Research Article

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Cryopreservation of kernel and zygotic embryos of oil palm (*Elaeis guineensis* Jacq.)

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Abstract

The seed storage behaviour of oil palm (*Elaeis guineensis* Jacq.) is enigmatic. Earlier, it was categorised as recalcitrant or orthodox and later, it was confirmed to be exhibiting intermediate seed storage behaviour. Considerable difference in the water content between zygotic embryo and the seed/kernel exists which is proportionally maintained even after desiccation of kernel, which qualifies it to be intermediate storage behaviour. To attempt long term conservation of oil palm germplasm grown in India, studies on germinability/viability in relation to moisture content was conducted for 'dura' palm using seed kernel, zygotic embryos and endosperm plug as explants. Based on desiccation and cryopreservation experiments in kernels and embryos, it showed intermediate storage behavior. Zygotic embryo cryopreservation was possible between the moisture content of 10-20 per cent of embryos, and was achieved using silica gel desiccation method of either kernels or embryos or both. Four hours of desiccation under laminar air flow was found optimum to maintain viability after cryo-exposure. A new type of explant, endosperm plug, of 0.1 g weight and 2-5 mm length could offer advantage for the desiccation and cryopreservation of oil palm as it could be dehydrated up to 6 per cent moisture level and retained 20 per cent viability after cryo-exposure. Initial attempt to use encapsulation-dehydration and vitrification-desiccation methods for embryos of oil palm did not yield positive results, necessitating further experiments.

Keywords: Cryopreservation, embryos, kernel, oil palm, seed

Introduction

Oil palm (*Elaeis guineensis* Jacq.) has been the most recently domesticated tree species for commercial use. It is perennial monocotyledonous tropical palm species, the primary source of edible vegetable oil and most traded in the international market. Oil palm is the most productive of all oil crops, with an average yield about 4-5 t ha^{-1} $year^{-1}$ of oil in the major producing countries. Palm oil is versatile oil, used for food products and non-food derivatives.

Cryopreservation as a method of *ex situ* long term conservation of oil palm has been attempted employing various explants like somatic embryos (SEs), embryogenic cell suspensions and friable tissues, zygotic embryos, kernels, seeds and pollen (Silva and Engelmann, 2017). As oil palm micro-propagation is based on somatic embryogenesis, the vast majority of cryopreservation studies in oil palm have thus employed SEs and few studies exist on the cryopreservation using other explants (Chaudhury and Malik, 2017; Silva and Engelmann, 2017).

King and Roberts (1979, 1980) considered oil palm seeds as recalcitrant based on their desiccation intolerance, but reported that data available to support this tentative conclusion was insufficient. Grout *et al*. (1983) reported that oil palm seeds were orthodox and not recalcitrant on the basis of desiccation experiments. Ellis *et al*. (1991) hermetically stored seeds of four cultivars of oil palm at 15 ºC with 10-12 per cent moisture content up to 12 months. Seed viability was found to be reduced at lower temperature and intact seeds at 6.1-7.4 per cent moisture showed rapid loss in viability. Rajanaidu and Ainul (2013)

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reported that seeds could be stored viable for up to two years only. These reports confirmed that oil palm seeds were neither recalcitrant nor orthodox and therefore was considered intermediate between the two categories. Engelmann *et al*. (1995) performed a detailed study on the effect of rehydration on the cryopreservation of oil palm zygotic embryos and kernels and reported high recovery of up to 65 per cent after cryopreservation of desiccated embryos preceded by rehydration of kernels. Chaudhury and Malik (2004) reviewed genetic conservation methods for plantation crops and categorised oil palm seeds as intermediate storage behaviour. Recently, Norziha *et al*. (2017) attempted cryopreservation of oil palm zygotic embryos following silica gel, room temperature as well as laminar air desiccation. The highest germination percentage (74%) of cryopreserved embryos was obtained for those with moisture contents between 17.5-13.0 per cent. Silva and Engelmann (2017) recently reported cryobanking of 68,550 zygotic embryos from 457 oil palms by Malaysian Palm Oil Board.

