CRYOPRESERVATION OF SOLANUM LASIOCARPUM DUNAL USING VITRIFICATION TECHNIQUE

PAULA MACCIE ANAK PETER

UNIVERSITI MALAYSIA SARAWAK
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Paula Maccie Anak Peter
Resource Biotechnology Programme
Faculty Science and Technology

ABSTRACT

A study was conducted to evaluate the effectiveness of vitrification technique in cryopreserving of *Solanum lasiocarpum* seeds. The seeds were immersed in Plant Vitrification Solution 2 (PVS2) with 0.0, 0.2, 0.4, 0.6, 0.8 and 1.0M of sucrose concentrations for 0, 30, 60, 90 and 120 minutes. Initial germination and moisture content of the seeds used were 51% and 37.9% respectively. Vitrification solution with sucrose concentration of 0.6M exposed for 30 minutes gave the highest viability of 70% with 26% germination and 35.53% moisture content Viability of the seeds decreased slightly after vitrification and stored in liquid nitrogen. Regression analysis showed that *S. lasiocarpum* seeds can be stored for 10 months and 2 weeks and 4 days after vitrification and kept in liquid nitrogen.

Keyword: *Solanum lasiocarpum* Dunal, vitrification, viability, germination and regression analysis.

ABSTRAK

Kajian dijalankan untuk menilai keberkesanan vitrifikasi bagi menyimpan biji benih Solanum lasiocarpum. Biji benih diletakkan di dalam larutan ' Plant Vitrification Solution 2' (PVS2) dengan kepekatan sukrosa 0.0, 0.2, 0.4, 0.6, 0.8 dan 1.0M selama 0, 30, 60, 90 dan 120 minit. Peratus percambahan dan kandungan kelembapan awal bagi biji benih masing-masing adalah 51% dan 37.9%. Vitrifikasi menggunakan PVS2 dengan larutan sukrosa berkepekatan 0.6M memberikan keputusan kebolehidupan terbaik iaitu70% dan percambahan terbaik sebanyak 26% pada 35.53% kandungan kelembapan dengan tempoh pendedahan selama 30 minit. Kebolehidupan biji benih didapati menurun setelah dirawat melalui vitrifikasi dan diletakkan di dalam cecair nitrogen. Analisis regrasi menjangkakan biji benih S. lasiocarpum boleh disimpan selama 10 bulan dan 2 minggu dan 4 hari selepas dirawat melalui vitrifikasi dan disimpan di dalam cecair nitrogen.

Kata kunci: *Solanum lasiocarpum Dunal*, vitrifikasi, kebolehidupan, percambahan dan analisis regrasi.

INTRODUCTION

Solanum lasiocarpum Dunal or known as Terung Dayak (Dayak Brinjal) among local people is a species in the Solanaceae family. It is a species in the genus Solanum, the biggest genus in family Solanaceae, consisting of over 1000 species. *S. lasiocarpum* is believed to originate from South East Asia and South Asia (Voon *et al.*, 1988).

S. lasiocarpum is a wild, herbal plant that grows up to 100-250 cm. The stem and branches are thorny with leaves are wavy and broad. It has white flowers and green fruits at immature stage. Matured fruits ranged from yellow, reddish yellow, purplish yellow colour and different shapes depending on varieties (Voon *et al.*, 1988).

S. lasiocarpum consists of many varieties such as AC. No.1 to AC. No.10. Classification of the varieties depends on colour, size or shape of the fruits. Four varieties: AC.No.2, AC. No.3, AC. No.9 and AC. No.10 have been developed for commercial purpose because these varieties contain less seeds and size of the fruits are large (Anonymous, 1994). The Sarawak Government has officially changed the name of Terung Dayak to Terung Emas (Golden Brinjal) during the Farmers and Breeders International Day 1998 in order to promote its commercial value.

Due to its sour taste, sometimes local population called it as 'Terung Asam' (Sour brinjal). Malaysian Agriculture Research and Development Institute (MARDI) have diversified the food product from *S. lasiocarpum* such as juice and syrup (Anonymous, 1993). Like other wild fruit and vegetables, it is rich in nutrients with low fat and high carbohydrates and fibers (Table 1 in Appendix). *S. lasiocarpum* grows wild in the jungle in moderate humid habitat as it requires a lot of water. It thrives well in temperature around 25° - 35°C.
In vitro conservation is employed where the more conventional *ex-situ* methods involving seed storage or field collections are inappropriate or impractical. *In-vitro* conservation offers the possible techniques for the preservation of plant germplasm that otherwise has to be maintained with difficulty and limited success. Hence, the use of *in vitro* conservation techniques such as cryopreservation represents an important option for the medium and long-term conservation of recalcitrant seeds and vegetative propagated plant species (Towill, 1991).

