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VITRIFICATION OF EXCISED EMBRYOS OF JACKFRUIT (ARTOCARPUS HETEROPHYLLUS LAMK)

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VITRIFICATION OF EXCISED EMBRYOS OF JACKFRUIT (*ARTOCARPUS HETEROPHYLLUS* LAMK)

By

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

VITRIFICATION OF EXCISED EMBRYOS OF JACKFRUIT (*ARTOCARPUS HETEROPHYLLUS* LAMK)

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This study was carried out to evaluate the potential of vitrification technique for germplasm conservation of jackfruit. The effects of loading solutions and vitrification solutions on survival of jackfruit embryos were evaluated.

The first experiment was to study the effects of different loading solutions during vitrification. The embryos were treated with six different loading solutions, followed by exposure to Plant Vitrification Solution 2 (PVS2) before plunging into liquid nitrogen. Results show that without freezing, all the loading solutions did not show deleterious effect on jackfruit embryos as high survival ranging from 81-96% was obtained. On freezing, embryos loaded with 25% PVS2 gave the highest viability (43.3%) and thus selected as the best loading solution for jackfruit embryos for the ensuing experiments.

In the second experiment, the embryos were loaded with 25% PVS2 for 0, 8, 12, 14, 16, 18 and 20 hours to determine the optimum time of loading. On

vitrification, 12, 14 and 16 hours of loading were equally advantageous, but 14 hours showed the highest with 18.3% viability and 13.3% survival. This was then chosen as the optimum time of exposure for the following experiments.

The third experiment was to evaluate the effects of different vitrification solutions on survival of jackfruit embryos. On freezing, embryos treated with L Solution gave the highest viability (30%) and survival (24%), which were significantly better than PVS2 and Watanabe Solution. This was thus selected as the most effective vitrification solution for jackfruit embryos.

Having determined the best vitrification solution, the fourth experiment assessed the different time of exposure to L Solution, whereby, the embryos were treated for 0, 15, 30, 45, 60, 75, 90, 105 and 120 minutes before freezing. Without freezing, high viability (94.6-100%) was obtained up to 120 minutes exposure. Following freezing, percentage viability increased with increasing time and reached an optimum of 47.4% after 75 minutes before decreasing to only 6.8% viability after 120 minutes exposure.

It was concluded that loading with 25% PVS2 for 14 hours, followed by exposure to L Solution for 75 minutes was optimum for vitrification of jackfruit embryos. Viability and survival 47.4% and 35.4% respectively can be obtained.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains Pertanian.

VITRIFIKASI EMBRIO NANGKA (*ARTOCARPUS HETEROPHYLLUS* LAMK)

Oleh

WONG LAY YIENG

November 2000

Pengerusi: Profesor Madya Dr. Hor Yue Luan

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Kajian ini telah dijalankan untuk menilai potensi teknik vitrifikasi dalam pemeliharaan germplasma nangka. Kesan larutan "loading" dan vitrifikasi ke atas kemandirian embrio nangka telah dikaji.

Eksperimen pertama dijalankan untuk mengkaji kesan jenis larutan "loading" semasa vitrifikasi. Embrio dirawat dengan enam jenis larutan "loading", diikuti dengan pendedahan kepada Larutan Vitrikasi Tumbuhan 2 (PVS2) sebelum dicelupkan ke dalam cecair nitrogen. Keputusan menunjukkan tanpa penyejukan, semua larutan "loading" tidak memberi sebarang kesan kemerosotan ke atas embrio nangka kerana peratus kemandirian di antara 81-96% telah diperolehi. Selepas penyejukan, embrio nangka yang dirawat dengan larutan 25% PVS2 memberi peratus kebernasan yang paling tinggi, iaitu 43.3% dan seterusnya dipilih sebagai larutan "loading" yang paling baik untuk embrio nangka dalam eksperimen berikutnya.

Untuk eksperimen kedua, embrio nangka telah dirawat dengan larutan "loading" selama 0, 8, 12, 14, 16, 18 dan 20 jam untuk menentukan masa optima rawatan. Selepas vitrifikasi, didapati 12, 14 dan 16 jam adalah sama baik, tetapi 14 jam rawatan memberi peratus kebernasan dan kemandirian yang paling tinggi dengan 18.3% dan 13.3% masing-masing. Oleh yang demikian, 14 jam telah dipilih sebagai masa optima dalam eksperimen berikutnya.

