Initiation and Proliferation of Globular Somatic Embryos of Oil Palm (Elaeis guineensis, Jacq.) observed by inverted and transmission electron microscopy

Mariani, T.S1, Sjafrullatif2, Wardjo, N.G.1, Miyake, H3
School of Life Science and Technology, Institut Teknologi Bandung, Jalan Ganesha 10, Bandung 40132, Indonesia1, Indonesian Oil Palm Research Institute, Medan, Indonesia2, Laboratory of Plant Resources and Environment, Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa-ku, Nagoya 464-8601, Japan3

Abstract-- Oil palm (Elaeis guineensis, Jacq.) 635 clone from Marihat Research Station – North Sumatra was used in this research. Embryogenic callus was transferred into suspension initiation medium (SIM) with two variations of 2,4-D concentrations: 100 ppm (SIM 1) and 80 ppm (SIM 2), BAP (1 ppm) was also added into each medium to help initiation process of somatic embryos. After four weeks in SIM, the single embryogenic cells proceeded an asymmetrically division and differentiated into globular embryos with a suspensor. The optimum number of globular embryos cultured in SIM 1 was 100±1, 5 embryos/20 ml. A 2 mm-nylon mesh was used to synchronize globular embryos from SIM 1. Proliferation of synchronized globular embryos occurred in suspension proliferation medium (SPM) supplemented with 50 ppm 2,4-D (SPM 1) and 30 ppm 2,4-D (SPM 2). Optimum proliferation occurred in SPM 1 with number of proliferation was 28 embryos in average. The effects of embryo density to embryo proliferation were observed using three variations of initial embryo densities in SIM 1 medium, i.e: 10, 30, and 60 embryos/20 ml. One-way ANOVA and Lsd test (p<0,05) showed that 10 embryos/20 ml variation was the optimum density with significantly increased of proliferation in SPM 1. Observation by transmission electron microscopy (TEM) on proliferating globular somatic embryo showed the ultrastructure of proteinaceous material in the vacuole. The optimum initiation and proliferation of globular embryos of oil palm with synchronized development as a result of somatic embryogenesis are very potential for the onset of artificial seed production of oil palm.

Index Term-- Oil palm, somatic embryogenesis, globular somatic embryo, TEM.

I. INTRODUCTION

Oil palm (Elaeis guineensis Jacq.) is a plant with highest productivity of oil among another oil-producing plants with total product per year is 5-6 ton/ha. Oil palm is widely used in industries such as food, soap, textile, steel, medicine, cosmetics and as an alternative biodiesel. Its propagation using seeds produces variations in yield between individual palms reflecting genetic heterogeneity of an outbreeding species. Since oil palm belongs to a monocotyledon, conventional vegetative propagations such as grafting, cutting, an budding are not possible. Therefore, oil palm propagation by tissue culture technique has an obvious potential [1].

In the eighties, some in vitro researchers have successfully gained the planlet of oil palm which can produce the best clone in order to scale up the productivity of oil palm per ha [2]. Somatic embryogenesis is one of in vitro method that widely used for micropropagation of oil palm. The advantage of somatic embryogenesis is for mass propagation, plant genetic improvement program and synthetic seed production [3].

In this study, we use liquid medium for initiation and proliferation of oil palm globular somatic embryo. According to Tahardi [4] the use of liquid or cell suspension culture system offers an alternative approach to solving this problem because of the the potential for enhancement of embryo multiplication an synchronizal development of embryo development. Thus, in a liquid culture system there is the potential for substantially reducing the time frame, labour input and consequently the cost for plantlet production. However, Tahardi [4] reported that germination of the somatic embryo derived from cell suspension was not uniform. Therefore, in this study we reported the effect of 2,4-D on the initiation and proliferation of oil palm globular somatic embryos and effect of embryo density on the proliferation of globular somatic embryo by previously synchronize them. The synchronization of the somatic embryos was to produce uniform somatic embryos. Moreover, we performed transmission electron microscopy (TEM) study on proliferating globular somatic embryo. The result of this study is expected to fulfill the long term objective, i.e., for production of synthetic seed. To our knowledge, TEM observation on oil palm somatic embryo has not been reported.

II. MATERIAL AND METHODS

Material

The material of this study is embryogenic callus obtained from Marihat Research Station, Pematang Siantar, North Sumatra.

METHODS

Medium

Medium used were, suspension initiation medium (SIM 1 and SIM2) and suspension proliferation medium (SPM1 and SPM2). Basal medium was Touchet medium [5]. The composition of the medium was shown in Table I.
0.5 g friable embryogenic calli were inoculated into 20 ml suspension initiation medium (SIM 1 and SIM 2). The cultures were shaken at 80 rpm, maintained in 25°C and 12 hr photoperiod for 4 weeks.