Cryopreservation refers to the placing and holding of biological materials at a very low temperature in a manner such that viability is retained after thawing. Cryopreservation in liquid nitrogen is the most convenient techniques for long storage. At liquid nitrogen temperature (-196°C) all cellular divisions and metabolic events are ceased. The plant material can be stored without alteration or modifications for a theoretically unlimited period of time (Towill, 1995).

The historical roots of plant cryopreservation lie partly in studies of cold-hardiness and freezing injury in whole-plant materials (Levitt, 1966, Li & Sakai, 1978; 1982). The ability of plant tissues to recover after exposed to ultra-low temperature such as

that of liquid nitrogen (-196°C) was recognized by Sun in 1958. The first success was reported in 1968 by Quatrano, who froze cells of flax (Linum *usitatissimum*) to a temperature of -50°C while maintaining viability at a level of 14% (Whithers, 1983). Most recently, qualitative aspects of recovered cultures have received particular attention, with care being given to their biochemical evaluation and physiological performance (Yeoman, 1986).

All indications available to date are that cryopreservation will offer a very high degree of biochemical stability, paralleling the few examples of stability of karyotype. According to Kartha (1987), the evidence available so far indicates that cryopreservation is the most reliable approach for the long-term preservation of cell accumulation of secondary metabolites. In addition, cryopreservation is one of the important methods to preserve germplasm because it reduce operation cost, need less space and little changes occurred in the genetic constituents.

Vitrification is one of the new cryopreservation methods, which is, a more simple, practical and inexpensive approaches as compared to standard cryopreservation protocols. Many researchers using cell suspensions (Uragami *et al.*, 1989), protoplasts (Langis and Steponkus, 1990), somatic embryos and meristems (Towill, 1990) of various species have utilized this technique successfully.

Vitrification refers to solidification ('glassification') of the system during cooling without ice formation. Large concentration of cryoprotectant and appropriate (usually rapid) cooling and warming rates are required (Fahy *et al*, 1984). Potential advantages of vitrification strategies include application to larger pieces of tissue, convenience in cooling, and avoiding or minimizing 'solution effects' injury (Towill, 1991).
Survival using vitrification has been reported for animal embryos (Rall, 1987) and some animal tissues, including human monocytes (Takahashi *et al*, 1986) and human islets of Langerhans (Jutte *et al*, 1987). Vitrification accounted for the survival of liquid nitrogen-treated cold-hardy plants (Hirsh *et al*, 1985, Hirsh, 1987).

Since seeds of *S. lasiocarpum* are a recalcitrant seed, it is difficult to store because of their sensitivity to drying (and thus to drying, since desiccation occurs during the freezing process) and their general short-lived nature. Recalcitrant (desiccation sensitive) seeds lose viability after drying below relatively high water content (20-30% water). This type of seeds is produced by many tropical and subtropical plants, some aquatic plant and temperate zone trees with large seed (Chin & Roberts, 1980). Combination of tissue culture and cryopreservation techniques may enhance germplasms conservation for *S. lasiocarpum* once suitable methodologies are established. Currently, species with recalcitrant seeds are maintained as clones.

Until now, there are no known researches conducted to preserve *S. lasiocarpum* seeds via vitrification technique. This study is to determine the effectiveness of vitrification as a suitable method to conserve germplasms of *S. lasiocarpum* in long-term storage.

MATERIALS AND METHODS

Material

Fresh and matured seeds of *S. lasiocarpum*, harvested from a farm in Sri Aman Division was used as the material in these studies. Well-developed matured (yellowish colour) seeds were selected and the pericarp removed after two hours of soaking in running tap water. The surface of seeds immersed in 70% ethanol solution for about ten minutes to sterile. The seeds were air-dried overnight under sterile air in a laminar flow to remove the excess water before kept in an air-tight bottle.

Plant Vitrification Solution 2 (PVS2): PVS2 consists of glycerol (30%), ethylene glycol (15%),Dimethylsulfoxide (DMSO) (15%) was used as cryoprotectant. PVS2 was prepared in water with sucrose. The pH was adjusted to 5.8 and then was autoclaved for 20 minutes at 120°C. The solution was stored at 4°C and used within a 2 month-period.

