochemical processes of the cell (Hopkins, 1999; Burritt, 2008). Further advantages include small storage space requirements, low maintenance costs, and only a modest number of replicates is required to conserve a plant effectively (Shibli and Al-Juboory, 2000). Conventionally liquid nitrogen (LN) is used as the coolant to attain the temperature of -196^{o}C in liquid phase and -150^{o}C in vapour phase. The plant material can be stored without alterations or modifications for a theoretically unlimited period of time (Engelmann, 2004).

Cryopreservation is a tool for long-term preservation of plant germplasm as applied in a series of plant systems. The encapsulation-dehydration method has proved to be highly successful for meristems of many plant species, such as grape vine (*Vitis vinifera*) (Plessis *et al.*, 1993), mulberry (*Morus bombysis*) (Niino and Sakai, 1992), Kiwifruit (Susuki *et al.*, 1994) and sugar cane (*Saccharum species*) (Gonzalez *et al.*, 1993). The cryopreservation of shoot tips of some plants such as strawberry, carnation, tomato, pea, and wild potato is now routine (Sakai, 1985). Successful cryopreservation methods have been reported for a number of root crops including sweet potato (Towill and Jarret, 1992), yams (Mandel *et al.*, 1996), taro (Thinh, 1997) and cassava (Escobar *et al.*, 1997).

Several protocols have been standardized even for the preservation of medicinal plants. The most commonly used techniques for cryopreservation include encapsulation dehydration, vitrification, encapsulation-vitrification and droplet-freezing (Engelmann, 2004). Encapsulation-dehydration is proved as an effective method to preserve shoot tips (Decruse and Seeni, 2002) somatic embryos (Nair and Reghunath, 2008), or axillary buds (Nair and Reghunath, 2009).

Vitrification is also an equally effective method for cryopreservation of embryogenic calli (Ming-Hua and Sen-Rong, 2010), shoot tips (Sharma *et al.*, 2009) and orchid protocorms (Ishikawa *et al.*, 1997). Encapsulation vitrification is an alternative method successfully applied for Protocorm-like bodies (PLBs) of *Dendrobium candidum*, embryogenic calli of *Dioscorea bulbifera* L. (Sen-Rong and Ming-Hua, 2012) and shoot-tip meristems of mint (*Mentha spicata* L.) (Hirai and Sakai, 1999). In species like *Chrysanthemum* (Halmagyi *et al.*, 2004) droplet freezing method is proved effective.

2.2.1 CRYOPRESERVATION OF HAIRY ROOT CULTURE

Progress in the last decade on the cryopreservation of plant systems has made possible the regeneration of entire plants from frozen cells (Panis *et al.*, 1990; Lu and Sun, 1992; Nishizawa *et al.*, 1992), embryos (Lecouteux *et al.*, 1991; Radhamani and Chandel, 1992) and shoot tips (Niino *et al.*, 1992; Demeulemeester *et al.*, 1993; Yoshimatsu *et al.*, 1994) of a number of species.

Cryopreservation of roots, in general, has received very little attention, and the development of protocols for root cryopreservation is still in its infancy. The situation may change with increasing interest in transformed roots as potential production systems for secondary metabolites, thus leading to the development of cryopreservation protocols useful for a variety of plant species for both germplasm conservation as well as commercial purposes (Teoh *et al.*, 1996).

Three main techniques can generally be used for plant cryopreservation; vitrification, encapsulation-dehydration and controlled rate freezing. Despite the progress that has been made in the last decade concerning cryopreservation of plant material, there are only few reports on cryopreservation of hairy root cultures.

Benson and Hamill (1991) first reported the cryopreservation of hairy roots for *Beta vulgaris* and *Nicotiana rustica*. Vitrification protocols are described for hairy roots of *Atropa belladonna* (Touno *et al.*, 2006), *Panax ginseng* (Yoshimatsu *et al.*, 1996) and *Angelica acutiloba* (Yoshimatsu, 2000), while the encapsulation-dehydration method was used for *Vinca minor* (Hirata *et al.*, 2002) and *Armoracia rusticana* (horseradish) hairy roots (Hirata *et al.*, 1998). Both encapsulation-dehydration and vitrification methods were used for *Maesa lanceolata* and *Medicago truncatula* (Lambert *et al.*, 2009). One single report describes a slow freezing method for *Artemisia annua* hairy roots (Teoh *et al.*, 1996) and droplet vitrification method for *Rubia akane* hairy roots (Kim *et al.*, 2010).

The low success rate of the cryopreservation of root cultures is due to because vitrification is not favorable to the roots. In such a viscous solution, like PVS_2, handling of free fine root tips is much more difficult than handling of other materials such as shoot tips and somatic embryos. Therefore, the addition of, exchange of, and repeated washings are very time consuming. Moreover, these chemicals must be washed out or considerably diluted after rewarming of the cryopreserved material to eliminate their toxic effect. The encapsulation-dehydration technique is more suitable for the cryopreservation of roots in comparison with the vitrification technique because encapsulated root tips can be easily handled with tweezers, and the washing step after rewarming can be eliminated because no toxic chemicals are used (Hirata *et al.*, 2002).

2.2.1.1 Cryopreservation by Encapsulation-Dehydration

The encapsulation-dehydration procedure is based on the technology developed for the production of artificial seeds. Explants are encapsulated in sodium alginate, pre-grown in liquid medium enriched with sucrose for several days, partially desiccated down to 20 percent water content (fresh weight basis), then frozen rapidly.

Survival rates are high and growth recovery of cryopreserved samples is generally rapid and direct, without callus formation. This technique has been developed for apices of various species from temperate origin such as apple, pear, grape, eucalyptus, and of tropical origin such as sugarcane and cassava (Dereuddre, 1992; Engelmann, 1997). A modified protocol has been developed by Sakai *et al.* (2000) in which encapsulation and pre-growth in medium with sucrose and glycerol are performed simultaneously.

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

` Hirata *et al.* (2002) reported that *Vinca minor* roots, pre-cultured for one day with a mixture of 0.3*M* sucrose and 10 μM ABA shows a sufficiently high survival rate of more than 70 percent after cooling. Horseradish hairy root cultures were encapsulated in calcium alginate beads containing 0.5 *M* glycerol and 0.3 *M* sucrose pre-cultured in the presence of 2 μM ABA was effective in increasing the survival rate to about 60 percent (Hirata *et al.*, 1998)

Lambert *et al.* (2009) reported that encapsulated hairy roots of *Maesa lanceolata* shows 90 percent and *Medicago truncatula* shows 53 percent survival rates after cooling.

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

2.2.1.1.1 Preconditioning

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

In the case of tropical plant species, which are cold-sensitive, sucrose-enriched medium is primarily used for culturing mother plants instead of cold-hardening in order to produce high level of recovery growth after cryopreservation (Engelmann, 1991; Dumet *et al.*, 1993a; Dumet *et al.*,1993b; Panis, 1995). The actively growing tissues required special treatments before they could withstand freezing stresses (Sakai and Nishiyama, 1978; Kartha *et al.*, 1979; 1980). Culturing of plant material on a medium supplemented with high sucrose concentrations to induce desiccation and chilling tolerance is considered essential to the success of encapsulation- dehydration method (Grenier-de March *et al.*, 2005).

In *Chrysanthemum cinerariaefolium* cell cultures, preconditioning with high sucrose is shown to induce accumulation of endogenous ABA and proline, which are directly related to freezing tolerance (Hitmi *et al*., 1999).

A long preconditioning phase of 30 days in 0.5*M* sucrose significantly reduced tissue water and improved post-freeze recovery of *Holostemma annulare* shoot tips (Decruse and Seeni, 2002).

Wang *et al*. (2002) reported that sucrose concentration in the preconditioning medium significantly influenced the growth and dry matter percentage of the stock shoots as well as subsequent survival of the cryopreserved 'Troyer' citrange shoot tips.

Negative effect of ammonium and nitrate in the recovery of cryopreserved material is also known. Ammonium nitrate in the culture medium is shown to reduce post-freeze recovery and regeneration of shoot tips of *Holostemma annulare* (Decruse and seeni, 2002). Substituting KNO_3 for NH_4NO_3 is proved to enhance recovery of cryopreserved shoot tips of *Beta pendula* (Ryynanen and Haggman, 1999). A modified Murashige & Skoog (MS) medium containing 2.6 m*M* $NH_4H_2PO_4$ instead of NH_4NO_3 to produce callus free shoots has been preferred for the successful cryopreservation of *Holostemma annulare* shoot tips (Decruse *et al.*, 1999).

Factors like NH4+, glycine and leucine adversely affect the recovery of cryopreserved *Lavandula vera* cells (Kuriama *et al.*, 2000; Kuriama *et al*., 1996). High nitrogen content in medium can decrease the frost hardiness of plants by increasing cell size and vacuolation (Levitt, 1980). Kuriama *et al*. (1989) showed that NH4+ ions in the post thaw recovery medium are deleterious to freeze damaged rice cells.

2.2.1.1.2 Encapsulation

For preparation of alginate beads, the plant material is suspended in calcium-free liquid basal medium with 3 percent sodium alginate (low viscosity, 250 cps) (Gonzalez-Arnao and Engelmann, 2006). This solution generally contains sucrose at the level used in the normal culture medium. In some case, explants are encapsulated in a medium with a slightly higher sucrose concentration. The mixture is dropped using a pipette into the liquid culture medium containing a high concentration of Calcium chloride (usually 100 m*M* $CaCl_2$). This induces the polymerization of alginate in the presence of an elevated concentration of calcium, thereby producing calcium alginate beads of about 4 or 5 mm around the explants. They are held in the calcium solution for 20-30 min after the last bead to guarantee a good polymerization. Beads become opaque as polymerization progresses. Beads are transferred to the sucrose pre-culture medium when they are completely opaque (Reed, 2008).

Lambert *et al.* (2009) used root tips (2-3mm) of *Maesa lanceolata* and *Medicago truncatula* hairy root cultures for encapsulation using 100 ml of 3 percent (w/v) sodium alginate solution containing 0.1 *M* sucrose and 100 ml 0.1 *M* $CaCl_2$ containing 0.1 *M* sucrose. The roots were kept in the solution for 10 min to allow solidification of the calcium alginate beads.

Hirata *et al.* (2002) demonstrated the use of 2 percent (w/v) sodium alginate solution containing 0.3 *M* sucrose + 0.5 *M* glycerol and 100 ml 50 m*M* $CaCl_2$ solution containing the same concentrations of sucrose and glycerol for the encapsulation of *Vinca minor* root tips. The roots were kept in the solution for 5 min to allow the formation of calcium alginate beads of about 5 mm diameter.

2.2.1.1.3 Pre-culture and Osmoprotection

Pre-culture corresponds to the culture of the explants for several hours or days after excision and before encapsulation. Preconditioning or pre-culture stage refers to the culturing of plant material on medium supplemented with osmotic agents at different concentrations and at different durations depending on plant species and type of plant material used (Shibli *et al.,* 2006). Osmoprotection refers to the treatment of encapsulated explants in medium with sucrose for several hours or days immediately before desiccation and cryopreservation. During pre-culture, the osmotic agent (i.e. sucrose, mannitol and/or sorbitol) enters into plant tissue and their concentrations increases considerably (Reed *et al.,* 2008) ultimately enhancing the efficiency of cryopreservation in many species (Shibli *et al.,* 2006).

The most common cryoprotective substances are dimethylsulfoxide (DMSO), polyethylene glycol (PEG), sucrose, sorbitol and mannitol. These substances have the osmotic actions; however some of them such as DMSO can enter into the cells and protect cellular integrity during cryopreservation (Rajasekharan, 2006).

DMSO is the most common chemical pretreatment and it is used both for pregrowth and during cryoprotection. The typical DMSO pretreatment for pea and strawberry meristems is with 5 percent DMSO in the medium for 48 hours. This was determined by meristems exposed to levels lower than 5 percent DMSO did not survive cryopreservation while those at higher concentrations survived (Kartha *et al.,* 1979; 1980).

De Carlo and Lambardi (2005) successfully cryopreserved embryogenic callus of *Citrus* pre-cultured with cryoprotectants, mainly DMSO (3 percent).

Embryogenic tissue of *Pinus patula* Scheide et Deppe was cryopreserved for 8 weeks using sorbitol and dimethylsulfoxide (DMSO) as cryoprotectants. Results

indicate that 0.3*M* sorbitol and 5 percent DMSO had the best cryoprotecting effect (Ford *et al.*, 2000)

DMSO was successfully used in *Zea mays* cells (Withers and king, 1979). Suspension cultures of *puccinellia distans* had 95 percent viability using 12.5 and 15 percent (v/v) DMSO (Heszky *et al.*, 1990).

High sucrose levels within a plant cell protect against excessive dehydration and cell collapse that may result to lethal freezing damage (Hirsh, 1987; Koster, 1987). Fowler *et al.* (1981) found a significant correlation between increased cellular sucrose concentrations and improved freezing tolerance in winter wheat cultivars in the field. The freezing tolerance of apple and *Rubus* shoot tips is significantly improved with sucrose (Caswell *et al.*, 1986; Palonen and Junttila, 1999).

ABA is a well known phytohormone which facilitates the acquisition of tolerance to environmental stresses (Chandler and Robertson, 1994, Ingram and Bartels, 1996, Shinozaki and Yamaguchi-Shinozaki, 1997). Exogenously added ABA can increase tolerance to freezing (Keith and McKersie, 1986, Robertson *et al.*, 1994) and ABA treatment has been used to maintain high survival rates during the cryopreservation of shoot tips and embryogenic tissues (Kendall *et al.*, 1993, Li *et al.*, 1999, Shiota *et al.*, 1999). ABA induces late embryogenesis-abundant (lea) genes, which encode LEA proteins. These proteins have increased levels during drought and are involved in dehydration tolerance in plants (Ingram and Bartels, 1996).

Pre-culture on medium supplemented with 0.3 M sucrose for three days is useful for successful cryopreservation as reported in *Maesa lanceolata* and *Medicago truncatula*. Plant growth inhibitors like ABA and PBZ are also used to prevent root tip outgrowth in beads during pre-culture in *Medicago* sp. (Lambert *et al.*, 2009)

Hirata *et al.* (2002) reported sufficiently high survival rate of more than 70 percent after cooling of excised root tips of *Vinca minor* subjected to pre-culture for one day in solution containing 10 μM ABA and 0.3 M sucrose prior to encapsulation and cryopreservation. They used the same concentrations of ABA and sucrose in addition to 0.5 M glycerol in the encapsulation medium.

Root tips excised from Horseradish hairy root culture encapsulated in calcium-alginate beads containing 0.5 *M* glycerol and 0.3 *M* sucrose and pre-cultured in the presence of 2 µM ABA is shown to be effective in increasing the survival rate as regeneration of hairy roots being observed in about 60 percent of the tips dehydrated and cooled in liquid nitrogen (Hirata *et al.*, 1998).

