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Cryopreservation of embryogenic callus of hybrid tenera oil palm by dehydration technique and evaluation of somaclonal variation by SSR marker

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Cryopreservation of oil palm embryogenic callus (EC) was investigated using dehydration techniques. Haustorium embryos (HEs) derived from cryopreserved EC were assessed genetic instability by simple sequence repeat (SSR) marker. The results revealed that EC were successfully cryopreserved by dehydration. After preconditioning the tissue on Murashige and Skoog (MS) medium supplemented with 0.25 M sucrose for 7 d, EC were dehydrated with desiccators at 67.3% RH for 24 h, followed by storing in liquid nitrogen for 1 h gave the highest percentage of somatic embryo formation at 44.44. However, dehydrated callus by desiccators at 20.20% RH for 36 h had the highest average number of SEs at 1.67 embryos/explants after thawing and culturing on MS medium supplemented with 0.1 mg/L dicamba. Those SEs at haustorium stage from the best treatment was uniform by nine primers of SSR marker. The results suggest that cryopreservation using dehydration does not affect genetic stability of oil palm EC.

Keywords: Embryogenic callus, cryopreservation, dehydration, SSR marker

Introduction

The Oil palm (*Elaeis quineensis* Jacq.) is a valuable economically important source of vegetable oil, the most traded vegetable oil in the international market, and increasingly used in the food industry (Corley and Tinker, 2003), bio-diesel and a fuel to be burnt in power stations to produce electricity. Moreover, the high and increasing yields of oil palm have led to a rapidly expanding world industry. Because this palm has only a single growing point, and does not produce suckers like some other palm species, it can be propagated by seed and tissue culture which is possible to clones (Corley and Tinker, 2008). The growing tissue may form callus through somatic

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embryogenesis (Chehmalee and Te-chato, 2008; Sanputawong and Te-chato, 2011). For embryogenic callus cultures it requires periodic subculturing to maintain both a high proliferation potential and the capacity of cells to develop into somatic embryos. Repeated subculturing is not only labor intensive and time consuming, but also increases the risk of losing the embryogenicity through contamination, human errors or technical failures (Lambardi *et al*., 2008). The loss of embryogenic potential of culture lines and the occurrence of genetic alteration due to long-term subculturing is frequently reported (Bhatti *et al*., 1997). A combination of advanced biotechnological methods of plant cell culture and cryopreservation may facilitate the long-term conservation of a variety of forest plant genetic resources (Tsai and Hubscher, 2004). This method may also limit the amount of contamination and somaclonal variation resulting from routine subculturing of embryogenic tissue (Schrijnemakers and van Iren, 1995). It has been used for the storage of many different types of plant material, such as seeds, embryonic axes isolated from seeds, and vegetative propagated plant material, including apical or axillary buds, pollen, somatic embryos, and embryogenic tissues (ET) (Engelmann, 2004). Effective protocols for the induction and the establishment *in vitro* of embryogenic cultures were developed for a large number of plant species of Citrus (Perez *et al*., 1999; Gonzalez-Arnao *et al*., 2003) and Quercus (Martinez *et al.*, 2003; Valladares *et al*., 2004; Fernandes *et al*., 2008). Nowadays, there are two available methods. Both methods are different in terms of techniques employed and the physical mechanism. A first method is classical techniques involve with freeze-induced dehydration, though new method based on vitrification (Engelmann, 2000). For oil palm, Dumet *et al*. (1993) showed that, by introducing a desiccation stage, drying the embryoids to between 19 and 35% moisture content after preconditioning of any type of embryoids on solidified medium enriched with sucrose was very important in producing a high regrowth (93%) after cryopreservation however, the percentage of development of explants were still variable.

Recently, molecular marker techniques based on the polymerase chain reaction (PCR) has been one of the most commonly used for characterization of genetic variability, genotype identification, genetic instability analyses and selection and breeding purposes (Tingey and Tufo, 1993). Among those techniques simple sequence repeat (SSR) was proved to be very commonly used in identification hybridity of oil palm F1 hybrid (Thawaro and Te-chato, 2009), verification of genetic instability in oil palm plantlets from tissue culture (Sanputawong and Te-chato, 2011). To make sure that cryopreservation of embryogenic callus (EC) of elite clone of oil palm produce no effect on genetic instability of developed somatic embryos (SEs) and plantlets it is necessary to

use marker assist selection (MAS) for evaluation the uniformity of those materials. Thus, in this study, simplify technique for cryopreservation using ECs of *E. guineensis* was discussed, and assessment the uniformity of ECs-derived SEs or plantlets using SSR marker with nine primers was described.

