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## Simple vitrification protocol for cryopreservation of oil palm using embryogenic culture

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Friable embryogenic tissue (FET) of oil palm was successfully cryopreserved by vitrification. After preconditioning the tissue on Murashige and Skoog (MS) medium supplemented with 0.25 M sucrose for 7 days, FET was dehydrated for 60 min at 0°C with a highly concentrated vitrification solution (PVS2) and plunged directly into liquid nitrogen gave the highest percentage of somatic embryo (SE) formation at 66.77, fresh weight at 177.5 mg and number of SE at 2.33 SE/clumps. SE at haustorium stage from different treatment were detected their uniformity using SSR technique with primer EgCIR008. The results showed that DNA profiles were unique and no polymorphism between cryopreserved explants and controls, suggesting that cryopreservation using vitrification does not affect genetic stability of oil palm.

**Key words:** cryopreservation, vitrification, oil palm (*Elaeis quineensis* Jacq.), SSR

### Introduction

Oil palm (*Elaeis quineensis* Jacq.) is a perennial oleaginous monocotyledonous plant predominantly cultivated in humid tropical regions of Africa, Latin America, and Southeast Asia. It 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), and is used as an ingredient in bio-diesel and as a fuel to be burnt in power stations to produce electricity. This plant can be propagated by seed and tissue culture, since it only has a single growing point, and does not produce suckers like some other palm species. Several studies for *in vitro* regeneration through somatic embryogenesis on callus have been developed (Rival and Parveez, 2004; Te-chato and Hilae, 2007; Chehmalee and Te-chato, 2008). For embryogenic callus cultures require periodic subculturing to maintain both a high proliferation potential and the capacity of cells to develop

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into somatic embryo primordial. Repeated subculturing is not only labor intensive and time consuming, but also increases the risk of losing the embryogenic cultures 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).

Cryopreservation also provides a means of cutting costs incurred by the regular subculturing of embryogenic lines. It may also limit the amount of contamination and somaclonal variation resulting from routine subculturing of embryogenic tissue (Schrijnemakers and van Iren, 1995). This method 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). Protocols have been developed for the induction of ET and somatic embryos for use in the micropropagation, via somatic embryogenesis, of a number of selected species of *Picea* (Norgaard *et al.*, 1993; Touchell *et al.*, 2002) and *Quercus* (Martinez *et al.*, 2003; Valladares *et al.*, 2004; Fernandes *et al.*, 2008). For cryopreserved embryoid of oil palm, Dumet *et al.* (1993) showed that, by introducing a desiccation stage, drying the embryoids to between 19 and 35% moisture content, a proportion of any type of embryoids would survive, although success rates were still variable. Pregrowth on sucrose was shown to reduce the water content of embryoids, but for good survival rate it was necessary to dry the embryoids further, to a point where ice crystals no longer formed in the cells during freezing. This paper reports on an effective method for the cryopreservation (-196°C) of friable embryogenic tissue (FET) of *E. guineensis* following preconditioning the tissues on agar medium supplemented with different concentrations of sucrose by simple vitrification process followed by evaluation of somaclonal variation after cryopreservation by SSR marker.

## **Materials and methods**

### ***Plant material***

FET initiated from young leaves of elite “Tenera” tree were cultured on callus induction medium by the methods described by Te-chato *et al.* (2002). Cultures were maintained on MS medium supplemented with 3% sucrose, 200 mg/l ascorbic acid and 0.3 mg/l dicamba, and adjusted pH to 5.7 with 0.1 N HCl before adding agar and autoclaving at 1.05 kg/cm<sup>2</sup>, 121°C for 15 min. The cultures were placed at 28±0.5°C under 14 h photoperiod at 1,300 lux illumination and subcultured monthly intervals.

