# Comparison between Gelled Culture and Temporary Immersion System for Somatic Embryos Proliferation of Oil Palm (*Elaeis guineensis* Jacq.)

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***Abstract*—Oil palm *in vitro* propagation was developed using both solid (gelled) and liquid media. Many restrictive factors remain in oil palm *in vitro* propagation such as long culture duration, a high degree of genotype dependence, somaclonal variation, low rate of somatic embryogenesis and also followed by low rate of somatic embryos proliferation. Therefore, it is necessary to find alternative ways to increase somatic embryo proliferation to obtain more shoots without compromising the quality of *in vitro* plantlets. In this study, somatic embryos proliferation and germination were evaluated through Temporary Immersion System (TIS) in comparison with gelled culture. Globular primary somatic embryos were selected and cultured in TIS and gelled culture. After six months of culture, somatic embryos in TIS were multiplied and germinated on solid medium. Parameters observed were fresh weight and number of somatic embryos and shoots production amount. The results shown that TIS produced more clumps (186 clumps) and fresh weight (12.79g) of somatic embryos compared to gelled culture (81 clumps; 3.60 g). TIS also produced more shoots and therefore resulting in more plantlets. The quality of plantlets derived from TIS was not different from gelled culture.**

***Index Terms*—oil palm, temporary immersion system, somatic embryogenesis, proliferation**

## I. Introduction

Oil palm *(Elaeis guineensis* Jacq.) is a monocotyledonous species that has a single dominant vegetative apex and does not produce axillary shoot. Therefore, reliable vegetative propagation of oil palm can only be achieved through somatic embryos formation. One of the limiting factors in commercial oil palm vegetative propagation is inefficiency protocol of *in vitro* propagation, including low rate of somatic embryos formation. Somatic embryo formation rate is very difficult to be predicted because of random induction occurrence [1] and genotype dependent [2]. Somatic embryos formation rate varied from 3-6% and even 50% of them failed to develop [3], [4].

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Various *in vitro* propagation methods of oil palm are evaluated and improved continuously to bring out its maximum potential. Two protocols, semi-solid medium (gelled culture) and suspension culture, have been widely applied in *in vitro* propagation of oil palm. Suspension culture is continuous immersion of explants in liquid medium which leads to increase nutrient uptake and assimilation [5]. Suspension culture is more ideal for commercial production because it provides more uniform culture and reducing production cost of gelled culture. However, this last protocol is still hampered by low plantlet production rates, vitrification or hyperhydricity, and some cultures lost their embryogenic potential.

Recently, many strategies have been investigated and developed to overcome the constraints of suspension culture. One of the most effective ways is Temporary Immersion System (TIS). TIS is highly suitable for semi-automated *in vitro* propagation. The explants are not constantly immersed in liquid media, which often negatively affects plant growth and morphogenesis. TIS allows for control of contamination, adequate nutrient and oxygen supply and mixing, relatively infrequent subculturing, ease of medium changes and limited shear damage [6].

In term of the efficiency and the benefits of the use of TIS, this study aims to evaluate proliferation rates of somatic embryos through TIS for mass production of oil palm plantlets and compared to gelled culture.

## II. Materials and Methods

### A. Plant Materials and Media Preparation

The oil palm type of DxP or tenera was used for all experiments. Immature leaves were surface sterilized in 5% of sterilant (brand Bayclin®) solution two times for 10 minutes followed by three times rinses in sterile distilled water. The culture media was MS medium [7] consisted of MS mineral salts and vitamins supplemented with 3% sucrose, 100 mg/L casein hydrolysate, plant growth regulators 2.4-D and NAA at 0.5 mg/L and 10 mg/L, and with the addition of an gelling agent 0.6% agar. For TIS culture was without adding of gelling agent. The pH of culture media was adjusted about 5.8 and



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autoclaved at temperature 121 ℃, pressure 1.5 psi for 15 minutes.

### B. Induction of Embryogenic Callus

Sterilized leaves were cut about 1 cm and cultured in test tube containing 10 mL media for induction of embryogenic callus. The leaf explants were subcultured every 3 months for one year culture period in dark room at temperature ±25 ℃ and humidity ±50%. Explants that have produced callus were selected for somatic embryos formation.

