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RESEARCH ARTICLE

Study of Oil Palm (*Elaeis guineensis* Jacq.) *In Vitro* Embryogenesis using Young Leaf Explants

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Abstract

This study reported *in vitro* embryogenesis of oil palm using young leaves as explants. Explants were grown in solid modified MS or Eeuwens medium containing different concentrations of NAA and 2,4-D, i.e. media C1, C2, C3, C4 and C5, M1, M2, M3 and M4, to induce embryogenic calli. Compact and pearly-white, globular calli were obtained from the youngest leaf explants 28 weeks after culture.

C1 media (MS medium + 107.41 µM of NAA + 100 $mg.L^{-1}$ of asparagine + 100 $mg.L^{1}$ of glutamine) produced the highest percentage of calli formation (30.56%), whereas C4 media (C1 supplemented with 67.86 µM 2,4-D) was the optimal media for embryogenic callus induction. Direct embryoids were obtained from slightly older leaf explants on the C3 media containing NAA after 36 weeks of culture. However, four subcultures using the same medium with gradual reduction of auxin concentration were not successful to develop embryogenic callus and embryoid cells during the course of this study.

Key words: Somatic embryogenesis, 2,4-D, NAA, immature leaves.

Introduction

Kernel oil from palm (*Elaeis guineensis* Jacq.) is the highest yielding oil-bearing crop, producing more than five times the yield of oil per year per hectare of any annual oil crop. Indonesia emerged as the world's largest producer of palm oil in 2007. Palm oil is a perennial monocot plant, originating from West and Central Africa (Hartley, 1988) that belongs to Arecaceae family (Dransfield et al., 2005).

Several explant sources have been used to establish propagation protocols of oil palm *in vitro,* including mature embryos (Rabechault et al., 1970), inflorescences (Smith and Thomas, 1973), roots and seedlings (Ong, 1977), and young leaves (Schwendiman et al. 1988; Staritsky, 1970). Earlier studies to produce clonal plantlet reported growth abnormalities of the *in vitro* plants (Corley et al. 1986). However, oil palm tissue culture techniques have undergone continuous improvement in recent years and produced clonal palms with minimal abnormality (Jones et al., 1995; Rival et al., 1998; 1999; Smith et al., 2013) from various explants. Plant propagation using explants from flower organs either anther or ovary through embryogenesis techniques have been successful although their regeneration is more difficult than that using young leaves (Perera et al., 2007, 2008; Texeira et al., 1994). However, the complete plant regeneration using the current protocols are still inefficient. The embryogenesis responses were influenced mainly by genotype and donor plant age (Ruslan, 1993), media, and source of explants. Further development of new protocols are still need to be developed for propagation of oil palm *in vitro*.

The objective of this study is to determine the optimum medium for callus induction and somatic embryo formation using young leaf explants. This study is a part of a long term project to establish a protocol of *in vitro* oil palm regeneration using young leaves, female flower, and zygotic embryo explants.

Materials and Methods

Source of explants

Young leaves were collected from 9 to 13-year-old oil palm trees growing at the IPB experimental station of Cikabayan, Darmaga, West Java, Indonesia.

A spear from oil palm "Tenera" was used as source of explants. The spear was removed from the tree and sterilized by spraying with 96% alcohol, wrapped and kept in a sampling bag for protection. The outermost leaves were carefully removed under sterile condition and discarded. Young leaves were isolated per cluster and sterilized in 10% sodium hypochlorite (5.25% w/v) for 10 minutes, followed by rising three times in sterile distilled water. Each leaf cluster was soaked in 10% sterile sucrose solution. The young leaflets were sliced into 10 mm length and planted into the callus induction media with four explants per jar.

Media

Two basal medium, Murashige and Skoog (1962) and Eeuwens and Blake (1976), supplemented with Naphthaleneacetic acid, glutamine, asparagine, picloram and 2,4-D were used for callus induction and embryo proliferation for all experiments. The media compositions tested in this study are described below:

C1: MS medium + 107.41 µM of NAA + 100 mg.L^{-1} of asparagine + 100 mg.L^{-1} of glutamine
C2: C1 supplemented with 22.62 µM of 2,4-D.
C3: C1 supplemented with 45.24 µM of 2,4-D.
C4: C1 supplemented with 67.86 µM of 2,4-D.
C5: MS + 450 µM of picloram + 500 mg.L^{-1} of glutamine + 0.3 g. $L^{-1}$of activated charcoal
C6: MS + 450 µM of 2,4-D + 500 mg.$L^{-1}$of glutamine + 0.3 g. $L^{-1}$of activated charcoal
M1: Y3 + 107.4 µM of NAA + 500 mg.$L^{-1}$of glutamine + 0.3 g. $L^{-1}$of activated charcoal
M2: Y3 + 22.62 µM of 2,4-D + 107.4 µM of NAA + 500 mg.$L^{-1}$of glutamine + 0.3 g.$L^{-1}$of activated charcoal
M3: Y3 + 45.24 µM of 2,4-D +107.4 µM of NAA + 500 mg.$L^{-1}$of glutamine + 0.3 g.$L^{-1}$of activated charcoal
M4: Y3 + 67.86 µM of 2,4-D +107.4 µM of NAA + 500 mg.$L^{-1}$of glutamine + 0.3 g. $L^{-1}$of activated charcoal

Media pH was adjusted to 5.7-5.8 before adding purified agar and charcoal. The treatments were arranged in a factorial randomized complete block design with three replications. The first factor is the leaf order within the explant, i.e. the 5^{th}, 6^{th}, 7^{th}, 8^{th}, and 9^{th}; the second factor is the types of media (described above). Cultures were incubated in the dark at 28±1 °C for 48 weeks and subcultured 12 weeks afterwards. The resulting calli were transferred to the same basal formulation medium every eight weeks.

