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ORIGINAL ARTICLE

Optimisation of Encapsulation-dehydration Protocol for the Orchid Hybrid *Ascocenda* 'Princess Mikasa'

[1, 2]Ranjetta Poobathy, [2] Helen Nair and [1]Sreeramanan Subramaniam

[1]School of Biological Sciences, Universiti Sains Malaysia (USM), Minden Heights, 11800, Penang, Malaysia, [2]Department of Biotechnology, AIMST University, Batu 3½ , Jalan Bukit Air Nasi, Bedong , 08100, Kedah, Malaysia,

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ABSTRACT

Ascocenda 'Princess Mikasa' is a commercially important orchid hybrid. Long-term storage of its germplasm through cryostorage is a promising option of propagating clonal plants of this hybrid. In this study, protocorm like-bodies (PLBs) of the orchid pretreated with sucrose or sorbitol with an encapsulation-dehydration technique were cryopreserved for 24 hours, prior to monitoring recovery through viability observations and the 2,3,5-triphenyltetrazolium chloride (TTC) assay. PLB sizes of 3 mm and 6 mm and varying concentrations of sucrose and sorbitol were tested to determine the best conditions for the method. The viability of the PLBs after the cryopreservation were determined after a two-day dark and five-day light recovery period in Part I of the experiment, and after a one-week dark recovery period in Part II of the experiment, conducted on defined culture media. It was found that the best PLB size for cryopreservation was 6 mm, and that 0.50 M sucrose or 0.25 M sorbitol pretreatments gave the highest viabilities after the cryopreservation protocol. The best encapsulated-dehydrated beads retained 40% of its water content during cryostorage. It appeared that either sucrose or sorbitol, at appropriate concentrations, were equally good as pretreatments to ensure viable PLBs were recovered after cryopreservation.

Key words: Encapsulation-dehydration . protocorm like-bodies (PLBs). *Ascocenda* 'Princess Mikasa' orchid . 2,3,5-triphenyltetrazolium chloride (TTC)

Introduction

The family Orchidaceae is classified as one of the largest and most diverse group of plants, containing almost 20,000 to 25,000 species [46]. Over 100,000 commercial hybrids are registered presently, most of them grown as cut flowers and potted plants [83]. A range of attractive hybrids, varieties or cultivars of sympodial orchids, for instance, the genus *Oncidium* or *Ascocenda*, is important in the cut-flower and potted-plant industries [10]. Their popularity is attributed to their bewildering colour schemes, shapes and sizes, bloom persistence, and their ability to travel long distances, hence their position as one of the top 10 cut flowers in the international market [46].

The orchid hybrid, *Ascocenda* 'Princess Mikasa', is a man-made epiphyte derived from the hybridization between the genera *Ascocentrum* and *Vanda* (Fig. 1). This flower is highly desirable due to the combination of traits expressed by the parents: large flower size from *Vanda*, which is considered as a promising progenitor for synthesizing a variety of cut flower hybrids and the interesting colour combinations from *Ascocentrum*, also considered as an important parent in the production of miniature *Vanda* hybrids. The hybrid plant possesses upright and narrow oviform leaves, with inflorescences

Corresponding Author

Sreeramanan Subramaniam, School of Biological Sciences, Universiti Sains Malaysia (USM), Minden Heights, 11800, Penang, Malaysia.;Tel:604-6533528 Fax:604-6565125
E-mail:sreeramanan@usm.my / sreeramanan@gmail.com

occuring twice or thrice a year. Just like the parents, this hybrid is able to proliferate in warm, sunny areas and in tropical climates, especially in Thailand, Philippines, India and Myanmar [36].

Germplasm preservation is important to safeguard biodiversity and to store elite plants, the latter important to develop and maintain new cultivars [7]. Important crops have been preserved for long periods through the establishment of either field or *in vitro* gene banks [17]. *In vitro* germination of orchids has been long established among orchid growers, and most orchid seeds are usually germinated immediately after harvest from the parent plant, or stored for later propagation [83]. The storage of seeds, a method of *ex situ* conservation, has always been a favourite among breeders, as this method is considered as one of the best ways to preserve valuable genetic resources [28], especially for species with limited reproductive capabilities. Orchid seeds are usually stored at low temperatures for this purpose [28].

Thus, the development of a long-term preservation method for embryos or protocorms is important in the conservation of the orchid germplasm, breeding programs, and the orchid floricultural industry [31]. Cryopreservation has been described as the most valuable method used for long-term germplasm conservation, as cryopreserved materials require very limited space, low maintenance, and are protected from contamination [66]. Cryopreservation arrests metabolic and biochemical processes of cells and causes energy to be unavailable for kinetic or dynamic reactions, hence halting normal cell division and growth [77,21]. Thus, the explant can be stored without any deterioration or modification for unlimited periods [1,38] as its genetic stability and regeneration potential is maintained [4,61,52]. Cryopreservation involves cryogenic (cryoprotectant and low temperature treatments) and non-cryogenic (pre- and post-storage culture) protocols. The success of the technique depends on germplasm tolerance and sensitivity to the stresses incurred and accumulated at each stage of the cryopreservation procedure. The basic cryopreservation protocol involves the application of cryoprotectants and treatments prior and subsequent to freezing, to protect and recover the germplasm material during and after storage in liquid nitrogen [85]. There must be minimized levels of crystallisable water within the plant material to ensure high recovery percentages after the cryostorage [85].

