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Effect of Sucrose and ABA on Survival Rate of Cryopreserved Protocorms of *Dendrobium gratiosissimum* Rchb.f.

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ABSTRACT

Dendrobium gratiosissimum Rchb.f. is a rare wild orchid. Cryopreservation is an alternative and important tool for *ex situ* preservation of orchid germplasm. In this study, protocorms of *D. gratiosissimum* were induced and cultured on solidified New Dogashima (ND) medium containing 0.3 mg/l NAA for two months. These protocorms were used as explants in cryopreservation using an encapsulation – dehydration method. For treatment with ABA, encapsulated protocorms were precultured in liquid ND medium containing various concentrations of ABA made up of 0, 0.01, 1 and 10 mg/l. For the sucrose treatment, beads were precultured in a semi-solid ND medium containing 0.25 M sucrose in the dark for one week at 4°C and then transferred into liquid ND medium containing 0, 0.5, 0.75 and 1 M of sucrose and then cultured for two days before dehydration. The highest percentage regrowth of cryopreserved explants was observed when precultured in the liquid ND medium supplemented with 1 mg/l ABA for one week and then dehydrated with silica gel in a laminar air-flow for five hours prior to immersion in liquid nitrogen for one hour. Regrowth ability testing performed after two weeks of treatment produced a rate of regrowth of 67% ± 0.5. Consequently, a simple and reliable protocol for the cryopreservation of *D. gratiosissimum* was clearly revealed in this study.

Key words: *Dendrobium gratiosissimum* Rchb.f., cryopreservation, encapsulation-dehydration, sucrose treatment, ABA

INTRODUCTION

Dendrobium gratiosissimum is an epiphytic orchid which is native in Thailand. This species is also considered to be a rare species. Harvested extensively in recent years, it is in danger of extinction in its natural environment. Therefore, *in vitro* propagation and cryopreservation of this species are urgently needed. Cryopreservation is an important method recognized by the FAO, IPGRI, IBPGR and CGIAR for the conservation of plant genetic resources (Teixeira da Silva, 2003). Storage of orchid genetic resources has been achieved through *ex vitro* and *in vitro* propagation. There are several reports on the cryopreservation of *in vitro* orchid cultures (Na and Kondo, 1996; Ishikawa *et al*., 1997; Thammasiri, 2000).

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Several different techniques of cryopreservation (two-step freezing, vitrification and encapsulation-dehydration) have been used to develop cryogenic protocols. The encapsulation-dehydration method was the selected protocol for this research, as it is easy to handle and avoids the use of costly programmable freezers (Martinez *et al.*, 1999). Moreover, encapsulation of explants in alginate beads for cryopreservation has some merit over non-encapsulation, with the advantage of alginate beads being the protection of dried material from mechanical and oxidative stress during storage (Niino and Sakai, 1992). This paper is the first report of a simple and reliable encapsulation-dehydration method for the cryopreservation of *D. gratiosissimum*.

MATERIALS AND METHODS

Plant materials and encapsulation step

The mature capsules (fruits) of *D. gratiosissimum* were collected 60 days after artificial self-pollination at the Department of Biology, Faculty of Science, Khon Kaen University, Thailand. Capsules were surface-sterilized by dipping in 70% ethyl alcohol and flaming immediately four to five times in a laminar air-flow. After surface sterilization and complete drying, the capsules were dissected longitudinally and the seeds were scraped off and transferred to solid ND medium (New Dogashima medium) (Tokuhara and Mii, 1993) with 1% (w/v) sucrose and 0.85% (w/v) agar powder (pH 5.4). Seeds were cultured at 25±2°C under a photoperiod of 16 h light/8 h darkness with a photon dose of 40 μmol m^{-2} s^{-1}. Protocorms derived from seeds reaching a diameter of 1-5 mm were used as explants in all experiments. For the encapsulation step, protocorms were immersed in a 3% (w/v) sodium alginate (Fluka) solution prepared with ND medium free of calcium, growth regulator and iron. Each protocorm was dropped into 100 mM $CaCl_2$ solution, and allowed to polymerize for 10 min. The resulting beads (3-5 mm in diameter) were washed three times in sterile distilled water.