Synchronization of globular somatic embryo
Suspension cultures were sieved using 2 mm nylon mesh. The result of observation and sieving resulted in 3 kinds of embryo density, namely 10, 30, 60.

Proliferation of globular somatic embryo
After three kinds of embryo density in SIM were obtained, the 2 mm globular somatic embryos at each density were subcultured into SPM1 for 4 weeks and subsequently in SPM2 for 4 weeks. The cultures were shaken at 80 rpm, maintained in 25°C and 12 hr photoperiod. Proliferation of the globular somatic embryo was counted.

Transmission electron microscopy (TEM)
Globular somatic embryos were prefixed in 0.1 M cacodylate buffer pH 7.2 containing 5% glutaraldehyde for 24 h, rinsed 3 times in the same buffer, postfixed in 2% osmium tetroxide in cacodylate buffer for 12 h, rinsed in the same buffer once and distilled water twice, and gradually dehydrated in an alcohol series, all at 4 degree C. The samples were then infiltrated and embedded with Spurr’s resin at room temperature. The embedded samples were polymerized in an oven at 70 degree C for 24 h. Ultrathin sections were made with an ultramicrotome at a thickness of 70-90 nm. These sections were stained using aqueous 2% uranyl acetate for 30 min, and with lead citrate for 10 min. Then, they were observed by TEM.

Table I. Composition medium of SIM and SPM

<table>
<thead>
<tr>
<th>Medium</th>
<th>Plant growth regulator</th>
<th>Activated charcoal</th>
<th>Gelling agent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2,4-D (ppm)</td>
<td>BAP (ppm)</td>
<td></td>
</tr>
<tr>
<td>SIM 1</td>
<td>100</td>
<td>1</td>
<td>0,1%</td>
</tr>
<tr>
<td>SIM 2</td>
<td>80</td>
<td>1</td>
<td>0,1%</td>
</tr>
<tr>
<td>SPM 1</td>
<td>50</td>
<td>1</td>
<td>0,1%</td>
</tr>
<tr>
<td>SPM 2</td>
<td>30</td>
<td>1</td>
<td>0,1%</td>
</tr>
</tbody>
</table>

Statistical analysis
Effect of embryo density on the proliferation of embryo were analyzed statistically using one-way analysis (ANOVA). Lsd test was used for multiple comparisons of categorical means. The software used was SPSS 13.

III. RESULT AND DISCUSSION
4.1 Embryogenic Callus
Creamy and friable embryogenic callus with nodules on its surface was observed on ECIM supplemented with 100 ppm 2,4-D (Figure 1.) Touchet et al [5] found that nodular embryogenic callus consisted of single embryogenic cells. The aggregates were composed of typical meristematic cells containing soluble proteins and sometimes starch granules. They had a round prominent nucleus, and a dense cytoplasm with small vacuoles. These embryogenic masses were dividing actively.

4.2 Globular somatic embryo initiation
Creamy friable embryogenic calli were inoculated into liquid suspension initiation medium (SIM) to obtain embryogenic cell suspension culture. SIM have 2 variation of 2,4-D concentration, 80 and 100 ppm. SIM I and SIM II were supplemented with 100 ppm 2,4-D and 80 ppm 2,4-D, respectively. Both of SIM I and SIM II were supplemented with 1 ppm BAP. The average number of embryos was shown in Table II.

Table II. Number of embryo in SIM (Suspension Initiation Medium) after 4 weeks of culture

<table>
<thead>
<tr>
<th>MEDIUM</th>
<th>Average Number of embryo</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIM I</td>
<td>108 ± 1,5</td>
</tr>
<tr>
<td>SIM II</td>
<td>73 ± 1,2</td>
</tr>
</tbody>
</table>

The result showed that single embryogenic cell was obtained in both medium. The single embryogenic cell is round and small, has dense cytoplasm, large nucleus, little vacuole and thin cell wall (Fig. 2). This single embryogenic cell divided asymmetrically within 5 days of culture as shown in Fig. 3. The two smaller cell will become embryo and the bigger cell will become suspensor. This asymmetric division is an important character of somatic embryogenesis. Fig. 4 shows globular somatic embryo with suspensor within 2 weeks of culture. The existence of the suspensor gave the
evidence that the embryo derived from a single cell. Mariani et al. [6] reported that at an early stage, suspensors were observed on the elongated rice somatic embryo, because the somatic embryo was unicellular origin. Unicellular origin means that the somatic embryo was derived from a single cell.