Three cryopreservation methods for orchids are available presently: desiccation (air-drying), vitrification, and encapsulation-dehydration [28]. Encapsulation techniques, either using alginate or agar, may be useful for this purpose as germplasm immobilization may aid in regeneration and the subsequent orchid conservation by protecting the developing embryo or the dividing tissue mass [42]. Encapsulation of vegetative propagules and subsequent retrieval of plantlets have been reported in several orchids [69,11,56,15,46] as well, showing that encapsulation is an agreeable method of conserving the plant germplasm in vitro, as reported in many endemic and endangered orchids such as *Geodorum densiflorum* [15,46]. Encapsulation-dehydration works through the extensive desiccation of plant tissues prior to freezing, and relies heavily on the best combination of techniques that most effectively minimize ice formation during freezing [17].

Encapsulation-dehydration involves the placement of explants in sodium-alginate beads, and the subsequent progressive or non-progressive dessication in the presence of high sucrose concentrations, which beneficially affect the germplasm tolerance to the air-drying and ice crystal growth during freezing [16]. The technique does not require the addition of toxic cryoprotectants such as dimethylsulphoxide (DMSO) and ethylene glycol (EG) [29], which are known to kill cryopreserved propagules during thawing. Alginate encapsulation protects the desiccated germplasm from mechanical and oxidative stresses during storage, analogous to an embryo sac, and allows easier handling of the plant material due to their small sizes, hence often superior to naked buds which are susceptible to fragility [58]. Alginate is commonly used for encapsulation purposes due to the polymer's inertness, non-toxicity, cheapness, easy manipulability, and its availability in large quantities [19].

Trials on the preservation and propagation of plants through encapsulation-dehydration have been promising. The ability to store encapsulated PLBs for long periods and at different temperatures will greatly enhance the efficiency of micropropagation by this system. The various parameters involved in the encapsulation of *Dendrobium* sonia, such as the stage of PLBs suitable for encapsulation, concentration of gelling agents, and nutrient concentration in the matrix has been standardized, with these conditions imposed on studies with three orchid genera: *Dendrobium, Oncidium*, and *Cattleya* [63]. There is great potential in investigating the use of encapsulation-dehydration as a technique of conserving endangered and commercially useful germplasm of orchids. More studies must be conducted on optimizing the techniques and propagules used in encapsulation-dehydration to provide the best possible outcome in plant regeneration experiments, especially those of orchids. Although currently riddled with uncertainty and setbacks, more studies in this particular cryopreservation method will beneficially affect how commercially viable and endangered species of

orchids are propagated in the future. The objectives of this present study are:

to determine the optimal size of protocorm like-bodies (PLBs) that gives the best results with the encapsulation-dehydration method, to test the suitability of various concentrations of sucrose and sorbitol for preculture in the encapsulation-dehydration of the PLBs, to observe the effects of encapsulation-dehydration and cryopreservation on the water contents of the PLBs.

Materials and methods

Plant Materials

The *in vitro*-grown protocorm like-bodies (PLBs) of *Ascocenda* 'Princess Mikasa' were used in this study. The entire research was conducted in two parts: Part I, to observe and choose the best-performing PLB size in cryopreservation involving both sucrose and sorbitol pretreatments; and Part II, to run the entire experiment using the chosen PLB size, followed by viability testing using the 2, 3, 5-triphenyltetrazolium chloride assay.

Preparation of culture media

All the MS [54] media and the necessary solutions that were required in the experiments were prepared in advance. The media prepared included half-strength liquid MS medium for subcultures, half-strength solid MS media not supplemented with sucrose or sorbitol as control, and half-strength solid MS media supplemented with sucrose or sorbitol for pretreatment of the PLBs using the following concentrations of 0.25 M, 0.50 M, 0.75 M, and 1.0 M. Half-strength solid MS medium containing 0.30 M sucrose was prepared for the growth recovery step, and half-strength liquid MS medium containing 3.0% sodium alginate, but devoid of hydrated calcium chloride ($CaCl_2.2H_2O$) for the encapsulation step. A separate solution of 0.1 M $CaCl_2.2H_2O$ was prepared as the polymerizing agent for encapsulation. None of the media prepared contained hormones, except for the subculturing media, which was required to encourage the division and proliferation of the PLBs. This was a precautionary step to avoid the treated PLBs from experiencing severe shock form cryopreservation. The pH values of all media were adjusted to 5.7–5.8 prior to autoclaving.

Excision and Pretreatment of the PLBs

The PLB clumps were aseptically teased apart and measured into $3mm^2$ and $6mm^2$ single PLBs using a millimetre grid graph paper placed under a sterile glass Petri plate. They were placed in plastic Petri plates containing half-strength MS solid media supplemented with sucrose or sorbitol according to their predetermined pretreatment concentrations using 0 M (control), 0.25 M, 0.50 M, 0.75 M, and 1.0 M. The excised PLBs were then pretreated by placing the plates under continuous light (Osram, 1600 ± 100 lux) for 18 hours.

Encapsulation of the pretreated PLBs

After the 18 hours pretreatment period, the PLBs were suspended in universal bottles containing half-strength liquid MS medium supplemented with 3.0% sodium alginate, but devoid of hydrated calcium chloride ($CaCl_2.2H_2O$). The PLBs were pipetted with 150µL of the alginate medium using a 1ml volume micropipette fitted with a tip having a modified diameter of six mm. The mixture was then dropped into 0.1 M $CaCl_2.2H_2O$ solution, and left to polymerize for 30 minutes, with occasional agitation. The beads were then rinsed with liquid half-strength MS medium devoid of sucrose, followed by a one-hour incubation period in liquid half-strength MS medium supplemented with 0.5 M sucrose.

