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Original Article

Characterization of the Second Generation Cryopreserved *Dendrobium* Bobby Messina Using Histological and RAPD Analyses

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Abstract

Key words: morphology, histology, molecular analysis, *Dendrobium*, cryopreservation

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This study was conducted to detect the morphological, histological and molecular differences in the second generation of the PVS2 cryopreserved *Dendrobium* Bobby Messina [DBM] (18 months old culture) plantlets. Morphological analyses indicated that similarities and differences in cryopreserved DBM plantlets comparing to control stock culture based on selected morphological criteria. Morphological criteria, such as root length, number of shoot per explant and shoot length displayed differences, while the other three criteria, leaf diameter, leaf length and PLBs size were similar in cryopreserved compared to the control stock culture plant. Higher amount of homogenous cell population and denser cytoplasm were observed in cryopreserved PLBs compared to control stock culture PLBs based on histological analysis. This suggests the existance of somatic embryogenesis development mechanism taking place during the recovery and regeneration of the cryopreserved PLBs. However, RAPD analyses based on 10 primers indicated that cryopreserved DBM regenerated from vitrification method generated a total of 20 to 39.9% polymorphic bands as compared to stock culture indicating potential somaclonal variation. Hence, an increase percentage of polymorphics bands in cryopreserved plantlets 18 months post cryopreservation as compared to previous report of 10% polymorphic bands in cryopreserved DBM 3 months post cryopreservation.

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Introduction

Orchids are widely distributed and can be found in all apart from the most extreme terrestrial environment. Recently, orchids are became main ornamental plants in the world as cut flowers or blooming potted plants (Sujjaritthurakarn & Kanchanapoom, 2011). *Dendrobium* orchids are the largest genus in the family Orchidaceae, have become a major cut flower crop for export (Sujjaritthurakarn & Kanchanapoom, 2011).

Many orchid species are now at the edge of extinction as a result of extensive interruption of their natural habitat and unsystematic harvesting of naturally growing plants (Poobathy *et al*., 2012). Therefore, immediate action should be taken to ensure the continuation of the orchid species, especially wild orchid. So, critical mission nowadays is to protect as well as

restore and use sustainably of orchids. Tissue culture technique has been established to mass produce orchid species (Griesbach, 2002). Micropropagation by tissue culture technique provides long-term germplasm storage and production of difficult-to-propagate species with *in vitro* germination. However, maintenance of *in vitro* germplasm will always face the risk of contamination.

Cryopreservation is an ideal technique for long-term conservation of plant genetic resources, using ultra-low temperature (liquid nitrogen, -196°C) (Kaviani, 2011). Cryopreservation is secure and low-budget for long-term conservation of germplasm. The entire metabolic processed and cellular division are ceased at this ultra-low temperature (Engelmann, 2004). Thus, the plant material can be kept without any changes for a theoretically unlimited period of time. Additionally, cultures are stored in a little volume, protected from contamination, and require negligible maintenance (Engelmann, 2004).

Consequently, there is a great potential in develop a method to detect somaclonal variation of orchids regenerated following cryopreservation procedure. It is important to have an appropriate management of a regenerated cryopreserved plant collection including determination of the genetic composition of the plant material and a periodical assessment of the preserved material. According to Antony *et al*. (2012), the RAPD results demonstrated that the genetic stability of *Dendrobium* Bobby Messina PLBs was retained following cryopreservation based on from the 6 chosen primers.

Thus, the aim here is to detect any possibilities of periodical changes in genetic changes of the 2nd generation of the regenerated cryopreserved PLBs after 18 months. In addition, there is limited number of genetic studies in *Dendrobium* Bobby Messina. Therefore, the genetic fidelity of the cryopreserved explants of *Dendrobium* Bobby Messina is important to be studied.

