Abstract: Ascocenda Wangsa Gold is a new and fascinating orchid hybrid in the Malaysian flower industry. An efficient plant vitrification solution 2 (PVS2) cryopreservation technique was developed for protocorm-like bodies (PLBs) of Ascocenda Wangsa Gold orchid. Parameters assessed included the effect of PVS2 exposure periods, thawing duration, temperature, and culture conditions based on 2,3,5-triphenyltetrazolium chloride absorbance readings and regrowth rates. A regrowth rate of 33.3% was obtained after 2 months when the PLBs were dehydrated in PVS2 for 30 min. The growth rate was improved to 47% when thawing was conducted at 45 °C for 85 s. The highest growth rate (53.3%) was obtained when the PLBs were subjected to a 7-day dark treatment before being transferred to a 16-h/8-h light/dark photoperiod. Histological analyses were conducted to study the morphology of cryopreserved and noncryopreserved PLBs of Ascocenda Wangsa Gold. The vitrification protocol developed in this study is a feasible and safe method for strengthening the germplasm conservation of this orchid for commercial purposes.

Key words: Plant vitrification solution 2, liquid nitrogen, 2,3,5-triphenyltetrazolium chloride assay, Ascocenda Wangsa Gold, histology

1. Introduction
Orchids are among the most diversified of the angiosperm families, with over 800 described genera, 25,000–30,000 species, and more than 150,000 registered artificial hybrids (Yue et al., 2006). Their unique floral characteristics place the orchid species in a different category from other flowering plants. Large-scale multiplication of species and hybrids using in vitro plant micropropagation techniques has positioned orchids among the top 10 cut and potted flowering plants in the world (Chugh et al., 2009). Many orchid species, however, are in danger of extinction due to extensive disturbances of their natural habitat and indiscriminate harvesting of naturally occurring plants. Thus, the conservation of orchids is of vital importance. To that end, frequent maintenance is required through tissue culture techniques.

However, orchid plants under in vitro micropropagation or regular subculture are at an increased risk of tissue loss due to microbial contamination, adverse culture conditions, and somaclonal variation (Mohanty et al., 2012). Cryogenic storage offers a stable and secure means of long-term storage while protecting against genetic erosion (Engelmann, 2011). Ascocenda Wangsa Gold is a hybrid between Vanda Ram Indra Gold and Ascocentrum Rasri Gold. It is valuable in the orchid floriculture industry due to its unique flower structure and can be found in Thailand, Malaysia, Myanmar, India, and the Philippines. Several studies have reported on cryopreservation of different PLBs (protocorm-like bodies): Dendrobium Walter Oumae (Lurswijidjarus and Thammasiri, 2004), Bletilla striata (Hirano et al., 2005), Geodorum densiflorum (Luo et al., 2008), and Ascocenda Princess Mikasa (Ranjetta et al., 2009). A PLB is a structure that has the capability of easily developing into a whole plant (Ishikawa et al., 1997). Cryopreservation of shoot tips, seeds, protocorm, and PLBs of many orchids has been successfully used for short- and long-term conservation. It is a promising strategy for providing long-term germplasm conservation of plant tissues, living cells, and tissue organs via storage at the ultralow temperature of liquid nitrogen (LN) (−196 °C). Under cryogenic storage, all metabolic activities and cellular division of cryopreserved cells are halted, and the cells will not undergo genetic changes during storage (Gonzalez-Arnao et al., 2008). Factors influencing the
effectiveness of plant cryopreservation include cell type, size, phase and rate of growth, cell incubation temperature, medium density and pH, cooling rate, storage temperature and duration, thawing temperature, plant recovery, and growth medium. Dehydration tolerance and prevention of injury from chemical toxicity are major contributing factors to successful cryopreservation (Vendrame et al., 2007).

