Early detection of somaclonal variation in oil palm callus culture through cytological and SDS-PAGE protein analysis

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Abstract. The major problem of oil palm in vitro propagation is the occurrence of somaclonal variation. This study aimed to obtain somaclonal variation analytical method based on chromosome activities and protein molecular weight level using SDS-PAGE technique on callus culture. Generally, three types of callus were observed, i.e. rooty, nodular friable, and nodular aggregate callus. During mitotic analyses, normal and abnormal cell division of each callus was successfully observed in several phases of mitotic. Rooty callus showed the highest rate of abnormal mitotic divisions up to 7.0 %. In contrast, nodular friable resulted in 3.2 % abnormality rate which is even less than normal plant cell division that was 3.7 %. The most mitotic abnormality type was a reduction-grouping mechanism of the chromosome during metaphase and prophase. SDS-PAGE revealed different protein molecular weight between nodular friable, nodular aggregate, and rooty callus. Protein 20 kDa is a specific protein found in nodular friable callus; this protein was known as a biochemical marker to distinguish embryogenic and non-embryogenic callus.

Keywords: Chromosome, mitotic, clonal propagation, tissue culture, rooty callus, protein 20kDa

1. Introduction

Oil palm (Elaeis guineensis Jacq.) is a high oil-bearing plant. This species is the largest source of vegetable oil compared to soybean and sunflower [1]. Studies to increase oil yield productivity will fulfill the vegetable oil demand in the future. One of the studies is the propagation of elite palms through tissue culture technique with the aim to improve its yield of 20 % to 30 % higher than the parental plant [2].

Clonal propagation has been conducted to generate elite planting materials with higher yield, more uniform, and faster production than conventional seedling method. Unfortunately, oil palm clonal propagation has several constraints whereas one of them is somaclonal variation [3]. Somaclonal variation produces mantled fruits and reduces potential oil yield up to 30 %. Several approaches have been done to study this phenomenon including cytological analysis although the information on this analysis is inadequate. The small size of the chromosome and the difficulty in spreading them were probably reason to this [4].
Most of oil palm tissue culture laboratories, somatic embryogenesis is initiated by callus formation. Callus is unorganized cell masses in response to stresses, formed by the vigorous divisions of plant cells [5]. Callus derived from oil palm leaf explant culture containing somaclonal variation [6]. Generally, three types of callus were produced, i.e. nodular friable, nodular aggregate, and rooty. In oil palm tissue culture propagation, callus was formed through several subculture process in MS medium with the addition of growth regulators.

The somaclonal variation could be detected with different approaches such as morphological, cytological, cytochemical, and biochemical analysis [7]. Cytological screening of a large number of oil palm callus cultures that is having different origins can be done as an early detection technique to determine the possible mechanisms leading to abnormal variants. A comparative study at the biochemical level of callus type especially to their storage proteins is important to obtain high-quality somatic embryoids with criteria of high formation of storage reserves [8]. Comparative of the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein profile can indicate the somaclonal variation at the biochemical level of callus type.

Cytological and SDS-PAGE approaches are an efficient method for early detection of a somaclonal variation. It has some advantages compared to other methods such as morphological and molecular DNA (direct DNA sequencing, single nucleotide polymorphisms, and microsatellites). The cytological analysis in the chromosomal structure is direct and give strong evidence of genetic mutation of an organism. Although, it is time-consuming and chromosomes are difficult to observe. Relative to morphological detection, the use of SDS-PAGE for detecting variants is faster and economical. However, the application is complex and requires high expertise. Direct DNA sequencing, single nucleotide polymorphisms and microsatellites are now available for more informative marker systems, but it is not an ideal technique for commercial application due to cost implications [7].

Morphological differences of callus might result in a variation of embryoid’s or shoot’s performance. The morphological change was reported that had been correlated with cytological analysis such as chromosome aberrations [9]. In this study, to detect somaclonal variation, the cytological analysis was done by observing chromosome activities during cell mitosis in rooty, nodular friable, and nodular aggregate callus culture. In addition, the protein molecular weight separation was done using SDS-PAGE to observe any protein differentiation among samples.