Materials and methods

Plant material

EC initiated from young leaves of elite "Tenera" tree by the methods described by Te-chato *et al*. (2002). Briefly, the leaves were cultured on callus induction medium (CIM) which was MS medium supplemented with 3% sucrose, 200 mg/L ascorbic acid and 2-5 mg/L dicamba, and solidified with 0.75% agar. Proliferation of the callus/embryogenic callus was maintained on CIM but decreased the concentration of dicamba to 0.1-1 mg/L. This culture medium was designated as proliferation medium (PM). Those calli/EC were maintained on solidified PM medium with regularly subculture at four week intervals for ten years. All cultures were placed at 28±0.5ºC under a 14 h photoperiod at 15 µmol/m^2/sec illumination.

Dehydration: preliminary experiment

ECs at approximately 60-80 mg fresh weight were dehydrated by two different methods. The first method was air-drying in laminar air flow cabinet for 1-5 h. The flow rate of air in this cabinet was 80-90 ft/min. The second method was desiccated in desiccators together with silica gel for 6-36 h. After dehydration EC was cultured on solid MS medium supplemented with 0.1 mg/L dicamba, 3% (w/v) sucrose and 0.75% (w/v) agar. The cultures were incubated at 28±0.5ºC under a 14 h photoperiod at 15 µmol/m^2/sec illumination.

Cryopreservation procedure

EC were precultured on solid MS medium supplemented with 0.25 M sucrose under the same conditions as described in PM for 7 d (Khawnium and Te-chato, 2011) and then dehydrated by two different methods as the same mention in preliminary experiment. After dehydration, the EC were plunged into liquid nitrogen (-196ºC) for at least 1 h.

Thawing and unloading

For recovery, the cryo-tube containing EC was rapidly thawed in a warm water bath at 40˚C for 2 min. Then, EC was placed onto sterile filter paper and transferred to recovery media composed of solid MS medium supplemented with 0.1 mg/L dicamba, 3% (w/v) sucrose and 0.75% (w/v) agar. The cultures were incubated at 28±0.5ºC under a 14 h photoperiod at 15 µmol/m^2/sec illumination.

SSR analysis

SEs at haustorium stage was cut into half. The first halves (150–200 mg fresh weight: mgFW) were collected and used to isolate DNA by the protocol described by Te-chato (2000). In case of regenerated plantlets, young first and second leaves of those plantlets at approximately 200 gFW were collected and subjected to extract DNA according to the method described by Doyle and Doyle (1990) with some modification. SSR analysis of genomic DNA was carried out using 1 microsatellite loci amplified in oil palm using 9 primers primers (EgCIR0008, EgCIR0243, EgCIR0337, EgCIR0409, EgCIR0446, EgCIR0465, EgCIR0781, EgCIR0905 and EgCIR1772) which was proved to be generally used for identify somaclonal variation in plantlet derived from tissue culture (Thawaro, 2009). Amplification of genomic DNA was carried out in a 10 µl mixture containing AmpliTaq Gold 360, 0.5 mM of each primer and 35 ng of template DNA. PCR amplifications were performed on a thermocycler (TC-XP-G, Japan) using the following program: denaturation at 95°C for 10 min, 35 cycles of 95°C for 30 s, 52°C for 45 s, 72°C for 60 s, and a final elongation step at 72°C for 7 min. The products were separated with MultiNA.

Data recording and statistical analysis

The percentage of SE formation, fresh weight (mg) and number of SE were recorded after 2 months of culture. Completely randomized design (CRD) with 8 replicates was designed. Data of mean were analyzed using a one-way analysis of variance (ANOVA). Significant differences among treatments were detected using Duncan's multiple range tests (DMRT) at the 0.01 or 0.05 level of probability.

