UNIVERSITI PUTRA MALAYSIA

DETERMINATION OF HYDRATION WINDOW FOR CRYOPRESERVATION OF THREE CITRUS SPECIES

AMYITA WITTY UGAP

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By

Amyita Witty Ugap

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree Master of Science

April 2008

DEDICATION

This manuscript is especially dedicated to my parents, Ugap Ganai and Moureen Dasim, to my brothers; Collin, Rayner, Melven and Earven, to my sisters; Flourita, Adriana, Viviana and Rozyiana and to my dear friend, Andrew Kong.

An abstract of the thesis presented to the Senate of Universiti Putra Malaysia as partial fulfilment of the requirements for the degree of Master of Science.

DETERMINATION OF HYDRATION WINDOW FOR CRYOPRESERVATION OF THREE CITRUS SPECIES

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AMYITA WITTY UGAP

April 2008

Chairman : Associate Professor Uma Rani Sinniah, PhD

Faculty : Agriculture

The objective of this study was to determine the optimal hydration window for successful cryopreservation of *Citrus madurensis*, *C. grandis* and *C. reticulata* seeds in relation to their tolerance to desiccation and freezing.

Initially, seed tolerance to desiccation and freezing were empirically investigated based on the seed survival, before and after freezing in liquid nitrogen (LN). Seeds were desiccated in a laminar air flow (LAF) (55% RH) and over silica gel (SG) (5% RH) for different periods (0, 12, 24, 36, 48, 60, 72, 96 and 120 hours). The dehydration rates were similar for both methods employed for the first 48 hours of desiccation but differed thereafter, with seeds desiccated using SG reaching water content below 0.10 $gH_2O.g^{-1}$ fresh weight (fw) earlier than using LAF. Significant decline in seed survival was observed with decrease in water content of the three citrus species. Water content for desiccation sensitivity (WC_{DS}) measured as the water content when significant decline in survival was observed. WC_{DS} for seeds desiccated in LAF was 0.14, 0.18 and 0.07 $gH_2O.g^{-1}$ fw for *C. madurensis*, *C. grandis* and *C. reticulata*, respectively. As for SG, it was 0.17, 0.23 and 0.08 $gH_2O.g^{-}$

[1] fw for *C. madurensis*, *C. grandis* and *C. reticulata*. In relation to freezing tolerance, the water content at the highest survival achieved or termed the high moisture freezing limit (HMFL) was 0.09, 0.13 and 0.11 gH_2Og^{-1} fw for *C. madurensis*, *C. grandis* and *C. reticulata* when seeds were desiccated in LAF. As for seeds desiccated over SG, the HMFL was 0.10 $gH_2O.g^{-1}$ fw for *C. madurensis* and 0.08 $gH_2O.g^{-1}$ fw for *C. grandis* and *C. reticulata*. This resulted in WC_{DS} having greater value than HMFL. Therefore WC_{DS} was determined based on 50% reduction in seed survival and the values were 0.07 $gH_2O.g^{-1}$ fw for *C. madurensis* and 0.06 $gH_2O.g^{-1}$ fw for *C. grandis* and *C. reticulata* when desiccated in LAF. As for SG, it was 0.06, 0.08 and 0.05 $gH_2O.g^{-1}$ fw for *C. madurensis*, *C. grandis* and *C. reticulata*. Seeds exposed to LN, subsequent to desiccation to ~0.10 $gH_2O.g^{-1}$ fw water content using the LAF resulted in better survival (40 – 50%) as compared to SG (<40%). However, the water content obtained would depend on the initial water content and the seed-to-seed variation.

In the second study, seeds were equilibrated over various saturated salt solutions (8 – 92% RH) at 25°C for three weeks to allow seeds to reach their equilibrium water content in order to minimize seed-to-seed variation. Seed desiccation sensitivity was measured based on the water content at which 50% survival was achieved (WC_{50}) using the fitted pattern of the desiccation sensitivity model which ranged between 0.06 – 0.11 $gH_2O.g^{-1}$ fw for the three citrus species. The expression of water content in terms of water activity obtained from water sorption isotherm showed that irrespective of the water content for desiccation sensitivity the water activity was similar (~0.65). As for seed freezing tolerance, a high variation was observed between seeds of the citrus species when measured as HMFL (HMFL=0.13, 0.15 and

0.10 $gH_2O.g^{-1}$ fw for *C. madurensis C. grandis* and *C. reticulata*). When determined by thermal analyses, the unfreezable water content (WC_U) of *C. madurensis*, *C. grandis* and *C. reticulata* were 0.19, 0.20 and 0.15 $gH_2O.g^{-1}$ fw as indicated by Differential Thermal Analysis (DTA) and it was 0.11, 0.14 and 0.09 $gH_2O.g^{-1}$ fw when determined by Differential Scanning Calorimetry (DSC). The DTA was not sensitive enough to detect freezable water at lower water contents. A highly significant correlation was found for those species between the WC_U and the HMFL. The values for WC_U (DSC) and HMFL coincided indicating that the removal of all freezable water is absolutely essential for the survival of the three citrus species studied. Therefore, the use of DSC for determination of WC_U is reliable. Seeds desiccated in 70 – 80% RH consistently resulted in removal of freezable water.

