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ORIGINAL ARTICLE

A Novel Approach for Preliminary PVS2 Vitrification Optimization Parameters of *Dendrobium* Sonia-28 Orchid with Evan Blue Staining

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ABSTRACT

Protocorm-like bodies (PLBs) of *Dendrobium* sonia-28, an orchid hybrid which is a commercial ornamental plant was cryopreserved by vitrification technique. The effects of PLB sizes, sucrose concentrations in preculture media and exposure to PVS 2 duration were the main parameters assessed in vitrification technique. The viability of the cryopreserved PLBs were determined by 2,3,5-triphenyltetrazolium chloride (TTC) and Evan Blue Staining assay after two weeks of recovery. In vitrification technique, PLBs with the size of 3-4 mm, precultured in half strength semi-solid MS media supplemented with 0.6M sucrose and dehydrated in PVS 2 solution for 20 minutes at 0°C showed the best viability rate. The best parameters were chosen for positive and negative storage in LN and further biochemical content analysis (total soluble protein and peroxidases activities) were conducted to investigate the physiological responses of the PLBs after cryopreservation.

Key words: PVS2. Orchid. Protocorm-like bodies. Evan Blue

Introduction

Cryopreservation is a modern technique which is used to preserve endangered or commercially important germplasm. The main purpose of cryopreservation is to conserve good quality cell as well as multiply specific traits of the species in a large quantity for ecological and economical purposes. Generally, the cultivation of plants is based on unique characteristics of the plants such as unique flower shapes, fragrances, colours, and keeping quality of several orchid genera which is commercially important.Orchid is one of the commercially important plants. From variety orchid species, *Dendrobium* sonia-28 has been cultivated for decade as it is widely known as commercial cut flower cultivars through direct organogenesis from *in vitro* derived foliar explants [11,22-23].

Cryopreservation is the preservation of cells by arresting the cell division and biochemical process of the plant [14]. Cryopreservation technique implies the storage of plant material in liquid nitrogen. There are two methods of plant cryopreservation, namely plant vitrification method and encapsulation-dehydration method. Plant vitrification method is done by using plant vitrification solution such as PVS2 and PVS3 which act as a cryoprotectant to the plant cells before being stored at the temperature of liquid nitrogen (-196°C). The cryopreserved plant can be stored without any genetic changes theoretically inside the liquid nitrogen as long as 100 years provided without any shortage of liquid nitrogen in the nitrogen tank. In cryopreservation, cryoprotectants such as sucrose, DMSO, Ethylene glycol, glycerol are used to protect

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the plant material from damaging effects due to freezing. However, the use of cryoprotectant can be responsible for loss of viability on the cryopreserved cells. Therefore, the period of cryoprotectant exposure to the cells need to be studied so that it does not bring any side effect to the cryopreserved cell. The sucrose concentration of preculture treatment must be taken into consideration.

There is no report so far on cryopreservation of *Dendrobium* sonia-28 using Evan Blue method and therefore the aim of this study is to develop a simple cryopreservation protocol for *Dendrobium* sonia-28 with PVS2 method.

Materials and methods

In vitro cultures of protocorm-like bodies (PLBs) of *Dendrobium* sonia-28 (Fig. 1) were used as the starting material to initiate multiplication of PLBs for this study. The cultures were incubated at 25±2ºC in a 16-h photoperiod under cool white fluorescent lamps (Philips TLD, 36 W) at 150 µmol $m^{-2}s^{-1}$. In this study, the age of the PLBs explant used was 3-4 weeks old.

Fig. 1: Stock culture of *in vitro* grown *Dendrobium* sonia-28 orchid protocorm- like body. (Bar = 1.0cm)

Preculture

For optimization of PLBs size range, PLBs with size range of 1-2 and 3-4 mm were selected from 4 weeks old culture and precultured respectively in half strength semi-solid MS media [13] supplemented with 0.5M sucrose at 25°C for 24 hours under 16 hours photoperiod. For optimization of preculture sucrose concentration, PLBs selected from 4 weeks old culture were precultured in half strength semi-solid MS media supplemented with different concentration of sucrose (0.0, 0.2, 0.4, 0.6 and 0.8) at 25°C for 24 hours under 16 hours photoperiod.