Embryogenic calli and embryoids were maintained on the same basal medium with gradually reduced of 2,4-D and NAA concentrations during three subcultures of six week intervals. The 2,4-D and NAA were reduced by 50% in each subculture, followed by transferring to a new medium containing 2-iP without auxin. The explants were kept under light intensity of 1000 lux and maintained at 30^{0}C.

Scoring were conducted weekly for the percentage of contamination, and every eight weeks for the percentage of callus formation, the percentage of callus producing embryoid, the percentage of embryoid. The evaluation of callus formation, and culture response was performed at the end of each experimental period. Explants which were at least one-third oxidized or brown were scored as fully oxidized. Cultures which demonstrated cell proliferation on at least one-third of explants were recorded as undergoing callus induction. In addition, the callus structure (nodular, root-like, embryogenic), and the shapes of the resulted embryos were recorded.

Data analysis

Data were analyzed using ANOVA, and the means were separated with Duncan's multiple range tests (DMRT) at the 0.01 and 0.05 level of probability.

Results and Discussion

Callogenesis

Callus induction was first observed from young leaf number nine explants on medium C1, i.e. media containing NAA without 2,4-D, at 8 to 12 weeks after culture. These nodular and root-like calli had grey to dark grey and white color, and had a friable consistency.

Callus production varied from 1.39% to 30.56% depending on the combination of NAA and 2,4-D concentration, leaf order of the explant, and the type of basal media at week 39 (Table 1). Similar type of callogenesis was reported by Chehmalee and Techato (2007; 2008) in zygotic embryo of oil palm. C1 media gave the best percentage of explant bearing callus and the young leaf rank (leaf no 9 in this experiment) was more responsive than older leaf explants (leaf no five to eight in this experiment). Eighty-seven percent of calli were induced from MS media whereas Eeuwens media induced 13%.

Callus production in oil palm culture has been reported to be extremely slow for palms (Tisserat, 1987), and this was consistent with the results of our study. Type of media significantly affected the number of young leaf explants bearing callus (P=0.0001). The C1 media composed of MS as

basal medium and plant growth regulator NAA was optimal for callogenesis from oil palm immature leaf explants. However, no significant differences were observed amongst the order of oil palm leaves for callus production (P=0.53; Table 1).

Table 1. Effects of media types and young leaf rank of oil palm (*E. guineensis*) "Tenera" on percentage of explant bearing callus at 39 weeks after culture.

Leaf rank within the spear	Percentage of callogenesis (%)									
	MS medium**						Eeuwens medium**			
	C1	C2	C3	C4	C5	C6	M1	M2	M3	M4
5th leaf	11.11	0	0	2.78	0	0	1.39	0	0	0
6th leaf	1.39	0	0	0	0	0	0	0	0	0
7th leaf	6.94	0	0	1.39	0	0	0	0	0	0
8th leaf	12.50	0	0	0	0	0	1.39	0	0	0
9th leaf	30.56	0	0	0	0	0	6.94	0	0	0

** Media compositions are described in the Materials and Method.

Embryogenesis

Small, compact and pearly-white, globular calli was observed in culture derived from leaf number five at 28 weeks after culture. These pearly-white cells were obtained from C4 media containing both NAA and 2,4-D. Branton and Blake (1983) reported similar results from immature inflorescences of coconut explants, and by Teixeira et al. (1994) in immature inflorescences of oil palm. From all the obtained calli 3.63% of embryogenic callus were produced. Steinmacher et al. (2007) reported that crossings of plants or genotypes had important roles in somatic embryogenesis.

Different mass cells were observed from the explants at 36 weeks after culture. The cells consisted of direct large, compact and pearly-white globular callus (direct embryoids). These calli were obtained from leaf number six explants grown on C3 media, i.e. C1 media supplemented with 45.24 µM of 2,4-D. Indirect embryogenesis is more common in oil palm culture (George 1993). Direct embryogenesis is more desirable but more difficult to achieve than embryogenic callus.

No further development was observed in these embryogenic callus and embryoids after four subsequent subcultures onto the same medium with gradual reduction of auxin concentrations. These tissues were then transferred to new media containing 2ip (N^6-[2-isopentenyl] adenine) without auxin. These tissues maintained this characteristics when cultured in the same medium without changing the medium compositions during the course of this study.

Future studies have been conducted to determine the conditions of embryoid multiplication to ensure mass ramet production, and the results will be reported in the next paper.

Conclusion

MS medium + 107.41 µM of NAA + 100 mg.L^{-1} of asparagine + 100 mg.L of glutamine^{-1} (C1) was the optimal medium for callus induction, whereas C1 media supplemented with 45.24 µM of 2,4-D (C3) was the best media for direct embryoids production from young leaf explants. C1 supplemented with 67.86 µM of 2,4-D (C4) resulted in embryogenic callus formation from young leaf explants.

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