Dehydration and cryostorage of the beads

The incubated beads were placed upon sterile filter papers in glass Petri dishes, and dehydrated under the sterile air flow of the laminar flow hood for 100 minutes. The dehydrated beads were then placed in sterile 2mL cryovials (Nalgene Cryowares). The vials were first placed in a Dewar flask for 2minutes, followed by storage in LN_2 for 24 hours.

Thawing and growth recovery

The cryovials were retrieved from the LN_2 tank after 24 hours and immediately thawed in a water bath at 40°C for 90 seconds. The thawed beads were then placed on hormone-free half-strength solid MS media supplemented with 0.3 M sucrose, and immediately incubated in the dark at room temperature (25°C). In Part I of the research, the beads were removed from the dark after 48 hours and placed under a 16 hour/8 hour photoperiod for 5days. On the other hand, the beads in Part II of the research were placed in the dark continuously for a week. Observations were made everyday on the colour of the PLBs encapsulated within the beads, with the final observation recorded prior to the 2, 3, 5-triphenyltetrazolium chloride (TTC) viability assay.

2,3, 5-Triphenyltetrazolium chloride (TTC) viability assay

In a TTC assay, cell survival is estimated by the amount of formazan produced from the reduction of TTC due to the action of dehydrogenases in living

cells or tissue [72]. In this research, the TTC assay was conducted according to the protocol described by Verleysen *et al*. [85]. After a week of incubation, the beads were sliced open to remove the PLBs from within. Each PLB in a replicate were weighed as a group, and then immersed in 1.5 ml of the TTC solution consisting of 0.18 M TTC buffered by 0.05 M KH_2PO_4. The PLBs were incubated in the dark for 15 hours at room temperature (25°C). Next, the TTC solution was drained off, followed by rinsing the PLBs thrice with distilled water, and placing them in 7 ml of 95% ethanol in test tubes. The test tubes were then boiled in a water bath at 100°C for 10 minutes. The extract obtained was cooled, and the intensity of the redness of the extract was measured with a spectrophotometer at 530 nm, using 95% ethanol as the blank.

Determination of the dry weight of the PLBs

After the TTC assay was conducted, the residual PLBs from the assay were rinsed thrice with distilled water and placed upon filter papers in glass Petri dishes according to their replicates. The uncovered plates were placed in an oven for 24 hours at 80°C. The dried PLBs were then cooled in a desiccator, and weighed. The PLBs were continuously weighed and replaced in the desiccator until a constant weight was observed [84].

Experimental Design

Each replicate consisted of 5 PLBs, with three replicates employed in Part I and 5replicates in Part II for each pretreatment concentration. One set of control, consisting of three replicates that had not been pretreated, was cryopreserved using the exact protocols employed for the other pretreated replicates in Part I of the research. In Part II, two sets of controls, each consisting of 5 replicates, were run throughout the entire cryopreservation protocol with the rest of the pretreated PLBs. 4 out of 5 PLBs were randomly selected for the TTC assay in Part II, with one PLB remaining in each replicate for observation. The data obtained were subjected to analysis of variance using SPSS, at the 0.05 probability level. Variation among treatments was analyzed using Tukey's test.

Results and discussion

Part I Experiment Results

The viability observations of the PLBs in Part I of the research, based on the colours observed after growth recovery, showed that there was no significant difference between the 3mm and 6mm PLBs in terms of the type of pretreatment and the concentrations employed, although the 6mm PLBs seemed to have higher viabilities (Fig. 2). However, there was a difference in the absorbance obtained for both the sizes when each size was compared as a whole as the 6mm PLBs were shown to have higher absorbance when compared to the 3mm PLBs. No significant differences were obtained between the sizes when they were compared according to their pretreatment concentrations (Fig. 3). Based on these observations, the 6mm PLBs were selected to continue Part II of the research. However, the first part of the research was plagued with high variances in the data obtained due to insufficient replicates, as only 3 replicates were employed for each treatment. Furthermore, it was evident that the absorbance obtained for the PLBs was a function of the size, the surface area and the amount of the tissue present in the sample, with 6mm PLBs giving higher absorbance when compared to the minute 3mm PLBs. Immediate browning (Fig. 4a) and bleaching (Fig. 4b) of the PLBs were also observed when the PLBs were exposed to light (1600±100 lux), usually occurring 24 hours after the exposure. Hence, based on these irregularities, it was decided that 5 replicates consisting of 5 PLBs each were to be used for each treatment in Part II of the research, and absorbance at 530 nm was to be recorded with respect to the dry weight of the tissue present in the sample. The PLBs were stored in the dark during the growth recovery phase in Part II of the research to prevent bleaching and browning of the PLBs.

Part II Experiment Results

Among all concentrations of sucrose tested, the highest absorbance per milligram of PLBs was recorded by 0.50 M sucrose, followed closely by 0.25 M sucrose (Fig. 5). The lowest absorbance value was recorded by 1.00 M sucrose. A statistically significant difference was found was among the 5 sucrose pretreatment concentrations. However, further experiment showed that there was no significant difference in using 0.25 M sucrose and 0.50 M sucrose in pretreating the PLBs, suggesting that both the concentrations yield similar viabilities in the pretreatment of the PLBs. There was no significant difference between the controls and 0.75 M sucrose in their capability as pretreatment agents as well, although being significantly lower in their prowess compared to both 0.25 M and 0.50 M sucrose. Pretreatment using 1.0 M sucrose yielded significantly lower results when compared to all the other sucrose concentrations (Fig. 4a).

