UNIVERSITI PUTRA MALAYSIA

CRYOPRESERVATION OF RUBBER (HEVEA BRASILIENSIS MUELL. -ARG.) ZYGOTIC EMBRYOS USING VITRIFICATION TECHNIQUE

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CRYOPRESERVATION OF RUBBER (*Hevea brasiliensis* Muell. –Arg.) ZYGOTIC EMBRYOS USING VITRIFICATION TECHNIQUE

By

SAM YEN YEN

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LIST OF ABBREVIATIONS

g	gram
g/l	gram per litre
w/v	weight per volume
mg/l	milligram per litre
BAP	6-benzylaminopurine
DMSO	dimethysulphoxide
EG	ethylene glycol
GA_3	gibberellic acid
LN	liquid nitrogen
MS	Murashige and Skoog
M	Molar
NAA	alpha-naphthalene acid
PEG	polyethylene glycol
PEG-800	polyethylene glycol 800
PVS	plant vitrification solution
PVS2	plant vitrification solution 2
PVS3	plant vitrification solution 3

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Agriculture Science.

CRYOPRESERVATION OF RUBBER (*HEVEA BRASILIENSIS*) ZYGOTIC EMBRYOS USING VITRIFICATION TECHNIQUE

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Novenber 1999

Chairman : Associate Professor Hor Yue Luan, Ph. D.

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The study was carried out to develop a vitrification procedure for the cryopreservation of *Hevea* zygotic embryos. Vitrification generally involves three main steps, namely, loading, freezing in vitrification solution and unloading. This study evaluated the optimum loading and vitrification treatments for *Hevea* embryos for liquid nitrogen exposure and also to compare the effectiveness of vitrification with other cryopreservation techniques.

The loading solutions tested were relatively toxic as the loaded embryos showed lower viability and survival compared to the fresh embryos. However, the effects of the four loading solutions up to 60 minutes exposure were similar. On exposure to liquid nitrogen, none of the embryos survived.

The vitrification solution PVS2 was more effective than PVS and L solution in vitrifying *Hevea* tissue in liquid nitrogen because only embryos treated with PVS2 could survive after eight weeks culture. Longer time of exposure to vitrification solution significantly increased the viability suggesting that more tissues were vitrified with longer exposure. The optimum time of exposure to PVS2 was 80 minutes with 28.0% viability and 13.4% survival. The moisture content of embryos after PVS2 exposure stabilised around 43.3-46.6%. Survival of frozen *Hevea* embryos at such relatively high moisture confirmed the potential of PVS2 for vitrifying the tissue in liquid nitrogen.

Desiccation using fast and slow drying methods to reduce tissue moisture did not improve survival of frozen embryos. Viability and survival of desiccated embryos following vitrification treatment declined dramatically compared to the control. For *Hevea* embryos, desiccation following vitrification treatment caused more injuries to the tissues.

Comparison of the vitrification technique with other methods of cryopreservation showed vitrification technique to be more effective with 81.1% viability and 51.2% survival after liquid nitrogen exposure. Naked desiccation, desiccation following sucrose preculture and encapsulation dehydration, were relatively ineffective.

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PENGKRIOAWETAN EMBRIO GETAH (*HEVEA BRASILIENSIS*) MEGGUNAKAN TEKNIK VITRIFIKASI

Oleh

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November 1999

Pengerusi : Profesor Madya Hor Yue Luan, Ph.D.

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Kajian ini dijalankan untuk menghasilkan satu prosidur vitrifikasi bagi pengkrioawetan embrio *Hevea*. Tiga langkah vitrifikasi ialah, 'loading', penyejukan dalam larutan vitrifikasi dan 'unloading'. Kajian ini menilai rawatan 'loading' dan vitrifikasi yang optimum untuk embrio *Hevea* dalam simpanan cecair nitrogen serta membandingkan keberkesanan rawatan vitrifikasi ini dengan rawatan-rawatan pengkrioawetan yang lain.

Pendedahan embrio kepada larutan 'loading' yang dikaji menunjukkan kesan toksik ke atas embrio *Hevea* kerana embrio yang didedahkan kepada larutan "loading" mencapai viabiliti dan kemandirian yang lebih rendah berbanding dengan embrio segar tanpa rawatan. Walaubagaimana pun, empat jenis larutan "loading" dan jangkamasa pendedahan sehingga 60 minit tidak memberi kesan bererti. Selepas dimasukkan ke dalam ceçair nitrogen, tiada embrio berupaya hidup.