Negative effect of sucrose in pre-culture is also reported. Cryopreservation of pineapple apices by encapsulation dehydration technique is not successful due to the high sensitivity of pineapple apices to sucrose and dehydration (Martínez-Montero, 2005). *Citrus madurensis* embryonic axes are relatively sensitive to sucrose since pre-culture in sucrose concentrations higher than 0.3 *M* had a negative effect on survival, both before and after LN exposure (Cho *et al.*, 2001).

2.2.1.1.4 Dehydration

After osmoprotection beads are rapidly surface dried on sterile filter paper to remove any remaining liquid medium and are subjected to evaporation at room temperature. Two desiccation methods are usually employed: dehydration under the air current of a laminar flow cabinet or dehydration in sealed containers with dry silica gel (Gonzalez-Arnao and Engelmann 2006). In general, the bead water content that ensures highest re-growth after cooling in LN is around 20 percent, which corresponds to the amount of unfreezable water in the samples. At such water contents, only glass transitions are recorded by

differential scanning calorimetry, when samples are plunged in LN (Sherlock *et al*., 2005).

Dehydration to very low intracellular moisture levels, so that crystallization cannot occur, is essential for the survival of cryopreserved hairy roots. As per the published reports, water content in the encapsulated root tips is 23 percent for *Vinca minor* hairy root tips (Hirata *et al*., 2002), 35 percent for *Maesa lanceolata*, 36 percent for *Medicago truncatula* (Lambert *et al*., 2009) and 33 percent for Horseradish hairy roots (Hirata *et al*., 1998).

2.2.1.1.5 Cryopreservation in liquid nitrogen

After dehydration, the beads are placed in sterile cryotubes, plunged directly in LN and recommended to hold there for a minimum of one day. Hirata *et al.*, (2002) provided 24 h exposure in LN for encapsulated and dehydrated root tips of *Vinca minor*. Lambert *et al.* (2009) provided three days exposure of *Maesa lanceolata* and *Medicago truncatula* root tips to LN.

2.2.1.1.6 Thawing or Re-warming

With the encapsulation-dehydration technique, re-warming is usually carried out slowly at room temperature since samples are sufficiently dehydrated before freezing and there is no risk of ice re-crystallization upon warming (Gonzalez-Arnao and Engelmann 2006). Thus re-growth becomes independent of the re-warming method. For slow re-warming, beads are taken out of the cryotubes and placed in open petri dishes in the laminar air flow cabinet for about 5 min or they are transferred directly onto the recovery medium. In cases where rapid warming is performed, the cryotubes are stirred in water bath for 2-3 min at 40°C as in the case of *Maesa lanceolata* , *Medicago truncatula*, *Vinca*

minor and Horseradish hairy root cultures (Hirata *et al.*, 1998; Hirata *et al.*, 2002; Lambert *et al.*, 2009).

2.2.1.1.7 Re-growth

Re-growth generally takes place on standard semi-solid culture medium. Lambert *et al*. (2009) observed no morphological abnormalities for *Maesa lanceolata* and *Medicago truncatula* after cryopreservation of the hairy roots. The growth rates were also the same for non frozen and frozen samples.

The re-growth of cryopreserved ABA treated *Vinca minor* hairy roots after 5 weeks was slightly slower than that of ABA treated non-cryopreserved roots, but morphological differences were not observed between them (Hirata *et al*., 2002)

2.2.1.2. Cryopreservation by vitrification

Vitrification procedure for cryopreservation has been applied to a wide range of plant meristems (Sakai, 1993). Vitrification refers to the physical process by which a highly concentrated cryoprotective solution supercools to very low temperatures and finally solidifies into a metastable glass, without undergoing crystallization at a practical cooling rate (Fahy *et al*., 1984). Thus, vitrification is an effective freeze-avoidance mechanism. As a glass is exceedingly viscous and stops all chemical reactions that require molecular diffusion, its formation leads to metabolic inactivity and stability over time (Burke, 1986).

In vitrification method, freezable water in samples is osmotically removed by incubating them in highly concentrated cryoprotector solutions (Sakai and Engelmann, 2007). Matsumoto *et al.* (1994) developed procedure for cryopreservation of hairy roots by vitrification.

Glycerol based vitrification solutions designated as PVS2 (Sakai *et al.*, 1990; 1991) and PVS3 (Nishizawa *et al.*, 1993) are mostly used in different plant systems. The PVS2 solution contains 30 percent (w/v) glycerol, 15 percent (w/v) ethylene glycol, 15 percent (w/v) DMSO and 0.4 *M* sucrose (pH 5.8). PVS_3 consists of 40 percent (w/v) glycerol and 40 percent (w/v) sucrose in basal culture medium. The PVS2 solution easily supercools below -100°C upon rapid cooling and solidifies into a metastable glass at about -115°C (Sakai *et al.*, 1990).

Successful reports on cryopreservation of roots/hairy roots through vitrification methods are scanty. *Panax ginseng* adventitious roots are reported as more sensitive to cryopreservation showing 60 percent recovery after being cryopreserved by vitrification method (Yoshimatsu *et al.*, 1996). The only successful report of hairy root cryopreservation through vitrification is in *Atropa belladonna,* where upto 83 percent survival has been reported (Tauno *et al.*, 2006). But in certain other species like *Maesa lanceolata* and *Medicago truncatula,* hairy roots did not survive freezing in liquid nitrogen after vitrification (Lambert *et al.*, 2009).

Preconditioning is essential for vitrification methods as that of encapsulation-dehydration method. Although a high level of resistance to dehydration and subsequent freezing can be induced by a sucrose pre-culturing treatment, there have been many cases in which 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).

Thinh (1997) clearly demonstrated that taro shoot tips cryopreserved by vitrification using the PVS2 solution produced nearly 100 percent shoot growth recovery, when the donor plantlets were pre-cultured on MS medium containing 60-120 g/l sucrose for one month before freezing of the excised shoot tips.

The same preconditioning was successfully applied by Thinh (1997) to about 20 tropical monocotyledonous plants such as taro (8 cultivars (cvs)), banana (about 10 cvs), pineapple (2 cvs) and orchids (2 cvs). It is very interesting to note that the same procedure could be applied to a wide range of tropical plants belonging to different families and genera with only little modification.

Paclobutrazol is a growth retardant that has a negative effect on root growth but it is also an inhibitor of ABA catabolism, so addition of paclobutrazol may increase the endogenous ABA levels during desiccation and as a consequence lea genes may be induced which might make the hairy roots less susceptible to drought stress (Krizan *et al.*, 2006; Cha-Um *et al.*, 2007). Growth retardation effect of paclobutrazol and its role to induce hardiness in an array of plants have been well studied. Snir (1988) reported that paclobutrazol give better survival after cold storage. Its property to protect plants from injury due to drought, heat, chilling, ozone and SO_2 is also understood (Asare-Boamah and Fletcher, 1986; Fletcher and Hofstra, 1985; Hayat, 1978; Lee, 1985; Wang, 1985). Preconditioning citrus microshoots with paclobutrazol resulted higher *ex vitro* survival by intensive internode length, thickening of roots and reducing dehydration by regulating the stomatal function and increasing epicuticular wax per unit area of leaf (Hazarika *et al.*, 2001). PBZ treated *in vitro* shoots improves *ex vitro* survival of *Lilium* plantlets (Rajesh *et al.*, 2006; Hazarika *et al.*, 2002)

2.2.1.2.1 Pre-culture prior to vitrification

Sucrose is commonly added in the pre-culture medium to stimulate the acquisition of dehydration and freezing tolerance (Sakai and Engelmann, 2007; Yoshimatsu *et al.*, 1996; Xue *et al.*, 2008). Inclusion plant growth regulators like 2, 4-D also enhance recovery after cryopreservation (Yoshimatsu *et al.*, 1996; Touno *et al.*, 2006).

An average survival rate of 83 percent observed when *Atropa belladonna* hairy root tips pre-cultured with 2, 4-D before cryopreservation, and the highest of 96 percent than those pre-cultured without phytohormone (Touno *et al*., 2006).

Yoshimatsu *et al*., (1996) reported that root tips of *Panax ginseng* pre-cultured with 0.3*M* sucrose and 0.1mg/l 2,4-D for 3 days prior to immersion into liquid nitrogen had 60 percent survival rate.

Angelica acutiloba root tips is also shown to give high recovery rates if pre-cultured with a combination of 0.3*M* sucrose and 0.1 mg/l 2, 4–D for one day (Yoshimatsu, 2000)

Three-day pre-culture of alginate-embedded root tips of *Eruca sativa* with 0.3*M* sucrose is known to improve their post-cryo recovery (Xue *et al*., 2008), while one day pre-culture is sufficient for adventitious roots of *Hyoscyamus niger* (Jung *et al*., 2001).

Kim *et al*. (2010) reported that step-wise increase of sucrose concentration in pre-culture medium from 0.3 to 0.5 *M* or pre-culture with 0.3 *M* sucrose alone produce better regeneration of *Rubia akane* hairy roots undergone droplet vitrification than non-pre-cultured controls. Post-cryo root regeneration in the later case is enhanced to 48-53 percent simply by extending the duration of pre-culture with 0.3 *M* sucrose from 29 to 54 h with other parameters set at the same value.

2.2.1.2.2 Osmoprotection (Loading treatment)

For many species, pre-culture with sucrose appears to be unsatisfactory to produce a high level of survival by vitrification (Sakai, 2000). For many others, direct exposure to highly concentrated vitrification solutions is toxic. Loading implies a short (20-60 min) exposure of samples to cryoprotectant mixture with moderate

concentration prior to dehydration with highly concentrated vitrification solutions (Nishizawa *et al*., 1993).

Among the various loading solutions tested, a mixture of 2.0 *M* (18 percent) glycerol plus 0.4 *M* (14 percent) sucrose was very effective in inducing tolerance to dehydration by the vitrification solution (Nishizawa *et al.*, 1992; Sakai *et al.*, 1991). The procedure aims at preparing cells to further osmotic stress, thus mitigating toxicity of vitrification Solutions and minimizing sample injury during the dehydration step (Sakai and Engelmann, 2007)

Loading is particularly important, for hairy roots, which is highly susceptible to the toxic effects of vitrification solutions (Lambert *et al*., 2009). Optimization of both loading solution composition and loading duration before treatment with vitrification solution is required. Lower recovery of cryopreserved roots before and after the optimum loading period may be due to osmotic stress rather than to ice crystallization

Hairy roots of *Rubia akane* is damaged by direct exposure to vitrification solutions. Loading solution (a mixture of 17.5 percent glycerol and 17.5 percent sucrose) showing higher post-cryo regeneration suggests that balancing weights of glycerol and sucrose might be necessary for hairy roots (Kim *et al*., 2010)

Xue *et al*. (2008) reported that optimum loading solution treatment for *Eruca sativa* hairy roots found to be 20 min at 25°C is valid for the cryopreservation. Loading solution treatment is valid for the preservation of *Eruca sativa* hairy roots, whereas completely ineffective on the hairy roots of *Astragalus membranaceus* and *Gentiana macrophylla* Pall.

Pretreatment of the roots with loading solution (LS) had a clear positive effect on viability of the *Maesa lanceolata* hairy roots, maintaining the survival rate at 66 percent after 5 min of PVS2 treatment (Lambert *et al.*, 2009).

2.2.1.2.3 Exposure to Vitrification solutions

The one of the keys to successful cryopreservation by vitrification is the careful control of dehydration and prevention of injury by chemical toxicity or excess osmotic stresses during treatment with PVS_2 (Reed, 2008). Optimization of exposure time to vitrification solution is vital to reduce cytotoxicity. When vitrification is applied to hairy roots, difficulty is to overcome the high toxicity of concentrated vitrification solutions. Thus, the duration of the dehydration treatment should be very short (Kim *et al.*, 2010)

The optimal duration of incubation with PVS_2 is proved as 8 min for hairy roots of *Panax ginseng* and 15 min for their adventitious roots (Yoshimatsu *et al.,* 1996; Oh *et al.,* 2009). For *Hyoscyamus niger* adventitious roots and *Atropa belladonna* hairy roots, the optimal duration of PVS_2 treatment at 0ºC is shown as 15 and 20 min, respectively (Jung *et al.,* 2001; Touno *et al.*, 2006).

Recovery of *Eruca sativa* hairy roots cryopreserved using encapsulation-vitrification decreased from 73.3 to 12.3 percent when PVS_3 was replaced by PVS2 under the same treatment conditions (Xue *et al.,* 2008).

Kim *et al.* (2010) reported that *Rubia akane* exhibited highest post-cryo regeneration of 79.5 percent with 80 percent PVS_3 as vitrification solution (w/v, 40 percent glycerol and 40 percent sucrose).

However, despite these encouraging reports, hairy roots of some plant species appear to be very sensitive to PVS_2. Lambert and Geelen (2007) noted that survival of hairy root tips of *Maesa lanceolata* dropped down to 5 percent after 20 min incubation with PVS_2. Cytotoxicity of PVS_2 was the main barrier, which limited the application of vitrification to hairy roots of *Maesa lanceolata* and *Medicago truncatula* (Lambert *et al*., 2009).

2.3 PHYTOCHEMICAL ANALYSIS

Phytochemistry deals with the various chemical constituents accumulated in plants. Plants have potent biochemicals and have components of phytomedicine. Plant based natural constituents can be derived from any part of the plant like bark, leaves, flowers, roots, fruits, seeds, etc (Gordon and David, 2001) *i.e*. any part of the plant may contain active components. The beneficial medicinal effects of plant materials result from the combinations of secondary metabolites present in the plant. The medicinal actions of plants are unique to particular plant species or groups and are consistent with this concept as the combination of secondary products in a particular plant is taxonomically distinct (Wink, 1999). The plants are rich in secondary metabolites like flavonoids, alkaloids, pyrethrins and sterols etc. These pigments can be readily detected with a range of spectrophotometric and chromatographic separation techniques (Charlwood and Rhodes, 1990).

2.3.1 Stability of regenerates from cryopreserved material with regard to secondary metabolites

It is particularly important that cryopreserved plant cells remain capable of producing cells or tissue identical with untreated ones. The re-growth and conservation of biosynthetic potential and genetic stability are three important criteria for the application of cryopreservation. There are an increasing number of reports

indicating that no changes are observed in the material regenerated from cryopreservation (Engelmann, 1997)

In *Hyoscyamus niger* adventitious roots, tropane alkaloids production of cryopreserved roots is reported as same compared to untreated ones even first subculture after cryopreservation (Jung *et al*., 2001).

Xue *et al*. (2008) reported that no visible abnormalities in morphology and growth rates are observed between cryopreserved and non-cryopreserved hairy root cultures of *Astragalus membranaceus, Eruca sativa* and *Gentiana macrophylla* Pall.

Panax ginseng hairy roots regenerated from cryopreserved root tips grew well and showed the same ginsenoside productivity and patterns as those of the control hairy roots (Yoshimatsu *et al.*, 1996).

