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# Regeneration of viable oil palm plants from protoplasts by optimizing media components, growth regulators and cultivation procedures

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Oil palm protoplasts are suitable as a starting material for the production of oil palm plants with new traits using approaches such as somatic hybridization, but attempts to regenerate viable plants from protoplasts have failed thus far. Here we demonstrate, for the first time, the regeneration of viable plants from protoplasts isolated from cell suspension cultures. We achieved a protoplast yield of $1.14 \times 10^6$ per gram fresh weight with a viability of 82% by incubating the callus in a digestion solution comprising 2% cellulase, 1% pectinase, 0.5% cellulase onuzuka R10, 0.1% pectolyase Y23, 3% KCl, 0.5% $CaCl_2$ and 3.6% mannitol. The regeneration of protoplasts into viable plants required media optimization, the inclusion of plant growth regulators and the correct culture technique. Microcalli derived from protoplasts were obtained by establishing agarose bead cultures using Y3A medium supplemented with 10 μM naphthalene acetic acid, 2 μM 2,4-dichlorophenoxyacetic acid, 2 μM indole-3-butyric acid, 2 μM gibberellic acid and 2 μM 2-γ-dimethylallylaminopurine. Small plantlets were regenerated from microcalli by somatic embryogenesis after successive subculturing steps in medium with limiting amounts of growth regulators supplemented with 200 mg/l ascorbic acid.

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## 1. Introduction

Palm oil is the largest source of edible oil before soybean oil in the world, which contributes 31.8% of the world's production of oils and fats [1]. The demands of palm oil will double in the year 2020 exceeding 57 million tons (62%) of the world's production of oils and fats. This is due to the increasing world population which is expected to be seven billion in the year 2020. Thus, larger oil palm plantation and more workers are needed, and this is expected to be a major problem by the competition with population area and modernization of the jobs than agriculture sectors.

Oil palm plantations in Malaysia are cultivated with the eight generation of *Elaeis guineensis*, tenera oil palm. The tenera is hybrid oil palm derived from crosses between dura and pisifera using conventional breeding approach. The breeding of first tenera oil palm requires almost 12 years and 40 years for eight generations. This was achieved by using large planting area due to open pollinating behavior of oil palm. Therefore, the production of new traits or varieties of oil palm by conventional breeding is significantly slow. Consequently, the oil palm has a major problem with incomplete dominance inheritance. For example, the tenera fruits showed all three fruit forms, dura: tenera: pisifera, in a ratio of 1:2:1, indicates the incomplete dominance dura over pisifera. This means only 50% of the fruits maintained the property of tenera. This problem makes the propagation of oil palm through seeds germination is unsatisfactory.

As above, limited land resources for oil palm plantation, labor shortage and problems with conventional breeding and seeds propagation, it is important to find the strategies to solve the mentioned aspects. One of the strategies is already on the way as the propagation oil palm through tissue culture. Since oil palm is a single growing apex and basal shoots cannot be produced, the propagation of oil palm through vegetative tissue culture is impossible. Thus, the oil palm tissue culture depends exclusively by somatic embryogenesis approach. By using somatic embryogenesis, since

*Abbreviations:* 2,4-D, 2,4-dichlorophenoxyacetic acid; 2iP, 2-γ-dimethylallylaminopurine; BAP, 6-benzyl aminopurine; GA3, gibberellic acid; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; NAA, naphthalene acetic acid; PGRs, plant growth regulators.

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1974, significant progress has been made in the propagation of oil palm through tissue culture [2–7]. Furthermore, the factors influenced somatic embryogenesis of oil palm such as plant growth regulators, explants genotypes and molecular mechanisms have been intensively investigated [8–11] and [12]. At Malaysian Palm Oil Board (MPOB), generally, conventional oil palm tissue culture was based on solid media culture. However, the long process (52–55 months) of solid media culture produced high percentage (~10%) of the abnormality of oil palm [13] which was mainly due to somaclonal variation. Tissue culturists of MPOB have made extensive improvements, particularly for oil palm suspension cultures which the oil palm propagation has been reduced as a minimum as 35 months. With the availability of oil palm suspension cultures as well as Malaysia is the world largest collection of oil palm germplasma, the production of new traits or varieties of oil palm by somatic hybridization using protoplast fusion is the promising approach. It is postulated the new traits could be obtained less than five years compared conventional breeding. However, the regeneration of viable oil palm plants from protoplasts remains a challenge and there are only a few reports describing the generation of microcalli from protoplasts.

Oil palm protoplasts were first isolated from cell suspension cultures, 4–14 days after subculture, by digestion with 10% (w/v) driselase in half-strength MS medium supplemented with 0.3 M glucose, yielding up to $10^4$ protoplasts/ml with a viability of >90% [14]. The protoplasts were cultured either in thin-layer liquid medium or in nurse culture medium comprising half-strength MS supplemented with 1 g/1 casein hydrolysate, 25 g/1 sucrose and either 2.0 mg/1 2,4-dichlorophenoxyacetic acid (2,4-D) or 2.5 mg/1 naphthalene acetic acid (NAA). After one month, approximately 1% of protoplasts in the nurse culture developed into colonies comprising 7–10 cells, but no further results have been reported.