Preculture and desiccation regime; ABA and sucrose treatment

The encapsulated protocorms were precultured based on two methods: ABA; and sucrose. For treatments with ABA, the encapsulated protocorms were precultured in liquid ND medium containing various concentrations of ABA at 0, 0.01, 1 and 10 mg/l for three and seven days. For sucrose treatments, beads were precultured in a semi-solid ND medium containing 0.25 M sucrose in the dark for one week at 4°C and then transferred into different liquid ND media containing 0, 0.5, 0.75 and 1 M of sucrose and cultured for two days (Figure 1). The treated explants were then dried for five hours by placing them in a laminar airflow on filter paper in a Petri dish which contained 20 g of silica gel (30 beads/Petri dish).

Water content, freezing and thawing

The water content of dehydrated explants was determined [water content = (fresh weight – dry weight)/dry weight] (Dumet *et al.*, 2002). After dehydration, the desiccated beads were transferred to 1.8 ml cryotubes and directly immersed into liquid nitrogen and kept in the dark for one hour. The cryotubes were then rapidly warmed in a water bath at 38±2°C for 2 min. The cryopreserved explants were transferred to liquid ND medium containing 0.3 mg/l NAA in the dark. After being cultured in liquid medium for two weeks in the dark, the cryopreserved protocorms were transferred to solid ND medium supplemented with 0.3 mg/l NAA and 1%(w/v) potato extract. Cryopreserved plants were cultured at 25±2°C under a photoperiod of 16 h light/8 h darkness with a photon dose of 40 mmol m^{-2} s^{-1}. (There were ten encapsulated protocorms for each treatment and the experiments were repeated three times).

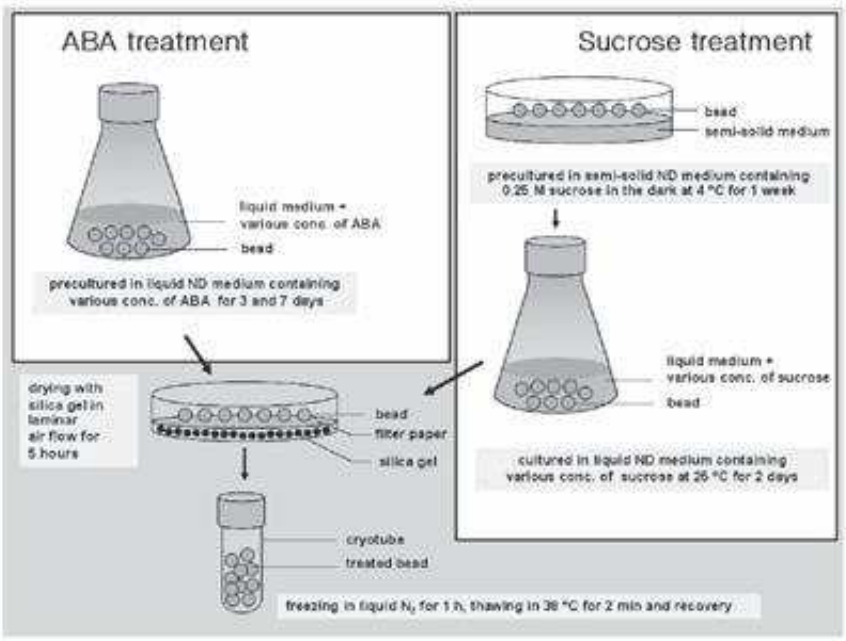

Figure 1 Precultural regime of encapsulated protocorms.

Survival rates and statistical analysis

Survival rates of cryopreserved samples were also determined by the regeneration of cryopreserved protocorms on ND medium for two weeks. The numbers of surviving protocorms were recorded. Each experiment was replicated three times with ten samples per treatment.

Electrolyte leakage

After dehydration, freezing and thawing, each plant sample was transferred into a test tube which contained 10 ml of deionized water. The tissue samples were incubated at 32°C for 2 h and the initial electrical conductivity of the medium (EC_1) was measured using an electrical conductivity meter (WTW TetraCon®325, inoLab pH/Cond Level 1). After EC_1 had been measured, all samples were autoclaved at 121°C for 20 min. The final electrical conductivity of the medium (EC_2) was measured after cooling each sample in a water bath at 25°C. Freeze damage of the tissue was calculated as a percentage of the total electrolyte leakage (EL) ($EL = EC_1/EC_2 \times 100$).

