CRYOPRESERVATION TECHNIQUES FOR THE LONG-TERM STORAGE OF MAHOGANY (*SWIETENIA MACROPHYLLA*) SEEDS

M. Marzalina

Forest Research Institute Malaysia, Kepong, 52109 Kuala Lumpur, Malaysia

&

M. N. Normah

Department of Botany, Faculty of Life Sciences, Universiti Kebangsaan Malaysia, 43600 UKM, Bangi, Selangor, Malaysia

Received April 2001

MARZALINA, M. & NORMAH, M. N. 2002. Cryopreservation techniques for the long-term storage of mahogany (*Swietenia macrophylla*) seeds. Research on cryopreservation of mahogany seeds (with and without testa) and embryonic axes was carried out using liquid nitrogen either by direct immersion or slow cooling. The best treatment for seeds was six hours desiccation, without testa, using a slow cooling regime, which resulted in 77% survival. The moisture content after six hours desiccation was 5.59%. The initial moisture content of the excised embryos was 11.52% and viability at this moisture was 97%. The best pretreatment for axes before cryopreservation was six hours desiccation (resulting in a 4.59% moisture content) in which 63% survival was obtained after direct immersion in liquid nitrogen. By comparison 87% of the embryonic axes survived cryopreservation using the slow cooling technique.

Key words: Embryonic axes - desiccation - moisture content - survival - viability

MARZALINA, M. & NORMAH, M. N. 2002. Teknik krioawetan untuk penyimpanan jangka masa panjang biji benih mahogani (*Swietenia macrophylla*). Penyelidikan mengkrioawetkan biji benih mahogani (berkulit dan tanpa kulit) dan paksi embrionya telah dilakukan sama ada secara memasuk terus ke dalam cecair nitrogen atau dengan menggunakan teknik penyejukan berkadar. Kajian mendapati bahawa teknik terbaik adalah secara mengeringkan biji benih selama enam jam, tanpa kulit dengan menggunakan teknik penyejukan berkadar. Kaedah ini menghasilkan 77% kebolehidupan dengan kandungan lembapan 5.59% selepas pengeringan. Kandungan lembapan asal paksi embrio ialah sebanyak 11.52% dan kebolehidupannya ialah 97%. Kaedah prarawatan yang terbaik untuk paksi embrio sebelum dikrioawetkan adalah dengan mengeringkannya selama enam jam (menghasilkan kandungan lembapan 4.59%). Keputusan menunjukkan bahawa teknik memasuk terus ke dalam cecair nitrogen telah menghasilkan 63% kebolehidupan paksi embrio, sementara teknik penyejukan berkadar menghasilkan 87% kebolehidupan.

Introduction

Malaysia is one of the twelve countries of the world categorised as a centre of megadiversity. These countries together contain 60% of the world's known species. The terrestrial flora here are found in habitats and ecosystems ranging from the lowlands to the top of the highest mountains, thus include a spread of forest types. These forests are considered as the cradle of the world's biological diversity. As a result, there is an increasing awareness regarding the importance of conserving forest trees and plant genetic resources. However, most tropical timber species produce recalcitrant seeds and are irregular and unpredictable fruit bearers (Marzalina & Krishnapillay 1999). Cyclic mass fruiting usually happens once every three to six years (Ashton *et al.* 1988, Yap & Marzalina 1990). Thus, the development of long-term storage techniques for seeds is highly important to maintain seed availability.

Mahogany was chosen as a case study because it is one of the main tropical timber species traded in the world. Several reports mentioned that the centers of origins of mahogany are being threatened, resulting in germplasm depletion (Uetsuki 1988, Rodan *et al.* 1992). Considerable efforts are being undertaken to conserve the germplasm of this species. Mahogany seeds have been considered intermediate and unable to tolerate drying to a low moisture content (Lopez 1938, Harrington 1972). To date, various storage conditions have been investigated including containers, temperature of storage and seed moisture content (Vivekanandan 1978, Martini 1985, Pena & Montalvo 1986, Uetsuki 1988, Pukittayacamee 1991). By and large, the conventional seed storage techniques is the most widely used (Mayhew & Newton 1998). This involves storing the seed in a cold room at temperatures between - 20 and 25 °C (room temperature). The maximum storage period reported thus far is two years, with seeds placed in polyethylene bags at 10 °C; however, the seed viability dropped over the period (Pukittayacamee *et al.* 1995). Cryopreservation technique offers the possibility of preserving germplasm, especially for seed that has limited success in *ex situ* long-term storage using conventional methods. As a complementary method to the conventional techniques, cryopreservation is now increasingly used to conserve endangered species (Benson 1999). The objective of this study was to determine the desiccation sensitivity of mahogany seeds and embryonic axes and establish cryopreservation techniques for these two types of explants.