2. Materials and methods

2.1. Plant material

This experiment analyzed three types of callus, i.e. rooty, nodular friable, and nodular aggregate that originated from immature leaf explants of 10 yr to 5 yr Tenera and Dura oil palm types. According to Murashige and Skoog [in 10], callus was maintained at MS media. Following this callus was maintained at MS medium with the addition of 5 % sucrose, and 0.65 % agar for callus induction and differentiation stages. At cytological approach, roots from Tenera seedlings and root from abnormal plantlets were used as negative and positive control respectively.

2.2. Culture maintenance

Callus induction was conducted through sub-culturing immature leaf explants three times every 3 mo in 12 mo of the incubation period. After callus was formed, the callus was subcultured every 2 mo during 12 mo of incubation period to produce somatic embryoid. The somatic embryoids were subculture every 2 mo to produce plantlet. Abnormal plantlets were identified by observing leaf or stem morphology.

2.3. Cytological analysis

Callus samples were treated according to chromosomes squash method. Callus and roots were cut into ± 1 cm and fixated in Carnoy solution (one part of acetic acid : three parts of ethanol) for at least 24 h followed by hydrolysis in 1 N HCl for 1 h. Samples were stained with 2 % aceto-orcein and incubated for 30 min. Stained samples were placed on object glass and added with drops of 2 % of aceto-orcein. The sample was squashed and observed under a light microscope using 1 000 times magnification.
The number of chromosome and number of the abnormal chromosome for each cell were counted. At least 250 cells were analysed for each sample. The chromosomal instability was identified within four group including chromosome reduction mechanism, lagging chromosome, poliploidization, and bridge chromosome at anaphase [11].

2.4. Analysis of protein molecular weight using SDS-PAGE

In this study, SDS-PAGE was used to obtain protein profiles with different molecular weights of each callus type. It comprised of three main steps, i.e. protein extraction, protein concentration measurement, and protein separation according to its molecular weight. Protein extraction was conducted by grinding two gram of callus samples in a mortar using liquid nitrogen into a fine powder. This was dissolved in 300 μL phosphate buffer saline (PBS) based on Bollag method [in 12] and centrifuged at 13 000 g for 30 s. The supernatant was stored at -20 ºC. Protein concentration was determined using Bio-rad assay method as described in the following. About 2 μL of protein samples were mixed with 200 μL Bio-rad dye and 798 μL distilled water. Protein concentration was measured using spectrophotometer at optical density 595 nm through BSA protein standard curve function. The protein separation was done through SDS-PAGE method that was developed by Maniatis [in 13]. Resolving gel 12 % was consisted of 3.3 mL distilled water; 4 mL polyacrylamide; 2.5 mL 1.5 M Tris pH 8.8; 100 μL 10 % Sodium dodecyl sulphate (SDS); 10 μL tetra methylene diamine; and 90 μL of 10 % ammonium persulphate that was polarized at flat glass. Then, 0.1 % running buffer (distilled water 1 L, Tris Base 15 g, glycine 72 g, SDS 5 g) was filled into the electrophoresis container. About 10 μg of protein’s samples were mixed with 2 μL of sample buffer, while 10 μL of protein weight marker (Fermentas, Waltham, USA) were mixed with 2 μL of buffer’s sample. All solutions were heated in hot water for 2 minutes and cooled in ice immediately. These solutions were then filled into the 12 % acrylamide gel. Electrophoresis at 100 V was conducted for 1.5 h to 2.5 h. Afterward, the gel was stained using silver nitrate solution and was washed with a solution containing 50 mL distilled water, 40 mL methanol, and 10 mL glacial acetic acid, finally was stored at 10 % acetic acid glacial and kept in room temperature for 24 h.

2.5. Experimental design

This study used three types of callus taken from two genotypes, i.e. Tenera and Dura. Each genotype consists of three callus lines. Root from Tenera seeds was used as a negative control while root from abnormal plantlet Tenera as a positive control for cytological analysis. Cytological data from the individual sample was analyzed using descriptive statistics and odds ratio to obtain the highest abnormality chance from callus types. Protein content was analyzed using ANOVA test and DMRT at 5 % confidence level.