Total lipid content in seeds was determined in order to see the relationship between seed total lipid content and WC_U. The amount of total lipid content in the three citrus species studied differed significantly, namely 41.9% fw for *C. madurensis*, 30.7% fw for *C. grandis* and 48.6% fw for *C. reticulata* but was negatively correlated to WC_U. The negative correlation can be explained by the equation; $WC_U = 30.41 - 0.47x$. Therefore, the possibility of obtaining the safe water content for cryopreservation of citrus species is based on total lipid content.

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

PENENTUAN JULAT KANDUNGAN AIR YANG OPTIMUM UNTUK PENGKRIOAWETAN TIGA SPESIES CITRUS

oleh

Amyita Witty Ugap

April 2008

Pengerusi : Professor Madya Uma Rani Sinniah, PhD

Fakulti : Pertanian

Kajian ini dijalankan untuk menentukan julat kandungan air yang optimum untuk pengkrioawetan (HWC) bagi *Citrus madurensis*, *C. grandis* dan *C. reticulata* berdasarkan ketoleransian biji benih terhadap pengeringan dan penyejukan.

Pada permulaannya, kajian secara empirikal dijalankan untuk menentukan kemandirian biji benih, sebelum dan selepas didedahkan kepada cecair nitrogen. Pengeringan dijalankan di dalam kebuk aliran laminar (LAF) (55% RH) dan di dalam desikator yang terkandung gel silika (SG) (5% RH) untuk jangkamasa yang berbeza (0, 12, 24, 36, 48, 60, 72, 96 dan 120 jam). Kadar pengeringan biji benih adalah sama bagi kedua-dua jenis pengeringan untuk 48 jam yang pertama, di mana kandungan air kurang daripada 0.10 $gH_2O.g^{-1}$ fw dicapai lebih awal apabila SG digunakan berbanding pengeringan menggunakan LAF. Kemandirian biji benih berkurangan dengan penurunan kandungan air selepas pengeringan. Nilai kandungan air bagi sensitiviti terhadap pengeringan (WC_{DS}) adalah berdasarkan kepada kemerosotan awal kemandirian biji benih. Bagi pengeringan menggunakan LAF, WC_{DS} adalah 0.14, 0.18 dan 0.07 $gH_2O.g^{-1}$ fw bagi *C. madurensis*, *C. grandis* dan *C.*

reticulata. Manakala bagi pengeringan menggunakan SG, ianya adalah 0.17, 0.23 dan 0.08 $gH_2O.g^{-1}$ fw bagi *C. madurensis*, *C. grandis* dan *C. reticulata*. Bagi toleransi terhadap penyejukan, nilai kandungan air pada kemandirian biji benih yang tertinggi atau kandungan air bagi had penyejukan (HMFL) adalah 0.09, 0.13 dan 0.11 gH_2Og^{-1} fw bagi *C. madurensis*, *C. grandis* dan *C. reticulata* apabila dikeringkan di dalam LAF. Bagi biji benih dikeringkan di atas SG, nilai HMFL adalah 0.10 $gH_2O.g^{-1}$ fw bagi *C. madurensis* dan 0.08 $gH_2O.g^{-1}$ fw bagi *C. grandis* dan *C. reticulata*. Ini menyebabkan nilai WC_{DS} adalah lebih tinggi berbanding nilai HMFL. Oleh itu, nilai WC_{DS} adalah berdasarkan kepada 50% kemerosotan biji benih selepas pengeringan dan nilainya adalah 0.07 $gH_2O.g^{-1}$ fw bagi *C. madurensis* dan 0.06 $gH_2O.g^{-1}$ fw bagi *C. grandis* dan *C. reticulata* apabila dikeringkan dalam LAF. Bagi pengeringan menggunakan SG, ianya adalah 0.06, 0.08 dan 0.05 $gH_2O.g^{-1}$ fw bagi *C. madurensis*, *C. grandis* dan *C. reticulata*. Biji benih yang dikeringkan kepada ~0.10 $gH_2O.g^{-1}$ fw menggunakan LAF dan didedahkan kepada nitrogen cecair menunjukkan kemandirian yang lebih memuaskan (40 – 50%) berbanding SG (<40%). Namun, kandungan air tersebut dicapai bergantung kepada kandungan awal air dan variasi antara biji benih.