Larutan vitrifikasi PVS2 didapati lebih berkesan dalam mengvitrifikasikan tisu *Hevea* dalam cecair nitrogen berbanding dengan larutan-larutan PVS dan L. Ini adalah kerana hanya embrio yang dirawat dengan PVS2 dapat hidup selepas tempoh kultur lapan minggu. Pendedahan yang lebih lama kepada larutan vitrifikasi meningkatkan viabiliti embrio *Hevea* dengan bererti. Masa pendedahan yang optimum kepada PVS2 ialah 80 minit dengan pencapaian 28.0% viabiliti dan 13.4% kemandirian. Selepas rawatan PVS2, kandungan kelembapan embrio *Hevea* kekal dalam linkungan 43.3% hingga 46.6%. Keupayaan embrio Hevea untuk hidup pada peratus kelembapan yang agak tinggi ini membuktikan potensi PVS2 untuk mengvitrifikasikan tisu dalam cecair nitrogen.

Pengeringan secara cepat dan perlahan untuk mengurangkan kandungan kelembapan dalam embrio tidak berjaya untuk meningkatkan peratus kemandirian. Kadar viabiliti dan kemandirian berkurangan dengan banyak selepas rawatan vitrifikasi dan pengeringan. Rawatan vitrifikasi diikuti dengan pengeringan mengakibatkan kerosakan ke atas embrio *Hevea*.

Perbandingan teknik vitrifikasi dengan teknik-teknik krioawetan yang lain menunjukkan teknik vitrifikasi lebih berkesan dalam mengkrioawetkan embrio *Hevea*, dengan pencapaian 81.1% viabiliti dan 51.2% kemandirian selepas didedahkan kepada cecair nitrogen. Teknik-teknik pengeringan embrio, pengeringan

embrio dengan pra-rawatan sukrosa dan pengeringan ke atas embrio berselaput adalah kurang berkesan berbanding dengan teknik vitrifikasi.

CHAPTER I

INTRODUCTION

Hevea brasiliensis originates from the tropical rain forest of the Amazon. It was first introduced into Malaysia in 1877 from the original 22 Wichkam seedlings received in Singapore in the same year. The original gene pool was therefore very narrow. Realising the importance of widening the gene base in order to sustain further genetic improvement such as disease resistance, a number of introduction of *Hevea* materials were carried out. These included importing *Hevea* seedlings and collecting wild *Hevea* germplasm from Brazil (Benong *et al.*, 1996). An important ingredient of these efforts is that the collected and existing germplasm must be conserved for maximum genetic diversity in future breeding programmes.

Conservation of *Hevea* genetic resources is presently through field banks. However, field conservation can be endangered by many field factors, such as pest and disease outbreak, besides competing for limited land resources for other development. Furthermore, *Hevea* seeds are difficult to conserve as they are recalcitrant (King and Roberts, 1980) and have a relatively short longevity, varying from a few weeks to a few months (Normah, 1987).

In recent years cryopreservation of plant cells, meristems and organs has become an important tool for long term preservation of germplasm without genetic alteration. Much success has been achieved with the recently developed and modified methods such as desiccation of naked tissue, freezing after encapsulation and the use of vitrification solutions.

Preliminary study on *Hevea* cryopreservation was reported by Normah *et al.*, (1986). The highest percentages of survival (69-71%) was obtained when excised embryonic axes were desiccated for 2 and 3 hours, cooled by stepwise or direct immersion into liquid nitrogen and rapidly thawed. Yap (1997) recorded 59% survival of *Hevea* embryonic axes using encapsulation dehydration technique. Encapsulated embryos were precultured with 0.3M sucrose, desiccated to 16% moisture content, followed by direct freezing in liquid nitrogen. As only moderate survival was achieved in the previous studies, the potential of other newly developed techniques should be considered. This project aims to investigate the feasibility of using vitrification solution for cryopreservation of *Hevea* zygotic embryos.

Vitrification generally involves three main steps, namely loading, vitrification in liquid nitrogen and unloading (Matsumoto *et al.*, 1994). This study evaluates the optimum loading and vitrification treatments for *Hevea* embryos for liquid nitrogen exposure. The unloading step is a relatively standard procedure and is not investigated in this study.