Additional advantages of plant cryopreservation as compared with conventional storage approaches include genotypic and phenotypic character stability, minimal storage space requirements, and minimal maintenance (Sakai et al., 2000; Gonzalez-Arnao et al., 2008). Cryopreservation is the only technique that ensures safe and cost-efficient long-term conservation of various categories of plants, including nonorthodox seed species, vegetatively propagated plants, and rare or endangered species.

There are 3 types of cryopreservation techniques currently in use: vitrification, droplet-freezing, and encapsulation-dehydration. They differ based on their physical mechanisms. Vitrification is the most common technique and capitalizes on the use of plant vitrification solutions such as plant vitrification solution 2 (PVS2) and PVS3, which act as cryoprotectants to the plant cells prior to liquid nitrogen exposure. Cryoprotectants such as sucrose, sorbitol, ethylene glycol, trehalose, and glycerol are used as vitrifying agents in order to protect plant cells from injuries due to freezing. For successful cryopreservation, lethal intracellular freezing has to be avoided during rapid cooling in LN. During vitrification, the highly concentrated aqueous solution solidifies into a noncrystal and glassy state structure after tissues are plunged into liquid nitrogen. Subsequently, ice crystal nucleation is impeded and the vitrified cells are free from the danger of intracellular freezing (Reed and Uchendu, 2008). To date, there are no studies reported on cryopreservation of PLBs of *Ascocenda* Wangsa Gold using a PVS2 vitrification technique. Therefore, the aim of this study was to develop a simple and efficient cryopreservation based on the vitrification approach for long-term storage of *Ascocenda* Wangsa Gold orchid plant using PLBs.

2. Materials and methods

2.1. Plant material and culture conditions

PLBs of *Ascocenda* Wangsa Gold induced from in vitro protocorm cultures were obtained from a commercial orchid nursery, and the PLBs were used as explant tissue to establish cultures and form a clump tissue in this experiment. Protocorm-like bodies were induced in Vacin and Went (VW) medium (Vacin and Went, 1949) supplemented with 2% sucrose, 150 mL of coconut water, and 20 g/L homogenized banana and potato. Cultures were proliferated for 3 months in order to obtain a sufficient number of PLBs in the study (Figure 1a). The pH of the medium was adjusted to 4.8 before sterilization by autoclaving for 15 min at 120 °C. The cultures were incubated at 25 ± 2 °C under a 16-h photoperiod using cool white fluorescent lamps (Philips TLD, 36 W, 150 µmol m⁻² s⁻¹). Uniformly sized PLBs measuring 3–4 mm (Figure 1b) in diameter were used.

2.2. Preculture treatment

The cryopreservation of PLBs was conducted according to the method of Sakai et al. (1990), with slight modifications. PLBs (4-week-old culture) were excised at 3–4 mm in diameter and pretreated in VW semisolid medium supplemented with 0.75 M sucrose, 150 mL of coconut water, and 20 g/L banana and potato at 25 °C for 24 h under a 16-h photoperiod.

2.3. Treatment of PLBs with PVS2 and LN storage

Following preculture treatment, the PLBs were dehydrated with 1.5 mL of loading solution (2 M glycerol supplemented with 0.4 M sucrose in VW medium) in 2-mL cryotubes at 25 °C for 20 min. After 20 min, the PLBs were dehydrated in 1.5 mL of PVS2 containing 30% (w/v) glycerol, 15% (w/v) ethylene glycol, and 15% (w/v) dimethyl sulfoxide (DMSO) supplemented with 0.4 M sucrose in VW medium (Sakai et al., 1990) at 0 °C for 0 min, 10 min, 20 min, 30 min, 40 min, and 50 min. Finally, the PLBs were dehydrated in 1.5 mL of fresh ice-cold PVS2 and directly plunged into liquid nitrogen for at least 1 h.

2.4. Treatment of PLBs with thawing temperature and duration

Cryotubes stored in LN were removed from the tank and rapidly rewarmed in a water bath at 25, 30, 35, 40, 45, and 50 °C for 80, 85, 90, 95, 100, and 105 s before being rinsed with an unloading solution supplemented with VW medium and 1.2 M sucrose for 20 min.

