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LONG-TERM STORAGE OF OIL PALM GERMPLASM ZYGOTIC EMBRYO USING CRYOPRESERVATION

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ABSTRACT

The MPOB germplasm collections from various parts of the world were planted mainly at MPOB Research Station, Kluang, Johor, Malaysia. The collections were maintained in the form of field gene bank. Conserving the genetic materials in ex-situ *living collections requires high cost of maintenance, large land area and the palms are exposed to diseases and extreme weather conditions. Experiments were conducted to study the possibility of preserving oil palm tissues for long-term storage at ultra-low temperature (-196°C) in liquid nitrogen. This technique, known as cryopreservation requires lower cost of maintenance, lesser space and protects the genetic materials from pests and diseases. Oil palm seeds are intermediate between orthodox and recalcitrant. Oil palm seeds that have high levels of moisture lose viability when stored in liquid nitrogen. Experiments were conducted to study the possibility of cryopreserving smaller tissues such as oil palm zygotic embryos. Simple desiccation methods were applied to reduce the moisture levels of the embryos namely room temperature, laminar flow and silica gel. These methods were shown to be useful for zygotic embryos. Silica gel, however, is advantageous as it removes the moisture in relatively shorter period than the other two methods. The same approach has potential to be applied to cryopreserve oil palm pollen.*

Keywords: cryopreservation, oil palm.

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INTRODUCTION

MPOB has the largest oil palm germplasm collection in the world (Rajanaidu, 1994; Rajanaidu and Jalani, 1994). The germplasm collections are being conserved in *ex situ* field genebank. However, these plots require high maintenance cost and large amount of land. Apart from being exposed to diseases and extreme climate conditions, the materials need to be regenerated every 25 years (Williams and Hsu, 1970). Therefore, new technique for oil palm germplasm conservation was explored to preserve a part of the germplasm in the form of cryopreservation.

There are three types of seed storage behaviour; orthodox, intermediate and recalcitrant seeds. Orthodox seeds can be dried without damage at low moisture contents while recalcitrant seeds do not survive drying to any large degree and are thus not amenable to long-term storage (Hong *et al*., 1998). Oil palm seeds show intermediate seed storage behaviour. Intermediate seeds can be desiccated to around 10%-12% moisture content and can tolerate freezing temperatures (Ellis *et al*., 1991).

Cryopreservation refers to the non-lethal storage of biological tissues at ultra-low temperature,

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usually that of liquid nitrogen (-196°C). At low temperatures, biological and biochemical activities in the cells are stopped. Therefore, theoretically, tissues can be stored for unlimited period of time. It causes no change in viability, vigour and genetic make-up of the cryopreserved materials (Mandal *et al*., 2000).

Cryopreservation technique offers minimum space and low maintenance and has turn out to be very essential tool for long-term storage of germplasm materials. It appears to be the most practicable method for storing recalcitrant seeds and species that are vegetatively propagated. Cryopreservation using a desiccation procedure is the simplest technique as it involves dehydrating the plant material to a suitable moisture content followed by rapid freezing through direct immersion in liquid nitrogen. Desiccation has been utilised mainly with zygotic and somatic embryos (Grout *et al*., 1983; Chaudhury *et al*., 1991). Panis *et al*. (2001) reported that desiccation using a closed vial containing silica gel is more reproducible.

Embryo is preferred as the first tissue for oil palm cryopreservation because of its relatively smaller in size as compared to seeds and kernels. Bigger tissues are more constrained by desiccation and freezing sensitivity. Several publications on recalcitrant and intermediate species (*e.g*. oil palm, cocoa, rubber) reported that excised embryos are relatively more tolerant to dessication and cryoexposure than whole seeds (Bajaj, 1984; Radhamani and Chandel, 1992; Normah *et al*., 1994; Makeen *et al*., 2005).

