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ESTABLISHMENT OF SHOOT MULTIPLICATION SYSTEM AND CRYOPRESERVATION OF JACKFRUIT (Artocarpus heterophyllus LAMK) SHOOT TIPS

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By

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Chairman : Professor Uma Rani a/p Sinniah, PhD
Faculty : Agriculture

Jackfruit produces recalcitrant seeds that are dessication-sensitive and cannot be stored by conventional seed storage. This poses a major problem for conservation of valuable genetic diversity of this plant species. Thus, sustainable conservation of its germplasm is imperative. Cryopreservation is the only viable option for long-term storage of recalcitrant plant species. The aim of this study, therefore, is to develop *in vitro* multiplication system using shoots obtained from seedlings and cryopreservation protocol for *in vitro* shoot tips. The results presented in this thesis ascertain that the physiological and developmental state of explant and suitable *in vitro* culture system coupled with optimization of the various steps involved in cryopreservation protocols are critical factors that influence successful cryopreservation of jackfruit shoot tips. To establish an efficient *in vitro* culture, suitable seeds were selected for seedling establishment. Seeds from top, middle and basal section of the fruit were extracted and germinated. The seeds from the middle and basal sections germinated earlier than those from the top and had 100% germination. *In vitro* culture was established using shoot tips from the germinated seedlings at 4, 6, 8 and 12 weeks with variation in terminal shoot developmental stage *viz* shoot with unexpanded leaf, shoot with partially expanded leaf and shoot with expanded leaf. The results showed that terminal shoot obtained from 6-week-old seedlings in MS medium supplemented with 3.0 mg/L benzylaminopurine performed best compared with 4, 8 and 12-week-old seedlings. Shoots with partially expanded leaf and with expanded leaf obtained from 6 weeks old seedlings induced 100% new shoots and produced up to seven multiple shoots than shoots with unexpanded leaf. The shoot tips form the multiple shoot produced *in vitro* were used to establish the cryo-protocol. Successful cryopreservation was achieved by using 6-week *in vitro* cultured shoot tips of sizes 1.5 mm - 2.0 mm as the explant. Two cryopreservation protocol *viz* vitrification and encapsulation-vitrification techniques using plant vitrification solutions: PVS2, PVS3 and PVS4 were carried out. The findings of the study on encapsulation shows that stepwise exposure to PVS2 [30%

(w/v) glycerol 15% (w/v) ethylene glycol, 15% (w/v) DMSO, 0.4 M sucrose] resulted in survival of 82.1% and 23.5% in non-cryopreserved and cryopreserved encapsulated shoot tips. In contrast, survival of 88.9% and 35.2% was obtained with direct exposure to PVS3 [50% (w/v) glycerol, 50% sucrose] while PVS4 [35% (w/v) glycerol, 20% (w/v) ethylene glycol, 0.6 M sucrose] resulted in 77.1% and 37.0% survival respectively. Moreover, 28.7% survival was obtained with PVS2 at 0°C while a higher percentage survival of 35.8% and 37.9% was obtained at 25°C with PVS3 and PVS4 in cryopreserved shoot tips respectively. In non-encapsulated shoot tips, sucrose concentration at 0.5 M exposed for 48 h induced cryotolerance to dehydration. Elevated sucrose concentration (0.7 - 0.9 M) and extended period of exposure (72 - 240 h) was detrimental. The different plant vitrification solution used had varied responses on the shoot tips at different times of exposure. The critical hydration window range 49.7% - 52.4% water content was attained in all the vitrification solutions with survival range of 26.8% - 37.4% at different time of exposure. Histological observation revealed that structural changes occurred in the cell during incubation with plant vitrification solution and freezing. Sucrose preculture and PVS4 treatment for 50 minutes (52.4% WC) was able to preserved some living cells against damage during freezing. This study was successful in establishing an efficient system for *in vitro* shoot tip multiplication and cryopreservation protocol by vitrification using PVS4. Thus, long-term storage of explants of *Artocarpus* species is possible by focusing on the critical factors influencing successful cryopreservation.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PEMBANGUNAN SISTEM PENGGANDAAN PUCUK DAN KRIOAWETAN HUJUNG PUCUK NANGKA (*Artocarpus heterophyllus* LAMK)

