Development of *in vitro* Technique to Preserve Oil Palm Genetic Materials[1]

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Abstract

The MPOB germplasm collections accumulated from various parts of the world are planted mainly at MPOB Research Station, Kluang, Johore, Malaysia. The collections are maintained in the form of field genebank. 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 are being 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, smaller space and protects the genetic materials from diseases. Oil palm seeds have intermediate characteristic. Oil palm seeds that have high levels of moisture lose viability when stored in liquid nitrogen. Therefore, preliminary 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. All three methods are shown to be useful for zygotic embryos. Silica gel however is advantageous as it removes the moisture in relatively shorter time than the other two methods. The progress of the present work is summarised in this paper.

Keywords: moisture content, cryopreservation, oil palm germplasm

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INTRODUCTION

MPOB has the largest oil palm germplasm collection in the world. The germplasm collections are being conserved in *ex situ* field plots. However, these plots require high maintenance cost and large amount of land. Apart from being exposed to diseases and extreme climate condition, MPOB has to put up plans for replanting programmes every 25 years. Therefore, new technique for oil palm germplasm conservation such as cryopreservation needs to be developed.

Oil palm seeds show intermediate seed storage behaviour (Ellis *et al*., 1991). Intermediate seeds can be desiccated to around 10-12% moisture content and can tolerate freezing temperatures.

Cryopreservation refers to the non-lethal storage of biological tissues at ultra-low temperature, usually that of liquid nitrogen (-196^{o}C) (Mandal, 2000). At low temperatures, any 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 makeup of the cryopreserved materials (Mandal *et al.,* 2000).

Cryopreservation method offers minimum space and low maintenance and has become very important tool for long term storage of germplasm materials. It appears to be the most feasible method for storing recalcitrant seeds and species that are vegetatively propagated. Cryopreservation using a desiccation protocol is the simplest procedure as it consists of dehydrating the plant material down to a suitable moisture content followed by rapid freezing through direct immersion in liquid nitrogen. Desiccation has been utilized mainly with zygotic and somatic embryos (Grout *et al*., 1983; Chaudhury *et al*., 1991).

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

MATERIALS AND METHODS

Plant Materials

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

Preparation of the seeds

The harvested bunches were labelled and depericarped to remove the mesocarp from the seeds. The clean seeds were then cracked and the kernels obtained were surface-sterilized for 20 minutes 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, laminar flow and silica gel (15 grams, 5 hours), respectively.

Room temperature method

The seeds were subjected to room temperature method. The moisture contents of the excised embryos were measured at 10, 20 and 30 days after treatment.

Air laminar flow method

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 of treatment.

Silica gel method

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

For each experiment, three replicates (five embryos/replicate) were used. The moisture contents of all treated embryos were measured using oven method described by 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 hours.

Dehydrated embryos for all treatments using silica gel method were sealed in propylene cryovials and directly immersed in liquid nitrogen (-196^{o}C). After 16 hour (at least), the embryos were thawed in 40^{o}C water bath for one minute. Embryos were then transferred onto MS basal medium to determine rate of

germination. The embryos were incubated at a temperature of 24 - 25^{o}C under 16/8 (light/dark) photoperiod.

RESULTS AND DISCUSSIONS

Room temperature method

The initial 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 in room temperature, the moisture contents decreased to 20.8% ranging from 11.3 to 33.2%. Moisture contents of 12.4% and 10.4% were obtained after 20 days and 30 days of exposure in room temperature, respectively.

TABLE 1: REDUCTION OF MOISTURE CONTENT FOR OIL PALM EMBRYOS DEHYDRATED USING ROOM TEMPERATURE METHOD.

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}

Note: D: Dura, T: Tenera, P: Pisifera. Means within the same column with the same letter are not significantly different at P ≤ 0.05 from Tukey's Studentized Range (MSD) Test. Figures in the row are minimum and maximum values.

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 from 37.6% 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 contents of embryos has not stabilised and the data was not useful and should be discarded. It is suggested that 30 days is sufficient to obtain moisture content of 10 – 20% for air laminar flow method.

TABLE 2: REDUCTION OF MOISTURE CONTENT FOR OIL PALM EMBRYOS DEHYDRATED USING AIR LAMINAR FLOW METHOD.

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]

Note: D: Dura, T: Tenera, P: Pisifera. Means within the same column with the same letter are not significantly different at P ≤ 0.05 from Tukey's Studentized Range (MSD) Test. Figures in the row are minimum and maximum values.

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 one hour 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 hours), there was an increase in viability up to 74.7% at 4 hours. After four hours in the silica gel, the germination rate of cryopreserved embryos ranged from 53.3% to 93.3%. Lower germination rate was observed for embryos that have moisture content of 8.8% after five hours desiccated on silica gel (Graph 1). This indicates that at moisture content of less than 10% the cryopreserved embryos has 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. It is also evident that drying the embryos longer than 4 hours resulted in reduced rate of viability. The amount of moisture removed after 5 hours of treatment was probably too high that affect the ability for the embryos to survive after cryopreservation.

TABLE 3: REDUCTION OF MOISTURE CONTENT FOR OIL PALM EMBRYOS DEHYDRATED USING SILICA GEL METHOD (15 GRAMS, 5 HOURS).

