CRYOPRESERVATION OF *Vanda wightii* Rchb.f PROTOCORMS

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(2009-09-112)

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KERALA, INDIA

2014

DECLARATION

I, hereby declare that this thesis entitled **"CRYOPRESERVATION OF *Vanda wightii* Rchb.f PROTOCORMS"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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LIST OF ABBREVIATIONS

%	per cent
@	at the rate of
µg	microgram
^{0} C	Degrees Celsius
SD	Standard Deviation
cm	centimeter
et al.	and others
Fig.	Figure
g	gram
i.e.	that is
KAU	Kerala Agricultural University
kg	kilogram
LN	Liquid nitrogen
m	Meter
mg	Milligram
RBD	Randomized block design
M	Molar
M.C.	Moisture Content
Etc.	and other things
PVS2	Plant Vitrification Solution 2
DMSO	Dimethyl Sulfoxide
h	Hour

PLBs	Protocorm like bodies
ml	Milli litre
min	Minute
Kg	Kilogram
Cm	Centimetre
mM	Milli molar
w/v	Weight by volume
CH	Caesin Acid Hydrolysate
ANOVA	Analysis of Variance
VW	Vacin and Went

1. INTRODUCTION

Variety of life on earth is defined by the term biodiversity. This can refer to the genetic variation, species variation, or ecosystem variation within an area, biome, or planet. Green plants are the main component of the ecosystem, and there will be no life without them on earth. Total plant population is constituted by about 250 – 350 thousand species of flowering plants, 200 thousand species of algae (Groombridge, 1992), as well as many species of ferns, conifers, mosses and liverworts.

Orchidaceae is a diverse family of flowering plants with colourful and fragment blooms, commonly known as orchid family. The family, Orchidaceae is one among the largest and most highly evolved family of angiosperms (IUCN, 1996). *Vanda wightii* is an orchid species which is endemic to the Southern Western Ghats. It was first collected from the Walayar forest of Palakkad District in 1849 by Robert Wight (Sathish *et al*., 2006). It laid undiscovered for about 150 years after its first discovery, and later it was rediscovered by Sathish *et al*, 2006 from the regions of Kerala and Karnataka. Main host of this plant is *Ficus religiousa* and it is found at about 60-80m altitude. The stem is about 15-22cm having profuse rooting at the base. Its inflorescence is simple and axillary raceme having 2-3 flowers which are dirty brownish greenish yellow.

Several species of the orchid distributed in the Western Ghat region are declining day by day. Presently, the family Orchidacea is having high proportion of threatened generas, with most threatened species (Swarts and Dixon, 2009). The plant *Vanda wightii* is found in places mainly Palakkad, and Kannur districts of Kerala,, Coimbatore in Tamil Nadu and in Subramanyam, Belthangadi of Karnataka. Their presence in Sri Lanka is also known. *V. wightii* is currently distributed mostly in inhabited lands where anthropogenic pressures are very high.

Now the species is treated as endangered and restricted collection as notified by Government of India (MOEF, 2009) Therefore, conservation and improvement of this high value orchid species is one of the main concern and to accomplish these objectives, the present work is formulated.

Though a single orchid produces a large number of seeds only a few germinate in the nature with the help of a symbiotic fungus. Thus habitat destruction for developmental activities and conversion of land for agricultural purposes have severely hampered their natural spread leading to severe genetic erosion and subsequent narrowing of genetic base. Therefore, an urgent measure of conservation of the remaining germplasm in the species is of at most important. Conventional method of conservation of the plant needs high cost of land and labour; moreover there are risks of deterioration from natural disaster and plant pests during conservation. *In vitro* conservation is an alternative method for plant conservation that eliminates the obstacles of field conservation.

Cryopreservation is the commonly used *in vitro* method for the long term conservation of plant germplasm. This technique uses the liquid/vapour form of most abundant gas, Nitrogen (-196°C) for preserving the materials of choice. This is a globally acclaimed conservation method. The principle behind this method of preservation is, at -196°C the cellular divisions as well as metabolic processes are arrested. Theoretically this process allows the conservation of plant material for unlimited period of time (Gonsalez *et al*., 2008). This method requires low cost and limited space and provide phenotypic and genotypic stability (Suzuki *et al*., 2008). Other advantage of cryopreservation is the limitation of the risk of microbial contamination and somaclonal variation in stored cultures (Malabadi and Nataraja, 2006). Desiccation (air drying), encapsulation-dehydration and vitrification are the main three cryopreservation techniques applied in the case of orchid species (Hirano *et al*., 2006; Tsai *et al*., 2009) and moisture content manipulation is the main principle behind the above mentioned methods.

Considering the advantages of cryopreservation and the need of *ex situ* conservation in gene bank, the present study is framed to test whether protocorm cryopreservation is effective for germplasm conservation of the endangered species of orchid *Vanda wightii* Rchb.f. The following objectives were defined to test the hypotheses.

Objectives of the study

- Standardisation of the protocol for protocorm cryopreservation of *Vanda wightii.*
- Comparison of encapsulation-dehydration and vitrification methods of cryopreservation for the protocorms.

2. REVIEW OF LITERATURE

Orchid is one of the largest group of flowering plants in the plant kingdom and a total estimate of about 779 genera and 22,500 species are present in 5 different subfamilies (Dressler, 1993; Mabberley, 1997). These are ancient groups of plants whose survival is known from the date way back as the 125 million years ago and it is still evolving actively (Raven and Axelrod, 1974; Chase, 2001). The bio-geographical areas of Western Ghats have high endemicsm, taxonomic uniqueness, possibly yet to discover flora and fauna. Among the 924 wild species of orchids listed from the present boundaries of India ranging in about 132 genera, nearly 287 species distributed over 71 genera are endemic (Rao, 1998). Out of the 310 species inhabiting the Western Ghats, many of them are included in extinct (1 species), critically endangered (27 species), endangered (46 species) and vulnerable species (14 species) (Kumar *et al*., 2001). The main reason for the loss of germplasm of orchids is habitat loss. Many of them are threatened, endangered or extinct, usually due to habitat destruction or poaching. Orchid smuggling is contributing to the loss of many species of orchid in the wild (Nigel and Kingsley, 2009). Concern about diminishing populations, loss of genetic diversity and local extinctions has made the adoption of conservation strategies inevitable.

Nearly 250 species of Indian orchids are facing the threat of extinction which includes *Vanda coerulea, Aphylorchis golani, Paphiopedilam charlesworthii, Pleione langenaria* and *Vanda wightii* (Singh *et al*., 2001). *Vanda wightii* Rchb.f, first collected in 1849 is once considered as extinct from wild (Singh *et al*., 2001) but re-discovered from Kerala and Karnataka after 150 years (Sathish *et al*., 2006).

Vanda wightii is an endemic species of Indian orchid distributed in Western Ghats regions of Kerala, Karnataka and Tamil Nadu at 60-80m above mean sea

level (Sathish *et al.*, 2006). Its presence also has been reported in Sri Lanka. This is one of the orchid species remained unknown after its original description in 1849. When re-collected after 150 years in 2000, their natural localities have been almost completely lost. Consequently, their population has been reduced to limited numbers in restricted localities. Therefore, it is now considered as endangered (MOEF, 2009).

V. wightii have characteristic leaf length of 35cm and 2-3 flowers in its inflorescence. The characteristic beautiful flowers with white lip make it easily distinguishable from other Vandas of Western Ghats. Its flowering and fruiting are in the months of September – December. The plant holds high potentialities in recombination breeding which has not been utilised yet.

The present study 'Cryopreservation of *Vanda wightii Rchb.f* protocorms', involves standardization of cryopreservation protocol using encapsulation-dehydration as well as vitrification procedure for long term conservation of *V. wightii Rchb.f.*

In this chapter, literature on cryopreservation of orchids as well as other plants has been reviewed.

2.1. CRYOPRESERVATION

Cryopreservation is a cost effective and safe mechanism for the conservation of plant germplasm by storing them under ultra-low temperature of liquid nitrogen (-196°C). At this teamperature, metabolic and cellular divisions of plant tissues are suspended and genetic stability is maintained (Tandon and Kuamria, 2005; Engelmann, 2004).

Sakai (1960) reported first successful cryopreservation procedures in silver birch twigs. Quatrano (1968) could freeze *in vitro* cultured flax cells successfully. The first protocol developed in the 1980s included pre-treatment with cryoprotectants followed by controlled rate of cooling. Plant parts used for cryopreservation included axillary buds, shoot tips, pollen grains, pieces of callus and aliquots of cell suspension cultures. Before immersion and storage in liquid nitrogen, the explants needed preparation and pre-conditioning to withstand desiccation and freezing at ultra-low temperature. Re-growth of a frozen and thawed callus culture of *Populus euramericana* was observed by Sakai and Sugawara (1973). Later many techniques have been developed for the cryopreservation of cell suspensions, protoplasts, pollen, somatic embryos, protocorms, zygotic embryos, meristems and even seeds (Hirano *et al*., 2005; Ishikawa *et al*., 1997; Ito, 1965; Popov *et al*., 2006; Pritchard, 1984; Pritchard *et al*., 1999; Thammasiri, 2000; Wang *et al*., 1998; Bajaj, 1995). Many biological materials, such as some seeds and pollen, that can tolerate extensive desiccation could be plunged directly in LN (Akihama and Omura, 1986).

Seeds, the natural regenerating organs of plants, are the preferred choice of material, as used in a range of *Piper spp.* (Chaudhury and Chandel 1994; Decruse and Seeni 2003) and *Rauvolfia micrantha* (Decruse *et al.,* 1999). Those plants which are clonally propagated, producing recalcitrant large seeds or otherwise do not set seeds necessitates the application of cryopreservation for their effective conservation. Excised zygotic embryo as utilized in other recalcitrant seed crops are used for the cryopreservation purpose (Engelman, 2000).

2.1.1. Cryopreservation by Encapsulation-Dehydration

The encapsulation – dehydration procedure is the most widely used technique of cryopreservation which is based on the technology developed for the production of artificial seeds. The samples which are to be cryopreserved are encapsulated in alginate beads, pre-grown in liquid medium enriched with

sucrose for several days, partially desiccated down to a water content around 20 per cent (fresh weight basis), then frozen rapidly. This method is simple, inexpensive and maintains the genetic stability of cryopreserved materials (Kartha and Engelmann, 1994). The most widely used sugar in the encapsulation-dehydration procedure is sucrose (Antony *et al*., 2011). Sucrose has been reported to maintain the plasma membrane integrity by substituting for water on the membrane surface, thus stabilising protein under dry and freezing condition (Crowe *et al*., 1987). This is a rapid and direct technique which does not involve the formation of the callus. Apices of various species from temperate origin such as apple, pear, grape, eucalyptus, and of tropical origin such as sugarcane and cassava have been conserved with this method sucessfully (Dereuddre, 1992; Engelmann, 1997).

The protocol for the encapsulation – dehydration was modified by Sakai *et al.* (2000) where encapsulation as well as pre-growth in medium with sucrose and glycerol was performed simultaneously. This method was used to cryopreserve the apices of numerous species of temperate and tropical origin and cell suspensions, as well as somatic embryos (Engelmann and Takagi, 2000).

Meristems and somatic embryos have been cryopreserved using encapsulation-dehydration method which is relatively practical and easy to perform (Dereuddre *et al.,* 1991; Fabre and Dereuddre, 1990). Sucrose enriched medium is used for the gradual extraction of the water from the encapsulated plant tissue. Further, beads were air desiccated, to reduce the molarity of the sucrose in the beads, and reach/exceed the saturation point of the sucrose solution resulting in a glass transition during cooling to –196°C. The key for the success of the cryopreservation is the increase in the desiccation tolerance which is provided by the subsequent increase in the concentration of the sucrose.

Encapsulation dehydration technique was found to be effective in cryopreserving the shoot tips of the *Dendrobium walter oumae* (Lurswijidjarus and Thamasiri, 2004) and protocorms of *Vanda coerulea (*Jitsopakul*et al*., 2008), *Brassidium* shooting star orchid hybrid (Yin *et al*., 2011) *and Dendrobium nobile* (Mohanty *et al*., 2012).

Protocorm-like bodies (PLBs) of *Phalaenopsis bellina* and *Dendrobium* Bobby Messina were successfully cryopreserved by the encapsulation-dehydration approach where the PLBs were precultured in half-strength MS medium supplemented with 0.75 M sucrose for 3 days (Khodadamzadeh *et al*., 2011; Zainudin *et al*., 2011).

The shoot tips, nodal segments, axilliary buds and micro-shoots were subjected to encapsulation and was used for the protection from cryoinjuries in case of many rare and threatened medicinal plants like *Ocimum americanum, O. basilicum, Ananas comosus, Allium sativum, Rauwolfia serpentina, Vitex negundo, Withania somnifera, Zingiber officinale* and *Glycyrrhiza glabra* (Mandal *et al*., 2000; Bekheet, 2006; Ray and Bhatacharya, 2008; Ahamad and Anis, 2010; Singh *et al*., 2006; Sundararaj *et al.*, 2010).

Lily meristems pre-cultured in 0.8M sucrose plus 1.0M glycerol produced considerably high rate of post thaw shoot recovery while 0.8M sucrose used alone for pre-culture gave 57 per cent recovery (Matsumoto *et al.*, 1995). However, a mixture of 0.8 *M* sucrose in combination with ethylene glycol and DMSO produced toxic effects during the dehydration process. It is considered that glycerol contributes to minimizing the injurious membrane changes resulting from severe dehydration. Hirai *et al.* (1998) confirmed that strawberry could be conserved using the revised procedure.

Encapsulation-dehydration techique was also used for cryopreserving shoot tips of *Holostemma annulare* (Decruse and Seeni, 2002), somatic embryos derived from leaf induced callus of *Clitoria ternatea* (Nair and

Reghunath, 2007) and axillary buds of *Indigofera tinctoria* (Nair and Reghunath, 2009).

Cold acclimatisation for a period of 1 to 4 week was combined with the encapsulation-dehydration procedure in *Cyanodon* which proved the method to be more effective than the standard procedure used normally (Reed *et al.,* 2005).

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

Preconditioning, encapsulation, pre-culture and cryoprotection, desiccation/dehydration, freezing, thawing, recovery and plant regeneration are the different procedures involved in the cryopreservation. The success of the cryopreservation is directly linked to the tightly coupling of the events like cryopreservation, freezing and thawing. Each aspect of cryopreservation procedure may affect the recovery of tissues and predispose cells to genetic change (Harding and Benson, 1994).