The objectives of the present study are:

(1) To evaluate the optimum loading treatment for the vitrification of *Hevea* zygotic embryos in liquid nitrogen.

(2) To establish an optimum vitrification procedure for *Hevea* embryos using the most effective loading solution.

(3) To compare the optimum vitrification technique with other cryopreservation methods, namely: naked desiccation, desiccation with sucrose preculture and encapsulation dehydration.

CHAPTER II

LITERATURE REVIEW

Germplasm conservation of cultivated species and their wild relatives is important for sustainable exploitation and maintenance of species and genetic diversity. Basically, conservation of plant gerplasm can be classified into two categories: *in situ* and *ex situ*. *In situ* involves conserving plants within their natural habitat such as forest reserves, nature parks and sanctuaries for wild relatives of crops. Most crop plants are conserved by *ex situ* means: their seeds, plants, plant parts, tissue or cells are preserved in an artificial environment. There are three main methods of *ex situ* conservation, namely, seed banks, field gene banks and *in vitro* conservation (Chin, 1994).

The most convenient way to maintain plant germplasm is by storing seeds, but this form of storage has its limitation. For example, species that rely on vegetative propagation and which either do not produce viable seed or produce heterogeneous seed which does not reflect the clonal nature of the species. In such species, it may be appropriate to consider alternative methods of conservation, such as the cryopreservation of non-seed tissues: meristems, embryos, pollen or cultured cells.

In addition, dry seeds of some species are sensitive to low temperatures of gene banks. Such intermediate recalcitrant seeds may be suited to cryogenic storage. Moreover, cryopreservation may be the only viable option for the long term stable storage of recalcitrant seeds. Cryopreservation can also be an alternative storage method for orthodox seeds that are inherently short-lived or those with a very limited seed stock.

Cryopreservation

Cryopreservation is the storage of biological material at ultra-low temperatures, usually at –196°C in liquid nitrogen. According to Engelmann (1991), cryopreservation is, at present, the only suitable alternative for long term storage of gemplasm. It is the safest alternative because at this temperature, biochemical processes are so reduced that no cell division, cell deterioration or mutation occurs (Stanwood and Bass; 1981; Stanwood ,1985). Hence, there is little loss of viability over time and germplasm can be stored, theoretically, for unlimited length of time.

Cryopreservation requires minimum space, low maintenance and is non-dependent on electricity. Because of its economic and reliability, cryopreservation techniques has also been recommended for orthodox seeds as an alternative storage strategy to the widely used electrical refrigeration procedures involving storage at –20°C (Stanwood, 1985).

Cryopreservation has been successfully utilized for more than 70 plant species, among which around 40 species are from tropical origin (Engelmann *et al*., 1997). Stanwood and Bass (1981) tested cryopreservation on seeds of 120 plant species and many were shown to be able to withstand the ultra-low temperatures. Other parts of plants have also been successfully stored cryogenically; for example, shoot tips (Maruyama *et al*., 1997), pollen (Jorgensen, 1990), zygotic embryos (Brearly *et al*., 1995), cell suspensions (Gazeau *et al*., 1998), and embryogenic tissues (Blakesley *et al*., 1997).

There is an obvious need to develop long term conservation technique especially for intermediate and recalcitrant seeds. Although recalcitrant seeds cannot tolerate low moisture, their excised embryos have survived low moisture and temperatures (Normah *et al*., 1986). In practice, the smaller the tissue volume, the better it can be conditioned for cryopreservation. The excised zygotic embryos are relatively small and are more amenable and practical for conservation. It has better potential to regenerate a whole plant from its root and shoot meristems (Chin *et al*., 1989). Furthermore, the embryos are reasonably uniform genetically and are highly regenerative (Kendall *et al*., 1993). However, due to their relatively high moisture, they must be subjected to specific treatment before cryopreservation in order to avoid the formation of lethal ice crystals. The most critical parameter in the cryopreservation procedure is the removal of the intracellular water fraction which is capable of conversion to ice crystals during freezing or rewarming. This can be achieved in a number of ways such as treatment with cryoprotectants, slow cooling,

desiccation or partial desiccation, encapsulation and vitrification. In some cases, two or more approaches have been combined.