For successful cryopreservation, many elements such as status of source-plant, materials, personnel, culture conditions, pre-treatment conditions, cryopreservation techniques, cryogenic facilities, organisations and post-thawing are involved (Reinhoud *et al*., 2000; Reed *et al*., 2004). At MPOB, some five-year cryopreserved embryos have been germinated on MS basal media. The success rate was 70%. Similar results were obtained in citrus at the National Bureau of Plant Genetic Resources (NBPGR) in India where after an average of 6.3 to 8.4 years of cryo-storage, between 69% and 81% of accessions per species retained more than 70% of the viability after desiccation (Malik *et al*., 2012). In some germplasm conservation centres, 20% recovery is sufficient for long-term preservation (Golmirzaie and Panta, 2000). Reed (2001) reported that many authors consider that survival should be higher than 40%. It is crucial that those percentages be reproducible. In this study, experiments were conducted to examine the possibility of cryopreserving smaller tissues such as oil palm zygotic embryos. Simple desiccation methods were applied to reduce the moisture levels of the embryos namely room temperature, laminar flow and silica gel.

MATERIALS AND METHODS

Plant Materials

Experiments were carried out on oil palm from various MPOB germplasm collections planted at MPOB Research Station Kluang, Johor, Malaysia. Embryos were excised from oil palm fruits collected at random from five germplasm collections namely Angola, Cameroon, Ghana, Senegal and Guinea. Fruits from a standard cross (DxP) were used as the control. For each germplasm, one *dura* and one *tenera* open-pollinated bunch was collected at random.

Preparation of the Seeds

The harvested bunches were labelled and the mesocarp manually removed from the fruit. The clean seeds were then cracked and the kernels obtained were surface-sterilised for 20 min using 0.05% Tween 20 and 0.01% mercuric chloride, followed by three times rinsing with sterile water.

Methods to Reduce Moisture Content

Desiccation of seeds, kernels and embryos was carried out using room temperature, air laminar flow and silica gel, respectively.

Room Temperature

The seeds were subjected to controlled room temperature with an average of 25°C and RH of 70%. The moisture content of the excised embryos was measured at 10, 20 and 30 days after treatment.

Air Laminar Flow

The kernels obtained from the seeds were kept in air laminar flow and the moisture contents of the excised embryos were measured after 30, 60 and 90 days.

Silica Gel

The embryos excised from fresh kernels were exposed to 15 g silica gel in sealed Petri dishes. The moisture content of the excised embryos was measured at 0, 1, 2, 3, 4 and 5 hr of desiccation. Embryos at 0 hr of exposure are considered untreated and used as control. For every hour, some embryos were cryopreserved and germinated in MS basal medium to monitor the viability rate.

For each experiment, three replicates (five embryos/replicates) were used. The moisture contents of all treated embryos were measured using oven method described by the International Seed Testing Association, ISTA (1985). Moisture

content is expressed by the difference between fresh and dry weight of the samples. Dry weight was obtained after drying of embryos at 105°C for 16 hr. The moisture content is then calculated using the following formula:

$$\text{Percentage of moisture content} = \frac{M2 - M3}{M2 - M1} \times 100\%$$

where,
M1 is aluminium foil weight;
M2 is aluminium foil weight + sample fresh weight; and
M3 is aluminium foil weight + sample dry weight.

Dehydrated embryos for all treatments using silica gel method were sealed in propylene cryovials and directly immersed in liquid nitrogen (-196°C). After 16 hr (at least), the embryos were thawed in 40°C water bath for 1 min. Embryos were then transferred onto MS basal medium to determine rate of germination. The embryos were incubated at a temperature of 24°C - 25°C under 16/8 (light/dark) photoperiod.

RESULTS AND DISCUSSION

Room Temperature Method

The initial mean moisture contents of the embryos excised from the fresh kernels were approximately 36.4% ranging from 31.5% to 40.0% (*Table 1*). After 10 days of exposure at room temperature, the mean moisture contents decreased to 20.8% ranging from 11.3% to 33.2%. Mean moisture contents of 12.4% and 10.4% were obtained after 20 days and 30 days of exposure in room temperature, respectively.

Air Laminar Flow Method

The initial mean moisture content for air laminar flow method was 37.6%. After 30 days, the mean moisture content of embryos excised from kernels was reduced to 17.6% whereas, embryos from DxP progenies was decreased from 41.8% to 9.9%, respectively (*Table 2*). However, after 90 days the moisture content of embryos has not stabilised and the data was not useful and should be discarded. Experiment conducted at 90 days where drying beyond critical moisture may impose a desiccation stress which led to solute effect such as pH changes, increasing electrolyte concentrations, protein denaturation, membrane phase transition and macromolecular interaction and then damage of the cell (Dumet and Benson, 2000). It is suggested that 30 days is sufficient to obtain moisture content of 10% - 20% for air laminar flow method.

