Effect of Storage Temperature on Seed Viability and *In Vitro* Germination of Nobile *Dendrobium* Hybrids

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ABSTRACT

Nowadays, seed storage plays an important role for long-term conservation for plant genetic resources including orchids. Besides, asymbiotic germination provides an essential tool for commercial propagation and conservation purpose. Nobile-type *Dendrobium* hybrids are in high demand as decorative pot plants due in part to their showy flowers. In this study the effect of storage temperatures on seed viability and subsequent germination capability of nobile *Dendrobium* hybrids was evaluated. Mature seeds from ripe capsules were collected from three cross combinations of nobile *Dendrobium* hybrids; *D.* Spring Dream 'Apollon' x *D.* Hamana Lake 'Dream', *D.* Golden Tower x *D.* 012-3, and *D.* 184-1 x *D.* 140-1 and air-drie*D.* The dried seeds were stored at either room temperature (25+2 ˚C) or -20 ˚C for twenty four months. *In vitro* seed germination and viability test by TTC stain were investigated. The germinability and viability varied on storage temperature as well as hybrid combinations and duration of seed storage. The viability of seed stored at -20 ˚C was higher than at room temperature as revealed by their germination percentage. Stainability percentage of seed stored at room temperature was severely low after 9 months of storage, while seed stored at -20C could maintain viability longer than at room temperature. Seed germination decreased rapidly after 6 months at room temperature, whereas seeds stored at -20 ˚C the germination decreased after 20 months. Also, the seeds rarely developed to advance seedling stages (stage 5 and 6) after 20 months of storage at -20˚C.

Key-words: nobile *Dendrobium*, TTC staining, seed storage, low temperature

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INTRODUCTION

Increased demand for orchids in the global floriculture trade is creating interest among orchid growers in producing new high-quality hybrids (Ngampanya and Homla-aor, 2010). Orchid hybridization is a common and essential practice among orchid growers, its main goal is to generate new and improved material with characteristics of commercial interest such as new flower colors, color patterns, and flower size and number (Vendrame *et al*., 2008). Dendrobium hybrids are considered to be one of the most globally popular orchids since their high number of flowers per inflorescence, longlasting and recurrent flowering (Martin and Madassery, 2006; Kananont *et al*., 2010). Among them, the high heterozygosity of nobile type Dendrobium hybrids has attracted the attention of orchid industry for large scale clonal propagation of elite genotypes due to their strong vigor and showy flowers (Faria *et al*., 2004). *In vitro* germination of hybrid seeds is a common practice among orchid growers and most orchid seeds can either be readily germinated after harvest from the mother plant or stored for later germination (Vendrame *et al*., 2007; 2008). According to Kamemoto *et al.* (1999), the success of Dendrobium breeding program has depended on the utilization of amphidiploids which were produced from seed propagation method. Observation and evaluation of progenies are usually carried out for a minimum of two years of flower production. Therefore, seed storage could obtain seeds for the germination of selected amphidiploid progenies. Seed storage is of great importance for plant breeding as well as an efficient means for conservation of plant genetic resources and germplasm preservation of rare or endangered species (Pritchard and Seaton, 1993; Vendrame *et al*., 2007; Ngampanya and Homla-aor, 2010).

Seaton and Pritchard (2011) demonstrated that the time of banking orchid seeds has started to be used as a key component of an effective ex situ conservation strategy. Seed banking, either short-term (seeds lasting for 1-5 years) or long-term (seeds lasting >5 years) persistent (Bakker *et al*., 1996), has been recognized as being the most efficient way of storing large numbers of living plants in one place (FAO, 1996; Linington and Pritchard, 2001). There are

currently very few genebanks that preserve orchid germplasm and this creates the need to develop procedures to improve orchid seed storage for germplasm conservation (Mweetwa *et al*., 2007).