There are also instances where cryopreserved hairy roots exhibits lower alkaloid contents than the untreated control as reported in *Atropa belladonna* clone M8 (Touno *et al*., 2006).

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The present study, 'Cryopreservation of hairy root culture of *Decalepis arayalpathra* (Joseph & Chandras.) Venter' was carried out at the Biotechnology and Bioinformatics Division, Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode during 2013-2014. The study aimed to find an efficient cryopreservation protocol for hairy root culture of *Decalepis arayalpathra* and a comparative analysis of secondary metabolite profile.

The four major components of the study are (1) Maintenance of hairy root culture through regular subculture (2) Optimization of pretreatment and desiccation conditions for cryopreservation and (3) Growth study of control and cryopreserved root culture (4) Comparative analysis of phytochemical profile between the culture derived from control and cryopreserved root culture for the assessment of secondary metabolite production potential of post-freeze recovered hairy roots.

3.1 PLANT MATERIAL

Hairy roots of *D. arayalpathra* (Plate I) established and maintained at Biotechnology and Bioinformatics Division, JNTBGRI were used as the plant material in the present study.

3.2 SUBCULTURE AND MAINTENANCE OF HAIRY ROOTS

3. 2.1 Induction and establishment of hairy roots

The hairy root culture of *Decalepis arayalpathra*, is already developed in the Plant Biotechnology and Bioinformatics division, JNTBGRI. The procedure is briefed below:

Hairy roots were induced from cotyledon and hypocotyls explants. The explants were pre-incubated on half strength MS basal medium (half-MSB) (solid) for 2–3 days prior to infection. The pre-incubated explants were wounded with sterile scalpel and dipped into the overnight grown bacterial cultures (*Agrobacterium rhizogenes* strains A4, TR-105, LBA-9402, and MTCC 532) for the induction of hairy roots. Wounds were made gently on the explants without

bacteria and the treatments served as controls. The infected and control explants were placed on 15 ml half-MSB medium in petridishes and incubated under dark. After 3 days of co-cultivation, the explants were rinsed with sterile distilled water, blotted on sterile filter paper and transferred onto fresh half-MSB medium containing 500 mg l^{-1} cefotaxime and ampicillin individually and incubated under dark until the infected sites responded and thereafter cultured at 24±2°C under a 16 h photoperiod.

To eliminate the *Agrobacterium*, the explants were transferred onto fresh half-MSB medium containing the same concentration of antibiotics at 1 week intervals for 10 weeks. Along with the induced roots or galls, part of the explant was retained during the initial 3 passages into the medium containing antibiotics. Later, roots and galls were excised from the explants, segmented and transferred onto half-MSB medium with antibiotics. When the bacteria-free, well-established roots were obtained, they were transferred to 80 ml of half-MSB liquid medium in 250 ml Erlenmeyer flasks and kept on a gyrotory shaker (New Brunswick Scientific, Co, USA.) at 100 rpm (Sudha *et al.*, 2013).

3.2.2 Subculture

Subculture was done by inoculating root segments alternatively in half strength MS liquid and solid medium (Plate1). Actively grown 5-6 week old contamination free cultures were selected for subculturing. Established hairy root cultures in half strength MS solid medium was subcultured in half strength liquid MS medium and kept on a gyrotory shaker (New Brunswick Scientific, Co, USA.) at 100 rpm. From half strength liquid medium, roots were subcultured to half strength MS solid medium. Cultures were incubated at 24±2°C under dark condition.

3.3 CULTURE MEDIA

3.3.1 Hairy root subculture and maintenance

Murashige and Skoog (MS) (1962) was used with half the strength of both macro and microsalts. Macro and micro nutrients were prepared as stock solutions

Plate 1. *Decalapis arayalpathra* and its hairy root cultures. (A) *D. arayalpathra* in a native locality. (B) & (C) Hairy root cultures developed and maintained at JNTBGRI in half strength MS (solid). (B) and half strength MS (liquid) (C) medium.

as detailed in Appendix-I and stored at 4°C. Half strength MS medium was prepared by taking half the volume of macro and micro nutrients under continuous stirring and the other ingredients (sucrose and inositol) added in full strength and made up to the final volume using distilled water. After adjusting pH to 5.8 with either 0.1 *N* NaOH or 0.1 *N* HCl agar was added (0.6 percent; w/v) and dissolved by uniform heating. Aliquots (80 ml) of nutrient medium were dispensed into 250 ml Erlenmeyer flasks and tightly closed with non-absorbent cotton plugs before being autoclaving under 1.1 Kg/cm^2 pressure at 121°C for 18 min. Liquid media were prepared by the same manner without adding agar.

3.3.2 Preconditioning media - Modified MS

For preconditioning of root cultures, some modifications of MS medium were used.

1. Modified half strength medium: MS medium with half the strength of macronutrients without having ammonium nitrate and glycine but containing increased sucrose concentrations (0.088, 0.2, 0.3, 0.4, and 0.5 *M*).

2. Modified MS: It is as per the modification by Decruse and Seeni (2002)**.** It contains all the components of MS medium; not having ammonium nitrate and glycine but having 300 mg/l ammonium phosphate and increased sucrose concentration (0.2, 0.3, 0.35 and 0.43 *M*).

3. Half strength MS with paclobutrazol: It is by adding 0.2 *M* sucrose and different concentration of PBZ (1, 2 and 3 mg/l) to the above mentioned modified half strength MS medium. After adjusting pH to 5.8 with either 0.1 *N* NaOH or 0.1 *N* HCl agar was added (0.6 percent; w/v) and dissolved by uniform heating.

3.3.3 Encapsulation media

3.3.3.1 Encapsulation solution

Sucrose at the concentration of 102.6 g/l (0.3 *M*) and 171 g/l (0.5 *M*) added to half strength MS devoid of calcium chloride and made up to the final volume (50 ml) and then adjusted the pH to 5.8. Sodium alginate (2.5 percent) was added and dissolved

by stirring. The medium was dispended in 10 ml aliquots in 25 ml screw cap bottles and autoclaved at 121° C and 1.1 kg/Cm^2 pressure for 18 minutes.

3.3.3.2 Calcium chloride solution

Sucrose at the concentration of 102.6 g/l (0.3 *M*) or 171 g/l (0.5 *M*) and 14.7 g/l Calcium chloride added to `half strength MS and made up to the final volume, pH was adjusted to 5.8, dispensed in 50 ml aliquots in 100 ml screw cap bottles and autoclaved at 121° C and 1.1 kg/Cm^2 pressure for 18 minutes.

3.3.4 Pre-culture solutions

3.3.4.1 Encapsulation dehydration (Half strength MS +0.3 /0.5 M sucrose)

Sucrose at the concentration of 102.6 g/l or 171 g/l was added to half strength MS medium, made up to the final volume and pH adjusted to 5.8, dispensed as 50 ml aliquots into 50 ml screw cap bottles and autoclaved.

3.3.4.2 Pre-culture medium for Vitrification experiments (Half strength MS +0.3 Msucrose+0.l mg/l 2, 4-D)

Sucrose at the concentration of 102.6 g/l and 0.1 mg/l 2, 4-D was added to half strength MS medium. The medium was made up to the final volume, pH adjusted to 5.8, added agar to 0.6 percent and dissolved by uniform heating. The solution was then autoclaved at 121° C and 1.1kg/Cm^2 pressure for 18 minutes.

3.3.5 Loading solution (Half MS + 0.4 *M* sucrose + 2 *M* glycerol)

Sucrose at the concentration of 136.9 g/l was added to half strength MS medium containing 0.4 *M* sucrose. Glycerol was added to this solution to 2 *M* concentration and made up to the final volume. The solution was then autoclaved at 121°C and 1.1 kg/Cm^2 pressure for 18 minutes.

3.3.6 PVS_2 (Plant Vitrification Solution, Sakai *et al*., 1990)

A mixture of 30 percent (w/v) glycerol, 15 percent (w/v) ethylene glycol and 15 percent (w/v) dimethyl sulfoxide was prepared and adjusted the final volume to 100 ml using half MS medium containing 0.4 *M* sucrose (pH 5.8). The mixture is extremely viscous and takes some time to completely dissolve in solution. Thus the mixture was

added gradually under vigorous agitation on a magnetic stirrer for 15 min. The solution was then filter sterilized under vacuum through 0.22 µm nitrocellulose filter membrane (Millipore) using a Millipore filtration apparatus and Millipore vacuum pump. The filtered solution was dispensed into sterile screw cap bottles and refrigerated for storage.

3.3.7 Washing solution (half MS + 1.2 *M* sucrose)

Sucrose at the concentration of 410 g/l was added to half MS medium before adjusting to the final volume. The medium was dispended in 50 ml aliquots in 100 ml screw cap bottles then autoclaved at 121° C and 1.1 kg/Cm^2 pressure for 18 minutes.

3.3.8 Recovery medium

Sucrose at the concentration of 30 g/l was added to half strength MS medium, made up to the final volume, pH adjusted to 5.8, added agar (0.6 percent), dissolved by uniform heating, dispensed as 150 ml aliquots in 250 ml flasks, plugged using cotton plug and autoclaved. After autoclaving, the solution was dispensed into 8 mm diameter sterile glass petriplates under aseptic conditions, cooled and sealed using cling film.

3.4 OPTIMIZATION OF CRYOPRESERVATION PROTOCOL

3.4.1 Optimization of root tip size

Hairy roots after 30 day of culture in half-MSB were transferred to sterile petridishes and root tips of 1, 2 and 3 mm size were dissected out and inoculated onto half-MSB solid medium. Observations were made after 1-2 weeks and percentage survival as well as increase in length of the root tips recorded. The optimum size was selected based on higher survival rate and growth rate.

3.4.2 Preconditioning treatments

Preconditioning treatments are often practiced to harden the tissue for cryoprotection prior to dehydration and LN exposure. It is usually done through culture of source material in presence of osmoticum active agents like sucrose, mannitol, sorbitol etc. In the present study, we used sucrose as the osmotic agent.

3.4.2.1 Culture of hairy roots in Half-MSB with different concentration of sucrose

In vitro established hairy roots (after 30 day of culture in half-MSB) were cut into 5 cm segments and inoculated onto half strength MS medium supplemented with different sucrose concentration (0.088, 0.2, 0.3, 0.4 and 0.5 *M*) and 0.6 percent agar. Cultures were incubated at 24±2°C under dark. Observations on root growth and branching were made after 3 weeks and data on root elongation, number of branches and length of branches were recorded.

3.4.2.2 Culture of Hairy roots in modified half strength MS medium with different sucrose concentrations

In vitro established hairy roots (5 cm segments; after 30 day of culture in half-MSB) were inoculated onto modified half MS medium (devoid of ammonium nitrate and glycine) supplemented with different sucrose concentration (0.088, 0.2, 0.3, 0.4, and 0.5 *M*) and 0.6 percent agar. Cultures were incubated at 24±2°C under dark condition. Observations were made after four weeks and data on root length and number of branches recorded.

3.4.2.3 Hairy roots cultured in modified half strength MS supplemented with ammonium phosphate and different sucrose concentrations

Growing root tips from *in vitro* established hairy roots (after 30-day of culture in half-MSB) were cut into 5cm segments and inoculated onto modified half MS medium (devoid of glycine; ammonium nitrate replaced with 300 mg/l ammonium phosphate) supplemented with different sucrose concentration (0.2, 0.3, 0.35 and 0.43 *M*) and 0.6 percent agar. Cultures were incubated at 24±2°C under dark condition. Observations were made at weekly intervals and data on root growth, number of branches and length of branches were recorded after three weeks.

3.4.2.4 Hairy roots cultured in presence of paclobutrazol, a growth retardant

Growing root tips from *in vitro* established hairy roots (after 30-day of culture in half-MSB) were cut into 5 cm segments and inoculated onto modified half MS medium (devoid of glycine; ammonium nitrate replaced with 300 mg/l ammonium phosphate) supplemented with 0.2 *M* sucrose, 0.6 percent agar and three concentrations

of paclobutrazol (1, 2 and 3 mg/l). Cultures were incubated at 24±2°C under dark. Observations were made at weekly intervals and data on root growth, number of branches and length of branches were recorded after three weeks.

3.4.3 Cryopreservation through Encapsulation- Dehydration Technique

The method described by Fabre and Dereuddre (1990) and Hirata *et al.* (2002) was followed for cryopreservation of *D. arayalpathra* hairy roots.

3.4.3.1 Encapsulation

Root tips (2-3 mm) were excised aseptically from preconditioned hairy root cultures using sterile blade and suspended in 2.5 percent sodium alginate prepared in calcium free half MS medium supplemented with 0.3*M* or 0.5 *M* sucrose as the case may be and immediately dropped into 0.1 *M* calcium chloride solution containing the same concentration of sucrose, using a sterile pasteur pipette. Sucrose concentration in the alginate solution and calcium chloride was maintained equal to that of preconditioning culture. The encapsulated root tips kept in the solution for 30 min to allow formation of calcium alginate beads.

3.4.3.2 Pre-culture

3.4.3.2.1 Encapsulation and pre-culture

Root tips encapsulated in calcium alginate were pre-cultured in half MS liquid medium supplemented with different concentrations of sucrose (0.3 and 0.5 *M*) taken in 50 ml screw cap bottle (50 beads in 25 ml medium) and incubated at 24±2°C under dark for one day prior to dehydration and LN exposure.

3.4.3.2.2 Pre-culture and encapsulation

Root tips excised from root cultures preconditioned in half MS and supplemented with 0.35 *M* sucrose were pre-cultured in half MS liquid medium supplemented with 0.3 *M* or 0.5 *M* sucrose as the case may be, (50-10 toot tips in10 ml medium) and incubated at 24±2°C under dark for one day. The pre-cultured root tips were then encapsulated in alginate beads as described above keeping sucrose concentration in the alginate and calcium chloride solution equal to that in the pre-culture solution (0.3 *M*

or 0.5 *M*). Immediately after encapsulation, the beads were subjected to dehydration and LN exposure.

3.4.3.2.3 Effect of pre-culture with DMSO concentration on encapsulated and cryopreserved root tips

DMSO was included in the pre-culture medium to test whether it can improve recovery after cryopreservation. Root tips encapsulated in calcium alginate beads were pre-cultured in half MS liquid medium supplemented with 0.3 or 0.5 *M* sucrose and 0, 1 or 3 mg/l DMSO for one day before dehydration and LN exposure.