2.1.1.1. Encapsulation

The samples used for the cryopreservation are very much prone to the damages caused by the pre-culture with high sucrose concentrations and desiccation to low moisture contents. 3 per cent (w/v) sodium alginate (low viscosity, 250 centipoises) is usually used for the preparation of the synthetic beads (Gonzalez-Arnao and Engelmann, 2006). The mixture is dropped with a pipette in liquid culture medium containing a high concentration of calcium chloride (usually 100 mM $CaCl_2$). Alginate polymerizes in presence of the elevated concentration of calcium, thereby producing calcium alginate beads containing the explants. These spherical beads are usually about 4 or 5 mm in diameter, depending on the size of explants, and contain one explant or more.

They are held in the calcium solution for 20-30 min after the last bead has been formed in order to guarantee complete polymerization (Engelmann, 2012).

Dendrobium nobile Lindl., protocorms were made into synthetic seeds using 3 per cent (w/v) sodium alginate and 0.1M calcium chloride. Similarly the shoot tips of *Dendrobium* walter oumae beads were formed using 3per cent (w/v) sodium alginate as well as by using 0.1M calcium chloride (Lurswijidjarus and Thamasiri, 2004).

Protocorms of *Vanda coreulea* were used for encapsulation-dehydration after preparing the synthetic beads which were encapsulated in alginate matrix composed of 2 per cent sodium alginate, 2M glycerol plus 0.4M sucrose in modified VW liquid medium (Jitsopakul *et al.,* 2008).

Four week old protocorms of *Brassidium* shooting star orchid were encapsulated using 2.5, 3.0 and 3.5 per cent sodium alginate and 0.1M calcium chloride. Best result was observed in the protocorms encapsulated in 3.5 % sodium alginate (Yin *et al*., 2011).

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

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

The maximum survival percentage of cryopreserved leaf segment-derived PLBs of *Aerides odorata* was achieved by encapsulating PLBs with 2 per cent sodium alginate combined with 2 *M* glycerol and 0.4 *M* sucrose (Hongthongkham and Bunnag, 2014)

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

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

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

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

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

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

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

2.1.1.2 Pre-culture and Cryoprotection

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

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

cryopreservation. According to Dereuddre *et al.* (1988) pre-culture of carnation shoot tips with 0.25 - 0.75 *M* sucrose and 5 per cent DMSO enhanced their survival after LN treatments. Sucrose treatment durations may vary between 16-18 h as for sugarcane (Paulet *et al.,* 1993) to 7-10 days in the case of coffee (Mari *et al.,* 1995) and yam (Malaurie *et al.,* 1998).

Protocorms of *Vanda coerula* were successfully cryopreserved using encapsulation-dehydration procedure by preconditioning the encapsulated beads in Vacin and Went medium having 0.7M sucrose (Jitsopakul *et al*., 2008). *Dendrobium* Walter Oumae shoot tips were preconditioned in Vacin and Went medium supplemented with 0.3M, 0.5M and 0.7M sucrose and highest growth rate was observed due to 0.3M sucrose preconditioning (Lurswijidjarus and Thamasiri, 2004).

Dendrobium nobile Lindl., protocorms like bodies were osmoprotected with a mixture of 0.4M sucrose and 2M glycerol where 53.3% survival and 50.2% re-growth was recorded (Mohanty *et al*., 2012).

Four week old protocorms of *Brassidium* shooting star orchid was used for cryopreservation by pre-culturing the protocorms in varying sucrose concentrations of 0.0M, 0.2M, 0.4M, 0.6M, 0.8M and 1.0M and the best results were got due to 0.8M sucrose pre-culture (Yin et al., 2011).

Protocorm-like bodies (PLBs) of *Phalaenopsis bellina* were successfully cryopreserved by the encapsulation-dehydration approach where the PLBs were precultured in half-strength MS medium supplemented with 0.75 M sucrose for 3 days (Khodadamzadeh *et al*., 2011).

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

2.1.1.3 Dehydration

After pre-culture and cryoprotection, beads are submitted to an additional physical dehydration by evaporation at room temperature. To avoid lethal intracellular ice crystallization during rapid ultra-cooling, explants have to be sufficiently dehydrated prior to a plunge into LN. *In vitro* materials are not inherently tolerant to dehydration as they contain large amount of water. Hitmi *et al*. (1999) demonstrated that sucrose decreased the water content of *Chrysanthemum cinerarifolium* shoot tips and thus enhanced their freezing tolerance. The explants are dried in the laminar air flow cabinet by the sterile air flow (Kavani, 2011). The method of dehydration in the laminar air flow was mainly used in the case of orthodox seeds, zygotic embryos and pollen of many common agricultural and horticultural species (Panis and Lambardi, 2005). Dehydration plays an important role in the successful recovery of the explants from the LN storage. The dehydration after encapsulation is also important for the maximum survival of plants under cryostorage. Gonzalez-Arnao and Engelmann (2006) described two desiccation levels *viz.,* dehydration under the air current of a laminar flow cabinet or dehydration in sealed containers with dry silica gel.

Encapsulated protocorms of *Vanda coerula* were dehydrated in a sterile air-flow in a laminar air-flow cabinet at 25 ± 3°C for 0-10 h and then directly plunged into liquid nitrogen for 1 day. With the decrease in water content, re-growth without freezing remained at about 61% as water contents decreased from 77% to 48% over 5 h of dehydration; thereafter, re-growth fell to 43% after 10 h dehydration to 25% water content (Jitsopakul *et al*., 2008).

Encapsulated PLBs of *Aerides odorata* were dehydrated on a sterile filter paper laid down on an open Petri dish (40 beads/Petri dish) and exposed to sterile air flow in a laminar air flow cabinet for 0-8 h (Hongthongkham and Bunnag, 2014). In the cryopreservation experiment of shoot tips of *Dendrobium* Walter Oumae by Encapsulation/Dehydration, the encapsulated protocorms were

dehydrated in the laminar air flow for a period of 0-10h and then plunged into the liquid nitrogen. Water content in visible cryopreserved PLBs ranged from 10 to 35% (Lurswijidjarus *et al*., 2004). The pre-treated beads containing the PLBs of *Dendrobium nobile* Lindl. were dehydrated in the laminar airflow for a period of 0-8 h. Maximum survival of PLBs (53.3%) was achieved after cryopreservation when precultured beads were dehydrated for 5 h reducing water content to 28.3% and gradually decreased (37.4%) with 6 h of dehydration with bead water content of 16.7%. As the water content of the beads containing PLBs decreased, the survival rates after cryopreservation increased whereas survival of encapsulated non-cryopreserved PLBs declined with increased dehydration (Mohanty *et al*., 2012).

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

The embryonic axes of *Nothapodytes nimmoniana* lost M.C. rapidly in the laminar air flow cabinet to 43.7, 31.3, 24.4, 19.6 and 15.4% on fresh weight basis respectively after ½, 1, 1 ½, 2 and 2 ½ h desiccation.. Reduction in the m.c of axes was accompanied by reduction in the survival rate. Cryopreservation was effective only for the axes desiccated up to 19.6% M.C., whereas embryonic axes having moisture content below this lost the viability gradually when exposed to LN (Radha *et al.,* 2010).

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

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

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

2.1.1.4 Cryopreservation in Liquid Nitrogen

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

2.1.1.5 Thawing or Rewarming

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).

Protocorm-like bodies of *Vanda* Kaseem's Delight orchid which were subjected to the LN treatment were taken and rapidly thawed in the water bath before the recovery step. After thawing at 40°C of *Vanda coerulea* were cultured on modified VW agar medium for re-growth (Jitsopakul *et al*., 2008).

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

Mandal and Sharma (2007) reported that, following 1 h storage of *Dioscorea deltoidea* in LN, rewarming was performed by placing cryovials in a 40° C water-bath for 2 min. Damiano *et al.,* 2011 reported that re-warming was performed by placing the cryotubes in the air current of the laminar flow cabinet. For thawing and rewarming of cryopreserved seeds of *Lilium iedebourii,* cryotubes were thawed in a water-bath at 37-38° C for 3 min (Kaviani, 2011). After dehydration, the beads containing the PLBs of *Dendrobium* Bobby Messina were placed in 2 ml cryovials and were directly plunged into liquid

nitrogen for 24 h. Cryopreserved PLBs were thawed by rapid warming in a water bath at 40 ± 2°C for 90 s.

The cryopreserved embryos of *Byrsonima intermedia* were thawed by plunging the cryotubes into a water bath for 5 minutes at 37±2°C (Nogueira *et al.,* 2011). After cryopreservation of *Artemisia herba-alba,* cryogenic vials containing beads were thawed in a water bath at 38° C for 2–3 min (Sharaf *et al.,* 2012).

2.1.1.6 Recovery

Re-growth generally takes place on standard semi-solid culture medium. However, the composition of the recovery medium may be transitorily altered to eliminate phenolic compounds produced by dead cells by adding activated charcoal to the medium as with sugarcane apices (Paulet *et al.,* 1991) or to stimulate re-growth of frozen explants by modifying the growth regulator content of the medium as with sugarcane and yam apices. Environmental conditions also influence the recovery of plant tissues after cryopreservation. Organized structures such as meristems, are subjected to post-thaw recovery in the dark for a short period (around one week) (Gonzalez-Arnao and Engelmann, 2006). Exposure to dark period during post-thaw recovery stage helps to prevent or decrease detrimental photo oxidation of frozen samples.

Brassidium Shooting Star orchid hybrid protocorms were tested for its recovery in the MS medium (Yin *et al*., 2011). *Dendrobium gratiosissimum* Rchb.f. protocorms recovery was studied in the New Dogshima media and a recovery percentage of 67 % was got (Maneerattanarungroj *et al*., 2007). Protocorm like bodies of *Dendrobium* Sonia 28 was recovered after cryopreservation using vitrification method with recovery medium consisting of semisolid pour plates (Brandon, 9 cm) of half-strength MS components, supplemented with 2% sucrose and 2.75 g L–1 gelrite (Poobathy *et al*., 2012). For the recovery of cryopreserved embryonic axes of *Melia azedarach* L. and *Camellia*

sinensis L. and cryopreserved seeds of *Lily ledebourii* (Baker) Bioss. were cultured on solid basal MS medium (0.8% Agar) with 3% sucrose in 9-cm plastic petri dishes were used (Kaivani, 2010). The beads containing the PLBs of *Dendrobium* Bobby Messina were thawed in a 40°C water bath for 90 s and were placed in recovery media composed of half strength semisolid MS media supplemented with 2% sucrose for four days under dark condition (Zainuddin *et al*., 2011). In the experiment involving *Vanda coerulea ,* where it was cryopreserved using droplet-vitrification method, after thawing the samples these were cultured on to New Dogashima medium for checking the regeneration (Jitsopakul *et al*., 2008). The plant material i.e the seed of *Bletilla striata* after vitrification treatments were immersed in liquid nitrogen, rewarmed rapidly and cultured on solidified ND medium supplemented with 3% sucrose for recovery (Jitsopakul *et al*., 2008).

2.1.2 Cryopreservation through Vitrification

Vitrification based methods involves the removal of most or all freezable water by physical or osmotic dehydration of explants, followed by ultra-rapid freezing which forms intracellular solutes into an amorphous glassy structure without occurrence of ice crystals (Kaviani, 2011). Fahy *et al*., 1984 first presented this method on rabbit kidney and later has been used successfully with various storage forms of plant groups (Towill *et al*., 2002).

This method was applied to a broad range of plant materials from various species, including complete organs like embryos and shoot apices (Sakai, 1995; Wang *et al*., 2006; Takagi, 2000). The best results in the cryopreservation of PLBs of Vanda Kaseem's Delight was obtained when 3-4mm PLBs were precultured in VW medium supplemented with 0.1M sucrose for 24 hours, followed by a loading treatment, and 20 minutes of dehydration in PVS2 at 0°C, prior to cryostorage, and 30 minutes of unloading treatment after 90 seconds of thawing (Poopathy *et al*., 2012). Vitrification was carried out for V*anda coerulea* Griff. Ex Lindl. seeds at 25 ± 2°C for 0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 min, before plunging the

cryotubes into LN where they were kept for one day. There was no survival when exposed for 0- 20 min, but the survival percentage increased continuously up to the exposure time of 70 min and the survival decreased sharply upon longer exposures to about 11% when exposed for 100 min (Thammasiri and Soamkul, 2007). Similarly, the protocorms of the medicinal orchid *Dendrobium nobile* Lindl. were cryopreserved using encapsulation-vitrification procedure using PVS2 solution. The beads were directly dehydrated with PVS2 at either 25°C or 0°C for various time periods (5–145 min) prior to their direct immersion in LN for 1 h. Maximum frequency of survival (78.1%) was achieved at 0°C with 115 min of dehydration (Mohanty *et al*., 2012).

Seeds of *Dendrobium cruentum* Rchb.f. obtained after 2, 3 and 4 months after pollination were cryopreserved by vitrification. The seeds were exposed to PVS2 for 0-90 min at 25±2℃before being plunged into liquid nitrogen (LN). Protocorms of *Dendrobium cruentum* Rchb. f. were cryopreserved with the vitrification method, protocorms were precultured on modified VW medium supplemented with 0.06 (control), 0.3, 0.5, and 0.7 M sucrose for 1-5 days and then exposed to loading solution, and PVS2 for 0- 420 min at 0±2℃before being plunged into LN. Protocorms of *Doritis pulcherrima* orchid was cryopreserved using vitrification using PVS2 for 0 to 90 min, but there was no survival in any treatment (Thammasiri, 2000). Protocorms of *D.cruentum* orchid were subjected to cryopreservation through vitrification method using PVS2 solution. Different exposure time were provided with the PVS2 and the best one was identified as 240 min PVS2 exposure, where 33% survival was recorded (Thammasiri, 2008). *Seidenfadenia mitrata* protocorms were subjected to cryopreservation by vitrification using PVS2. Different exposure times were provided with PVS2 and 80 min exposure gave the maximum survival (67 per cent) considering the entire experiment. *Dendrobium candidum* Wall. orchid species was tested for cryopreservation taking the protocorms as the material for cryopreservation. Here PVS2 exposure was provided for different time period for dehydrating the sample, the highest survival rate was 85 per cent (Yin and Hong, 2009). The seeds of the

orchid *Dendrobium candidum* was subjected to cryopreservation through vitrification procedure using PVS2 with 95% survival (Wang *et al*., 1998.). Seeds of *Doritis pulcherrima* orchid species was experimented for cryopreservation taking seeds as the material. The method followed was vitrification method of cryopreservation using PVS2. Different period of exposure to PVS2 solution was given to dehydrate the seeds before plunging in LN. Out of the treatments performed, the best was identified as 50 min exposure to PVS2 which provided 62% survival (Thammasiri, 2000). 82 per cent survival was recorded for the seeds of *Bletilla striata* orchid species using vitrification method of cryopreservation (Hirano *et al*., 2005). When seeds of *Dendrobium chrysotoxum* were subjected to the vitrification procedure of cryopreservation using PVS2, the highest survival of 99 percentage was obtained for 50 min exposure (Thammasiri, 2008). Zygotic embryos of *Bletilla striata* Rchb. f. was treated with PVS2 to get 60% survival after cryopreservation (Ishikawa *et al*., 1997).