Materials and methods

Mahogany seeds were freshly collected and extracted from fruits gathered from plantations in Sg. Buluh, Selangor and at the Forest Research Institute Malaysia (FRIM)'s research plots. The experiments were carried out at the Seed Technology Laboratory at FRIM.

The initial moisture content (fresh weight basis) and germination capacity of the seeds were determined after 0, 2, 4, 6 and 8 hours desiccation periods. All desiccation treatments were carried out under laminar flow bench (Gelman

Sciences Pty. Ltd., New South Wales, Australia) at a temperature of 25 °C and RH of 55%. Three replicates of 10 seeds per treatment were used in all experiments. Germination tests for whole seeds were performed using containers with pure sand placed in a 28 ± 2 °C germination room (Anonymous 1993).

Cryopreservation experiments were divided into two parts, i.e. whole seeds and embryonic axes.

Whole seeds

Seeds were processed for storage with or without the testa and after each desiccation period, they were exposed to liquid nitrogen (- 196 °C) using direct immersion or slow cooling techniques. Desiccation controls were also performed to determine the viability of seeds after desiccation.

For the direct immersion, seeds were placed in aluminium packets, tied onto cryocanes and directly inserted into a cryotank (model: 34HC, Union Carbide Corp. Danbury, USA) filled with liquid nitrogen. Those seeds that underwent the slow cooling procedures were also put into aluminium packets, tied onto the cryocanes before being placed inside a cooling chamber of a programmable freezer (Cryomed, model: 1010, New Baltimore, Michigan, USA). Using the microcomputer programmable freezing controller, the cooling parameters were set as follows: 0 °C (initial temperature), 1 °C min^{-1} (cooling rate), - 35 °C (final precooling temperature) and 35 min of holding period at that temperature. The aluminium packets were then immersed directly in liquid nitrogen. The minimal storage duration tested was 24 hours. The duration of storage time in liquid nitrogen is irrelevant because the success of the techniques applied depends on pre- and post-treatment strategies. Storage of 24 hours is considered long enough to reflect an actual long-term storage. Storage time has been proven to have no significant effect during cryopreservation storage period (Gonzales-Benito *et al.* 1999).

As for the thawing procedure, the packets were withdrawn from the dewar and placed under the laminar flow bench for 45 min to allow slow thawing until the temperature of the seeds stabilised at ambient temperature. Seeds were then processed for germination tests.

Embryonic axes

Embryonic axes were dissected out aseptically from fresh seeds. The size of the axes were approximately 2 × 2 mm, with some parts of cotyledons still attached. These axes were placed under the air current of the laminar flow and removed at two hour intervals. After each desiccation period (from 0 to 8 hours), the axes were either transferred onto semi-solid medium (desiccation controls) or placed in cryovials, immersed directly in liquid nitrogen or cooled slowly using the protocols previously described for whole seeds. After 24 hours of storage in liquid nitrogen, the cryovials were taken out, and the embryos thawed slowly under the

laminar flow bench and aseptically placed onto the culture medium. The culture medium comprised salts (MS) (Murashige & Skoog 1962) + charcoal (2.0 g l^{-1}) and various combination of α-naphthaleneacetic acid (NAA) (0, 0.5, 1.0 mg l^{-1}) and 6-benzylaminopurine (BAP) (0, 0.5, 1.0 mg l^{-1}).