Materials and Methods

Plant materials. *In vitro* cultures of regenerated plantlets and protocorm-like bodies (PLBs) of *Dendrobium* Bobby Messina (DBM) used for histological and RAPD analysis. Samples taken were based on 18 months old culture from the second generation of DBM regenerated from PVS2 vitrification method by Antony *et al*. (2012). Three cryopreserved DBM and control stock culture were chosen for morphological study, histological analysis and RAPD analysis. Three cryopreserved DBM were labelled as DBVG2P1, DBVG2P2 and DBVG2P3, while the control stock culture was labelled as C. The full explanation of plant identification is as below:

DBVG2P1, DBVG2P2, DBVG2P3 and C
DB = *Dendrobium* Bobby Messina
V = PVS2 Cryopreservation
G2 = Generation No.2
P1 = Plant Line 1
P2 = Plant Line 2
P3 = Plant Line 3
C = Control stock culture of *Dendrobium* Bobby Messina

Morphological analysis. DBVG2P1, DBVG2P2, DBVG2P1 and stock culture C plantlets were selected to study its morphology. Morphology of the chosen plant was studied based on leaf morphology (leaves diameter and leaves length), root morphological (root length), shoot morphology (number of shoot per explants and shoot length) and PLBs size.

Histological analysis. Fixation, dehydration, clearing, impregnation, embedding, microtomy, staining and mounting were the important steps in preparation of histological slides of PLBs for histological analysis. Three cryopreserved PLBs (DBVG2P1, DBVG2P2 and DBVG2P1) and one control (stock culture C) PLBs were chosen.

DNA isolation. PLBs from DBVG2P1, DBVG2P2, DBVG2P3 and stock culture C of DBM were chosen for DNA extraction procedure prior to RAPD analysis. The DNA extraction kit, Genomic DNA Mini Kit (Plant; Geneaid Biotech Ltd., Taipei Country, Taiwan, Republic of China) was used to extract the genomic DNA from the samples.

RAPD analysis of DBVG2P1, DBVG2P2, and DBVG2P3 plants compared to stock culture C. For RAPD analysis, 20 oligonucleotide primers were chosen based on the production of both highly scoring and polymorphic bands in the molecular analysis of DBM by Antony *et al*. (2012). However, only 10 primers favoured good results in producing bands in DBM

Table 1. Primers used for RAPD analysis of cryopreserved and stock culture of *Dendrobium* Bobby Messina.

Primers	Sequence (5' – 3')	G + C Content (%)	T_M (°C)
OPA04	AATCGGGCTG	60	35.1
OPAW13	CTACGATGCC	60	31.9
OPAW17	TGCTGCTGCC	70	36.7
OPB02	TGATCCCTGG	60	32.2
OPB12	CCTTGACGCA	60	35.7
OPB13	TCCCCCCGCT	70	46.3
OPB18	CCACAGCAGT	60	29.8
OPG14	GGATGAGACC	70	30.0
OPB06	TGCTCTGCCC	70	39.8
OPZ09	CACCCCAGTC	70	35.8

(Table 1). The primers were selected and made by Integrated DNA Technology (Coralville, IA, U.S.). The total volume of RAPD-PCR reaction will be 20 µL. The mixture consists of 3 µL DNA, one unit of *Taq* DNA polymerase (DreamTaqTM DNA Polymerase, 5 U/µL), 0.5 µL of 10 mM deoxyribonucleotide triphosphate (dNTP; dNTP Mix), 2 µL of 10x PCR buffer (10x DreamTaqTM Buffer) containing 100 mM Tris-Hydrochloride (Tris-HCl) at pH 8.8, 500 mM potassium hydrochloride (KCl), 0.8 & (v/v) Nonidet P40 and 20 mM magnesium chloride ($MgCl_2$), 1 µL from 10 µM of a single primer and nuclease-free distilled deionised water was be used to top up the PCR reaction volume to 20 µL.

The MyCyclerTM Thermal Cycler (Bio-Rad Laboratories, Inc., USA) was used to amplify the DNA samples. The PCR conditions was set with initial denaturation at 95°C for 3 minutes, temperature profile was performed for 35 cycles with denaturation at 95°C for 30 seconds, annealing at 5°C below each primer's melting temperature (Tm) for 30 seconds and extension at 72°C for 1 minute followed by a final extension cycle at 72°C for 10 minutes. The PCR product was kept at 4°C and finally stored at -40°C prior to electrophoresis. Agarose gel at 1.5% was used for analyzing the RAPD-PCR products by electrophoresis with 1x TBE buffer in the electrophoresis machine (Wide Mini SubCell® GT Agarose Gel Electrophoresis System, Bio-Rad Laboratories, Inc., USA). All the samples were loaded in the gel wells with a 100 bp DNA ladder GeneDirex (USA). The evaluation of the DNA fragment patterns of each sample in the cryopreserved PLBs was performed by calculating the Similarity Indices of the cryopreserved PLBs as compared to the DNA bands of control PLBs. The reproducible bands were scored manually as 1 or 0 for the presence or absence of the bands.