Cryopreservation

After preculture, the PLBs were dehydrated with 1.5 ml of loading solution (2M glycerol supplemented with 0.4 M sucrose in half strength liquid MS media) in 2 ml cryotubes at 25 ºC for 20 minutes. Then, the PLBs were then dehydrated in 1.5 ml PVS2 solution [17] at 0 ºC for 0, 5, 10, 20 minutes. PVS2 solution contains 30 % (w/v) glycerol, 15% (w/v) ethylene glycol and 15% (w/v) DMSO supplemented with 0.4 M sucrose in half strength liquid MS medium. After dehydration in PVS2 solution at 0ºC for 0-20 minutes, the PLBs were resuspended in fresh 1.5 ml of ice cold PVS2 and were directly plugged into liquid nitrogen for minimum of 24 hours. Cryopreserved PLBs were thawed at 40°C for 90 seconds. After thawing, PVS2 solution was drained from the cryotubes and replaced with 1.5 ml of half strength liquid MS media supplemented with 1.2M sucrose in which the PLBs were washed at 25 ºC for 20 minutes. In the non-cryopreserved experiments, the PLBs were subjected to all treatment except PVS2 treatment, cryostrorage and thawing procedures. Both cryopreserved and non cryopreserved PLBs were then transferred onto a layer of sterilized filter paper disc over half strength semi-solid MS media supplemented with 2% sucrose and 2.75 g/L gelrite without the presence of any growth regulators at 25°C for 24 hours. After 1 day, the PLBs were transferred onto half strength semi-solid MS media supplemented with 2% sucrose and 2.75 g/L gelrite without the presence of any growth regulators. At the 1st week, the PLBs were kept in dark condition; 2nd week were kept in dim light condition and the 3rd week were exposed to 16 hours photoperiod.

Determination of viability and regrowth of the cryopreserved PLBs: 2,3,5-triphenyltetrazolium chloride (TTC) method

Viability of cryopreserved cells was determined by using TTC assay [7].

Evans Blue assay

The Evans Blue assay method was used to determine the viability of PLBs.

Total soluble protein

The Bradford method was used to determine the protein content of the PLBs extract [2].

Peroxidase activity

The same extracts for protein determination was used for the assay of peroxidase and was adapted from Flocco and Giulietti [5].

Statistic analysis

Each experiment was repeated twice and consisted of 3 replicates per treatment with 10 samples each. All data were subjected to independent sample t-test, one way ANOVA and means were compared using tukey HSD test.

Results and Discussion

Importance of suitable PLBs size

Protocorm-like body (PLBs) is a somatic embryo which is widely used in cryopreservation [22]. In cryopreservation, the sizes of the PLBs have to be taken into consideration. TTC viability test has been used instead of Evans Blue method since it has relatively low extinction coefficients. In this study, TTC viability test was used as the tiny sized PLBs cannot be shown clearly under Evans blue stain. Among the various assays based on enzymatic function, reduction of TTC has been used most frequently as a biochemical test for the viability of an explant. TTC will be absorbed by living cells, where it reacts with hydrogen atoms released by the dehydrogenase enzymes during cellular respiration [9]. Usual enzymatic tests measure the ability of viable cells in reducing 2,3,5-triphenyltetrazolium chloride (TTC) to red formazan in mitochondria [19]. In this study, according to TTC viability assay, 3-4 mm of cryopreserved and non-cryopreserved PLBs withstood cryopreservation better comparative to 1-2 mm cryopreserved and non-cryopreserved PLBs (Fig. 2, 3). Besides, PLBs with size range of 3-4 mm appeared more viable compared to 1-2 mm PLBs. This morphological observation suggested that the optimised PLBs size was 3-4 mm instead of 1-2 mm. In addition, this also suggested that TTC viability test which was enzymatic based appear to be more suitable viability test in this study.

Importance of suitable preculture sucrose concentration

The most suitable preculture sucrose concentration was determined by Evans Blue viability test. Evans Blue viability test was chosen for the following study as it is not enzymatic based test. However, it is dependent on not only cell viability but also on the metabolic state of the cells [12]. In addition, some of the enzymes involved (i.e., dehydrogenases) are not informative with respect to the growth ability of a cell because their activity can persist even when cellular growth is no longer possible [18]. Moreover, previous studies have shown that sucrose and glucose induce desiccation tolerance which were very effective by means of osmotic dehydration [21].Therefore, it is more suitable to use non-enzymatic test on the study which involves chemical exposure (PVS2 exzposure duration) and effect of concentration (sucrose concentration in preculture treatment).