RESULTS

ABA treatment

The effect of ABA concentration and dehydration on the water content and survival rate of cryopreserved protocorms are shown in Figures 2 and 3, respectively. When encapsulated protocorms were treated with various concentrations of ABA for three and seven days without dehydration, a high level of water content was observed ranging from 18.14–22.78 g water/g dry weight, while a low percentage of plants survived after cryopreservation (Figures 2A and 3A). After dehydration of the treated explants for 5 h, the survival of cryopreserved explants improved (Figures 2B and 3B). The water content of dehydrated explants was lower than that of non dehydrated explants. From this result, the highest percentage of survival was achieved (67%) when explants were precultured in 1 mg/l ABA for seven days prior to dehydration by the air drying method, with silica gel for 5 h (Figure 3B). This indicated that adding ABA to the culture medium in

combination with dehydration by the drying method could increase the survival rate of frozen explants.

Sucrose treatment

The effect of the sucrose concentration on the water content and survival rate of plant samples was investigated. The water content of the treated beads was dramatically decreased when precultured in high concentrations of sucrose (Figure 4A). The highest survival percentage (9%) was obtained when the explants were treated with 0.75 M sucrose and air dried in a laminar flow for 5 h (Figure 4B). Under these conditions, the average water content of the treated beads was 0.69 g water/g dry weight. Reducing the water content

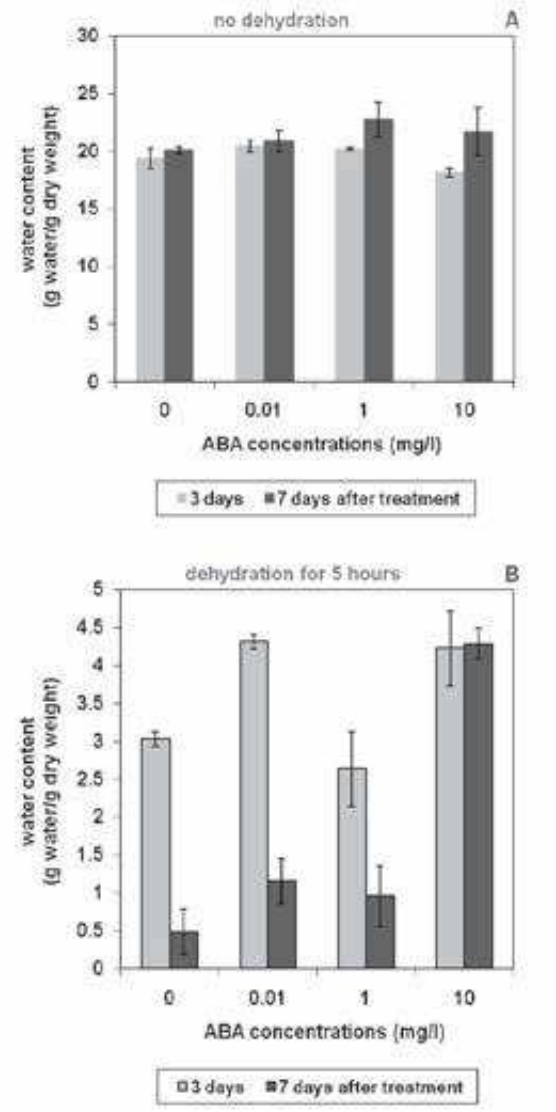

Figure 2 Water content of treated explants in various concentrations of ABA . Vertical bars indicate the standard error of means from n = 3.