The response of explants was observed at once every week and the experiment was terminated after no further response was observed after three months. Germination was defined as when the seeds started to produce root and shoots. Viability was defined as when the explants maintained the creamy green colour and produced either only roots or shoots. Survival was defined as when the embryos started to produce root and shoots. Results were analysed using Statistical Analysis System (SAS) software, where average results were compared using Duncan's multiple range test (DMRT).

Results

Whole seeds

The average initial moisture contents of seeds with testa and seeds without testa were 16.09 and 10.55% respectively. As tested at every two hours desiccation, the moisture content of seeds with testa reduced at the rate of 1.2% $hour^{-1}$ compared with seeds without testa which reduced only at 0.8% $hour^{-1}$ (Figure 1). After eight hours desiccation, seeds with testa retained 7.04% moisture while seeds without testa have only 2.22%.

The viability (as germination) of the whole seeds is shown in Figure 2. Control seeds (both with and without testa) indicated high viability at 0 and 2 hours desiccation (> 90%). Viability of seeds with testa was reduced to 70% after 8 hours desiccation and seeds without testa maintained only 65% viability after the same period.

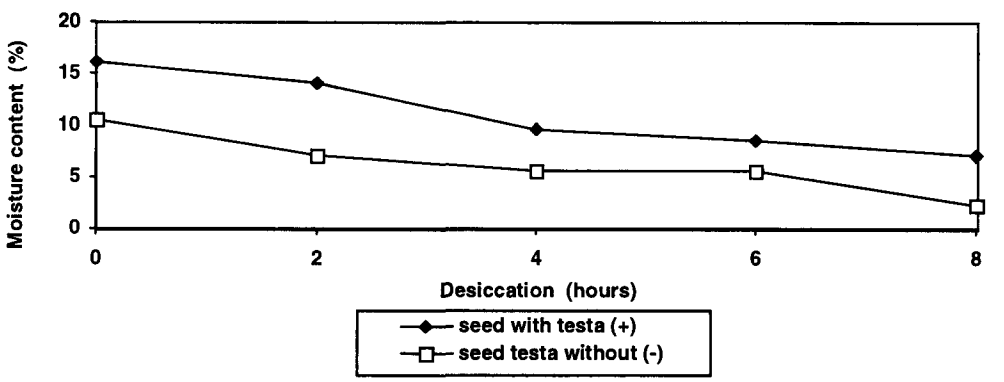

Figure 1 Average moisture content of 30 mahogany seeds, each with testa and without testa, after desiccation in laminar flow

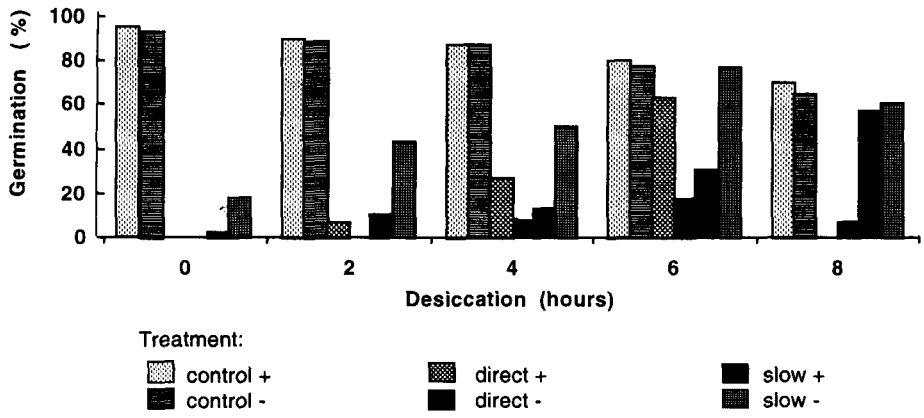

Figure 2 Average germination percentage of 30 mahogany seeds, each with testa (+) and without testa (-), after cryopreservation. LSD (p = 0.05) between two points = 6.42. (direct: Direct immersion in liquid nitrogen; slow: slow cooling)

None of the seeds survived direct plunging into liquid nitrogen at 0 hour desiccation. However, the survival increased with increased desiccation time but only up to six hours. This resulted in percentage survival of 63% for seeds with testa and 18% for seeds without testa. As for the slow cooling technique, the survival of seeds with testa increased from 3 to 57% after 0 to 8 hours desiccation respectively. However, seeds without testa subjected to slow cooling were able to survive much better from 0 to 2, 4, 6 and 8 hours desiccation periods, resulting in 18, 43, 50, 77 and 60% survival respectively. Interestingly, seeds with and without testa in various treatments survived at 6 hours desiccation (Figure 2).