Silica Gel Method

The moisture contents of embryos at different hour of dehydration are shown in *Table 3*. The initial mean moisture content was 37.1%. The moisture content decreased to 28.3% after 1 hr and gradually dropped to 22.9%, 17.5%, 13.0% and 8.8% for subsequent hours of desiccation. Embryos with no treatment showed the lowest viability and survival when cultured *in vitro*. Only 16.7% of survival rate was obtained for the fresh embryos extracted from kernels (*Table 4*). However, with increasing duration of desiccation (1 - 5 hr), there was an increase in viability up to 74.7% at 4 hr. After 4 hr in the silica gel, the germination rate of cryopreserved embryos ranged from 53.3% to 93.3%. A similar result was obtained in oil palm, survival of cryopreserved

TABLE 1. MOISTURE CONTENT FOR OIL PALM EMBRYOS DEHYDRATED USING ROOM TEMPERATURE

No.	Country	Palm No.	Fruit type	Moisture content of treated embryos (%)			
				0 day	10 days	20 days	30 days
1	Angola	0.311/46	D	38.4	11.3	8.0	6.2
2	Angola	0.311/20	T	40.1	11.4	10.8	8.5
3	Cameroon	0.219/893	D	32.5	28.2	8.9	2.5
4	Cameroon	0.219/789	T	40.0	25.4	12.9	8.5
5	Ghana	0.397/589	D	39.5	27.6	7.9	9.4
6	Ghana	0.397/2068	T	31.5	24.0	8.4	10.6
7	Guinea	0.353/140	D	37.1	17.6	12.4	11.5
8	Guinea	0.353/88	T	32.4	16.0	14.4	10.8
9	Senegal	0.352/22	D	33.4	13.8	13.6	13.4
10	Senegal	0.352/5	T	39.5	33.2	26.2	23.2
11	DxP	0.418/571 x 0.174/655	T	35.4	21.1	13.5	10.7
		Mean		36.4[a]	20.8[b]	12.4[c]	10.4[c]
		CV		9.8%	37.4%	44.1%	51.6%

Note: D - *Dura*, T - *Tenera*, P - *Pisifera*. Means within the same column with the same letter are not significantly different at $P \leq 0.05$ from Tukey's Studentised Range (MSD) test. Figures in the row are minimum and maximum values. CV – coefficient of variations.

TABLE 2. MOISTURE CONTENT FOR OIL PALM EMBRYOS DEHYDRATED USING AIR LAMINAR FLOW

No.	Country	Palm No.	Fruit type	Moisture content of treated embryos (%)			
				0 day	30 days	60 days	90 days
1	Angola	0.311/46	D	42.8	29.5	7.0	14.1
2	Angola	0.311/20	T	40.6	34.6	12.9	6.4
3	Cameroon	0.219/893	D	35.1	21.0	14.2	7.8
4	Cameroon	0.219/789	T	30.6	15.6	12.4	12.3
5	Ghana	0.397/589	D	50.8	9.9	2.6	6.8
6	Ghana	0.397/2068	T	41.0	11.1	5.3	4.2
7	Guinea	0.353/140	D	36.3	11.1	7.4	6.6
8	Guinea	0.353/88	T	28.8	9.8	5.4	15.8
9	Senegal	0.352/22	D	33.1	8.9	6.9	12.8
10	Senegal	0.352/5	T	37.4	24.3	10.7	13.1
11	DxP	0.418/571 x 0.174/655	T	41.8	9.9	6.3	18.3
		Mean		37.6[a]	17.6[b]	8.5[c]	9.9[c]
		CV		17.2%	52.7%	45.3%	40.4%

Note: D - *Dura*, T - *Tenera*, P - *Pisifera*. Means within the same column with the same letter are not significantly different at $P \leq 0.05$ from Tukey's Studentised Range (MSD) test. Figures in the row are minimum and maximum values. CV – coefficient of variations.