The normal shoot tips as well as clumps of strawberry were treated with PVS2 solution, and highest survival obtained were 70% for 50 min exposure and 70% for 30 min exposure respectively (Niino *et al*., 2003). The *Ribes nigrum* shoot tips were subjected to vitrification treatment with PVS2 solution and the procedure turned to be having little toxic effect on regeneration. The maximum survival obtained was 80 per cent for 120 min exposure to the PVS2 solution (Benson *et al*., 1996). To determine the potential of vitrification on freeze protection of date palm, cultures were exposed to PVS2 solution for 20 – 100min. The best survival rates were obtained for those cultures which were treated for 80 min followed by 40 min (Bekheet *et al*., 2007). Studies of Sarkar and Naik (1998) on potato showed the shoot tips precultured on medium containing 0.3 M sucrose plus 0.2 M mannitol, and loaded with PVS2 for 30 min followed by 15 min incubation in 60% PVS2 and 5 min incubation in 100% PVS2 at 0°C resulted up to 54% survival after vitrification. Successful cryopreservation of the apical meristems of wasabi (*Wasabia japonica*) was done using vitrification method (Matsumoto *et al*., 1994). To determine the optimal time of exposure to PVS2 solution, precultured wasabi

shoot tips (1 mm in size) were treated with PVS2 solution at 25 or 30°C for different lengths of time prior to immersion in LN. Exposure to PVS2 solution produced time dependent shoot formation. In wasabi shoot tips cooled to -196°C after treatment with PVS2 at 25°C, shoot formation increased rapidly in line with increasing dehydration periods and reached its highest value (about 95%) at 10 min. Shoot formation was slightly lower after 20 min, and then decreased rapidly due to the toxicity of the PVS2 solution.

In many herbaceous plants, the optimal exposure time to PVS2 solution was reported to be 10 to 25 min at 25°C (Matsumoto *et al*., 1995; Takagi *et al*., 1997). However, for excised gentian node segments (2 x 2 mm) bearing two axillary buds, the optimal exposure time to the PVS2 solution was 45 min at 25°C. For apple shoot tips (2 mm long, 1.5 mm base diameter) consisting of the apical shoot tips and 4 to 5 leaf primordia, the optimal exposure time to PVS2 solution was 80 to 100 min at 25°C.

In this method, the explants are treated with highly concentrated cryoprotective solution for variable periods of time, which make them resistant to lethal osmotic and evaporative dehydration stresses (Reed *et al*., 2005). Loading solution (2M glycerol) treatment for the explants provided the explants or tissues with desiccation tolerance (Sakai, 2000). This loading solution treatment has shown to increase the capacity of the cells to tolerate dehydration with PVS2 (Hirai and Sakai, 2003; Matsumoto and Sakai, 2003; Kobayashi *et al*., 2006). The vitrification method eliminates the method of slow freezing and only main problem associated with it is the toxicity which could be overcome by the cold and sugar hardening.

The vitrififcation method of cryopreservation was also found to be effective in the cryopreservation of *in vitro* raised shoot tips of sweet potato which gave high regeneration percentage (Pennycooke and Towill, 2000). Embryogenic cells of asparagus (*Asparagus officinalis* L.) were successfully cryopreserved by vitrification and subsequently regenerated plantlet, where PVS3 solution was used for the vitrification procedure (Nishizawa *et al*., 1993). Matsumoto *et al. (*1995)

and Hirai *et al.* (1998) reported that vitrification procedure was more effective than encapsulation – dehydration procedure with respect to root formation rate and recovery and regrowth of meristems after LN treatment.

3. MATERIALS AND METHODS

3.1. PLANT MATERIAL

Vanda wightii Rchb.f. is an endangered species of orchid endemic to the southern Western Ghats. It is mainly seen in the Kerala, Karnataka and Tamil Nadu regions of the Western Ghats and also in Sri Lanka. The leaves are arranged very closely and the inflorescence is simple axillary raceme with 2-3 flowers. The flowering season is between October and December. The flowers are greenish yellow in colour. Its main host is *Ficus religiosa* and these are found at an altitude of 60-80 m.

The plant material for cryopreservation of *Vanda wightii* Rchb.f. i.e. mature capsule (Plate.1.) containing the seeds were collected from field conservatory of the Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode. The capsule was about 14.1cm in length and was 12 months old.

3. 2. GLASSWARES AND CHEMICALS

Petri plates, conical flasks and glass bottles were the main glasswares used in this study. The chemicals used for the preparation of the media were from Sigma (USA), SRL (Mumbai), Merck (Mumbai) and HiMedia Laboratories Pvt. Ltd. (Mumbai). The common decontamination agents used include Tepol, mercuric chloride and alcohol.

3.3. EQUIPMENTS AND OTHER REQUIREMENTS

Sl. no	Name of equipment	Uses
1	Surgical blade and blade holder	Dissection of capsule
2	Forceps	Picking the capsule, encapsulated beads etc.
3	Micropipette 0.2 and 1ml.	Used for taking the stock solution, pH adjusting etc.
4	Pasteur pipette	Encapsulation of the protocorms
5	Electronic balance	Weighing of capsule, chemicals, encapsulated beads etc.
6	Magnetic stirrer	Preparation of culture medium, encapsulation medium, vitrification medium etc.
7	pH meter	pH calibration of all the culture media.
8	Autoclave	Disinfection of glasswares, sterilisation of culture media and all equipment.
9	Distillation unit	For production of distilled water.
10	Laminar air flow chamber	Inoculation of explants, dehydration of the encapsulated protocorms.
11	Microscope	For analysing the viability of seeds, the survival and regeneration of the protocorms.
12	Cryovials 2ml and 4ml.	Holding protocorms, beads etc.

13	Cryo canes	Holding cryovials in liquid nitrogen
14	Liquid nitrogen plant	Production of liquid nitrogen
15	Liquid nitrogen container	Sample storage
16	Fibre illuminator	Surface illumination
17	Air conditioner	Maintaining the humidity for the encapsulation dehydration of the protocorms.
18	Refrigerator	For keeping the stock solution, PVS2 etc.at 4°C.
19	Oven	Drying glasswares, M.C. determination of the protocorms etc.
20	Millipore filtration unit	Filtering PVS2
21	Water bath	Thawing of the cryopreserved material.
22	Screw cap bottles	Keeping the encapsulation medium, vitrification solutions, Pre-culture solutions etc.

3.4. CULTURE MEDIA

The present study used Mitra medium (Mitra *et al.,* 1976) as the culture medium for raising protocorms and for other cryopreservation experiments.

3.4.1. Media Preparation

3.4.1.1 *Mitra Medium*

For the preparation of culture medium standard procedures were used. The medium was prepared adding macronutrients (Annexure-1) as salts and micronutrients and vitamins from stock solutions. The stocks were stored at 4° C in a refrigerator.

Glassware for *in vitro* experiments were washed thoroughly using commercial detergents like Laboline or Extran, rinsed in distilled water and dried for 2 h in hot air oven. The required amount of macronutrients was added after weighing accurately in an electronic balance (Shimadzu AW220) and stocks of micronutrients and vitamins added by pipetting. After dissolving required amount of sucrose (20 g/l) and casein hydrolysate (CH; 0.01%, w/v) if required, the medium was made up to final volume and pH adjusted to 5.6 using either 1.0 M NaOH or HCl. Agar (SRL 0.65%) was then added into the medium and melted in a microwave oven in case of the solid medium and dispensed into culture bottles/conical flasks. Liquid culture media used for raising protocorms were dispensed to conical flasks (250 ml) and closed with cotton plug. Plastic lid of the culture bottles were closed and the media were sterilized at 121° C/ 1.1 kg/cm² for 18 min in a horizontal autoclave. In such cases, medium containing 0.65 per cent agar was prepared in conical flasks taking 1/3rd volume of its full capacity, autoclaved and dispensed into pre-sterilized glass petri-plates under sterile conditions, in a laminar air-flow.

3.4.1.2 *Encapsulation Media (Fabre and Dereuddre, 1990; Decruse et al., 1999)*

Sodium alginate solution: Mitra medium with 3 per cent sucrose but not having $CaCl_2$ was prepared, made up to the final volume and pH adjusted to 5.6. Sodium alginate (3 per cent) was added, stirred to dissolve, autoclaved and kept under ambient conditions.

Calcium Chloride solution (100 mM): Salts and vitamins of Mitra medium were dissolved in distilled water and added $CaCl_2$ at the rate of 100 mM followed by 3 per cent sucrose. The solution was made up to the desired final volume, pH adjusted to 5.6, dispensed into screw capped bottles, autoclaved and stored under ambient conditions.

3.4.1.3. ***Pre-culture Medium (Mitra medium + 0.5M and 0.75M sucrose)***

In order to dehydrate the encapsulated protocorms, culture media having 0.5 and 0.75 M sucrose concentrations were prepared. Salts and vitamins of Mitra medium were dissolved in distilled water and sucrose was added to make the concentration to 0.5 M and 0.75 M. The pH of the medium was adjusted to 5.6 and 50 ml aliquots of the medium dispensed into screw capped bottles, autoclaved and stored under ambient conditions.

3.4.1.4 ***Loading Solution (Mitra medium + 0.4M sucrose + 2M glycerol)***

Loading solution was prepared for the initial dehydration of the protocorms prior to the PVS2 treatment. After dissolving the salts of Mitra medium, sucrose (w/v) was added to get 0.4 M concentration and after completely dissolving it glycerol (w/v) added to 2 M concentration. After mixing completely, pH was adjusted to 5.6 and dispensed into screw capped bottles as 50 ml aliquots, autoclaved and stored under ambient conditions.

3.4.1.5. ***PVS2 (Plant vitrification solution, Sakai et al., 1990)***

A mixture of 30 per cent (w/v) glycerol, 15 per cent (w/v) ethylene glycol and 15 per cent (w/v) dimethyl sulfoxide was prepared and adjusted the volume to 100 ml using Mitra medium containing 0.4 M sucrose at pH 5.6. After thorough mixing, the solution was filter sterilised under vacuum through 0.22 µm nitrocellulose filter membrane (Millipore) using a Millipore filtration unit. The filtered solution was transferred to screw capped bottles and stored in a refrigerator.

3.4.1.7 *Washing Solution (Mitra medium + 1.2M sucrose)*

Washing solution (also known as unloading solution) is used for washing the protocorms after the treatment with PVS2 solution during cryopreservation through vitrification protocol. After dissolving the salts and vitamins of Mitra medium in distilled water, sucrose added to get 1.2M concentration before making up to the final volume. The pH was adjusted and the solution was dispensed to the screw capped bottles. It is then autoclaved and kept under ambient conditions.

3.4.2 Surface Sterilisation of Capsules and Seed Culture

Mature and green capsule of *V. wightii* collected from mother plants was washed thoroughly under running tap using a few drops of commercial detergent, (Laboline), followed by washing 4-5 times with distilled water. Further, the capsules were de-contaminated by dipping in 70 per cent alcohol for 2-3 s and subsequent flaming using a spirit lamp. The capsule was placed in a sterile Petri-plate and cut longitudinally into two halves using a sterile scalpel blade under aseptic conditions. The seeds were scraped into the petriplate and a small quantity transferred to pre-sterilized liquid Mitra medium. The seeds from one capsule were equally divided and transferred to 16 flasks. Cultures were incubated in a culture room maintained at 25^{o} C with 10/14 light/dark period provided by cool fluorescent tubes. The flasks were swirled manually twice in a day.

3.5 CRYOPRESERVATION OF PROTOCORMS

3.5.1 Encapsulation dehydration of the Protocorms

Protocorms were cryopreserved through encapsulation – dehydration protocol as described earlier (Fabre and Dereuddre, 1990; Decruse *et al*., 1999).

3.5.1.1 *Encapsulation*

Protocorms which have developed from the seeds after 30days of culture was used for encapsulation. Encapsulation media used were (1) liquid Mitra medium containing 3 per cent (w/v) sodium alginate and (2) liquid medium

containing 0.1M $CaCl_2$. Protocorms were suspended in sodium alginate solution and a uniform suspension is made. Using a sterile pasture pipette (3 ml, disposable), 1 ml of the suspension was pipetted and transferred drop-wise into $CaCl_2$ solution (50 ml, in a screw capped bottle) with uniform pressure while holding the pipette vertically. On contact with the calcium ions, the droplets solidified and formed a gel encapsulating the protocorms. The solution was drained off and allowed 30 min, to complete solidification.

3.5.1.2 *Pre-culture of the Encapsulated Protocorms*

One – step preculture

The encapsulated protocorms were transferred to the liquid culture media (Mitra *et al* + 0.01% CH) containing 0.5 M sucrose and incubated at normal culture conditions for 1-3 days. Another preculture treatment was also used in which 3 per cent DMSO was added in addition to 0.5 M sucrose, to which protocorms were transferred and incubated at normal culture conditions for 1-3 days.

Two – step preculture

The encapsulated protocorms were transferred to liquid culture media (Mitra *et al* + 0.01 % CH) containing 0.5M sucrose and incubated for one day; followed by transfer to liquid culture media containing 0.75 M sucrose and incubated for one day under normal culture conditions.

3.5.1.3 *Dehydration and Cryostorage*

After the respective day of pre-culture, the beads were taken out and were placed in a petri plate lined with a filter paper to completely blot the pre-culture solution. To determine the optimum dehydration time, the beads were desiccated up to 5h in a sterile air laminar flow cabinet. After every one hour, 5 beads containing the protocorms were transferred to cryovials (liquid nitrogen treatment) as well as to solid culture media (control). The samples for cryostorage were plunged into liquid nitrogen and held there overnight.

3.5.1.4 *Moisture content Determination*

Empty beads (approximately 20 numbers without protocorms) were prepared and subjected to pre-culture as that of test samples were placed in the laminar airflow after initial weight determination. At one h intervals (1-5 h), the beads were weighed and finally dried in an oven maintained at 130°C for one hour. Dry weight of the beads was determined and the moisture content estimated in relation to fresh weight as given below:

$$\% \text{ mc} = \frac{\text{Initial weight} - \text{Dry weight}}{\text{Fresh weight}} \times 100$$

Where initial weight is the weight of the beads after dehydration treatment, dry weight is the weight of the beads which were dried in the oven and fresh weight is the weight of the beads before the dehydration.