Embryonic axes

The average moisture contents of mahogany embryonic axes after subsequent desiccation are shown in Table 1. The axes initial moisture of 11.52% was reduced to 3.26% at 8 hours, with an average reduction rate of 1.03% hour^{-1}. However, the rate of loss of moisture content was highest (3.22%) after the first two hours of desiccation.

Table 2 shows that the average percentages of viability and survival of cultured controlled mahogany embryonic axes at the age of two months after they were given desiccation treatments. Observation of plantlet survival was based upon positive growth from which healthy seedlings were produced. Without cryopreservation, at 0 hour desiccation, 97% of the axes survived on just basic MS medium, without the need of extra hormonal supplements.

Table 1 Average moisture content of 30 embryonic axes when desiccated in the laminar flow

Desiccation hour	Moisture content (%)
0	11.52 ± 0.42
2	8.30 ± 0.36
4	6.21 ± 0.38
6	4.59 ± 0.25
8	3.26 ± 0.48

Table 2 The average percentage viability (V) and percentage survival as seedlings (S) of mahogany embryonic axes at two months after desiccation treatment

Desiccation hour	BAP (mg l^{-1})	NAA (mg l^{-1}) 0		0.5		1.0	
		%V	%S	%V	%S	%V	%S
0	0	100^{a}	97^{a}	90^{bcd}	77^{bc}	87^{cde}	50^{hij}
	0.5	90^{bcd}	73^{fg}	87^{cde}	53^{ghi}	87^{cde}	47^{ijk}
	1.0	90^{bcd}	70^{cde}	87^{cde}	63^{efg}	87^{cde}	33^{jkl}
2	0	100^{a}	90^{bcd}	90^{bcd}	43^{jkl}	87^{cde}	67^{def}
	0.5	87^{cde}	73^{fg}	87^{cde}	37^{jkl}	93^{abc}	27^{l}
	1.0	83^{def}	47^{ijk}	90^{bcd}	53^{ghi}	90^{bcd}	50^{hij}
4	0	97^{ab}	73^{bcd}	93^{abc}	43^{jkl}	90^{ab}	37^{jkl}
	0.5	90^{bcd}	47^{ijk}	90^{bcd}	57^{fgh}	93^{abc}	27^{l}
	1.0	90^{bcd}	53^{ghi}	87^{cde}	30^{kl}	83^{def}	37^{jkl}
6	0	93^{abc}	80^{bc}	87^{cde}	53^{ghi}	87^{cde}	33^{jkl}
	0.5	97^{ab}	43^{jkl}	73^{fg}	53^{ghi}	73^{fg}	33^{jkl}
	1.0	83^{def}	47^{ijk}	77^{efg}	47^{ijk}	80^{def}	47^{ijk}
8	0	80^{def}	77^{bc}	83^{def}	33^{jkl}	73^{fg}	33^{jkl}
	0.5	83^{def}	47^{ijk}	83^{def}	33^{jkl}	67^{g}	27^{l}
	1.0	73^{fg}	40^{jkl}	73^{fg}	30^{kl}	67^{g}	37^{jkl}

Values within V columns and S columns followed by the same letter(s) do not differ significantly ($p \leq 0.01$) according to DMRT.

Table 3 shows the effects of cryopreservation on excised embryos at different desiccation periods. Overall, the results showed that embryos desiccated for 6 hours had the highest survival rate when placed on MS medium consisting of 1.0 mg l^{-1} BAP. More than 85% axes that survived 6 and 8 hours desiccation were able to produce roots and shoots. On the other hand, after 0, 2 and 4 hours desiccation, the seedlings grew less vigorously; they were slow to revive and most formed callus.