Eksperimen ketiga telah dijalankan untuk menilai kesan jenis larutan vitrifikasi ke atas kemandirian embrio nangka. Selepas penyejukan dalam cecair nitrogen, embrio yang dirawat dengan Larutan L memberi peratus kebernasan (30%) dan kemandirian (24%) yang paling tinggi yang berbeza secara bererti dengan larutan PVS2 dan Larutan Watanabe. Dengan itu, Larutan L telah dipilih sebagai larutan vitrifikasi yang paling berkesan untuk kemandirian embrio nangka selepas penyejukan.

Selepas menentukan larutan vitrifikasi yang paling baik, eksperimen keempat dijalankan untuk menilai kesan jangkamasa pendedahan yang berlainan terhadap Larutan L. Embrio nangka telah didedahkan kepada Larutan L selama 0, 15, 30, 45, 60, 75, 90, 105 dan 120 minit sebelum dicelupkan ke dalam cecair nitrogen. Tanpa penyejukan, didapati peratus kebernasan (94.6-100%) telah diperolehi sepanjang jangkamasa pendedahan tersebut. Selepas penyejukan, peratus kebernasan embrio nangka meningkat dengan meningkatnya masa pendedahan dan kebernasan optima sebanyak 47.4% diperolehi selepas 75 minit. Peratus kebernasan mula menurun selepas itu dan hanya 6.8% kebernasan didapati selepas 120 minit pendedahan.

Kesimpulannya, rawatan dengan larutan 25% PVS2 selama 14 jam, diikuti dengan 75 minit pendedahan kepada Larutan L adalah optima untuk vitrifikasi embrio nangka di mana 47.4% kebernasan dan 35.4% kemandirian boleh dicapai.

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I certify that an Examination Committee met on 7 November 2000 to conduct the final examination of Wong Lay Yieng on her Master thesis entitled "Vitrification of Excised Embryos of Jackfruit (*Artocarpus heterophyllus* Lamk)" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously of concurrently submitted for any other degree at UPM or other institutions.

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LIST OF ABBREVIATI

BAP	Benzylaminopurine
DMSO	Dimethyl sulfoxide
NAA	α-Napthalene Acetic Acid
MS	Murashige and Skoog
MC	Moisture content
GA_3	Gibberellic Acid
PVS	Plant Vitrification Solution
p.s.i.	Pounds per Square Inch
w/v	Weight by volume
wt	Weight
M	Molar
mM	Millimolar
-LN	without liquid nitrogen exposure
+LN	With liquid nitrogen exposure
SAS	Statistical Analysis System
ANOVA	Analysis of Variance

CHAPTER 1

INTRODUCTION

Jackfruit (*Artocarpus heterophyllus* Lamk) is indigenous to Western Ghats, India and was introduced and widely cultivated as fruit crops in the South-East Asia region. Jackfruit has many uses as the pulp of the young fruits can be cooked as a vegetable and the ripe fruit can be eaten fresh or made into jam, jelly, paste and cake. The seed is consumed as an aphrodisiac or a nutritious tonic while the leaves, seed starch, latex and root are used to heal ulcers, asthma, snakebite, glandular swellings, skin diseases, fever and diarrhoea. The wood of the tree was also reported to have medicinal properties and is also used for furniture, construction, implements, oars and musical instrument (Verheij and Coronel, 1991).

In Malaysia, jackfruit is planted mainly in home gardens and mixed orchards and the canned fruits are exported to Australia and Europe. Because of its economic importance, conservation of genetic resources of jackfruit is a vital factor for future crop improvement. However, seed storage is problematic as the seeds are recalcitrant which are sensitive to desiccation and low temperature (Stanwood, 1985). Present field conservation is costly as it requires much land and human resources, besides being threatened by diseases and environmental

stresses. Therefore, *in vitro* conservation especially cryopreservation is an important alternative.

Cryopreservation has become a valuable means for germplasm conservation as it provides physical and genetical stability and requires little management and low input of consumable materials. At the low temperature of liquid nitrogen (-196°C), metabolic processes are greatly reduced, resulting in minimal deterioration of cells thereby prolonging storage life to near eternity. Krisnapillay (1989) reported 60% viability of jackfruit embryos after treatment with a mixture of cryoprotectants prior to partial desiccation and prefreezing to -40°C before plunging into liquid nitrogen. It involved complicated cryoprotective procedure and expensive prefreezing equipment.