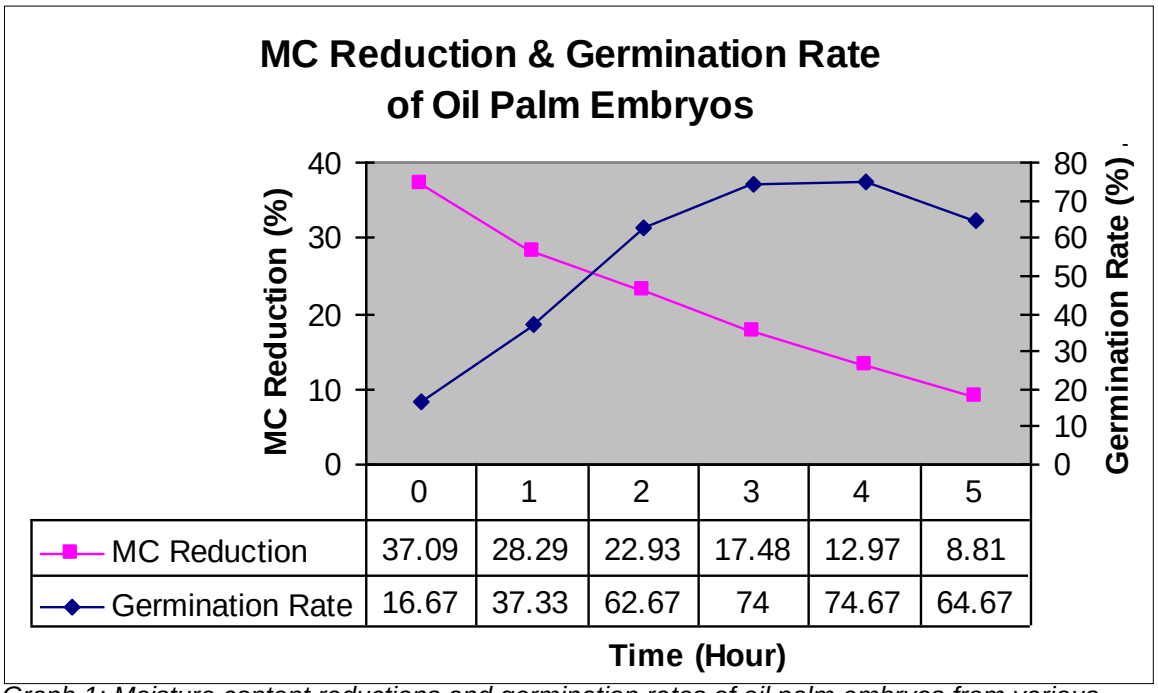
No.	Country	Palm No.	Fruit Type	Moisture Content of Treated Embryos (%)					
				0 hr	1 hr	2 hrs	3 hrs	4 hrs	5 hrs
1	Angola	0.311/46	D	36.0	27.3	23.3	19.0	13.9	9.0
2	Angola	0.311/20	T	40.9	29.9	24.6	20.6	15.8	12.0
3	Cameroon	0.219/893	D	33.0	24.0	22.4	18.3	15.2	2.2
4	Cameroon	0.219/789	T	34.3	23.1	17.6	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]	12.97[e]	**8.8**[f]

Note: D: Dura, T: Tenera, P: Pisifera. Means within the same column with the same letter are not significantly different at P ≤ 0.05 from Tukey's Studentized Range (MSD) Test. Figures in the row are minimum and maximum values.

TABLE 4: GERMINATION RATE OF OIL PALM EMBRYOS DEHYDRATED USING SILICA GEL METHOD (15 GRAMS, 5 HOURS).

No.	Country	Palm No.	Fruit Type	Germination Rate of Treated Embryos (%)					
				0 hr	1 hr	2 hrs	3 hrs	4 hrs	5 hrs
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]

Note: D: Dura, T: Tenera, P: Pisifera. Means within the same column with the same letter are not significantly different at P ≤ 0.05 from Tukey's Studentized Range (MSD) Test. Figures in the row are minimum and maximum values.



Graph 1: Moisture content reductions and germination rates of oil palm embryos from various germplasms using silica gel method (15 grams, 5 hours).

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 all embryos had moisture contents below 20% after 4 hours silica gel treatment. From the present study, it was indicated that moisture content of 10 - 20% gave higher survival rate of cryopreserved oil palm embryos.

REFERENCES

BAJAJ, Y P S (1984) Introduction of growth in frozen embryos of coconut and ovules of citrus. *Cryoletters* 23:1215-1216

CHAUDHURY R; RADHAMANI J & CHANDEL K P S (1991) Preliminary observation on the cryopreservation of desiccated embryonic axes of tea (*Camellia sinensis* (L.) O. Kuntze) seeds for genetic conservation. *Cryoletters* 12:31 - 36

ELLIS R H, HONG, T D, ROBERT, E H & SOESTISNA U (1991) Seed storage behavior in *Elaeis guineensis*. *Seed Science Research* 1:99-104

GROUT B W W; SHELTAN K & PRICHARD H W (1983) Orthodox behaviour of oil palm seed and cryopreservation of the excised embryos for genetic conservation. *Ann. Bot*. 52:381 - 384

ISTA (1985) International Rules for Seed Testing. *Seed Science & Technology* 13:299-355

MAKEEN A M; NORMAH, M N; DUSSERT, S & CLYDE, M M (2005) Cryopreservation of whole seeds and excised embryogenic axes of Citrus suhuiensis cv. Limau langkat in accordance to their desiccation sensitivity. *Cryoletters* 26:259-268

MANDAL B B; CHAUDHURY R; ENGELMANN F; BHAG MAL; TAO K L & DILLON B S (2000) Proceeding of a regional training course on *in vitro* conservation and cryopreservation of plant germplasm, 12 – 25 October 2000, NBPGR, New Delhi, India. pp. 283

NORMAH, M N; REED, P M & YU, Y L (1994) Seed storage and cryoexposure behaviour in hazelnut (*Corylus avellana* L. cv. Barcellona). *Cryoletters* 15:315-322

RADHAMANI, J & CHANDEL, K P S (1992) Cryopreservation of embryonic axes of trifoliate orange (*Poncirus trifoliate* (L) Raf.). *Plant Cell Report* 11:204-206