Method

Standard Germination Test

Standard Germination Test was conducted based on the Rules for Testing Seed (AOSA, 1985). Four replications of 25 seeds in each replication, were used and seeds were arranged in petri dishes each consisted of three layers of filter paper (Whatman No.1, diameter 90mm), and moistened with distilled water. The petri dishes were placed in a Plant Growth Chamber at 29°C. Germination percentage of seeds were calculated using the formula:

$$\text{Germination (\%)} = \frac{\text{Total of seeds germinated}}{\text{Total of seeds used}} \times 100\%$$

Moisture Content Test

Moisture content of the seeds was evaluated by wet-weight basis. Four replicates of one-layer seeds placed in crucibles were weight-using precision Electronic Balance (Model FY 300, A&D Company, Japan). Then dried in oven (Memmert Oven, model 500) at 60°C for 48 hours. After this drying period, the seeds again were weighed. Moisture content weight was then calculated (International Seed Testing Association, 1976).

$$\text{Moisture Content (\%)} = \frac{(b) - (c)}{(b) - (a)} \times 100\%$$

(a)= weight of empty crucible

(b)=weight (a) + weight of the seeds before put in the oven

(c)= weight (a) + weight of the seeds after put in the oven

Viability Test

Viability test (Tetrazolium Test) was conducted using tetrazolium chloride solution. Respiration from dehydrogenase enzymes in living cells can be estimated by their capacity to reduce 2,3,5-triphenyl tetrazolium chloride (TTC) (Towill and Manzur, 1974). Four replications of 25 seeds in each replication were used and immersed in the TTC solutions of 0.1, 0.5, or 1.0% and incubated in an oven at 35°C for durations of 0, 30, 60, 90, 120, 150 and 180 minutes. After each incubation periods, seeds were withdrew from the oven and TTC solution rinsed at least 4 to 5 times. The red colour (formazan) indicated reactions of the solution of TTC with dehydrogenase enzymes in the living cells of seeds.

Vitrification

This technique is based on the modification of experiment that has been improved by Sakai *et.al*, 1991. Four replications of 25 seeds in each replication were used. The optimum concentration of the sucrose was determined. The seeds were immersed in Plant Vitrification Solution 2 (PVS2) mixed with sucrose at concentrations of 0.0, 0.2, 0.4, 0.6, 0.8 and 1.0 M for periods of 0, 30, 60, 90 and 120 minutes.

In subsequent treatment vitrified seeds were put in cryovials, then placed and held in liquid nitrogen for an hour. Thawing of samples was carried out in a water bath at 35°-40°C for about 15 minutes. After which seeds were rinsed with 1.2M sucrose and thereby evaluated for viability and germination.

Storage

Three lots of seeds were kept in three different environments namely: air-conditioned room (20°-23°C), refrigerator (3°-5°C) and in liquid nitrogen (-196°C). For air-conditioned room and refrigerator environments the seeds lots were kept in covered petri dishes and left in an air-conditioned room and a refrigerator respectively. A concurrent treatment was run with seed materials stored in liquid nitrogen. Evaluation was made at an interval of a week for a period of 8 weeks where seeds were drawn out for germination and viability tests.

Experimental design used was complete randomized design (CRD). Data were analyzed using Analysis of Variance (ANOVA). For storage of *S. lasiocarpum* seeds regression analysis was conducted.

RESULT AND DISCUSSION

The initial moisture content and germination of *S. lasiocarpum* used *was* 37.19 % and 51% respectively. This indicated that the seed lot used was of moderate quality. The low percentage of germination might be attributed to the fact that the seed lot exhibited a degree of dormancy, which contributed to this low percentage of germination as the seeds used were matured and was newly harvested.

Tetrazolium (TZ) Staining

In Figure 1, showed that the viability of seeds was generally higher when stained for a period between 90 to 120 hours for the three concentrations of TZ solution. Maximum germination of 87% was obtained at 0.5% concentration of tetrazolium solution exposed for 2 hours. As such this concentration of tetrazolium solution and period of exposure was used for viability evaluation in the subsequent experiments.