Vitrification

The physical and biochemical mechanisms by which plant parts are able to survive severe dehydration and low temperatures are poorly understood. Burke (1986) suggested that such ability might be associated with cytoplasmic vitrification (glassy state). It is proposed that the glass, a liquid of high viscosity, stops or slows down all chemical reactions requiring molecullar diffusion (Franks, 1985; Leopold *et al.*, 1992). In so doing, a glassy state may assure quiescence and stability over time.

Vitrification is the formation of a glass-like, noncrystalline substance at the freezing point of an aqueous solution. In cryopreservation, it is achieved in practice using high concentration of cryoprotectants which prevent crystallization of water during freezing (Bajaj, 1985). Ice crystallization will cause tissue injury, but the metastable glass formed is relatively non-injurious to tissues, provided the cryoprotectants used are also relatively non-toxic.

The vitrification procedure consists of (1) addition of cryoprotective solution; (2) vitrification in liquid nitrogen; (3) rapid thawing; and (4) unloading. During exposure to highly concentrated cryoprotective solutions, cells are dehydrated sufficiently without causing osmotic injuries, and later vitrified in liquid

nitrogen. Certain penetrating solutes, such as dimethysulphoxide (DMSO) and ethylene glycol (EG), will permeate into the cells and provide some antifreeze benefits, thereby widening the window for survival between excessive cell dehydration and intracellular freezing (Meryman and Williams, 1985).

The most commonly used vitrification solution in the cryopreservation of plant germplasm is PVS2 which contains (w/v) 30% glycerol + 15% EG + 15% DMSO + 0.4 M sucrose (Sakai *et al.*, 1990). Many species have been effectively cryopreserved using this solution (Matsumoto *et al.*, 1995a, b; Kohmura *et al.*, 1992,1994, Niino *et al.*, 1992). Other vitrification solutions have also been developed, such as PVS (Uragami *et al.*, 1989), PVS3 (Nishizawa *et al.*, 1993), (w/v) 25% glycerol + 15% sucrose + 15% EG + 13% DMSO + 2% polyethylene glycol (PEG) (Maruyama *et al.*, 1997), and (w/v) 35% EG + 1 M DMSO + 10% PEG-800 (Towill, 1990). The type of vitrification solution used appears to be species-specific. This is probably due to variations in drought-tolerance and cryoprotectant-permeability of different species (Huang *et al.*, 1995).

Successful cryopreservation using vitrification was obtained by various techniques. For some, directly exposing tissues to the vitrification solution prior to freezing in liquid nitrogen was adequate. Some of these examples are bud clusters of *Guazuma crinita* (Maruyama *et al.*, 1997) using (w/v) 25% glycerol, 15% sucrose, 15% EG, 13% DMSO and 2% polyethylene glycol; meristems of garlic (Niwata, 1995) and cell suspensions of tobacco (Reinhoud *et al.*, 1995), both using a PVS2 solution.

The one-step or direct vitrification method has limited success especially on some less resistant cultured cells. This is due to the harmful effects of osmotic stress or chemical toxicity as a result of direct exposure to the highly concentrated vitrification solution. This limitation was almost completely overcome by a mild cryoprotective treatment, namely loading, with a less concentrated loading solution. In 1994, Matsumoto and colleagues improved the survival of vitrified wasabi meristems by loading with a mixture of 2 M glycerol and 0.4 M sucrose before vitrification. Huang *et al*., (1995) also loaded rice cells with 25% PVS2 to enhance the survival of vitrified cells. He suggested that loading may be capable of enhancing the permeation of individual solutes of the vitrification solution and provide desired degree of desiccation for plant cells.

Because of the toxic effects cryoprotectants may have on growth, it is necessary to dilute or remove the compounds after they have been frozen and thawed. For this purpose, the unloading step was also introduced after thawing, by exposing the vitrified cultures to mild cryoprotectants such as 1.2 M sucrose to dilute the toxic substances in the cells (Matsumoto *et al*., 1994,1995, Kohmura *et al*., 1994).

The key to successful cryopreservation by vitrification is to carefully control the procedure for dehydration and cryoprotectant permeation during exposure to highly concentrated vitrification solution. This will prevent injury by chemical toxicity or excess osmotic stress during dehydration.