3. Results and discussion

3.1. Cytological analysis

Normal cell division goes through four phases, i.e. prophase, metaphase, anaphase, and telophase. Prophase was indicated by condensation of chromosomes which gather into one group. Subsequently, the chromosomes achieved maximum condensation at metaphase and aligned in the middle of the cell, this phase called metaphase and become the ideal period for counting chromosome. In the next phase, chromosomes were separated, and chromatids were moved to the opposite poles, which is the characteristic of anaphase. This was followed by the formation of a new nucleus membrane at the telophase. At the end of telophase, cytoplasm divided into two cells and the new cell wall was formed in equator [14]. In this study, all phases were observed in the majority of callus samples (figure 1).

However, some cells showed the abnormal mitotic process. These abnormalities were categorized as a reduction of chromosomes grouping, polyploidy, the formation of chromosome bridge at anaphase and chromosome lagging at metaphase generating aneuploidy (figure 2). Reduction grouping mechanism began with nuclei split, then after prophase directly in numerous arrested metaphase chromosomes arranged in groups in the same cell [11]. Polyploidy happened when chromosome
duplication was not followed by nuclear division. Polyploidy can naturally arise in some different ways.

**Figure 1.** Four phases of oil palm callus cell division. (a) prophase; (b) metaphase with 32 chromosomes; (c) anaphase; and (d) telophase.

In some cases a somatic mutation can occur, due to a disruption in mitosis, resulting in chromosome doubling in a meristematic cell that will give rise to a polyploid plant [15]. The chromosomal bridges abnormality occurred when the attached chromatids failed to separate or separated with significant delay formed a chromosome bridge during anaphase. The nuclear division does not seem to prevent the reorganization of the nuclear membrane [16]. The lagging chromosomes occurred when one or more chromosome was a delay to grouping likely lost during the division. Two last abnormalities indicated severe genomic imbalances [11]. These mitotic abnormalities shifted genome rearrangement in the chromosome and impacted on the gene regulation.

**Figure 2.** Abnormality mitotic cell division; reduction grouping of chromosome (a, b, & c); polyploidy (d); chromosome bridge at anaphase (e) and chromosome lagging at metaphase (f).

The longer the cells that remains in culture, the greater their chromosomal instability. Prolonged culture period in vitro, especially the callus stage shows a higher frequency and degree of abnormality [17]. Cytological analyses showed that the majority of oil palm callus cells (93.0 % to 96.8 %) undergo normal mitosis and 3.2 % to 7.0 % displayed abnormal mitosis (table 1). Most dominant abnormality type was the reduction of grouping mechanism. Other mitotic process abnormalities occurred rarely. Based on odd ratio analyses, rooty callus possessed abnormal cell mitotic probability
up to 1.6 times higher than nodular aggregate callus and 2.3 times greater than nodular friable callus (table 2). Based on the result, a similar cell type was not followed by a similar mitotic activity.

### Table 1. Abnormality rate of mitotic division and types of abnormality

| Callus type | Abnormality (%) | Types of chromosome abnormality (%) |
|-------------|-----------------|-------------------------------------|
| (-) Control | 3.7             | 98.8 Reduction grouping mechanism   |
| (+) Control | 6.2             | 98.2 Reduction grouping mechanism   |
| N. Aggregate| 4.6             | 98.6 Polyploidy                      |
| N. Friable  | 3.2             | 99.2 Bridge                          |
| Rooty       | 7.0             | 98.8 Lagging                         |

### Table 2. Odds ratio analysis of callus types based on mitotic abnormalities

| Vs (column/row) | (-) Control | (+) Control | N. Aggregate | N. Friable | Rooty |
|-----------------|-------------|-------------|--------------|------------|-------|
| (-) Control     | 1.0         | 1.7         | 1.3          | 0.9        | 1.3   |
| (+) Control     | 1.0         | 1.3         | 0.9          | 1.0        | 1.1   |
| N. Aggregate    | 1.0         | 0.7         | 0.7          | 1.6        |       |
| N. Friable      | 1.0         | 1.0         |              | 2.3        |       |
| Rooty           | 1.0         |             |              |            |       |

Several factors affected the mitotic abnormality of cells such as plants' genotype. The t-test resulted that rooty callus of Dura has a higher significantly mitotic abnormality than Tenera (table 3). This abnormality might be affected by the genotype of Dura. Subsequently, the abnormalities were produced in long term cultures by prolonged exposure of the tissues to 2,4-D which is known to induce such aberrations [18]. It is probably due to the synthetic media must be developed if stable cell populations are to be maintained successfully in vitro. Only in a chemically defined nutrient medium which permits all the normal cell processes to proceed without over stimulation or under nourishment will be possible to establish and maintain stable cell populations.