In view of enigmatic seed storage behaviour of *E. guineensis*, present investigations were carried out for evaluating the feasibility of cryobanking for long-term conservation, especially for germplasm adapted to the Indian conditions.

Materials and methods

Plant materials

About 1000 seeds of dura type oil palm *(E. guineensis)* were collected from ICAR-Indian Institute of Oil Palm Research (ICAR-IIOPR), Regional Station Palode, Kerala and brought to Cryo-laboratory at ICAR-National Bureau of Plant Genetic Resources (ICAR-NBPGR), New Delhi for the experiments. Kernels were extracted after breaking the hard endocarp of seeds. Morphological studies were carried out and data recorded for size, weight, colour and other parameters for ten random samples per accession. Embryos were extracted using lab grade blade and scalpel. Additionally, endosperm plug was tried as a new explant for desiccation experiments. From the extracted kernel, a cylindrical endosperm plug (containing embryo) measuring 2-5 mm length and weighing approximately 0.1 g was scooped/cut using scalpel blade. This was a practical approach since the kernel of oil palm is very hard and size is too big to handle for long term cryobanking of germplasm. These endosperm plugs also offer advantage for space constraints in cryotanks as well as ease of handling. The three explants namely kernel, zygotic embryos and endosperm plug were subjected to various desiccation experiments. Moisture content was determined gravimetrically by the low constant temperature oven dry method where seeds were dried at 103±2 °C for 17 hours (ISTA, 1985). Viability of embryos and endosperm plug were tested by *in vitro* culturing using MS medium in combination with different growth hormones. Embryos were extracted from desiccated as well as cryo-exposed kernels (after thawing at 40±2 ºC water) and cultured *in vitro* after surface sterilization and germination percentage recorded at 15 days interval. Cultures were monitored at regular intervals after culturing. Each experiment was repeated thrice and the standard errors (SEs) of the arithmetic means were determined for each treatment.

Oil palm kernel and embryo desiccation in silica gel and cryopreservation

Oil palm kernels were desiccated for 24, 48, 72 and 96 hours by enclosing in muslin cloth and placing over charged silica gel in desiccators. Desiccated kernels were subjected to cryo-exposure while placed in 50 mL cryo-vials. Extracted embryos were desiccated after putting in stainless steel closed strainer in silica gel desiccators and desiccated embryos were enclosed in small cryo-vials (1 mL) and fast frozen in liquid nitrogen. The following scheme was followed for cryopreservation experiments under silica gel desiccation.

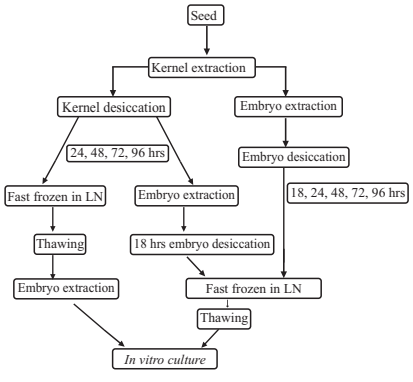

Fig. 1. Flow chart for oil palm kernel and embryo desiccation over silica gel and cryopreservation methods

Oil palm endosperm plug desiccation and cryopreservation

Excised endosperm plugs (containing embryo) were packed in stainless steel strainer and placed in desiccator containing charged silica gel for various periods.

Cryopreservation of embryos by different methods

Embryos were subjected to three different desiccation methods *viz.* air-desiccation, encapsulation-desiccation and vitrification and survival and growth data were recorded.

Air desiccation method: Aseptically excised embryos were kept in the sterile air of laminar flow cabinet and desiccated for 1 to 6 hrs. After each desiccation interval, moisture content and viability of embryos were determined. Desiccated embryos were then sealed in 1.0 mL polypropylene cryo-vials and fast frozen by direct plunging in liquid nitrogen (LN). After minimum 24 hr of storage, the cryo-vials were rapidly thawed in water bath at 38 °C for 2-5 min and embryos were cultured *in vitro*.