In addition, Evans Blue which acts as non-permeating or exclusion dyes can leak through ruptured membranes and stain the contents of dead cells. Hence, it is often favored for indicating cell death [1]. Evans blue, however, has been compared in several studies and has been used extensively for indicating cell death in microscopic studies [18]. Evans blue stains the dead cell wall. Hence, the samples which have higher absorbance value indicating the higher rate of mortality. In this context, samples which obtained lower absorbance value indicating higher viability rate.

Preculture is used to increase tolerance of explants to dehydration and subsequent freezing in liquid nitrogen. The type and concentration of sugar compound used in the preculture medium appears to be important in cryopreservation.

Peculture with a high sucrose concentration (0.75–1.0 M) increases total soluble protein and sugar contents in the treated tissues, resulting in a twofold increase in the survival of cryopreserved cells as compared to preculture with a low sucrose concentration (0.25 M) [26]. The increase in protein level is considered as one of the early physiological responses of osmotically stressed cells, which may be related to the improved survival after cryopreservation. In this study, the most suitable preculture sucrose concentration is 0.6M (Fig. 4).

Importance of suitable PVS2 exposure duration at 0°C

One of the key to successful cryopreservation by vitrification is the careful control of dehydration and prevention of injury by chemical toxicity [16,24]. Optimization the time of exposure or the temperature during exposure to PVS2 is important for producing a high level of shoot formation after vitrification [16]. PVS2 has a remarkable ability to limit freezing of water. This prevents water freezing without forming glass as an indication that PVS2 imparts its effect in 'pre-vitrified' solutions. These solutions have sufficient mobility to permeate cells at 0°C and allow water to be displaced. At lower temperatures, these cryoprotective solutions restrict molecular reorganization necessary to nucleate water and allow ice crystals to grow [25].

However, PVS2 solution which contains DMSO is toxic to plant cell. This cryoprotectant toxicity may cause cell injury in association with distinct ultrastructural changes, especially in the plasma membranes [10]. Therefore, it is vital to optimize the PVS2 exposure duration in order to reduce toxicity to the plant. In addition, the optimal exposure time with PVS2 varies with plant species and depends on the temperature during exposure [26].

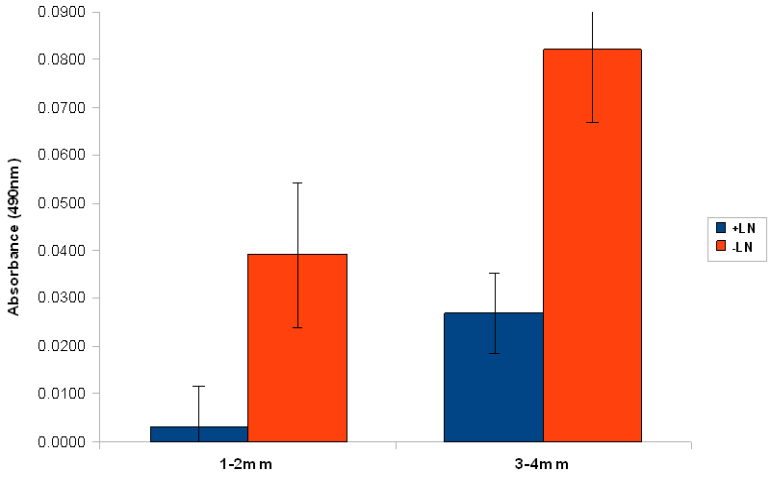

Fig. 2: PLBs viability test by TTC method at wavelength 490nm. TTC assay stainability of the PLBs in different sizes (1-2, 3-4 mm).Errors bars represent the standard error of TTC assay stainability. +LN, Cryopreserved PLBs ; -LN, Non-cryopreserved PLBs.