### Table 3. The effect of genotype on callus mitotic abnormality

| Callus type | Mitotic abnormality rate (%) |
|-------------|-----------------------------|
| Dura        | Tenera                      |
| N. Aggregate| 5.0 ± 2.7                   | 4.2 ± 0.2                  |
| N. Friable  | 2.9 ± 2.4                   | 3.4 ± 1.5                  |
| Rooty*      | 9.1 ± 1.4                   | 4.8 ± 1.7                  |

*significant with t-test (α = 5 %)

3.2. SDS-PAGE analysis

The protein content was analyzed for each callus type. The ANOVA test resulted that callus type and genotype were not significantly affected total protein concentration. There was no interaction between callus type and genotype on the total concentration of protein (table 4). Protein contents are more
affected by physiological stress related to diminishing nutrition in the medium and cell degradation [22].

**Table 4.** The protein concentration of callus types of Dura and Tenera genotypes

| Callus type | Protein content (mg mL\(^{-1}\)) | Dura | Tenera | Average |
|-------------|----------------------------------|------|--------|---------|
| N. aggregate| 1.4 ± 0.7                        | 0.7 ± 0.5 | 1.1 ± 0.5 |
| N. friable  | 1.1 ± 0.5                        | 1.4 ± 0.4 | 1.2 ± 0.5 |
| Rooty       | 1.4 ± 1.3                        | 0.9 ± 0.2 | 1.1 ± 0.7 |
| Average     | 1.3 ± 0.8                        | 1.0 ± 0.4 |          |

![Figure 3](image)

**Figure 3.** Analysis of molecular protein weight of callus using SDS-PAGE of Tenera (A) and Dura (B) callus. M = Marker, NF = Nodular Friable, NA = Nodular Aggregate, R = Rooty.

SDS-PAGE revealed different protein patterns of expression between each callus type (figure 3). As shown in figure 3, there were three unique protein bands that were specifically expressed in nodular friable and nodular aggregate callus of Tenera that was 56, 42, and 20 kDa protein. Protein 20 kDa was reappeared on nodular friable callus of Dura. Those proteins were not detected on rooty callus. Protein 20 kDa is a specific protein found in embryogenic callus of *Diantulhus caryopyllus* L. and used as a biochemical marker to distinguish non-embryogenic callus [23]. This result was similar as described earlier; only a nodular callus could induce somatic embryoid.

Currently, this 20 kDa protein is still unidentified. In the future, the marker proteins will be purified and characterized by using protein sequencing method in order to utilize them as a marker for analysing the somaclonal variation of callus culture, especially for distinguishing embryogenic and non-embryogenic callus. There is literature described a purified 25 kDa protein from embryogenic culture, and its identity was determined as glycosylated osmotin-like protein [24]. The marker protein
could also be glycosylated osmotin-like protein due to the similarity of the molecular protein weight although further analysis is needed to confirm the assumption. The close similarity observed in the total protein may be due to the fact that clusters have a common origin. However the presence of specific proteins in callus type could indicate that these may be involved in the determination process of embryoid formation. The specific proteins could thus serve as biochemical markers for assessing the embryogenic capacity of in vitro cultures.

4. Conclusion
Cytological and SDS-PAGE analysis are two potential methods for early detection of somaclonal variation. Rooty callus showed the highest rate of abnormal mitotic divisions at the level of 7.0 %. The dominant abnormality type was a reduction grouping mechanism. Thus, the rooty callus should be avoided or no longer used in sub-culture process. Protein 20 kDa was a specific protein that appeared on nodular friable callus as biochemical markers for assessing the embryogenic capacity.

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