2.5. Treatment of PLBs under dark culture condition during growth recovery

Both cryopreserved and noncryopreserved PLBs were transferred onto a layer of sterilized filter paper over VW medium supplemented with 2% sucrose after the unloading treatment. The PLBs were placed under 3 different photoperiod conditions: complete darkness (0, 7, and 10 days), exposure to dimmed lighting (3.4 µmol m⁻² s⁻¹), and a 16-h photoperiod using cool white fluorescent lamps (Philips TLD, 36 W, 150 µmol m⁻² s⁻¹), with all incubations conducted at 25 ± 2 °C.

2.6. Tissue preparation for histology

Histological studies were conducted on cryopreserved and noncryopreserved PLBs 2 months after thawing. The PLB samples were fixed in formalin–acetic acid–alcohol at a ratio of 10:1:2:7 (95% ethyl alcohol/glacial acetic acid/formaldehyde/water) for 1 week. The PLBs were washed...
under running tap water for 24 h after the fixation process and then dehydrated with a graded ethanol series. During the wax absorption step, the PLBs were immersed in a mixture of xylene substitute and wax for 6 h and then transferred to a pure wax solution and left overnight. The next day, the PLBs were treated twice with a new wax solution and left overnight. Following blocking, the blocks were cut into thin slices using a microtome (6 Micron Microtome Leica RM 2135) and the slices were placed on clean glass slides. These slides were then stained and viewed under a light microscope.

### 2.7. 2,3,5-Triphenyltetrazolium chloride assay

After 3 weeks of recovery, the survival of noncryopreserved and cryopreserved PLBs was assessed based on growth observations and a viability assay via 2,3,5-triphenyltetrazolium chloride (TTC) spectrophotometry analysis at 490 nm (Verleysen et al., 2004). The viability of PLBs was determined by the reduction of TTC to formazan as a result of dehydrogenase activity in cells and tissues (Figure 1c). The treated PLBs were immersed in 2 mL of TTC buffer under completely dark conditions for 18–24 h at 30 °C. After incubation, the TTC solution was discarded and the cells were washed with distilled water 3 times. The formazan color complex was extracted using 7 mL of 95% (v/v) ethanol in each tube for 20 min at room temperature and then 80 °C in a water bath for 5 min. The extract was cooled at room temperature and the volume was adjusted to 10 mL with 95% (v/v) ethanol. The absorbance of the extract was measured by using a spectrophotometer (UVmini-1240 UV-VIS spectrophotometer) at 490 nm with 95% ethanol used as the blank.

### 2.8. Assessment of PLB survival and plant regeneration

Percentage of regrowth was recorded after 30 days of postcryopreservation treatment on the basis of the number of PLBs showing signs of life. The PLBs were considered to have survived if they remained green, started to divide, and showed signs of growth; brown PLBs were considered dead.

![Figure 1. a) Initial PLB stock culture before isolation; b) isolated PLB, 3–4 mm in diameter; c) TTC-stained PLBs. Bars = 1 cm.](image-url)
2.9. Experimental design and statistical analysis
Each experiment was repeated 3 times and the experiment consisted of 6 replicates containing 10 PLBs. All data were subjected to an independent sample t-test and one-way analysis of variance (ANOVA). Means were compared using Tukey’s test. The probability value of both tests was set at P ≤ 0.05 and SPSS was used.

3. Results
3.1. Treatment of PLBs with PVS2 and LN storage
To determine the optimal time of exposure to PVS2, PLBs were treated with PVS2 solution for 0 to 50 min at 0 °C prior to LN exposure. In the absence of PVS2 dehydration treatment (0 min) at 0 °C, cryopreserved PLBs had very low viability. However, PLBs precultured in 0.75 M sucrose for 1 day showed the highest viability when exposed to PVS2 solution at 0 °C for 40 min (Figure 2). The PLBs showed considerable improvement in regrowth (33.3%) when dehydrated with PVS2 for 30 min (Figure 2). The PLBs lost their green color within 3 days after the thawing treatment. New green PLBs formed on the surface of cryopreserved PLBs after 2 months of regrowth (Figure 3). The cryopreserved PLBs were clearly red-colored using the TTC assay method. The best TTC absorbance reading was obtained at 30 min of PVS2 exposure time.