Encapsulation-dehydration method: Aseptically excised embryos were encapsulated in alginate beads by first suspending in calcium-free MS basal liquid medium containing 3 per cent (w/v) Na-alginate. Individual drops each enclosing one explant, were dispensed with a pipette into MS basal liquid medium supplemented with 100 mM calcium chloride. Beads were swirled intermittently and were solidified by 15 min incubation at 25 ºC, and then pre-cultured on a rotary shaker in liquid MS basal medium supplemented with different sucrose concentrations (0.3, 0.5 and 0.75) at 100 rpm for 24 hours. Later the beads were removed from liquid medium, blotted dry and dehydrated for up to 6 hrs in a laminar flow cabinet. The moisture content of the beads was determined by drying at 103±2 ºC for 17 hrs. Beads were finally enclosed in 1.0 mL cryo-vials and fast frozen in liquid nitrogen. After minimum 24 hrs, the cryo-vials were thawed in water bath at 38 ºC for 2-5 min, and the beads cultured *in vitro*.

Vitrification method: Aseptically excised embryos were pre-cultured for three days on basal MS pretreatment media supplemented with 1.2 M sucrose. Embryos in batches of 15-25 were then transferred to 1.0 mL sterile cryo-vials and treated with loading solution (0.4 M sucrose, 2 M glycerol in basal MS medium) for 20 min at 25 ºC. The loading solution was replaced by plant vitrification solution 2 [PVS2 - 30 per cent (w/v) glycerol, 15 per cent (w/v) ethylene glycol, 15 per cent (w/v) dimethyl sulphoxide (DMSO) with 0.3 M sucrose] for 20 min at 25 ºC. Explants were then frozen by fast freezing in liquid nitrogen. Frozen cryo-vials were thawed after a minimum of 24 hrs storage by 2-5 min immersion in a water bath at 38±2 ºC, following which the vitrification solution was replaced with unloading solution (1.2 M sucrose) by keeping for 20 min.

Recovery medium: For *in vitro* recovery of embryos and endosperm plugs following media combinations were used.

- OP1: MS medium + 30 g L^{-1} sucrose + 0.01 mg L^{-1} NAA + 0.1 mg L^{-1} kinetin + 2 g L^{-1} gelrite
- OP2: MS medium + 30 g L^{-1} sucrose + 0.1 mg L^{-1} NAA + 0.1 mg L^{-1} kinetin + 7 g L^{-1} agar
- OP3: MS medium + 30 g L^{-1} sucrose + 0.5 mg L^{-1} IAA + 0.1 mg L^{-1} kinetin + 7 g L^{-1} agar
- OP4: MS medium + 30 g L^{-1} sucrose + 0.1 mg L^{-1} NAA + 1 mg L^{-1} BAP + 7 g L^{-1} agar
- OP5: ½ MS medium + 30 g L^{-1} sucrose + 2 mg L^{-1} 2iP + 0.25 per cent phytagel

Results

Oil palm fruit is botanically a drupe, varying in shape from nearly spherical to ovoid or elongated. In general, exocarp is included with mesocarp (comprising pericarp) while endocarp together with kernel forms the seed. The seeds are endospermic, spherical to ovoid to ellipsoid in shape. The drupaceous fruit usually are one seeded; sometimes two seeded kernels are also present. Wide variation in the size, shape and weight of the kernels were recorded. The kernel size was in the range of 12.50-17.81 mm length, 8.82-13.02 mm diameter and 0.92-1.68 gm weight. The kernel consists of dark brown testa covered with a fibrous network and inside is a greyish-white endosperm. A straight small embryo (2-3 mm long) was embedded in the endosperm anteriorly, beneath the operculum.

Moisture content of kernel and embryos of oil palm

The average moisture content of fresh kernel and embryo were 23.4 and 37.9 per cent, respectively. Moisture content of embryo was found to be always higher compared to that of kernels at each of the desiccation levels. Kernels kept for desiccation in open trays at room temperature (27±3 ºC, 70% RH) up to six months showed slow and steady decline in moisture content (Table 1).

