CRYOPRESERVATION OF WHOLE SEEDS AND EXCISED EMBRYOS OF *PTEROCARPUS INDICUS*

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KRISHNAPILLAY, B., MARZALINA, M. & ALANG, Z.C. 1994. Cryopreservation on whole seeds and excised embryos of *Pterocarpus indicus*. A study was conducted to show the feasibility of using liquid nitrogen for the long term storage of both whole seeds and excised embryos of *Pterocarpus indicus*. A woody plant medium containing 2-3 mg l^{-1} of either benzylaminopurine (BAP) or kinetin was found suitable for the normal development of cryopreserved embryos. Whole seeds dried to a moisture content of 4 - 6% could be successfully cryopreserved with a good recovery percentage. The critical moisture content for excised embryos was found to be around 5%.

Key words: *Pterocarpus indicus* - whole seeds - excised embryos - desiccation - cryopreservation - growth medium

KRISHNAPILLAY, B., MARZALINA, M. & ALANG, Z.C. 1994. Krioawetan biji benih dan keratan embrio *Pterocarpus indicus*. Satu ujian ke atas biji benih dan keratan embrio *Pterocarpus indicus* telah dijalankan untuk mengkaji kemungkinan penggunaan nitrogen cecair bagi tujuan penyimpanan jangkamasa panjang. Medium jenis 'woody plant (WPM)' yang telah ditambahkan dengan 2-3 mg l^{-1} benzylaminopurine (BAP) atau kinetin didapati sesuai digunakan untuk pertumbuhan normal embrio yang telah dikrioawetkan. Sementara kandungan lembapan biji benih yang dikurangkan kepada 4-6 % telah mencapai peratusan pemulihan yang tinggi setelah dikrioawetkan. Tahap kritikal kandungan lembapan bagi keratan embrio pula di sekitar 5%.

Introduction

Pterocarpus indicus is a large deciduous tree, usually up to 30 m high or more. It is best known as a roadside tree, planted for its vast shady crown and its fragrant yellow flowers. There are about 30 species of *Pterocarpus* throughout the tropical countries; however, only *P. indicus* is common in Peninsular Malaysia (Whitmore 1972).

The fruit of *P. indicus* is a few seeded samara, corky-woody and flattened into a wing around the periphery, adapted for dispersal by wind as well as to float in water. Internally the fruit is divided by cross walls into four or five seed chambers of which a couple may contain developed seeds. The seeds have a leathery brittle

seed coat with the embryonic initials attached at one end of the cotyledons (Ng 1992).

Whole seeds and excised embryos of this species were tested for their critical moisture content, suitable medium for the *in vitro* growth and storability in liquid nitrogen for long term conservation of germplasm.

Materials and method

Mature fruits from fruiting trees were collected in the Forest Research Institute's compound at Kepong. A total of 3000 mature seeds were processed and thoroughly bulked, and random samples were taken for the various studies.

To establish a suitable combination of hormones in the medium for the normal growth and development of the excised embryos of *P. indicus*, Llyod and McCown (1980) woody plant medium formulation was used. The hormones tested were auxins, napthalene acetic acid (NAA) and 2,4-dichlorophenoxy acetic acid (2,4-D) at concentrations of 0.0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg l^{-1}; and cytokinins, benzylaminopurine (BAP) and kinetin at concentrations of 0, 1, 2, 3, 4, 5 mg l^{-1}. A factorial experiment using a completely randomised design was adopted to test the various hormone combinations. The plantlets were evaluated after six weeks of culture. They were removed from the culture medium and the root zone was cleaned on soft tissue paper to remove any adhering culture medium. Each seedling was then bisected at the base of the vestigial hypocotyl into shoot and root tissues. These two portions were dried separately in a 75 °C oven for one week and the dry weights noted separately.