3.2. Treatment of PLBs with thawing temperature and duration
Based on the TTC absorbance, the PLBs produced the lowest and highest viability when thawed at 25 °C and 45 °C, respectively. At 30 °C, 35 °C, 40 °C, and 50 °C, regrowth rates were not significantly different (Figure 4). The viability of the PLBs was the highest (47%) when thawing was conducted at 45 °C for 85 s (Figures 4–6).

3.3. Treatment of PLBs under dark culture conditions during growth recovery
In this experiment, the explants were cultured in recovery medium for 0 days, 7 days, and 10 days following exposure to the unloading solution. The highest regrowth (53.3%) of cryopreserved PLBs was obtained when they were cultured in the dark for 7 days, followed by continuous light exposure (16-h photoperiod) thereafter (Figure 7). Regrowth of cryopreserved PLBs was significantly reduced to 16% when they were cultured for 10 days under dark conditions.

3.4. Histological analysis
In the current study, histology was carried out to study cellular damage and plasmolysis on both cryopreserved...
and noncryopreserved PLBs of *Ascocenda Wangsa Gold*. The parenchymatous cells in cryopreserved PLBs were physically damaged (Figure 8). Even though the cryopreserved tissues could not preserve their cross-section of the original layered structure after thawing, the viable cells were still able to enhance the recovery of the plantlets. The nuclei within the cryopreserved meristematic cells were large and darkly stained (Figure 8), suggesting that the cells were still actively dividing following the cryopreservation treatment. The amount of water content in the tissues needs to be minimized in order to obtain a higher number of viable cells after cryopreservation treatment. The packed cell volume is higher in cryopreserved PLBs compared with noncryopreserved PLBs (Figure 9). In the present study, the noncryopreserved PLBs showed more compactness of cells and their recovery process was faster than that of the cryopreserved PLBs. This is because the noncryopreserved PLBs were not exposed to LN. However, some parts of the noncryopreserved PLBs appeared damaged due to the exposure of PVS2 treatment. PVS2 consists of glycerol, ethylene glycol, and DMSO, which could be detrimental to plant tissue.

4. Discussion
The optimization of each step of the vitrification protocol and the duration of exposure is crucial in order to obtain a high percentage of plant regeneration. In this experiment,
the effects of PVS2 exposure time, thawing temperature, and growth recovery culture conditions were evaluated based on TTC analysis and percentage of growth recovery. Vitrification prevents ice formation in cells through the use of highly concentrated cryoprotectants that increase the viscosity in target cells up to a point where glass formation is induced and water crystallization is avoided (Hong and Yin, 2009). PVS2 contained a mixture of cryoprotectants consisting of DMSO, ethylene glycol, and glycerol.

The key parameters for successful cryopreservation depend upon the freezing and dehydration tolerance and the prevention of injury from chemical toxicity during treatment with PVS2 solutions. Optimum dehydration of cultures is required to reduce the injurious effects of overexposure to PVS2 and to enhance their ability to be vitrified upon rapid cooling into LN (Toshikazu and Akira, 2003). In our study, an optimized PVS2 vitrification protocol was developed for cryopreservation of *Ascocenda* Wangsa Gold PLBs, whereby the PLBs were subjected to 0.75 M sucrose for 24 h and dehydrated with PVS2 solution at 0 °C for 40 min. Under these conditions, 33.3% regrowth was obtained for cryopreserved PLBs. Prolonged PVS2 exposure (40 min) led to excessive dehydration, resulting in cell death. Therefore, balancing the water content is crucial to minimize desiccation damage and freezing injury (Volk and Walters, 2006). Regrowth of *Dendrobium nobile* improved to 80% following thawing at 38 ± 2 °C for 2 min (Mohanty et al., 2012), while regrowth of *Anemarrhena asphodeloides* Bunge was observed at 60% after thawing was conducted at 35 °C for 5 min (Hong and Yin, 2012). Fang et al. (2008) reported 70%–80% survival of citrus shoot tips by thawing them in a 40 °C water bath for 90 s.

