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The evaluation of sterilization protocol for sprout explants in oil palm (*Elaeis guineensis* Jacq.) tissue culture

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Abstract. The objective of this study was to develop an efficient protocol for sterilization of sprout explants in tissue culture of oil palm. Plant materials used were plumulae and radicle from sprouts of the D x P Sriwijaya variety obtained from Seed Processing Unit PT Binawit Makmur, Palembang. The medium used was MS composition was supplemented with vitamins, myo-inositol and sucrose, and the pH was set at 5.8 ± 0.02 before being solidified with agar. Cultures were maintained under a light intensity of 1,500 lux and 16-hour photoperiod and temperature of 24 to 26°C. Five protocols for the eradication of explant contamination were tested. i.e. There were 5 (five) different methods of explant sterilization employed, ie: A) explants were washed with sterile aquadest followed by soaking in 70% alcohol for 1 minute; B) explants were washed with sterile aquadest followed by soaking in 1% Benlox 50WP solution for 30 minutes, 1% Agrept 20WP for 30 minutes, and soaking in 70% alcohol for 5 minutes; C) explants were washed with sterile water plus detergent, followed by soaking in 1% Benlox 50WP plus few drops of Tween-80 for 30 minutes, soaking in 1% Agrept 20WP plus few drops of Tween-80 for 30 minutes, soaking in 70% alcohol for 5 minutes; D) explants were washed with sterile water plus detergent, followed by soaking in 0.1% HgCl₂ solution for 30 minutes; and E) explants were washed with sterile water plus detergent, followed by soaking in 0.2% Dithane M-45 plus few drops of Tween-80 for 30 minutes, soaking in 1% NaOCl for 5 minutes, soaking in 0.1% HgCl₂ plus few drops of Tween-80 for 30 minutes. The results showed that treating the explants with 0.1% HgCl₂ for 30 minutes following 0.2% Dithane M-45 and 1% NaOCl applications was proven to be effective for the eradication of contamination.

1. Introduction

Among the world's palm oil producers, Indonesia is the largest producer of palm oil with a production of 33,229,381 tons in 2016, and is estimated to reach 35,359,384 tons in 2017 in the form of Crude Palm Oil (CPO). Even the Indonesian Palm Oil Entrepreneurs Association (GAPKI) is targeting Indonesian palm oil production of 40 million tons of CPO by 2020 [1]. Most of Indonesian palm oil is exported to China, Pakistan, Malaysia and the Netherlands.

In order to achieve the production target of 40 million tons of CPO as mentioned above, strategic steps that are well-directed and well-planned are required. One of important steps taken by the government was to encourage replanting (rejuvenation) of old oil palm plantations. In addition, land expansion is another effort that can also be done to increase production according to target. Bangun predicted the growth of Indonesia's oil palm area by 2020 ranges from 5% to 6% [2]. This figure is lower



than the average growth (10% - 12%) of palm plantation during 1990 - 2000, but still needs to be taken into account in order to fulfill the planting materials (seedlings).

Efforts to increase the production of oil palm, either through replanting or expansion of planting area will require plant material in large quantities. The provision of seeds through generative propagation (via seeds) is confronted with the occurrence of variability in the progeny as male and female flowers in oil palm are separated and reach maturity in different time, increasing the chances of cross-pollination among different individuals. Meanwhile, conventional clonal propagation is not possible because it is a monocotyledonous plant that cannot be propagated vegetatively through cutting, grafting or other conventional means. Therefore, an alternative technology that can be applied in mass propagation of oil palm is tissue culture technique. This method offers better prospect than conventional vegetative propagation since it can produce large quantity of clean and uniform progenies within short time [3].

Studies on clonal propagation of oil palm through tissue culture techniques had been started more than three decades ago [4]. The first success of plantlet regeneration from leaf tissue was first reported by Rabechault [5]. Since then, more researchers reported their success in clonal propagation of oil palm through in vitro technique, while making improvements to the protocol. Since the oil palm tissue culture was introduced in the 1970s, clonal propagation in these plants proved to be highly beneficial not only in order to obtain uniform plant materials, but also in the development of plant breeding programs through genetic engineering [6].

One of factors affecting the success of plant tissue culture protocol is the sterility of explants being cultured [7]. This is crucial since the materials obtained from *ex vitro* environment contain high level of microorganism contamination. Meanwhile, the presence of any microorganism in tissue culture system should be avoided to let the growth of cultured plant tissues.