Oleh

OLUWAGADE ADENIKE OKUNADE

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Pengerusi : Professor Uma Rani a/p Sinniah, PhD
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Buah nangka mempunyai biji benih jenis rekalsitran yang sensitif kepada pengeringan dan tidak boleh disimpan menggunakan kaedah penyimpanan biji benih secara konvensional. Ini menyebabkan masalah besar dalam pemuliharaan kepelbagaian genetik yang bernilai bagi spesies tumbuhan ini. Oleh itu, kaedah konservasi janaplasma yang mampan adalah penting. Krioawetan merupakan satu-satunya pilihan berdaya maju bagi penyimpanan jangka panjang bagi spesis tumbuham jenis rekalsitran. Oleh itu, tujuan kajian ini adalah untuk menghasilkan satu sistem bagi penggandaan pucuk bagi explan yang diperolehi dari anak benih dan mendapatkan protokol bagi krioawetan hujung pucuk in vitro nangka. Keputusan yang dibentangkan dalam tesis ini menunjukkan bahawa keadaan fisiologi dan tahap perkembangan explan dan sistem in vitro yang sesuai digabungkan dengan pengoptimuman pelbagai langkah yang terlibat dalam protokol krioawetan adalah faktor kritikal yang mempengaruhi kejayaan krioawetan nangka. Untuk mewujudkan sistem in vitro yang efisen, biji benih yang sesuai dipilih untuk mendapatkan anak benih. Biji benih dari bahagian atas, tengah dan basal buah telah diekstrak dan dicambahkan. Biji benih dari bahagian tengah dan basal bercambah lebih awal daripada yang dari atas dan mempunyai percambahan 100% Kultur in vitro dihasilkandenganmenggunakan pucukdarianakbenih yang berumur 4, 6, 8 dan 12 minggu selepas percambahan dengan variasi dalam tahap perkembangan pucuk iaitu dengan daun yang belum kembang, daun separa kembang dan daun kembang sepenuhnya. Keputusan menunjukkan bahawa pucuk yang diperolehi daripada anak benih berusia 6 minggu dalam medium MS ditambah dengan benzylaminopurine 3.0 mg / L memberikan respon terbaik dibandingkan dengan anak benih 4, 8 dan 12-minggu. Pucuk dengan daun yang kembang sepenuhnya yang diperolehi daripada anak benih berusia 6 minggu memberikan 100% pucuk baru dengan tujuh pucuk berbanding dengan pucuk dari daun yang tidak berkembang. Krioawetan telah berjaya dicapai dengan menggunakan hujung pucuk in vitro berusia 6-minggu yang bersaiz 1.5 mm - 2.0 mm yang digunakan

sebagai explan. Dua protokol krioawetan iaitu teknik vitrifikasi dan teknik pengkapsulan-vitrifikasi menggunakan larutan vitrifikasi tanaman: PVS2, PVS3 dan PVS4 telah dijalankan. Keputusan kajian pengkapsulan menunjukkan bahawa pendedahan langkah demi langkah terhadap PVS2 [30% (w/v) gliserol 15% (w/v) ethylene glycol, 15% (w/v) DMSO, 0.4 M sucrosa] memberikan kemandirian hujung pucuk yang telah dikapsulkan sebanyak 82.1% dan 23.5% tanpa dan dengan krioawetan. Sebaliknya, kemandirian sebanyak 88.9% dan 35.2% diperoleh dengan pendedahan langsung kepada PVS3 [50% (w/v) gliserol, 50% sukrosa] manakala PVS4 [35% (w/v) gliserol, 20% (w/v) ethylene glycol , 0.6 M sukrosa] masing-masing menghasilkan 77.1% dan 37.0% kemandirian. Selain itu, kemandirian sebanyak 28.7% diperoleh dengan PVS2 pada 0°C manakala peratus kemandirian yang lebih tinggi sebanyak 35.8% dan 37.9% diperolehi pada 25ºC dengan PVS3 dan PVS4 masing-masing selepas krioawetan. Kepekatan sukrosa optimum iaitu 0.5 M dengan pendedahan selama 48 jam memberikan toleransi kepada pengeringan dan menyebabkan teknik vitrifikasi berjaya. Peningkatan kepekatan sukrosa kepada 0.7 - 0.9 M dan tempoh pendedahan yang lanjut (72 - 240j) adalah memudaratkan. Jenis larutan vitrifikasi yang berbeza memberikan respon yang berbeza terhadap hujung pucuk mengikut jangkamasa pendedahan. Julat penghidratan kritikal dengan kandungan kelembapan sebanyak 49.7% - 52.4% dapat diperolehi dalam semua jenis larutan vitrifikasi dengan julat kemandirian sebanyak 26.8% - 37.4% pada masa pendedahan yang berbeza. Pemerhatian histologi mendedahkan bahawa terdapt perubahan pada struktur sel sewaktu pendedahan kepada larutan vitrifikasi dan penyejukan. Pendedahan kepada sukrosa dan larutan PVS4 selamat 50 min (52.4% WC) membolehlah sel hidup mengelakkan dari kerosakan semasa penyejukan. Kajian ini berjaya menghasilkan sistem yang cekap untuk penggandaan hujung pucuk in vitro dan protokol cryopreservation melalui keadah vitrification menggunakan PVS4. Oleh itu, penyimpanan jangka panjang spesies Artocarpus boleh dilakukan dengan memberi tumpuan kepada faktor kritikal yang mempengaruhi kejayaan krioawetan.