3.4.3.3 Dehydration and cryopreservation

To determine the optimum period of dehydration to attain safe moisture level, encapsulated root tips after pre culture were transferred to petriplates lined with 2-3 layers of sterile filter paper and blotted thoroughly by rolling over the filter paper. The blotted beads were then transferred to another petriplate lined with filter paper and desiccated for 0 to 5 h duration under sterile air flow of laminar flow cabinet. At one hour interval, samples of 10 beads were transferred into 2 ml cryovials, plunged into liquid nitrogen (LN) and held overnight. Simultaneously, 10 beads were transferred to recovery medium (solid) prepared in 7 mm diameter glass petriplates and incubated in a culture room under dark conditions. Samples of about 50 empty beads were subjected pre-culture and dehydration as that of test samples and their fresh weight as well as after each hour of desiccation was determined. After 5^{th} hour of desiccation, dry weight of the beads were determined by forced drought oven method (ISTA, 1985) by placing in an oven maintained at 130˚ C. Moisture content in the fresh beads and after every one hour dehydration was determined using the formula,

MC = [(Initial weight – Dry weight) / Fresh weight] * 100

3.4.3.4 Rewarming and Post-freeze recovery

On rewarming, cryotubes were removed from liquid nitrogen and transferred to a circulating water bath maintained at 40° C and allowed 30-60 s for complete thawing. The re-warmed root tips were transferred to recovery medium (half MS+ 3 percent sucrose + 0.6 percent agar) and incubated in a culture room at 24±2°C under dark.

Observation on root tip survival and elongation was made after 1-2 weeks and percent survival/ regeneration was determined.

3.4.4 Cryopreservation through Vitrification

3.4.4.1Vitrification

The method described by Tauno *et al.* (2006) for cryopreservation of hairy roots of *Atropa belladonna* was followed, root tips (2-3 mm) isolated from root culture preconditioned in modified half MS and normal half strength MS medium with 3 percent sucrose for 30 days were pre-cultured on half strength MS solid medium fortified with 0.3 *M* sucrose and 0.1 mg/l 2, 4-D at 24 ± 2^{o} C in the dark for (1, 2 or 3) days.

The pre-cultured root tips were transferred into 2 ml cryotube and added 2 ml of loading solution (2 *M* glycerol and 0.4 *M* sucrose in half MS medium) and kept for 10 min at 25°C. The loading solution was removed and 2 ml pre-chilled PVS_2 solution (30 percent w/v glycerol, 15 percent w/v ethylene glycol, 15 percent w/v dimethyl sulfoxide and 0.4 *M* sucrose in half MS liquid medium) was added and kept in a refrigerator. A total of 12-16 vials each with 5-10 root tips were maintained and after each treatment period (5 min-2 hour) one vial was directly plunged into liquid nitrogen and held there for a minimum of one hour. After loading treatment and each period of PVS_2 treatment, root tips (5-10) in one vial was washed (procedure described in the next section) and transferred to recovery medium to use as control.

3.4.4.2 Rewarming

On rewarming, cryotubes were removed from liquid nitrogen and immediately transferred to circulating water maintained at 40^{o}C without any disturbance to avoid the possibility of de-vitrification. For complete thawing, 30-60 s was given.

3.4.4.3 Washing and post-freeze recovery

The PVS_2 treated control samples (immediately after treatment) and those retrieved from liquid nitrogen were washed in half MS liquid medium containing 1.2 *M* sucrose. Initially, 1 ml of the PVS_2 was removed from the vial using a

sterile pasteur pipette, added 1 ml washing solution and kept for 3 min. This process was repeated 3 times and finally replacing with 2 ml washing solution. The whole washing process was done at 25°C and lasted for 10 min. The washed root tips were transferred onto half MS solid medium containing 3 percent sucrose and incubated at 24±2°C in the dark for 2 week. Observations were made after 2-4 weeks and data on percent survival and regeneration recorded.

3.5 GROWTH STUDY OF CONTROL AND CRYOPRESERVED ROOT CULTURE

Hairy root regenerated from cryopreserved root tips were multiplied in half- MSB liquid medium and kept on a gyrotory shaker (New Brunswick Scientific, Co, USA.) at 100 rpm. Control hairy roots were also grown as above to compare the growth between control and cryopreserved root cultures.

3.6 PHYTOCHEMICAL ANALYSIS

3.6.1 Phytochemical Stability of regenerated root culture from cryopreserved hairy roots

Assessment of growth and secondary metabolite production potential of post-freeze recovered hairy roots and non cryopreserved hairy roots was carried out.

3.6.1.1 Determination of secondary metabolite profile of normal and cryopreserved hairy roots

The phytochemical procedure was carried as per Sudha *et al.,* 2013. The dried root powder (3 g) of cryopreserved hairy root and control (hairy root) cultures of four week old were extracted using 30 ml petroleum ether (60–80 °C) for 48 h with agitation twice. The extract was filtered using presoaked whatman No. 1 filter paper. The extracts thus obtained were combined and concentrated using a rotavapour (Heidolph Laborota 4000, Germany) and chromatographed on silica gel TLC plates (Merck, 25 DC-Alufolien 20x20 cm Kieselgel 60 F_{254}). The chromatogram was developed in a solvent system consisting of petroleum ether (60–80°C): ethyl acetate (9:1). The secondary metabolite profile was observed under UV chamber and recorded the Rf values of each compound. Thereafter the

plates were sprayed with anisaldehyde and visualized the spots followed by heating at 105°C. Anisaldehyde reagent was prepared as per the procedure briefed below.

Freshly prepared 0.5 ml anisaldehyde in 10 ml glacial acetic acid, 85 ml methanol and 5 ml concentrated sulfuric acid and stored in amber bottles. Rf value was calculated using the formula

$$\text{Rf value} = \frac{\text{Distance from baseline travelled by solute}}{\text{Distance from baseline travelled by solvent (solvent front)}}$$

3.6.1.1.2 HPLC profile comparison and determination of MBALD

The qualitative comparison of secondary metabolite profile of cryopreserved hairy root and control (hairy root) and quantitative determination of MBALD was done by HPLC, which was performed on a Waters 600 series pump, Reprobond C18 column 4.6X250 mm, 7725 Rheodyne injector. The mobile phase was a mixture of methanol: water: 36% acetonitrile (45:55:2) at a flow rate of 1 ml/min and detection using 2487 UV Detector at λ max 278 nm with a known quantity of the reference compound.

3.7 EXPERIMENTAL DESIGN AND DATA ANALYSIS

The whole cryopreservation experiment was completely randomized and each experiment was repeated twice, but in a few instances could not be repeated. In each experiment, 5-10 root tips were utilized for each treatment. Thus percent survival/regeneration was calculated from the 5-10 explants used.

RESULTS

4. RESULTS

The results of the research work entitled "Cryopreservation of hairy root culture of *Decalepis arayalpathra* (Joseph & Chandras.) Venter'' conducted at the Biotechnology and Bioinformatics division of Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode during the academic year 2013-14 are presented in this chapter.

4.1 CRYOPRESERVATION OF HAIRY ROOT CULTURE OF *Decalepis arayalpathra*

4.1.1 Optimization of root tip size

Optimized root tip size is a pre-requisite to get maximum recovery after cryopreservation. As a preliminary step, 1-3 mm long root tips were evaluated to know their regeneration efficiency. The root tips cultured on half strength MS medium have exhibited differential response depending on their size (Table 1) The shortest roots (1.66±0.32 mm) exhibited poor regeneration so that only 52 percent of them showed elongation. Longer root tips (2-3 mm) exhibited better response so that 63.15-88.23 percent of them showed elongation. Therefore, 2-3 mm long root tips were used for all the cryopreservation experiments.

4.1.2 Preconditioning of hairy roots

Preconditioning is an essential step to harden the plant material for making tolerant to desiccation and LN exposure. In the present investigation, increased concentration of sucrose supplemented to half strength MS medium as well as some of its modifications was attempted.

4.1.2.1 Hairy roots grown in half MS with different concentration of sucrose

Hairy root tip cuttings (5 cm) cultured into half strength MS with 0.088, 0.146, 0.2 and 0.3 *M* sucrose showed their tolerance even up to 0.3 *M* concentration. However, the root tips showed elongation with significant

branching only up to 0.2 *M* concentration. Root cuttings cultured in 0.3 *M* sucrose showed less degree of branching but profuse callusing (Table 2; Plate 2F).

4.1.2.2 Hairy roots cultured in modified half MS medium with different sucrose concentrations

Hairy roots grown in half strength MS medium devoid of Ammonium nitrateand glycine but supplemented with different sucrose concentrations (0.088, 0.2, 0.3, 0.4 and 0.5 *M*) were investigated for their growth performance. The roots were thin (Plate 2 A-E) and high sucrose concentration was inhibitory to root growth (Table 3). The root tip segments exhibited appreciable elongation and branching up to 0.2 *M* sucrose concentration.

4.1.2.3 Preconditioning in modified half strength MS (w/o NH_4NO_3 + 300 mg/l $NH_4H_2PO_4$) supplemented with 0.2-0.43 M sucrose

Hairy roots grown in modified half MS (w/o NH_4NO_3 and glycine + 300 mg/l $NH_4H_2PO_4$) supplemented with 0.2, 0.3 and 0.35 *M* sucrose showed elongation and branching (Table 4)**.** As the sucrose concentration increased, the roots became thick and the branches short (Plate 3 A-D). At 0.43 *M* sucrose, the roots did not grow further but a few branches with retarded growth were observed (Plate 3E). Such roots tolerant to high sucrose were selected for cryopreservation experiments.

Table 1: Effect of root tip size on elongation and growth.

Root tip size (mm) Mean ± SD; n=8-11	Response after 4 days	
	Percent of roots showing root elongation	Root length (mm) Mean ± SD; n=8-11
1.66±0.32	52.38	2.84±1.74
2.22±0.35	63.15	4.53±2.23
3.00±0.64	88.23	5.68±1.40

Note: 1-3 mm size root tips were dissected out, categorized into three size groups and cultured into half strength MS medium.

Table 2: Response of roots subjected to preconditioning with different concentration of sucrose

Sucrose concentration (*M*)	Increase in length (cm) Mean ± SD; n=2-5	No. of branches Mean ± SD; n=5
0.088	4.13±1.65	08.6±03.58
0.146	4.50±0.70	11.6±10.04
0.200	2.63±1.44	07.6±04.11
0.300	00.0±0.00	01.2±01.11

Note: 5 cm size root segments were inoculated into half MS solid medium. Observations were recorded after three weeks.

Table 3: Response of root segments subjected to preconditioning in modified half strength MS medium with different sucrose concentration

Sucrose concentration (*M*)	Increase in length (cm) Mean ± SD; n=5-15	No. of branches Mean ± SD; n= 10-15
Control (Half MS)	3.45±1.46	08.73±4.96
0.088	3.30±1.48	12.20±5.84
0.200	2.42±1.74	09.73±3.88
0.300	0.00±0.00	07.00±4.81
0.400	0.00±0.00	02.20±2.37
0.500	0.00±0.00	00.00±0.00

Note: 5 cm roots were inoculated into modified half MS (devoid of Ammonium nitrate and glycine). Observations were noted after four weeks.

Table 4: Response of roots subjected to preconditioning in modified half strength MS supplemented with Ammonium phosphateand different sucrose concentrations

Note: 5 cm long root tip cuttings inoculated into modified half MS (w/o Ammonium nitrate and glycine + 300 mg/l Ammonium phosphate) supplemented with different sucrose concentrations. Observations were recorded after three weeks.

Sucrose Concentration (*M*)	Increase in length (cm) Mean ± SD; n=2-10	No. of branches Mean ± SD; n=10-15	Branch length Mean ± SD; n=9-18
0.20	3.06±1.18	13.27±6.44	1.82±0.48
0.30	2.50±0.71	08.00±4.29	No Data
0.35	2.50±0.71	06.40±3.33	1.11±0.40
0.43	0.00±0.00	02.10±1.91	0.31±0.15

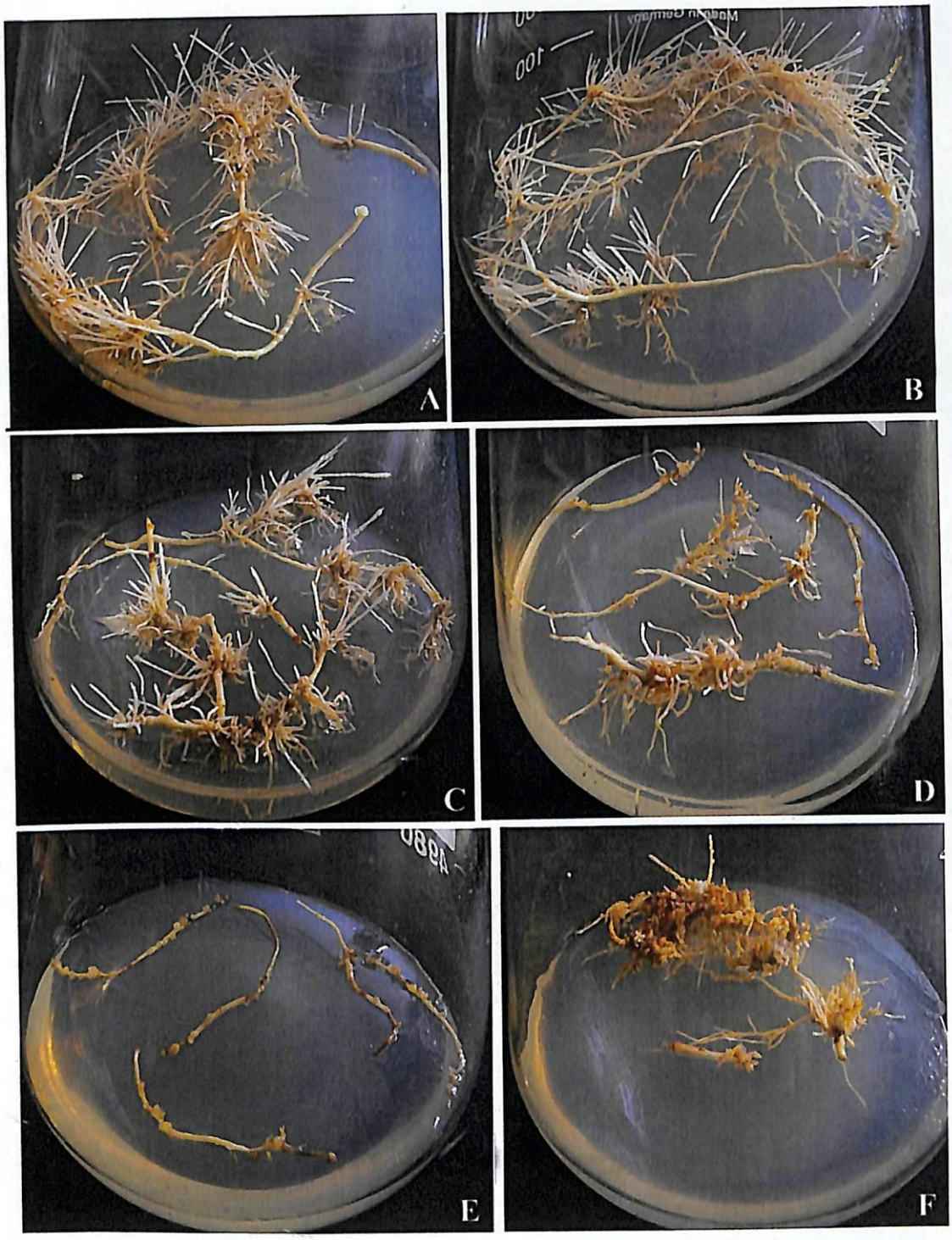

Plate 2. Effect of preconditioning of hairy roots in presence of different sucrose concentrations supplemented to half strength MS devoid of ammonium nitrate and glycine. (A) 0.08 *M* sucrose (B) 0.2 *M* sucrose (C) 0.3 *M* sucrose (D) 0.4 *M* sucrose (E) 0.5 *M* sucrose (F) Root cuttings cultured in half strength MS + 0.3 *M* sucrose showing less degree of branching but profuse callusing.