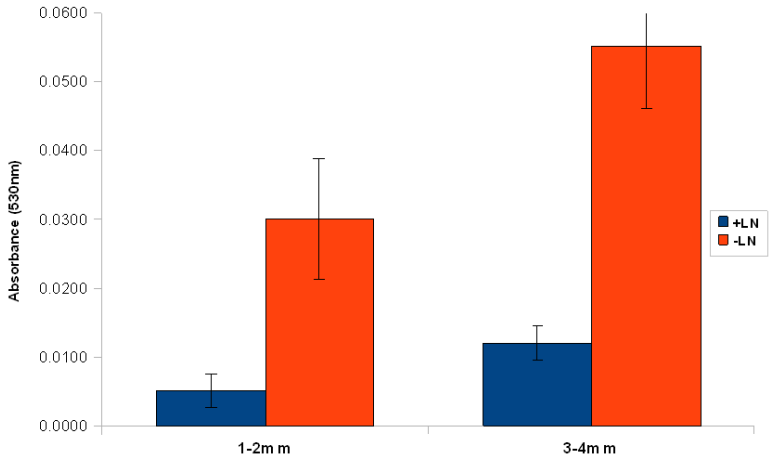

Fig. 3: PLBs viability test by TTC method at wavelength 530nm.TTC assay stainability of the PLBs in different sizes (1-2, 3-4 mm). Errors bars represent the standard error of TTC assay stainability. +LN, Cryopreserved PLBs ; -LN, Non-cryopreserved PLBs.

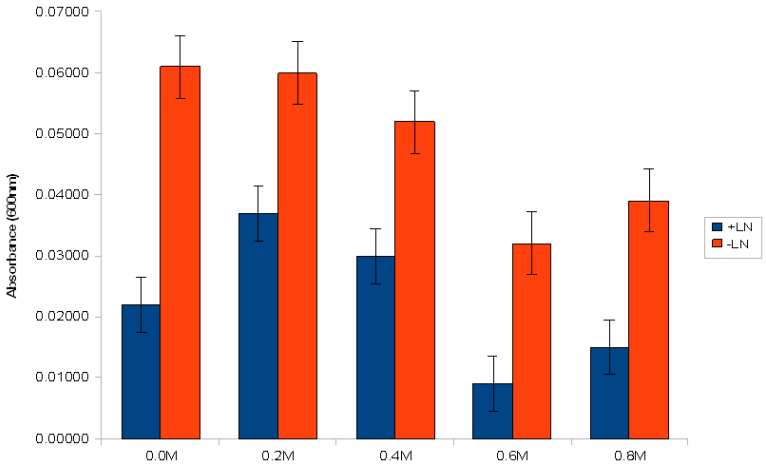

Fig. 4: Viability of PLBs with different sucrose concentration by using Evans Blue viability test. Errors bars represent the standard error of Evans Blue stainability. +LN, Cryopreserved PLBs; -LN, Non-cryopreserved PLBs

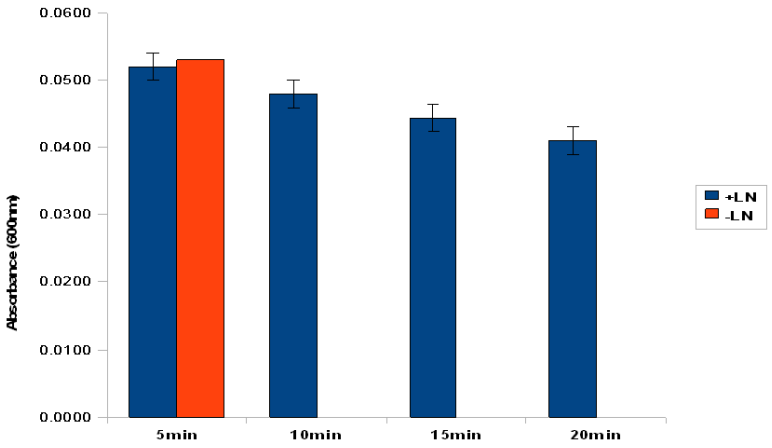

Fig. 5: Viability of PLBs with different PVS2 exposure durations. Errors bars represent the standard error of Evans Blue stainability. +LN, Cryopreserved PLBs; -LN, Non-cryopreserved PLBs