Table 1. Moisture content of kernel and embryos of oil palm after various desiccation periods of kernel in open air room temperature and over silica gel

Desiccation period	Kernel moisture content (%)	Embryo moisture content (%)
Fresh	23.4	37.9
24 hrs silica desiccation	12.3	31.5
48 hrs silica desiccation	8.6	23.5
72 hrs silica desiccation	7.2	20.0
96 hrs silica desiccation	6.1	18.1
15 days at room temperature	18.8	33.5
1 month at room temperature	15.3	28.5
3 months at room temperature	12.7	19.3
6 months at room temperature	8.1	14.4

Kernel and embryo desiccation, germination and cryopreservation

Fresh embryos of oil palm with 37.9 per cent moisture exhibited 56.5 per cent germination *in vitro*. Following results were achieved in three separate experiments using silica desiccation.

Desiccation of kernel: Desiccation of kernels for 24, 48, 72 and 96 hours over charged silica gel resulted in reduction in moisture content and *in vitro* germinability of the embryos respectively (Table 2). Fresh kernels, which were desiccated for up to 48 hours showed germinability between 33-56 per cent, but failed to survive LN exposure. Kernels desiccated for 72 hours and 96 hours, with 20.0 and 18.1 per cent moisture level respectively and 20-25 per cent germinability, after LN exposure gave 11.5 and 9.5 per cent *in vitro* germination, respectively.

Desiccation of embryo: On desiccation of excised embryos for 18, 24, 48, 72 and 96 hours, moisture content reduced significantly up to 9.5 per cent moisture (Fig. 2A). Concomitantly, there was a decline in germinability to 40.0, 33.3, 25.0, 16.6 and 10.0 per cent, respectively (Table 2, Fig. 2B). On cryo-exposure of variously desiccated embryos, those with moisture levels of 18.0 and 12.4 per cent alone survived albeit with low 7.7 and 9.3 per cent germinability, respectively (Fig. 2C).

Table 2. Moisture content and germination after desiccation and cryopreservation of kernel and embryos of oil palm

	Embryo moisture (%)	Germination (%) (-LN)	Germination (%) (+LN)
Fresh embryo	37.9	56.5 (±0.3)	0 (±0.0)
Kernel desiccation			
24 hrs	31.5	42.5 (±0.4)	0.0 (±0.0)
48 hrs	23.5	33.3 (±0.1)	0.0 (±0.0)
72 hrs	20.0	25.0 (±0.1)	11.5 (±0.0)
96 hrs	18.1	20.0 (±0.5)	9.5 (±0.1)
Embryo desiccation			
18 hrs	30.2	40.0 (±0.0)	0.0 (±0.0)
24 hrs	26.4	33.3 (±0.1)	0.0 (±0.0)
48 hrs	18.0	25.0 (±0.0)	7.7 (±0.8)
72 hrs	12.4	16.6 (±0.0)	9.3 (±0.6)
96 hrs	9.5	10.0 (±0.6)	0.0 (±0.0)
Kernel + embryo Desiccation			
24 hrs	21.5	45.5 (±0.8)	12.5 (±0.3)
48 hrs	16.3	30.0 (±0.5)	15.4 (±0.1)
72 hrs	11.4	18.0 (±0.1)	6.5 (±0.0)

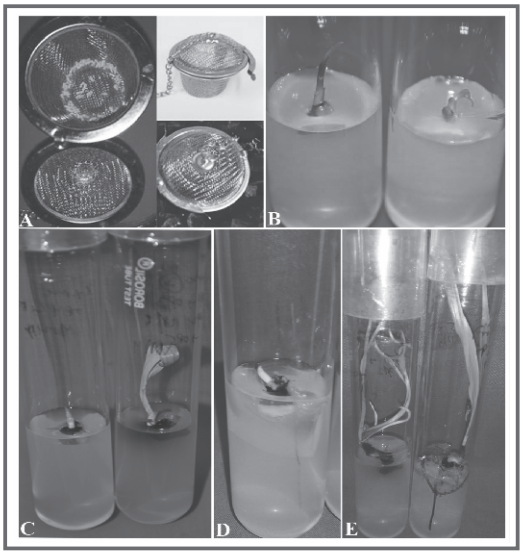

Fig. 2. (A) Oil palm embryos placed for desiccation in stainless steel strainer over silica gel, (B) *in vitro* germination of desiccated embryos, (C) freshly excised endosperm plug germination, (D) cryopreserved endosperm plug germination and (E) plantlets developed from cryopreserved embryos