3.5.1.5 *Thawing and Post Freeze Recovery*

The cryovials were removed from liquid nitrogen and transferred to a circulating water bath maintained at 40^{o} C and allowed for 30-60 s to re-warm. The rewarmed beads having the protocorms were transferred to solid recovery medium (Mitra + 0.01% CH). The cultures were incubated in the culture room at 25 ± 2^{o}C away from direct light for initial two weeks followed by normal light conditions. Two replications were given each having five beads. Observations were made at 30 days interval and up to 4 - 6 months. The results were expressed as survival percent and/or shoot regeneration. Survival was estimated as percent of treated protocorms remaining green and showing early symptoms of *in vitro* response (swelling/enlargement) in 30 - 45 days. Regeneration was estimated as percentage of the survived protocorms transformed into shoots in 2 - 4 months.

3.5.2 Cryopreservation through Vitrification (Sakai *et al*., 1990)

3.5.2.1 *Pre-culture of the Encapsulated Protocorms*

One – step pre-culture: Protocorms obtained after 30 days of seed culture were subjected to pre-culture in liquid culture media (Mitra *et al* + 0.01% CH) containing 0.5 M sucrose for one day under normal culture conditions.

3.5.2.2 *Vitrification*

The pre-cultured protocorms were transferred to 4 ml cryovials and added loading solution (Mitra medium + 0.01% CH + 0.4 M sucrose + 2 M glycerol). After 20 min exposure, the loading solution was pipette out and added 2 ml PVS_2 solution using a sterile Pasteur pipette. The samples were exposed to PVS_2 solution for 1, 2, 3 and 4 h at 4^{o} C by keeping in a refrigerator. After each duration of exposure, two cryovials were taken, one as control and the other plunged into liquid nitrogen (-196°C). The control samples were washed, removing 1.0 ml of PVS2 from the vial and adding 1.0 ml of washing solution (Mitra medium + 1.2M sucrose) and kept for 5 min. The washing process was repeated thrice and finally the protocorms were suspended in 2 ml of washing solution. The washed protocorms were transferred to Mitra medium + 0.01 % CH + 0.65 % agar for recovery and regeneration.

3.5.2.3 *Thawing and Post-freeze Recovery*

The cryovials were taken out of the liquid nitrogen without disturbance and immediately transferred to circulating water bath maintained at 40° C and kept for 30 – 60 s. The vials were taken into the laminar air flow chamber, the protocorms were washed as mentioned above using washing solution and transferred to recovery media i.e. Mitra medium + 0.01% CH+0.65% agar.

3.5.3 Cryopreservation through Vitrification Using Loading Solution

Thirty days old protocorms were subjected to cryopreservation using a modified protocol of vitrification, using loading solution alone.

3.5.2.1 *Pre-culture of the Encapsulated Protocorms*

The 30 days old protocorms were subjected to pre-culture in liquid culture media (Mitra *et al* medium + 0.01% CH) containing 0.5M sucrose, under normal culture conditions for one day.

3.5.2.2 *Vitrification*

The pre-cultured protocorms were transferred to 4ml cryovials, added 2 ml loading solution (Mitra medium + 0.01% CH + 0.4M sucrose + 2M glycerol) and kept for 0 - 4 h at 25° C. After one hour interval, two cryovials were taken, one as control and the other plunged into liquid nitrogen (-196° C). The control vials were washed to remove the loading solution using the washing solution (Mitra medium +0.01% CH + 1.2 M sucrose) as did for other vitrification experiment using PVS2. The washed protocorms were transferred to Mitra medium + 0.01 % CH+0.65 % agar for recovery and regeneration.

3.5.2.3 *Thawing and Post-freeze Recovery*

The frozen samples were re-warmed, washed and subjected to post-freeze recovery culture as mentioned for vitrification experiment using PVS2 solution.

3.6. EXPERIMENTAL DESIGN AND DATA ANALYSIS

Two methods of cryopreservation were used in this study i.e. cryopreservation through encapsulation-dehydration and cryopreservation through vitrification. For encapsulation – dehydration, the protocorms were taken from the same source and the experiment was conducted with 2 replications. The experiment was completely randomised and thus one way ANOVA was done using SPSS

software for comparison of means. For cryopreservation using vitrification procedure, the protocorms were taken from different sources and the experiment was replicated thrice at different time. Thus the experiment was performed in a Randomized Block Design (RBD) and a 2 - way ANOVA and Duncan's multiple range test performed using SPSS software to select best treatment(s). In a single replication, a uniform suspension of protocorms from same source was used.

A

B

C

A-Vanda wightii Rchb.f capsule (matured 12 months old)

B-Vanda wightii Rchb.f seeds

C-Protcorms

Plate.1. *Vanda wightii* Rchb.f (A capsule, seeds and protocorms)

4. RESULTS

4.1. SEED CULTURE AND PROTOCORM PRODUCTION

Capsules (Plate.1) of *V. wightii* collected from the field gene bank of JNTBGRI were 12 months maturity possessing more than 95% seeds with viable embryos. The seeds cultured in Mitra *et al* medium supplemented with 0.05% CH germinated in 15 days and 0.1-3 mm size protocorms were obtained after 30 days. The protocorms thus obtained were pale yellow in colour but turned to yellow or yellowish green in another 30 days.

4.2 CRYOPRESERVATION OF *VANDA WIGHTII* PROTOCORMS USING ENCAPSULATION DEHYDRATION TECHNIQUE.

Protocorms of *V. wightii* at maturity stage (30 days) were cryopreserved through encapsulation dehydration technique, having encapsulation, pre-culture, dehydration, thawing and post-freeze recovery steps. Both one-step and two-step preculture were attempted to optimize recovery after cryopreservation.

4. 2.1 One-step Preculture

Protocorms encapsulated in sodium alginate,pre-cultured with 0.5M sucrose for 1, 2 and 3 days and dehydrated for 0- 5 h exhibited 5.93 to 43.5% regeneration when observed after 30 days (Fig. 1). The fresh beads after 1 day preculture possessed 83% moisture content which was reduced to 72.06 and 70.2% after 2 and 3 days preculture respectively. Regneration exhibited by encapsulated protocorms before dehydration was significantly less than that of dehydrated samples (Fig. 1;Fig.2;Fig.3). Dehydration for 3-4 h reduced the moisture to 10-14 per cent considered safe for satisfactory dehydration as well as liquid nitrogen tolerance. But, dehydration for 5 h to reduce m.c less than 10 per cent did not reduce regeneration of the protocorms. However, increase of preculture duration to 3 days did not improve desiccation tolerance.

Table. 1. Effect of one-step preculture on regeneration of encapsulated protocorms of Vanda wightii

Preculture solution	Preculture duration (days)	Duration of desiccation (h)	Moisture content (%)	Per cent regeneration after 30days (mean±SD;n=2)	
				DC	LN
Mitra + CH + 0.5M sucrose	1	0	83.00	12.00 ± 2.83^{d}	0.00 ± 0.00^{b}
		1	47.40	20.61 ± 2.28^{c}	0.00 ± 0.00^{b}
		2	31.33	32.26 ± 1.51^{b}	0.00 ± 0.00^{b}
		3	13.33	43.50 ± 2.12^{a}	17.5 ± 10.61^{a}
		4	10.25	33.00 ± 4.24^{b}	0.00 ± 0.00^{b}
		5	9.80	40.50 ± 71^{a}	0.00 ± 0.00^{b}
	2	0	72.06	10.05 ± 1.49^{d}	0.00 ± 0.00^{b}
		1	42.46	26.23 ± 5.34^{c}	0.00 ± 0.00^{b}
		2	25.00	34.48 ± 1.629^{bc}	0.00 ± 0.00^{b}
		3	14.85	38.65 ± 5.16^{ab}	5.00 ± 7.07^{b}
		4	13.10	43.00 ± 4.24^{ab}	36.11 ± 9.64^{a}
		5	9.96	47.77 ± 2.21^{a}	15.0 ± 7.07^{ab}
	3	0	70.61	5.93 ± 1.03^{b}	0.00 ± 0.00
		1	47.06	11.16 ± 0.59^{b}	0.00 ± 0.00
		2	32.21	27.83 ± 0.59^{b}	0.00 ± 0.00
		3	12.095	30.27 ± 0.51^{a}	0.00 ± 0.00
		4	10.06	26.49 ± 2.14^{a}	0.00 ± 0.00
		5	9.60	31.77 ± 0.62^{a}	0.00 ± 0.00

Note: The encapsulated protocorms were pre – cultured in Mitra medium+ 0.01%CH +0.5M sucrose for 1- 3 days and subjected to dehydration and LN exposure. For post recovery, agar–gelled Mitra + CH medium was used. Different letters in a data series represent significant difference at 5% level based on Ducan's multiple range test.

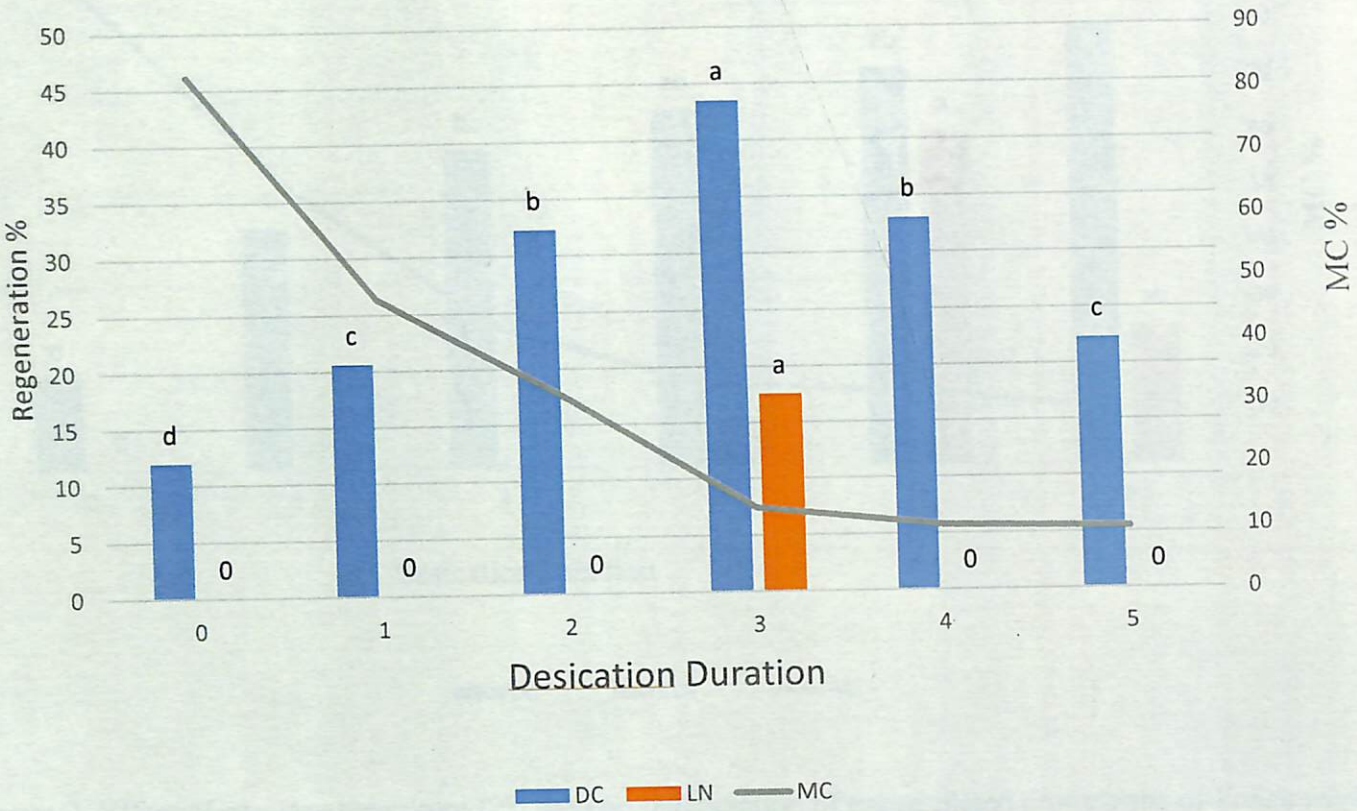


Figure.1. Effect of one-step preculture (1 day) on regeneration of encapsulated protocorms of *Vanda wightii*

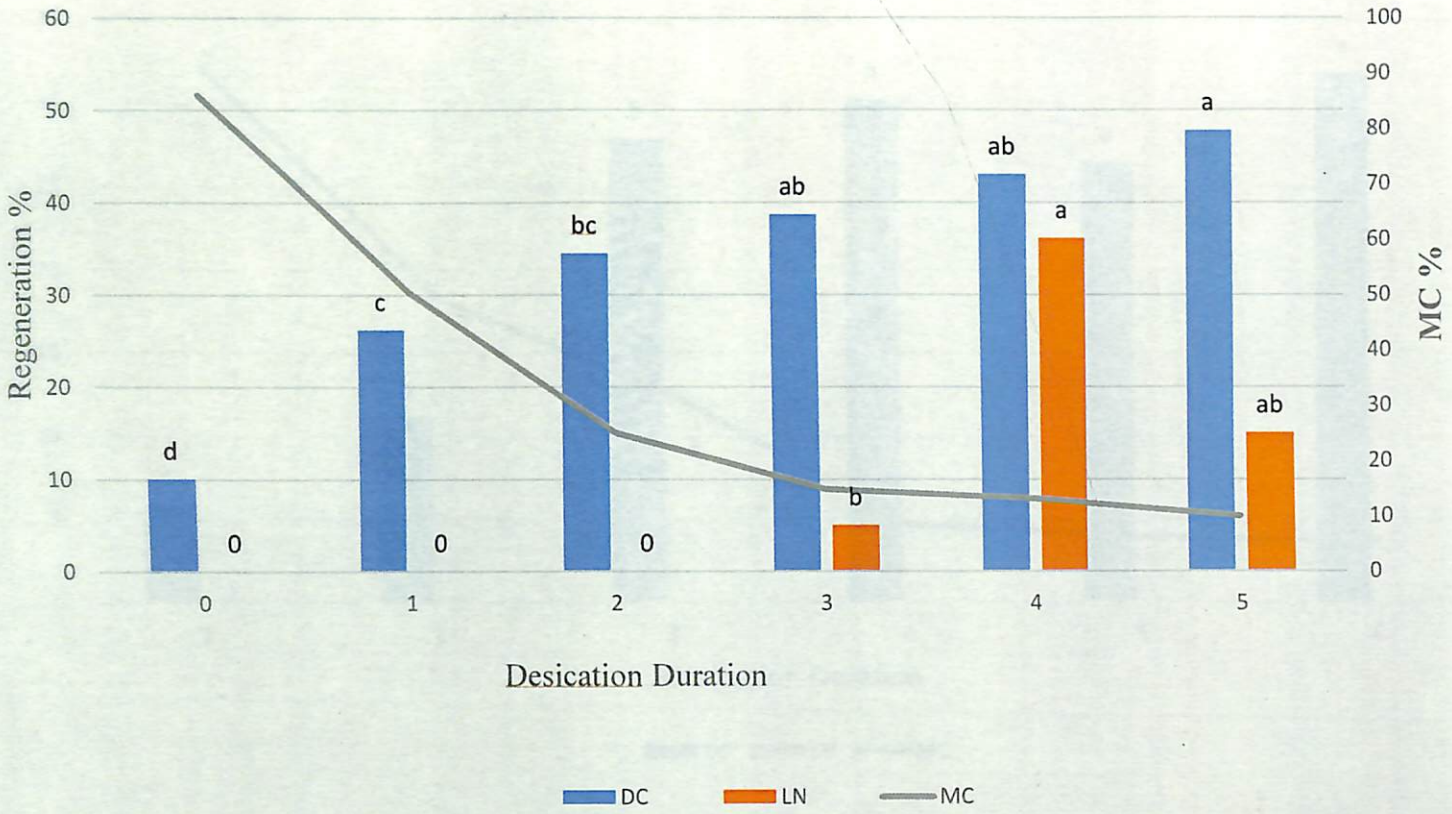


Figure.2. Effect of one-step preculture (2nd day) on regeneration of encapsulated protocorms of *Vanda wightii*