Even though, cryopreservation has been reported as a tool for long-term seed conservation in various of orchids such as Spathoglottis plicata (Khor *et al*., 1998), Bratonia spp. (Popova *et al*., 2003), Bletilla striata (Hirano *et al*., 2005), Brassia and Phalaenopsis spp. (Mweetwa *et al*., 2007) and Phaius tankervilleae (Hirano *et al*., 2009) but it faces significant obstacles because of methodological problems involved with determination of seed germination rate *in vitro* and selection of optimum freezing conditions (Pritchard, 1984). Moreover, seeds of some species do not tolerate desiccation or cold storage so lower temperature storage may be more suitable for long-term storage (Machado-Neto and Custodio, 2005; Seaton and Pritchard, 2008). According to Seaton and Pritchard (2003), orchid seeds can be stored successfully at either 5 °C, the temperature of a domestic refrigerator, or -20 °C, the temperature of a domestic deep freezer.

Although plenty of Dendrobium hybrids have high commercially value in the global floriculture trade, there is rarely information on low temperature seed storage of nobile Dendrobium hybrids are described. There are only some reports demonstrated seed cryopreservation in Dendrobium hybrids (Vendrame *et al*., 2007), *D. crystallinum* Rchb.f. and *D. virgineum* Rchb.f. (Huehne and Bhinija, 2012) and *D. candidum* PLBs by encapsulation (Yin and Hong, 2009) and air-drying method (Bian *et al*., 2002). Therefore, establishment of an efficient method for nobile type Dendrobium hybrids seeds storage has been needed for breeding programs and conservation purpose. In this study, we investigated the effect of storage temperature and duration on viability and germination ability of mature seeds of nobile Dendrobium hybrids.

MATERIALS AND METHODS

Plant materials

Three cross combinations of nobile type Dendrobium hybrids; *D.* Spring Dream 'Apollon' x *D.* Hamana Lake 'Dream', *D.* Golden Tower x *D.* 012-3, and *D.* 184-1 x *D.* 140-1 were hand-pollinated

under greenhouse in National Pingtung University of Science and Technology. Mature seeds from ripening capsules were collected and seeds were stored in 1.5 ml centrifuge tubes provided by wrapping in heavy-duty aluminum foil and stored at two different conditions; 25±2 °C (room temperature) and -20 °C (low temperature) over silica gel desiccant until experimentation.

Seed sterilization, germination and culture conditions

Seeds from 0, 1, 2, 3, 6, 9, 16, 20 and 24 months after storage were surface-sterilized with 0.6% sodium hypochlorite for 10 min, and then washed with sterile water for 3 times. Sterilized seeds were resuspended in a small amount of sterile water and the seeds were extracted with a sterile glass Pasteur pipette spreading thinly as possible over the surface of medium in 90x15 mm Petri plates (Alpha Plus Scientific Corp., Taiwan). Basal medium consisted of half-strength Murashige and Skoog (1962) supplemented with 20 g/L sucrose and solidified with 8 g/L Sigma agar (Sigma-Aldrich, St Louis). The pH of the media was adjusted to 5.6-5.8 prior to autoclaving at 121 °C and 1.21 kg/cm^2 for 30 min. Petri plates were sealed with clear plastic tape and maintained at 25±2 °C under a 12/12-h (day/night) photoperiod provided by cool white fluorescent lamps (StarcoatTM F28W/T5/ 840,170 MA, Hungary) at photosynthetic photon flux (PPF) of 40+10 mol/m^2/s. Seed germination was observed weekly under stereomicroscope (Stemi SV6 Zeiss, Germany) and germination percentage was calculated by dividing the number of germinating seeds by total number of seeds in the sample 6 weeks after sowing. Four replicates (each replicate includes 300-400 seeds approximately) were performed for all hybrids.

Seed viability test

Seed viability was assessed using the 2,3,5-triphenyltetrazolium chloride (TTC) staining method. A small amount of seeds were placed in 1.5 ml centrifuge tubes and surface-fertilized as above methods before being resuspended in 0.5% TTC aqueous solution and incubated for 24 h at 25±2 °C in darkness. The 0.5% TTC aqueous solution was prepared by dissolving 0.5 g TTC in solution with 0.2 M NaH_2PO_4 30.5 ml, 0.2M NaH_2PO_4 19.5 ml and total volume

to 100 ml by water. Then seeds were examined under a fluorescence microscope (Olympus CX40, USA); seeds containing embryos with any degree of pink to red staining throughout the embryos were considered viable while wholly unstained embryos were considered non-viable. Three replicates (each replicate includes 150-200 seeds approximately) were performed for all genotypes and percent viability was calculated by dividing the number of seeds with viable embryos by the total number of seeds with embryos.