Desiccation of whole kernel followed by 18 hours desiccation of excised embryos: When embryos from kernels desiccated for 24, 48 and 72 hours were extracted and subjected to further desiccation of 18 hours over silica gel, moisture content of embryos got reduced to 21.5, 16.3 and 11.4 per cent with germinability of 45.5, 30.0 and 18.0 per cent, respectively (Table 2). After LN exposure of desiccated embryos, germination of 12.5, 15.4 and 6.5 per cent was recorded respectively.

Endosperm plug as explant for desiccation and cryopreservation

Fresh endosperm plugs (containing embryo, with initial moisture of 28.6 per cent showed 36.6 per cent germination *in vitro* (Fig. 2C); however, it did not survive cryo-exposure. These endosperm plugs on desiccation for 24, 48 and 72 hours in silica gel showed drastic reduction in moisture to 7.8, 6.1 and 5.3 per cent, respectively with *in vitro* germinability of 21.4, 33.3, and 9.5 per cent, respectively. On fast freezing, plugs with 6.1 and 7.8 per cent moisture level exhibited 20 per cent and 14.3 per cent germination, respectively (Table 3, Fig. 2D). In another experiment, when endosperm plugs were extracted from 24, 48 and 72 hours desiccated kernels and further subjected to desiccation for 18 hours in silica gel, moisture content got reduced to 11.15, 10.8 and 9.7 per cent respectively; however, germinability *in vitro* was lost completely.

Table 3. Desiccation and cryopreservation of endosperm plugs of oil palm

Endosperm plug desiccation period	Moisture (%)	Germination (%) (-LN)	Germination (%) (+LN)
Fresh plug	28.6	36.6 (±0.6)	0.0 (±0.0)
24 hrs	7.8	21.4 (±0.1)	14.3 (±0.0)
48 hrs	6.1	33.3 (±0.1)	20.0 (±0.5)
72 hrs	5.3	9.5 (±0.0)	0.0 (±0.0)

Laminar air flow desiccation and cryopreservation

When freshly excised embryos were exposed to laminar air flow desiccation, moisture content reduced drastically to 11.4 per cent until 3 hours of desiccation and further moisture reduction was very slow as it got reduced to 8.2 per cent in the next 3 hours. Germination steadily declined at each successive desiccation period (Fig. 3) and viability was completely lost at about 8 per cent moisture. Maximum germinability of 10 per cent was observed for LN exposed desiccated embryos at 4 hours of desiccation at 10.3 per cent moisture (Fig. 2E).

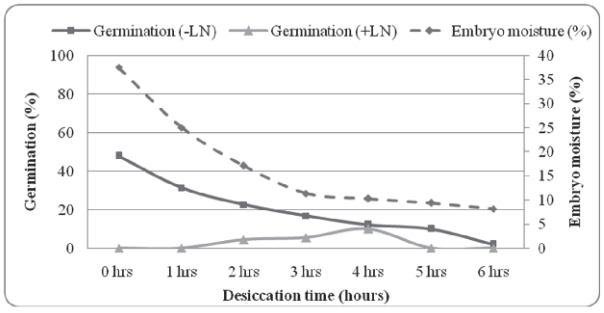

Fig. 3. Laminar air flow desiccation and LN exposure of oil palm embryos

Encapsulation-desiccation and vitrification of excised embryo

Excised embryos from fresh kernels were attempted for cryopreservation using encapsulation-desiccation and vitrification. Moisture content of encapsulated beads after preculture using 0.3, 0.5 and 0.75 M sucrose was between 81 to 85 per cent moisture which on 6 hrs desiccation got reduced to 17.4, 20.8 and 18.7 per cent, respectively (Table 4). Fresh control beads exhibited 35.5 per cent germination. Survival *in vitro* of 13.3 per cent was observed only for beads pre-cultured in 0.5 M sucrose and air desiccated which on LN exposure failed to germinate.