Dalam kajian yang kedua, biji benih citrus dikeringkan dalam beberapa larutan kimia tepu (8 – 92% RH) pada suhu 25°C selama tiga minggu untuk mencapai ekuilibrasi dan perbezaan kandungan air di antara biji benih adalah minimum. Toleransi terhadap pengeringan diukur pada kandungan air apabila 50% kemandirian biji benih dicapai (WC_{50}) berdasarkan corak pemadanan model sensitiviti pengeringan dan ianya adalah di antara julat 0.06 – 0.11 $gH_2O.g^{-1}$ fw bagi ketiga-tiga spesies citrus tersebut. Penentuan nilai aktiviti air adalah berdasarkan kandungan air dari isoterma penyerapan air menunjukkan kandungan air bagi toleransi terhadap pengeringan adalah hampir sama (~0.65) walaupun kandungan air asal adalah berbeza. Manakala toleransi tehadap cecair nitrogen adalah berbeza bagi setiap spesies citrus apabila diukur sebagai HMFL (HMFL=0.13, 0.15 dan 0.10 $gH_2O.g^{-1}$ fw bagi *C. madurensis* *C. grandis* dan *C. reticulata*). Apabila diukur menggunakan 'Differential Thermal Analysis' (DTA), kandungan air tak beku (WC_U) bagi *C. madurensis*, *C. grandis* dan *C. reticulata* adalah 0.19, 0.20 dan 0.15 $gH_2O.g^{-1}$ fw dan ianya adalah 0.11, 0.14 dan 0.09 $gH_2O.g^{-1}$ fw apabila diukur menggunakan 'Differential Scanning Calorimetry' (DSC). DTA tidak boleh mengesan kehadiran air boleh beku pada kandungan air yang rendah. Korelasi di antara WC_U dan HMFL adalah sangat tinggi. Nilai WC_U (DSC) dan HMFL yang sama menunjukkan kepentingan mengeluarkan air boleh beku untuk kemandirian biji benih citrus. Oleh itu, penggunaan DSC untuk mengukur nilai WC_U adalah lebih tepat. Biji benih yang dikeringkan di antara 70 – 80% RH menyebabkan air boleh beku dikeluarkan dari biji benih.

Jumlah keseluruhan kandungan lipid dalam biji benih diukur untuk melihat kaitan di antara lipid dan WC_U. Ianya menunjukkan bahawa terdapat perbezaan kandungan lipid bagi setiap spesies citrus iaitu 41.9% fw bagi *C. madurensis*, 30.7% fw bagi *C. grandis* dan 48.6% fw bagi *C. reticulata* dan terdapat kaitan negatif di antara lipid dan WC_U. Korelasi negatif tersebut adalah berdasarkan formula; $WC_U = 30.41 - 0.47x$. Oleh itu, kebarangkalian untuk menentukan kandungan air yang selamat bagi pengkrioawetan biji benih citrus adalah melalui kandungan lipid.

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Last but not least, I would like to express my deepest appreciation to my family for their support and understanding throughout my study.

I certify that an Examination Committee has met on 17 April 2008 to conduct the final examination of Amyita Witty Ugap on her Master of Science thesis entitled "Determination of Hydration Window for Cryopreservation of Three Citrus Species" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The committee recommends that the student be awarded the Master of Science.

Members of the Examination Committee were as follows:

Mihdzar Abdul Kadir, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairman)

Saleh Kadzimin, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Internal Examiner)

Mohamad Mohd. Lasim, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Internal Examiner)

Normah Mohd. Noor, PhD
Professor
Faculty of Science and Technology
Universiti Kebangsaan Malaysia
(External Examiner)

HASANAH MOHD. GHAZALI, PhD
Professor and Deputy Dean
School of Graduates Studies
Universiti Putra Malaysia

Date: 22 July 2008

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Master of Science. The members of the Supervisory Committee were as follows:

Uma Rani Sinniah, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Chairperson)

Maheran binti Abd. Aziz, PhD
Associate Professor
Faculty of Agriculture
Universiti Putra Malaysia
(Member)

AINI IDERIS, PhD
Professor and Dean
School of Graduates Studies
Universiti Putra Malaysia

Date: 14 August 2008

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 or concurrently submitted for any other degree at UPM or other institutions.

AMYITA WITTY UGAP

Date: 17/6/08

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degree Celsius
DMRT	Duncan Multiple Range Test
DSC	Differential Scanning Calorimetry
DTA	Differential Thermal Analysis
dw	Dry Weight
e.g.	As an example/ for instance
etc.	et cetera/ and so on/ forth
fw	Fresh Weight
HL	High Limit
HMFL	High Moisture Freezing Limit
HWC	Hydration Window for Cryopreservation
i.e.	id est/ that is to say/ in other words
ISTA	International Seed Testing Association
LAF	Laminar Air Flow
LL	Low Limit
LN	Liquid Nitrogen
-LN	Without Freezing in Liquid Nitrogen
+LN	With Freezing in Liquid Nitrogen
RCBD	Randomized Complete Block Design
RH	Relative Humidity
SAS	Statistical Analysis System
SG	Silica Gel

SSS	Saturated Salt Solution
viz.	As follows
WC_{50}	Water Content at which 50% survival was lost
WC_{DS}	Water Content for Desiccation Sensitivity
WC_{U}	Unfreezable Water Content
AOSA	Association of Official Seed Analysts
FAO	Food and Agricultural Organization of the United Nations
IBPGR	International Board for Plant Genetic Resources