### ***Pre-conditioning***

FE (16-1400 m) were precultured on solid MS medium supplemented with different concentrations of sucrose (0.0874, 0.10, 0.25, 0.5, 0.75 and 1 M) under the same conditions as described in proliferation medium (MS+0.3mg/l dicamba) for 1,3 ,5 , 7 and 9 days. After the pre-conditioning FET was divided into two parts, one part of FET was directly plunged into liquid nitrogen for at least 1 hour. After rapid warming in a water bath at 40°C for 2 min, FET was transferred to solid MS medium supplemented with 0.1 mg/l dicamba, 3% (w/v) sucrose and 0.75% (w/v) agar. The somatic embryo formation was recorded after 2 month of culture. Factorial in completely randomized design (CRD) with 8 replicates was designed.

### ***Vitrification procedure and rapid cooling***

After pre-conditioning, FET was sufficiently dehydrated by modified plant vitrification solution 2 (PVS2) at 0 and 25°C for various durations (15-90 min). The solution consisted of 30% (w/v) glycerol, 15% (w/v) ethylene glycol and 15 % (w/v) dimethylsulfoxide without sucrose in liquid MS medium. The cultures were transferred into cryo-tube contained vitrification solution and plunged into liquid nitrogen (-196°C) for at least 1 hour.

### ***Thawing and unloading***

For recovery, the cryo-tube containing FET was rapidly thawed in a warm water bath at 40°C for 2 min. The vitrification solution was drained and replaced with 1.2 M sucrose solution for 20 min at room temperature. Then, FET 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±0.5°C under a 14 h photoperiod at 1,300 lux illumination.

### ***SSR analysis***

FET was cultured on MS medium supplemented with 0.1 mg/l dicamba for 4 weeks. Somatic embryos at haustorium stage were cut into half. The first halves (15–20 mg) were collected and used to isolate DNA by the protocol described by Te-chato (2000). SSR analysis of genomic DNA was carried out using 1 microsatellite loci amplified in oil palm using 1 primer (EgCIR008) which was proved to be generally used for identify somaclonal variation in plantlet derived from tissue culture (Thawaro, 2009). Amplification of genomic

DNA 25 was carried out in a 10 µl mixture containing 2.5 mM MgCl<sub>2</sub>, 10× *Taq* buffer, 100 µM of each dNTP, 0.3 mM of each primer, 1.5 units of *Taq* polymerase and 20 ng of template DNA. PCR amplifications were performed on a thermocycler (TC-XP-G, Japan) using the following program: denaturation at 95°C for 1 min, 35 cycles of 94°C for 30 s, 52°C for 60 s, 72°C for 120 s, and a final elongation step at 72°C for 8 min. An equal volume of loading buffer (98% formamide, 0.025% bromophenol blue, 0.05% xylene cyanol) was added to the amplified products, following denaturation at 94°C for 5 min. The products were separated in 6% (w/v) denaturing polyacrylamide gels and visualized with silver staining which was conducted according to the protocol provided by Bassam *et al.* (1991).

### ***Statistical analysis***

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.

## **Results**

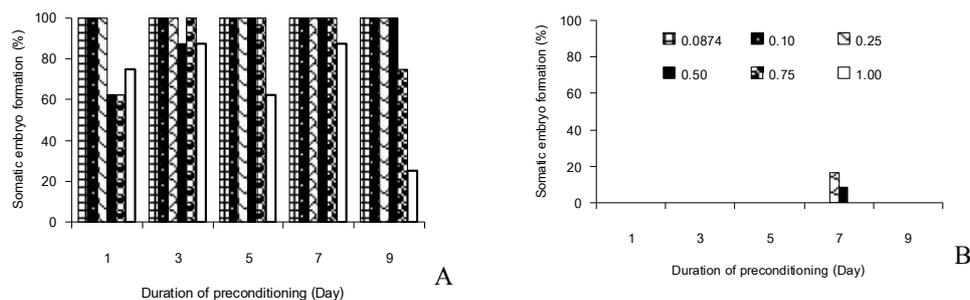
### ***Effect of pre-condition of on somatic embryo formation***

Preliminary experiment was conducted where FET was precultured on MS medium supplemented with different concentration of sucrose for various times and freezing. After 2 months of culture, the result indicated that SE formation was gradually increased by increasing duration of preconditioning and concentration of sucrose, and decreased when using sucrose concentrations at 0.75 M for 9 days with further increased in this parameter (Fig. 1A). However, FET precultured with 0.25 M sucrose for 7 days and stored in liquid nitrogen gave the highest SE formation at 16.67% (Fig. 1B).