In the assessment of the shoot to root ratio, the ratio by dry matter of the shoot to root was calculated. The convention adopted to quantify the best shoot to root development for jackfruit, *Artocarpus heterophyllus* (Krishnapillay 1990), was used in this study. In this convention an equal shoot and root weight by dry matter content would give a ratio with a value of 1. This value of 1 is given a score of 100. The ratios of shoot to root from the various treatments falling above and below the value of 1.0 are scored using the following arbitrary scale:

Shoot to root ratio	Score
2.0000	0.00
1.9000	10.00
1.8000	20.00
1.7000	30.00
1.6000	40.00
1.5000	50.00
1.4000	60.00
1.3000	70.00
1.2000	80.00
1.1000	90.00

1.0000 Balanced Ratio	100.00 Highest score
0.9000	90.00
0.8000	80.00
0.7000	70.00
0.6000	60.00
0.5000	50.00
0.4000	40.00
0.3000	30.00
0.2000	20.00
0.1000	10.00
0.0000	0.00

In this convention those ratios with their scores closest to 100 will indicate a balanced development of the shoot and root while those deviating away from 100 will indicate unbalanced development. The calculated score values for each treatment were used in the analysis of data to determine the medium in which a balanced shoot and root development was obtained. For this study as the number of embryos required was large, only two replicates of 10 embryos each were cultured per treatment combination.

To determine the drying rate and critical moisture content for whole seeds, two methods of drying were tested. One using silica gel and the other involving drying in a laminar air flow cabinet. For aseptically excised embryos, only the laminar air flow cabinet was used. For the whole seeds, for each method of drying, 425 seeds were used. Moisture loss in the seeds was recorded continuously at intervals of 4 h over 48 h using 4 replicates of 25 seeds. The other 325 seeds were divided into batches of 25 and from these seeds, at the end of each interval of drying one set of 25 seeds were placed for germination in petri-dishes over moist filter paper. For excised embryos, 220 seeds were taken, the embryos attached to a small portion of the cotyledon were excised aseptically and these were used. Moisture loss from the embryos was recorded continuously at intervals of 1 h for a period of 6 h using 4 replicates of 20 embryos. The other 140 embryos were divided into batches of 20 and from these embryos, at the end of each hour of drying, one set of 20 embryos were cultured on a woody plant medium formulation (Llyod & McCown 1980) containing 3 mg l^{-1} of BAP to evaluate survival.

For the cryopreservation studies, both whole seeds and excised embryos were tested. For whole seeds, they were dried to three moisture levels of around 14 - 16%, 8 - 10% and 4 - 6%. At these moisture levels, the seeds were wrapped in aluminum foil and plunged directly into liquid nitrogen. After 48 h of storage, the packings were removed from the tank and allowed to thaw slowly to attain room temperature (26 ± 2 °C). The seeds were then rinsed in distilled water and germinated in germination boxes on moist filter paper and incubated in a germination chamber at 29 °C and with a relative humidity of 70%. Four replicates of 25 seeds per replicate were used for each moisture level. In the study on cryopreservation of excised embryos, fresh seeds were first surface sterilized

with 80% alcohol for 10 min. The seeds were then swapped dry in the laminar flow cabinet and the embryonic axes were carefully excised. The excised embryos were allowed to desiccate over a period of 4 h. At the end of each hour of drying, 2 replicates of 10 embryos each were taken for moisture content testing while another two replicates of 10 embryos each were placed in sterile cryovials, then mounted on cryocanes and plunged into liquid nitrogen. The embryos were held in liquid nitrogen for one week. At the end of the storage period, the canes were removed from the tank and the vials were rapidly thawed in a water bath at 40 °C. After thawing the embryos were cultured in a medium formulation of Llyod and McCown (1980) containing 3 mg l^{-1} of BAP and solidified using a 0.2% gelrite. All embryos cultured *in vitro* were maintained at 26 - 28 °C in a temperature controlled room with 12 h photoperiod provided by fluorescent lights of approximately 2000 lux intensity.

Results

Results on best hormone combinations for the normal growth of excised embryos are shown in Tables 1 - 4. Tables 1 and 2 show the various combinations of NAA with BAP or kinetin. Results showed that a medium containing 2 mg l^{-1} of BAP or 2 - 3 mg l^{-1} of kinetin and in the absence of NAA gave a good shoot to root ratio after 6 weeks of growth. In those combinations where NAA was present growth was either stunted or swelling with some callus was observed.

Table 1. Score values of shoot to root ratio of plantlets grown *in vitro* on medium containing NAA/BAP

NAA (mgl $^{-1}$)	BAP (mgl $^{-1}$)					
	0	1	2	3	4	5
0.0	34.80^{i}	63.00^{c}	75.80^{a}	66.70^{b}	59.93^{d}	36.60^{i}
0.1	20.20^{h}	21.97^{h}	40.73^{e}	8.80^{j}	23.93^{g}	42.27^{e}
0.2	14.33^{i}	10.73^{j}	22.30^{h}	12.97^{i}	32.93^{f}	20.43^{h}
0.3	41.64^{e}	26.34^{g}	26.83^{g}	16.23^{i}	22.83^{h}	24.50^{g}
0.4	18.23^{i}	27.77^{g}	16.47^{i}	8.70^{j}	21.63^{h}	16.00^{i}
0.5	13.74^{i}	19.40^{i}	10.70^{j}	13.60^{i}	13.03^{i}	10.36^{j}

(Values having the same superscripts are not significantly different at p = 0.05 based on DNMRT).