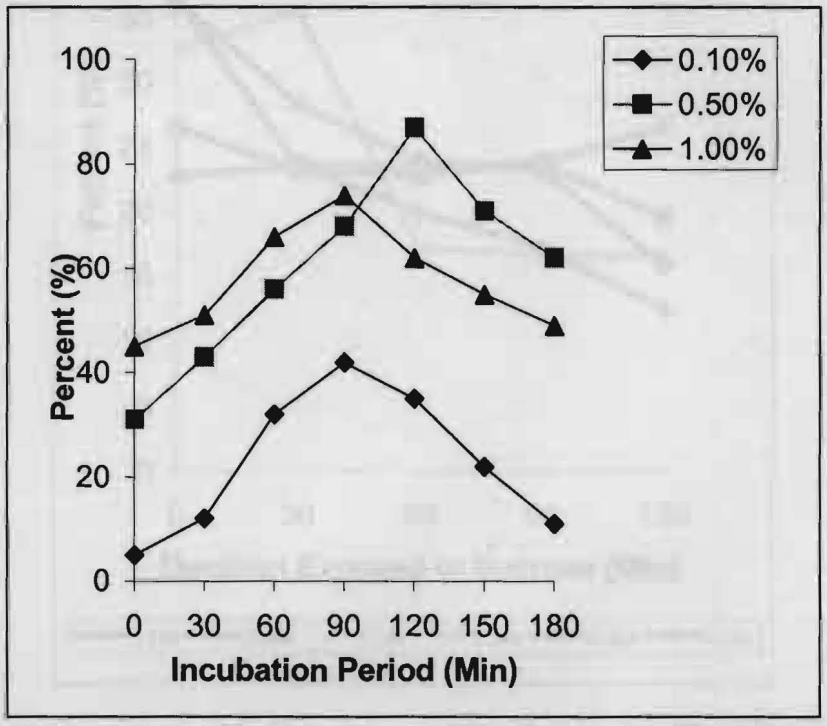

Figure 1: Viability of *S. lasiocarpum* seeds in different terazolium concentrations at 35°C.

Vitrification of Seeds

The survival of germplasms in vitrification depended on the exposure duration to PVS2 utilized. This included osmosis dehydration process and also avoidance of toxicity of PVS2 solution. Sakai *et al.* (1991) introduced a one-step dehydration procedure that reduced the period of exposure.

Figure 2, exhibited the moisture content of *S. lasiocarpum* seeds after vitrification, which was 35.53%. The moisture content of seeds decreased with increased in concentrations of sucrose solution and with lengths of exposed period.

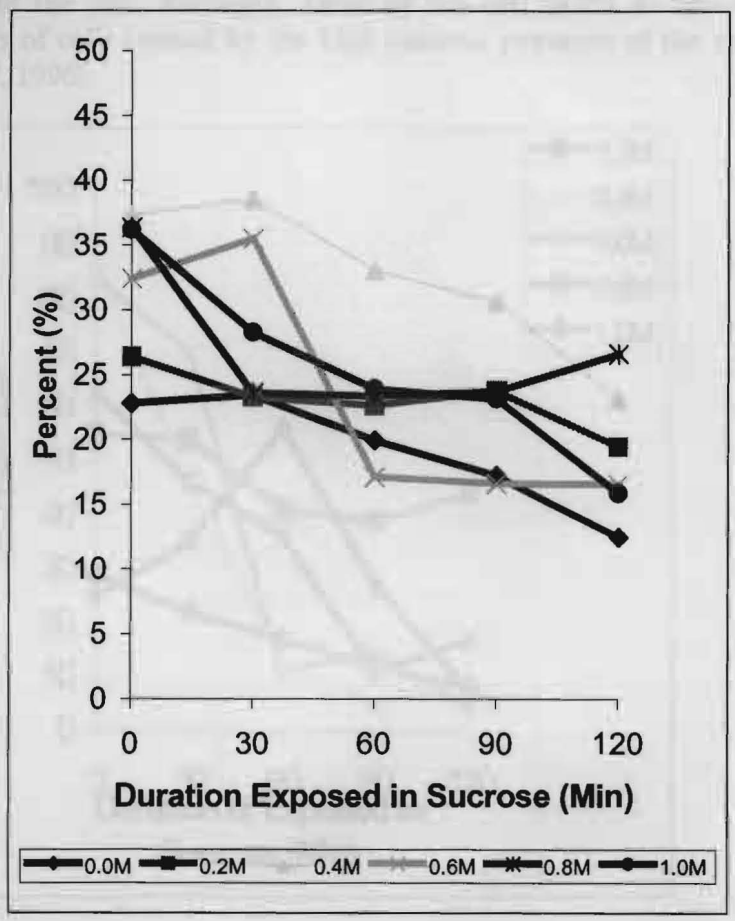

Figure 2: Moisture content of *S. lasiocarpum* seeds after exposed in different concentrations of sucrose.

Different concentrations of sucrose were used to determine the most suitable concentration that would then be added with PVS2 as a cryoprotectant for seeds. As demonstrated in Figure 3, the highest percentage of viability for seeds, which were vitrified, was using 0.6 M with seeds prior to exposure in liquid nitrogen. PVS2 treatment with the concentration of 0.6 M of sucrose was used in the subsequent experiments as it gave 70% viability. Percentage of viability of *S. lasiocarpum* decreased above 0.6 M of sucrose due to immediate absorption of the liquid-cell by sucrose, which made the cell, damaged. Most of the cell failed to recover due to excessive plasmolysis of cells caused by the high osmotic pressure of the surrounding solutions (Panis *et al*, 1996).

