Adding Ascorbic Acid to Reduce Oxidative Stress during Cryopreservation of Somatic Embryos of *Paphiopedilum niveum* (Rchb.f.) Stein, an Endangered Orchid Species

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**Paphiopedilum niveum** (Rchb.f.) Stein, an endangered species, has been listed in CITES Appendix I and its germplasm conservation is required. To improve the regeneration of cryopreserved somatic embryos (SEs), adding 0.1 mM ascorbic acid (AsA) at a critical step during cryopreservation was investigated. The reactive oxygen species (ROS) and malondialdehyde (MDA) contents were also assessed during five steps (preconditioning, 1st preculture, 2nd preculture, osmoprotection, and dehydration) of a developed V cryo-plate technique as described briefly. Two-month-old SEs were preconditioned on modified Vacin and Went medium (MVW) containing 0.1 M sucrose for seven days. These SEs were precultured on MVW containing 0.2 M sucrose for one day (1st preculture) before being transferred to the same medium with 0.6 M sucrose for one day (2nd preculture). Precultured SEs were embedded on a cryo-plate, incubated in loading solution (LS) with 1.2 M sucrose for 30 min at 25°C and dehydrated with plant vitrification solution 2 (PVS2) for 60 min at 25°C. It was found that applying AsA on day 7 after culture (before the 1st preculture) could reduce total ROS and MDA levels, leading to a high regeneration percentage (39%) of cryopreserved *P. niveum* SEs.

**Key Words:** antioxidants, malondialdehyde, reactive oxygen species, V cryo-plate method, vitamin C.

**Introduction**

*Paphiopedilum niveum* (Rchb.f.) Stein, an endangered species protected by CITES Appendix I, is endemic to the Peninsula Malaysia (Pedersen et al., 2011). The conservation status of this species is a serious concern because of the declining wild population from human interference and climate change (Seaton et al., 2010). Thus, cryopreservation is an appropriate method to conserve this endangered species. The V cryo-plate method was developed from the vitrification method using an aluminium cryo-plate (Yamamoto et al., 2011). This technique is carried out using treatment of a loading solution (LS) and a plant vitrification solution 2 (PVS2) without damaging plant materials ( Sekizawa et al., 2011) because the cryo-plate can facilitate rapid and uniform thermal exchange (Yamamoto et al., 2012b). The successful high regrowth percentage of cryopreserved explants using the V cryo-plate method was confirmed in carnation (*Dianthus caryophyllus*) (93–97%) ( Sekizawa et al., 2011), mulberry (*Morus spp.*) (73–97%) (Yamamoto et al., 2012c), and potato (*Solanum tuberosum*) (93.3–100%) (Yamamoto et al., 2012a).

Generally, osmotic dehydration and freezing stresses can induce high reactive oxygen species (ROS) production during cryopreservation (Chen et al., 2015). The excessive ROS synthesis causes cellular damage to plant cells leading to cell death (Mittler, 2002). The production of antioxidants, known to reduce the oxidative stress in plant cells, is a physiological response against toxic ROS (Bhattacharjee, 2010). Moreover, important antioxidant enzymes including catalase (CAT) and ascorbate peroxidase (APX) could enhance the chilling tolerance during cryopreservation (Kuk et al., 2003).

Recently, the benefit of antioxidant application, especially ascorbic acid (AsA), during the cryopreservation procedure was confirmed in many plant species. Chua and Normah (2011) reported that the addition of AsA (0.28 mM) in LS could improve the survival of cryopreserved shoot tips of *Nephelium ramboutan-ake*. The
AsA application was firstly reported to improve the regrowth of cryopreserved shoot tips of *Rubus hybridra* ‘Chehalem’ and *R. hybridra* ‘Hull Thornless’ (Uchendu et al., 2010). It was found that adding AsA (at 0.14–0.58 mM) at any time during cryopreservation (pretreatment, loading (osmoprotection), and rinsing or regrowth) could reduce lipid peroxidation as indicated by the decrease in malondialdehyde (MDA) content. Thus, the total ROS and MDA content could be used to evaluate the level of oxidative stress induced by the cryopreservation process. This useful information also indicated that filling AsA in the correct step of the cryopreservation protocol could prevent oxidative damage in recalcitrant tropical plant species. The present study aimed to determine whether applying AsA at the precondition step (the step before preculture) of the developed V cryo-plate procedure could improve the regeneration percentage of cryopreserved somatic embryos (SEs) of *P. niveum*. The total ROS and MDA content during each cryopreservation step were also examined.