The vitrification method developed by Sakai et al. (1990, 1991) has helped to simplify the protocol for cryopreservation. Vitrification involves treatment of the embryos with a concentrated vitrification cocktail which form an amorphous glass during rapid cooling in liquid nitrogen without crystallization. Vitrification also eliminates the need for controlled freezing equipments and expensive apparatuse.

During vitrification, plant tissues are generally treated with loading solutions containing cryoprotective properties which help to reduce the harmful effects of direct exposure to vitrification cocktail due to osmotic stress or chemical toxicity. The loaded tissues are then partially dehydrated using a vitrification cocktail to promote metastable glass formation during freezing.

The loading solution and vitrification cocktail need to be evaluated for individual plant species as they appear to be species-specific in term of their chemical components and incubation time (Huang et al., 1995). Thus, this project was carried out to study the potential of the vitrification method for germplasm conservation of jackfruit embryos and the objectives of the study are:

(1) To investigate the effects of different loading solutions and time of exposure on viability and survival of jackfruit embryos in liquid nitrogen.

(2) To assess the effects of different vitrification solutions and time of exposure on viability and survival of jackfruit embryos in liquid nitrogen.

CHAPTER 2

LITERATURE REVIEW

Seed Storage Behaviour

Roberts (1973) described two major groups of seed based on their storage physiology, that is orthodox and recalcitrant seeds. Orthodox seeds follow the Rule of Thumb postulated by Harrington (1959) which specify that:

1) For each 1% reduction in moisture content, the storage life of the seed is doubled when the moisture content is between 5 and 14%;
2) For each 5°C reduction of storage temperature, the storage life of the seed is doubled when the temperature is between 0 and 50°C.

Hence, orthodox seeds remain viable for many years at ultra-low moisture content and their longevity can be further increased by storing the seeds at low temperature (Ellis and Roberts, 1980).

On the other hand, a number of tropical or subtropical tree species such as rubber (*Hevea brasiliensis*), jackfruit (*Artocarpus heterophyllus* Lamk), rambutan (*Nephelium lappaceum* L.) and cocoa (*Theobroma cacao*) have seeds showing recalcitrant behaviour. Recalcitrant seeds do not undergo maturation drying, but remain hydrated and metabolically active. They are shed at relatively high moisture content and are generally poised for continuous

development or germination after being shed (Chin & Pritchard, 1988; Pammenter et al., 1994). Storage of such seeds is therefore difficult unlike that of orthodox seeds. Recalcitrant seeds are generally killed if dried below a relatively high critical value, usually between 12 and 35% moisture content (Stanwood, 1985) and are also sensitive to low temperature. Such seeds have to be kept in moist and relatively warm conditions where their longevity is limited from a few weeks to a few months.

Chin (1975) found that storage of rambutan seeds in moist charcoal or sawdust could prolong seed viability for up to one month. Surface-dried jackfruit seeds stored in sealed plastic bag at temperature above 15°C retained viability for eight weeks (Hanson, 1984). Normah et al. (1986) reported that survival of rubber seeds was limited to around one year storage at room temperature.

Before storage, the seeds could be pretreated with a mixture of fungicides to reduce viability loss caused by the activity of microorganisms, and thereby increasing survival. The shelf life of cocoa seeds was prolonged from a few days to twenty four weeks after treatment with 0.2% w/w Benlate-Thiram mixture and partial desiccation to 33-35% moisture content before storing in loosely packed perforated polythene bags (Hor, 1984).

However, recalcitrance is not an absolute phenomenon (Berjak and Pammenter, 1994). Recent investigations have identified species which exhibit an intermediate form of seed storage behaviour (Ellis et al., 1990). They can

tolerate desiccation to fairly low moisture content but the dry seeds are injured by low temperature. Some economically important species such as coffee (*Coffea liberica*) and oil palm (*Elaies guinensis*) are included in this category.

Cryopreservation

Cryopreservation is a valuable means for germplasm conservation at ultra-low temperature of liquid nitrogen (-196°C). The method provides physical stability, requires little management and low input of consumable materials. At temperatures below –120°C, all biochemical and physical processes causing biological deterioration are slowed down or have come to a stand still (Kartha, 1985). Thus, the storage life of the preserved tissues are greatly prolonged and appears to remain genetically stable.

Resistance to freezing in liquid nitrogen has been studied for more than 100 plant species of temperate as well as tropical origin, in various culture forms. In the past decades, there has been varied degree of success with different plant tissues such as callus, cell suspension, protoplast, buds and shoot tips (Chandel and Pandey, 1991) as well as somatic, pollinic and zygotic embryos (Charrier et al., 1991). Cryogenic storage is also reported to provide a secure method for long-term storage of endangered plants in Australia (Touchell and Dixson, 1996).