### ***Effect of dehydration with vitrification solution on somatic embryo formation***

To determine the optimal time of exposure and temperature to vitrification, pre- conditioning FET on 0.25 M sucrose, loaded FET was dehydrated with PVS2 for various lengths of time and temperature prior to a plunge into LN. Without a plunge into LN found that fresh weight, number of SE and SE formation were decreased when increasing of exposure time of PVS2 (Table 1, 2 and Fig. 2A). The best result of fresh weight (177.5 mg) and number of SE (2.33 SE/clumps) significantly difference with another treatments was obtained from FET treated with PVS2 for 60 min at 0°C (Table 1 and 2).

Moreover, this treatment can produced SE alone and gave the highest percentage of SE formation at 66.67 (Fig. 2B), and SE can be observed when FET was cultured on recovery medium for 45 days (Fig. 3).



**Fig.1.** Effect pre-conditioning period and various concentrations of sucrose containing medium on somatic embryo formation.) A) Without LN (B) with LN.

**Table 1.** Effect of exposure time to vitrification solution at 0 and 25°C on fresh weight of cryopreserved FET at -196°C. The FET was cultured on MS medium supplemented with 0.1 mg/l dicamba for 2 month.

Exposure time (min)	Fresh weight (mg)			
	-LN		+LN	
	0°C	25°C	0°C	25°C
15	475b	706.3a	69.5e	72.33e
30	247.8cd	460.7b	72.83e	74.33e
60	223.2cd	324.3c	177.5de	71.17e
90	87.83	175de	75.67e	78.17e

C.V. (%) : 65.58. Mean sharing letter in common within column and row are not significantly difference ( $P \leq 0.05$ ) by DMRT.

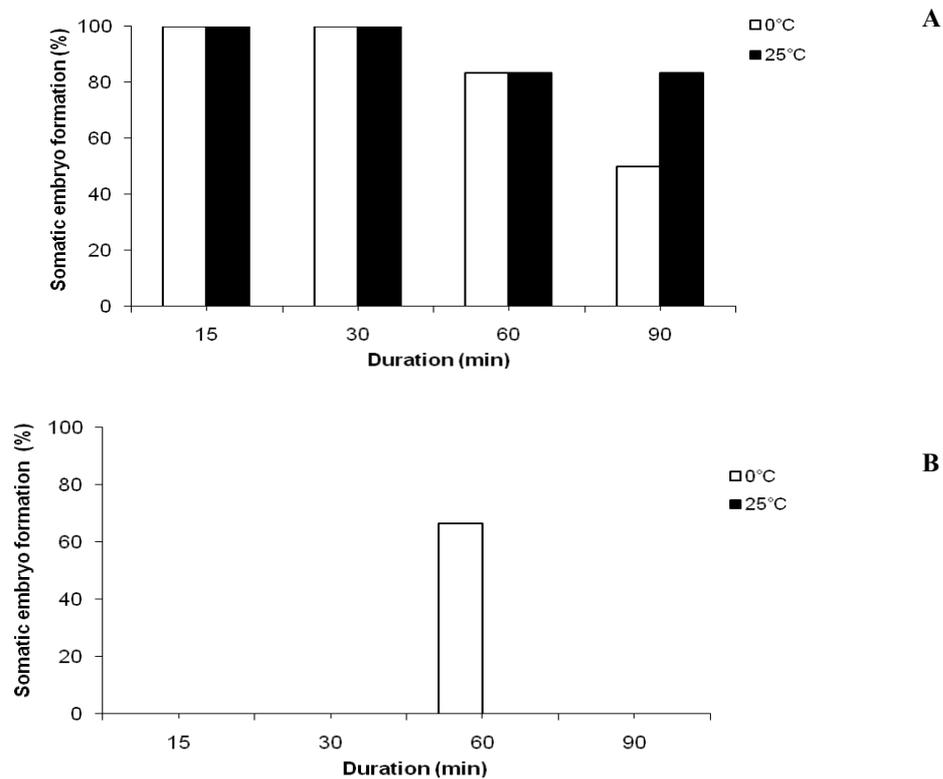
**Assessment of somaclonal variation by SSR analysis**