### C. Primary Somatic Embryos Formation

Selected callus was subcultured every two months to form somatic embryos. Every callus culture was observed monthly to obtain primary somatic embryo. Ten lines of globular primary somatic embryos were selected in this study. Each line of somatic embryo was divided into two parts, one for culturing in TIS and one for gelled media.

### D. Proliferation of Somatic Embryos in TIS and Gelled Culture

Selected somatic embryos were cultured in TIS and gelled culture for further growth and proliferation. TIS in this study used the RITA® system (recipient for automated temporary immersion system) [8]. The RITA system is an apparatus composed of a container with two compartments which are placed on top and bottom side. The top compartment contains the embryos culture and the bottom compartment contains the liquid medium. They are linked together so that when the air pressure is applied to the bottom compartment, the liquid medium is pushed into the top compartment and reaches the embryos culture. When the air pressure drops, the liquid medium returns to the bottom compartment. Thus, the embryos culture in the top compartment are temporarily immersed when it is flooded with liquid medium. The frequency of immersion was every six hours for three minutes. Subculture was conducted every two months for 6 months culture period. Cultures were incubated in a room with 16/8 hours photo-periods (light/dark) at temperature 28 ℃ ± 2 ℃ and humidity 50% ± 10%. Data were recorded on initial fresh weight at every subculture to calculate the average fresh weight addition.

### E. Multiplication and Germination of Somatic Embryos on Solid Media

After six months in TIS, somatic embryos were multiplied and germinated on solid MS medium consisted of MS mineral salts and vitamins supplemented with 3% sucrose, 100 mg/L casein hydrolysate, plant growth regulators NAA at 7.5 mg/L and solidified by 0.6% agar. Cultures were incubated in a room with 16/8 hours photo-periods (light/dark) at temperature 28 ℃ ± 2 ℃ and humidity 50% ± 10%. Subculture were conducted every two months for 12 months culture period. The parameter recorded every subculture were number of embryos (multiplication rate) and number of shoots production.

### F. Statistical Analysis

SPSS software (version 16.0) was used for whole data analysis. The results were analyzed using one-way ANOVA followed by Tukey's test at the 5% level. When ANOVA and Tukey's test could not be applied, the mean of ten replicates, as well as standard error, was applied to analyze the data.

## III. Results and Discussion

### A. Proliferation of Somatic Embryos in TIS and Gelled Culture

Explants from immature leaf tissue successfully formed embryogenic callus and then differentiated into somatic embryos. Ten embryonic lines were selected at the globular stage. Each embryonic line was divided into two equal parts, both in number and by weight, and then cultured in TIS and gelled culture.

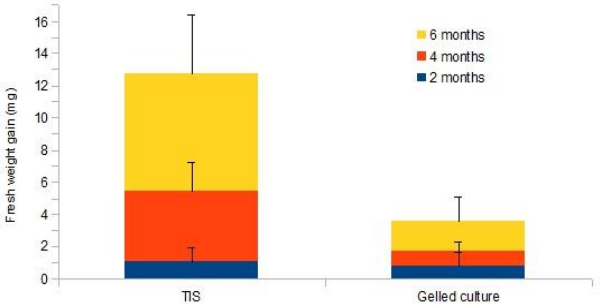


Figure 1. Comparison of somatic embryos fresh weight gain in TIS and gelled culture. Vertical bars indicate the standard deviation from ten embryonic lines. Different letters indicate significant differences at P<0.05 between treatments after six months of culture.

After a month of culture period all embryonic lines proliferated well. This could be seen by an increased the embryo clumps number. After two months of culture period, subculture and fresh weight measurement of embryos were performed. Fresh weight of ten embryonic lines in TIS and gelled culture were also increased. On average, the embryoids fresh weight in TIS was slightly higher than on gelled culture. After 4 months, somatic embryo proliferation was highly increased in TIS than on gelled culture (Fig. 1). Embryo fresh weight from TIS reached 12.79g, which was significantly different compared to embryo fresh weight from gelled culture (only reached 3.60g) (Table I). Similar results were reported by Heringer *et al.* (2014) [9] who reported that the greatest number of plants was obtained using both RITA® and TIS bioreactor models in the conversion of somatic embryos of *Bactris gasipaes* compared to those in traditional cultivation systems. Furthermore, Georgieva *et al.* (2016) [10] also reported that strawberry and raspberry plants multiplication ratio for 4 weeks was higher in TIS than on gelled culture.

