Evaluation of 2.4-D and NAA Concentrations for Callus and Somatic Embryos Formation in Oil Palm

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***Abstract*—The evaluation of 2.4-D and NAA concentrations for oil palm micropropagation were conducted in this study. Callus was initiated from immature leaflet of ten oil palm trees. Callus formation began two months after culture and increased at the subsequent subcultures. On average, the best callus induction rate was obtained in a culture medium without 2.4-D in combination with 6 mg/L of NAA. After 11 months of culture, somatic embryos were found mostly from nodular aggregate and nodular friable callus. At the end of culture time, the results showed that 6 mg/L of NAA slightly increase somatic embryos formation by the addition of 0.5 mg/L 2.4-D in both explants and callus culture. When 2.4-D was only added during callus culture, the formation of somatic embryos was low. Different results were shown in treatment with 10 mg/L of NAA. The formation of somatic embryos were much better by the addition of 0.5 mg/L 2.4-D only during callus culture. When 0.5 mg/L 2.4-D was added during explants and callus culture, the formation of somatic embryos were very low. In general, treatment of 10 mg/L NAA added with 0.5 mg/L 2.4-D during callus culture was the best media for oil palm propagation.**

***Index Terms*—auxin, plant growth regulators, somatic embryogenesis, micropropagation**

I. Introduction

The oil palm (*Elaeis guineensis* Jacq.) is a perennial cross-pollinating oleaginous monocotyledon, mostly cultivated in tropical regions of Latin America, Southeast Asia and Africa. It originally comes from the Northwest region of Africa (Guinea-Bissau) and belongs to the Arecaceae family [1]. Generally, oil palm is propagated by seeds therefore a great variation in the plantation is expected due to the heterozygosity of the seedlings. In addition, seed germination of some cultivars such as *Pisifera* (Shell-less; embryo rarely form) is very poor [2].

Clonal propagation of oil palm has been studied for many years as a potential way to develop high-yielding collections while circumventing the long generation time required with traditional breeding techniques [3]. The reliable method of vegetative propagation is only through tissue culture system. Various sources of explants has been used to initiates the somatic embryos formation. Furthermore, the influence of plant growth regulators has been evaluated in many research works. Touchet *et al.* [4] was successfully initiated embryogenic cells and protruding proembryos of oil palm in a medium with 80 or 100 mg/L 2,4-dichlorophenoxyacetic acid (2.4-D) and 1 g/L activated charcoal. Patcharapisutsin and Kanchanapoom [5] suggested that half strength MS medium enriched with either NAA or 2,4-D, sucrose and activated charcoal can be used to initiate callus cultures from matured embryos of oil palm. Guedes *et al.* [1] reported that on average, the best results for percentage of embryogenic explants from immature oil palm inflorescences were obtained in a culture medium with 225 μM of 2,4-D.

Although many research on oil palm tissue culture have been conducted since 1970, it is still difficult to obtain embryogenic callus from certain genotypes. In addition, true to types were limited by high concentration of plant growth regulators. The use of plant growth regulators may lead to somaclonal variation. To minimize somaclonal variation in regeneration of oil palm through tissue culture, plant growth regulators in callus and embryos induction medium should be as low as possible. Therefore our purposes in these studies were to minimize the concentration of auxin and optimize the propagation protocol to obtain genetic stability and true to type of oil palm culture.

II. Materials and Methods

A. Plant Materials and Media Preparation

There are ten *Tenera* palms used in this study. Immature leaflet from those palms were surface sterilized in 5% of Chlorox solution two times for 10 minutes followed by three times rinses in sterile distilled water. The culture media is MS medium (Murashige and Skoog, 1962 [6]) consisted of MS mineral salts and vitamins supplemented with 3% sucrose, 100 mg/L casein hydrolysate, plant growth regulators according to experiment design, and solidified by 0.6% agar. The pH of culture media was adjusted to 5.8 and 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 callus.

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The leaf explants were kept for one year in dark room at temperature ±25 ℃ and humidity ± 50%. Subculture on the fresh same media was conducted every three months. Callus were selected from the explants every month.

C. Somatic Embryos Formation

Selected callus were kept for one year in a dark room at temperature ±25 ℃ and humidity ± 50% to form somatic embryos. Subculture on the fresh same media was conducted every two months. Observations and data recording were conducted every month to obtain primary somatic embryo.