TABLE 3. MOISTURE CONTENT FOR OIL PALM EMBRYOS DEHYDRATED USING SILICA GEL METHOD

No.	Country	Palm No.	Fruit type	Moisture content of treated embryos (%)					
				0 hr	1 hr	2 hr	3 hr	4 hr	5 hr
1	Angola	0.311/46	D	36.0	27.3	23.3	19.0	13.9	9.0
2	Angola	0.311/20	T	40.9	29.9	24.6	20.6	15.8	12.0
3	Cameroon	0.219/893	D	33.0	24.0	22.4	18.3	15.2	2.2
4	Cameroon	0.219/789	T	34.3	23.1	17.6	15.4	14.1	11.7
5	Ghana	0.397/589	D	37.1	32.4	27.3	24.2	14.4	9.0
6	Ghana	0.397/2068	T	33.5	27.7	19.8	13.3	11.8	10.9
7	Guinea	0.353/140	D	40.3	32.6	25.1	19.9	15.0	9.6
8	Guinea	0.353/88	T	35.2	29.7	24.7	9.7	5.8	3.9
9	Senegal	0.352/22	D	41.2	33.7	26.7	19.0	9.0	8.7
10	Senegal	0.352/5	T	39.3	22.5	17.8	15.6	14.6	11.0
11	DxP	0.418/571 x 0.174/655	T	40.3	25.7	24.0	15.0	13.6	8.5
		Mean		**37.1[a]**	28.3[b]	22.9[c]	17.5[d]	13.0[e]	**8.8[f]**
		CV		8.5%	14.4%	15.2%	23.5%	24.7%	37.2%

Note: D - *Dura*, T - *Tenera*, P - *Pisifera*. Means within the same column with the same letter are not significantly different at $P \leq 0.05$ from Tukey's Studentised Range (MSD) test. Figures in the row are minimum and maximum values. CV – coefficient of variations.

TABLE 4. GERMINATION RATE OF OIL PALM EMBRYOS DEHYDRATED USING SILICA GEL METHOD

No.	Country	Palm No.	Fruit type	Germination rate of treated embryos (%)					
				0 hr	1 hr	2 hr	3 hr	4 hr	5 hr
1	Angola	0.311/46	D	26.7	73.3	93.3	86.7	80.0	60.0
2	Angola	0.311/20	T	33.3	80.0	86.7	93.3	93.3	100.0
3	Cameroon	0.219/893	D	13.3	26.7	33.3	86.7	80.0	26.7
4	Cameroon	0.219/789	T	20.0	33.3	73.3	80.0	80.0	86.7
5	Ghana	0.397/589	D	0	0	40.0	40.0	66.7	46.7
6	Ghana	0.397/2068	T	0	0	26.7	66.7	80.0	80.0
7	Guinea	0.353/140	D	0	13.3	40.0	46.7	80.0	46.7
8	Guinea	0.353/88	T	33.3	73.3	93.3	60.0	53.3	53.3
9	Senegal	0.352/22	D	33.3	33.3	53.3	86.7	66.7	66.7
10	Senegal	0.352/5	T	6.7	40.0	86.7	93.3	66.7	80.0
11	DxP	0.418/571 x 0.174/655	T	20.0	13.3	26.7	33.3	53.3	33.3
		Mean		**16.7[d]**	37.3[c]	62.7[b]	74.0[a]	**74.7[a]**	64.7[b]
		CV		86.9%	79.5%	2.6%	26.3%	15.0%	34.4%

Note: D - *Dura*, T - *Tenera*, P - *Pisifera*. Means within the same column with the same letter are not significantly different at $P \leq 0.05$ from Tukey's Studentised Range (MSD) test. Figures in the row are minimum and maximum values. CV – coefficient of variations.

embryos increased with increasing desiccation periods from 13% without desiccation to nearly 90% after 4 hr of dehydration (Engelmann *et al*., 1995). Lower germination rate was observed for embryos that have moisture content of 8.8% after 5 hr desiccated on silica gel (*Figure 1*). This indicates that at moisture content of less than 10% the cryopreserved embryos have less ability to survive when cultured *in vitro*.