Table 4. Encapsulation-desiccation in excised embryos of oil palm

Pre-culture medium	% moisture of beads	% moisture of beads after 6 hrs of desiccation	% viability before LN	% viability after LN
Control	-	-	35.5 (±5.0)	0
0.3 M	81.0	17.4	0.0 (±0.0)	0
0.5 M	83.7	20.8	13.3 (±0.8)	0
0.75 M	84.6	18.7	0.0 (±0.0)	0

For vitrification experiment, excised embryos were pre-cultured for three days before subjecting to vitrification by fast freezing. Embryos at this stage showed 35.5 per cent germination. Unfrozen controls after loading and after PVS2 treatment exhibited 22.5 per cent and 16.67 per cent germinability *in vitro* (Table 5). However, none of the embryos survived LN exposure.

Table 5. Vitrification in excised embryos of oil palm

Treatment	Viability (%) before LN	Viability (%) after LN
Control	35.5 (±5.0)	0
Loading solution (20 min)	22.5 (±2.8)	0
PVS2 (20 min)	16.67 (±1.3)	0

Growth and recovery media optimization for oil palm embryos and endosperm plug

There were five growth medium used for *in vitro* culture of explants. These media were selected based on previous work reported by other researchers in oil palm (Grout *et al.*, 1983; Engelmann *et al.*, 1995). Based on results with various experiments, medium OP3 was found optimum as it resulted in 42.10 per cent germination followed by OP4 with 37.54 per cent germination (Fig. 4).

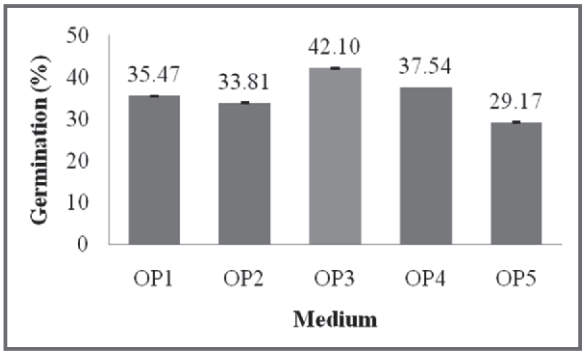

Fig. 4. Media optimization for oil palm embryos and endosperm plugs

Discussion

Controversial views exist regarding the storage behaviour and classification of oil palm seeds. It has been classified under different categories by different authors. Initially, King and Roberts (1979,1980) classified *E. guineensis* as recalcitrant because of the desiccation sensitivity. However, categorization of seed storage behaviour remain inconclusive. Later, after desiccation experiments, Grout *et al.* (1983) suggested oil palm seeds to be orthodox based on recovery of viable seeds after cryopreservation. Again, oil palm seeds were proven to be of intermediate category between orthodox and recalcitrant on the basis of storage behaviour under different temperatures and moisture content (Ellis *et al.*, 1991). In their experiment, four cultivars of oil palm maintained seed viability up to 12 months when hermetically stored at 15 ºC with 10-12 per cent moisture content. However, at lower temperature and lower moisture (6.1-7.4%) intact seeds showed much reduction in seed viability. These reports confirmed that oil palm seeds were neither recalcitrant nor orthodox in storage behaviour but were intermediate between the two categories.

In the present experiment, seed kernel moisture was recorded to be 23.4 per cent and that of embryo to be higher as 37.9 per cent. Thus, there was significant difference in the water content between the embryo and that of seed kernel and the difference was found maintained even after desiccation of kernels. When kernels were desiccated to various periods in silica gel to moisture levels in the range of 6-8 per cent, the enclosed embryo moisture was still higher in the range of 18-23 per cent. This result is in agreement with work by Grout *et al.* (1983) who reported that the embryos always contained more moisture than the average for whole kernels. The authors recorded kernel moisture of about 21 per cent and that of the embryos as 48 per cent which on drying of kernels to about 7 per cent moisture content, showed retention of as high as 20-21 per cent moisture in embryos. This high moisture of embryos may have resulted in the failure of storage of whole seeds at sub-zero or freezer temperatures. Thus, despite the 'orthodox' behaviour reported for oil palm seed, the use of conventional low temperature seed storage techniques was found inappropriate (Grout *et al.*, 1983). In our experiments, on cryo-exposure of desiccated kernels at moisture of less than 7 per cent having embryo moisture less than 20 per cent, some viability was retained for embryos when tested *in vitro.*