D. Experiment Design

The experiment was to evaluate the usage of plant growth regulators of 2.4-Dichlorophenoxyacetic (2.4-D) and 1-naphthaleneacetic acid (NAA) during callus induction medium (at explant stages). Culture medium was added without and with 0.5 mg/L 2.4-D in combination with three different level of NAA concentration (6, 10, 12 mg/L). In somatic embryos formation (at callus stages), all treatments were added with 0.5 mg/L 2.4-D.

E. Data Analysis

Data were collected as the frequency of callus induction and embryos formation. Data is statistically analyzed with one way ANOVA followed by Duncan's multiple range test (DMRT) that was performed at the level of *p* value less than 0.05 ($p<0.05$) using SAS 9.0.

III. Results and Discussion

A. Callus Induction

All palms were successfully produced callus. The first callus formation was about two months after culture. In general, three types of callus were found in all palms, i.e. nodular friable callus, nodular aggregate callus and chunky callus (Fig. 1). Among these three types of callus, nodular aggregate callus was mostly found. One leaf explant (1 cm^2) can be found more than one type of callus. Therefore, it is imperative to select routinely for embryogenic callus in effort to achieve a higher rate of somatic embryos formation. In addition, the phenotypic fidelity of regenerants depends on the nature of embryogenic callus used in the micropropagation process. Nodular compact callus have been found to produce on average 5% variant palms, this rate reaches 100% in plantlets derived from fast growing callus [7].

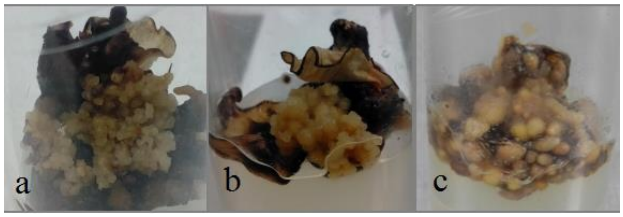

Figure 1. Three types of callus formation. (a) Nodular friable callus. (b) Nodular aggregate callus. (c) Chunky callus

Callus induction rates were still increase at the end of time of culture (Fig. 2). In the first three months of culture, there was only 0.78% of explants formed callus. The highest rate was during 10 to 12 months of time of culture (5.16%). It indicates that a long exposure of explants in auxin increase the ability of cells to differentiate into callus. However, long exposure explants in media containing plant growth regulator auxin is not recommended. It might increase the potential risk of somaclonal variation in oil palm. As explained by Saieed *et al*. [8] that somaclonal variation may be a consequence of the stress inherent in cellular deprogramming induced by plant growth regulators such as the synthetic auxin analog 2,4-D.

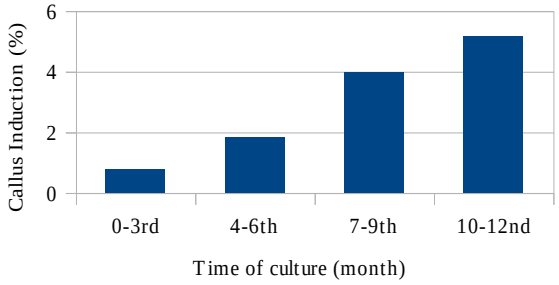

Figure 2. Callus induction rates for 12 months of time of culture.

B. Effect of Presence and Absence of 2.4-D on Callus Induction

The frequency of callus induction among treatments did not significantly different, but on average, the best results for callus induction was obtained in a culture medium without 2.4-D in combination with 6 mg/L of NAA. Increasing of NAA concentration and presence of 2.4-D showed a decreasing of callus induction rates (Fig. 3). This indicated that increasing concentration of auxin in media did not increase callus induction rate. 2.4-D and NAA are relatively strong auxin [9]. High levels of auxin are especially implicated in somaclonal variation, particularly epigenetic variation caused by methylation [10]. Thus, the result of this experiment can be recommended to reduce somaclonal variation in oil palm culture.

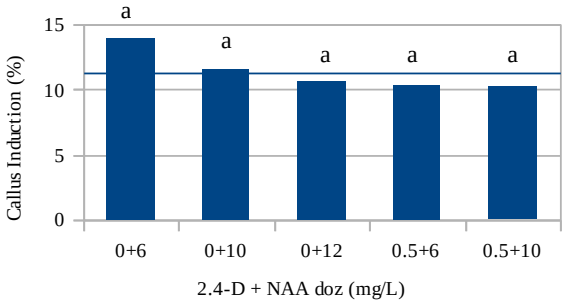

Figure 3. Effect of 2.4-D and NAA on callus induction.