Somatic embryos fresh weight increment was correlated with embryo clumps number increment. Number of somatic embryos in TIS had increased about 7.2-fold (186 clumps) and on gelled culture were about 3.2-fold (81 clumps) (Table I). Some of somatic embryos in TIS cultures had larger size than others. This was probably the result of increased nutrient uptake and water as a consequence of better availability of water and nutrients. Somatic embryos looked enlarge and slightly

translucent or glassy. These symptoms were often the case in liquid culture and indicates the occurrence of hyperhydricity. Hyperhydricity is responsible for poor growth and substantial losses during and after *in vitro* culture [11]. There were some embryos that form chlorophyll (greenish), but there was no formation of plumule. When somatic embryos were cultured continuously in TIS, it resulted severe hyperhydricity and embryos failed to develop further. There were some embryos still able to germinated but most of them were browned, even blackened. Rojas-Martínez *et al*. (2010) [12] explained that hyperhydricity as the result of the stressful conditions brought about by waterlogging of the apoplast. This causes hypoxia and thereby leads to severe oxidative stress. In this study, to avoid severe occurrence of the hyperhidricity, after six months in TIS culture, the embryos were transferred to solid media.

TABLE I. SOMATIC EMBRYOS PROLIFERATION AFTER SIX MONTHS OF CULTURE PERIOD

| System of culture | Fresh weight (g) | No. Embrio (clumps) |
|---|---|---|
| TIS | 12.79 ±6.23 | 186 ±0.7 |
| Gelled culture | 3.60 ±2.88 | 81 ±0.3 |

± Standard deviation

### B. *Multiplication and Germination of Somatic Embryos from TIS on Solid Media*

In solid media, somatic embryos from TIS and gelled culture showed the same embryonic developmental pattern. It developed further, from globular phase and differentiated into scutellar, coleoptilar and germinating phases, until they finally produced shoots. However, the developmental stage of somatic embryos were varied in time, even in the same vessel. Several embryos were already germinated and produced leaf shoots while others embryos were just formed. Therefore, it is very important to do culture selection with the same phases of developmental stages when doing subculture to obtain a more uniform culture.

After a month in solid media, the translucent symptoms of somatic embryos from TIS started to disappear. Somatic embryos were capable to develop further to produce shoots. However, there were also some translucent somatic embryos that did not develop further. Surprisingly, on the outer surface occurred the formation of secondary somatic embryos (Fig. 2). It seems that those embryos recovered from stress during in TIS culture. TIS could create different forms of stress, such as temporary hypoxia, which is sensed by the cells as a sort of stress triggering short-term metabolic adaptations [13]. Stress is an important factor related to the acquisition of embryogenic competence [14]. In present study, secondary somatic embryos proliferated well in solid medium and developed further and produced shoots.

Proliferation of somatic embryo from TIS and gelled culture after transferred to solid medium showed a similar pattern. The embryo multiplication rates were slightly increased at the beginning of culture, and then subsequently decreased after several months of the culture period (Fig. 3). The decrease in multiplication capability was due to the presence of numerous embryos that had entered the shoot formation stage. Multiplication rates of somatic embryos from TIS culture started to decrease after 8 months culture period, whereas from gelled culture was after 6 months culture period (Fig. 3). This result was in line with the formation of shoots, in which somatic embryos from gelled cultures germinated and formed shoots faster than somatic embryos from TIS culture.