Fig. 1. Friable embryogenic callus of oil palm Elaeis guineensis, Jacq. 635 clone 12 weeks after culture. Fig. 2. Single embryogenic cell (SEC) of oil palm Elaeis guineensis, Jacq. 635 clone. Figure 3. Asymmetric division of oil palm (Elaeis guineensis, Jacq.635 clone) cell (BE = Becoming Embryo ; BS = Becoming Suspensor). Figure 4. Oil palm (Elaeis guineensis, Jacq,Clone 635) globular somatic embryo with suspensor ( S = Suspensor ; E = Embryo )

Number of embryo in SIM and SPM can be seen in Table 3. Result of optimisation showed that concentration of 100 ppm 2,4-D produced optimum number of embryo i.e. 108 ± 1.5 which is higher than others variety of oil palm. Ginting and Fatmawati [7] reported that optimum concentration of 2,4-D for producing globular somatic embryo from others clone was 50 ppm. The difference of the concentration may be because of the difference of genetic factor [8], [9].

<table>
<thead>
<tr>
<th>Embryo density</th>
<th>SIM 1</th>
<th>SPM 1</th>
<th>SPM 2</th>
<th>Proliferation 1</th>
<th>Proliferation 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>10a</td>
<td>59b</td>
<td>84.5b</td>
<td>49</td>
<td>25.5</td>
</tr>
<tr>
<td>30</td>
<td>30a</td>
<td>44b</td>
<td>84a</td>
<td>14</td>
<td>40</td>
</tr>
<tr>
<td>60</td>
<td>60a</td>
<td>81a</td>
<td>84a</td>
<td>21</td>
<td>3</td>
</tr>
<tr>
<td>Average</td>
<td>28.00</td>
<td>22.83</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Note:
Values followed by different letters are significantly different at the 5% level (p<0.05) in Lsd test
SIM 1 : initiation medium with 100 ppm 2,4-D
SPM 1 : proliferation with 50 ppm2,4-D
In this result, the somatic embryo can be produced within 2 weeks of culture, which is faster than research performed by Ginting and Fatmawati [7]. They reported that the somatic embryos were produced within 2-4 months of culture. It could happened because of the suitable medium and the use of friable embryogenic calli so that the calli were easy to disperse in the liquid medium. Optimum dispersion was also helped by agitation that caused good aeration and uniform distribution of nutrition.

4.3 Synchronization of embryo

Synchronization of embryos were conducted by selecting the globular somatic embryos in suspension initiation medium (SIM) 4 weeks of culture by using 2 mm nylon mesh sieve. Sieving process was needed to obtain efficient and synchrone embryo size. [10], [3], [11], [4], [12]. The key point in establishing such systems is the initial materials used, which should be homogenous cells having high competency [13]. A high-frequency, synchronous embryogenic systems in liquid culture is needed to take full advantage of somatic embryogenesis as it is essential for automation and for investigating physiological, biochemical and molecular aspects of a process for which there is still limited information concerning woody tree species [14].

4.4 Proliferation of embryo

Sieved globular embryos were subsequently transferred to proliferation medium (SPM 1) by batch cultures method. SPM 1 consists of 50 ppm 2,4-D and 1 ppm BAP. Lower 2,4-D concentration compared to suspension initiation medium (SIM) was to support proliferation of globular somatic embryos. Kreuger [10] stated that once embryogenic cell lines are obtained the globular somatic embryos have to be proliferated in order to produce the number of embryos wanted. SPM 1 was also supplemented with low concentration of cytokinin, in this case was BAP. Hartmann et al. [3] reported that BAP can help to induce somatic embryogenesis. It further suggested that cytokinins could function as a signal regulating when and where cell proliferation occurs in the plant [15]. The addition of 2,4-D and BAP in the proliferation medium was expected to optimize the proliferation of globular somatic embryo of oil palm Elaeis guineensis, Jacq. 635 clone.

To optimize the proliferation, 4 weeks SPMI cultures were subcultured to SPM2 medium containing 30 ppm 2,4-D and 1 ppm BAP. Subculturing the culture to the medium with lower 2,4-D concentration was a strategy to optimize the proliferation and avoid further embryo development. [16], [10], [12], [3]. It was happened because the globular somatic embryo in proliferation stage continue to divide and disintegrated in the medium.

Proliferating globular somatic embryos in SPM1 were also observed by transmission electron microscopy (TEM) as seen in Figs. 5-8. Fig 5 shows small cells in globular somatic embryo that accumulated protein in the vacuoles. The accumulation of protein in the vacuoles occurred due to maturing process of the cells. Subculturing the globular somatic embryos from SIM to SPM1 with lower 2,4-D concentration triggered maturation of the embryos. Fig. 6 was the magnification of Fig. 5 shows one cell of the globular embryo that accumulated protein in the vacuole. This storage protein have the characteristic of secreted proteins. According to Fosket [15], the protein are transported to the tonoplast and secreted from the cytoplasm into the vacuole.