This investigation aimed at developing an efficient protocol for sterilization of sprout explants in tissue culture system of oil palm.

2. Materials and methods

2.1. Plant material

Plant materials used in this study were plumule and radicle obtained from sprouts of the D x P Sriwijaya variety. The sprouts were supplied by Seed Processing Unit PT Binasawit Makmur, Palembang. The superiority of D x P Sriwijaya variety are: high production of fruit bunches (28 - 33 tons per hectare per year), high oil extraction (27.3% Crude Palm Oil and 4.1% Palm Kernel Oil), slow growth rate (56 cm per year), and highly adaptive to drought [8].

2.2. Sterilization agents

Materials needed for sterilization are sterile water, alcohol (70% and 96%), Dithane M-45, Benlox 50WP, Agrept 20WP, Tween-80, sodium hypochlorite (5.25% NaOCl), HgCl₂ and liquid detergent.

2.3. Culture medium

The medium used was MS composition was supplemented with vitamins, myo-inositol and sucrose 3% (w/v) [9]. The acidity (pH) of the medium was set at 5.8 ± 0.02 before being solidified with agar 0.8% (w/v), and poured into culture flasks with volume of 10 mL per each. The medium was sterilized in an autoclave at a pressure of 1.06 kg cm⁻² at a temperature of 121°C for 15 minutes.

2.4. Treatments tested

Five different methods of explant sterilization protocol were tested, i.e.:

A: sterile water → 70% alcohol (1 min.) → sterile aquadest (3 times).

B: sterile water → 1% Benlox 50 WP (30 mins) → sterile aquadest (3 times) + 1% Agrept 20 WP (30 mins) → sterile aquadest (3 times) → 70% alcohol (5 mins) → sterile aquadest 3 (times).

- C: sterile water plus detergent → sterile aquadest (3 times) + 1% Benlox 50 WP plus Tween-80 (30 mins) → sterile aquadest (3 times) → 1% Agrept 20 WP plus Tween-80 (30 mins) → 70% alcohol (5 mins) → sterile aquadest (3 times).
- D: sterile water plus detergent → sterile aquadest (3 times) → 0.1% HgCl₂ (30 mins) + sterile aquadest (3 times).
- E: running water → sterile aquadest plus detergent (10 mins) → sterile aquadest (3 times) → 0.2% Dithane M-45 plus Tween-80 (30 mins) → sterile aquadest (3 times) → 1% NaOCl solution (5 mins) + sterile aquadest (3 times) → 0.1% HgCl₂ plus Tween-80 (30 mins) → sterile aquadest (3 times).

2.5. Culture conditions

Each culture flask contain one explant only. Cultures were kept on culture rack in culture room with a light intensity of 1,500 lux from white-fluorescent light and 16-hour photoperiod. Room temperature was set at 25 ± 1 °C.

2.6. Data analysis

Observation were made on the percentage of contaminated explants, incubation period, and type of contaminating microorganism.

Data obtained were analyzed statistically by Descriptive Statistics method using Microsoft Excel computer applications and mean values plus standard errors was calculated [10]. In addition, qualitative data such as visual performance of explants were also captured and presented in the form of image to support quantitative data.

3. Results and discussion

In the study of seeking for effective protocol for explant sterilization, each method consisted of 10 replications, and each replication consisted of 4 explants cultured in separate flasks. Thus, there were 50 experimental units with 200 culture flasks. The results of the observation showed that the use of A, B, C and D methods were found to be ineffective in sterilizing oil palm sprouts from microorganism contamination. This can be seen on the rate of contamination that reached 100% of cultured explants. Contamination was first observed within 4 days after culture initiation (Table 1). However, in the E method which employed HgCl₂ following fungicide and NaOCl treatments, the contamination rate could be reduced to a maximum of 45%, depending on the concentration of HgCl₂ used (Table 2).

Table 1. The effectiveness of different sterilization methods on the suppression of contamination in sprout explants of oil palm (observation was made 2 weeks after culture initiation).

