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# Phloroglucinol enhances recovery and survival of cryopreserved *Dendrobium nobile* protocorms

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ABSTRACT

The aim of the present study was to evaluate the effect of phloroglucinol in the recovery and survival of cryopreserved *Dendrobium nobile* protocorms. The exposure of protocorms to 2 M glycerol osmoprotective solution for 20 min followed by 10 min in PVS2 vitrification solution with 1% phloroglucinol resulted in the highest protocorm recovery and survival (68%). A positive effect of phloroglucinol was observed when combined with glycerol and PVS2. Phloroglucinol added at 1% provided an increase of over 100% in protocorm recovery and survival as compared to the same treatment without phloroglucinol. However, when sucrose was added to treatments, a negative effect was observed with a reduction in survival by 90%. Protocorms that survived cryopreservation were successfully regenerated into plants and acclimatized with 100% survival in greenhouse. Survival of cryopreserved *D. nobile* protocorms was a determining factor for seedling survival and growth into normal and fully functional plants. This study demonstrated an efficient procedure for cryopreservation and subsequent recovery and survival of cryopreserved *D. nobile* protocorms using phloroglucinol as a cryoprotectant additive.

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## 1. Introduction

*Dendrobium nobile* is an epiphytic orchid which is valued for its attractive flowers (Nayak et al., 2002). This species has been referenced in the Chinese literature as a valuable medicinal plant (Hu, 1970) and used in traditional Chinese medicine (Ye et al., 2002). In fact, 5 alkaloids from *D. nobile* had already been identified in 1965 (Okamoto et al., 1966), followed by a number of additional compounds in related species, including alkaloids (Suzuki et al., 1973), fluorenones and sesquiterpenoids (Talapatra et al., 1985, 1992), bibenzyls and phenathrenes (Majumder and Chatterjee, 1989), and sesquiterpene glycosides (Zhao et al., 2001). Reports indicate antibiotic effects and possible anti-cancer properties (Hu, 1970), as well as antitumor and antimutagenic activities (Lee et al., 1995; Miyazawa et al., 1997) of compounds isolated from crude plants extracts.

The genus *Dendrobium* (Orchidaceae) comprises more than 1100 species widely distributed throughout the world from Southeast Asia to New Guinea and Australia (Puchooa, 2004; Xu et al., 2006). Potted Dendrobiums have become very popular in China, Germany, Japan, the Netherlands, Philippines, Taiwan, Thailand, and the Unites States (Puchooa, 2004). Dendrobiums are commercially desirable due to the large number of flowers per inflorescence and recurrent flowering (Martin and Madassery, 2006), making them also attractive for the cut flower market. However, habitat destruction and over collection from the wild are the main factors threatening the species survival, thus conservation is a major issue that needs to be addressed (Malabadi et al., 2005).

The potential value of cryopreservation as a conservation tool has been widely reported (Stacey et al., 1999; Engelmann and Dussert, 2000; Engelmann, 2004), being a viable option for storage of plant cells, tissues, seeds, pollen and embryos. Cryopreservation techniques commonly used are controlled rate cooling, vitrification, encapsulation dehydration, and dormant bud preservation (Reed, 2008).

In orchids, cryopreservation has been reported (Pritchard, 1984; Pritchard et al., 1999; Popova et al., 2003) and vitrification has been the most common method used (Ishikawa et al., 1997; Wang et al., 1998; Tsukazaki et al., 2000) to cryopreserve orchid protocorms, zygotic embryos, and cell suspensions. In Dendrobium, cryopreservation has been reported for in vitro shoot tips of Dendrobium Walter Oumae (Lurswijidjarus and Thammasiri, 2004), seeds and pollen of Dendrobium hybrids (Vendrame et al., 2007; Vendrame et al., 2008) and protocorm-like bodies of *Dendrobium candidum* (Yin and Hong, 2009).

Even though vitrification has been reported as a successful method for cryopreservation of orchid cells, organs and tissues, vitrification solutions include cryoprotectants that are toxic to many plants, requiring careful handling. Phloroglucinol (1,3,5-trihydroxybenzene) is a benzenetriol known for its growth-

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regulating property (Sarkar and Naik, 2000). Phloroglucinol has been reported to enhance growth and rate of axillary shoots in several woody plants, to initiate adventitious roots in in vitro shoots of different woody species, to enhance survival of meristems and/or shoot tips in vitro (Jones, 1976; James, 1979, 1983; Goudarzi et al., 1997; Demiralay et al., 1998), and to have a synergistic effect with auxin during root initiation (James and Thurbon, 1981; Sharifian et al., 2009). Phloroglucinol is also known to protect cells against oxidative damage by free radicals and to possess cryoprotective effect against oxidative stress and related metabolism (Benson and Bremner, 2004; Kang et al., 2006).

The aim of the present study is to evaluate the effects of phloroglucinol in the cryopreservation, and subsequent recovery and survival of cryopreserved *D. nobile* (Orchidaceae) protocorms.

## 2. Materials and methods

### 2.1. Plant material

Mature seed capsules from self-pollinated *D. nobile* were obtained from greenhouse grown plants. The capsules were collected in the spring 2008 and surface sterilized in a solution of 3% sodium hypochlorite for 20 min, under a sterile laminar flow hood. Sterile capsules were dissected using a scalpel. Seeds were removed from the capsule and placed for germination in Petri dishes containing half-strength MS medium (Murashige and Skoog, 1962) with 58.5 mM sucrose (pH 5.7) and solidified with 0.6% agar (Fisher®, Chicago, IL, USA). Petri dishes were maintained under controlled environmental conditions; 27 ± 2 °C; 120 μmol $m^{-2}$ $s^{-1}$; 16/8 light/dark; 2 × 9A Philips® fluorescent bulbs. After 60 days, protocorms (1–2-mm diameter) were obtained and used for cryopreservation.