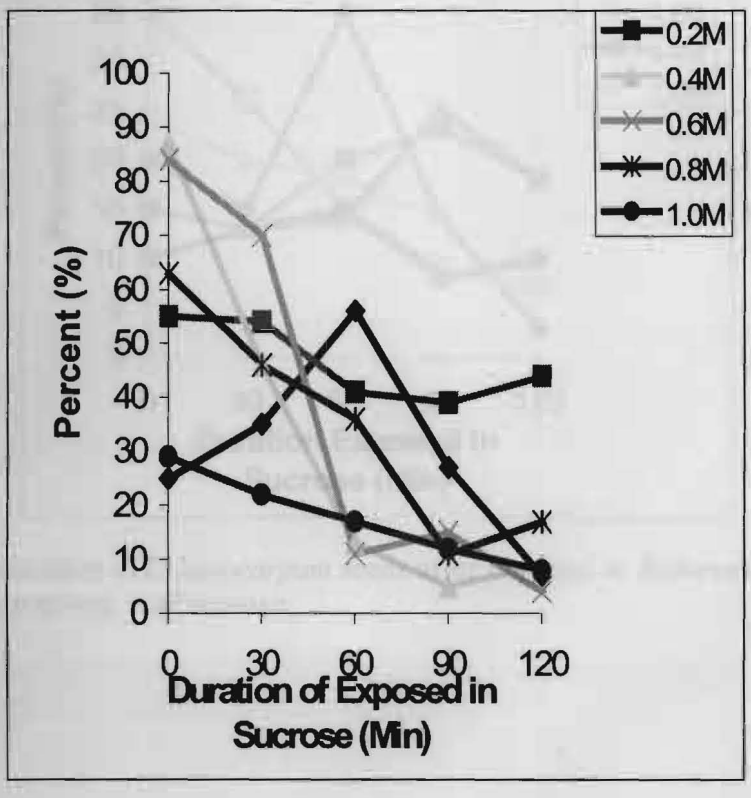

Figure 3: Viability of *S. lasiocarpum* seeds after exposed in different concentrations of sucrose.

Results obtained in Figure 4, showed that seeds exposed to PVS2 solution and sucrose concentration of 0.6 M gave the highest percentage of germination. This indicated that toxicity of the PVS2 solution may be encountered and with insufficient periods of dehydration occurred. According to Finkle *et al.* (1985), a mixture of cryoprotectants were less damaging during cooling and rewarming of the sample then a high concentration of cryoprotectant such as DMSO. This method also has been successfully applied for the cryopreservation of germplasms of white clover (Yamada *et al.*, 1991), apple and pear (Niino *et al.*, 1992), chrysthemum (Kohmura, 1993) and carnation (Langis *et al.*, 1990).

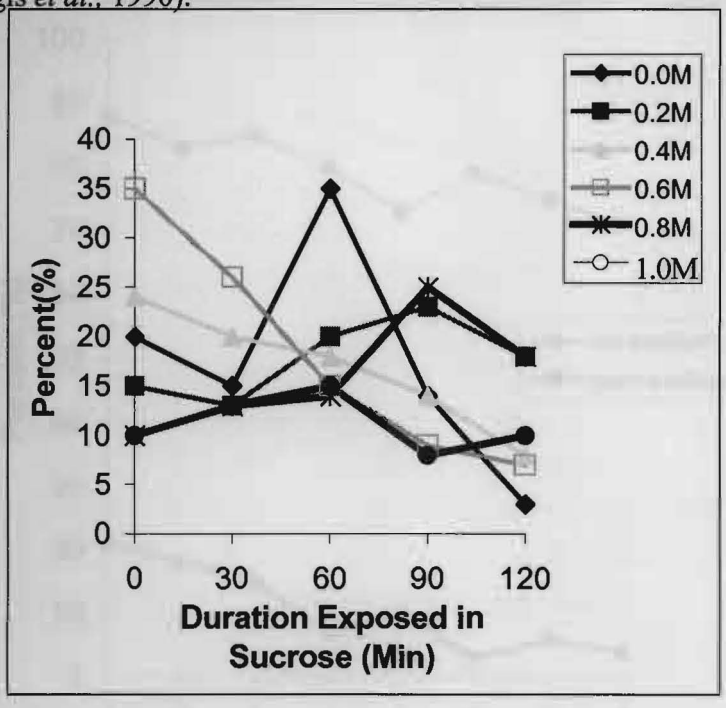

Figure 4: Germination of *S. lasiocarpum* seeds after exposed in different concentrations of sucrose.