4.1.2.4 Effect of paclobutrazol, a growth retardant on root segment cultured in modified half strength MS

To get hardened root tips for cryopreservation, root tip cuttings (5 cm) were cultured in modified half strength MS (w/o Ammonium nitrate and glycine + 300 mg/l Ammonium phosphate) supplemented with 1, 2 and 3 mg/l paclobutrazol. At the three concentrations tried, PBZ caused profuse branching of roots with retarded growth so that the roots obtained was thick and short (Table 5; Plate 3 F-H).

4.1.3 Cryopreservation by Encapsulation- Dehydration method

4.1.3.1 Effect of encapsulation followed by pre-culture in 0.3 M sucrose on cryopreserved root tips.

The root tips encapsulated in calcium alginate followed by pre-culture in 0.3 *M* sucrose for one day possessed 86.33 percent moisture. The moisture content (m.c.) was reduced down to 3.76 by 4 h desiccation. Regeneration capacity of the encapsulated root tips was observed only up to 2 h desiccation. Regeneration capacity was completely lost due to desiccation beyond 2 h (Table 6). None of the root tips subjected to LN exposure showed regeneration. Initially, half MS liquid medium was used as the recovery medium, but after a particular period, the roots showed retarded growth (Plate 4 A and B). Nevertheless, the roots transferred to solid medium showed better growth (Plate 4C). Therefore, solid medium was selected as recovery medium for further experiments.

4.1.3.2 Effect of encapsulation followed by pre-culture in 0.5 M sucrose on cryopreserved root tips.

None of the encapsulated root tips subjected to pre-culture in 0.5 *M* sucrose for one day survived. Even the encapsulated root tips possessing 81.02 percent m.c failed to regenerate (Table 7). The root tips turned brown after pre-culture in 0.5 *M* sucrose. The root tips seem to be more sensitive to increased sucrose concentration.

Table 5: Effect of paclobutrazol, a growth retardant on root segment cultured in modified half strength MS

Concentration of Paclobutrazol mg /l	Increase in length(cm) Mean ± SD; n=2-8	Number of branches Mean ± SD; n=10-15
1	2.50±0.93	16.93±11.42
2	1.25±0.65	14.20±08.33
3	2.75±0.35	11.90±11.57

Note**:** 5 cm long root tip cuttings were inoculated into modified half MS (devoid of Ammonium nitrate and glycine) supplemented with 300 mg/l Ammonium phosphate+ 7 percent sucrose + Paclobutrazol. Observations were recorded after three weeks.

Table 6: Effect of encapsulation followed by pre-culture in 0.3 *M* sucrose on cryopreserved root tips.

Desiccation Duration (h)	Moisture content (percent)	Regeneration (percent)	
		-LN	+LN
0	86.33	73	0
1	49.36	83	0
2	22.16	70	0
3	07.25	0	0
4	03.76	0	0

Note: 2-3 mm long root tips excised from root cultures raised in half strength MS (liquid) supplemented with 3 percent sucrose were used. Observations were recorded after two weeks.

Table 7: Effect of encapsulation followed by pre-culture in 0.5 *M* sucrose on cryopreserved root tips.

Desiccation duration (h)	Moisture Content (percent) (Mean of two replications)	Regeneration (percent)	
		-LN	+LN
0	81.02	0	0
1	59.30	0	0
2	42.17	0	0
3	28.93	0	0
4	20.54	0	0
5	15.28	0	0

Note: 2-3 mm long root tips excised from root cultures raised in half strength MS (liquid) supplemented with 3 percent sucrose were used. Observations were recorded after one week.

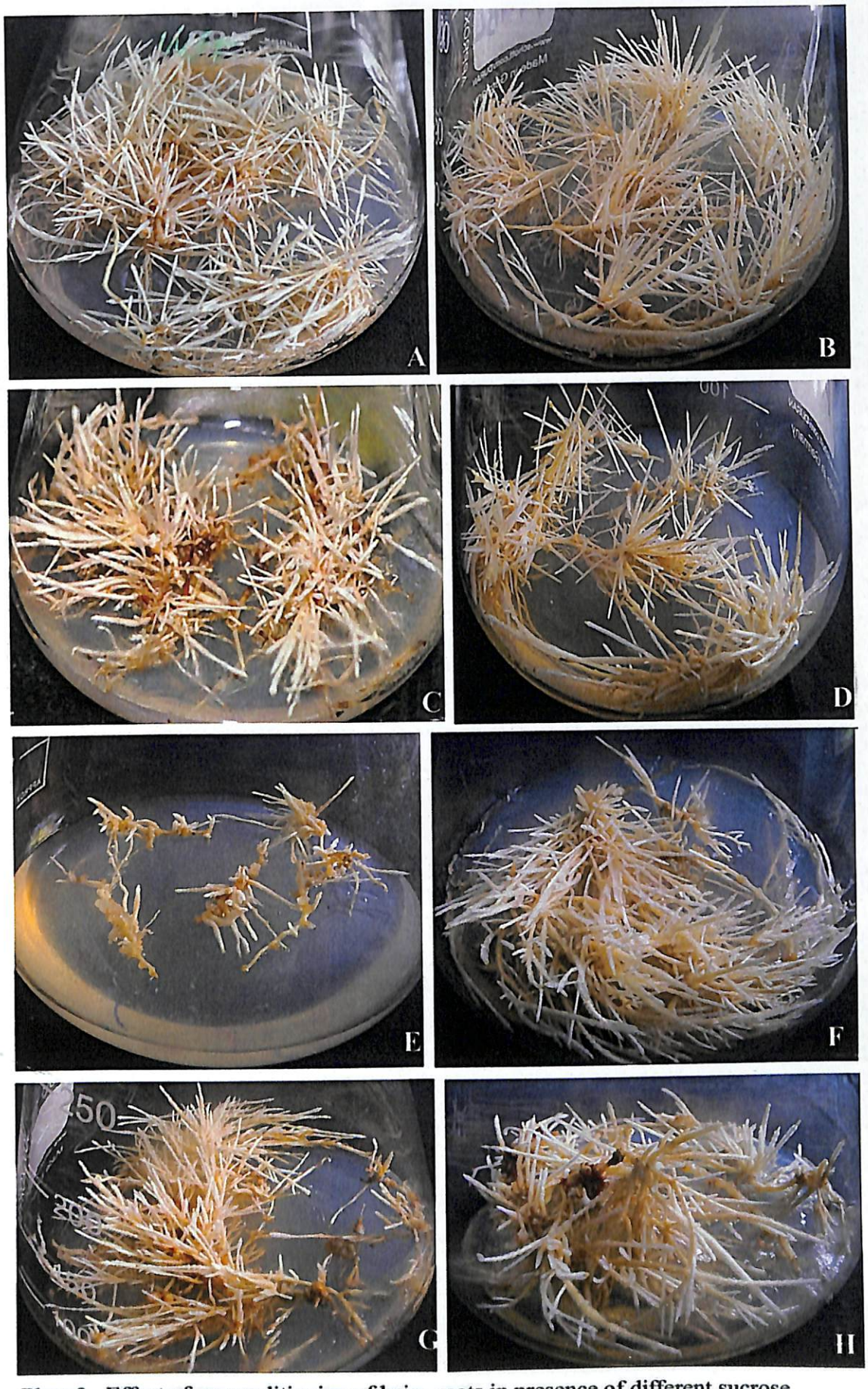

Plate 3. Effect of preconditioning of hairy roots in presence of different sucrose concentrations supplemented to modified half strength MS (no ammonium nitrate and glycine + 300 mg/l ammonium phosphate) (A) 0.15 *M* sucrose (B) 0.2 *M* sucrose (C) 0.3 *M* sucrose (D) 0.35 *M* sucrose (E) 0.43*M* sucrose (F-H) Modified half strength MS + 0.2M sucrose + 1, 2, and 3 mg/l PBZ.

4.1.3.3 Effect of pre-culture in 0.3 and 0.5 M sucrose followed by encapsulation on cryopreserved root tips

In this method, the root tips were first pre-cultured in 0.3 *M* as well as 0.5 *M* sucrose pre-culture before encapsulation and LN exposure. Even though the root tips subjected to pre-culture in 0.3 *M* sucrose and dehydration of encapsulated root tips for 3 h to attain 15 percent m.c. retained 40 percent viability, none of them tolerated LN exposure. None of the root tips pre-cultured in 0.5 *M* sucrose survived.

4.1.3.4 Cryopreservation of root tips preconditioned in modified MS supplemented with 0.3 M and 0.2 M sucrose

Root tips (2-3 mm long), separated from root tip cuttings subjected to preconditioning culture in modified half strength MS containing 0.2 *M* sucrose, encapsulated and pre-cultured in 0.3 *M* sucrose showed enhanced dehydration tolerance. Even after 4 h dehydration, at 3 percent m.c., 16 percent of the root tips showed regeneration. Root tips from cultures preconditioned in 0.3 *M* sucrose encapsulated and pre-cultured in 0.5 *M* sucrose also showed regeneration (40 percent) up on 1 h desiccation (Table 9). Even though preconditioning in high sucrose concentration increased the percentage of regeneration of root tips in desiccated control root tips, none of them survived LN treatment.

4.1.3.5 Effect of preconditioning in 0.35 M sucrose and short pre-culture in 0.5 M sucrose

Root cultures raised in presence of 0.35 *M* sucrose and root tips isolated from the later culture up on encapsulation and pre-culture in 0.5 *M* sucrose for 1 h did not give any positive response with regard to LN tolerance (Table 10).

Table 8: Cryopreservation of root tips subjected to preconditioning culture in 0.2 *M* and 0.3 *M* sucrose

Desiccation Duration (h)	MC %	0.3 *M*		0.5 *M*	
		Regeneration (%)			
		-LN	+LN	-LN	+LN
0	83	60	0	16	0
1	52	50	0	40	0
2	27	25	0	0	0
3	11	20	0	0	0
4	03	16	0	0	0

Note: Root tip cuttings grown in modified half strength MS (w/o Ammonium nitrate and glycine + 300 mg/l ammonium phosphate) supplemented with 0.2 and 0.3 *M* sucrose for 30 days were used as the explants source. Root tips (2-3 mm long) separated from the preconditioned culture were encapsulated and pre-cultured either in 0.3 *M* (preconditioned in 0.2 *M* sucrose) or in 0.5 *M* sucrose (preconditioned in 0.3 *M* sucrose) for one day. Observations were made after one week.

Table 9: Cryopreservation of root tips preconditioned in 0.35 *M*

Desiccation Duration (h)	MC %	0.5 *M*	
		Regeneration (%)	
		-LN	+LN
0	77.78	20.0	0
1	53.52	30.7	0
2	33.65	00.0	0
3	18.40	00.0	0
4	10.00	00.0	0

Note: Root tip cuttings grown in modified half strength MS (w/o Ammonium nitrate and glycine + 300 mg/l ammonium phosphate) supplemented with 0.35 *M* sucrose for 30 days were used as the explants source. Root tips (2-3 mm long) separated from the preconditioned culture were encapsulated and pre-cultured in 0.5 *M* for 1 h before dehydration and LN exposure. Observations were made after one week.

4.1.3.6 Effect of DMSO in pre-culture solution as a cryoprotectant

4.1.3.6.1 Cryopreservation of root tips preconditioned in 0.2 M sucrose and pre-cultured in 0.3M and 0.5 M sucrose

Regeneration percentage of encapsulated and desiccated root tips was significantly enhanced by the addition of DMSO into the pre-culture medium (Table 11). Due to 4 h desiccation, the m.c. came down to 16.76 and only 11 percent of the root tips pre-cultured in the absence of DMSO showed regeneration (Plate 4D). Regeneration of the corresponding root tip samples pre-cultured in the presence of 1 and 3 percent DMSO were however enhanced to 60 and 44 percent respectively (Plate 4 E and F). Therefore, 1 percent DMSO is adjudged as the best to improve desiccation tolerance of root tips. However, the treatments were not enough to get LN tolerance as none of the root tips subjected to LN exposure showed regeneration. Root tips pre-cultured in 0.5 *M* sucrose showed slow desiccation so that even after 4 h, the m.c. reached 26.66 percent retaining regeneration capability even in the absence of DMSO (Table 12).

4.1.3.6.2 Cryopreservation of root tips preconditioned in 0.35 M sucrose and pre-cultured in 0.3 and 0.5 M sucrose + DMSO

Addition of DMSO did not improve dehydration tolerance of encapsulated root tips that were isolated from root culture preconditioned in presence of 0.35 *M* sucrose (Table 13). However, encapsulated root tips pre-cultured in presence of 0.5 *M* sucrose showed reduced rate of regeneration even in presence of DMSO (Table 14). Moreover, none of the treatments were good enough to retain viability of root tips after LN exposure.

Table 10: Cryopreservation of root tips preconditioned in 0.35 *M*

Desiccation Duration (h)	MC %	0.5 *M*	
		Regeneration (%)	
		-LN	+LN
0	77.78	20.0	0
1	53.52	30.7	0
2	33.65	00.0	0
3	18.40	00.0	0
4	10.00	00.0	0

Note: Root tip cuttings grown in modified half strength MS (w/o Ammonium nitrate and glycine + 300 mg/l ammonium phosphate) supplemented with 0.35 *M* sucrose for 30 days were used as the explants source. Root tips (2-3 mm long) separated from the preconditioned culture were encapsulated and pre-cultured in 0.5 *M* for 1 h before dehydration and LN exposure. Observations were made after one week.