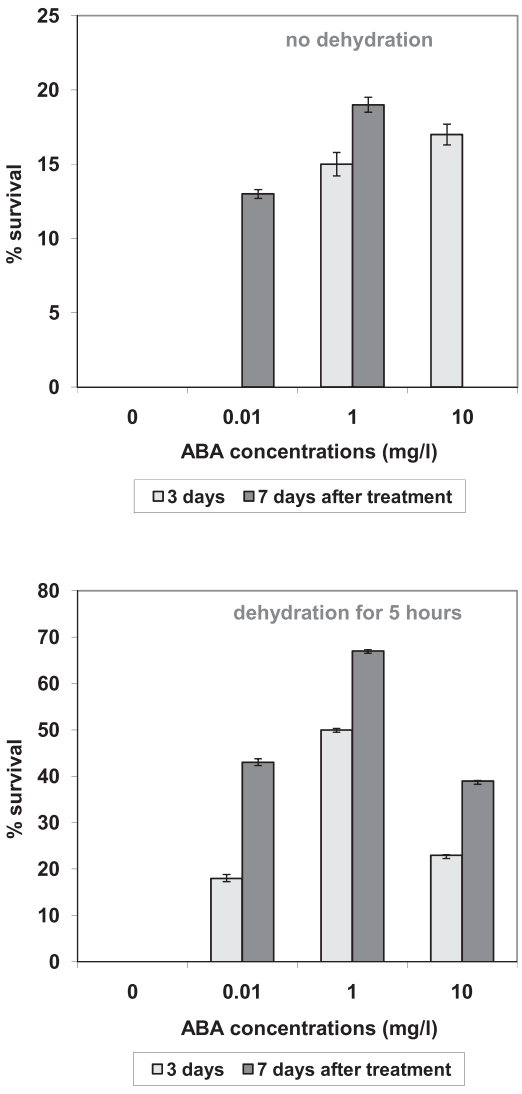

Figure 3 Effect of ABA and dehydration on survival rate of cryopreserved protocorms.

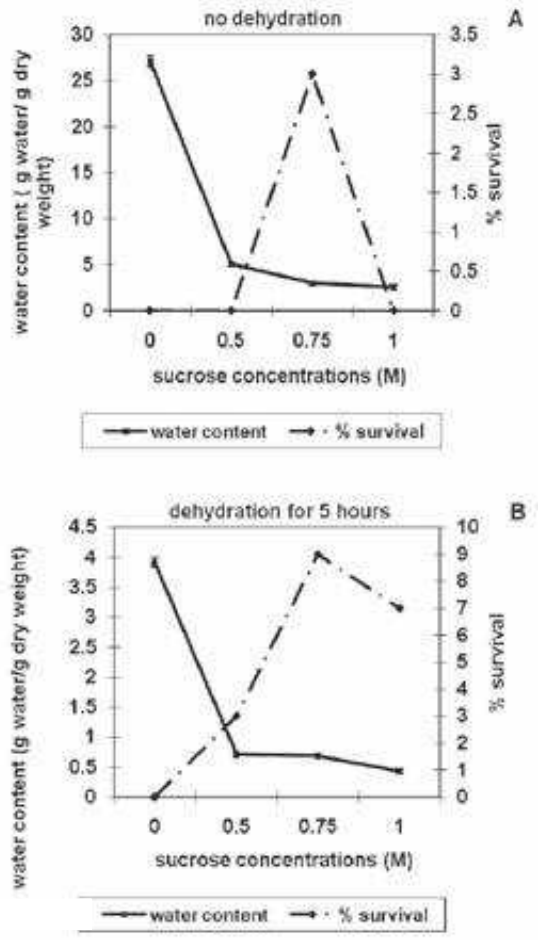

Figure 4 Effect of sucrose and dehydration on water content and survival rate of cryopreserved protocorms. Vertical bars indicated the standard error of means from n = 3.

in plant cells and tissues was also important before freezing. This result was similar to that observed in the ABA experiment. The water content of dehydrated plant cells and tissues was significantly decreased. However, a low level of water content could not improve the survival rate of the cryopreserved plants in this experiment.

Electrolyte leakage

Measurement of the electrolyte leakage for *D. gratiosissimum* samples (treated with 1 mg/l ABA for seven days and dehydrated with silica gel for 5 h) was investigated. The percentage of EL under the three conditions is shown in Table 1, indicating it was significantly decreased in the

Table 1 Electrolyte leakage percentage of treated beads with 1 mg/l ABA for seven days and dehydration for 5 h.

Treatments	% Electrolyte leakage
control (no dehydration, non freezing)	82.4 ± 1.2 ^{a}
dehydration without freezing	69.8 ± 2.9 ^{b}
dehydration with freezing	77.1 ± 2.5 ^{ab}

Means with the same letter are not significantly different at $p < 0.05$ (LSD test)