Among the various sorbitol pretreatment concentrations, 0.25 M sorbitol recorded the highest absorbance at 0.123, followed closely by 0.50 M at 0.116 (Fig. 6). The lowest value, 0.077, was obtained from 1.00 M sorbitol. A statistically significant

difference was found among the 5 sorbitol pretreatment concentrations. All sorbitol concentrations used in the pretreatment recorded significantly different results from each other in their absorbance at 530 nm, except for the mean differences between 0.25 M with 0.50 M, and 0.50 M with 0.75 M respectively. Both groups of pretreatments yielded PLBs which remained green when stored continuously in the dark.

The measurement of the dry weights of the PLBs showed that the PLBs were composed almost entirely of water, constituting a mean of 98.8% of the total weight of each PLB. The final water contents of the PLBs were not significantly different when compared according to their pretreatment concentrations (Fig. 6), as both groups retained about 40% water, but were different when compared according to the type of pretreatment they underwent–either sucrose or sorbitol (Fig. 7). PLBs pretreated with sucrose generally lost more water than those treated with sorbitol. The results as a whole implied that both sucrose and sorbitol can be used as pretreatment prior to the encapsulation and cryostorage of the PLBs of *Ascocenda* 'Princess Mikasa', and that they both have their own mechanisms that ensured the effectiveness of the pretreatment.

Discussion

Effects of the Sizes of PLBs on Viability

Synthetic seed technology is currently considered an effective alternative for propagating commercially important agronomic and horticultural crops, such as seedless grape, seedless watermelon, seedless jack, seedless cucumber, corn, cotton, soybean, hybrid tomato, hybrid cereals, forage legumes, pines, potato and banana. [62,63]. Synthetic seeds of orchids are frequently produced by encapsulating protocorm like-bodies (PLBs) in an alginate matrix, serving as a low-cost, high-volume propagation system. Advantages of synthetic seeds over somatic embryos for propagation include ease of handling during storage and transportation, potential long-term storage without losing viability; and maintenance of the clonal nature of the resulting plants [25,63]. However, the viability of the selected explant or tissue must be taken into account prior to any encapsulation and cryostorage experiments, as the success or failure of the entire experiment depends on the tissue. There is always the threat of contamination and undesirable variations in explants obtained *in vivo*. Hence, in many experiments, plantlets raised in vitro were the source of explants for encapsulation [44], as conducted in this research.

The PLB is the earliest structure formed during embryo development in orchid seed germination, and is unique to orchids [30,46]. Proliferations of protocorms and protocorm like-bodies (PLB) are usually the only means of increasing the number of orchid species that do not germinate well or produce few seeds [55]. PLBs that have been subjected to subcultures, transfers, and cryostorage rarely display the characteristic green colour that indicate viability, but instead generate three different appearances: white or bleached, light yellow and brown [93]. However, shoots of the African Violet *(Saintpaulia ionantha* Wendl.) recovered from cryopreserved tissues of the plant were either pale green or yellow [52], with greenish appearance indicating a quicker regrowth and higher viability. The yellowish appearance, as well as the bleaching and browning conditions, could be attributed to osmotic shock or unfavorable regrowth conditions [74,52,93] reported that the light yellow calli of the orchid *Dendrobium candidum* Wall ex Lindl maintained their initial fast growth potential and bleached calli gradually turned moist while brown calli proliferated into lighter coloured tissues followed by browning. These tissues needed hormone supplementations to continue proliferation and development of healthy green tissues. All the cryostored PLBs recovered from this research were initially light green and remained light green when immediately recovered from the liquid nitrogen and incubated in the dark, but underwent either bleaching or browning within 24 hours of exposure to light, as observed in Part I of the research. No yellowing PLBs were observed. On the other hand, all the recovered PLBs in the second phase of the research maintained their light green colour when incubated in the dark continuously, with the remaining beads not subjected to the TTC assay remaining green up to 6 weeks after the growth recovery step. Hence, a higher degree of PLB viability can be obtained if the PLBs are incubated in the dark continuously. This observation has been cited by Shatnawi and Johnson [67] and Moges *et al*. [52] in the cryopreservation of the seeds of the 'Christmas bush' (*Ceratopetalum gummiferum*) and the shoot tips of the African violet (*Saintpaulia ionantha* Wendl.) respectively, with both stating that this step is essential to reduce shock to the cryopreserved plant tissue.

The size of tissues to be manipulated also play an important role in the survivability of the tissues in the subsequent steps of an experiment. Throughout the research, the 6mm PLBs had displayed better viability compared to the 3mm PLBs, irrespective of the type of pretreatment chemical applied. This implies that the 6mm PLBs are able to withstand the entire encapsulation-dehydration protocol better than the 3mm PLBs, and hence were selected to continue with the second part of the research. Generally, PLBs are selected for tissue culture manipulations when they are in the range of 3mm to 5mm, with protocorms of 3mm and 4mm shown to be suitable for optimum conversion of the encapsulated PLBs of *Cymbidium giganteum* Wall. PLBs smaller than this size range displayed poor conversion frequencies,

Fig. 1: Ascocenda 'Princess Mikasa' (Wikimedia, 2008)