Sterilization Method	Percentage of Contamination	Incubation Period (days after culture initiation)
A	100	3 - 6
B	100	5 - 7
C	100	5 - 6
D	100	2 - 4
E	0	0

In A method, contamination began to take place within 3 days after culture (DAC), and all cultures were contaminated within 5 DAC. In B method, contamination started to show up within 5 DAC, and all cultures were contaminated within 7 DAC. In C method all explants were contaminated within 5 – 6 DAC. Meanwhile, in D method the contamination took place within 2 – 4 DAC. In addition to contamination, explants sterilized using D method showed tissue damage in the surface of cut section. The damage was characterized by tissue browning, especially in the plumule. During the first week, all

explants sterilized using A, B, C and D methods appeared to be yellow to green even though they were surrounded by hyphae from the surrounding fungus. Within 2 weeks, however, all explants died due to the contamination. In contrast to previous methods, there was no contamination found during the first two weeks of culture on explants sterilized using E method. However, following 3 weeks of culture initiation there were 2 explants were found contaminated and 16 explants died. This contamination could be attributed to the source of the oil palm sprouts, which were obtained from seed producer where there was not enough sterilization treatment were taken during seed germination.

We proved that the use of fungicide, NaOCl and HgCl₂ were effective to eradicate microorganism contamination on sprout explants of oil palm. This finding was in accordance with the experiment reported by Altaf where NaOCl and HgCl₂ were used in sterilizing Kinow tree explants [11]. Badoni and Chauhan added that NaOCl was better for controlling contamination on potato sprouts [12], and Maina *et al.* found that HgCl₂ was more effective as sterilizing agent for seed explants of *Arachis hypogaea* compared to NaOCl [13]. However, due to the rate of tissue death was quite high in present study, we carried out further screening to seek for appropriate duration of explant soaking in HgCl₂ solution to kill microorganisms but not explant tissues.

Based on the results of E method, screening was carried out on the duration of soaking of explants in HgCl₂ solution. Five soaking duration were tested, i.e.: 5, 10, 15, 20 and 30 minutes. Results showed that the incubation period (time needed to observe the first contamination) was much longer than the previous 4 methods (without HgCl₂). It was also found that there was a correlation between the duration of soaking, the contamination rate and the incubation period of microorganisms (Table 2). Observation made at 4 weeks after culture initiation showed that all explants soaked in HgCl₂ for 5 – 15 minutes were thoroughly contaminated. Whereas with 20 and 30 minutes of soaking, the number of contaminated explants was lesser and the incubation period was also longer.

Table 2. The effect of various immersion times in HgCl₂ solution on the percentage of explant contamination (observation was made 4 weeks after culture initiation).

Soaking Duration (minutes)	Percentage of Contamination	Incubation Period (days after culture initiation)
5	100	9.24 ± 0.35
10	100	11.67 ± 0.22
15	100	17.09 ± 0.44
20	91	25.33 ± 0.55
30	45	29.86 ± 0.84

Table 1 shows that 100% explants sterilized using A, B, C and D methods were contaminated. The contaminations were first detected on the day 2 after culture initiation, and within 7 days all explants were totally contaminated. The main cause of the contamination was fungus (Figure 1).



Figure 1. Ineffective sterilization protocol resulting in serious explant contamination, mostly by fungi.

Meanwhile, Table 2 shows that the incubation period of microorganisms is much longer (reaching 17 days after culture initiation), although all explants sterilized by soaking in HgCl₂ 5 – 15 minutes were completely contaminated. While in the 20-minute soaking not all explants were contaminated until 4 weeks after culture initiation, but the contamination rate is still very high, i.e. 91% with 25-day incubation period. However, in the 30-minute soaking the percentage of contaminated explants is reduced to only 45% until the end of the trial period, or 55% of cultured explants are clean, healthy, and grow.

Our findings are somewhat contradictory to the reports of Sharma and Anjaiah and Maina *et al.* who successfully obtain clean explants from *Arachis hypogaea* seeds treated with 0.1% (w/v) aqueous HgCl₂ for 8 minutes [13,14]. Jan *et al.* used 0.1% HgCl₂ for only 4 minutes to sterilize runner tips and nodal segments of strawberry which resulted in highest percentage of surviving explants [15]. And even, Zulkarnain *et al.* used only 0.02 % HgCl₂ solution for 5 minutes to sterilize crown bud slicing of field grown pineapple [16]. However, although HgCl₂ is highly effective against fungi and bacteria, but it frequently also kills explant materials if used inappropriately. At low concentrations (up to 0.1%) HgCl₂ perhaps the most effective disinfectant for seeds with soil-borne and epiphytic fungi. Nevertheless, HgCl₂ should be used with high caution (if cannot be avoided) as it is highly toxic to every organism. It is also quickly volatilizes, and as such significant quantities of its vapor form could be inhaled during measurement and preparation.