Result

Effect of kinds and duration of dehydration on SE formation

The EC of oil palm dehydrated by air-drying for 1-5 h or silica gel for 6-36 h before culturing on MS medium supplemented with 0.1 mg/L dicamba for 1 month revealed that dehydration by silica gel for 24 h promoted the highest fresh weight at 550.89 mg while dehydration by air-drying for 4 h gave the highest number of somatic embryos at 8.33 embryos/explants significantly different with other treatment (Table 1).

Table 1. Effect of duration time and kinds of dehydration on fresh weight and number of SE development on MS medium supplemented with 0.1 mg/L dicamba for 4 weeks.

Dehydration	Duration (h)	Water content (%)	FW(mg)	No. of SE (embryos/clump)
Air drying	1	86.25	443.33^{bcd}	3.11^{bc}
	2	83.38	438.89^{bcde}	2.44^{bc}
	3	70.03	392.56^{de}	4.22^{bc}
	4	61.58	361.56^{e}	8.33^{a}
	5	41.46	364.11^{e}	5.31^{b}
Desiccated	6	90.44	$456.33b^{cde}$	2.00^{c}
drying	12	84.35	413.94^{bcde}	2.22^{c}
	18	79.19	508.00^{ab}	2.39^{bc}
	24	74.04	550.89^{a}	1.72^{c}
	30	64.93	488.39^{abc}	3.56^{bc}
	36	55.83	431.33^{bcde}	3.56^{bc}
F-test			**	**
C.V. (%)			9.24	37.31

** Significant difference ($p \leq 0.01$)

Mean sharing letter in common within column and row are not significant difference ($p \leq 0.01$) by DMRT.

Effect of kinds and duration of dehydration on cryopreservation

The EC pre-cultured with 0.25 M sucrose for 7 d and then dehydrated by desiccators for 24 h (water content of EC was adjusted to 67.3%), followed by storing in liquid nitrogen for 1 h gave the highest percentage of SE formation at 44.44 (Fig.1). However, maximum average number of somatic embryos (1.67 embryos/explants) was observed in EC that were desiccated for 36 h (20.2% water content) (Table 2). After culturing for 2 months, SEs at haustorium stage can be observed (Fig. 2).

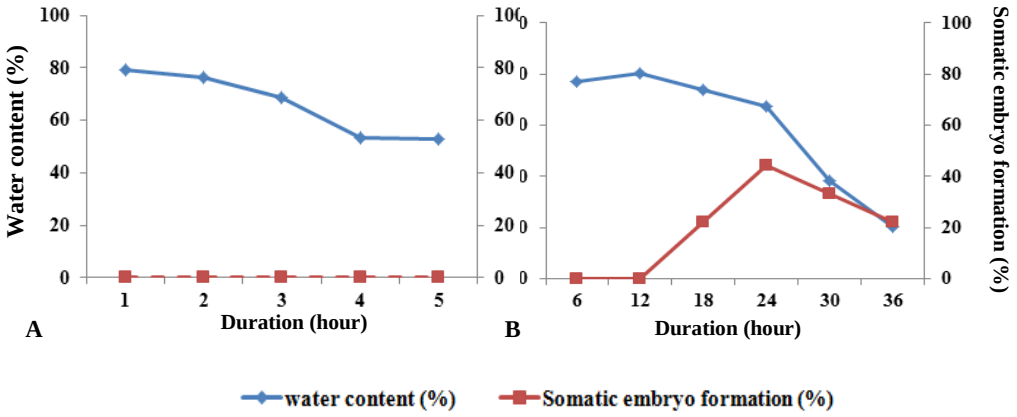


Fig. 1. Effect of duration time of dehydration by laminar flow (A) and silica gel (B) after preconditioning on MS medium supplemented with 0.25 M sucrose for 7 days on moisture content (%) and SE formation (%) after storing in liquid nitrogen.

Table 2. Effect of duration time and kinds of dehydration on fresh weight and number of SE after cryopreservation at -196ºC. The EC was cultured on MS medium supplemented with 0.1 mg/L dicamba for 2 months.