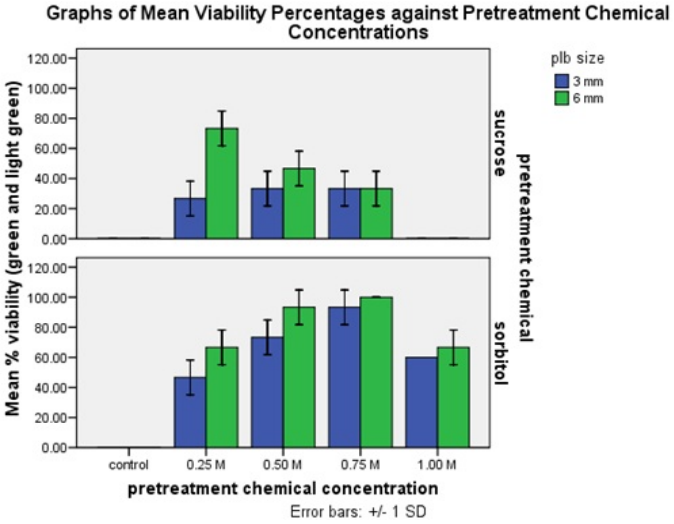

Fig. 2: Viability observations based on the colour of the PLBs encapsulated in the alginate matrix after being placed on growth recovery medium. Only green and light green PLBs were deemed as viable, based on their ability to grow on hormone-free media. Bleached, yellowing and brown PLBs were considered as not viable.

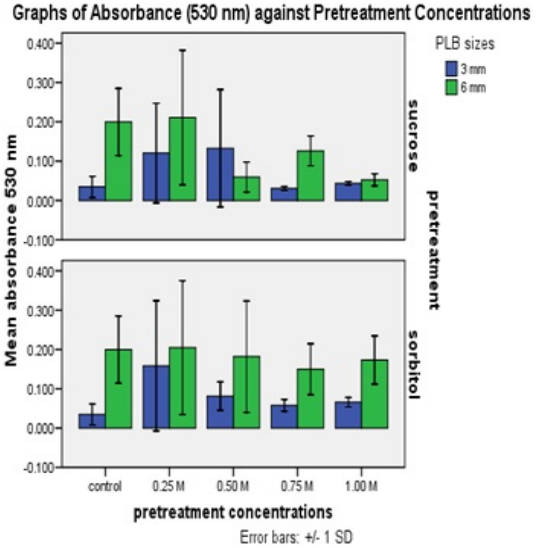

Fig. 3: Viability observations based on the absorbance of the PLBs subjected to the TTC assay at 530 nm. The data showed large variances for PLBs with higher absorbance and hence regarded as viable, while PLBs with low absorbance showed lower variances.

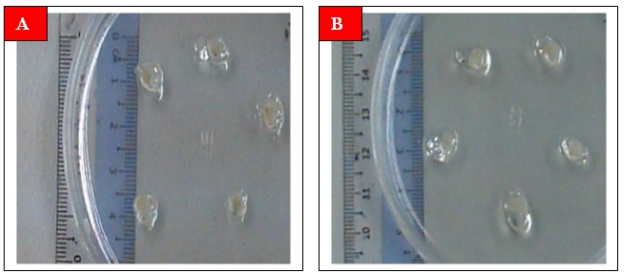

Fig. 4: (A) The encapsulated PLBs browning after exposure to light during the growth recovery step in Part I ; (B) The encapsulated PLBs bleaching after exposure to light during the growth recovery step in Part I.

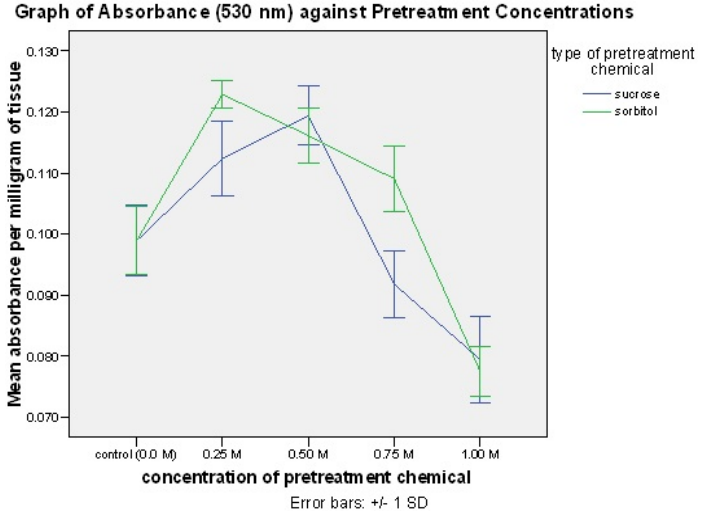

Fig. 5: The absorbance value per milligram of PLB against the pretreatment concentration for both sucrose and sorbitol. No significant difference was observed in the absorbance of the PLBs in the TTC assay when both sucrose and sorbitol were compared as a whole, hence suggesting that both the chemicals may possess similar efficacies in preserving the PLBs of the orchid hybrid Ascocenda 'Princess Mikasa'.

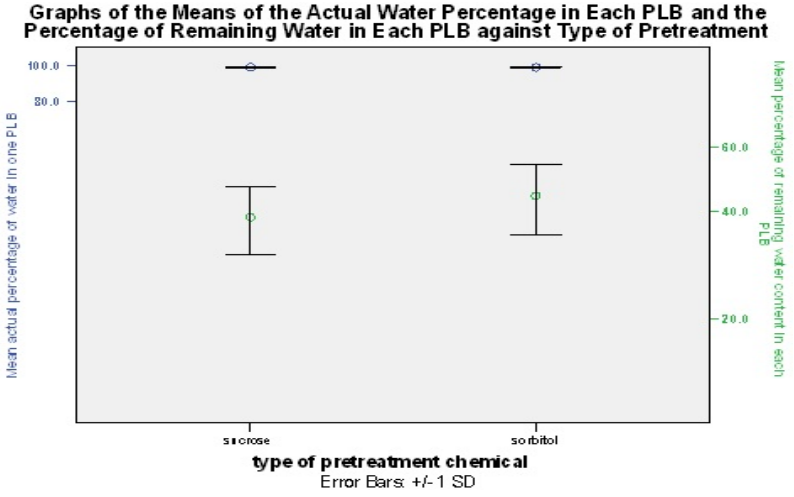

Fig. 6: The final water content in the PLBs after the entire cryopreservation protocol, prior to the TTC assay. No significant differences were found when the values were compared across the concentrations of sucrose or sorbitol employed, but a difference was observed when sucrose was compared to sorbitol as a whole, with sucrose pretreatment causing more water loss than sorbitol pretreatment.