The treated embryos have higher viability than the untreated ones which indicates that removal of certain amount of moisture helps the embryos to remain viable during cryopreservation storage. This was already reported for oil palm embryos which have been dried to 10.4% moisture content (fresh-weight basis) showed no loss in viability during 8 months storage at -196°C (Grout *et al*., 1983). It is also evident that dehydrating the embryos longer than 4 hr caused in reduced rate of viability. Desiccation must be sufficient to ensure survival after freezing but not too intense to induce prolonged desiccation injury.

Ideal survival rates are generally achieved when samples are frozen with a water content of between 10% and 20% on fresh weight basis (Engelmann, 1992). The amount of moisture removed after 5 hr of treatment was possibly too high that affect the ability for the embryos to survive after cryopreservation. Studies of recalcitrant seeds such as tea and jackfruit revealed that partially and fully matured embryonic axes could be desiccated to about 14% moisture content and successfully cryopreserved (Chandel *et al*., 1995). Similar successes in cryopreservation using zygotic embryos were reported in coffee (Pinto *et al*., 2016), rubber (Sam, 1996) and coconut (Sisunandar *et al*., 2010). Additional studies are required in order to simplify, standardise and develop cryopreservation techniques for oil palm germplasm collections.

CONCLUSION

It is apparent that moisture content affects embryo viability in oil palm. Among the three methods tested, silica gel method offers the shortest time to desiccate the embryos. Results showed that embryos had moisture contents below 20% after 4 hr silica gel treatment. Present study showed that moisture content of 10% - 20% gave higher survival rate to cryopreserve oil palm embryos. The cryopreservation approach has potential to be applied to other type of oil palm samples such as pollen, kernels, somatic embryos which complements the long-term conservation of oil palm germplasm as alternative from the field genebank. Therefore, the next step is to apply cryopreservation to other type of oil palm samples besides embryos by using silica gel method and thus, the results can be compared.

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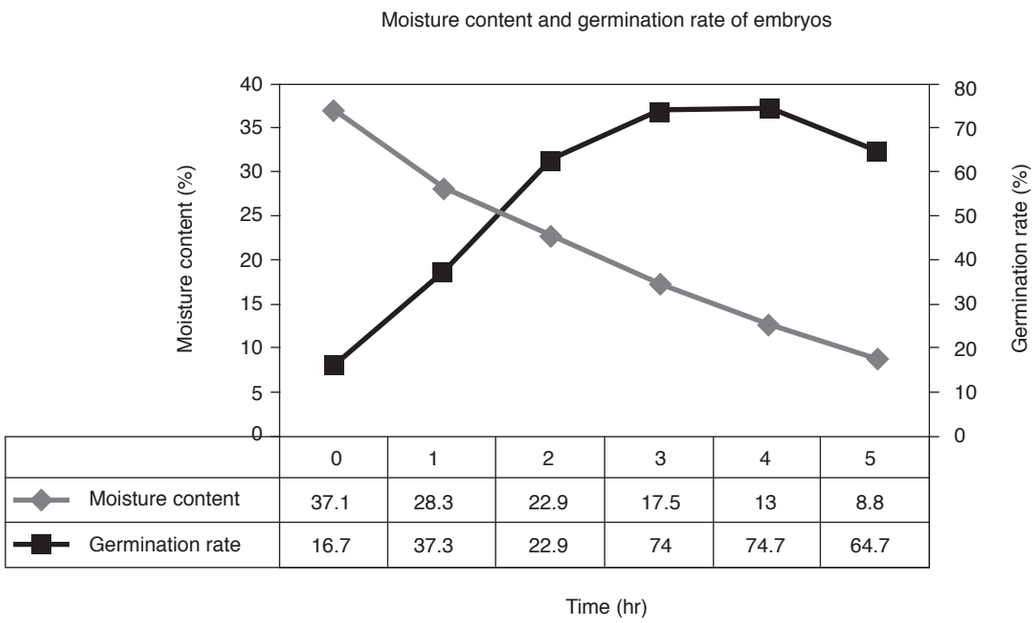

Figure 1. Moisture content reduction and germination rates of oil palm embryos from various germplasms using silica gel method (15 g, 5 hr).

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