Somatic embryos at haustorium stage from different treatment were detected their uniformity using SSR technique with primer EgCIR008. The results showed that SSR did not reveal any polymorphism between cryopreserved explants and control. All somatic embryos provided no variation of DNA profiles (Fig. 4).

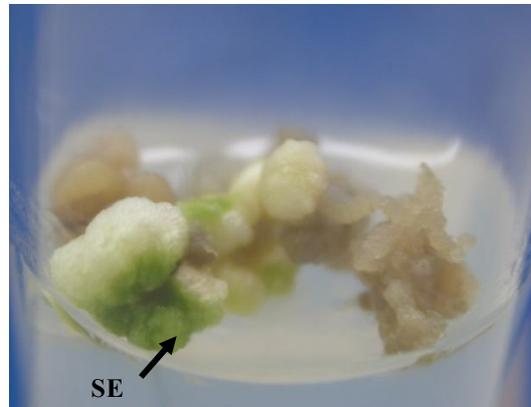
**Table 2.** Effect of exposure time to vitrification solution at 0 and 25°C on average number of somatic embryos. The FET was cultured on MS medium supplemented with 0.1 mg/l dicamba for 2 month.

Exposure times (min)	Number of somatic embryos			
	-LN		+LN	
	0°C	25°C	0°C	25°C
15	12.83a	12a	0e	0e
30	6.5bc	7.33b	0e	0e
60	3.67cd	3de	2.33de	0e
90	0.83de	1de	0e	0e

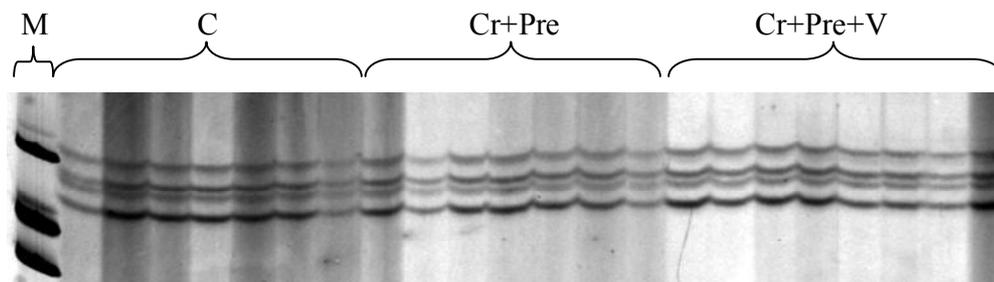
C.V. (%) = 80.69, Mean sharing letter in common within column and row are not significant difference ( $P \leq 0.05$ ) by DMRT.



**Fig. 2.** Effect of exposure time to vitrification solution at 0 and 25°C on somatic embryo formation of cryopreserved FET. The FET was cultured on MS medium supplemented with 0.1 mg/l dicamba for 2 month.) A) Without LN (B) with LN.



**Fig. 3.** Development of SE from cryopreserved FET on recovery medium after 45 days of culture. FET was pre-cultured with 0.25 M sucrose for 7 days, dehydrated with PVS2 for 60 min at 0°C and stored in liquid nitrogen.