The average percentages survival of excised embryos subjected to slow cooling techniques are shown in Table 4. Desiccation for 6 hours and recovery on MS medium supplemented with 0.5 mg l^{-1} BAP gave the highest percentage survival of 87%.

Table 3 The average viability (V) and percentage survival as seedlings (S) of mahogany embryonic axes at two months after direct plunge cryopreservation treatment

Desiccation hour	BAP (mg l^{-1})	NAA (mg l^{-1}) 0 %V	0 %S	0.5 %V	0.5 %S	1.0 %V	1.0 %S
0	0	23^{k}	3^{ij}	37^{ijk}	7^{hij}	53^{def}	10^{hij}
	0.5	27^{jk}	3^{ij}	43^{ghi}	3^{ij}	33^{ijk}	10^{hij}
	1.0	43^{ghi}	7^{hij}	40^{hij}	7^{hij}	57^{cde}	10^{hij}
2	0	47^{fgh}	3^{ij}	47^{fgh}	13^{hij}	43^{ghi}	17^{ghi}
	0.5	53^{def}	17^{ghi}	43^{ghi}	17^{ghi}	30^{ijk}	17^{ghi}
	1.0	40^{hij}	13^{hij}	33^{ijk}	17^{ghi}	40^{hij}	17^{ghi}
4	0	33^{ijk}	3^{ij}	47^{fgh}	7^{hij}	47^{fgh}	13^{hij}
	0.5	43^{ghi}	10^{hij}	43^{ghi}	7^{hij}	47^{fgh}	23^{efg}
	1.0	40^{hij}	7^{hij}	37^{ijk}	17^{ghi}	37^{ijk}	7^{hij}
6	0	57^{cde}	23^{efg}	43^{ghi}	27^{def}	53^{def}	33^{cde}
	0.5	50^{efg}	20^{fgh}	53^{def}	50^{efg}	47^{fgh}	33^{cde}
	1.0	70^{a}	63^{a}	60^{bcd}	47^{fgh}	50^{def}	37^{cd}
8	0	50^{efg}	17^{ghi}	50^{efg}	10^{hij}	57^{cde}	20^{fgh}
	0.5	63^{abc}	17^{ghi}	67^{ab}	13^{hij}	67^{ab}	17^{ghi}
	1.0	60^{bcd}	13^{hij}	67^{ab}	23^{efg}	67^{ab}	13^{hij}

Values within V columns and S columns followed by the same letter(s) do not differ significantly ($p \leq 0.01$) according to DMRT.

Table 4 The average viability (V) and percentage survival as seedlings (S) of mahogany embryonic axes at two months after slow cooling cryopreservation treatment

Desiccation hour	BAP (mg l^{-1})	NAA (mg l^{-1}) 0 %V	0 %S	0.5 %V	0.5 %S	1.0 %V	1.0 %S
0	0	47^{f}	3^{mno}	43^{fg}	10^{mno}	47^{f}	27^{jkl}
	0.5	33^{g}	10^{mno}	53^{def}	20^{lmn}	47^{f}	30^{ijk}
	1.0	33^{g}	10^{mno}	50^{ef}	27^{jkl}	50^{ef}	30^{ijk}
2	0	53^{def}	17^{mno}	57^{def}	43^{fg}	67^{bcd}	53^{def}
	0.5	70^{bc}	27^{jkl}	63^{cde}	47^{efg}	53^{def}	43^{fg}
	1.0	63^{cde}	53^{def}	53^{def}	43^{fg}	57^{def}	27^{jkl}
4	0	77^{ab}	27^{jkl}	77^{ab}	47^{efg}	73^{ab}	63^{bc}
	0.5	63^{cde}	33^{g}	73^{ab}	40^{fgh}	73^{ab}	63^{bc}
	1.0	67^{bcd}	37^{ghi}	63^{cde}	40^{fgh}	67^{bcd}	57^{cde}
6	0	73^{ab}	60^{bcd}	73^{ab}	63^{bc}	73^{ab}	63^{bcd}
	0.5	87^{a}	87^{a}	77^{ab}	60^{bcd}	70^{bc}	63^{bcd}
	1.0	67^{bcd}	40^{fgh}	67^{bcd}	47^{efg}	73^{ab}	57^{cde}
8	0	63^{cde}	40^{fgh}	63^{cde}	43^{fg}	57^{def}	20^{lmn}
	0.5	73^{ab}	27^{jkl}	67^{bcd}	33^{g}	57^{def}	13^{mno}
	1.0	70^{bc}	33^{g}	67^{bcd}	23^{klm}	47^{f}	13^{mno}

Values within V columns and S columns followed by the same letter(s) do not differ significantly ($p \leq 0.01$) according to DMRT.