Table 11: Cryopreservation of root tips preconditioned in 0.2 *M* sucrose and pre-cultured in 0.3 *M* sucrose + DMSO

Desiccation Duration (h)	MC %	Regeneration (%)					
		DMSO Concentration (%)					
		0		1		3	
		-LN	+LN	-LN	+LN	-LN	+LN
0	85.78	85.71	0	100	0	80.00	0
1	64.56	25.00	0	66	0	40.00	0
2	46.53	28.00	0	75	0	66.66	0
3	29.99	16.00	0	66	0	42.00	0
4	16.76	11.00	0	60	0	44.00	0

Note: Root tip cuttings grown in modified half strength MS (w/o Ammonium nitrate and glycine + 300 mg/l ammonium phosphate) supplemented with 0.2 *M* sucrose for 30 days were used as the explants source. Root tips (2-3 mm long) separated from the preconditioned culture were encapsulated and pre-cultured in

0.5 M sourse + 0-3 percent DMSO for one day before dehydration and LN exposure. Observations were made after 4 weeks.

Table 12: Cryopreservation of Root tips preconditioned in 0.2 *M* sucrose and pre-cultured in 0.5 *M* sucrose + DMSO

Desiccation Duration (h)	MC %	Regeneration (%)					
		DMSO Concentration (%)					
		0		1		3	
		-LN	+LN	-LN	+LN	-LN	+LN
0	77.62	60.0	0	66	0	60.00	0
1	64.50	50.0	0	66	0	37.50	0
2	49.27	75.0	0	44	0	57.00	0
3	36.82	54.5	0	55	0	80.00	0
4	26.66	100.0	0	58	0	68.75	0

Note: Root tip cuttings grown in modified half strength MS (w/o Ammonium nitrate and glycine + 300 mg/l ammonium phosphate) supplemented with 0.2 *M* sucrose for 30 days were used as the explants source. Root tips (2-3 mm long) separated from the preconditioned culture were encapsulated and pre-cultured in 0.5 *M* sucrose + 0-3 percent DMSO for one day before dehydration and LN exposure. Observations were made after four weeks

Table 13: Cryopreservation of root tips preconditioned in 0.35 *M* sucrose and pre-cultured in 0.3 *M* sucrose + DMSO

Desiccation Duration (h)	MC %	DMSO Concentration (%)					
		Regeneration (%)					
		0		1		3	
		-LN	+LN	-LN	+LN	-LN	+LN
0	88.00	75	0	25	0	60.0	0
1	73.00	60	0	40	0	00.0	0
2	55.20	50	0	33	0	60.0	0
3	42.48	90	0	62	0	41.6	0
4	29.00	50	0	50	0	27.0	0

Note: Root tip cuttings grown in modified half strength MS (w/o Ammonium nitrate and glycine + 300 mg/l ammonium phosphate) supplemented with 0.35 *M* sucrose for 30 days were used as the explants source. Root tips (2-3 mm long) separated from the preconditioned culture were encapsulated and pre-cultured in 0.3 *M* sucrose + 0-3 percent DMSO for one day before dehydration and LN exposure. Observations were made after four weeks.

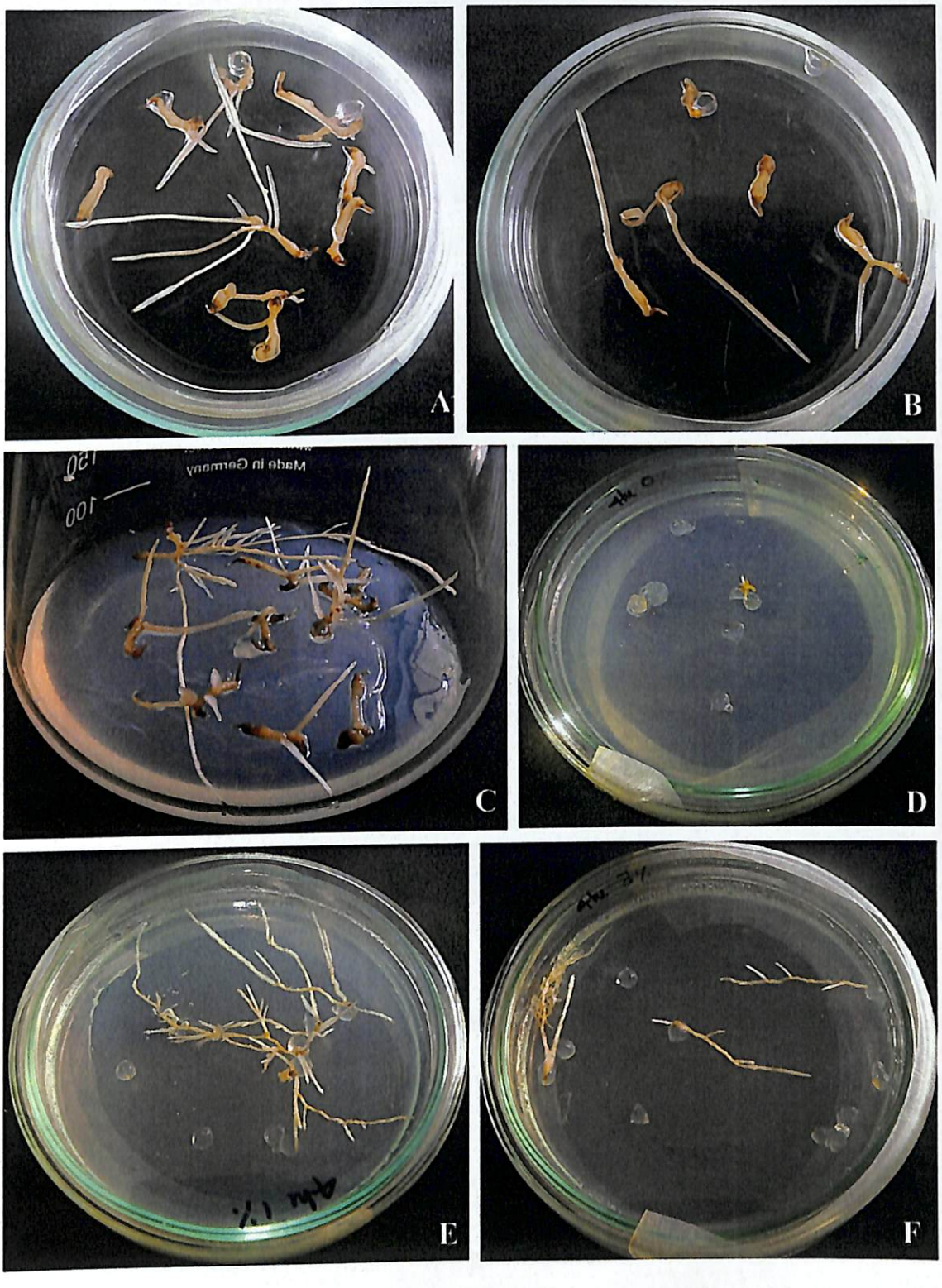

Plate 4. Encapsulation dehydration method (A)-(C) Encapsulated and dehydrated root tips showing recovery and regeneration. (A) & (B) After 1 and 2 h desiccation and transfer to liquid culture medium (HMSB), after 30 days (C) 1h desiccated root tips transferred to solid culture medium showing fast growth, after 7 days. (D)-(F) Effect of DMSO included in the preculture medium on recovery of encapsulated and dehydrated (4 h) root tips, after 30 days of culture in solid HMSB (D) Control, no DMSO (E) 1% DMSO (F) 3% DMSO.

4.1.4 Cryopreservation through vitrification method

4.1.4.1 Cryopreservation of root tips pre-cultured with 0.3 M sucrose and 0.1 mg/l 2, 4-D

As the encapsulation-dehydration experiments using pre-culture and preconditioning with high sucrose concentration were ineffective to get recovery after cryopreservation, root tips isolated from root culture raised in modified MS (w/o Ammonium nitrate and glycine + 300 mg/l ammonium phosphate) with normal sucrose concentration (3 percent) were used in further experiments using vitrification method. Root tips (2-3 mm) isolated from the source culture pre-cultured for three days in half strength MS supplemented with 0.1 mg/l 2, 4-D gave positive results upon cryopreservation through vitrification method. Exposure of pre-cultured root tips to PVS_2 for 30 and 45 minutes gave 40 and 12.5 percent recovery respectively after cryopreservation (Plate 5). Early sign of recovery were detected by microscopic observation as swelling of the meristematic region (Plate 5B), elongation of the meristematic tip, and doubling of the length of the root tips (Plate 5C) in 7 days. Root tips that did not show re-growth turned brown and later died. However, the results (Table 15) shows some discrepancies as evident in the regeneration of control root tips subjected to PVS_2 exposure alone.

4.1.4.2 Effect of pre-culture duration on root tips cryopreservation through vitrification

Root tips pre-cultured for 1- 3 days in 0.3 *M* sucrose with 0.1 mg/l 2, 4-D gave some indication that 30-45 min exposure to PVS_2 is better to get better recovery after cryopreservation (Table 16). Even though two day pre-culture seems to be good to get maximum recovery, the results are inconsistent to make any final conclusion. Therefore, repetition of the experiment is required to confirm the results.

Table 14: Cryopreservation of root tips preconditioned in 0.35 *M* sucrose and pre-cultured in 0.5 *M* sucrose + DMSO

Desiccation Duration (h)	MC %	DMSO Concentration (%)					
		0		1		3	
		Regeneration (%)					
		-LN	+LN	-LN	+LN	-LN	+LN
0	81.40	0	0	50	0	0	0
1	65.19	40	0	57	0	28.5	0
2	51.60	25	0	20	0	22.0	0
3	37.20	22	0	30	0	41.0	0
4	26.70	10	0	20	0	04.1	0

Note: Root tip cuttings grown in Modified half strength MS (w/o Ammonium nitrate and glycine + 300 mg/l ammonium phosphate) supplemented with 0.35 *M* sucrose for 30 days were used as the explants source. Root tips (2-3 mm long) separated from the preconditioned culture were encapsulated and pre-cultured in 0.5 *M* sucrose + 0-3 percent DMSO for one day before dehydration and LN exposure. Observations were made after four week.

Table 15: Cryopreservation through vitrification of root tips pre-cultured with 0.3 *M* sucrose and 0.1 mg/l 2, 4-D

Duration of PVS2 exposure (Minutes)	Regeneration (%)	
	-LN	+LN
0	60.0	00.0
15	16.7	00.0
30	28.6	40.0
45	00.0	12.5
60	00.0	00.0
120	14.2	00.0

Note: Root tips (2-3 mm) isolated from root cultures raised in Modified half strength MS (solid; w/o Ammonium nitrate and glycine + 300 mg/l ammonium phosphate) supplemented with 3 percent sucrose were pre-cultured in 0.3 *M* sucrose + 0.1 mg/l 2, 4-D for three days prior to PVS_2 exposure. Observations were made after one week.

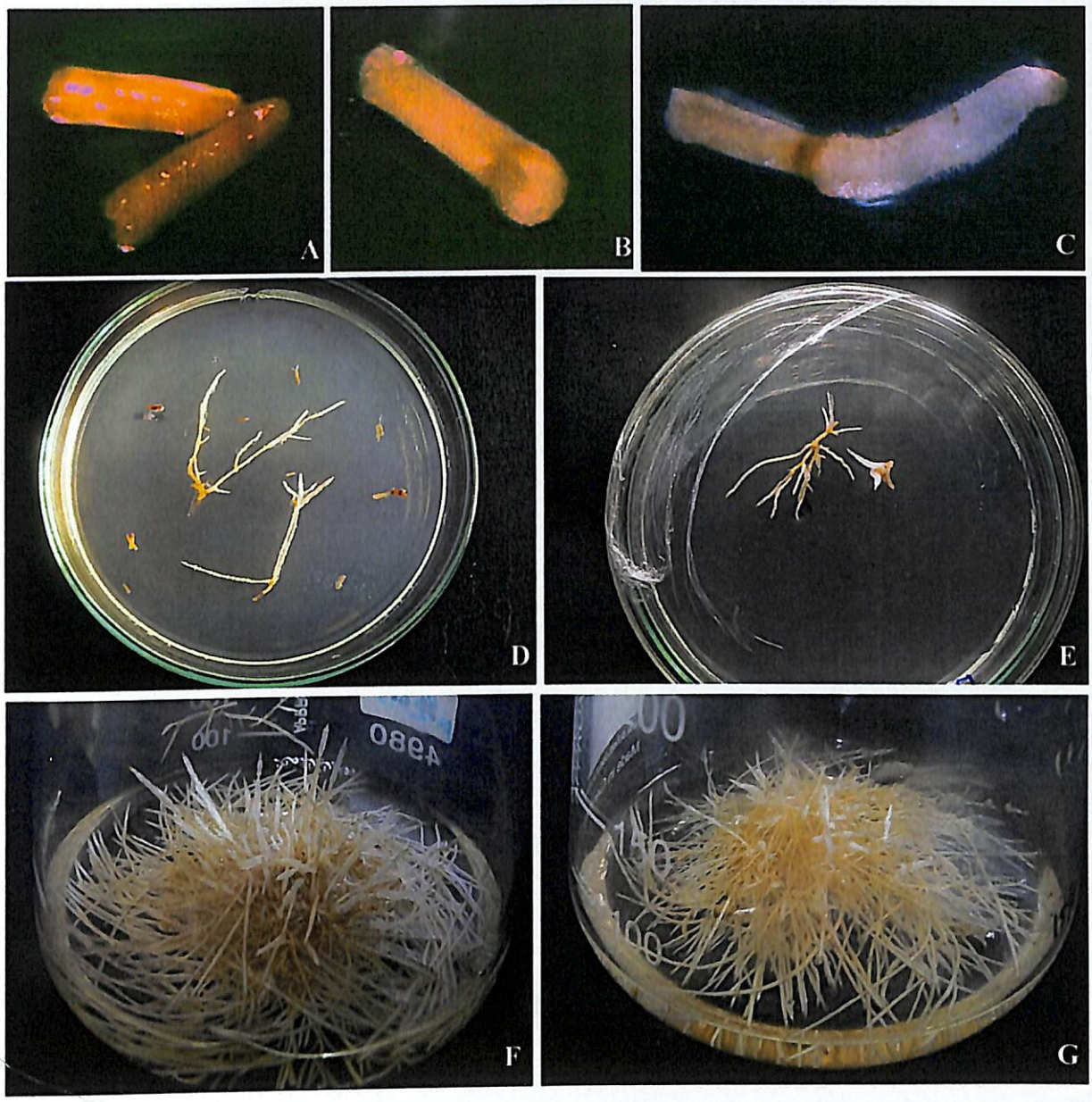

Plate 5. Recovery of cryopreserved hairy root tips of *D. arayalpathra* (A) Root tips subjected to vitrification treatment and transferred to recovery medium. (B) Cryopreserved root tips showing swelling of root tips as the initial symptom of regeneration, after 3-5 days. (C) Elongation of cryopreserved root tips with root hairs, after 7 days. (D) Recovery of roots tips subjected to one hour exposure to PVS2, after 30 days. (E) Recovery of root tips subjected to 45 min exposure to PVS2, and LN after 30 days. (F) Hairy roots regenerated from vitrified control cultured in half strength MS (liquid) medium, after 4 weeks.(G) Hairy roots regnerated from vitrified and LN exposed samples cultured in half strength MS (liquid) medium, after 4 weeks.

4.1.4.3 Cryopreservation through vitrification of root tips preconditioned in 0.2 M sucrose + paclobutrazol

Paclobutrazol (PBZ) is a growth retardant sometimes used to inhibit growth of root tips during pre-culture. In this experiment, PBZ was included in the preconditioning medium to know whether it have any cryoprotectant activity. The results obtained (Table 17) are inconsistent even though the roots tips isolated from those cultures raised in presence of 1 mg/l PBZ showed up to 22 percent recovery after 15-45 min PVS2 exposure and cryopreservation.