**Fig. 4.** SSR patterns of somatic embryo at haustorium stage obtained with primer EgCIR0008. The amplification products were compared on the basis of molecular size. Abbreviations: C, control; cr+pre, cryopreserved+preconditioning; cr+pre+vi, cryopreserved + preconditioning + vitrification; M, molecular marker.

## Discussion

The effect of high concentration of sucrose on the osmotic potential of the cells during somatic embryogenesis has been shown by some workers (May and Trigiano, 1991; Alkhateeb, 2008; Karami *et al.*, 2006). Therefore, the role of sucrose in the present study could be interpreted as both nutritional and osmotic regulatory functions of this carbohydrate. A sugar treatment of plants with sucrose was shown to be very important in improving the survival of cryopreserved embryogenic tissue from several plants (Huang *et al.*, 1995; Chmielarz, 2005; Gonzalez-Arno *et al.*, 2003). The accumulation of sucrose inside tissues helps maintain cell viability during dehydration and cryopreservation by stabilization of membranes (Oliver *et al.*, 1998; Gonzalez-Arno *et al.*, 1996). An indirect effect of sucrose could be due to the

accumulation of endogenous compounds induced by a mild osmotic stress which induces metabolic changes and enhances chilling and desiccation tolerance. In addition, the absorbed sugar stabilizes the membranes by replacing water and forming hydrogen bonds with the phospholipids (Turner, 2001).

For using vitrification technique, Kohmura *et al.* (1992) mentioned that the keys to success on cryopreservation by vitrification are to carefully control the procedures for dehydration and cryoprotectant penetration and to prevent injury by chemical toxicity or excess osmotic stresses during treatment. So that optimizing the time of exposure, or the temperature during exposure to PVS2, is important for producing a high level of SE formation after vitrification. Cryoprotectants that concentrations are higher increase the intracellular concentration and thus inhibit intracellular ice formations which cause physical damage to tissue (Tsai *et al.*, 2009). Penetrating PVS2 cryoprotectants therefore have a colligative role, moderating the effects of osmotically active, non-penetrating components of the mixture. The solution is effective because protection is imparted before exposure to cryogenic treatments as some components of PVS2 are sufficiently mobile to permeate cells at 0°C and displace water. When the temperature is subsequently reduced, the penetrating components of PVS2 cryoprotect the cells by restricting the molecular mobility of water molecules and prevent them from nucleating ice crystals (Benson, 2008). Thus, successful vitrification requires the use of a highly concentrated yet nontoxic solution of cryoprotectants and the optimum exposure time to it.

In this study, we used SSR to evaluate the genetic stability of the cryopreserved FET. Also this method showed no differences between the cryopreserved FET and controls. Similarly, no sign of somaclonal variation was detected in embryogenic cultures of sweet orange (Marin *et al.*, 1993) and *Picea glauca* (Park, 1994) after storage in LN. However, de Verno *et al.* (1999) found genetic alterations in embryogenic cultures of *Picea glauca*, 2 and 12 months after they were re-established in culture after cryopreservation. Nevertheless, as plantlets regenerated soon after callus recovery from cryostorage so that the evidence of genetic variation was not observed. Thus, the authors concluded that the observed genetic instability was reasonable a consequence of repeated subculturing rather than of the cryopreservation procedure itself.

FET was successfully cryopreserved by vitrification. After preconditioning the tissue on Murashige and Skoog (MS) medium supplemented with 0.25 M sucrose for 7 days, FET was dehydrated for 60 min at 0°C with a highly concentrated vitrification solution followed by plunging directly into liquid nitrogen gave the highest percentage of somatic embryo formation at 66.77, fresh weight at 177.5 mg and number of SE at 2.33

SE/clumps. SE at haustorium stage from different treatment were detected their uniformity using SSR technique with primer EgCIR008.

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