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Ascorbate Peroxidase Activity of *Aranda* Broga Blue Orchid Protocorm-like Bodies (PLBs) In Response to PVS2 Cryopreservation Method

Khor Soo Ping, Rahmad Zakaria and Sreeramanan Subramaniam*

School of Biological Sciences, Universiti Sains Malaysia, 11800 USM, Pulau Pinang, Malaysia

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Abstract: Throughout the cryopreservation process, plants were exposed to a series of abiotic stresses such as desiccation and osmotic pressure due to highly concentrated vitrification solution. Abiotic stress stimulates the production of reactive oxygen species (ROS) which include hydrogen peroxide, superoxide radicals, and singlet oxygen. Higher production of ROS may lead to oxidative stress which contributes to the major injuries in cryopreserved explants. Antioxidant enzymes in plant such as ascorbate peroxidase (APX) can protect plants from cell damage by scavenging the free radicals. This study was determined based on APX enzyme activity of *Aranda* Broga Blue orchid's protocorm-like bodies (PLBs) in response to PVS2 (Plant Vitrification Solution 2) cryopreservation treatments at different stages. PLBs that were precultured at 0.25 M sucrose for 3 days were subjected to vitrification cryopreservation method. Results obtained showed that the highest APX activity was achieved at PVS2 cryoprotectant treatment prior liquid nitrogen (LN) storage. This phenomenon indicating that accumulation of osmotic and dehydrating stress throughout the cryopreservation treatment resulted in oxidative burst which in turn leads to higher APX activity in order to control the excess production of ROS. To conclude, PVS2 treatment was revealed as the most detrimental step throughout cryopreservation treatment. Thus, this research also suggested that exogenous antioxidant such as ascorbic acid can be added throughout cryopreservation procedure especially at PVS2 treatment in the future experiments to aid in regrowth of cryopreserved explants by reducing oxidative stress.

Keywords: Cryopreservation, PVS2 Vitrification, Ascorbate Peroxidase, Protocorm-like Bodies

*Corresponding author: sreeramanan@usm.my

INTRODUCTION

When plants were exposed to abiotic and biotic stresses, ROS (reactive oxygen species) generation were triggered due to the activation of plant defence responses. However, overproduction of ROS such as superoxide radicals may cause oxidation and damages to the plant cells. To overcome this phenomenon, plants are able to detoxify the harmful ROS with a series of antioxidant enzyme mechanism. Ascorbate peroxidase (APX) which is synthesised in mitochondria is the main decomposition enzyme of hydrogen peroxide (H_2O_2) and is widely distributed in plant organelles. APX in conjunction with glutathione reductase in the glutathione-ascorbate cycle are responsible in keeping the ROS activity in plant cells under control. Cryopreservation treatments impose the plants to a series of oxidative burst caused by freezing temperature and high osmotic stress. The damaging factor that resulted poor regeneration in cryopreserved plantlets can be identified through monitoring the antioxidant enzyme activity throughout cryopreservation process. The objective of this study is to evaluate the oxidative level of PLBs using APX enzyme as stress marker at various cryopreservation stages.

MATERIALS AND METHODS

PVS2 Vitrification Cryopreservation

Selected PLBs (3–4 mm) were precultured in 0.25 M sucrose for 3 days. Precultured PLBs were then subjected to loading treatment (2 M glycerol and 0.4 M sucrose in VW medium) (Matsumoto *et al.* 1994) for 20 minutes at room temperature (RT), and subsequently treated with PVS2 which contains 30% (w/v) glycerol, 15% (w/v) ethylene glycol, 15% (w/v) DMSO (Sakai *et al.* 1990) in VW medium at 0°C for 20 minutes. Next, PLBs were transferred to cryovial containing fresh pre-chilled PVS2 prior plunging into LN for storage (minimum 1 hour). For thawing, cryovials were thawed directly in water bath at 40°C for 90 seconds, and PLBs were subsequently treated with unloading solution (1.2 M sucrose in VW medium) for 20 minutes at RT. After unloading treatment, PLBs were transferred to growth recovery medium (VW medium with 20 g/L sucrose) (Vacin & Went 1949) with a piece of filter paper on top and maintained in dark condition.