Discussion

In contrast to agriculture, cryopreservation techniques have limited application in tropical forest species. This could be due to the difficulties involved in obtaining the germplasm. Also, the viability of the seeds depends highly on maturity and moisture content (Krishnapillay *et al.* 1990, Krishnapillay & Marzalina 1993). This study has shown that in seeds with the testa removed, and with lower starting moisture content (10.55%), the loss of moisture was slower compared with seeds with the testa intact (starting moisture content 16%). Whole seeds with the testa intact exhibited a desiccation rate that was 1.2% higher compared with those seeds without the testa. This may be due to the fact that the seeds without testa already had a much lower initial moisture content. Thus, the differential loss of water from the seeds was far less.

Studies have indicated that more than 120 species of trees have seeds which retain a moisture content of within 3 to 14% and that the seeds can be cryopreserved successfully (Stanwood & Bass 1981). In this study, the optimum moisture content of seeds with testa was found to be 8.3% after 6 hours desiccation. The direct plunge technique gave higher survival percentage (63%) compared with slow cool technique (30% survival). This concurs with results from a study using *Coffea liberica* seeds (Normah & Vengadasalam 1992). The authors found that with 6 hours desiccation period, coffee seeds can be dried down to 16.7% moisture content and retained 57% survival after cryopreservation.

Seeds without testa survived better using the slow cooling technique (77%) compared with the direct plunge technique (17%). The same desiccation period (6 hours, moisture content of 5.6%) was effective to maintain higher survival rate. It appeared that seeds with intact testa required rapid freezing while those without testa, and having lower moisture content, required slow cooling. The testa probably slowed down the rate of cooling causing differential cooling effects within the seed. With slow cooling and slow thawing employed in the study, formation of lethal ice crystals or other lethal factors such as glass transitions and the formation of free radicals may have occurred. Seeds with testa will probably give better survival with rapid freezing and rapid thawing. The lower survival percentage in the seeds with testa compared with the seeds without testa may also be due to breakage of the testa causing physical damage to the seeds. Liquid nitrogen caused physical breakage to *Phaseolus vulgaris* seeds when they were rewarmed (Stanwood 1985).

With seeds without testa, more uniform cooling may have been obtained when slow-cooled. Direct cooling can cause cold injury as formation of ice within and between cells does not occur simultaneously (Meryman & Williams 1985). It has been suggested that, at a low moisture content, rapid cooling can cause vitrification of the membrane lipid which damages the membrane structure and consequently, reduces viability (Vertucci 1989).

As for the cryopreservation of excised embryos, it was found that by desiccating the embryos for 6 hours within the laminar flow to a 4.59% moisture content, the percentage of survival of the embryonic axes was significantly high for both direct plunge (63%) and slow cool (87%) techniques. The critical level of moisture

content that can withstand cryopreservation is slightly higher for mahogany excised embryos and is comparable with the study by Gonzalez-Benito & Perez (1994). The authors observed that when moisture content was reduced to the range of between 3 and 10%, viability of *Oleo europaea* embryos was high (70%) after direct plunging into liquid nitrogen. The moisture content range of the embryonic axes for best survival in the present study is lower than those of recalcitrant seeds such as jackfruit (Krishnapillay 1989), rubber (Normah *et al.* 1986), litchi, oak, neem and almond (Chaudhury & Malik 1999). However, the moisture content range for successful cryopreservation is similar to hazelnut (Reed *et al.* 1994).