**Materials and Methods**

**Culture medium**

The modified Vacin and Went medium (MVW) consisted of full-strength VW macronutrient (Vacin and Went, 1949) and half-strength MS micronutrient (Murashige and Skoog, 1962), full-strength MS vitamin, 100 mg L$^{-1}$ myo-inositol, 0.2% (w/v) peptone, 2% (w/v) sucrose, 5 mg L$^{-1}$ chitosan, 0.2% activated charcoal (AC), and 0.3% (w/v) phytagel. The pH of the medium was adjusted to 5.3–5.4 with 1 N NaOH or HCl prior to autoclaving at 121°C under 15 psi pressure for 20 min.

**Plant material preparation and SE induction**

Five-month-old capsules resulting from hand self-pollinated flowers of nursery-grown *P. niveum* were washed with detergent followed by rinsing with tap water. Each capsule was dipped into 70% ethanol for 20 sec and then flamed. The capsule was cut longitudinally and the seeds were then transferred to a 1.5 mL microcentrifuge tube. In order to shorten the seed germination time, seeds were pretreated in 1% (v/v) of Clorox with a few drops of Tween 20 for 60 min with shaking (Shimura and Koda, 2004; Lee, 2007). After that, the pretreated seeds were transferred to liquid MVW medium and maintained on an orbital agitator at 120 rpm in darkness at 25 ± 2°C for four months with subculture at monthly intervals (Kaewubon et al., 2010). SE induction followed the method of Yamamoto et al. (2011) as follows:

1. A single SE (1–1.5 mm in diameter) excised from the SE clumps with a scalpel tip was cultured on solid PGR-free MVW containing 2% sucrose, 0.2% (w/v) polyvinylpyrrolidone (PVP-40) and 0.2% (w/v) AC and maintained under a 16/8 h photoperiod condition at 23 μmol·m$^{-2}$·s$^{-1}$ with a cool white fluorescent tube for one week (Kaewubon et al., 2010; Yu et al., 2015).
2. These SEs were preconditioned in MVW medium with 0.1 M sucrose for seven days, followed by a two-step preculture with solidified MVW containing 0.2 M and 0.6 M sucrose in a petri dish for one day each.
3. 2.0 μL of 2% (w/v) Na-alginate solution (MVW medium containing 0.4 M sucrose) was dispensed into wells of the aluminum cryo-plate (7 mm × 37 mm × 0.5 mm with 1.5 mm diameter, depth 0.75 mm of 10 wells).
4. Precultured SEs were placed one by one into wells and CaCl$_2$ solution (0.1 M CaCl$_2$ in liquid MVW with 0.4 M sucrose) was fully added onto the cryo-plate. After complete polymerization (~15 min at room temperature), the CaCl$_2$ solution was removed by autopipette and the residual solution was absorbed with a piece of filter paper.
5. These SEs attached to the cryo-plate were then placed in the pipetting reservoir filled with 50 mL LS (2 M glycerol containing 1.2 M sucrose) for 30 min at 25°C.
6. The aluminum cryo-plate with embedded SEs was dehydrated by immersion in PVS2 for 60 min at 25°C. After that, the cryo-plate was placed into a 2-mL uncapped cryotube and rapidly plunged into LN for at least 1 h.
7. After LN storage, the cryo-plate was rewarmed in liquid MVW containing 1 M sucrose for 15 min at 25°C, and rinsed with liquid MVW with 0.0058 M sucrose.
8. Rewarmed SE was gently removed from alginate gel and the naked SE was then cultured on the regrowth medium, a modified Fe-free solid MVW medium containing 0.1 mg·L$^{-1}$ 1-naphthaleneacetic acid (NAA), 0.2% (w/v) PVP-40, and 0.2% (w/v) AC, under a dark condition for one week, followed by one week of a 16-h photoperiod (Soonthornkalump et al., 2019).