Successful cryopreservation depends on the avoidance of lethal ice crystal formation during freezing, and usually involves a dehydration step prior

to freezing. Factors known to affect the cryoability of storage materials include the physiological state, age and nature of the materials to be preserved, as well as cryoprotectants, storage temperature, freezing and thawing.

Much studies are now being carried out to conserve recalcitrant seeds produced by tropical or subtropical tree species of fruits and timbers. The recalcitrant seeds generally are large in size, chilling sensitive and not practical to store cryogenically. Chin et al. (1989) proposed that storage of recalcitrant seed species could be improved using excised embryos. Zygotic embryonic axes are reasonably uniform genetically and are highly regenerative (Kendall et al., 1993). They do not mutate easily since they have undergone meiosis and mitosis, but are easy to establish and more practical to store since they are small and more tolerant to dehydration and freezing.

Generally, conventional cryopreservation technique involves the use of mild cryoprotectant solutions with slow freezing before plunging into liquid nitrogen. For example, zygotic embryos of rambutan and jackfruit gave 40% and 60% survival respectively after cryoprotection in a mixture of cryoprotectants followed by partial desiccation and slow freezing to –40°C prior to liquid nitrogen exposure (Hiew, 1991; Krishnanpillay, 1989).

Recently, some new techniques or vitrification-based procedures such as encapsulation-dehydration (Dereuddre et al., 1991; Hirata et al., 1995), vitrification (Matsumoto et al., 1998a; Sakai et al., 1990), desiccation (Berjak and Dumet, 1996; Normah et al., 1986) and pregrowth-desiccation (Assy-Bah

and Engelmann, 1992; Dumet et al., 1993) have simplified the cryopreservation protocols. These methods eliminate the use of controlled freezing equipment and complicated cryoprotective procedures as required for conventional slow freezing method. They are based on partial dehydration of explants followed by rapid freezing to achieve vitrification.

Vitrification-based procedures have been used successfully to cryopreserve a wide range of plant tissues and organs of different species. For example; shoot tips (Benson et al., 1996; Martinez et al., 1999), apices (Mari et al., 1995; Shatnawi et al., 1999), shoot primordia (Hirata et al., 1995; Ogawa et al., 1997), meristem (Niwata, 1995); cell suspensions (Bachiri et al., 1995; Reinhoud et al., 1995), zygotic embryos (Gonzalez-Rio et al., 1994; Kioko et al., 1998), somatic embryos (de Boucaud et al., 1994; Uragami et al., 1989), embryogenic tissue/culture (Bhatti et al., 1997; Cyr et al., 1994) and nucellar cells (Sakai et al., 1991).

Vitrification

Vitrification is the physical process whereby at sufficiently low temperature, a highly concentrated cryoprotective solution called vitrification cocktail becomes so viscous that it solidifies into an amorphous metastable glassy (ice free) state at the freezing point of the cryoprotective solution. Therefore, vitrification avoids injuries due to intra and extracellular ice formation and solution effects occurring under conventional methods. It does not require controlled freezing equipment or sophisticated and expensive

apparatuse, whilst permits cells and meristems to be cryopreserved by direct immersion into liquid nitrogen.

Vitrification is more appropriate for complex organs, such as shoot tips and somatic embryos that contain a variety of cell types. This is because vitrification procedures dehydrate a major portion of freezable cell water of tissues at non-freezing temperatures, and permit them to be cryopreserved by direct plunge into liquid nitrogen without causing dehydration injury.

Loading

The key for successful vitrification is to induce osmo-tolerance of tissues to a highly concentrated vitrification cocktail. The harmful effects due to dehydration can be alleviated by adequate preconditionings, such as cold-hardening (Kohmura et al., 1994; Reed, 1990), preculture with sucrose or sorbitol (Niino et al., 1992; Yamada et al., 1991) and cryoprotective treatment (loading) (Langis and Steponkus, 1990; Matsumoto et al., 1994).

Preconditioning to enhance dehydration or freezing tolerance appears species-specific and is dependent on the natural property and the physiological state of the cells. Plant tissues are generally treated with various loading solutions containing cryoprotective properties which help to reduce the harmful effects of direct exposure to vitrification cocktail due to osmotic stress or chemical toxicity. It was suggested that loading solution may mitigate the excessive osmotic stress caused by the vitrification solution and protect the cell and tissues by minimizing injurious membrane changes caused by severe