Dehydration	Duration (h)	FW(mg)	No. of SE (embryos/clump)
Air drying	1	72.67^{bcd}	0^{b}
	2	69.67^{cd}	0^{b}
	3	71.78^{bcd}	0^{b}
	4	69.00^{cd}	0^{b}
	5	68.11^{d}	0^{b}
Desiccated drying	6	71.22^{bcd}	0^{b}
	12	72.22^{bcd}	0^{b}
	18	81.00^{abc}	0.33^{b}
	24	82.11^{ab}	0.67^{b}
	30	78.78^{abcd}	0.33^{b}
	36	84.56^{a}	1.67^{a}
F-test		**	*
C.V. (%)		7.15	191.48

* Significant difference ($p \leq 0.05$), ** Significant difference ($p \leq 0.01$)
Mean sharing letter in common within column and row are not significantdifferent by DMRT.

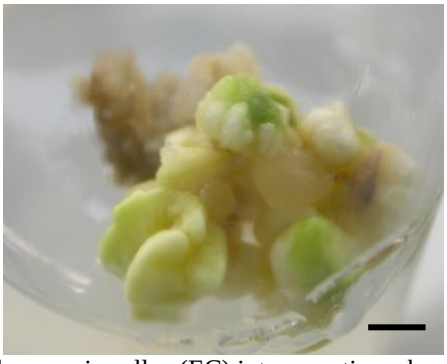
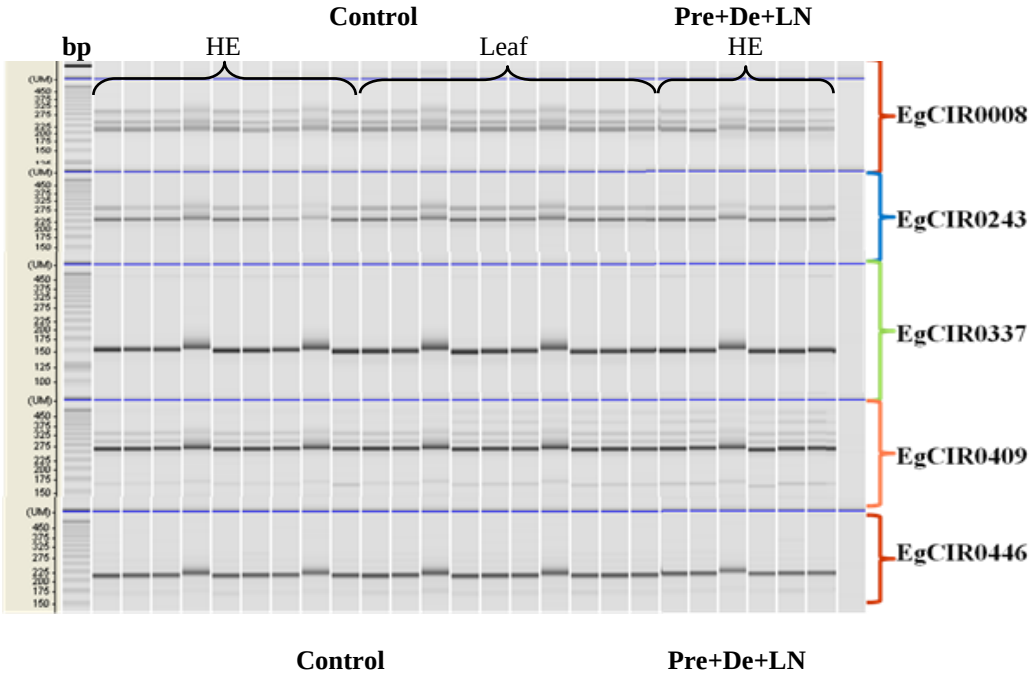


Fig. 2. Development of embryogenic callus (EC) into somatic embryos (SE) after cryopreservation in LN for 1 h. The EC was cultured on MS medium supplemented with 0.1 mg/L dicamba for 2 months. (Bar= 2 mm)

Assessment of genetic instability by SSR analysis

SEs at haustorium stage from treatments were detected their uniformity using SSR marker with 9 primers. The results showed that SSR did not have any polymorphism among cryopreserved EC and between cryopreserved EC and control. All SEs provided no variation of DNA profiles (Fig. 3) suggesting that there is no genetic instability obtained from cryopreservation of EC of oil palm by our protocols.