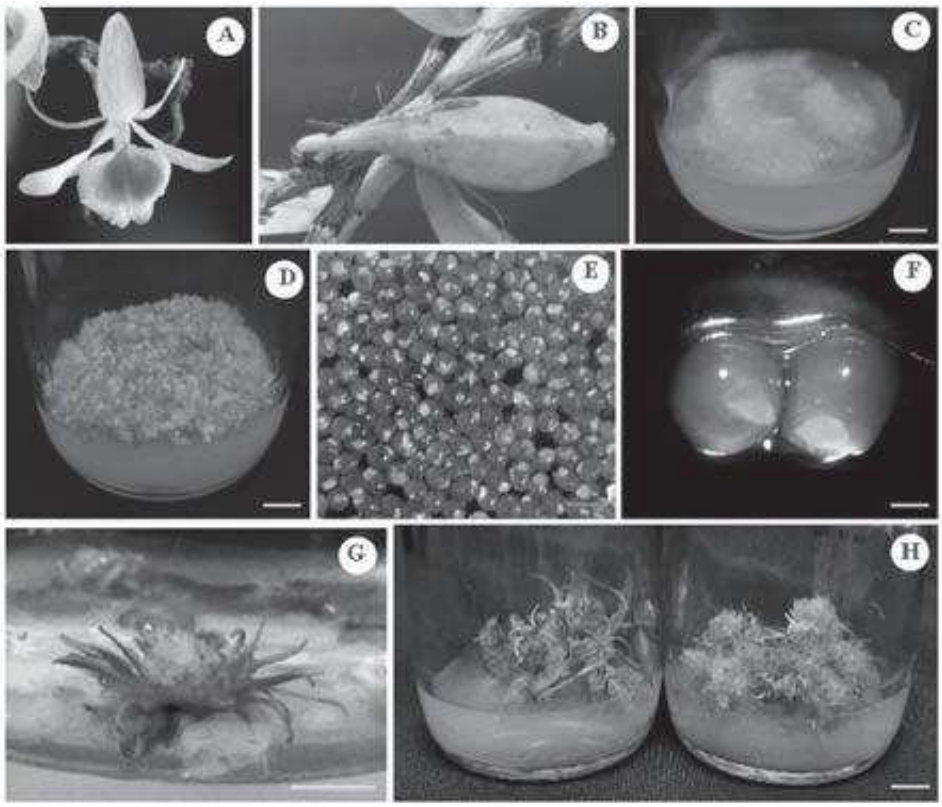

Figure 5 (A-H): Plant samples of *D. gratiosissimum* ; flowering stage (A), fruit stage (B), seed germination (C), protocorms used in this study (D), encapsulated protocorms (E-F), cryopreserved plantlets after recovery for three months (G), plantlets derived from non cryopreserved (H: left) and cryopreserved protocorms (H: right) after regrowth in ND medium containing 0.3 mg/l NAA for three months (C-D scale bar = 5 mm; E-F scale bar = 1 mm; G-H scale bar = 5 mm).

dehydrated plant samples. The percentage of EL in the dehydrated samples without freezing and with freezing was 69.8% and 77.1%, respectively, whereas the EL of the control samples was 82.4%. This result showed that treatment with ABA and dehydration was a suitable process for preparing plant samples for cryopreservation.

Regeneration of cryopreserved explants

In general, protocorms of *D. gratiosissimum* were easily regenerated to plantlets by using an ND medium supplemented with 10% (w/v) potato extract. The result was similar with the regeneration of cryopreserved protocorms. However, cryo-storage explants took a longer time for regeneration than non-frozen explants and this could be attributed to a partial desiccation during cryo-storage. Moreover, encapsulation might induce a short delay in the development of plant samples in comparison with non-encapsulated plants. Figure 5 shows the regeneration of non-frozen and frozen plants. Although control plants grew faster than cryopreserved plants, their morphology was still normal. However, repeating culturing several times may have an effect on somaclonal variation in the plant tissue.

DISCUSSION

The effects of ABA on the water content and survival ability of cryopreserved plants were investigated. It was found that a small amount of ABA could provide some degree of desiccation tolerance. In general, dehydration tolerance could be increased by adding ABA to the culture medium. ABA has been used to induce dehydration/desiccation tolerance and has also been related to the accumulation of various storage reserves such as starch, sucrose, proteins and lipids (Nieves *et al*., 2001). Moreover, ABA is considered to be one of the most important hormones involved in plant response to cold stress (Lee *et al*., 1997; Aroca *et al*., 2003). The protective mechanism of ABA against chilling for example has been identified with the induction of antioxidant enzymes (Prasad *et al*., 1994) and the modulation of polyamine levels (Lee *et al*., 1997). Although the addition of ABA to the medium has generally been found to promote desiccation tolerance, ABA has been shown to also inhibit embryo growth and development (Hoekstra *et al*., 2001).