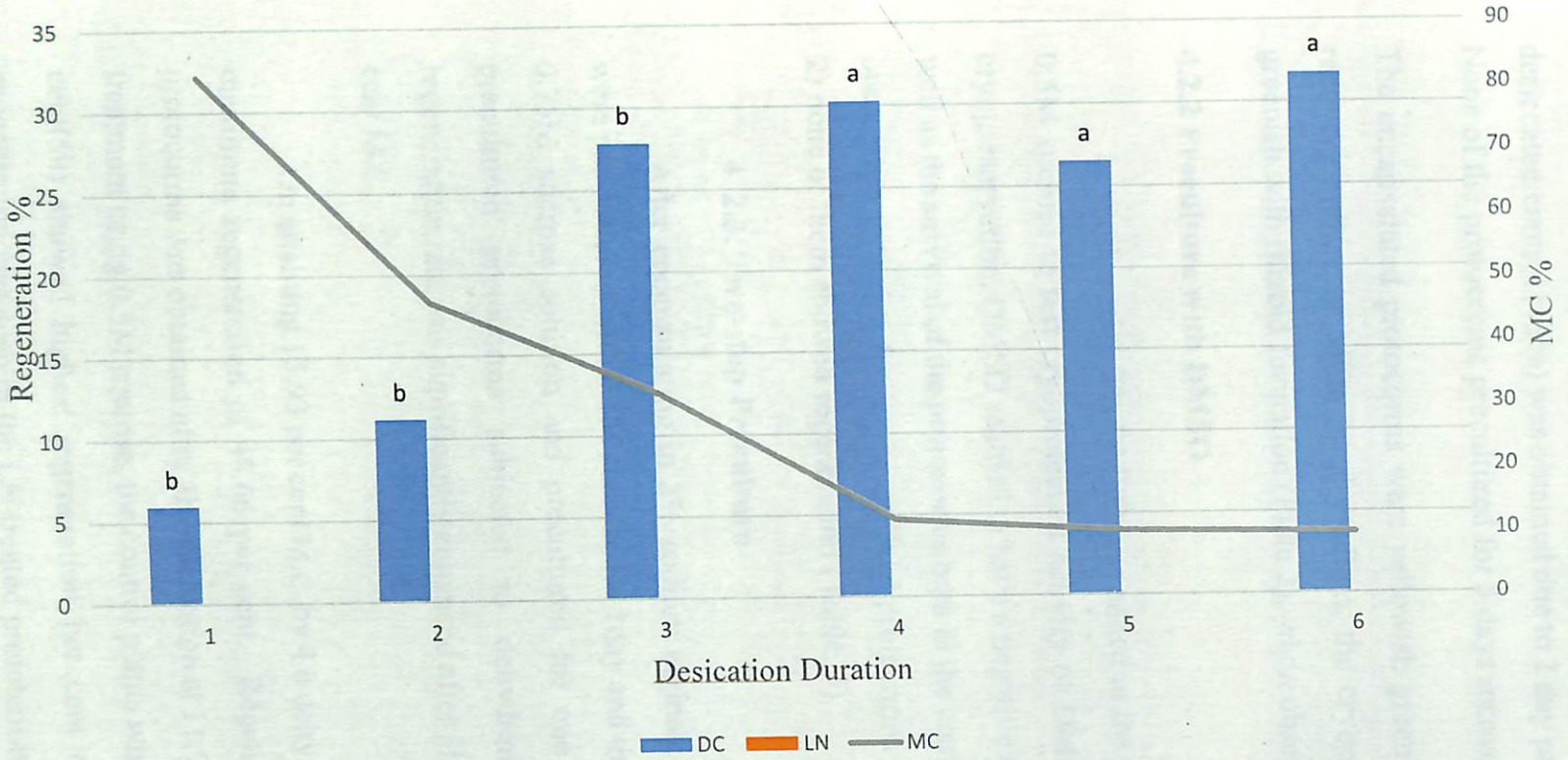


Figure.3. Effect of one-step preculture (3 day) on regeneration of encapsulated protocorms of *Vanda wightii*

Regeneration of cryopreserved protocorms was improved by 2 day preculture (Table. 1). Maximum 36.1% regeneration comparable to corresponding desiccated control (43%) was obtained due to 2 day preculture and 4 h dehydration. None of the protocorms precultured for 3 days recovered after cryopreservation.

The encapsulated protocorms were yellowish green in color. During post-freeze recovery culture in Mitra *et al* medium, the cryopreserved protocorms turned greenish with rhizoid formation (Plate.2), when observed after 30 days.

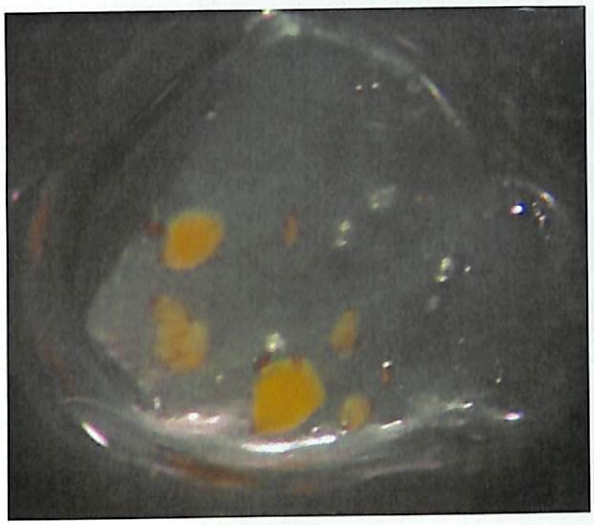
4.2.2 Preculture with DMSO

In another trial, 3% DMSO was added in the preculture medium containing 0.5M sucrose to test cryoprotectant activity of DMSO to improve recovery after cryopreservation. DMSO seemed to have a negative effective in the regeneration as well as the survival of the protocorms both in the control as well as in the LN treated ones. Even though a few cryopreserved protocorms survived after 20 days (Table. 2) none of them showed regeneration (Table. 3).

4. 2.3. Two-step Preculture

After encapsulating in 3% sodium alginate, the encapsulated protocorms were pre-cultured with 0.5M sucrose for 1day and the next day it was transferred to 0.75M sucrose solution and precultured for one day. The encapsulated and precultured protocorms subjected to dehydration for 0-5h. Nevertheless, regeneration rate was significantly enhanced after 3h desiccation at 11.95-13.95 per cent M.C.

On attaining 13.93 per cent M.C. by 4 h dehydration, the protocorms showed maximum regeneration of 48.66 per cent. Regeneration (12.5%) of LN treated protocorms was obtained after 5h desiccation at 11.95% m.c. In the 3 day preculture treatment using 0.5M sucrose, the control plants which were dehydrated to 9.66 per cent(5h) showed highest regeneration per cent of 31.77 (Fig.4.) and here no regeneration was seen in the LN treated prototcorms (Table.4).



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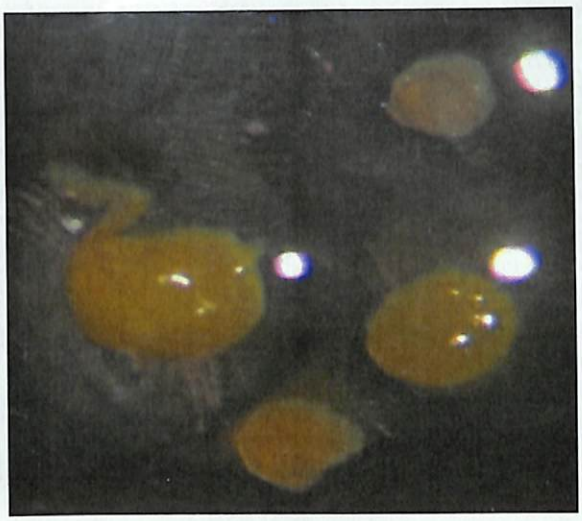

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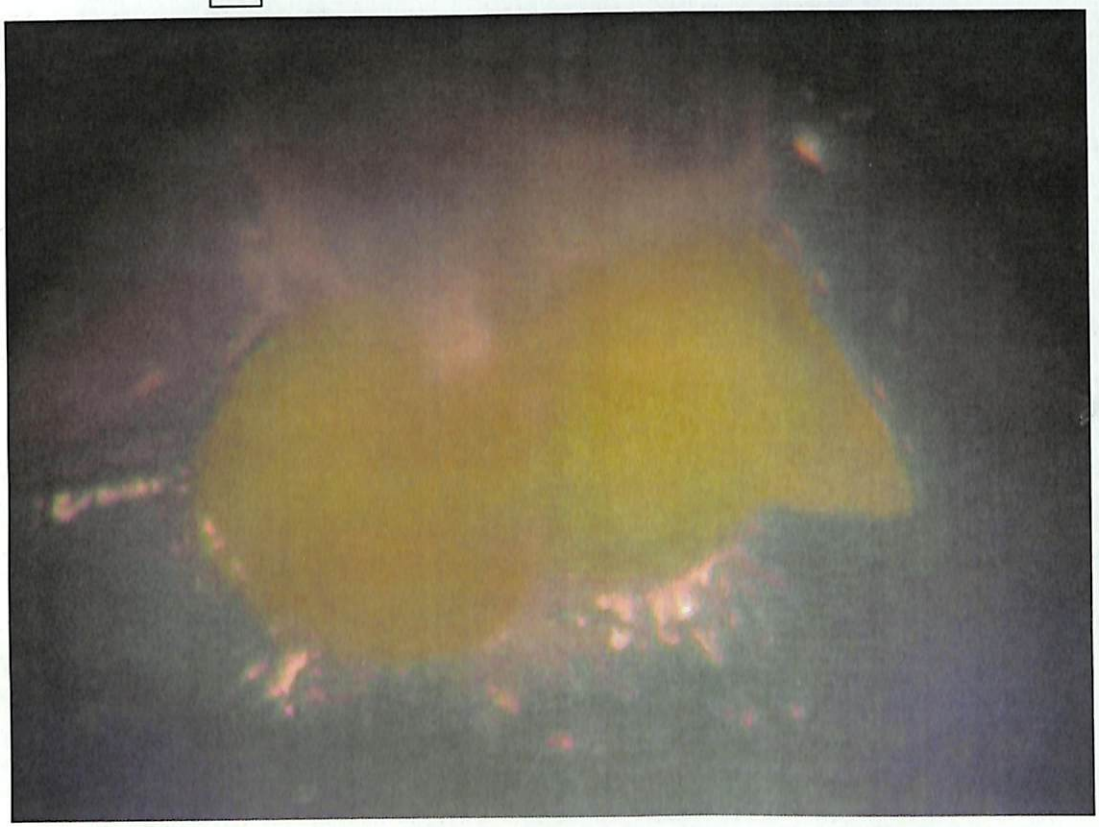


C

A- Encapsulated protocorms after recovery

B- Protocorms after 30 days showing recovery

C- Regenerated protocorms after 40 days

Plate.2. Cryopreservation of protocorms (*V. wightii*) through Encapsulation-Dehydration

Table. 2. Effect of 3% DMSO on survival of the encapsulated protocorms of *Vanda wightii*

Preculture solution	Preculture duration (days)	Duration of desiccation (h)	Moisture content (%)	Per cent survival after 20days (mean±SD;n=2)	
				DC	LN
Mitra + CH + 0.5M sucrose	1	0	83.27 ± 1.48	51.24 ± 1.78^{b}	0.00 ± 0.00^{b}
		1	39.80 ± 0.98	66.74 ± 5.78^{a}	0.00 ± 0.00^{b}
		2	19.4 ± 2.54	69.39 ± 3.86^{a}	8.37 ± 4.19^{a}
		3	15.50 ± 0.98	50.06 ± 5.75^{b}	11.54 ± 0.80^{a}
		4	13.10 ± 0.42	53.99 ± 3.77^{b}	0.00 ± 0.00^{b}
		5	12.55 ± 0.49	51.55 ± 2.37^{b}	0.00 ± 0.00^{b}
	2	0	80.10 ± 1.27	57.77 ± 1.57	0.00 ± 0.00
		1	41.11 ± 0.15	72.91 ± 8.84	9.42 ± 4.54
		2	18.20 ± 2.40	54.03 ± 7.25	10.67 ± 0.62
		3	16.65 ± 0.21	52.07 ± 2.96	11.27 ± 1.32
		4	14.95 ± 0.21	53.33 ± 9.43	6.46 ± 1.77
		5	13.10 ± 0.14	65.33 ± 1.89	8.99 ± 1.40
	3	0	76.1 ± 1.48	51.37 ± 6.72^{b}	0.00 ± 0.00^{b}
		1	35.26 ± 1.32	68.06 ± 2.74a	0.00 ± 0.00^{b}
		2	14.7 ± 0.56	51.10± 1.58^{b}	6.31 ± 8.93^{ab}
		3	12.81 ± 0.72	34.99± 2.35^{c}	10.51 ± 0.86^{a}
		4	10.95 ± 0.21	30.56± 2.23^{c}	0.00 ± 0.00^{b}
		5	9.70 ± 014	31.66 ± 2.35^{c}	0.00 ± 0.00^{b}

Note: The encapsulated protocorms were pre – cultured in Mitra medium+ 0.01%CH +0.5M sucrose + 3% DMSO for 1- 3 days and subjected to dehydration and LN exposure. For post recovery, agar–gelled Mitra + CH medium was used. Different letters in a data series represent significant difference at 5% level based on Ducan's multiple range test.