When the cells in the globular somatic embryo enlarged, the cells consisted of large vacuoles and the accumulated protein become digested (Fig. 7). It is assumed that the protein was digested for the development of globular somatic embryo. Fig. 8 is magnification of Fig. 7 shows one cell with digested protein in the vacuole.

In proliferation stage, concentration of 2,4-D was lowered so that inhibited cells growth and increased cell differentiation. Therefore, synchronized and optimal proliferation occurred. Besides nutrition composition, hormone and embryo size, embryo population density in the medium affected initiation and proliferation of the embryos as well. Kreuger [10] had proved it in Cucumic sativus (200 embryos / ml) and Daucus carota L. (100 embryos / ml).
4.4.1 Effect of embryo density on the proliferation of embryo

The density of somatic embryo in SIM1 was determined as 10 embryos/20 ml, 30 embryos/20 ml and 60 embryos/20 ml. After initiation step in SIM1 medium, the embryos were proliferated in SPM1 and subsequently in SPM2 with decreasing 2,4-concentration as follows: 100 ppm (SIM1); 50 ppm (SPM1) dan 30 ppm (SPM2). Proliferated embryos in each different initial density of embryo can be seen in Fig. 9, 10 and 11.

Based on one-way ANOVA and Lsd test (p<0.05), in initial density 10 embryo/20 ml (Fig. 9) it can be seen that increment of number of embryos in every medium occurred, i.e., 10, 59 and 84.5 In this case, number of embryo I SIM 1 significantly different with number of embryo in SPM 1. Whereas number of embryo did not increase significantly in the subculturing from SPM 1 to SPM 2.

Based on one-way ANOVA and Lsd test (p<0.05), in 30 embryo/20 ml initial density, increment of embryo number in every medium was as follows: 30, 44 and 84 (Fig. 10) The increment condition was the same as 10 embryo/20 ml initial density, i.e. number of embryo in SIM 1 significantly different with number of embryo in SPM 1. Whereas proliferation from SPM 1 to SPM 2 resulted in addition of 40 embryos. ANOVA and Lsd test indicated that number of embryo in SPM 1 was significantly different with number of embryo in SPM 2.

In 60 embryos/20 ml initial density (Fig. 11) non significant proliferation in all medium : SIM 1, SPM 1 dan SPM 2 (p<0.05) occurred.

Result in figs. 9-11 above revealed that initial embryo density affected the proliferation of embryo. According to Bhojwani and Razdan [16], growth of cell culture was affected by initial low density in medium due to the competition in nutrition, oxygen and space. Denser the embryos, tighter the competition in obtaining the nutrition and oxygen. Therefore, certain embryos will get an obstacle in growing and differentiation. High density of embryos created narrow space for the embryos to be exposed to the medium so that the nutrition and oxygen were not distributed well to the embryos.

Figure 12 showed that optimum density for proliferation of oil palm globular somatic embryo 635 clone was the 10 embryos/20 ml. The density of 10 embryos/20 ml have higher and stabil leap than the density of 30 embryos/20 ml and 60 embryos/20 ml. The density of 10 embryos/20 ml was more efficient compared to that of 30 embryos/20 ml and 60 embryos/20 ml because with fewer embryo density it can resulted the same number of embryos as the others two embryo density, i.e. 84 embryos in MPS2.
4.4.2 Effect of 2,4-D on the proliferation of embryos

Based on the observation on embryo proliferation number in three medium with different concentration of 2,4-D, i.e. 100 ppm (SIM 1); 50 ppm (SPM 1) and 30 ppm (SPM 2) (Table 3), it can be seen that high increment occurred after the embryos in SIM 1 was subcultured into SPM 1 (Proliferation 1) i.e. 49, 14, 21 in each density. The average proliferation in SPM 1 was 28 embryos. Whereas the proliferation of embryos from SPM 1 to SPM 2 with lower concentration of 2,4-D (Proliferation 2), were 25, 40 and 3 embryos for each density. The average proliferation in SPM 2 was 22.83 embryos. This proliferation was lower than proliferation 1. It can be seen that proliferation process occurred if the concentration of 2,4-D was decreased. In this study, we obtained optimum concentration of 2,4-D for proliferation of oil palm globular somatic embryo 635 clone, namely 50 ppm 2,4-D (SPM 1). The concentration of 2,4-D in this study (50 ppm) was lower than previous study conducted by Touchet [5] in the same species but with different variety. The differences occurred might be due to genetic differences in every clone of oil palm. The optimum initiation and proliferation of globular embryos of oil palm with synchronized development as a result of somatic embryogenesis are very potential for the onset of artificial seed production of oil palm.

ACKNOWLEDGEMENT

This study was supported by Indonesian Ministry of Research and Technology, RUT project.