ABA appears to restrict metabolism, thus reducing the uptake and utilization of sugars and allowing a high endogenous sugar content to be maintained in the slowly dried samples. In addition, the restriction of metabolism is expected to reduce the generation of free radicals during drying. The stressful conditions stimulate the expression of dehydration-linked proteins. Lack of either sugars or dehydration proteins has caused destabilization of the membrane (Hoekstra *et al*., 2001). There are many reports about the effect of ABA as a promotor of freezing avoidance in cells and organs (Na and Kondo, 1996; Nieves *et al*., 2001; Bian *et al*., 2002). The results of this study are similar to those in reports that consider ABA could possibly induce freezing avoidance. Cryopreserved explants can be easily regenerated to plantlets after being cultured in ND medium within two months (Figure 5).

Cryopreservation of *D. gratiosissimum* protocorms with the base protocol (Figure 1) resulted in only a 9% survival when precultured in 0.75 M sucrose and dehydrated for 5 h, as the high amount of crystallisable water in the tissue may have led to the formation of lethal ice crystals. The introduction of a dehydration step in the protocol was not sufficient to improve the result in this orchid species. For encapsulated protocorms not treated with sucrose, the water content after dehydration was high (approximately 3.9 g water/g dry weight), thus no survival was observed after cryopreservation. The water content of treated beads with 0.75 M sucrose was low (approximately 0.7 g water/g dry weight) and the survival percentage of treated samples after recovery from liquid nitrogen was 9%.

Generally, the water content of treated explants was also important in determining the survival rate after recovery from liquid nitrogen, although the water content of treated explants in this experiment seemed to be optimized for cryopreservation. The water content of cryopreserved explants was dependent on many conditions such as: plant species, developmental stage, preculture regime and recovery protocols.

Sucrose was important in the protection of the plant membrane and also the formation of a carbohydrate glass with a high melting temperature (Oliver *et al*., 2002). Moreover, Buitink and Leoprince (2004) also found that the formation of a glass during the dehydration step prevented serious damage to cells. Some types of sugar can protect cells or tissues from freezing damage. The high viscosity of sugar solutions inhibits chemical reactions within the cells, although vitrification during freezing is a key factor for cryopreservation.

In general, a lower water content in plant material prevented vitrification of the cytoplasm during freezing (Niino and Sakai, 1992). A similar result was found in this study, as additional drying was beneficial for *D. gratiosissimum* orchids. Protocorms in alginate beads that were not

sufficiently dried (more than 1 g water/g dry weight) could not readily survive. This may have been caused by a large amount of crystallisable water in the cytoplasm. It was concluded that the efficiency of cryoprotection was clearly related to the water content.

The freezing tolerance of orchid samples, as measured by the percent EL, was influenced by the dehydration period. The EL measurement has been commonly used to compare the oxidative stress tolerance of plant cultivars (Chai *et al*., 2005). In several other crops, assessing the membrane stability by measuring EL following a stimulated freeze-thaw stress was a rapid and reliable screening technique for freezing tolerance (Chai *et al*., 2005). Mohapatra *et al*. (1988) suggested that abscisic acid (ABA) was involved in plant adaptation to freezing stress. Similar to this study, the application of ABA can induce tolerance in whole plants and cell cultures. The stress-induced modifications in tissue with low water potential are converted into the following metabolic responses: active membrane transport (Palta, 1989); variation in synthesis and distribution of hormones (Mohapatra *et al*., 1988); variation in extension growth (Nomanie and Boyer, 1989); protein synthesis (Jonak *et al*., 1996); and sucrose partitioning (Oparka and Wright, 1988). Walton (1980) suggested that ABA may inhibit the passage of ions across cell membranes.

CONCLUSIONS

Protocorms of *D. gratiosissimum* were induced and cultured on solidified New Dogashima (ND) medium containing 0.3 mg/l NAA for two months. The preservation of protocorms of this orchid species was possible through a cryogenic procedure. The survival rate of cryopreserved plants in this study after treatment with sucrose was low in comparison with the ABA treatment. It was concluded that the ABA treatment and the air-drying method were suitable for cryopreservation of *D. gratiosissimum*. The addition of ABA in the preculture medium has proved to be the key to success in the cryopreservation method. A high survival rate was obtained after adding 1 mg/l ABA in the preculture medium and then culturing for 7 days in the dark prior to drying in a laminar airflow with silica gel for 5 h. However, more experimental work is needed to optimize the high survival rate of protocorms after recovery from liquid nitrogen. Moreover, DNA analysis or genetic fidelity of non-cryopreserved and cryopreserved explants should be studied in the future. This study will provide useful information for planning and implementing related projects in the future.

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