### 2.2. Cryopreservation procedures and treatments

For each treatment, 10 protocorms were placed in 2-ml cryovials containing a different loading solution (2 M glycerol alone, 0.4 M sucrose alone, or 2 M glycerol combined with 0.4 M sucrose, as indicated below) for 20 min at 25 °C, as described by Nishizawa et al. (1993). After that they were transferred to a plant vitrification solution, PVS2 (Sakai et al., 1990), consisting of 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) dimethyl sulfoxide (DMSO) in half-strength MS medium with 0.4 M sucrose (pH 5.7), for 10 min at 25 °C.

Treatments consisted of protocorms immersed in the different cryopreservation loading and vitrification solutions, respectively, with or without 1% phloroglucinol, prior to immersion in liquid nitrogen (LN) at −196 °C:

T1 – Control: no glycerol, no PVS2, no phloroglucinol
T2 – 2 M glycerol (20 min) + PVS2 (10 min)
T3 – 2 M glycerol (20 min) + PVS2 with 1% phloroglucinol (10 min)
T4 – 0.4 M sucrose (20 min) + PVS2 (10 min)
T5 – 0.4 M sucrose (20 min) + PVS2 with 1% phloroglucinol (10 min)
T6 – 2 M glycerol (20 min) + 0.4 M sucrose (20 min) + PVS2 (10 min)
T7 – 2 M glycerol (20 min) + 0.4 M sucrose (20 min) + PVS2 with 1% phloroglucinol (10 min)

### 2.3. Protocorm recovery and survival

After 60 days in LN, cryovials were removed and rapidly rewarmed in 40 °C water bath for 1.5 min. Cryopreservation solutions were removed from cryovials with a sterile plastic disposable transfer pipette under laminar flow hood. Protocorms were rinsed with half-strength MS medium with 1.2 M sucrose (pH 5.7) and transferred to Petri dishes containing half-strength MS medium with 58.5 mM sucrose (pH 5.7) solidified with 0.6% agar. Petri dishes with protocorms were maintained in dark for one week at 27 ± 2 °C, and then transferred to light under 27 ± 2 °C, 120 μmol $m^{-2}$ $s^{-1}$; 16/8 light/dark; 2 × 9A Philips® fluorescent bulbs.

After one month protocorm recovery and survival was determined as the percentage of protocorms showing green color and regrowth. The percentage of protocorms forming leaves and subsequently seedlings was evaluated after 40 and 60 days of culture, respectively. Rooted seedlings were transferred to 125-mL culture flasks for further growth and development using the same medium and under the same conditions as described above. After 30 days, fully developed seedlings were transplanted to trays containing coconut coir (Coco Gro-Brick, OFE International, Miami, FL, USA) as the growing mix. Trays were transferred to a Percival E30B incubator (Percival Scientific, Inc., Perry, IA, USA) at 27 ± 2 °C; 320 μmol $m^{-2}$ $s^{-1}$; 18/6 light/dark; 6 × 9A Philips® fluorescent bulbs for acclimatization. Trays were irrigated every other day with a solution of Peters Orchid Food (Spectrum Group, St. Louis, MO, USA) consisting of 30% total N, 10% $P_2O_5$, 10% $K_2O$, 0.5% Mg, 0.02% B, 0.05% chelated Cu, 0.1% Fe, 0.05% Mn, 0.0005% Mo, and 0.05% Zn. Plant survival was determined by growth and development of seedlings into normal plants, which were successfully transplanted to greenhouse.

### 2.4. Experimental design

The experimental design consisted of 6 treatments plus a control, with 10 replicates of 10 protocorms per treatment/control, with a total of 700 protocorms used per experiment. The entire experiment was repeated once. Protocorm recovery and survival percentage were calculated by the number of protocorms showing green color, forming leaves, and developing into seedlings, respectively. Data was analyzed using analysis of variance (ANOVA) and means were compared using Tukey's range test at $\alpha = 0.01$.

## 3. Results

### 3.1. Protocorm recovery and survival

Protocorms that were immersed in LN for 60 days, but were not submitted to any cryoprotectant treatment (control) did not survive and therefore no plants were obtained.

After 30 days of culture in MS medium, cryopreserved protocorms that were submitted to the different cryoprotectant treatments showed survival ranging from 6 to 68% (Table 1). Protocorms that survived cryopreservation increased to 2–3 mm in diameter and showed a distinct greenish color (Fig. 1A). Percentage of protocorm survival (PS), protocorms forming leaves (PL), protocorms generating seedlings (PGS), number of plants formed (NPF), and seedling height (SH) are shown in Table 1.

Among the treatments, the combination of 2 M glycerol for 20 min, followed by PVS2 for 10 min with 1% phloroglucinol (T3) provided the best results with 68% PS, 65.6% PL, and 62% PGS, with 62 plants formed (Table 1). T3 was significantly different from all other treatments.

The combination of 2 M glycerol for 20 min followed by PVS2 for 10 min alone (T2) returned the second best percentages, but significantly lower (about half the values) than T3. Percentages for T2 were 3- to 5-fold higher than and significantly different from all other treatments for PS (31%), PL (27%) and PGS (23.3%) (Table 1).

Percentages for treatments T4, T5, T6, and T7 were similar to each other and not significantly different. They ranged from 6.2 to 8.9% for PS, from 6.0 to 8.0% for PL, and from 5.3 to 7.8% for PGS (Table 1).

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