**Effects of ascorbic acid treatment at a critical step (precondition step) in the developed V cryo-plate procedure**

Ascorbic acid at 0.1 mM was sterilized by filtration with a sterile pore size 0.2 μm filter unit to warm autoclaved solid MVW (~50°C) and mixed by agitation. Added AsA medium (20 mL) was poured into each sterile Petri dish and then kept in the dark under a cold condition (4°C). The AsA treatment was conducted on day 7 of the precondition step for one day before the

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beginning of the 1st preculture. The total ROS and MDA contents were measured using the V cryo-plate procedure at the 1st preculture, 2nd preculture, osmoprotection and dehydration steps. Intact SE was used as the control to indicate normal production of total ROS and MDA.

When the SEs of P. niveum were cultured on the regrowth medium, they usually grew and turned into green masses in two weeks. Then, these SE masses formed shoot buds/small shoots after culture for one month, and developed shoots and plantlets after four months of culture (Soonthornkalump, 2019). Most SE masses with shoot buds/small shoots developed to form shoots and plantlets. Therefore, we defined survival and regeneration rates as follows. The survival percentage was confirmed by observation after 14 days of culture on the regrowth medium. All and partially green SEs (Fig. 4A) were evaluated for survival while, completely brown SEs (Fig. 4B) were evaluated for death. The regeneration percentage was evaluated based on whether the green SEs formed shoot buds/small shoots (Fig. 4C, D) after one month of culture. After culture for four months, the developing shoots and plantlets were also recorded (Fig. 4E, F). The treatment was performed with three replicates, each with six samples (SEs).

**Determination of total ROS content**

The total ROS content was measured according to the protocol described by Jambunathan (2010). The SEs were ground in LN. A ground powder of SEs was homogenized with 1 mL of 10 mM Tris buffer (pH 7.2) and centrifuged at 12,000X g for 20 min at 4°C. The 1 mL of sample mixture (100 μL supernatant and 900 μL Tris-buffer) was added to 10 μL of 1 mM dichlorofluorescin diacetate (DCFDA) and then vortexed. The sample mixture was incubated in darkness for 10 min prior to measurement. The sample mixture with DCFDA, the sample mixture without DCFDA (the control) and 1 mL Tris-buffer (the blank) were measured using a spectrofluorometer (FP-8200; JASCO) at 504 nm and 524 nm. The total ROS content was calculated using a standard protein curve determined by the Bradford reagent. The data are reported as the relative total ROS unit per mg of protein.

**MDA analysis**

Lipid peroxidation was measured by an MDA assay (Verleyen et al., 2004). Three replications of SEs were weighed (ca. 100 mg) and added to the reaction mixture containing 700 μL of deionized water and 750 μL of TBA reagent (0.5% (w/v) thiobarbituric acid (TBA) in 20% (w/v) trichloroacetic acid (TCA)). Then, the sample mixture was boiled at 95°C for 25 min, rapidly cooled on ice (5 min) and centrifuged at 1000 × g (10 min). The absorbance was measured at 532 and 600 nm against the TBA reagent (the blank). The concentration of MDA was calculated using the extinction coefficient of MDA (155·mM⁻¹·cm⁻¹) from Beer–Lambert’s equation (Heath and Packer, 1968).

**Water content determination (WC)**

The WC determination followed the protocol presented by Khoddamzadeh et al. (2011). The SEs were collected from the precondition, preculture, osmoprotection and dehydration steps. Intact SEs served as the control. Each step contained three replicates (each with six SEs). All samples were weighed and dried in a hot air oven (130°C/24 h), and then reweighed. The percentage of WC was calculated using the equation below:

\[
WC\% = \left(\frac{FW - DW}{FW}\right) \times 100
\]

FW = Fresh weight of SEs
DW = Dry weight of SEs

**Statistical analysis**

All experiments were organized using a completely randomized design (CRD) and repeated twice. Three replicates were performed for each treatment. The mean values were subjected to analysis of variance (ANOVA) and separated using Tukey’s honestly significant difference (Tukey’s HSD) test and the Least Significant Difference (LSD) at \( P \leq 0.05 \). The statistical tests were done with SPSS software ver. 17.0.