Protoplasts have also been isolated from oil palm polyembryogenic cultures, a procedure carried out in order to determine their fatty acid composition [15]. The same authors later attempted to isolate protoplasts from various tissues, including polyembryogenic cultures, the vegetative apices of clonal ramets, seed embryos, the embryonic axes of germinating seeds, and young inflorescences [16]. Among the tissues and enzyme combinations tested, the highest protoplast yield ($1.5 \times 10^6$ per g fresh weight) and viability (95%) was achieved using polyembryogenic cultures digested with a mixture of celluclast, pectinex, pectolyase Y23, hemicellulase and trypsin inhibitor. Four types of liquid media were tested: A medium, MS medium, KM medium [17] and WPM medium [18]. A medium promoted the most efficient formation of microcolonies (within 3–4 weeks) although the frequency was less than 0.1% and there was no further growth. Importantly, the addition of glutathione and catalase was essential to maintain protoplast viability. These authors also found that palmitoleic acid represented up to 27% of the fatty acid content of protoplasts compared to <0.1% in oil palm tissue.

We have developed a successful protocol for the regeneration of oil palm plants from protoplasts, originally derived from cell suspension cultures. The protocol represents a milestone in the use of protoplasts as a starting material for the production of new traits oil palm plants by somatic hybridization.

## 2. Materials and methods

### 2.1. Oil palm cell suspension cultures

Oil palm embryogenic cell suspension cultures were cultivated in 100-ml flasks containing 50 ml Y3 liquid medium (Supplementary Table 1) supplemented with 5 μM NAA, 5 μM 2,4-D and 2 μM 2iP. This medium was designated Y35N5D2iP. The suspension cultures were incubated in the dark at 28 °C on a rotary shaker and agitated at 120 rpm. Half of the medium was discarded and replaced with fresh medium every 14 days.

### 2.2. Protoplast isolation and purification

Protoplasts were isolated from embryogenic cell suspension cultures up to 14 days after the fresh medium was added. Cells were collected by filtration through a 300-μm nylon mesh, and 0.5 g fresh weight of cells was transferred into a 50-ml centrifuge tube containing 15 ml filter-sterilized enzyme solution (2% (v/v) cellulase (Sigma), 1% (v/v) pectinase (Sigma), 0.5% (w/v) cellulase onuzuka R10 (Duchefa), 0.1% (w/v) pectolyase Y23 (Duchefa), 3% (w/v) KCl, 0.5% (w/v) $CaCl_2$ and 3.6% (w/v) mannitol, pH 5.6). The cells were resuspended by inverting the tube 6–10 times and then incubated in the dark without shaking at 26 °C for 14 h. The mixture was diluted with 15 ml filter-sterilized washing solution (3% (w/v) KCl, 0.5% (w/v) $CaCl_2 \cdot 2H_2O$, 3.6% (w/v) mannitol, pH 5.6), resuspended by inverting the tube 3–5 times, filtered through a sterilized double layer of miracloth (22 μm) and collected in a 50-ml centrifuge tube. The filtration step was repeated 2–3 times until all undigested tissues, cell clumps and cell debris were removed. The mixture was centrifuged at $60 \times g$ for 5 min at 22 °C and the supernatant was discarded. The protoplast pellet was resuspended by adding 10 ml washing solution and mixing by inversion, followed by centrifugation as above. After three cycles, the supernatant was removed completely and the protoplast pellet was resuspended in 5 ml filter-sterilized rinse solution (3% (w/v) KCl, 3.6% (w/v) mannitol, pH 5.6).

### 2.3. Protoplast yield and viability

The yield and viability of the protoplasts were determined using a Nageotte hemocytometer. Three replicate measurements were taken for each independent experiment, and the yield was calculated using the formula $X = Y \times 10^5/Z$, where $X$ is the number of protoplasts per ml, $Y$ is the average quantity of protoplasts in $5 \times 1\ mm^2$ and $Z$ is the fresh weight of plant material in grams. The viability was calculated by counting the number of fluorescent protoplasts after staining with 5-carboxyfluorescein diacetate, acetoxymethyl ester (5-CFDA, AM; Invitrogen) and dividing this number by the protoplast yield in percent. Cell wall formation was evaluated by staining with fluorescent brightener 28 (Sigma).

### 2.4. Media optimization

Protoplasts were rinsed and allowed to settle for 20 min at room temperature, and were then cultivated in liquid media or embedded in solidified agarose media (Supplementary Table 1). For the liquid media, the rinse solution was replaced with liquid medium and 2 ml was dispensed into each well of a 24-well culture plate. For the solid media, the protoplasts pellet was resuspended in a double concentration of liquid medium. SeaPlaque agarose (Duchefa) was then dissolved to a concentration of 1.2% (w/v) in distilled water containing 0.1% (w/v) 2-*N*-morpholinoethanesulfonic acid (MES), the pH was adjusted to 5.7, and the agarose solution was filter-sterilized and maintained at 37 °C. Equal volumes of the protoplast suspension and agarose were mixed thus adjusting the final agarose concentration to 0.6% (w/v). We then dispensed 2 ml of medium into each well of a 24-well plate and allowed it to solidify for 1 h. The protoplasts embedded in agarose were covered with 500 μl of liquid medium of the same composition, and the culture plates were sealed and incubated at 28 °C in the dark. The culture was monitored daily under a microscope to record the first and second cell divisions, and at 7-day intervals to observe the formation of microcolonies and microcalli.

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