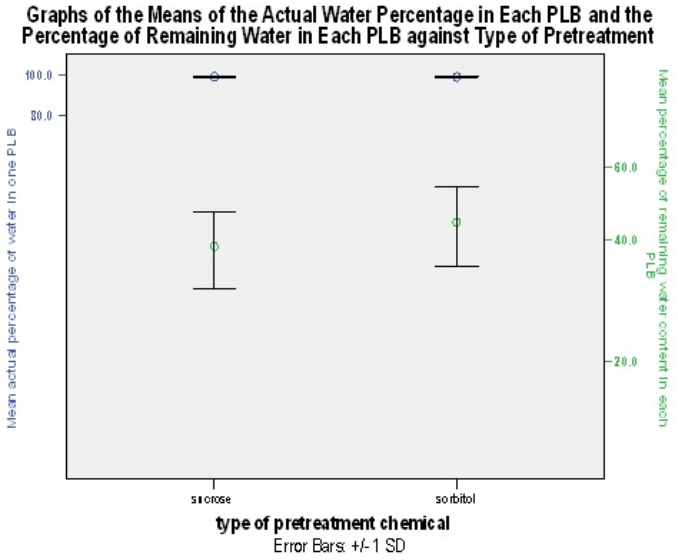

Fig. 7: The severity of water losses compared against the type of pretreatment, described as percentage of water weight. Although the graph implied that there was no difference in the amount of water lost in both type of pretreatments, the analysis of variance indicated otherwise.

possibly as a result of tissue immaturity, causing the inability of the PLBs to withstand encapsulation or lengthier emergence of the PLBs from the alginate capsule [13,63]. The success of a cryopreservation experiment may rely on the various stages of growth and age of plant tissues. For instance, the torpedo-stage embryos of *Medicago sativa* were shown to be suitable for encapsulation as the torpedo stage demonstrated a rapid increase in embryo mass and the deposition of majority of the storage reserves [49,63]. Similarly, younger or older somatic embryos failed to produce healthy plants before or after gel encapsulation in Geranium [25,63,]. selected PLBs at the leaf primordia stage for studies on the encapsulation of *Dendrobium* sonia, *Oncidium* 'Gower Ramsay', and *Cattleya leopoldii* as they discovered that PLBs at the leaf primordia stage gave earlier leaf and primary root formation than the pro-meristematic stage, and more complete germination than the first leaf stage PLBs, indicating that the developmental stage of PLBs used for encapsulation affected the germination percentage.

Effects of the Types and Concentrations of Pretreatment

In this research, 0.25 M sucrose, and both 0.25 M and 0.50 M sorbitol, the former producing higher absorbance than the other, had been proven to be effective as pretreatment in the encapsulation-dehydration of the PLBs of *Ascocenda* 'Princess Mikasa'. However, both the sugars might have been effective for different reasons entirely. Tokuhara and Mii [76] indicated that the morphogenetic responses of the *Doritaenopsis* PLB cells could be modified by the concentrations and the type of carbon sources applied in the media. Sucrose, when supplemented into a medium, is catabolized into the monosaccharides glucose and fructose by extracellular enzymes released during the *in vitro* culture, hence providing readily available nutrients for the explant [24,76]. A similar phenomenon may have occurred in the PLBs of the *Ascocenda* hybrid in this research during the pretreatment with sucrose, hence the optimal absorbance at 0.50 M sucrose, followed closely by 0.25 M sucrose. On the other hand, Hilae and Te-chato [27] discovered that sorbitol was a suitable osmoticum for shoot and root induction in oil palm as shoots and roots were formed simultaneously from somatic embryos of oil palm. This effect could be mimicry of the changes in osmolarity that occur in tissues surrounding the embryo within a real seed [50]. Hence, the impact of sorbitol in this study could have been the direct result of its osmotic potential [35]. However, they also reported that increasing concentrations of sucrose and sorbitol may heighten phenolic compound formation within the somatic embryos and promote leaf blight symptoms, similar to the effects of water stress. The oil palm plantlets died after being cultured on such medium for two to three months. This phenomenon may explain both the increased browning observed in PLBs pretreated under high concentrations of sucrose, and the low absorbance values obtained for PLBs placed under higher concentrations of sucrose and sorbitol pretreatments.