Table3. Effect of two-step preculture with 3% DMSO on regeneration of encapsulated protocorms of *Vanda wightii*

Preculture Solution	Preculture Duration	Duration of Dessication (h)	Moisture content (%)	% regeneration after 30days (mean±SD;n=2)	
				DC	LN
Mitra + 0.5M sucrose for hours and Mitra + 0.75M sucrose for the next hours	1	0	82.26 ± 1.50	29.86 ± 10.80^{ab}	0.00 ± 0.00
		1	44.18 ± 1.37	20.56 ± 2.35^{c}	0.00 ± 0.00
		2	21.21 ± 1.42	25.07 ± 5.53^{c}	0.00 ± 0.00
		3	16.16 ± 1.46	39.85 ± 3.33^{a}	0.00 ± 0.00
		4	13.95 ± 0.25	31.83 ± 2.12^{ab}	0.00 ± 0.00
		5	11.45 ± 0.91	25.68 ± 2.07^{c}	0.00 ± 0.00

Note: Note: The encapsulated protocorms were pre – cultured in Mitra medium+ 0.01%CH +0.5M sucrose + 3% DMSO for 24 hours and transferred to Mitra medium+ 0.01%CH +0.75M sucrose for another 24 hours. It was subjected to dehydration and LN exposure. For post recovery, agar–gelled Mitra + CH medium was used. Different letters in a data series represent significant difference at 5% level based on Ducan's multiple range test.

Table.4. Effect of two-step preculture on regeneration of encapsulated protocorms of *Vanda wightii*

Preculture Solution	Preculture Duration	Duration of Dessication (h)	Moisture content (%)	Per cent regeneration after 30days (mean±SD;n=2)	
				DC	LN
Mitra + 0.5M sucrose for hours and Mitra + 0.75M sucrose for the next hours	1	0	82.02 ± 1.42	13.50 ± 2.12^{d}	0.00 ± 0.00^{a}
		1	44.45 ± 1.62	26.11 ± 5.50^{c}	0.00 ± 0.00^{a}
		2	21.25 ± 1.48	33.00 ± 5.66^{bc}	0.00 ± 0.00^{a}
		3	16.16 ± 1.46	40.83 ± 5.42^{ab}	0.00 ± 0.00^{a}
		4	13.95 ± 0.21	48.66 ± 1.87^{a}	0.00 ± 0.00^{a}
		5	11.95 ± 1.19	44.22 ± 2.98^{a}	12.50 ± 17.68^{a}

Note: The encapsulated protocorms were pre – cultured in Mitra medium+ 0.01%CH +0.5M sucrose for 24 hours and transferred to Mitra medium+ 0.01%CH +0.75M sucrose for another 24 hours. It was subjected to dehydration and LN exposure. For post recovery, agar–gelled Mitra + CH medium was used. Different letters in a data series represent significant difference at 5% level based on Ducan's multiple range test.

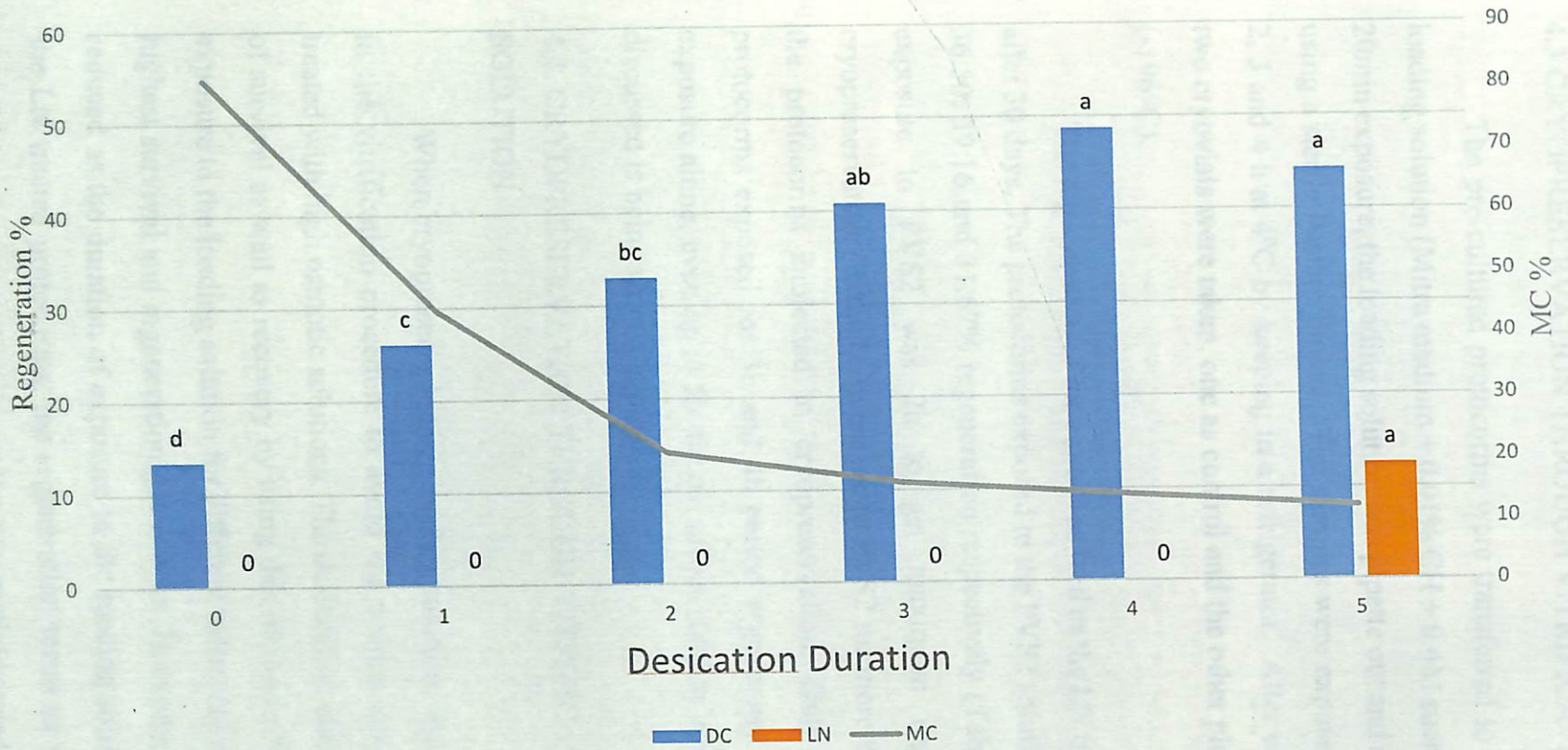


Figure.4. Effect of two-step preculture (1 day) on regeneration of encapsulated protocorms of *Vanda wightii*

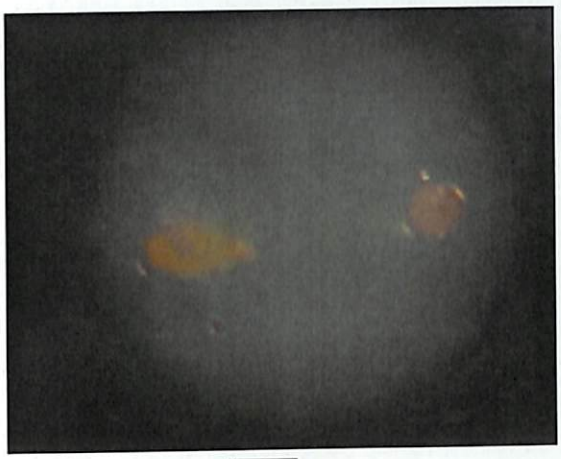
4.3 CRYOPRESERVATION THROUGH VITRIFICATION METHOD

The pre-cultured protocorms were transferred to 4ml cryovials and added loading solution (Mitra medium + 0.01% CH + 0.4M sucrose + 2M glycerol). After 20min exposure, the loading solution was pipette out and added 2 ml PVS2 solution using a sterile Pasteur pipette. The samples were exposed to PVS2 solution for 1, 2, 3 and 4 h at 4^{o}C by keeping in a refrigerator. After each duration of exposure, two cryovials were taken, one as control and the other plunged into liquid nitrogen (-196°C).

The regeneration of the control as well as the LN treated explants were noted after 30 days. The protocorms exposed to the PVS2 solution for 2, 3 and 4h showed 36.50, 39.16 and 31.67% regeneration respectively (Table. 5) Optimum period of exposure to PVS2 was 2h to get maximum regeneration (37.40) after cryopreservation (Plate.3). Exposure to PVS2 for more than 2h was detrimental to the protocorms subjected to cryopreservation. Only 12.50 and 9.17 of the protocorms exposed for 3h and 4h period regenerated (Fig.5). However, PVS2 exposure alone, even up to 5h did not affect viability of protocorms. The result as discussed is being shown in plate. 3.

4.4. CRYOPRESERVATION THROUGH VITRIFICATION USING LOADING SOLUTION

When cryopreserved using loading solution, which is being normally used in the vitrification procedure to avoid the sudden shock to the materials when treated with high osmotic solutions. The desiccation controls showed high per cent of survival as well as recovery by using this method of cryopreservation. For the exposure to the loading solution for different intervals i.e. from 0-4h, in the control highest survival and regeneration got was for 1h exposure. Gradually the survival reduced as the duration of exposure to the loading solution increased. In case of the LN treated protocorms, the regeneration result as well as the survival result showed improvement than the earlier two methods used. The data taken in case of



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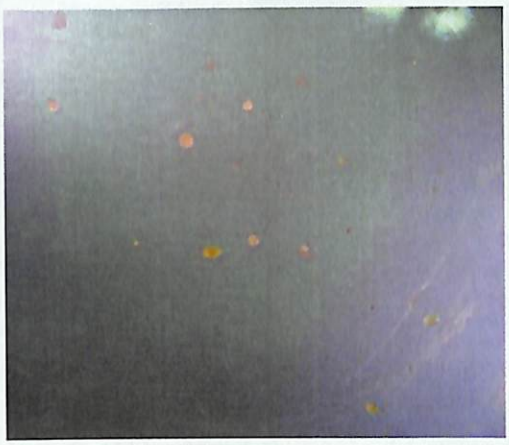

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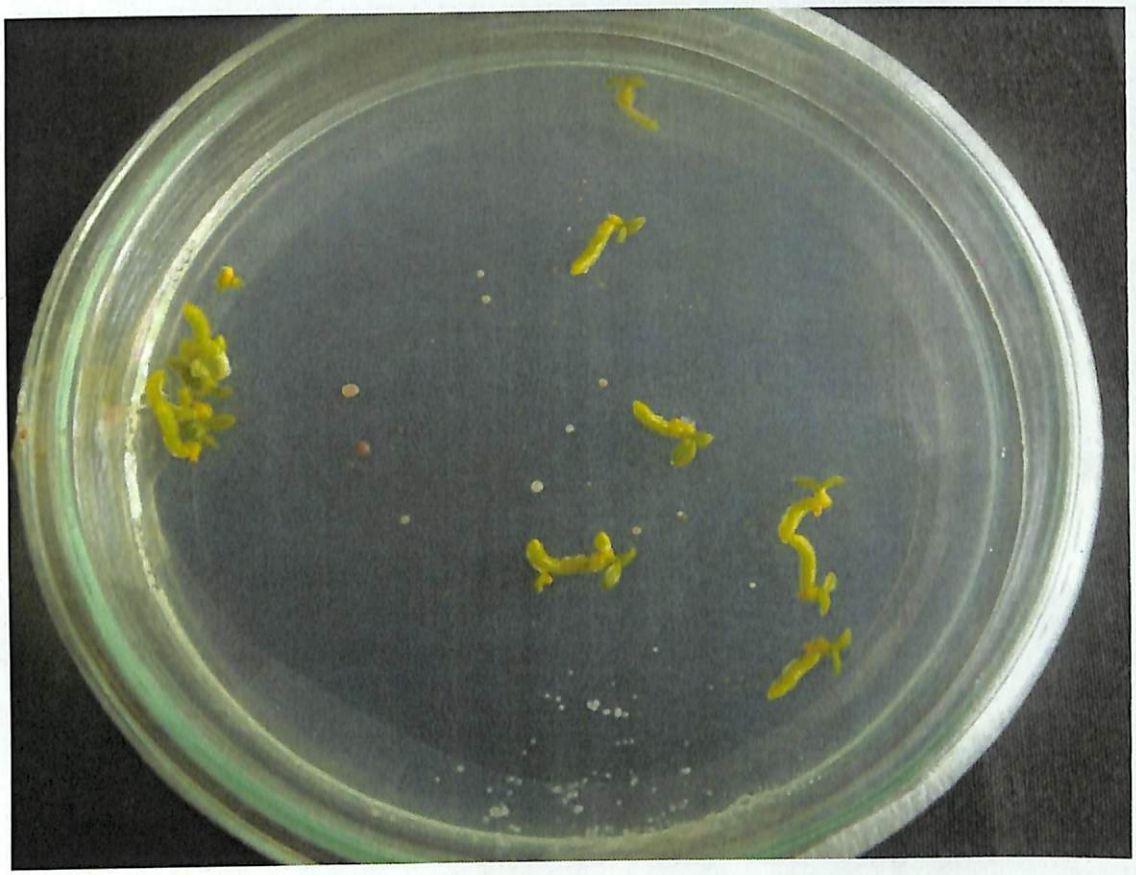


C

A – 20 days old protocorms after recovery.

B – 1 month old protocorms after recovery.

C – Regerated palntlets after 2 months

Plate.3. Cryopreservation of protocorms of *V. wightii* through vitrification

Table.5. Vitrification experiment of *Vanda wightii* protocorms.

Preculture solution	Preculture duration (days)	PVS 2 Exposure (h)	Per cent regeneration after 30days (mean ±SD; n=3)	
			DC	LN
Mitra + CH + 0.5M sucrose	1	0	29.44 ± 11.79[a]	0.00 ± 0.00[c]
		1	19.27 ± 7.80[a]	0.00 ± 0.00[c]
		2	36.50 ± 2.12[a]	37.40 ± 12.12[a]
		3	39.16 ± 10.62[a]	12.50 ± 17.68[ab]
		4	31.67 ± 15.08[a]	9.17 ± 12.96[ab]

Note: The protocorms used for the vitrification experiment was precultured in Mitra +0.01% CH + 0.5M Sucrose for one day and treated with loading solution for 20mins and various durations of exposure to PVS2 was given ie 0 – 4 hours.