Table 16: Cryopreservation through vitrification; of root tips preconditioned in 0.2 M sucrose + paclobutrazol(PBZ)

Duration of PVS2 exposure (Minutes)	Regeneration (%)					
	1 day pre-culture		2 day pre-culture		3 day pre-culture	
	-LN	+LN	-LN	+LN	-LN	+LN
5	0	0	20.0	16.7	20.0	00.0
10	0	0	25.0	00.0	25.0	00.0
15	33	0	16.7	33.0	28.5	16.6
20	25	0	50.0	00.0	20.0	00.0
30	50	50	00.0	25.0	00.0	20.0
45	0	25	20.0	100.0	25.0	33.0
60	0	0	20.0	50.0	0.0	00.0

Note: Root tips (2-3 mm) isolated from root cultures raised in modified half strength MS (solid; w/o Ammonium nitrate and glycine + 300 mg/l ammonium phosphate) supplemented with 3 percent sucrose were pre-cultured in 0.3 *M* sucrose + 0.1 mg/l 2, 4-D for 1- 3 days prior to PVS2 exposure. Observations were made after one week.

4.2 GROWTH STUDY OF CONTROL AND CRYOPRESERVED ROOT CULTURE

Growth of four week old hairy root cultures of *D. arayalpathra*, were compared with those recovered from cryopreserved root tips. Post-freeze recovered hairy roots didn't show any appreciable morphological difference (Plate 5G) from that of normal hairy roots (Plate 5F). However, growth of LN treated root was relatively slow, even though it was not much remarkable

4.3 PHYTOCHEMICAL ANALYSIS

4.3.1 Phytochemical Stability of regenerated root culture from cryopreserved hairy root

Assessment of growth and secondary metabolite production potential between post-freeze recovered hairy roots and non cryopreserved hairy roots was carried out.

4.3.1.1 Determination of secondary metabolite profile of normal and cryopreserved hairy roots

Petroleum ether extracts of four week old cultures of cryopreserved and non cryopreserved hairy roots metabolic profile were compared using TLC. The secondary metabolite profile of both cryopreserved and control roots were identical under UV and after derivatization using anisaledhyde. Two distinct bands were observed under UV (Plate 6A) and five bands were observed after anisaldehyde spraying (Plate 6B). The Rf (Retardation factor) values observed under UV and after derivatization using anisaldehyde were noted. Rf values of the compounds obtained were same for both control and hairy root regenerated from cryopreserved root tips (Table 18).

Table 17: Cryopreservation through vitrification of root tips preconditioned in 0.2 *M* sucrose and a growth retardant paclobutrazol (PBZ)

Duration (minutes)	Regeneration (%)					
	PBZ 1 mg/l		PBZ 2 mg/l		PBZ 3 mg/l	
	-LN	+LN	-LN	+LN	-LN	+LN
0	85.7	00.0	66.7	0	14.2	00.0
15	25.0	16.7	16.7	0	20.0	00.0
30	00.0	11.0	12.5	0	00.0	00.0
45	33.0	22.0	12.5	0	00.0	00.0
60	33..0	00.0	50.0	0	00.0	00.0
75	14.0	00.0	11.1	0	00.0	25.0
90	00.0	00.0	00.0	0	22.0	00.0
120	16.0	00.0	00.0	0	33.0	14.2

Note: Root tips (2-3 mm) isolated from root cultures raised in modified half strength MS (solid; w/o Ammonium nitrate and glycine + 300 mg/l ammonium phosphate) supplemented with 0.2 *M* sucrose + 1-3 percent PBZ were pre-cultured in 0.3*M* sucrose + 0.1 mg/l 2, 4-D for 2 days prior to PVS2 exposure. Observations were made after one week.

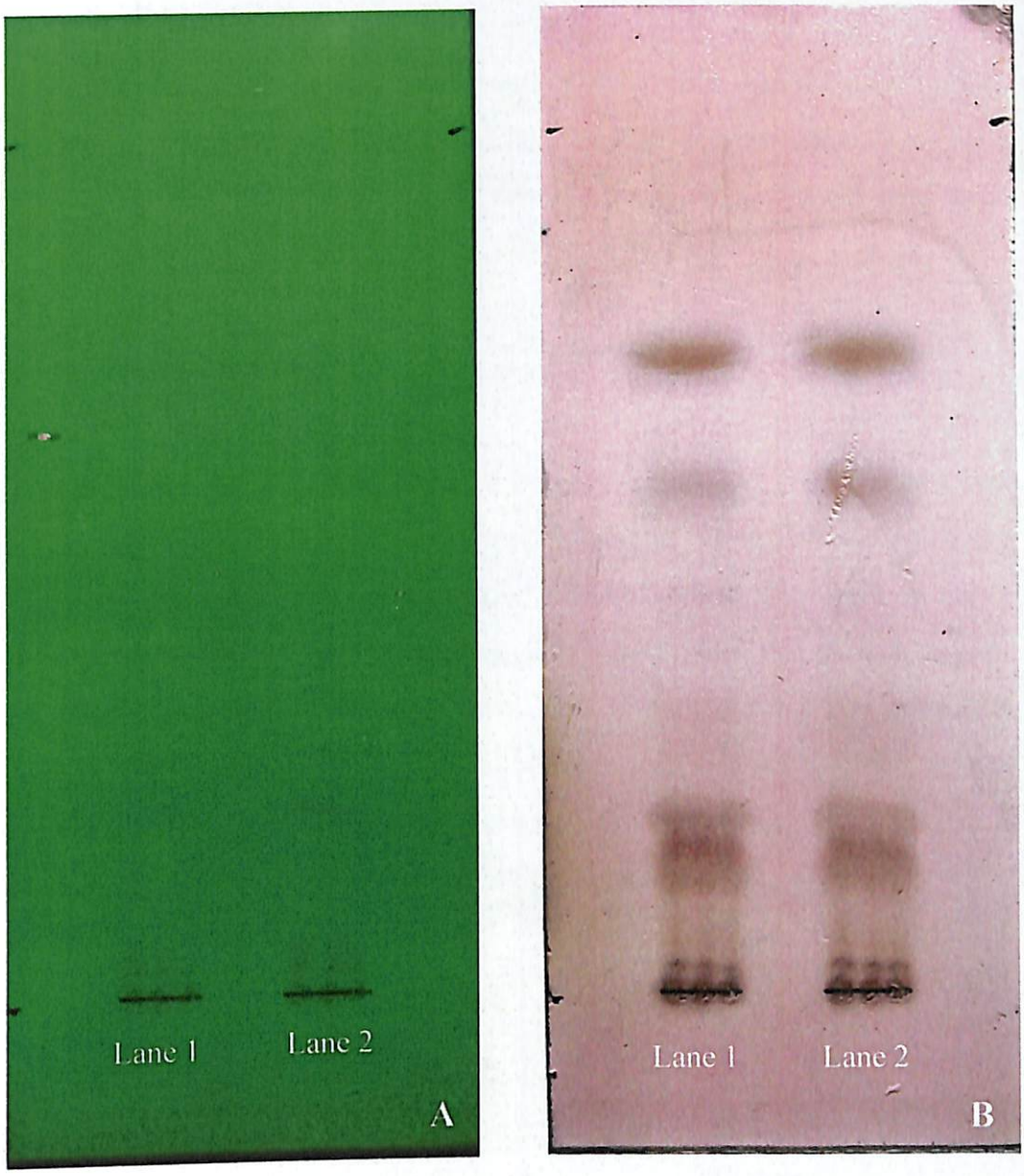

Plate 6. Phytochemical profile of hairy roots.
(A) Observed under UV illumination (B) After derivatization with anisaldehyde
Lane 1. Normal hairy roots, Lane 2. Hairy roots recovered from LN treated samples

4.3.1.2 HPLC profile comparison and determination of MBALD

4.3.1.2.1 Qualitative analysis

Qualitative analysis of secondary metabolite profile was done by HPLC. The HPLC profile of cryopreserved root showed four compounds at Retention Time (RT) 8.11, 9.31, 10.222 and 11.291 while control roots showed only three compounds at RT 8.211, 9.576 and 10.334.

4.3.1.2.2 Quantitative analysis

The retention time of the reference compound (MBALD) is 10.389 min which was compared in the extract of cryopreserved and non cryopreserved hairy roots. The quantitative analysis of compound MBALD using a calibration curve (Figure 1) showed that concentration was more in control hairy root culture (Figure 2) than in cryopreserved root (Figure 3). The normal hairy root culture at four weeks of growth showed MBALD concentration of 0.039 mg/g Dry weight (DW) while that of cryopreserved root was 0.018 mg/g DW. This difference was noticed in comparison with first subculture of cryopreserved root.

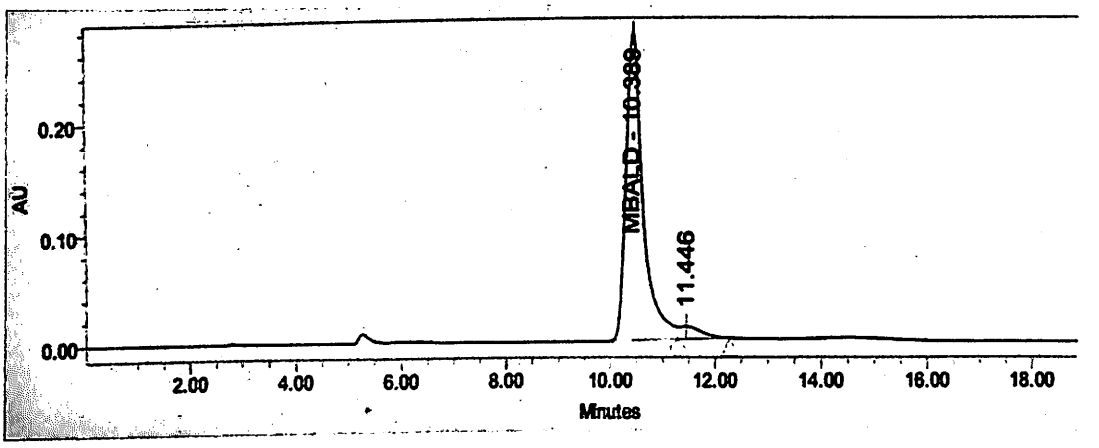

Figure 1. Chromatogram of standard (MBALD)

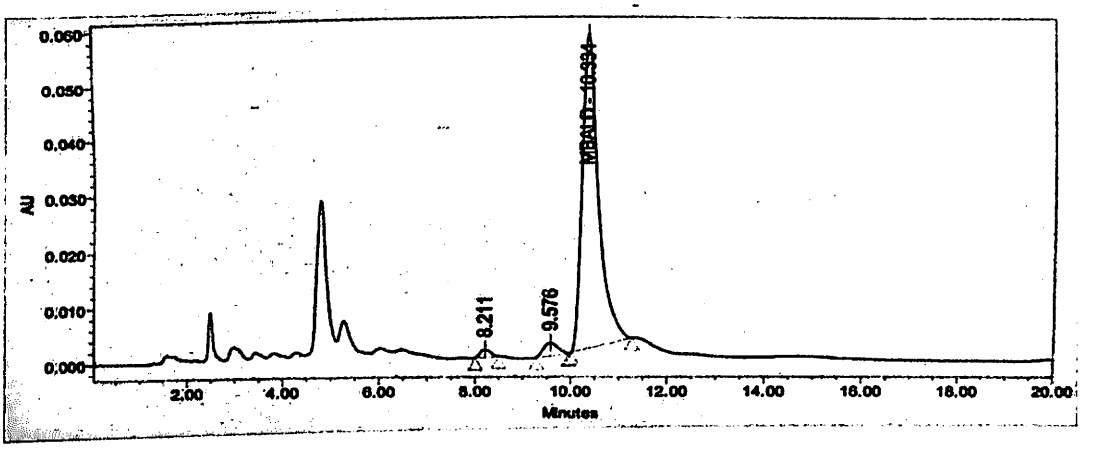

Figure 2. Chromatogram of Control hairy root

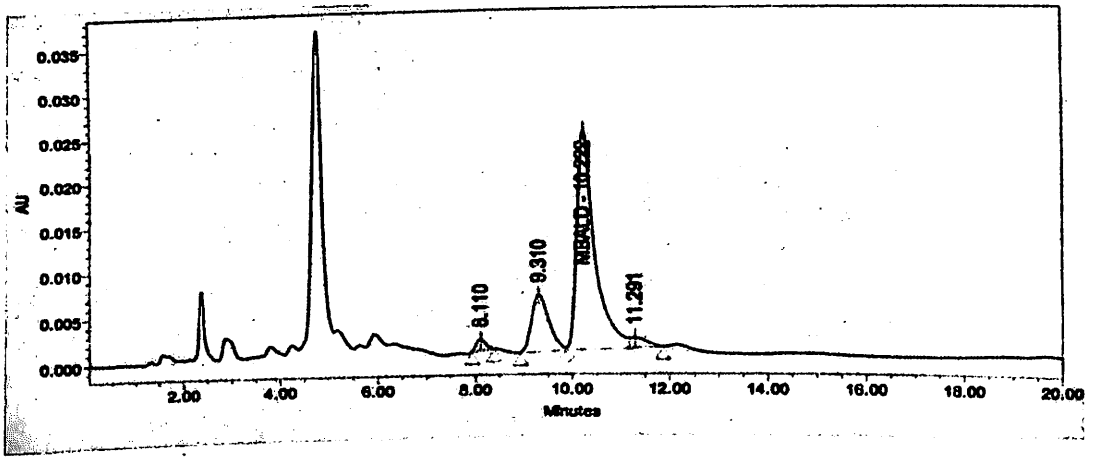

Figure 3. Chromatogram of cryopreserved hairy root

DISCUSSION

5. DISCUSSION

Decalepis arayalpathra, a critically endangered medicinal plant, which is used as an effective remedy for peptic ulcer and cancer like afflictions. Tuberous roots of the plant posses several bioactive molecules such as 2-hydroxy-4-methoxy benzaldehyde (MBALD), ά-amyrinacetate, 4-methoxy salicelaldehyde, magnificol, 3-hydroxy p-anisaldehyde, naringenin, kaempferol, and aroma dendrin (Susan *et al*., 2000) and are responsible for the medicinal value of the plant. The hairy root cultures are expected to serve as alternative source of those phytochemicals without disturbing natural populations of such endangered taxa. The present study is to develop an efficient cryopreservation protocol for the long term preservation of hairy root culture and to compare the secondary metabolite profile because maintenance of large numbers of cultures in the laboratory is laborious and costly.

Different cryopreservation protocols have been developed for isolated root cultures. Mostly, the protocols are developed by optimizing the conditions of explant preparation, preconditioning, loading treatment, dehydration duration, cooling and rewarming, and post-cryopreservation re-growth (Kim, 2011). In the present study, optimization of conditions like root tip size and preconditioning through culture of root tips in medium containing high sucrose have been attempted for cryopreservation through both encapsulation-dehydration and vitrification methods.