The coefficients of similarity between the treatment and stock culture were calculated using the formula below:

$$SI = 2N_{ij} / N_i + N_j$$

where,

SI = Similarity Index

N_{ij} = Number of monomorphic bands between the stock culture and cryopreserved PLBs

N_i = Total number of bands in the PLBs stock culture

N_j = Total number of bands in the cryopreserved PLBs

Results and Discussion

Cryopreservations by cold treatments have been reported to induce changes in plant tissues, which can adversely affect their viability and genetic fidelity (Berjak & Pammenter, 2008). The present study was undertaken in the aim of detecting induced changes at the morphological, histological or molecular level following 18 months of recovery from cryopreservation. In our analysis we discovered morphological differences of regenerated cryopreserved DBM (DBVG2P1, DBVG2P2 or DBVG2P3) comparative to the control stock culture C in some morphological criteria such as root length, number of shoot per explants and shoot length. However, we discovered some morphological similarities on the regenerated cryopreserved DBM (DBVG2P1, DBVG2P2 or DBVG2P3) comparative to the control stock culture C on

selected morphological criteria such as leaf diameter, leaf, and length and PLB sizes (data not shown).

As been reviewed by Rival *et al*. (2010), morphological analysis for three coconut cultivars Nias Yellow Dwarf (NYD), Sagerat Orange Dwarf (SOD) and Takome Tall (TKT), plantlets produced from cryopreserved embryos were morphologically similar to their non-cryopreserved embryos. Some changes were observed in morphology following cryopreservation including flower colour alterations in *Dendranthema grandiflorum* plants regenerated from frozen shoot tips (Fukai *et al*., 1994).

Histological observation revealed accumulation of mass homogenous cell population and denser cytoplasm in regenerated cryopreserved DBM (DBVG2P1, DBVG2P2 or DBVG2P3) comparative to the control stock culture C (Fig. 1). The mass cell population could be attributed due to selection process; elimination of non embryonic cells from cultures or it may be due to increase synchrony of development of embryonic cells (Salaj *et al*., 2011). Hence, similar finding in present study show that cryopreserved PLBs of DBM retain high meristematic cells growing property after 18 months regenerated from vitrification.

Total of polymorphic bands found in 10 primers tested in DBVG2P1, DBVG2P2 and DBVG2P3 were in the range of 20 to 39.9%, compared to Antony *et al*. (2012) which reported that polymorphic bands in cryopreseved PLBs were just 10% (data not shown).

These changes in profiles may be an indication of somaclonal variation associated with DMSO toxicity (PVS2), freezing or thawing induced injury or the regeneration phase. In this study, genetic variation could be detected in cryopreserved DBM after a period of 18 months. Antony *et al*. (2012)