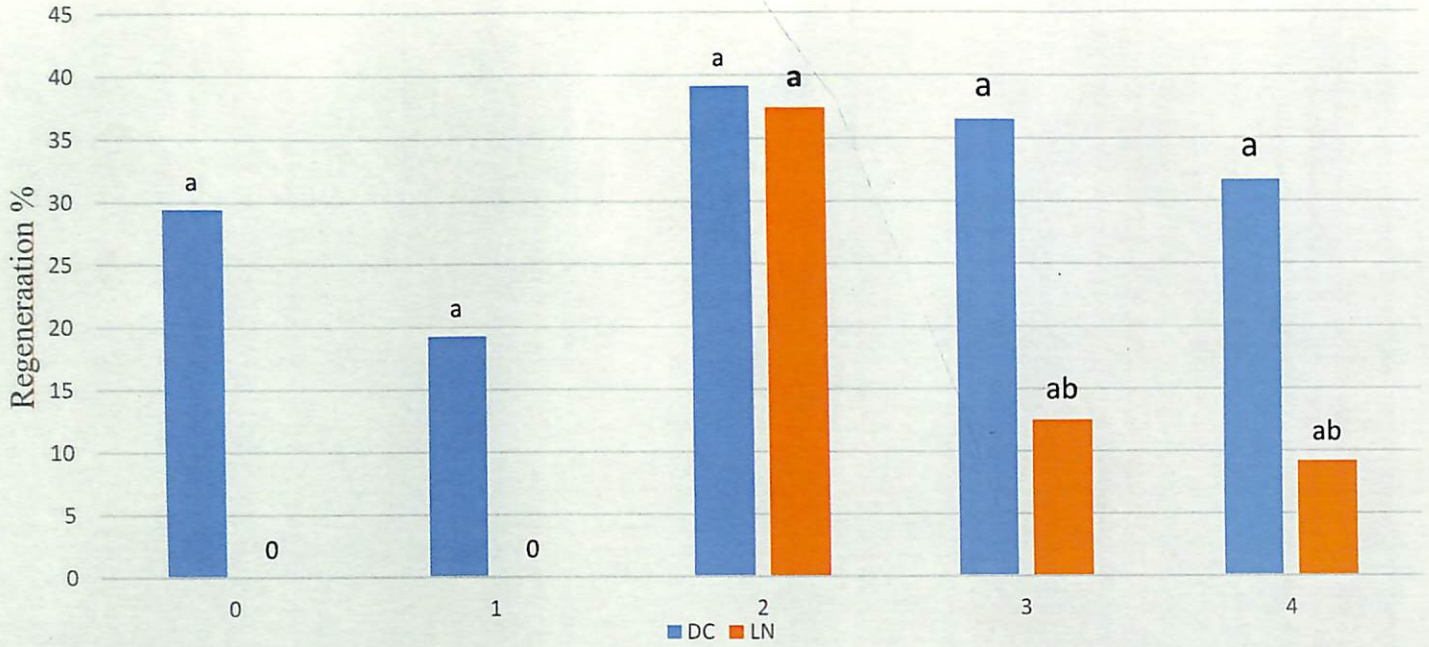
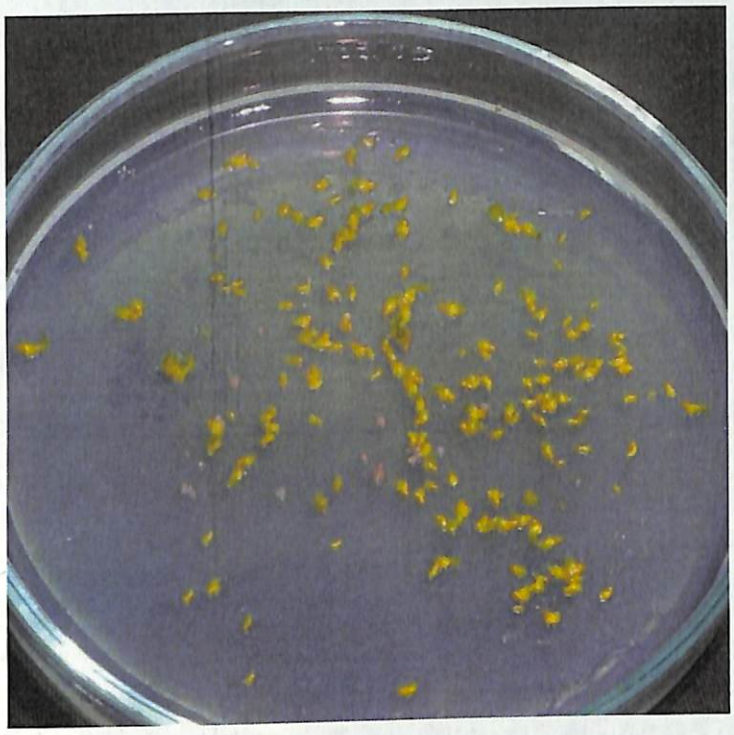


Figure.5. Cryopreservation using vitrification method

the LN treated protocorms were incomplete as contamination caused the discarding of some treatments. Survival as well as the regeneration data are given below in the Table.6 and Fig.6. The pictures depicting the result of the cryopreservation through vitrification using loading solution is being shown in Plate.4.



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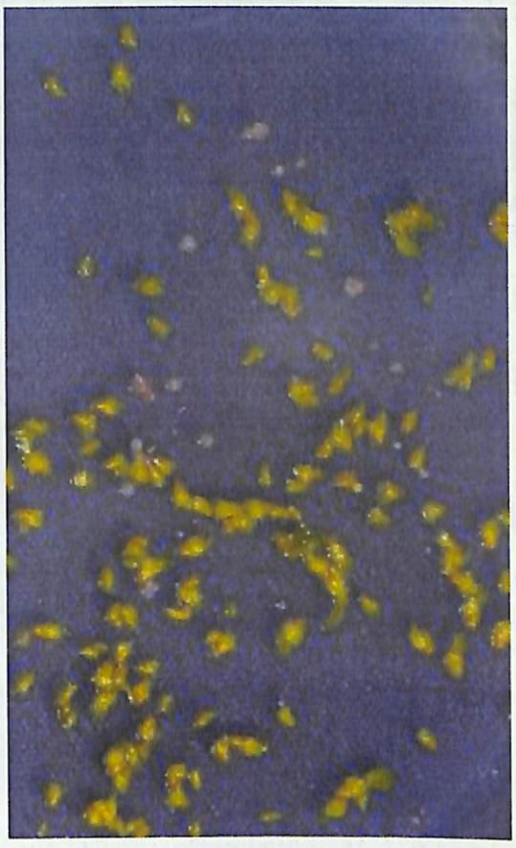

B

A- Regenerated protocorms after one month
B- Enlarged View.

Plate.4.Cryopreservation of protocorms of *V. wightii* through Vitrification Using Loading Solution

Table.6. Effect of loading solution on *Vanda wightii* protocorms in cryopreservation.

Loading solution Exposure (h)	% survival after 30days		% regeneration after 30days	
	DC	LN	DC	LN
0	56.10	40.33	*	*
1	68.48	61.95	31.03	34.95
2	57.14	52.38	*	*
3	51.33	42.42	*	*
4	46.00	33.33	42.62	31.66

Note: The protocorms were precultured in Mitra + 0.01% CH + 0.5M sucrose for one day and they were treated with loading solution for various durations ie from 0 – 4 hours. The values are from a single determination consisting of 10-50 protocorms (30 day old) for each treatment.

*- no data

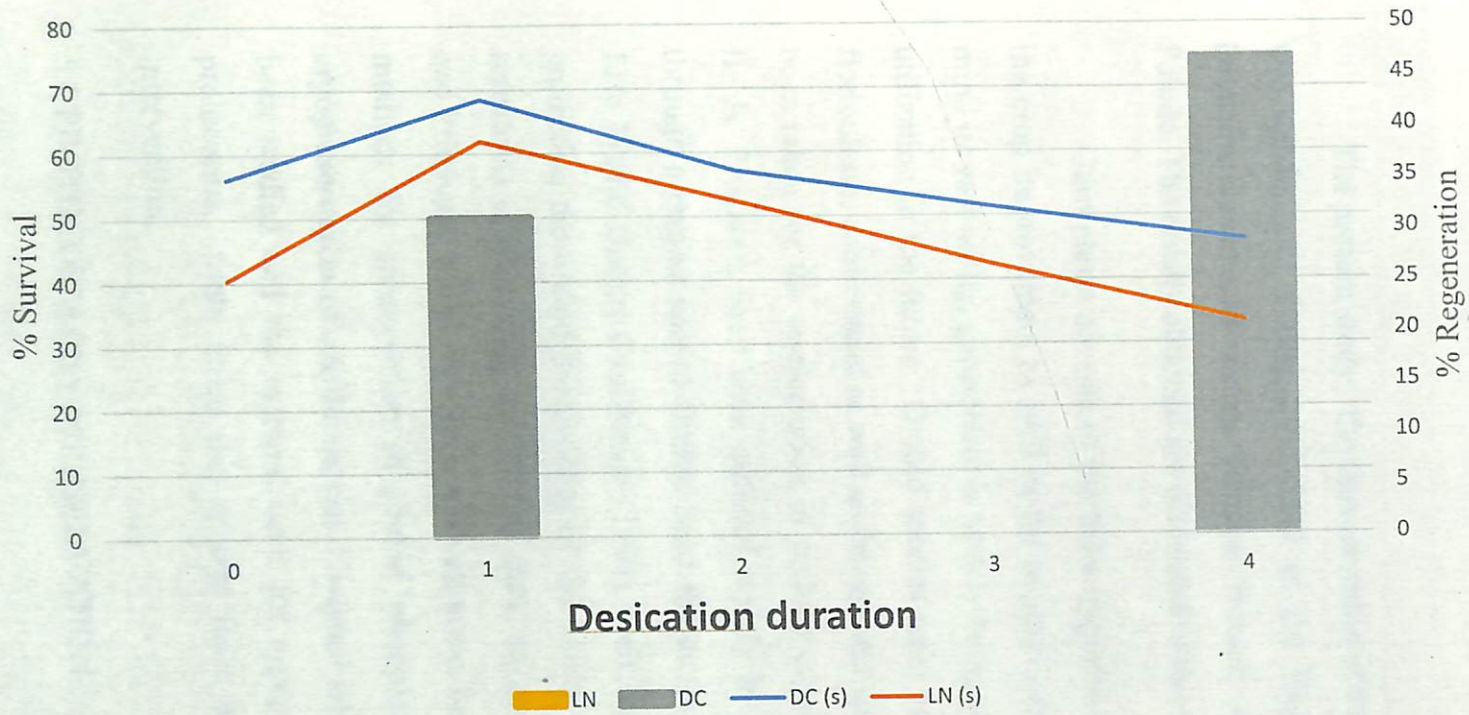

Figure.6. Effect of loading solution on survival of *V. wightii* protocorms subjected to LN exposure.

5. DISCUSSION

The present study, 'Cryopreservation of *Vanda wightii* Rchb.f protocorms', was carried out during 2013-2014 at the Biotechnology and Bioinformatics Division, Jawaharlal Nehru Tropical Botanic Garden and Research Instituite, Palode. The results obtained are discussed in this chapter.

Germplasm are one of the most important materials which can be used for the crop improvement as well as for reconstitution of new cultivars. Keeping in mind its vast utility, germplasm is usually being conserved in the gene banks for its utilization in the future. Orchid species have been recognized worldwide for its floriculture, commercial as well as therapeutic values. World-wide measures have been taken for the conservation of orchids considering its importance in various fields. Programs have been initiated mainly in the Royal Botanic Garden, Kew through a project named Orchid Seed Stores for Sustainable Use (OSSSU). The 11th World Orchid Conference (1984) itself has pointed out the significance in protecting the orchid biodiversity of the world. Some species of orchid showed sensitivity to the storage at -30ºC to -50ºC but showed tolerance to -196ºC (Seaton and Pritchard, 2008). So cryopreservation can be considered as one of the efficient methods for conservation of orchid germplasm. In this present study the cryopreservation of orchid species *V.wightii* which is an endangered orchid, has been studied and the material used for cryopreseravation are the 30 day old protocorms. Main threat this species faces is the habitat loss due to human intervention.

5.1 PROTOCORM CRYOPRESERVATION

Protocorms are the germinated organized structures which are arising from the seeds of orchids. For the conduction of the present study, 30-day old protocorms cultured in Mitra *et al*., 1990 medium was used.

In case of orchid species which are having less number of seeds or the seeds do not germinate properly, proliferation of the protocorms as well as protocorm like-bodies are usually the only means to increase the number of that particular species. Mainly two methods of cryopreservation has been followed for the protocorms i.e. vitrification method (Fahy *et al*., 1984; Sakai *et al*., 1990) or encapsulation-dehydration method (Fabre and Dereuddre, 1990, Decurse *et al*., 1999). In the above mentioned methods, encapsulation-dehydration is the most widely used method considering the involvement of toxic cryoprotectants used in the vitrification method of cryopreservation. The protocorms in this study has been subjected to precultures, transfers and cryogenic storage. The protocorms showed different appearances like some were yellow, green, white, browish etc. the yellowish as well as the bleaching and browning conditions could be attributed to osmotic shock or unfavorable regrowth conditions (Tahtamouni and Shibili, 1999; Moges *et al*., 2004; Zhoa *et al*., 2008).

5.1.1 Cryopreservation through Encapsulation Dehydration

5.1.1.1 Encapsulation

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

The protocorms of *Vanda wightii* were encapsulated using 3 per cent sodium alginate and 100mM calcium chloride in the present study. Lurswijidjarus and Thamasiri, (2004) reported shoot tips of *Dendrobium* Walter Omumae were cryopreserved by the encapsulation/dehydration with modified VW liquid medium supplemented with 3% (w/v) sodium alginate and 0.1 M CaCl2 solution. Mohanty *et al*., 2012 also used 3 per cent sodium alginate and 100mM calcium chloride

for the encapsulation of *Dendrobium nobile* Lindl. protocorms for cryopreservation.

5.1.1.2 Preculture treatments

Sucrose is an important pre-growth additive for most cryopreservation methods, which enhances desiccation tolerance during cryopreservation. Higher concentrations of osmoticum provide higher protection to the plant from desiccation injury. The tissues of orchids can be pre-cultivated in media containing high levels of sucrose, which is considered to be an important technique for long-term conservation without genetic alteration. Among different types of sugars (fructose, glucose, sorbitol, and sucrose) used as osmotic agents in pre-culture medium, sucrose was the best for the survival of cryopreserved samples (Bekheet *et al.,* 2007). It has long been reported that preculture duration influences high survival of different explants after cryopreservation in orchids (Ishikawa *et al*. 1997; Maneerattanarungroj *et al*. 2007).