C. Somatic Embryos Formation

Five out of ten palms used in this study successfully formed somatic embryos. The first embryos formation was found 11 months after culture. The embryos were formed on the primary callus and could be distinguished by the presence of white, opaque, and compact nodules. According to Rocha *et al*. [11] somatic embryos were originated from single polarized cell (unicellular) or a group of cells (multicellular). The question of a single- or multi-cell origin for somatic embryos is directly related to coordinate behavior of neighboring cells as a morphogenetic group [12].

Among three types of callus formation in this study, most of somatic embryos were initiated from nodular friable and nodular aggregate callus. The number of embryos increased and most of them enlarged in size when they were subcultured on new media. Different stages of somatic embryos development were observed during time of culture. Some of them were polarized and shoot-like growth development emerged from these embryoids (Fig. 4). In monocots, developmental stages of somatic embryo undergo globular, scutellar and then coleoptilar stages [9].

Figure 4. Somatic embryos development. Shoot-like growth development (arrow)

D. The Effect of 2.4-D and NAA on Somatic Embryos Formation

In study of somatic embryos formation, all callus were grown in media contain 0.5 mg/L of 2.4-D. After one year of culture, the results showed that 6 mg/L of NAA slightly increase somatic embryos formation when 0.5 mg/L 2.4-D was added in both explants and callus culture media (T4). When 2.4-D was only added in media of callus culture, the formation of somatic embryos was low (T1). However, different results were shown from treatment 10 mg/L of NAA. The formation of somatic embryos were much better by the addition of 0.5 mg/L 2.4-D only in callus culture media (T2). When 0.5 mg/L of 2.4-D was added in media of explants and callus culture, the formation of somatic embryos were very low (T5). The highest concentration of NAA (12 mg/L) without addition of 0.5 mg/L of 2.4-D in explant media also result a lower somatic embryos formation (T3) (Fig. 5). From all of the factors that influence the success of *in vtro* propagation protocols, the choice of plant growth regulators and concentration has the most profound effect [9]. Auxin serve to induce the formation of embryogenic cells, possibly by initiating differential gene activation, and also appear to promote increase of embryogenic cell populations through repetitive cell division, while simultaneously suppressing cell differentiation and growth into embryo [13].

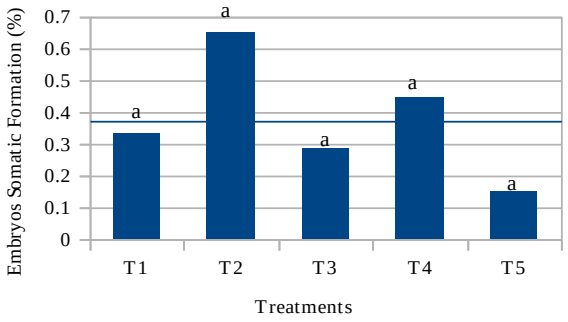

Figure 5. Effect of 2.4-D and NAA treatments on somatic embryos formation

Several studies has also been reported regarding the usage of 2.4-D in micropropagation of oil palm. The usage of zygotic embryos as explants sources produced the highest somatic embryos formation from callus grown on Y3 medium without or low concentration of 2.4-D [14]. The induction of somatic embryos in immature oil palm inflorescences of the *pisifera* palm occurred at a concentration of 500 µM 2,4-D [15]. Direct embryogenesis was achieved when the cotyledonary nodes of germinated immature zygotic embryos were cultured in dark for 8 weeks on Y3 medium supplemented with 40 µM of 2.4-D, 40 µM of NAA, 10 µM of 2,4,5-T, 10 µM of TDZ and 10 µM of BA [16]. In this experiment, although the frequency of somatic embryos formation among treatments did not different significantly, but on average, the highest percentage of embryo formation (0.65%) was found from treatment 10 mg/L NAA added with 0.5 mg/L of 2.4-D only in callus culture media (T2).

IV. Conclusion

Study of the usage of 2.4-D and NAA in oil palm micropropagation reveal that the frequency of somatic embryo formation was not only determined by the combination of 2.4-D and NAA concentration but also the phase/stages of the culture. When 2.4-D and NAA are given on the right phase of culture (explant or callus), the frequency of somatic embryo formation can be increased. Nevertheless, it should also consider the possibility of somaclonal variation of regenerants. Therefore, this result requires further study to evaluate the frequency of somaclonal variation of regenerants.