**Results and Discussion**

The profiling of total ROS and MDA levels at different steps in the V cryo-plate procedure showed the significantly highest total ROS was expressed in the 1st preculture with a continuous increase in MDA level in the same step (Fig. 1). Meanwhile, the WC result from the developed procedure demonstrated that a significant decrease in WC was initiated at the 1st preculture (64.5%) and gradually decreased to the lowest WC at 19.4% in the dehydration step (Fig. 2). Our results also showed the initiation of total ROS generation was associated with the reduction in WC in the 1st preculture step. Leprince et al. (2000) revealed that slightly osmotic dehydration could increase ROS generation. Subsequently, the ROS stimulate free radical-mediated lipid peroxidation, resulting in plasma membrane damage (França et al., 2007). According to these results, we assumed that the critical step would be the precondition step because this was when the earliest oxidative stress was observed.

Therefore, the prevention of oxidative stress is necessary for successful cryopreservation. To confirm this, the effect of addition of AsA on day 7 of the culture (AsA-treated SEs) before the start of the 1st preculture (oxidative stress emergence) was investigated. The AsA-treated SEs showed lower ROS levels (Fig. 1A) and MDA (Fig. 1B) production in all steps tested (1st preculture, 2nd preculture, osmoprotection, and dehydra-
The AsA treatment resulted in a significant increase in the regeneration percentage of cryopreserved SEs up to 39% compared to the non-AsA treatment control (8.5%), which was similar to the survival percentage result (Fig. 3). Also, the regeneration level of the AsA treatment cryopreserved SE was the same as in the non-cryopreserved treatment. However, the regeneration level of the non-AsA treatment cryopreserved SEs was significantly decreased compared to the non-cryopreserved one. These results indicated that AsA treatment for SEs was effective to obtain a higher regeneration level after cryopreservation. The surviving cryopreserved SEs had viable green parts after culture on regrowth medium for 14 days (Fig. 4A), while browning was detected in dead cryopreserved SEs within 24 h after culture on regrowth medium (Fig. 4B). Moreover, AsA-treated SEs exhibited greater vigor and proliferation of new tissue than the control after one month of culture (Fig. 4C, D). These SEs developed new shoots (or plantlets) after four months of culture (Fig. 4E, F).

Poobathy et al. (2013) revealed that orchid SE was attractive as a plant material for cryopreservation due to an appropriate material size and very fast proliferation with a vigorous SE-derived regenerant. However, orchid SE has been considered to be sensitive to vitrification-based cryopreservation because it contains a high water content and is tender (Jia et al., 2016). Jia et al. (2016) also reported that an increase in free radical (\(O_2^-\) and \(H_2O_2\)) and MDA levels, and a significant
Fig. 4. Visual observation of (A) a viable shoot of an AsA-treated cryopreserved SE exhibiting a light green color compared with (B) a completely brown cryopreserved SE obtained from a non AsA-treated specimen after culture on regrowth medium for 14 days. (C) AsA-treated cryopreserved SE exhibited high regeneration ability and vigorousness while (D) slow recovery growth was observed in a non AsA-treated cryopreserved SE after culture on regrowth medium for one month. Regenerated shoots from (E) AsA-treated cryopreserved and (F) non AsA-treated SE after culture on regrowth medium for four months, respectively.

decrease in AsA level during vitrification, may have caused the cryopreserved SEs of Dendrobium Hamana Lake ‘Dream’ very unlikely to survive.

AsA, an universal non-enzymatic antioxidant molecule, has been widely used to improve abiotic stress tolerance (Foyer and Noctor, 2011). AsA plays a key role as an electron donor for ascorbate peroxidases (APXs) to convert H$_2$O$_2$ to water and oxygen (Van Doorn and Ketsa, 2014). The application of exogenous AsA can improve endogenous AsA accumulation (Athar et al., 2009). Azzedine et al. (2011) reported that the free-radical scavenging activities of catalase (CAT) and superoxide dismutase (SOD) could be stimulated by exogenous AsA application. Moreover, AsA could increase endogenous osmolyte synthesis of proline and glycinebetaine in plant cells (Latif et al., 2016). These results demonstrated the role of AsA in minimizing and eliminating possible damage due to oxidative stress.