4. Conclusions

Selection of sterilization methods plays an important role for the success of obtaining aseptic cultures in tissue culture system of oil palm, even the explant source were sprouts germinated under controlled condition. The immersion of explants for 30 minutes in 0.1% HgCl₂ following fungicide treatment (0.2% Dithane M-45) was proven to be most effective to eradicate microorganism contaminating the surface of oil palm sprouts germinated under controlled condition.

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References

- [1] Indonesia Investments 2018 Palm Oil [Online] Retrieved from <https://www.indonesia-investments.com/business/commodities/palm-oil/item166?> (8th February 2018).
- [2] Bangun D 2015 Kebutuhan Minyak Nabati Dunia Bergantung pada CPO Indonesia [Online] Retrieved from <https://www.sawitindonesia.com/rubrikasi-majalah/kinerja/2020-kebutuhan-minyak-nabati-dunia-bergantung-kepada-cpo-indonesia/> (8th February 2018).
- [3] Tapingkae T, Zulkarnain Z, Kawaguchi M, Ikeda T and Taji A 2012 Somatic (asexual) procedures (haploids, protoplasts, cell selection) and their applications. In A Altman and P M Hasegawa [eds.], Plant biotechnology and agriculture: Prospects for the 21st century, 139-162. (Academic Press: Oxford)
- [4] Mariska I, Hutami S, Sukmadjaja D, Kosmiatin M, Rahayu S and Utami S 2013 Inovasi Kultur Jaringan Kelapa Sawit *Sinartani* **43** 2-16
- [5] Rabechault H, Martin J P and Gas S 1972 Recherches sur la culture des tissus de palmier a huile (*Elaeis guineensis* Jacq.) *Oleagineux* **27** 531-534
- [6] Low E-T L, Alias H, Boon S-H, Shariff E M, Tan C-Y A, Ooi L C, Cheah S-C, Raha A-R, Wan K-L and Singh R 2008 Oil palm (*Elaeis guineensis* Jacq.) tissue culture ESTs: Identifying genes associated with callogenesis and embryogenesis. *BMC Plant Biol.* **8**: 62.
- [7] Zulkarnain Z 2009 *Kultur Jaringan Tanaman: Solusi Perbanyak Tanaman Budidaya* (Jakarta: Bumi Aksara)
- [8] PT Binasawit Makmur 2018 [Online] Retrieved from <http://www.binasawitmakmur.com/product> (19th November 2018)

- [9] Murashige T and Skoog F 1962 A revised medium for rapid growth and bio assays with tobacco tissue cultures *Physiol. Plant* **15** 473-497
- [10] Microsoft-Corporation 2000 *Microsoft Office 2000 Professional Edition* (New York: Microsoft Corporation)
- [11] Altaf N 2006 In vitro bud culture of kinnow tree *Pak. J. Bot.* **38** 597-601
- [12] Badoni A and Chauhan J S 2010 In vitro sterilization protocol for micropropagation of *Solanum tuberosum* cv. 'Kufri Himalini'. *Acad. Arena* **2** 24-27
- [13] Maina S M, Emongor Q, Sharma K K, Gichuki S T, Gathaara M and de Villiers S M 2010 Surface sterilant effect on the regeneration efficiency from cotyledon explants of groundnut (*Arachis hypogaea* L.) varieties adapted to eastern and Southern Africa *Afr. J. Biotechnol* **9** 2866-2871
- [14] Sharma K K and Anjaiah V 2000 An efficient method for the production of transgenic plants of peanut (*Arachis hypogaea* L.) through *Agrobacterium tumefaciens* - mediated genetic transformation *Plant Sci.* **159** 7-19
- [15] Jan A, Bhat K M, Bhat S J A, Mir M A, Bhat M A, Wani I A and Rather J A 2013 Surface sterilization method for reducing microbial contamination of field grown strawberry explants intended for *in vitro* culture *Afr. J. Biotechnol* **12** 5749-5753
- [16] Zulkarnain N and Eliyanti E 2018 Plantlets regeneration from crown bud slicing of pineapple (*Ananas comosus*) *Biosaintifika* **10** 484-490