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References

[1] R. S. Guedes, T. L. Silva, Z. G. Luis, and J. E. Scherwinski-Pereira, "Initial requirements for embryogenic calluses initiation in thin cell layers explants from immature female oil palm

inflorescences," *Afr. J. Biotechnol*., vol. 52, no. 10, pp. 10774-10780, 2011.
[2] K. Kanchanapoom, A. Phongdara, and K. Kanchanapoom, "The effect of chitosan on the organogenesis of oil palm embryo-derived callus," *Bot. Hort. Agrobot. Cluj*., vol. 1, no. 38, pp. 213-217, 2010.
[3] N. Gorret, S. K. Rosli, S. F. Oppenheim, L. B. Willis, P. A. Lessard, C. Rha, and A. J. Sinskey, "Bioreactor culture of oil palm (*Elaeis guineensis*) and effects of nitrogen source, inoculum size, and conditioned medium on biomass production," *Journal of Biotechnology*, vol. 108, pp. 253–263, 2004.
[4] B. (de) Touchet, Y. Duval, and C. Pannetier, "Plant regeneration from embryogenic suspension cultures of oil palm (*Elaeis guineensis Jacq*)," *Plant Cell Rep.*, vol. 10, pp. 529–532, 1991.
[5] W. Patcharapisutsin and K. Kanchanapoom, "Somatic embryogenesis and plantlet regeneration from oil palm (*Elaeis guineensis* Jacq.) callus," *J. Sci. Soc Thailand.*, vol. 22, pp. 13-20, 1996.
[6] T. Murashige and F. Skoog, "A revised medium for rapid growth and bioassays with tobacco tissue culture," *Physiol. Plant*., vol. 15, pp. 473-497, 1962.
[7] E. Jaligot, A. Rival, T. Beul, S. Dussert, and J.-L. Verdeil, "Somaclonal variation in oil palm (Elaeis guineensis Jacq.): the DNA methylation hypothesis," *Plant Cell Reports.*, vol.19, pp. 684-690. 2000.
[8] N. T. Saieed, G. C. Douglas, and D. J. Fry, "Induction and stability of somaclonal variation in growth, leaf phenotype and gas exchange characteristics of poplar regenerated from callus culture," *Tree Physiol*., vol. 14, pp. 1–16, 1994.
[9] C. A. Beyl, "PGRs and their use in micropropagation," in *Plant Tissue Culture, Development and Biotechnology*, R. N. Trigiano and D. J. Gray, Eds., Taylor & Francis Group, New York: CRC Press, 2011, pp. 33-56.
[10] M. A. Norton and R. M. Skirvin, "Variation in tissue culture," in *Plant Tissue Culture, Development and Biotechnology*, R. N. Trigiano and D. J. Gray, Eds., Taylor & Francis Group, New York: CRC Press, 2011, pp. 543–550
[11] D. I. Rocha, E. Kurczynska, I. Potocka, D. A. Steinmacher, and W. C. Otoni, "Histology and Histochemistry of Somatic Embryogenesis," in *Somatic Embryogenesis: Fundamental Aspects and Applications*, V. M. Loyola-Vargas and N. Ochoa-Alejo, Eds., Springer, 2016, pp. 471-494.
[12] X. Yang and X. Zhang, "Developmental and molecular aspects of nonzygotic (somatic) embryogenesis," in *Plant Tissue Culture, Development and Biotechnology*, R. N. Trigiano and D. J. Gray, Eds., Taylor & Francis Group, New York: CRC Press, 2011, pp. 307–325.
[13] D. J. Gray, "Propagation from nonmeristematic tissues-nonzygotic embryogenesis," in *Plant Tissue Culture, Development and Biotechnology*, R. N. Trigiano and D. J. Gray, Eds., Taylor & Francis Group, New York: CRC Press, 2011, pp. 293–306.
[14] K. A. P. Bonetti, J. Nesi, R. C. Quisen, and M. Quoirin, "Somatic embryogenesis from zygotic embryos and thin cell layers (TCLs) of Brazilian oil palm (*Elaeis guineensis x Elaeis oleifera*)," *Afr. J. Biotechnol*., vol. 15, no. 37, pp. 2028-2037. 2016.
[15] J. B. Teixeira, M. R. Sondahll, and E. G. Kirby, "Somatic embryogenesis from immature inflorescences of oil palm," *Plant Cell Rep*., vol. 13, pp. 247-250, 1994.
[16] M. Jayanthi, N. M. Mohan, and P. K. Mandal, "Direct somatic embryogenesis and plantlet regeneration in oil palm," *J. Plant Biochem. Biotechnol*., vol. 2, no. 20, pp. 249-251. 2011.

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