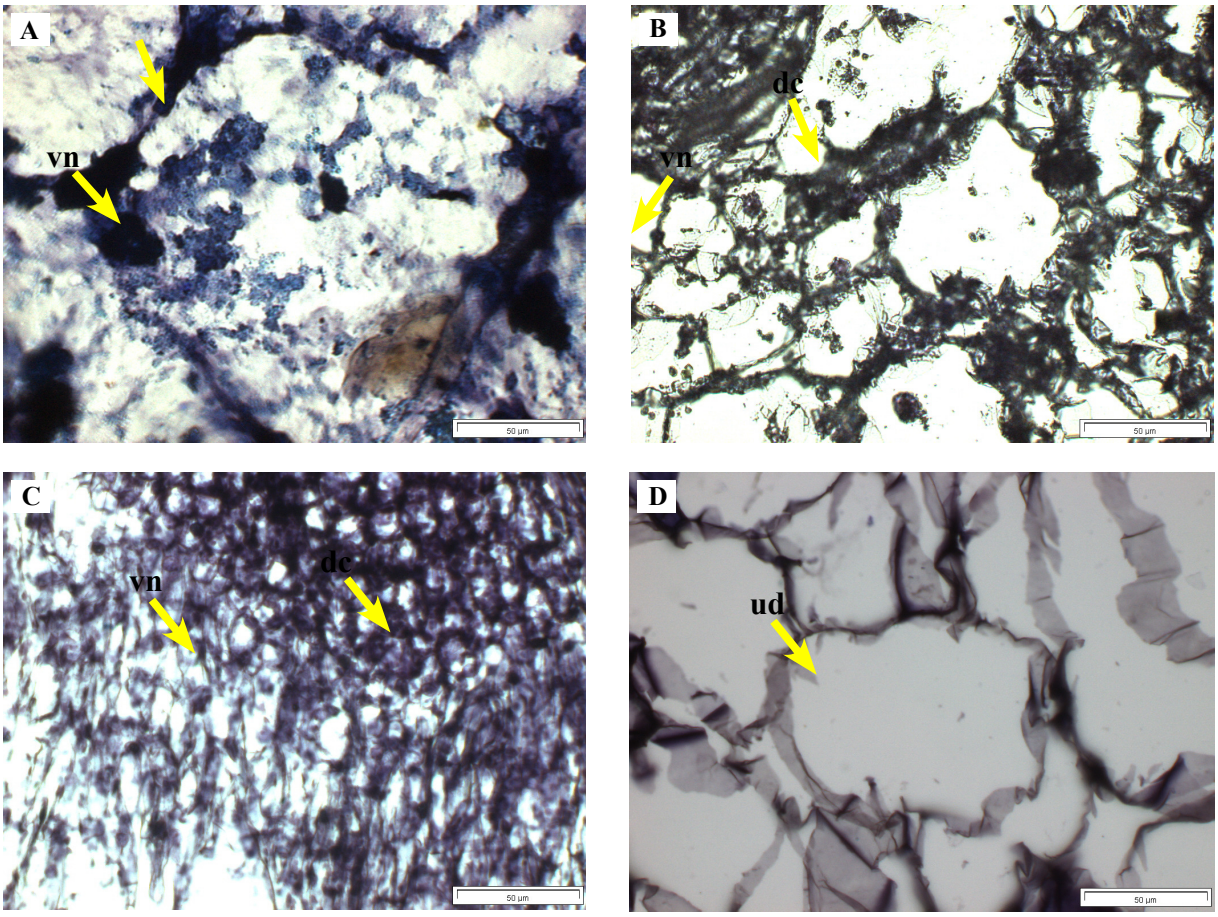

Figure 1. Histological section of *Dendrobium* Bobby Messina's PLBs (50µm). (A) The cross section of PLBs from DVBG2P1; (B) The cross section of PLBs from DBVG2P2; (C) The cross section of PLBs from DBVG2P3; (D) The cross section of PLBs stock culture C. dc – denser cytoplasm, ud – undamaged cells with polyhedral shaped, vn – voluminous nucleus.

reported that polymorphism was not detected in cryopreservation of DBM after three months of cryostorage. Changes in DNA methylation observed after cryopreservation could have occurred in response to the pre-treatment dehydration step, the cryopreservation step or the tissue culture recovery step. Indeed, water stress induced by tissue dehydration has been shown to cause an increase or a decrease in global DNA methylation rates (Lukens & Zhan, 2007; Johnston *et al*., 2009). The tissue culture steps needed for the recovery of plantlets after cryopreservation could also be responsible for measurable changes in DNA methylation status (Kaeppler & Phillips, 1993; Rani & Raina, 2000). It is conceivable that these genomic variations resulted from adaptive responses modulated by the binding or dissociation of regulatory proteins that can inhibit or permit the binding of primer and/or transcription factors to amplification start sites (Kaity *et al*., 2009).

This suggests that the somatic embryogenic tissue that recovered following cryopreservation composed of a mixture of genetically altered and unaltered cells, with different populations of genetically altered cells present at different stages of *in vitro* culture (DeVerno *et al*., 1994).

Conclusion

This study was carried out to detect morphological changes, histological changes and molecular changes of selected cryopreserved *Dendrobium* Bobby Messina (DBM) plantlets. Both morphological differences and similarities were detected in the regenerated cryopreserved DBM (DBVG2P1, DBVG2P2 or DBVG2P3) comparative to the control stock culture C. Histological analysis revealed accumulation of mass homogenous cell population and denser cytoplasm in regenerated cryopreserved DBM (DBVG2P1, DBVG2P2 or DBVG2P3) comparative to the control stock culture C.

In this study, there was an increase in percentage of polymorphics bands in cryopreserved DBM 18 months post cryopreservation comparative to the study that was previously done by Antony *et al*. (2012), which indicated 10% of polymorphic bands in cryopreserved DBM three months post cryopreservation.

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