As per the reports of Mohanty *et al*., (2012) the PLBs of *Dendrobium nobile* Lindl. orchid withstood sucrose concentration as high as 0.7M when precultured after encapsulation. In the present study the 30 day old protocorms after encapsulation were precultured in Mitra medium containing 0.5M (one-step preculture) sucrose for 1 to 3 days and in 0.75M (two-step preculture) preculture solution for one day. However increase of sucrose concentration in the preculture medium resulted in decrease of percentage survival of the encapsulated PLBs. This might have been due to sucrose creating harmful osmotic stress in the treated explants promoting excessive dehydration of the PLBs and hence incurring toxicity. Similar observations were found in the present study while precultureing the protocomrs in 0.75 M sucrose. Preculture in 0.5 M sucrose gave better results compared to 0.75M sucrose in agreement with the earlies studies in which encapsulated PLBs precultured with 0.5 M sucrose for 2 days and air dried for 5 h

or 6 h showed better percentage of survival than control (Mohanty *et al*., 2012). Maximum percentage survival (53.3%) of cryopreserved PLBs was recorded when pretreated with 0.5 M sucrose for 2 days and further.

DMSO was included in the preculture solution to observe the effect it produces on the regeneration of the protocorms, as Kaviani, (2011) reported that DMSO can enter into the cells and can protect the cells integrity during cryopreservation. DMSO can be included in the category of penetrating cryoprotectants. When DMSO was used, no regeneration was observed for the LN treated protocorms but the regeneration showed a decline compared to the preculture using sucrose alone. However some of the cryoprotectants used in preculture can cause cryotoxicity and osmotic stress to the samples. Surenciski *et al*., 2012 has reported that DMSO is having toxic effects to the samples which are being used for the cryopreservation.

5.1.1.3 Effect of dehydration

According to the studies performed by Zhang *et al.* (2001), the reduction of water content to a critical level before freezing is the key factor in developing successful cryoprotection protocols. If the cells are not sufficiently dehydrated, intracellular ice will be formed resulting in cryoinjury during cold storage in LN and if over-dehydrated, the osmotic stress can be damaging (Brian *et al.* 2002). Hence, dehydration must be long enough to ensure sufficient cell dehydration, without cytotoxic effects. Dehydration can be achieved by air drying of the encapsulated protocorms in laminar air flow, which is one of the common methods used for dehydration. The protocorms of *Grammatophyllum speciosum* were investigated for its successful cryopreservation where the protocorms were dehydrated in the sterile laminar air flow chamber (Sopalun *et al*., 2010).

In the present study, the encapsulated and pre-cultured protocorms of *V. wightii* were subjected to different hours of dehydration from 0 to 5 h to find the optimum dehydration level. Moisture content of the encapsulated protocorms prior

to dehydration was between 70 and 83 per cent. When cultured on, Mitra solid medium, the protocorms showedmaxium regeneration of 47.11 and 36.77 per cent in control and LN treated protocorms respectively.

In the present study, the M.C. value came down to as low as around 9 per cent after 5 h dehydration. Good regeneration was obtained between M.C. value of 9 – 14 per cent where the maximum regeneration per cent got was 36.77 for the LN treated protocorms. According to Abdelnour-Esquivel *et al*,. (1992), moisture content of 14 %t is optimum for getting high post-thaw recovery. According to the results obtained by Maneerattanarungroj *et al.* 2007 the beads having protocorms with high water content were dead after freezing in LN whereas beads with lower water content showed maximum viability. However optimal water content for the cryopreserved explants will depend on many conditions such as plant species, developmental stage, preculture regime and recovery protocol.

According to Reed *et al*. (2005), the optimum M.C. for germplasm of most plants before exposure to LN is normally about 19 to 23 per cent. Alginate beads dried to 20 per cent moisture vitrified on exposure to LN and formed stable glasses that do not form ice crystals on rewarming. Thus, a prerequisite for successful application of encapsulation dehydration technique is the avoidance of irreversible cell membrane damage caused by the formation of intracellular ice crystals. Ice crystallization can only be prevented through a reduction of the cellular water content (dehydration) to the strict minimum (Vertommeu *et al.,* 2008).

5.1.2 Cryopreservation through vitrification method

In this method of cryopreservation, the protocorms are precultured before vitrification. The vitrification method was successfully applied to mature seeds of *Bletilla striata* and *Doritis pulcherrima* (Thammasiri, 2000), and immature seeds of *Bletilla striata* (Hirano et al., 2005a) and *Ponerorchis graminifolia* var. suzukiana (Hirano *et al*., 2005b).

In this present study, one step preculture is followed where 30 days old protocorms are precultured in Mitra *et al*. medium containing 0.5M sucrose

for a single day, then subjected to vitrification procedures. According to Shuhaida *et al*., 2009, the best result for the cryopreservation of *Brassia rex.* orchid hybrid through vitrification method was obtained when the protocorms were precultured in 0.5M sucrose where as a sucrose concentration of 0.75 M reduced the viability of PLBs significantly.

During vitrification, the precultured protoroms were treated with loading solution for about 20min. This is used as a protective step before treating the samples with solutions with high osmoticum. Poobathy *et al*., (2012) reported vitrification and histological analyses of protocorm-like bodies of V*anda* Kaseem's Delight that the PLBs of the orchids were treated with the loading solution for 20 min which provided them with good results. Considering the case of *Brassica* rex Orchid Hybrid protocorm cryopreservation, 8 PLBs taken in each cryovials were immersed in a mixture of 2 M glycerol and 0.4 M (loading solution) sucrose prepared in MS medium (pH 5.8) for 20min at room temperature (Shuhaida *et al*., 2009). So the 20 min exposure to the loading solution can be considered as a standard exposure time.

Vanda wightii protocorms after loading treatment was treated with PVS2 for a duration of 0 – 4 h and then plunged into liquid nitrogen in the present study. In a similar study of *Blettilla striata*, embryos precultured in 0.3 M sucrose for 3 days, produced the highest survival when dehydrated in PVS2 for 3 h at 0°C prior to storage in LN (Hirano et al., 2005a, 2005b). Considering these early observations, the exposure time for the *V. wightii* protocorms were kept between 0 – 4 h. The best result was observed due to 2 h exposure to PVS2 ie 37.40 per cent and it decreased after that period of treatment. Our results are in agreement with the findings of Vendrame, (2008) which showed viability of pollinia of *Dendrobium* hybrids treated with PVS2 solution for 0 – 4 h, where 2 hr was the optimum. Poobathy, *et al*. (2013) also recorded highest regeneration using PVS2 between 60 and 120 min exposure time in *Dendrobium* hybrid.

None of the *Vanda wightii* protocorms without having any PVS2 treatment survived LN exposure possibly due to the high moisture content for

preserving them in the liquid nitrogen. Reduced regeneration beyond 2 h exposure to PVS2 solution may be due to over dehydration of the protocorms.

Vanda coerulea seeds showed highest survival (67%) with 70min exposure to PVS2 as reported earlier. There was no survival or regeneration of LN treated samples which were not treated with PVS2 solution, as the moisture content at the beginning was 33% not safe for LN tolerance. Exposure less than 20 min was not enough for *Vanda coerulea* seeds d, while dehydration beyond 70 min decreased the survival because of excessive dehydration and the inability to tolerate that dehydration level (Thammasiri and Soamkul, 2007). In the case of sucrose precultured proliferating banana cultivars, Panis, (2009) has observed that optimal post rewarming regeneration percentages are generally obtained after a 2 or 2.5 h PVS2 treatment. This findings support the result observed in the present study.

5.1.3 Cryopreservation using loading solution

Loading treatment (2M glycerol + 0.4M sucrose), which involves treating the explants with a moderately concentrated cryoprotectant solution, prior to vitrification treatment is to reduce the risk of sudden osmotic shock by highly concentrated vitrification solutions. However, exposure to loading solution alone appears to be effective for successful cryopreservation of *V. wighti* protocorms as observed in the present study. Protocorms subjected to a minimum 1h exposure to loading solution showing about 35% regeneration after LN exposure indicate the effectiveness of such treatment for successful cryopreservation. This is a new finding, but needs further confirmation. The experiment could not be repeated due to unavailability of seed samples and data on some of the treatments could not be gathered as those samples were lost due to contamination.

The objectives of this study was to device a cryopreservation protocol for the protocorms of Vanda wightii Rchb.f. Two methods of cryopreservation was followed i.e. Encapsulation dehydration and vitrification method of

cryopreservation where the vitrification method was found to be providing good and stable result than the encapsulation-dehydration method. The vitrification using loading solution treatment provided good results when comparing the other two methods but further optimization of this work has to be done as results of this was not confirmed by repeating due to shortage of material for the work as the material is seasonal. Further studies like optimization of different steps in cryopreservation to get better recovery per cent in encapsulation dehydration and other methods like encapsulation-vitrification, droplet immersion can be attempted to enhance the survival rate after long term storage.

6. SUMMARY

Cryopreservation is a cost effective and safe mechanism for the conservation of plant germplasm by storing them under ultra low temperature of liquid nitrogen (-196°C). At this temperature, metabolic and cellular divisions of plant tissues are suspended and genetic stability is maintained. This method can be considered as one of the best method for the conservation of the endangered germplasm compared with the conventional field conservation measures.

Vanda wightii Rchb.f is one of the endemic orchid species found in the Western Ghat regions of Kerala, Karanataka and Tamil Nadu. This plant faces risk of genetic loss due to habitat loss as it is found in areas where human population resides. Even though orchids produces millions of seeds, only few develop into plants in nature as suitable conditions are lacking. Effective conservation measures are thus necessary for this plant. In such instances where *in situ* conservation measures are quite insufficient due to intensive land and labour requirements cryopreservation is recommended effective tool for the conservation of germplasm of such endangered species of diverse habitat preferances in gen banks.. Different materials like the seeds, protocorms, meristems and embryos have been proved effective for the conservation of orchid species like *Vanda coerulea, Vanda spathulata* etc. The present study examines the possibilities of cryopreservation of protocorms of *Vanda wighti* Rchb.f. through encapsulation-dehydration as well as vitrification method..

As the source material, protocorms were raised from seeds extracted from 12 month old capsules in liquid Mitra medium. The seeds germinated by 2 weeks of culture and 30 days old protocorms were used for the present investigation. Colour of the protocorms at the time of experiments was yellow or yellowish green.

The protocorms generated were encapsulated in 3 per cent sodium alginate and subjected to one-step as well as two-step preculture. In the one-step method, protocorms were precultured in Mitra+0.5M sucrose for 1 – 3 days and in the two step preculture, protocorms precultured in 0.5M sucrose for one day was transferred to 0.75 M sucose and kept for one day. The precultured protocorms were subjected to dehydration under sterile laminar air flow for 0-5 h and plunged into liquid nitrogen.

Dehydration for 3-4 h reduced the moisture to 10-14 % safe for satisfactory dehydration as well as liquid nitrogen tolerance. Regeneration of cryopreserved protocorms was maximum after 2 day preculture so that 36.1% regeneration was obtained comparable to corresponding desiccated control (43). Three day preculture adversely affected viability of protocorms as none of them recovered after cryopreservation. Two step preculture did not improve regeneration after cryopreservation but gave significantly reduced regeneration (maximum 12.5%).

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Vitrification method was tried as an alternative method for the cryopreservation of protocorms.The protocorms treated with loading solution (Mitra medium + 0.4M sucrose + 2M glycerol) for 20 mins. followed by treatment with PVS2 solution for 0-4h revealed 2h exposure optimum to get maximum 37.4% regeneration after cryopreservation.. Exposure to PVS2 for more than 2h was detrimental to the protocorms subjected to cryopreservation so that only 12.50 and 9.17 of the protocorms exposed for 3h and 4h period regenerated.

In another modified procedure of vitrification method, protocorms of *V. wightii* was given exposure to loading solution for 0-4h and frozen in LN.. When exposed to loading solution for 1h the regeneration of protocorms was increased to

34.95%. The results revealed that treatment with loading solution alone is sufficient to cryoprotect the protocorms to get successful recovery after cryopreservation. However, the the results need to be confirmed through repeating the experiment.

The objectives of the present study was to device a cryopreservation protocol for the protocorms of *Vanda wightii* Rchb.f. Out of the two methods tested,. vitrification method of cryopreservation seems to be advantageous over encapsulation-dehydration giving consistent results.. Thus vitrification method is recommended for protocom cryopreservation of *V. wightii* for their effective germplasm conservation.

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Cryopreservation of *Vanda wightii* Rchb. F protocorms

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8. ABSTRACT

Investigations on cryopreservation of protocorms of *Vanda wightii* Rchb.f an endangered orchid of Western Ghats was carried out to devise a protocol for long-term conservation of their germplasm. The commonly used techniques encapsulation- dehydration and vitrification methods were compared.

Cryopreservation using encapsulation-dehydration gave maximum 36.1 per cent regeneration when one-step preculture was done in 0.5 M for 2 days and dehydrated for 4h. Optimum moisture content to get maximum recovery was 9.00 – 14%. Preculture in 0.5M sucrose for one day followed by 0.75M sucrose for one day did not improve regeneration of cryopreserved protocorms. Protocorms precultured in presence of 3 per cent DMSO did not recover after cryopreservation. Vitrification method tried as an alternative method gave 37.4 per cent regeneration when the protocorms were exposed to PVS2 for 2h prior to LN treatment. Exposure to PVS2 for more than 2h was detrimental to the protocorms so that Only 12.50 and 9.17 of the protocorms exposed for 3h and 4h period regenerated. Exposure to loading solution for 1h seems to give 34.95% regeneration after LN treatment suggesting that loading solution treatment alone is sufficient to cryoprotect the protocorms to get successful recovery after cryopreservation. Even though regeneration rates obtained through different methods are almost equal, consistent results and efficient direct plant regeneration make the vitrification method advantageous over encapsulation-dehydration method. Thus vitrification method is suggested as an efficient method for protocorm cryopreservation for germplasm conservation of *V. wightii* .

APPENDIX I

Mitra medium

Calcium Nitrate	200mg
Potassium Nitrate	180mg
Ammonium Sulfate	100mg
Sodium dihydrogen phosphate	150mg
Magnesium sulfate heptahydrate	250mg
Sodium EDTA	2.79mg
Iron sulfate	2.78mg
Potassium iodide	0.03mg
Magnesium chloride	0.4mg
Zinc sulfate	0.05mg
Boric acid	0.6mg
Copper sulfate	0.05mg
Sodium molybdate dehydrate	0.05mg
Cobalt nitrate	0.05mg
Thiamine HCl	0.3mg
Pyridoxine HCl	0.125
Sucrose	20g