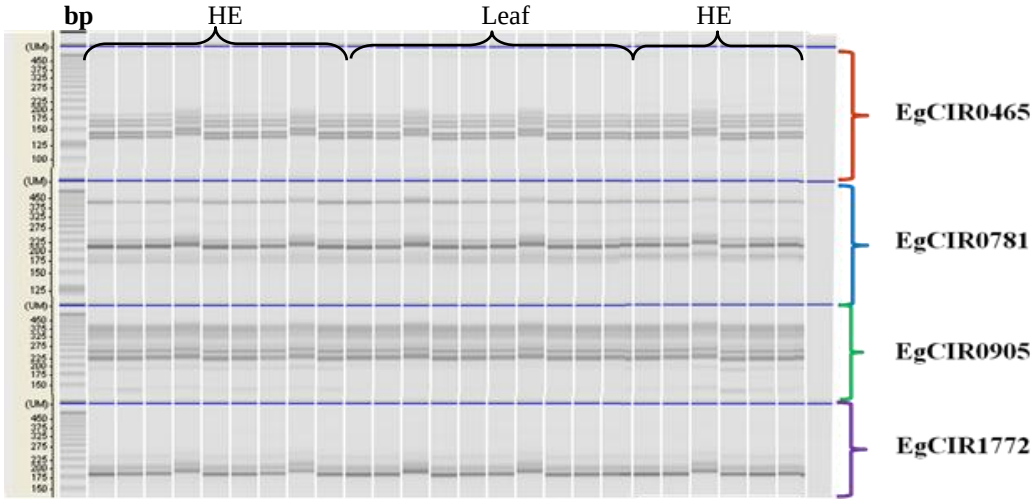


Fig. 3. DNA patterns in control and cryopreserved EC revealed by some SSR markers; EgCIR0008, EgCIR0243, EgCIR0337, EgCIR0409, EgCIR0446, EgCIR0465, EgCIR0781, EgCIR0905 and EgCIR1772.
HE: haustorium embryo, Pre: preculture, De: dehydration, bp: base pair
+LN: plunged in liquid nitrogen

Discussion

The present study found that combination of pre-culturing EC on enrich sucrose medium together with dehydration by desiccators improved survival rate of explants after storing in liquid nitrogen. Dehydration with desiccators containing silica gel may gradually adjust water content of EC leading to the peripheral cells and inner cells of the EC had nearly the same water content or critical content that couldn't form crystal whereas dehydration by laminar air flow caused a severe decrease in water content only at peripheral cells. Accordingly, dehydration EC by the second method (desiccators) gave the better results in terms of both survival rate of EC and SE formation after storage than dehydration under the laminar air flow. Similar results was also reported by Panis *et al*. (1996) who found that a reduction of the moisture content of banana meristem cultures by exposure meristematic clumps to sterile air flow did not result in an increment of survival rate of cryopreseved cultures.

As EC were subjected to several chemicals and physical environments during the processes of cryopreservation (preculturing, dehydration, cryopreservation, thawing), so, it is very risk to cause a change the genetic information of cell in EC. To ensure that cryopreserved plant materials are uniform and can be used for propagation purpose it is necessary to prove genetic instability by some molecular markers. Thus, in this study, SSR was performed to evaluate the genetic instability of the cryopreserved EC. This

marker showed no different in DNA profiles among cryopreserved EC-derived SEs and between those SEs and control EC.

Similar results were reported in embryogenic cultures of *Citrus sinensis* (Marin *et al*. 1993) *Pinus sylvestris* (Haggman *et al.*, 1998) *o*r *Picea glauca* (Park *et al.*, 1994) after the explants were recovered from storage in LN although de Verno *et al*. (1999) found genetic alterations in embryogenic cultures of *Picea glauca* after cryostorage and re-establishment in culture for 2 and 12 months. Rapid plantlet regeneration soon after callus recovery from cryopreservation might not show the evidence of somaclonal variation. In short, genetic instability was reasonably occur by long term or repeated subculturing rather than cryopreservation procedure.

Conclusion

EC was successfully cryopreserved by dehydration. After preconditioning the EC on MS medium supplemented with 0.25 M sucrose for 7 d, desiccators at 67.3% RH for 24 h, followed by storing in liquid nitrogen for 1 h gave the highest percentage of somatic embryo formation at 44.44. Increase in dehydration period to 36 h improved average number of SEs at 1.67 embryos/explants. This technique of cryopreservation did not affect genetic stability of oil palm EC as assess by SSR marker.

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