Similarly, in the combinations of 2,4-D with either BAP or kinetin (Tables 3 and 4) best shoot to root ratio was observed in those combinations where the auxin was absent and the cytokinins BAP or kinetin was present in the range of 2 - 3 mg l^{-1}.

Table 2. Score values of shoot to root ratio of plantlets grown *in vitro* on medium containing NAA/kinetin

NAA (mgl-1)	Kinetin (mg l^{-1}) 0	1	2	3	4	5
0.0	69.37^{b}	39.83^{d}	79.63^{a}	80.80^{a}	38.13^{d}	82.00^{a}
0.1	57.13^{c}	24.23^{e}	19.80^{e}	28.67^{e}	1.56^{h}	2.06^{h}
0.2	20.57^{e}	60.26^{b}	20.43^{e}	21.00^{e}	31.73^{d}	2.06^{h}
0.3	23.27^{e}	12.23^{f}	10.94^{f}	2.06^{h}	5.57^{g}	6.27^{g}
0.4	18.77^{e}	10.97^{f}	11.87^{f}	13.50^{f}	17.80^{e}	52.33^{d}
0.5	42.23^{d}	17.87^{e}	6.90^{g}	2.06^{h}	8.70^{f}	2.06^{h}

(Values having the same superscripts are not significantly different at p = 0.05 based on DNMRT).

Table 3. Score values of shoot to root ratio of plantlets grown *in vitro* on medium containing 2, 4-D/BAP

2,4-D (mg l^{-1})	BAP (mg l^{-1}) 0	1	2	3	4	5
0.0	34.03^{e}	45.60^{e}	81.53^{a}	80.73^{a}	77.73^{b}	35.33^{e}
0.1	35.00^{e}	15.30^{f}	23.90^{e}	47.43^{e}	41.73^{e}	7.37^{h}
0.2	17.53^{f}	35.07^{e}	66.87^{d}	41.77^{e}	15.50^{f}	14.07^{f}
0.3	13.73^{f}	37.70^{e}	35.67^{e}	6.63^{h}	9.27^{h}	43.97^{e}
0.4	12.47^{g}	8.10^{h}	2.06^{h}	15.53^{f}	2.06^{h}	69.50^{c}
0.5	2.06^{h}	5.37^{h}	2.06^{h}	41.13^{e}	2.06^{h}	2.50^{h}

(Values having the same superscripts are not significantly different at p = 0.05 based on DNMRT).

Table 4. Score values of shoot to root ratio of plantlets grown *in vitro* on medium containing 2, 4-D/kinetin

2,4-D (mgl-1)	Kinetin (mgl $^{-1}$) 0	1	2	3	4	5
0.0	53.73^{d}	44.03^{d}	23.70^{f}	85.73^{a}	51.03^{d}	45.53^{d}
0.1	20.63^{f}	25.33^{f}	31.67^{d}	85.47^{a}	19.83^{f}	80.77^{b}
0.2	15.07^{f}	26.33^{e}	9.27^{f}	4.03^{g}	22.10^{f}	63.07^{c}
0.3	11.73^{f}	2.36^{g}	22.80^{f}	3.23^{g}	27.03^{e}	35.10^{d}
0.4	1.76^{g}	2.27^{g}	2.86^{g}	2.60^{g}	25.40^{f}	18.10^{f}
0.5	2.06^{g}	2.13^{g}	1.73^{g}	2.06^{g}	6.70^{f}	10.40^{f}

(Values having the same superscripts are not significantly different at p = 0.05 based on DNMRT).

Hence, for the best development of the excised embryos of *P. indicus* a woody plant medium containing 2 - 3 mg l^{-1} of either BAP or kinetin was found suitable.

The study on desiccation of whole seeds either under a laminar flow cabinet or over silica gel for a period of 48 h and their subsequent viability is presented in Figure 1.