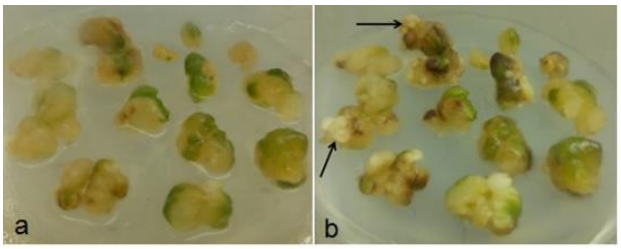


Figure 2. Somatic embryos (a) right after transferred on solid media and (b) one month after transferred on solid media showing the formation of secondary somatic embryos (*black arrows*)

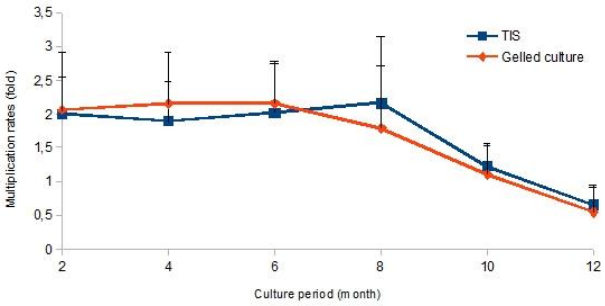


Figure 3. Multiplication rates of somatic embryos after transferred on solid media. *Vertical bars* indicate the standard deviation from ten embryonic lines.

### C. *Shoot Production and Performance*

After 12 months of culture period on solid medium, the amount of shoot produced by somatic embryos derived from TIS was 3.752 with 23.140 clumps still available for germination. Meanwhile the number of shoots produced by somatic embryos derived from gelled culture was 2.570 with only 7.890 clumps available for germination (Table II). These results indicated that the proliferation and multiplication of somatic embryos increased when using TIS which could ultimately increased the number of shoots and plantlets obtained. Similar results were also obtained from TIS *in vitro* propagation of pineapple [15], carnation [16], and eucalyptus [17]. The high number of somatic embryo clumps from TIS in this study originated from secondary embryos proliferation. Many authors reported that secondary somatic embryos were efficient to produce a larger number of somatic embryos than from primary somatic embryos [18]-[20]. In addition, transferring somatic embryos from TIS to solid culture medium showed high embryogenic capacity.

TABLE II. NUMBER OF SHOOTS PRODUCTION

| System of culture | No. Embryo (clumps) | No. shoots |
|---|---|---|
| TIS | 23140 | 3752 |
| Gelled culture | 7890 | 2570 |

From the acquisition of the number of shoots that have been produced, there were off-type shoots formation like grassy, curly, crinkle and others (Fig. 4). The percentage of off-type shoots from TIS cultures was approximately 0.18% and not significantly different with gelled cultures which were only about 0.14%. The highest percentage of off-type shoots came from grassy type (Table III). Normal shoots obtained from TIS and gelled cultures were maintained until root formation. The shoots from TIS and gelled culture showed similar growth and development stages. Roots formation observed after two weeks of culture period. Primary roots formed at the base of the shoot with the number of two to five roots per shoot. After four weeks of the culture period, formation of the fibrous roots begun along with the increase of primary roots length. Root hardening was about two to four weeks of culture before transferred to ex vitro acclimatization.


Figure 4. Off-type shoots, (a) grassy, (b) curly, (c) crinkle, (d) roll up, (e) white shoot.

TABLE III. PERCENTAGE OF OFF-TYPE SHOOTS

| System of culture | Grassy (%) | Curly (%) | Crinkle (%) | Roll up (%) | White shoot (%) | Total (%) |
|---|---|---|---|---|---|---|
| TIS | 0.11 | 0.03 | 0.02 | 0.01 | 0.01 | 0.18 |
| Gelled culture | 0.07 | 0.03 | 0.01 | 0.03 | 0.00 | 0.14 |

## IV. CONCLUSION

The results of this study provide methods to increase plantlets production of oil palm clones by somatic embryos proliferation in TIS culture. These methods are useful for mass *in vitro* propagation of oil palm to overcome low percentage of embryogenesis. Nevertheless, the development of methods to increase the percentage of embryogenesis is still needed. In addition, obtaining somatic embryos in a shorter period of time will also be beneficial to the improvement protocol of *in vitro* propagation of oil palm.

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