Experimental design and statistical analysis

All experiments were established in a completely randomized design (CRD). Analysis of variance (ANOVA) was performed by using SAS version 9.0 (SAS Institute Inc., Cary, NC, USA) and mean separation with the least significant difference (LSD) at $p < 0.05$.

RESULTS AND DISCUSSION

Effect of storage temperature on germinability of seeds

Storage temperature had varying effects on the germinability of seeds after storage with the results depending on genotype (Table 1). Before storage, seeds of *D.* Spring Dream 'Apollon' x *D.* Hamana Lake 'Dream' and *D.* Golden Tower x *D.* 012-3 showed higher germination percentage than *D.* 184-1 x *D.* 140-1. After one and two months storage, there is no significant different on germinability between 25°C and -20°C but significant different among species. The germination percentage of *D.* Golden Tower x *D.* 012-3 and *D.* 184-1 x *D.* 140-1 higher than *D.* Spring Dream 'Apollon' x *D.* Hamana Lake 'Dream' after one month storage. Whereas, the germination percentage of *D.* Golden Tower x *D.* 012-3 higher than *D.* Spring Dream 'Apollon' x *D.* Hamana Lake 'Dream' and *D.* 184-1 x *D.* 140-1 after two months storage. However, after 3 months storage the germination of seeds stored at -20°C was significantly higher than 25°C in all hybrids. After 6 months storage, the germination of all hybrids was rapidly decreased when stored at 25°C and no germination was found after 16 months. Whereas at -20°C the germination was rapidly decreased after 20 months, however the germination of *D.* Spring Dream 'Apollon' x Hamana Lake 'Dream' was more than fifty percent. Among three

cross combinations, the highest germination was found in the combination of *D.* Golden Tower x 012-3 (97.24%). However, the combination of *D.* Spring Dream 'Apollon' x *D.* Hamana Lake 'Dream' showed the highest germination rate (15.37%) after 24 months storage. In this study, the result showed that low temperature (-20˚C) storage was effective for preserving seed viability and germinability of mature seeds of nobile Dendrobium hybrids compared with room-temperature (25+2˚C) for almost 2 years of storage (Table 1 and 2). This result demonstrated that nobile Dendrobium hybrids were able to remain their viability for long periods at the storage temperature is reduced below refrigerator temperature (4˚C). Seaton and Pritchard (2011) reported that good-quality dry orchid seed has the potential to survive for many decades at conventional seed bank temperatures of around -20˚C. Although, there are previous studies about seed longevity during storage at different low temperature of orchid species such as *Phaius tankervilleae* stored at 4˚C (Hirano *et al*., 2009), *Caladenia arenicola* and *C. huegelii* (terrestrial Australian orchids) stored at -18˚C (Hay *et al*., 2010), and Cattleya species stored at -18˚C (Hosomi *et al*., 2012), however, the longevity from this study (2 years) is appeared to be the longest lived when stored at-25˚C for epiphytic orchid. Moreover, the results from this study showed that storage temperature had varying effects on the germinability of seeds after storage with the results depending on genotype. It has been demonstrated that genotypic differences may also have contributed for the differences in germination percentages among different hybrids (Vendrame *et al*., 2007; Mweetwa *et al*., 2007; Nikishina *et al*., 2001; Huehne and Bhinija, 2012). Meanwhile, Thornhill and Koopowitz (1992) suggested that the maturity must be considered prior to seed storage.