Sugar, when added in a culture medium, functions both as a carbon source and as an osmotic regulator of water stress. It has been reported that carbon sources such as glucose, fructose, mannitol and sorbitol play an important role in the germination of somatic embryos of asparagus [43] and cucumber

[41] further reported that a high concentration of sucrose (0.25 M or 0.50 M) could enhance germination of somatic embryos in cucumber. Osmoticum increased the water stress in palm oil somatic embryos, inducing shoots and/or root formation [27]. Osmotic adjustment is also a mechanism involved in drought tolerance. Sucrose, a disaccharide, is theoretically thought to functions as an osmoprotectant, by stabilising cellular membranes and maintaining turgor [53,80]. Islam and Ichihashi [32] had concluded that sucrose, a sugar easily metabolized by cells, was suitable for callus proliferation, while maltose and sorbitol, both not easily utilized by cells, were suitable for PLB proliferation and PLB growth respectively, based on the effects of the three carbohydrate sources on PLB formation and callus growth in *Phalaenopsis* embryogenic calli [76,33]. Higher concentrations of sucrose have also been demonstrated to be efficient in the morphogenesis of underground organs *in vitro* [73,65,68,57] and can be attributed to its nutritional effect . However, this particular phenomenon may also be partly caused by the low initial water potential of the medium, as the delay in the development of *Ipsea malabarica* bulbs in media containing low concentrations of sucrose was attributed to the high initial water potential of the media [47,91] theorized that high pretreatment concentrations, in their case, 0.75 M sucrose, allowed viability retention of encapsulated *Dactylorhiza fuchsii* seeds by reducing the bead drying rate, hence suggesting that rapid drying rates may hinder the survival of the orchid and its fungal symbiont. Higher concentrations of osmoticum were said to protect the plant from desiccation injury.

The Final Effect of Pretreatment and Dehydration on the Water Content of the PLBs

Although there were no significant differences in the final water content percentage of PLBs subjected to the various concentrations in the sucrose and sorbitol pretreatments, a significant difference was observed between the types of pretreatment applied as a whole, with sucrose-treated PLBs showing a greater water loss when compared to sorbitol-treated PLBs, both at 61.7% and 56.0% respectively. Jitsopakul *et al*. [34] had reported that the regrowth rate of non-cryopreserved and cryopreserved protocorms of *Vanda coerulea* depended on the water content of the precultured beads during dehydration, conducted from between zero to 10 hours. The group also reported that highest regrowth rate of the cryopreserved orchid beads, at 40%, was achieved by dehydrating the beads for eight hours, yielding a final water content of 35%. This result was almost similar to the results obtained from the sucrose-pretreated PLBs in this research (38.3%). The optimal water content of alginate beads is dependent to a large extent on the plant species, for instance, 33% for apple [58], 19% for Eucalyptus [59] and 20% to 25% for Citrus [26]. For azalea, shoots encapsulated in alginate beads with relatively high water content (38.6%) recorded 40% survival after cryopreservation [84].

Both sucrose and sorbitol act as osmoticum, drawing water out of the PLBs during pretreatment, but both may possess differing efficacies as a direct result of their chemical nature. The earlier mentioned enzymatic degradation of sucrose into glucose and fructose in a culture medium is known to increase the osmotic pressure in the medium, which in turn lowers the water potential of the medium, drawing more water out of the cultured explants [76]. This could be the reason behind the lower final water contents of the sucrose-pretreated PLBs when compared to the sorbitol-pretreated PLBs. Higher concentrations of sucrose may multiply this effect and hence promote excessive dehydration of the PLBs, incurring toxicity and extensive water stress on the PLBs and overriding the nutritional effects of the sugar [76]. Sorbitol, on the other hand, simply acts as an osmotic desiccator [27].

The control of water content of plant samples before freezing was the key factor in developing successful cryoprotection protocols [92]. When PLBs are not dehydrated sufficiently, freezing-injury can occur due to intracellular ice formation; on the other hand, when over-dehydrated, the osmotic stress can be damaging [5]. Hence, cells and tissues to be cryopreserved must be sufficiently desiccated in order to be vitrified before immersion into liquid nitrogen. The vitrification (glass formation) procedure for cryopreservation eliminates the controlled slow freezing step and allows cells to be cryopreserved by direct transfer into liquid nitrogen [22,39,64,5]. demonstrated that the application of exogenous ABA or dehydration caused accumulation of soluble sugars, followed by the accumulation of heat-stable proteins and dehydrin, a late-embryogenesis-abundant (LEA) protein in the PLBs of *Dendrobium candidum*, with the latter occurring at relatively low water contents (1.0g water/g DW). Soluble sugars are said to protect the cellular membrane through water replacement and to protect the cytoplasm by transit into a vitrified state [37]. The LEA proteins, a group of heat-stable proteins, are said to induce the ability to tolerate desiccation [23,3,18,6,8]. The dehydrin may achieve this by any of the three methods: the stabilization of the membrane [14]; *in vitro* cryoprotectant properties of the protein [40,90] or through inhibition of the coagulation of a range of macromolecules [12]. There were also theories that interactions between sugars and heat-stable proteins might play a role in improving the dehydration tolerance of plant cells. Oligosaccharides were found

to interact with LEAs to enhance the tolerance of developing soybean seeds [6]. Hence, with all these possibilities and evidence highlighted, there is a high chance of discovering similar molecules and pathways being expressed in the PLBs of Ascocenda 'Princess Mikasa' that are subjected to the pretreatment and dehydration steps, hence presenting opportunities for further research into this encapsulation-dehydration protocol.