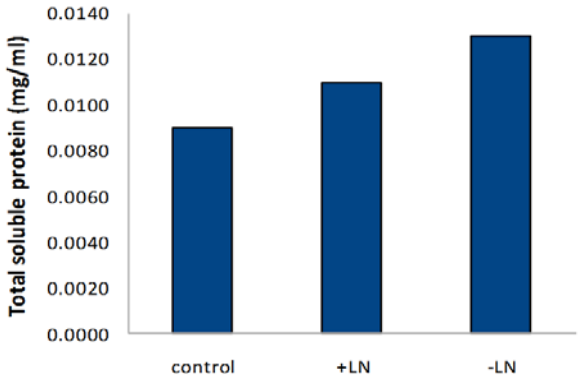

Fig. 6: Total protein content of PLBs in different conditions. Control, PLBs from stock cultures; +LN, Cryopreserved PLBs ; -LN, Non-cryopreserved PLBs

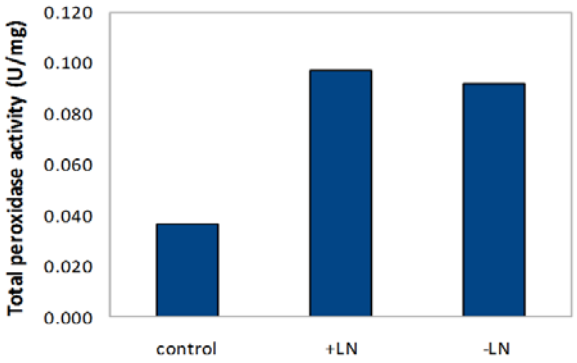

Fig. 7: Peroxidase activity of different treatment of PLBs samples. Control, PLBs from stock cultures; +LN, Cryopreserved PLBs; -LN, Non-cryopreserved PLBs

In this study, based on evans blue assay, dehydration with PVS for 20 minutes at 0°C was considered to be the best PVS 2 treatment for PLBs cryopreservation of *Dendrobium* sonia-28 by vitrification (Fig. 5). Cryoprotectant (PVS2 solution) added during cryopreservation are effective in preserving the PLBs. In addition, the PVS2 solution exposure was also critical to the cryopreserved PLBs as it directly influence the viability of the PLBs after recovery.

Total protein content

The determination of protein content is frequently required in cryopreservation. The test which is used to determine the total protein content

in this study is the Bradford method. In this study, the cryopreserved PLBs (+LN) obtained the lower total protein content comparative to non-cryopreserved PLBs (-LN) (Fig. 6). Cool storage in liquid nitrogen results in the loss of cell membrane integrity [20]. This was due to the activation of enzyme related to lipid degrading. Besides, the PLBs in control treatment showed the lowest protein content comparative to cryopreserved and non-cropreserved PLBs. Exogenous application or the increase in endogenous ABA level under stress conditions (osmotic or water loss) has been associated with synthesis of proteins [4]. Thus, cryopreservation has induced PLBs into stress condition which leads into PLBs to produce more protein compared to PLBs in control treatment.

Peroxidase activity

One of the most important aspects in plant physiology is the study of the changes in enzyme activity such as peroxidase (POD) enzyme which occurs in the several phases of development. The activity of peroxidase enzyme, also one of the stress parameters in plants, is directly or indirectly related to numerous physiological processes. The increase in POD activity in plant tissues has played an important role in many biochemical reactions such as lignin biosynthesis [3,8].

In this study, peroxidase activity was highest in protocorm-like bodies (PLBs) that were treated in the presence of liquid nitrogen (Fig. 7). This indicates that under stressed conditions, the PLBs secrete a higher amount of peroxidase enzyme. This is due to cryopreserved PLBs were exposed to many cryoprotectant such as loading solution, PVS2 solution, and unloading solution. This high rate of cryoprotectant exposure causes the PLBs to develop a stress condition. To overcome the stress condition, peroxidase activity was elicited. Peroxidase (POD) is one such enzyme associated with the plant defense pathway and is elicited when challenged with elicitors [6, 15].

Conclusions

In this study, the best PLB size and preculture condition for cryopreservation of *Dendrobium* sonia-28 was with the size of 3-4 mm, that were precultured in half strength semi-solid MS media supplemented with 0.6M sucrose. The best PVS2 exposure duration for *Dendrobium* sonia-28 was 20 minutes. However, alterations of biochemical contents such as total soluble protein in PLBs had been reported occurred after being cryopreserved. In addition, the highest total peroxidase activity was reported in PLBs after cryostorage Thus, further improvement and studies should be conducted in cryopreservation of *Dendrobium* sonia-28 in order to achieve higher survival rate of PLBs after cryostorage.

Acknowledgements

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