Initial moisture content of whole seeds was found to be around 16-17% on a fresh weight basis. Under laminar flow drying, over a period of 48 h the moisture in the seeds was reduced from around 17% to around 8% (Figure 1). Over the entire period of desiccation, the germination studies showed that the seeds maintained their viability even when they were dried to 8%, indicating that the seeds were indeed orthodox. Seeds dried over silica gel lost moisture less rapidly when compared to laminar air flow drying where over the 48 h, seeds maintained a moisture content of about 10% (Figure 1). Germination studies of the desiccated seeds also showed no serious loss to viability in those seeds desiccated to about 10% moisture content using this method.

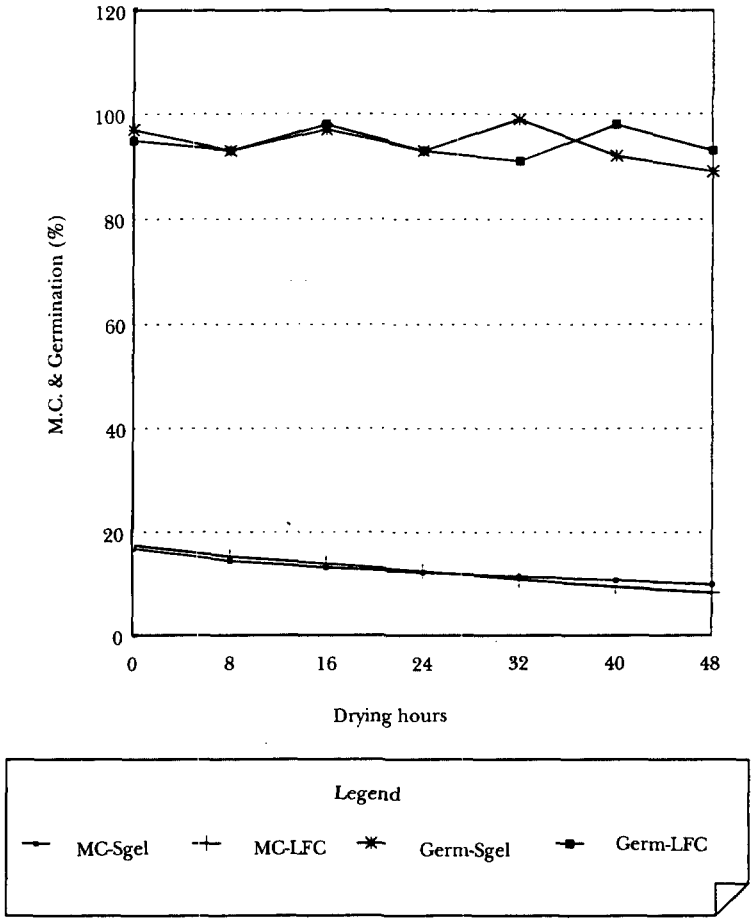

Figure 1. Moisture content and germination percentage of whole seed dried over 48 h in a laminar flow cabinet (LFC) and over silica gel (Sgel)

Results on the desiccation of excised embryos in a laminar flow cabine presented in Table 5. Initial moisture content of excised embryos was arc ..... 12%. Over the 6 h period of drying, the moisture in the embryos was reduced to around 3% (Table 5). Up to 4 h of drying, a good viability percentage was

obtained where the excised embryos developed into normal plantlets. In embryos dried to around 5 h (where moisture content was about 4%) abnormal development was observed in the plantlets. At 6 h of drying (3% moisture content) viability was reduced to around 30% and most of the embryos that survived showed abnormal development indicating that at around 3 - 4% moisture content, desiccation damage had began to occur in the excised embryos.

Table 5. Moisture content and germination percentage of cryopreserved excised embryos desiccated in a laminar flow cabinet over a period of 6 h

Drying (hours)	Moisture content (%)	Viability percentage
0	11.908 ± 0.642	90.00 ± 3.536
1	10.501 ± 0.439	88.75 ± 5.449
2	8.850 ± 0.136	93.75 ± 2.165
3	6.855 ± 0.432	73.75 ± 7.395
4	4.993 ± 0.104	80.00 ± 6.124
5	3.819 ± 0.186	65.00 ± 7.071
6	3.213 ± 0.152	28.75 ± 7.395

Table 6 presents the results of the cryopreservation of whole seeds desiccated to various moisture levels. Whole seeds with 14 - 16% moisture content were found unsuitable for cryopreservation as germination results showed only 26% survival. While seeds desiccated to between 8-10 % moisture content gave good recovery percentage (86%), best results were obtained when the seeds were desiccated to around 4 - 6%.