Ascorbate Peroxidase Assay

Enzyme extraction was carried out at every stages of PVS2 vitrification cryopreservation (Control, preculture, loading treatment, PVS2 dehydration, thawing, unloading, and growth recovery for 1 Day) and 4 weeks old PLBs without any treatment was used as control. Antioxidant enzyme was extracted based on the protocol described by Elavarthi and Martin (2010). APX activity of PLBs at different cryopreservation stages were assayed using modified method of Nakano and Asada (1981). The extinction coefficient of reduced ascorbate (2.8 $mM^{-1}\,cm^{-1}$) was used to calculate the enzyme activity of APX.

Experimental Design and Statistical Analysis

Each treatment contained 3 replicates and data was analysed using software IBM SPSS 21. Means of the data were analysed using one way ANOVA ($p<0.05$) and Tukey's test. Figure present mean values with standard error (SD).

RESULTS AND DISCUSSION

The changes of APX activity of PLBs were examined at every stages of cryopreservation. Control PLBs in the absence of cryopreservation treatment displayed relatively low APX activity. The level of APX activity increased gradually from preculture to PVS2 treatment and the highest APX activity was recorded at PVS2 treatment (Fig. 1). Preculture, loading, and PVS2 treatment are a series of dehydration process in cryopreservation to reduce water content in PLBs in order to avoid ice crystallisation during LN storage. These steps cause the accumulation of dehydration and osmotic stress thus triggered oxidative burst and production of ROS such as H_2O_2 which will further damage the plant tissue. To encounter oxidative damage caused by ROS, several antioxidant enzymes in the detoxification system are required to work in synchrony and APX is known to be the main antioxidant enzymes to detoxify hydrogen peroxides (Noctor & Foyer 1998). In this study, APX activity was further enhanced throughout preculture until PVS2 stages to deliberate the toxic effect of ROS. The similar finding was obtained by Rahmah *et al*. (2015) during cryopreservation of *Brassidium* Shooting Star orchid, where the APX activity increased from preculture until PVS2 dehydration and LN storage stages. Decreasing of APX activity was observed after thawing while unloading and growth recovery stage represents the lowest APX activity significantly. Unloading treatment dilutes cryoprotectants from PLBs and replace with water to help regaining the osmotic balance in plant cells. As a result, APX activity in PLBs has been reduced due to the reduction of osmotic stress.

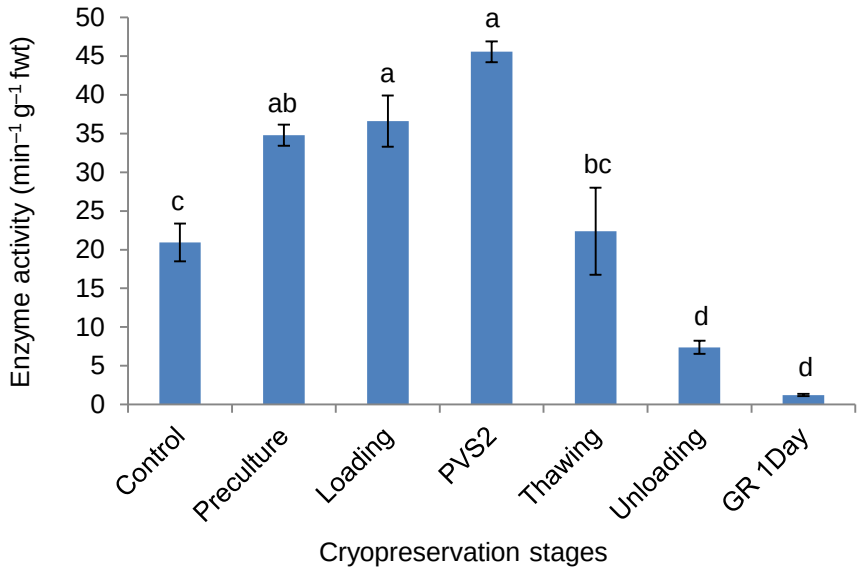

Figure 1: Ascorbate peroxidase activity of cryopreserved PLBs subjected to various stages of PVS2 vitrification cryopreservation experiment.

CONCLUSION

High osmotic stress that was imposed to PLBs throughout cryopreservation led to the production of high APX activity in order to cope with high ROS production. Hence, it is very crucial to reveal the effects of cryopreservation treatment to antioxidant activity such as APX since it acts as a stress marker to indicate the stress level encountered by PLBs. The key success to avoid cryopreservation damage is through supplementation of antioxidant and at the same time reduced ROS in plants. For future research, this study also suggested the addition of exogenous antioxidant such as ascorbic acid at the dehydrating steps (preculture to PVS2) in the future experiments to aids in reducing the damaging effect caused by cryopreservation treatment.

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