5.1 CRYOPRESERVATION

Hairy root grown on solid medium is known to be more beneficial than that grown in liquid medium (Withers and King, 1980; Chen *et al*., 1984; Benson and Hamill, 1991; Dussert *et al*., 1992) and thus were utilized in the present investigation. Half strength MS liquid

medium used as recovery medium supported poor growth of isolated root tips and thus transferred to solid medium for better growth in the initial trials. Explant size is

also a factor that determines successful recovery after cryopreservation. Root tips of 2-5 mm size are generally utilized (Teoh *et al.,* 1996; Tauno *et al.,* 2006; Lambert *et al.,* 2009) with successful recovery. As a trial, we also used 1 mm size root tips but showed poor regeneration capacity and thus 2-3 mm size exhibited higher regeneration than smaller root tip size (1 mm). Relatively too small or large sized materials may failed to withstand liquid nitrogen. Thus for cryopreservation experiments, the size of the root tip is optimized to 2-3 mm.

Encapsulation- dehydration and vitrification methods are the most commonly used methods in various plant systems for successful recovery after cryopreservation (Sakai and Engelman, 2007). As far as hairy root cryopreservation is concerned, both the methods have been applied in different systems. In *Maesa lanceolata* and *Medicago truncatula* where vitrification method of cryopreservation failed encapsulation-dehydration method has been proved effective (Lambert *et al.,* 2009). In other instances (Xue *et al.,* 2008), vitrification treatment has been proved effective in *Eruca sativa* but the same treatments and protocols not effective in *Astragalus membranaceus* and *Gentiana macrophylla*. Therefore, we used both the methods in the present investigation to test their relative efficiency.

Successful recovery after cryopreservation through encapsulation-dehydration is often imparted by different preconditioning and pre-culture steps. Preconditioning is a process often employed (Decruse *et al.,* 1996, Grospietsch *et al.,* 1999) to improve recovery after cryopreservation. It includes culture in the presence of osmotically active agents (Decruse *et al.,* 1996; Shibli *et al.,* 2006) or cold acclimation (Vandenbussche and De Proft, 1998). Increased sugar in pretreatment medium leads to the accumulation of solute inside the cells, which maintains the integrity of the plasma membrane and inner membranes during dehydration and freezing (Plessis *et al.,* 1993) and reduces the formation of ice crystals during cooling and thawing (Grospietsch *et al.,* 1999). Hairy roots without having any pre-culture treatment are ineffective for the recovery of *Eruca sativa* hairy roots after

cryopreservation (Xue *et al.*, 2008). In other systems, the encapsulation dehydration steps have been preceded with extensive preconditioning and pre-culture procedures. This includes culture of *Holostemma annulare* shoot tip explants in medium devoid of ammonium nitrate (Decruse and seeni, 2002) and culture of *Lavandula vera* cells in glycine free medium (Kuriama *et al.*, 1996; Kuriama *et al.*, 2000) due to their negative effect on post-freeeze recovery. However, such preconditioning cultures in medium devoid of ammonium nitrate and glycine but supplemented with high sucrose (up to 0.4 *M*) to harden hairy roots of *D. arayalpathra* did not give post-freeze recovery. Even though root tips processed through such preconditioning procedures gave thick and condensed root tips from profuse branching of original explants, such root tips failed to tolerate even 2 h dehydration (about 20 percent m.c.). Moreover, when sucrose concentration in the pre-culture medium is increased, desiccation tolerance of the root tips decreased substantially. The negative effect of sucrose on *D. arayalpathra* roots is thus evident. Therefore, sucrose not suitable for *D. arayalpathra*.

Positive effect of DMSO in improving the viability after LN exposure is well known. DMSO is reported to be useful in cryopreserving *Zea mays* cells (Withers and King, 1979). Suspension cultures of *Puccinellia distans* is shown to exhibit 95 percent viability after cryopreservation when used 12.5 and 15 percent (v/v) DMSO in the medium (Heszky *et al.*, 1990). However, pre-culture in the presence of 1 and 3 percent DMSO even though improved regeneration of desiccated control root tips to 60 and 44 percent respectively failed to tolerate LN exposure. Therefore, 1 percent DMSO is adjudged as the best to improve desiccation tolerance of root tips but further standardizations became essential to get LN tolerance. Encapsulation dehydration method suggested as more suitable for the cryopreservation of roots in comparison with the vitrification technique due to convenience of handling, elimination of washing step after rewarming and elimination of toxic chemicals (Hirata *et al.*, 2002) is thus not suitable of hairy roots of *D. arayalpathra*.

Nevertheless, the positive effect of DMSO imparting desiccation tolerance leads to carry out investigations on cryopreservation through vitrification.

The PVS_2 solution commonly used for vitrification lead to significant damage of some tissues and cell structures (Volk *et al.*, 2006). Vitrification is also reported as not suitable for cryopreserving hairy roots of *Maesa lanceolata*, *Medicago truncatula* (Lambert *et al.*, 2009), *Astagalus membraneous* and *Gentiana macrophylla* (Xue *et al.*, 2008). Also as suggested earlier (Hirata *et al.*, 2002) that success rate of cryopreservation of root cultures through vitrification is considered to be low, so this method was not tried. Vitrification involves less handling steps in comparison to other cryopreservation techniques (Engelmann, 2004). It is reported that *Panax ginseng* hairy roots pre-cultured with 2, 4-D before cryopreservation showed a high survival rate (Yoshimatsu, 1996). Similarly *Atropa belladonna* hairy root tips pre-cultured with 2, 4-D is reported to give 83% and the highest of 96% survival rates (Touno *et al.*, 2006). Based on these reports 2, 4-D is used in pre-culture medium. Root tips pre-cultured for 1- 3 days in 0.3*M* sucrose with 0.1 mg/l 2, 4-D indicates that 30-45 min exposure to PVS_2 seems to be better to get optimum recovery after cryopreservation. Root tip cuttings cultured in modified half strength MS medium (w/o Ammonium nitrate and glycine; 300 mg/l Ammonium phosphate and 3% sucrose) for 4 weeks, isolation of 2-3 mm root tips and pre-culture in 0.3 *M* sucrose + 0.1 mg/l 2, 4-D for 2 days and 45 min PVS_2 exposure gave as high as 100 percent regeneration of LN treated root tips. This result suggests that 45 min exposure to PVS_2 is the optimum duration. But the results obtained are inconsistent and thus a definite pattern could not be predicted. As the results are from a single determination and could not be repeated due to time constraints further work is necessary to repeat the experiment to make final conclusion. As per the available report in *Atropa belladonna* (Tauno *et al.*, 2006), very short duration (15 min) of treatment with PVS2 is sufficient to get 83 percent recovery. In other cases, hairy roots of *Eruca sativa* vitrified with PVS_3 is reported as fluctuating between 16 percent and 73 percent

reaching peak at 60 min exposure. Hairy roots of the same species are shown to give 12.3 and 25 percent recovery when vitrified with PVS_2 solution for 60 and 90 min respectively. Therefore, differential response to different vitrification solution by the same material is evident. Therefore, it is clear that a lot of standardizations are essential to recommend an efficient protocol for the long-term preservation of hairy roots of *D. arayalpathra*. However, as a mass of root material was regenerated from a few cryopreserved root tips it was sufficient to undertake further studies on phytochemical evaluation.

Paclobutrazol used as a growth retardant to precondition the root culture and the root tip isolated from that culture utilized for cryopreservation did not improve recovery after cryopreservation. Growth retardation effect of paclobutrazol and its role to induce hardiness in an array of plants are known. Snir (1988) reported that paclobutrazol give better survival after cold storage. PBZ treated *in vitro* shoots improves *ex vitro* survival of *Lilium* plantlets (Hazarika *et al.*, 2002; Rajesh *et al.*, 2006). However, the results obtained in the present study are from a single determination and thus the results are inconclusive.

5.3 PHYTOCHEMICAL ANALYSIS

The re-growth and conservation of biosynthetic potential and genetic stability are three important criteria for the application of cryopreservation. Therefore, the stability of cryopreserved roots in terms of their efficiency to produce the active phytochemicals is often tested. As known in *Atropa belladonna*, the regrowth of cryopreserved hairy roots is similar to that of untreated hairy roots and tropane alkaloid productivity became stable after 4th subculture (Tauno *et al.*, 2006). The present investigation, it was demonstrated that morphology of the cryopreserved root similar to that of normal cultures showing TLC profile exactly equal. Regrowth of cryopreserved root found to be relatively slow, even though it was not much remarkable. HPLC profiling of MBALD compound in cryopreserved root found to be less

compared to that of the normal root in the first subculture this may become stable after 4th subculture. Thus the results clearly suggest cryopreservation as an effective tool to preserve bioproductive hairy root cultures of *D. arayalpathra*.

SUMMARY

6. SUMMARY

The present study, "Cryopreservation of hairy root culture of *Decalepis arayalpathra* (Joseph & Chandras.) Venter" was carried out at the Biotechnology and Bioinformatics Division, Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode during 2013-2014. *Decalepis arayalpathra,* a critically endangered medicinal plant used by Kani tribes as an effective remedy for peptic ulcer and cancer like afflictions. The study aimed to find an efficient cryopreservation protocol for hairy root culture of *Decalepis arayalpathra* and to make a comparative analysis of secondary metabolite profile between cryopreserved and normal hairy roots. To explore the possibility of an effective long-term preservation of the hairy root of *Decalepis arayalpathra*, both encapsulation-dehydration and vitrification methods were attempted.

The size of the explant is very critical in controlling the regeneration after cryopreservation. For cryopreservation experiments the size of the root tips were found to be 2-3 mm.

Preconditioning and pre-culture treatments are essential for the success of encapsulation-dehydration method. But root tips of *D. arayalpathra* pre-cultured in 0.5 *M* sucrose failed to tolerate both desiccation and liquid nitrogen exposure. The root tips were highly sensitive to sucrose concentration during pre-culture as they did not tolerate desiccation for more than 2 h and moisture content less than 22 percent even pre-cultured in 0.3 *M* sucrose.

. Pre-culture with 1 percent DMSO enhanced the regeneration of root tips subjected to dehydration but could not support liquid nitrogen tolerance. Root tips isolated from root cultures subjected to various preconditioning culture in presence of 0.2 *M*-0.4 *M* sucrose encapsulated and pre-cultured in 0.3 *M* or 0.5 *M* sucrose prior to LN exposure did not recovered after cryopreservation suggesting that encapsulation-

dehydration method is ineffective for the preservation of hairy roots of *D. arayalpathra*.

Decalepis arayalpathra hairy roots were successfully cryopreserved through vitrification method. Hairy root tips (2-3 mm) pre-cultured on half strength Murashige and Skoog (MS) solid medium with 0.1 mg/l 2, 4-D for two days followed by 45-60 min exposure to PVS_2 gave maximum recovery and regeneration (50-100 percent) after LN treatment. Addition of paclobutrazol during preconditioning culture caused retarded growth giving thick roots but root tips isolated from such cultures pre-cultured and subjected to vitrification treatments did not improve recovery after cryopreservation.

Roots recovered from cryopreservation grew normally and shake flask cultures of cryopreserved roots could be established for phytochemical analysis. Secondary metabolites isolated from both cryopreserved and control roots by soxhlet extraction with petroleum ether showed similar pattern as revealed by thin layer chromatography. The results obtained in the present study indicate that cryopreservation of *D. arayalpathra* hairy roots is possible through vitrification method to preserve the secondary metabolite profile intact.

There are only a few reports regarding the cryopreservation of hairy root culture. In future this situation may change with increasing interest in transformed roots as potential production systems for secondary metabolites, thus leading to the development of cryopreservation protocols useful for a spectrum of plant species for both germplasm conservation as well as commercial purposes.

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APPENDICES

APPENDIX I

Murashige and Skoog medium

Components		**mg/l**
NH_4NO_3	-	1650
KNO_3	-	1900
$CaCl_2.2H_2O$	-	440
$MgSO_4.7H_2O$	-	370
$KH_2PO_4.3H_2O$	-	170
KI	-	0.83
H_3BO_3	-	6.2
$MnSO_4.4H_2O$	-	22.3
$ZnSO_4.7H_2O$	-	8.6
$Na_2MoO_4.2H_2O$	-	0.25
$CuSO_4.5H_2O$	-	0.025
$CoCl_2.6H_2O$	-	0.025
$FeSO_4.7H_2O$	-	27.8
Na_2EDTA	-	37.3
Nicotinic acid	-	0.5
Pyridoxine HCl	-	0.5
Thiamine HCl	-	0.1
Glycine	-	2
Inositol	-	100
Sucrose	-	3%
Agar		0.6%(w/v)
pH 5.8		

ABSTRACT

CRYOPRESERVATION OF HAIRY ROOT CULTURE OF AMRITHAPALA, *Decalepisarayalpathra* (Joseph &Chandras.) Venter

by

DHANYA C. S.
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Abstract of the Thesis
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M.Sc. (INTEGRATED) BIOTECHNOLOGY COURSE
DEPARTMENT OF PLANT BIOTECHNOLOGY
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ABSTRACT

The present study, "Cryopreservation of hairy root culture of *Decalepis arayalpathra* (Joseph & Chandras.) Venter" was carried out at the Biotechnology and Bioinformatics Division, Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode during 2013-2014. The objective of the study is to find an efficient cryopreservation protocol for hairy root culture of *Decalepis arayalpathra* and a comparative analysis of secondary metabolite profile. Hairy roots of *Decalepis arayalpathra*, a critically endangered medicinal plant used by Kani tribes as an effective remedy for peptic ulcer and cancer like afflictions were attempted to cryopreserve using encapsulation-dehydration and vitrification methods. A comparative analysis of secondary metabolite profile of cryopreserved and normal hairy roots was also made to examine practical utility of cryopreserved hairy roots. Encapsulation-dehydration method was not good for preserving hairy roots of *D. arayalpathra* in liquid nitrogen. The roots were highly sensitive to sucrose in pre-culture solution at least to get dehydration tolerance. Encapsulated root tips pre-cultured with 0.5 *M* sucrose did not survive even after one hour dehydration. Inclusion of DMSO in the pre-culture solutions improved dehydration tolerance of encapsulated root tips but did not support LN tolerance. Hairy root tips (2-3 mm) pre-cultured on half strength Murashige and Skoog (MS) solid medium with 0.1 mg/l 2, 4-D for two days followed by exposure to PVS_2 for 45 min gave 50-100 percent recovery and regeneration after cryopreservation. Roots regenerated from cryopreserved root tips grew like normal hairy roots showing similar secondary metabolite profile as revealed by thin layer chromatography. HPLC profiling of MBALD compound in cryopreserved root during first subculture passage was less compared to that of the normal hairy root culture. The study revealed cryopreservation by vitrification as a useful method to preserve hairy root of *D. arayalpathra*.