Table 6. Germination percentage of cryopreserved whole seeds desiccated to various moisture contents

Moisture content (%)	Germination percentage
14 - 16	26.000 ± 8.246
8 - 10	86.000 ± 4.472
4 - 6	90.000 ± 2.000

Results on the cryopreservation studies of excised embryos are presented in Table 7. Excised embryos having a moisture content of around 12% were found to be unsuitable for cryopreservation. Embryos dried to a moisture content of around 5% were most amenable to cryopreservation where after one week of storage in liquid nitrogen, about 85% of the embryos recovered and developed into normal plantlets (Table 7).

Table 7. Moisture content and germination percentage of cryopreserved excised embryos desiccated over a period of 4 h

Drying (hours)	Moisture content (%)	Germination percentage
0	12.018 ± 0.126	35.000 ± 5.00
1	10.765 ± 0.248	55.000 ± 5.00
2	8.886 ± 0.141	75.000 ± 5.00
3	7.104 ± 0.110	80.000 ± 0.00
4	5.194 ± 0.038	85.000 ± 5.00

Discussion

Cryopreservation of plant materials in the form of whole seeds, excised embryos, reproductive parts and vegetative tissues is gaining momentum as a method for the conservation of germplasm. Cryopreservation refers to preservation at -196 °C, the temperature of liquid nitrogen (LN). The basic concept of LN preservation is that, at this temperature, all metabolic processes in seeds are essentially stopped and held in suspended animation. Consequently, all sources of seed deterioration that are metabolically related are greatly reduced or stopped, thus providing 'indefinite' preservation.

The need for cryopreservation becomes evident when one examines conventional preservation systems and their limitations. Over long periods of storage, under current preservation standards of -18 °C and 4 - 6% seed moisture (IBPGR 1976), metabolism still occurs and viability eventually declines in the stored seeds. Reports show that many species decline in viability with time under such storage conditions (Stanwood & Bass 1981). Storage in LN could prevent or significantly reduce such viability loss (Committee on Germplasm Resources 1978). Thus, the nett result would be the improved maintenance of valuable genetic resources.

From the present desiccation and germination trials discussed in this paper, it is clearly seen that *P. indicus* is an orthodox seed. Through careful desiccation of either whole seeds or excised embryos this species is amenable to LN storage. Drying of the whole seeds either by using the laminar flow cabinet or silica gel method could be used to bring down the moisture in the seeds fairly rapidly. For excised embryos, as aseptic conditions had to be maintained, using a laminar flow cabinet for drying is recommended.

Various researchers (Ishikawa & Sakai 1978, Stanwood *et al.* 1978, Stanwood & Bass 1981, Karta 1985,Stushnoff & Fear 1985, Chin & Krishnapillay 1989), have cautioned, based on their experiences, that the cooling rate to -196 °C, rewarming rate and seed moisture content are critical factors to be considered for the successful application of LN preservation techniques to orthodox seeds. From our studies we observed that moisture content in the whole seeds as well as in the excised embryos was critical for successful cryopreservation. Seeds with moisture content of around 14 - 16% gave very poor recovery while those dried to around 4 - 6% was found to be most suitable for cryostorage. Direct plunging of the seeds,

wrapped in aluminum foil, into liquid nitrogen followed by slow thawing was found to be suitable for this species. For the excised embryos, however, the critical moisture content was around 5%. Embryos with moisture content of around 12% showed cryodamage (Table 7) and moisture content below 4% showed desiccation damage (Table 5). Direct plunging of vials containing the excised embryos into LN and subsequent rapid thawing in a water bath at 40 °C was found to be a suitable protocol for cryopreservation of the embryos.

The cryopreserved whole seeds could be germinated on moist filter paper and seeds began to germinate after about five days of planting to produce normal seedlings. The cryopreserved embryos on the other hand developed into normal plantlets over a period of six to eight weeks on the woody plant medium (Llyod & McCown1981) supplemented with 3 mg l^{-1} of BAP and solidified using 0.2% gelrite.

Conclusion

Pterocarpus indicus seeds or its excised embryos are amenable to LN storage and hence can be conserved using this technology for long term conservation of its germplasm. However, it is important that the seeds should be dried to around 4 - 6% while the excised embryos should be dried to around 5% for successful storage and recovery.

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