Effect of storage duration on germination and seeding development

Before storage seed germination and seedling development were observed. The germination and seedling were developed as following: viable seeds were swollen, color changed to green, embryo enlarged and testa ruptured at the first week of germination, and designated as

Table 1. Germination percentages of mature seeds of three combinations of nobile *Dendrobium* hybrids; Spring Dream 'Apollon' x Hamana Lake 'Dream', Golden Tower x 012-3, 184-1 x 140-1 after storage up to 24 months at room (25±2 °C) and low (-20 °C) temperature [1]

Hybrid combination	Storage temperature	Germination rate (%) Storage period (month)								
		0	1	2	3	6	9	16	20	24
D. Spring Dream 'Apollon' x Hamana Lake 'Dream'										
	25±2 °C	96.82 a	65.51 b	61.87 b	70.12 c	23.40 c	0.54 c	0 c	0 c	0 c
	-20 °C	96.82 a	60.52 b	66.41 b	83.78 b	84.25 ab	73.35 b	92.29 a	58.62 a	15.37 a
Golden Tower x 012-3										
	25±2 °C	97.24 a	79.48 a	87.90 a	84.93 b	50.99 b	0.12 c	0 c	0 c	0 c
	-20 °C	97.24 a	69.27 ab	89.65 a	96.34 a	96.22 a	90.13 a	86.84 ab	11.58 b	1.08 b
184-1 x 140-1										
	25±2 °C	76.41 b	80.44 a	53.03 b	51.86 d	6.58 d	0.08 c	0 c	0 c	0 c
	-20 °C	76.41 b	78.07 a	64.31 b	77.45 bc	84.34 ab	70.01 b	80.13 b	24.71 b	5.92 b

[1] Mean in the same column followed by the same letter are not statistically different at $p<0.05$ by Fisher's protected LSD test.

stage 1 (Figure 1a). Later, appearance of pro-meristem/trichomes on germinated protocorms within two weeks of culture, designated as stage 2 (Figure 1b). Subsequently, at stage 3 leaves emerged from protocorm and elongation occurred at the third week of culture (Figure 1c). At the fourth week or stage 4, protocorms with developing leaves and rhizoids were observed (Figure 1d). At stage 5 two leaves and one or more roots emerged after five weeks of culture (Figure 1e). Final stage, or stage 6, two or more leaves and roots were presented in seedlings at six weeks after sowing (Figure 1f). However, after sixteen months storage at low temperature seeds germinated slowly and seedling development was delayed (Figure 2). The germination (Stage 1) was observed after 2-3 weeks of culture and well developed to advance stages (stage 5 and 6) after six weeks of culture. In addition, after twenty month-storage the germination was observed at the fifth week of culture and rarely found advance stages (stage 5 and 6) at the sixth week. Moreover, some of these protocorms could develop to advance stages when culture for long. However, the seedlings of each stage