Grout *et al.* (1983) also suggested that the excised embryo might be used in attempts at cryogenic storage as the desiccated embryos do not lose viability after rapid cooling to the temperature of liquid nitrogen (-196 °C) and the size of embryo would be more amenable to controlled experiments than the intact seed. Authors extracted zygotic

embryos from kernels and dehydrated under laminar airflow cabinet to reduced moisture level of 10.4 per cent and exposed to -18 °C. When embryos were rewarmed immediately, there was no decline in viability but upon storage at -18 °C for one month resulted in a total loss of viability. Further, cryo-exposure of desiccated embryos in LN maintained viability for up to eight months.

In our experiments with embryos, moisture content between 11.4 to 21.5 per cent was found to be optimal for cryo-survival, although viability range varied from method to method. Norziha *et al.* (2017) however, found maximum germination percentage (74%) after cryopreservation when zygotic embryos were desiccated between 13.0-17.5% moisture. Similar results were obtained by Engelmann *et al*. (1995) where survival of cryopreserved oil palm embryos increased with increasing desiccation periods up to 4 hours. In one of our experiments, embryos were extracted from desiccated kernels at different moisture level and further desiccated to 18 hours under silica gel and then cryopreserved. The result showed that after partial desiccation of kernel (best being 48 hours) followed by embryo desiccation, there was reduction in the moisture of embryos to 16.3 per cent and after cryo-exposure it gave 15.4 per cent germination.

Overall, we have obtained lesser recovery of oil palm plantlets after various experiments compared to that reported previously in this crop. This may be due the fact that initial viability itself was very low in the received material. Fresh embryos germination was observed on an average of 56.5 per cent of total *in vitro* cultured embryo.

A new explant, cylindrical endosperm plug, measuring 2-5 mm in length and weighing approx. 0.1 g was attempted for cryopreservation in present study. Since the kernel of oil palm is very hard and size is too big to handle for long term cryobanking of germplasm, this endosperm plug offered advantage for space constraints of cryobanking as well as easy to handle. These endosperm plugs could be rapidly desiccated to low moisture of 6.1 per cent and on LN exposure gave germination values of 20 per cent which was maximum achieved by any of the methods used in the present study. This may be due to the fact that these embryos are better protected from cryo-injuries by the surrounding endosperm tissues in these plugs.

Encapsulation-desiccation and vitrification of oil palm zygotic embryos were not reported previously and hence attempts were made in the present study to explore its potential. However, no success was achieved by either of the methods. Sucrose dehydration in encapsulation method itself proved detrimental for the embryos as controls showed no or very low viability values. PVS2 treated controls during vitrification showed only a low average 16.67 per cent germination.

Conclusion

Desiccation and cryopreservation of seeds and/or embryos of dura *E. guineensis* showed intermediate storage behaviour with tolerance to reduced moisture level of embryos. Oil palm zygotic embryo cryopreservation was possible between the moisture content of 10-20 per cent of embryos using silica gel desiccation method of either kernels or embryos or both. Embryo desiccation up to 4 hours in Laminar Air Flow was found optimum to maintain viability after cryo-exposure. A new type of explant 'Endosperm plug' of 0.1 g weight and 2-5 mm length could offer advantage for the desiccation and cryopreservation of oil palm as it could be dehydrated up to 6.1 per cent moisture level and yet survived cryo-exposure with 20 per cent viability. Attempts to cryopreserve zygotic embryos using encapsulation-dehydration and vitrification methods in oil palm failed necessitating further refinements in future.

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