The 2,3,5-Triphenyltetrazolium Chloride Assay

Successful tissue cryopreservation depends on the technique and type of protection employed against damage from ultra-low temperature [21,51]. Tissue regrowth in recovery medium, although very sensitive in assessing cellular viability, is time-consuming. Hence, various staining methods such as the 2,3,5-triphenyltetrazolium chloride (TTC) test and vital staining with fluorescein diacetate (FDA) [88] are frequently employed to determine the viability of cells subjected to various stress factors such as cold, salinity and heat [86,31]. As observed in this research, assessing stress parameters for small explants used in cryopreservation can be a difficult affair. TTC staining is a reference method of the International Seed Testing Organisation (ISTA) for testing seed viability and can be used to test biochemical activity of plant tissues after cold treatment [82,85]. The TTC assay, suitable for large cell aggregates [31,71,86,87], is based on the enzymatic respiration of living plant cells, and indicate respiration levels in samples tested [86]. Active dehydrogenases in mitochondria reduce colorless TTC to red triphenylformazan [72,78]. Hence, living portions of tissue or single cells stain red. Although widely used as a fast and inexpensive test, there have been problems associated with its use as an indicator of post-thaw viability [60]. Abnormal inorganic reactions may interfere and intensify formazan production from TTC [51,86] pointed out that latent damage, which can destabilize the cell reaction, may be imperceptible shortly after thawing and suggested waiting for at least overnight after the tissue is thawed prior to the test. In this research, the thawed PLBs were allowed a recovery period of 48 hours under darkness, followed by another 48 hours of 16 hours/8 hours photoperiod in Part I and for a week under total darkness in Part II, to prevent such damages from occurring in the encapsulated PLBs.

Verleysen *et al*. [85] had demonstrated that viable tissues had relatively higher absorbance values when compared to non-viable tissues, when the absorbance of the TTC-treated azalea nodal segments was read at 490 nm. They also observed that the standard errors recorded for both type of tissues displayed similar traits: viable tissues had higher standard error values, while highly stressed tissue had lower standard error values. This exact observation was made in Part I of the research: lower sucrose and sorbitol pretreatment concentrations displaying high absorbance values had higher standard deviations, unlike higher pretreatment concentrations which displayed lower standard deviations. The group forwarded two explanations for this phenomenon: firstly, reducing components present in dead cells could reduce TTC. A second, more plausible explanation involves the metabolic activity of the tissues. Viable tissues may possess inconsistent metabolic activities due to a number of factors such as the age and stage of growth of the tissues, and number of viable cells remaining in the tissue that could contribute to the metabolic activity. Frozen or non-viable tissues, which are less metabolically active, will reduce less TTC, hence resulting in a lower standard error. Hence, the inconsistent absorbance values obtained in Part I of the research, involving the three mm and the six mm PLBs, could have been attributed to the factors mentioned above.

Advantages and disadvantages of Encapsulation-Dehydration

The encapsulation protocol employed in this research, as previously conducted by our lab member previously (unrecorded data) was found to be satisfactory as the beads containing the PLBs could be handled with ease and seemed to have shown the ability to protect the explants encapsulated within. In fact, some of the single PLBs left behind in each replicate were observed to have been proliferating, easily breaking through the alginate shell, three weeks after growth recovery. An optimal ion exchange between the sodium and calcium ions was achieved using 3% sodium alginate supplemented in half-strength MS medium and 0.1 M hydrated calcium chloride as the complexing agent, producing firm and clear isodiametric beads. Higher concentrations of sodium alginate (4–5%) was found to inhibit the conversion of encapsulated shoot tips of *Phyllanthus amarus*, a medicinally important plant, while lower concentrations (1–2%) caused the formation of unmanageable and fragile beads by prolonging the polymerization period [70]. The MS medium-supplemented alginate matrix served as artificial endosperm by providing nutrients to the encapsulated explants for plant regrowth [2,9] discovered that the addition of 1/2-MS nutrients in the gelling matrix of *Carica papaya*, as conducted in this research, enhanced its germination and conversion frequency [70]. The alginate coating helped in preventing the detrimental environmental effects on the encapsulated plant material [79,52,48]. demonstrated that encapsulated plant apices were able to withstand drastic treatments such as preculture with high sucrose concentrations and desiccation, naturally harmful to naked apices [52]. Formation of

intracellular ice crystals during freezing and/or thawing was also shown to be detrimental for viability, and was reduced by alginate encapsulation [81,52]. However, encapsulation have been said to increase the lag period in the germination of the Dactylorhiza fuchsia seed and the fungal symbiont. One possible explanation for this phenomenon is that the beads imposed a mechanical resistance to growth, with forces of up to 5 N cm^{-2} required to rupture the alginate beads [91]. These negative effects were not observed in this research. In fact, the PLBs within the beads seemed to be able to survive all the treatment applied prior to and after cryopreservation, making the protocols applied in this research feasible for further development and optimization for the cryopreservation of the orchid hybrid, *Ascocenda* 'Princess Mikasa'

Conclusions

This study has shown that an optimal encapsulation-dehydration protocol for the orchid hybrid *Ascocenda* 'Princess Mikasa' can be achieved using six mm PLB pretreated in either 0.50 M sucrose or 0.25 M sorbitol for 18 hours, followed by encapsulation in 3.0% sodium alginate supplemented in hormone-free half-strength MS medium. The PLBs, encapsulated and hardened in 0.1 M $CaCl_2.2H_2O$, dehydrated for 100 minutes under the laminar flow hood and cryopreserved for 24 hours before growth recovery, lost a mean 60.0% of their original water content regardless of the type of pretreatment and concentrations applied. The size of the PLB selected, the type and concentration of the pretreatment applied in the protocol, as well as the final water content in the PLBs after the end of the entire treatment had proven to be greatly influential in determining the success of the encapsulation-dehydration protocol for this orchid hybrid. In order to ensure greater success of this protocol, a more accurate viability test can be applied at each stage of the protocol to assess the effect of the particular stage on the PLBs. Controls that do not undergo cryopreservation can be evaluated to measure the effectiveness of the protocols in preserving the viability of the PLBs. The recovery of the PLBs can be assessed using different media or hormones. Finally, molecular studies can be performed to discover what genes are transcribed in the orchid during cryopreservation, and to observe interactions between proteins expressed during the cryopreservation.

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