The most suitable media for excised embryos after cryopreservation were those with hormone supplements. Embryonic axes that had undergone direct plunging in liquid nitrogen needed higher dosage of BAP (1.0 mg l^{-1}) while those going through slow cooling techniques needed only 0.5 mg l^{-1} BAP in basic MS medium to maintain normal growth of seedlings. The difference in hormonal requirement is probably due to the severity of the injury incurred during freezing. With direct plunging, more injury could have occurred, therefore, requiring more BAP to support recovery while with slow cooling, the injury of seeds without testa was probably less and thus, less BAP was required for recovery. An additional of 0.5 mg l^{-1} BAP was essential for *Brassica napus* shoot to regain growth after cryopreservation using direct plunge techniques (Withers *et al.* 1988). Besides BAP, cryopreserved *Solanum tuberosum* shoot tips needed NAA or zeatin to regenerate and grow successfully (Harding & Benson 1994).

In this study we found a novel approach in which desiccation treatments in combination with controlled cooling rates, resulted in the enhanced survival of seeds and embryonic axes of mahogany. The technique is simple with no requirement of cryoprotectants and thus, highly applicable to gene banks.

Acknowledgements

The authors wish to thank the Director General of FRIM and the Director of the Forest Plantation Division for permission to carry out this study. Our sincere thanks to the Selangor Forestry Department for the permission to collect mahogany seeds.

References

ANONYMOUS. 1993. International rules for seed testing. *Seed Science and Technology* 13: 299–355.

ASHTON, P. S., GIVNISH, T. J. & APPANAH, S. 1988. Staggered flowering in the Dipterocarpaceae: new insights into floral induction and the evolution of mass fruiting in the aseasonal tropics. *American Naturalist* 132: 44–66.

BENSON, E. E. 1999. Cryopreservation. Pp. 83–96 in Benson, E. E. (Ed.) *Plant Conservation Biotechnology.* Taylor & Francis Ltd., London.

CHAUDHURY, R. & MALIK, S. K. 1999. Cryopreservation and *in vitro* technology for conservation of recalcitrant seeds of some tree species. Pp. 119–125 in Marzalina, M., Khoo, K. C., Jayanthi, N., Tsan, F. Y. & Krishnapillay, B. (Eds.) *Proceedings of IUFRO Seed Symposium 1998: Recalcitrant Seeds.* Kuala Lumpur. 12–15 October 1998. Forest Research Institute Malaysia, Kepong.

GONZALEZ-BENITO, M. E. & PEREZ, C. 1994. Cryopreservation of embryonic axes of two cultivars of hazelnut (*Corylus avellana* L.). *Cryo-letters* 15: 41–46.

GONZALEZ-BENITO, M. E., MARTIN, C., IRIONDO, J. M. & PEREZ, C. 1999. Conservation of the rare and endangered plants endemic to Spain. Pp. 251–264 in Benson, E. E. (Ed.) *Plant Conservation Biotechnology.* Taylor & Francis Ltd., London.

HARDING, K. & BENSON, E. E. 1994. A study of growth, flowering and tuberisation in plants derived from cryopreserved potato shoot-tips. Implication of *in vitro* germplasm collections. *Cryo-letters* 15: 59–66.

HARRINGTON, J. F. 1972. Seed storage and longevity. Pp. 145–240 in Kozlowski, T. T. (Ed.) *Seed Biology: Insects, and Seed Collection, Storage, Testing and Certification.* Volume III. Academic Press Inc.

KRISHNAPILLAY, B. 1989. Towards the development of a protocol for cryopreservation of embryos of a recalcitrant seed (*Artocarpus heterophyllus* Lam.). Ph.D. thesis, University Pertanian Malaysia. 227 pp.

KRISHNAPILLAY, B. & MARZALINA, M. 1993. Cryopreservation of some tropical forest tree seeds. Paper presented at the Fifth National Biotechnology Seminar. 13–18 November 1993. Organised by University Malaya, Kuala Lumpur.

KRISHNAPILLAY, B., MARZALINA, M. & YAP, S. K. 1990. Is long-term storage of forest seeds possible? Pp. 66–78 in Appanah, S., Ng, F. S. P. & Roslan, I. (Eds.) *Proceedings Conference on Forestry and Forest Products Research FRIM.* Kepong. 3–5 October 1990. Forest Research Institute Malaysia, Kepong.