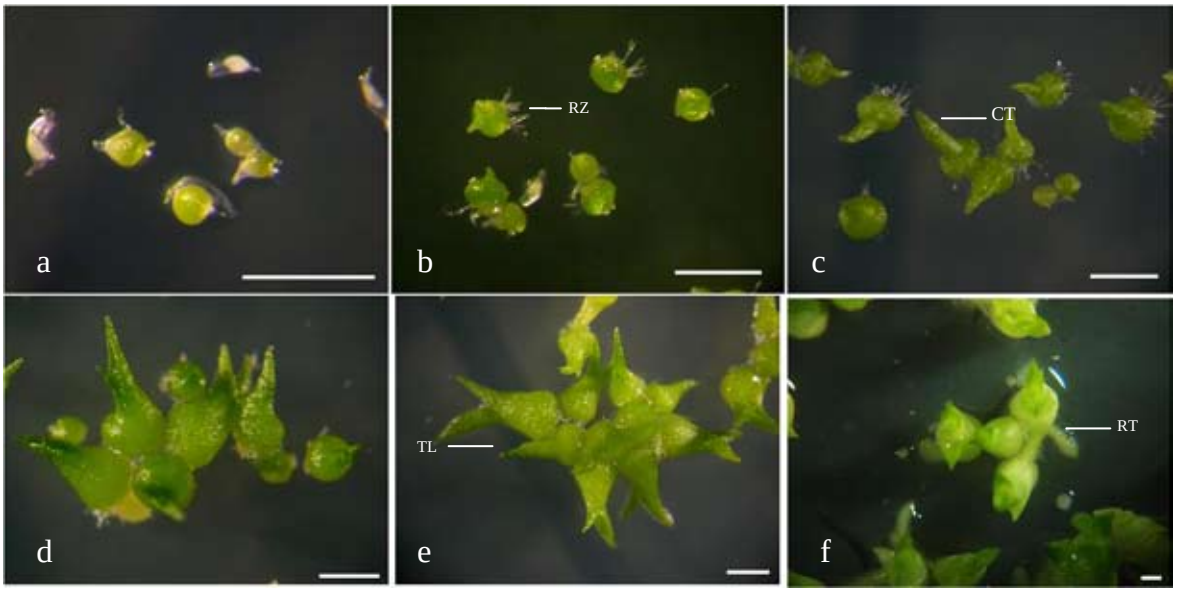

Figure 1. Different stages of protocorms and young seedlings of *D.* Spring Dream 'Apollon' x *D.* Hamana Lake 'Dream'. Stage 1: a swollen seed with yellow protocorm (a), stage 2: appearance of rhizoids (b), stage 3: emergence and elongation of cotyledon (c), stage 4: protocorm with developing leaves and rhizoids (d), stage 5: presence of two or more leaves and stage 6: presence of two or more leaves, with roots emerged (seedling) RZ: rhizoids, CT: cotyledon, TL: true leaf, RT: root. Scale bars = 1 mm.

were observed and estimated without counting. The use of mature seeds is generally a preferable approach as they remain viable for long periods under refrigeration (Kitsaki *et al.*, 2004). Therefore, seeds of current study were harvested at maturity and the germination percentage was high in all hybrids. Also, Seaton (2007) mentioned that immature embryos cannot be stored as successfully as mature seed. Although mature seeds have the advantage of remaining viable for long periods, they become dormant with maturation (Kitsaki *et al.*, 2004). Hirano *et al.* (2009) described that it is more probable that the decrease in germination rate after storage of P. tankervilleae seeds was caused by physiological changes leading to secondary dormancy. According to Mweetwa *et al.* (2007) demonstrated that in nature orchid, seeds may exhibit physiological (Rasmussen, 1995) or morphophysiological (Whigham *et al.*, 2006), dormancy can be broken by darkness, temperature, or atmospheric