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I certify that a Thesis Examination Committee has met on 2 June 2017 to conduct the final examination of Oluwagade Adenike Okunade on her thesis entitled "Establishment of Shoot Multiplication System and Cryopreservation of Jackfruit (*Artocarpus heterophyllus* Lamk) Shoot Tips" in accordance with the Universities and University Colleges Act 1971 and the Constitution of the Universiti Putra Malaysia [P.U.(A) 106] 15 March 1998. The Committee recommends that the student be awarded the Doctor of Philosophy.

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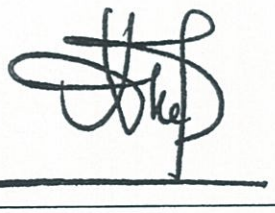

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This thesis was submitted to the senate of Universiti Putra Malaysia and has been accepted as fulfilment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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LIST OF ABBREVIATIONS

µM	Micromolar
ANOVA	Analysis of Variance
BAP	N^6-benzylaminopurine
$CaCl_2$	Calcium chloride
DMRT	Duncan's Multiple Range Test
DMSO	Dimethylsulfoxide
g.g-1	Gram per gram
g/L	Gram per litre
LN	Liquid Nitrogen
LP	Leaf Primordia
M	Molar
ml	Milliliter
mM	Milli molar
NaOCl	Sodium Hypochlorite
PGR	Plant Growth Regulator
PVS2	Plant Vitrification Solution 2
PVS3	Plant Vitrification Solution 3
PVS4	Plant Vitrification Solution 4
TDZ	N-phenyl-N-1, 2, 3-thiadazol-5-yl urea
WC	Water Content
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

Jackfruit (*Artocarpus heterophyllus* Lamk) is a monoecious tree that produces the largest tree-borne fruits in the world; yielding an average of 200 fruits per tree per year. The fruit can grow up to 50 kg in weight and 90 cm in length with an average of 250 seeds in a fruit. Jackfruit is widely cultivated in many countries in Asia and also found in some parts of Caribbean, Africa, America, and Australia (Sidhu, 2012). In South East Asia, jackfruit is cultivated due to its use in the food industry particularly as dessert owing to its rich source of phenolic compounds, flavonoid and various antioxidants (Swami et al., 2012).

Conservation of jackfruit valuable genetic diversity is vital not only to ensure food security but also to support breeding programmes (Haq, 2006). Jackfruits are mainly conserved in the field gene banks because the seeds are highly hydrated and sensitive to even slight drying, thus, cannot be stored under conventional conditions for orthodox seeds. The cost of maintenance of field bank is high and field accessions are vulnerable to adverse weather conditions, pests, and diseases. Currently, due to rapid land development and deforestation, genetic resources of jackfruit are being lost. Therefore, complementary conservation strategies via cryopreservation for long-term storage is of utmost importance (Mal et al., 2011).

For effective cryopreservation, it is important to establish a suitable system for the regeneration of the explant to be cryopreserved (Reed et al., 2013). Shoot tip is a suitable explant for cryopreservation of recalcitrant plant species (Ibrahim & Normah, 2013). *In vitro* culture is valuable in providing initial explants or stock cultures and the recovery of cryopreserved samples in appropriate growing media (Engelmann & Dussert, 2013; Engelmann, 2011). The success or failure of cryopreserved material is partly dependent on the appropriate *in vitro* culture method. A major step to successful cryopreservation is removal of free water from the tissue which is difficult to achieve in recalcitrant plant species like jackfruit. The sensitivity and intolerance to desiccation and freezing can be controlled with plant vitrification solutions. Vitrification-based method such as vitrification and encapsulation-vitrification is, thus, effective for recalcitrant plant species (Engelmann & Dussert, 2013).

Presently, there is limited knowledge on differential responses of tissues to the different plant vitrification solutions. Fundamental knowledge is required of the composition of the vitrification solution, time of exposure, method of application and prevailing temperature during vitrification process in order to establish a successful cryopreservation protocols. Furthermore, low levels of post-storage survival and regrowth still remains a challenge in recalcitrant plant species regardless of the protocol employed (Normah et al., 2012). Therefore, basic understanding relating to

the structural changes induced in cells during cryopreservation steps are required to improve responses of recalcitrant explants to cryopreservation.

Therefore the objectives of this study are:

1. To investigate the variation among seeds within the fruit in order to establish vigorous seedlings as source of explant for *in vitro* culture of jackfruit shoot tips.

2. To optimize an effective *in vitro* multiplication system for successful cryopreservation.

3. To establish a suitable protocol for cryopreservation of jackfruit shoot tips using vitrification and encapsulation-vitrification techniques.

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