The previous study revealed that adding AsA (0.14 to 0.58 mM) during cryopreservation in the preculture, LS and thawing steps separately could improve the survival percentage of blackberry shoot tips (Rubus hybrid ‘Chehalem’ and ‘Hull Thornless’) with a decrease in MDA level (Uchendu et al., 2010). Chua and Normah (2011) also reported that applying 0.28 mM AsA in the LS step could slightly enhance the survival percentage (3.3%) of pulasan shoot tips (Nephelium ramboutan-ake). The oxidative stress during callus cryopreservation of Agapanthus praecox was determined by Zhang et al. (2015). It was found that the ROS-induced oxida-

Fig. 5. Schematic diagram of cryopreservation of Paphiopedilum niveum SEs using the V cryo-plate method. (A) SEs (1–1.5 mm diameter) were preconditioned on PGR-free MVW containing 0.1 M sucrose and 0.1 mM ascorbic acid (one day). (B) After that, preconditioned SEs were placed on the 1st preculture medium (MVW containing 0.2 M sucrose) for one day followed by (C) the 2nd preculture (MVW containing 0.6 M sucrose) for one day. (D) Precultured SEs were embedded onto the cryo-plate using 2% (w/v) alginat gel and hardened with 0.1 M CaCl$_2$ solution. (E) Cryo-plates were immersed into LS containing 2 M glycerol with 1.2 M sucrose for 30 min at 25°C, (F) dehydrated by PVS2 for 60 min at 25°C (G) Cryotube containing the cryo-plate was affixed to cryocane and then (H) plunged into LN. (I) Cryopreserved SEs were rewarmed in 1 M sucrose solution and (K) cultured on regrowth medium (Fe-free MVW medium containing 0.1 mg·L$^{-1}$ NAA, 0.2% (w/v) PVP-40 and 0.2% (w/v) AC) for seven days in the darkness and then transferred to a light condition.
tive stress was initiated at preculture followed by the presence of H$_2$O$_2$-induced peroxidation in the osmo-protection step.

Chaireok et al. (2016, 2017) found that cryopreservation of *P. niveum* using the encapsulation-vitrification and vitrification methods led to 29.6% survival of calli and 22.2% of survival calli and SE clumps, respectively. Both previous studies employed larger plant materials without any dissection (approx. 3–4 mm calli and SE clumps) than the current study. Many previous studies also demonstrated the effect of explant size on the regeneration and regrowth ability after cryopreservation. Baek et al. (2003) reported that using larger garlic shoot apices by vitrification produced higher viability and regeneration percentages. Similar to many orchid species such as *Dendrobium* Bobby Messina (Antony et al., 2010), *Vanda* Kaseem’s Delight (Poobathy et al., 2012) and *Brassidium* Shooting Star (Mubbarakah et al., 2014), using larger (3–4 mm) SEs provided better viability compared to smaller SEs (1–2 mm) in post-cryopreserved SEs via vitrification. With the V cryo-plate method, large sugarcane shoot tips resulted in higher regrowth than smaller ones (Rafique et al., 2015). Melo et al. (2011) also found that large explants could accumulate carbohydrate in the form of starch grains that could serve as an energy source for the regeneration of sugarcane shoot tips after cryopreservation. Moreover, Chaireok et al. (2016) reported that vigor regrowth could be observed in large post-cryopreserved *P. niveum* calli with high carbohydrate accumulation. In our study, small *P. niveum* SEs (1–1.5 mm) were dissected from the SE clump. Therefore, these dissected SEs with wound injuries could make the post-cryopreserved SEs weak and reduce survivability. According to the results of previous studies, a reduction in mechanical injury and explant size could be key parameters to be considered to improve the survival of cryopreserved explants.

In this study, the use of a single SE (approx. 1 mm diameter) and effectiveness of AsA application as an antioxidant on day 7 of culture before the start of the 1st preculture was tested in cryopreservation using a V cryo-plate method. We applied a V cryo-plate procedure to a single SE of *P. niveum* (Fig. 5), and this resulted in a moderate regeneration rate (39%). The key factor was AsA application in the critical step of the V cryo-plate method. Further research is necessary to obtain higher regeneration rates after cryopreservation, especially by optimizing the exposure time by PVS2 and application of AsA to other steps in the V cryo-plate method. The V cryo-plate procedure could become an effective protocol for a single SE of *P. niveum*.

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