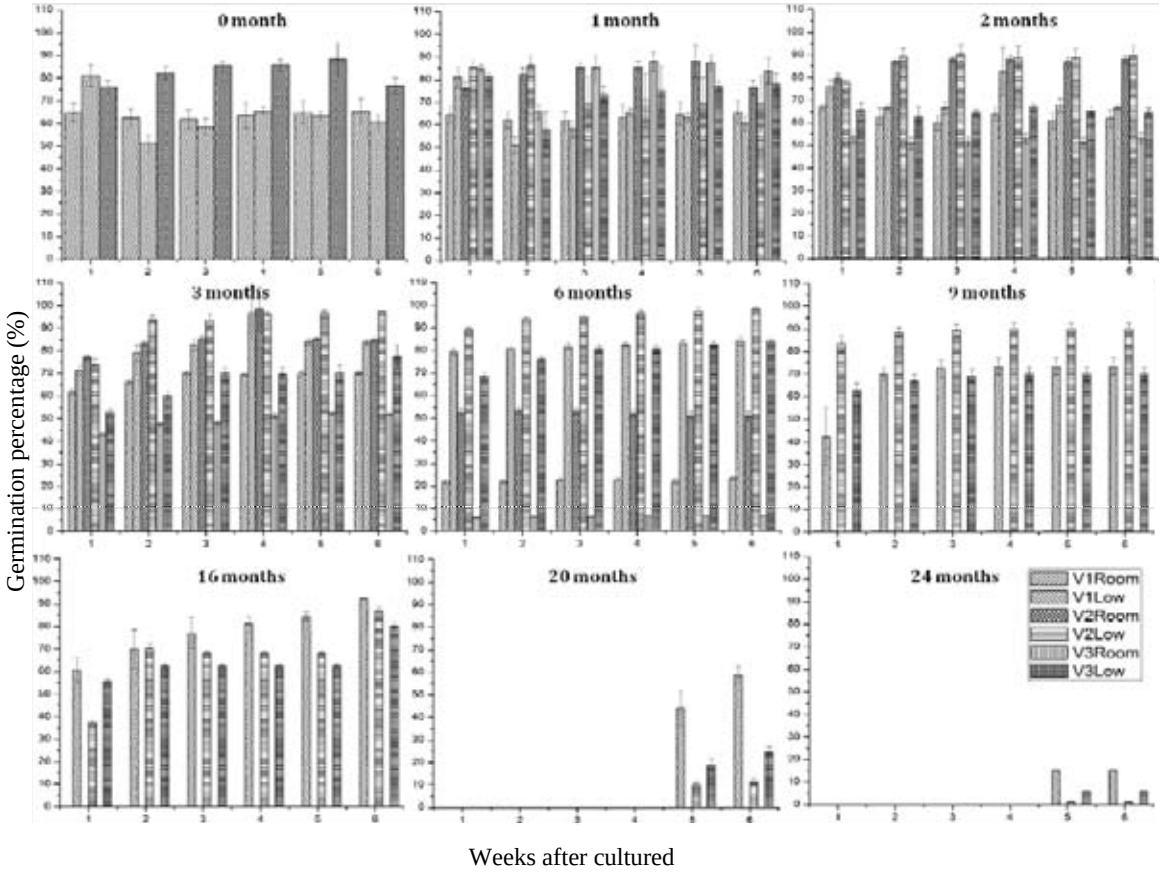

Figure 2. The weekly germination percentage of mature seeds of three crossed pollination of *Dendrobium* nobile types; *Dendrobium* hybrids; *D.* Spring Dream 'Apollon' x *D.* Hamana Lake 'Dream' (V1), *D.* Golden Tower x *D.* 012-3 (V2), and *D.* 184-1 x *D.* 140-1 (V3) at 25˚C (Room) and -20˚C (Low) in different storage time.

conditions. As shown in this study, the germination decreased after storage for few months and subsequently increased at low temperature storage compared with room temperature. Also, Mweetwa *et al.* (2007) reported that some Phalaenopsis species seeds have a chilling requirement for germination.

Seed viability test

The viability tests with TTC showed that it directly depends on the species and duration of seed storage (Table 2). After two month of storage, viability of seeds stored at -20 ˚C significantly higher than seeds stored at 25 ˚C in all combinations. The viability

Table 2. TTC stainability of mature seeds of three combinations of nobile *Dendrobium* hybrids; Spring Dream 'Apollon' x Hamana Lake 'Dream', Golden Tower x 012-3, 184-1 x 140-1 after storage up to 24 months at room (25±2°C) and low (-20°C) temperature [1]

Hybrid combination	Storage temperature	Germination rate (%) Storage period (month)								
		0	1	2	3	6	9	16	20	24
D. Spring Dream 'Apollon' x Hamana Lake 'Dream'										
	25±2°C	48.45 a	42.07 a	26.76 b	35.45 b	28.46 b	20.29 b	11.02 b	2.72 b	0.37 b
	-20°C	48.45 a	34.34 a	47.63 a	50.19 a	52.06 a	44.01 a	20.38 a	35.83 a	20.26 a
Golden Tower x 012-3										
	25±2°C	43.62 a	44.12 a	34.31 b	28.02 b	26.26 b	10.17 b	7.11 b	0.38 c	0.39 c
	-20°C	43.62 a	48.58 a	48.35 a	51.84 a	52.50 a	58.22 a	38.94 a	1.66 b	3.02 b
184-1 x 140-1										
	25±2°C	37.21 a	28.80 a	17.01 b	14.81 b	9.42 c	9.73 b	5.17 b	1.09 c	1.02 c
	-20°C	37.21 a	46.59 a	43.39 a	56.02 a	37.57 b	51.59 a	40.20 a	4.18 b	3.57 b

[1] Mean in the column followed by the same letter are not statistically different at $p<0.05$ by Fisher's protected LSD test.