Devitrification is the formation of damaging ice crystals from a previously vitrified solution upon rewarming. Thawing temperature and duration are the main factors significantly influencing the regrowth rate of cryopreserved plantlets. The transition of the vitrification solution from a vitreous to a crystalline phase during thawing could cause damage to the material (Hong and Yin, 2009). Thawing duration is thought to be one factor that could help avoid recrystallization and ensure the satisfactory recovery of vitrified material. Helenius et al. (2004) reported that high thawing temperatures (>15 °C) would thaw frozen root plugs rapidly and could result in the depletion of carbohydrates. In our study, regrowth was obtained at 47% when the PLBs were thawed at 45 °C for 85 s after 4 weeks of recovery.

The culture conditions had a significant influence on the regrowth rates of the cryopreserved PLBs. The highest regrowth of cryopreserved PLB (53.3%) was obtained under continuous light (16-h photoperiod) with a prior period of 7 days of darkness. Previous studies on culture conditions have indicated that postcryopreservation conditions affect the regeneration of plantlets. In particular, short periods in the dark after thawing allowed significant improvements in regrowth, presumably as a result of damage repair in the dark (Hong and Yin, 2009). Withers et al. (1988) suggested that cryopreserved in vitro plantlets should be maintained under dark conditions or under minimal light to reduce potentially harmful photooxidative effects.

It is particularly important that cryopreserved cells are capable of producing plants identical to the nontreated phenotype (Hong and Yin, 2009). In the present study, the TTC staining allowed us to make a prognosis of the viability of the explants. However, the rate of regrowth following cryopreservation treatment can be a complex process (Haskins and Kartha, 1980). We were able to
obtain a high regrowth rate of about 53.3%, but in most studies, the regrowth of explants can be poor and slow due to nonoptimized protocols or regeneration conditions such as culture medium and culture room (Touchell et al., 1992). Therefore, optimized protocols are required to achieve successful cryopreservation.

Various forms of stress imposed on plant tissues may potentially contribute to changes in the genomic content of explants (Harding et al., 2004; Martín and González-Benito, 2005). PLBs, having higher capabilities to regenerate into plantlets, are the preferred target tissues in cryopreservation due to the large number of PLBs that can be obtained within a relatively short period of time (Sreeramanan et al., 2008; Anthony et al., 2011). PLBs serve as models for somatic embryos in orchids (Hossain et al., 2013; Teixeira, 2013a). When PLBs are encapsulated, the resulting synthetic seeds (Sharma et al., 2013; Teixeira, 2013b) can be used for cryopreservation (Teixeira, 2012). PLBs are versatile orchid organs that can be induced from various orchid explants such as axillary buds, flower stalks, cell suspension, and callus cultures in the case of Doritaenopsis (Tsukazaki et al., 2000).

Histological observations revealed the accumulation of a homogeneous cell population in cryopreserved explants as compared with noncryopreserved explants. George et al. (2009) reported that the regeneration properties of Picea abies and Vitis vinifera increased following cryostorage. This could be due to the cellular selection process caused by the cryopreservation treatment. Microscopic observations provide a valuable insight into the degree of plasmolysis that occurs in cells. In the current experiment, the slow recovery of PLBs after cryostorage indicated that the survival of cryopreserved PLBs may have been compromised by DNA damage or by damage to the parenchymatous cells due to a possible reliance of the embryogenic cells on those cells for nutrition or metabolism (Ranjetta et al., 2013).

Acknowledgments
The authors are grateful to RU Grant USM 2011 for funding this project, to the Agricultural Crop Trust scholarship for financial support, and to MyBrain15 from the Ministry of Higher Education.

References