MARZALINA, M. & KRISHNAPILLAY, B. 1999. Recalcitrant seed biotechnology application to rain forest conservation. Pp. 265–274 in Benson, E. E. (Ed.) *Plant Conservation Biotechnology.* Taylor & Francis Ltd., London.

LOPEZ, F. M. 1938. Storage and germination of large leaf mahogany seeds. *Philippine Journal of Forestry* 1: 397–410.

MARTINI. 1985. Penyimpanan benih *Swietenia macrophylla* dan *Agathis loranthifolia. Buletin Penelitian Hutan* 468: 24–37.

MAYHEW, J. E. & NEWTON, A. C. 1998. *The Silviculture of Mahogany.* CABI Publ., Oxon. 226 pp.

MERYMAN, H. T. & WILLIAMS, R. J. 1985. Basic principles of freezing injury to plant cells. Natural tolerance and approaches to cryopreservation. Pp. 13–47 in Kartha, K. K. (Ed.) *Cryopreservation of Plant Cells and Organs.* CRC Press Inc., Boca Raton.

MURASHIGE, T. & SKOOG, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culture. *Physiologia Plantarum* 15: 473–497.

NORMAH, M. N. & VENGADASALAM, M. 1992. Effects of moisture content on cryopreservation of *Coffea* and *Vigna* seeds and embryos. *Cryo-letters* 13: 199–208.

NORMAH, M. N., CHIN, H. F. & HOR, Y. L. 1986. Desiccation and cryopreservation of embryonic axes of *Hevea brasiliensis* Muell-Arg. *Pertanika* 9: 299–303.

PENA, A. & MONTALVO, Y. J. M. 1986. Condiciones ambientales para pruebas de germinacion en cinco especies forestales. *Revista Forestal Baracoa* 16(1): 7–20.

PUKITTAYACAMEE, P. 1991. Drying effect on germinability of mahogany seed. Paper presented at the IUFRO Symposium on Seed Quality held on 14–17 October 1991 in Nanjing, China.

PUKITTAYACAMEE, P., SAELIM, S. & BHODTHIPUKS, J. 1995. Seed storage of *Swietenia macrophylla.* ASEAN Forest Tree Seed Centre Project. *Technical Publication AFTSC* 25. 11 pp.

REED, B. M., NORMAH, M. N. & YU, X. L. 1994. Stratification is necessary for successful cryopreservation of axes from stored hazelnut seed. *Cryo-letters* 15: 377–384.

RODAN, B. D., NEWTON, A. C. & VERISSIMO, A. 1992. Mahogany conservation: status and policy initiatives. *Environmental Conservation* 19(4): 331–338.

STANWOOD, P. C. 1985. Cryopreservation of seed germplasm for genetic conservation. Pp. 199–226 in Kartha, K. K. (Ed.) *Cryopreservation of Plant Cells and Organs.* CRC Press Inc, Boca Raton.

STANWOOD, P. C. & BASS, L. N. 1981. Seed germplasm preservation using liquid nitrogen. *Seed Science and Tech*nology 9: 423– 437.

UETSUKI, Y. 1988. Some characteristics of sixteen commercial tree species in the tropical forests in Peru-Amazon. *Bulletin of the Forest Tree Breeding Institute* 6: 1–45.

VERTUCCI, C. W. 1989. Effects of cooling rate on seeds exposed to liquid nitrogen temperatures. *Plant Physiology* 90: 1478–1485.

VIVEKANANDAN, K. 1978. Retention of viability of mahogany seed through cold storage. *The Sri Lanka Forester* 13: 3–4.

WITHERS, L. A., BENSON, E. E. & MARTIN, M. 1988. Cooling rate / culture medium interactions in the survival and structural stability of cryopreserved shoot-tips of *Brassica napus*. *Cryo-letters* 9: 114–119.

YAP, S. K. & MARZALINA, M. 1990. Gregarious flowering: myth or fact. Paper presented at the International Conference on Forest Biology and Conservation in Borneo. 1–3 August 1990. Sabah.