imaging of TTC-treated seeds showed not only viable (pink to red) and non-viable (white) seeds but also empty (no embryo) seeds (Figure. 3). According to Singh (1981), the TTC test has been successfully used for estimation of survival rate in orchid seeds. Also, using the TTC method enables testing the viability of seeds without long-term waiting for their germination (Nikishina *et al.*, 2001a). In this study, TTC staining indicated that seed viability of seeds stored at low temperature is higher than room temperature in all hybrids. Though the highest viability percentage was about 50% and it was lower than observed during germination experiments as similar as tetrazolium study of *S. nepalense* (Mahendran and Bai, 2009). In contrast, Vasudevan and Staden (2010) presented that TTC method tends to over estimate seed viability and is not a good indicator of germination ability of *D.* nobile seeds. Also, it has shown in several studies on the Orchidaceae such as *Bletia purpurea* (Johnson *et al.*, 2011), *D. bigibbum* var. compactum and *D. formosum* (Kanamont *et al.*, 2010) and *Cypripedium acaule* (Hirano *et al.*, 2009). Likewise, over estimation of seed viability was found when using TZ for *Phalaenopsis* and *Brassia* (Mweetwa *et al.*, 2007). The causes of over estimation are probably seeds subjecting to prolonged exposure

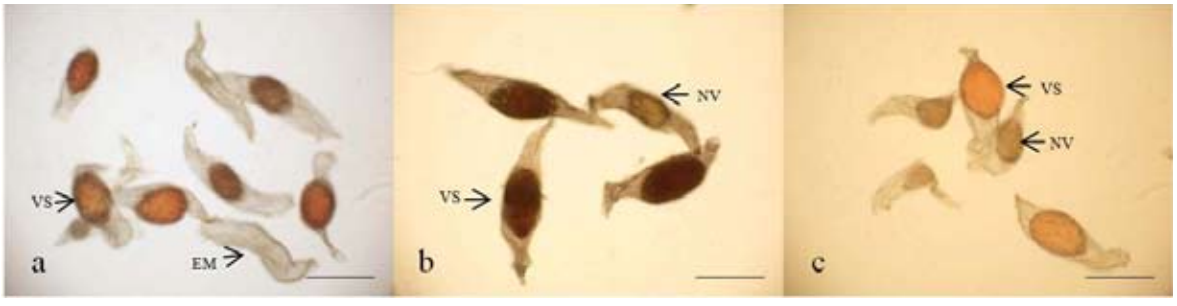

Figure 3. TTC stained seeds of crossed pollination of nobile type *Dendrobium* hybrids; *D.* Spring Dream 'Apollon' x *D.* Hamana Lake 'Dream' (a), *D.* Golden Tower x *D.* 012-3 (b), and *D.* 184-1 x *D.* 140-1 (c) showed viable (VS), non-viable (NV) and empty (EM) seeds without embryo. Scale bars = 0.5 mm.

to sodium hypochlorite, which caused high TTC stainability of the embryo because of release of dehydrogenase from damaged embryos (Lauzer *et al.*, 1994); also, dormant seeds were stainable with TTC (Hirano *et al.*, 2009) or they are capable of germination but had not germinated (Whigham *et al.*, 2006). Thus, the validity of viability estimates should be confirmed with germination tests. Although it might be better to use other methods to investigate seed viability such as fluorescein diacetate (FDA) as suggested by Pritchard (1985). In addition, this study was successfully found the seedling development after germination. According to Nowak and Shulaev (2003) demonstrated that it is necessary for seedling to reach to seedlings stage because with well developed root systems can absorb more water and nutrients in order to efficiently acclimate to *ex vitro* conditions.

CONCLUSION

In this paper, we have presented an appropriate simply method to preserve mature seeds of nobile *Dendrobium* hybrids by using low temperature (-20 °C) for short- and medium-term storage. The results indicated that hand pollinated-mature seeds of three cross combinations stored at low temperature (-20 °C) were able to germinate well on nutrient medium after 24 months of storage, although seed germination after 20 months was slowly. Nevertheless the viability of seeds by using TTC staining method was lower than the germination method, however, it indicated in the same direction as the

viability of seeds stored at low temperature which is higher than at room temperature. The principle of this study is not only for preserving genetic resources for *ex-situ* conservation but also for easy practice for orchid breeders who want to store seeds for later germination. Moreover, for the commercial outlook is to reduce labor costs and risks of handling errors, storage for long periods is preferable to storage under normal growth conditions.

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