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In Vitro Callus Induction and Embryogenesis of Oil Palm (*Elaeis guineensis* Jacq.) from Leaf Explants

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This research was to study *in vitro* callus induction and somatic embryogenesis in oil palm from leaf explants. Young leaf segments from mature oil palm were cultured on MS medium supplemented with different concentrations of 2,4-D with or without addition of 2 g/l activated charcoal (AC) or 2,4-D and picloram. Embryogenesis induction was done using MS medium containing 2,4-D 450 µM and benziladenine 4.4 µM with 3g/l activated charcoal. The treatment of 2,4-D 15 µM resulted in the highest percentage of callus induction. The treatment of 2,4-D and AC showed that 2,4-D 450 µM and AC led to higher percentage of callus induction than that of 2,4-D 400 µM and 2 g/l AC. Embryogenesis occured in 27 out of 250 clumps of primary callus was occurred after 2-3 times subcultures. Somatic embryo development occurred when the embryogenic callus was transferred on the same basal medium supplemented with casein hydrolysate with 1 µM BA or growth regulator free basal medium with 2 g/l activated charcoal.

Key words: 2,4-D, picloram, *in vitro*, calllus induction, somatic embryogenesis, oil palm

INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq. var. tenera) is the highest-yielding and the most efficient oil-bearing crop in the world. Currently, oil palm has surpassed soybean to be the ranked first of plants producing edible oils. The mature fleshy orange-red oil palm mesocarp of the fruits contain 45-55% oil (Tan 2009). Under optimum conditions, the most productive crops will yield up to 5-7 tons of oil/ha/year (Rajesh *et al*. 2003). With more than 8 millions hectares cultivated worldwide, it is the second world source of vegetable oil, represents after soybean (Corley 2003). In the near future, when palm oil is used for either food or biofuel extensification of high yielding oil palm plantation are essentially needed to boost up the crude oil production. Therefore, superior tenera seedling or clone demand will be increase. Success of *in vitro* clonal propagation of oil palm seedling received great enthusiasm from oil palm industries (Rival & Parveez 2005). Furthermore, oil palm tissue culture techniques have great continuous improvement during the last two decades, so that production of clonal palm seedling with minimal abnormality was achieved (Rival *et al*. 1998).

Some reports determined that oil palm *in vitro* propagation through indirect somatic embryogenesis consist of several steps, i.e., callus induction, establishment of embryogenic callus or suspension cultures, somatic embryo development, maturation and germination (Rival & Parveez 2005; Te-chato & Hilae 2007). However, there are very limited technical information published due to its high commercial value.

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The aim of this study was to investigate the effects of plant growth regulators on *in vitro* primary callus induction, to establish embryogenic callus cultures, and somatic embryo development of oil palm. This study was an effort to establish a protocol of rapid clonal propagation of oil palm through somatic embryogenesis.

MATERIALS AND METHODS

Plant Materials and Sterilization. Selected tenera varieties of oil palm explants were provided by PT Matahari Kahuripan Indonesia (MAKIN) and PTPN VII. The plantations are located in Kalimantan and Sumatera, respectively. Immature leaf segments of 1 x 1.5 cm in size were used as explants. The explants were surface sterilized by dipping them in 1.05% NaOCl containing several drops of Tween 20 for 15 minutes followed by three times rinse with sterile distilled water. Sterile explants were cultured on the callus induction media.

Primary Callus Induction. Three experiments were set up to investigate the effects of plant growth regulator on callus induction. In the first experiment, the explants were cultured on Murashige and Skoog (MS) medium (Murashige & Skoog 1962) supplemented with 100 mg/l myo-inositol, 1 mg/l Ca-panthothenate, 1 mg/l nicotinic acid, 1 mg/l pyridoxine-HCl, 1 mg/l thiamine-HCl, 0.01 mg/l biotin, 30 g/l sucrose, 200 mg/l ascorbic acid, 50 mg/l citric acid, 15% coconut water and 2,4-D with concentrations of 5, 10, 15, 20, 30, and 50 µM. In the second experiment, the explants were cultured on MS medium with 400 and 450 µM 2,4-D supplemented with 2 g/l activated charcoal (AC). In the third experiment, the explants were cultured on MS medium with 1, 2, 3, 4, and 5 µM 2.4-D

combined with 1 µM picloram. The media that could induce primary callus was designated as primary callus induction medium (PCIM). Percentage of callus formation in all treatments was observed monthly up to 7 months of culture.

Embryogenic Callus Induction and Somatic Embryo Initiation. To induce embryogenic callus, clumps of primary callus resulted from the best treatment were cultured on embryogenic callus induction medium (ECIM). The ECIM is the PCIM containing 450 µM 2,4-D, 4.4 µM benziladenine (BA), 18 mg/l adenine and 3g/l AC. Subculture of callus on the same medium was conducted 2-3 times with 6 weeks interval until embryogenic callus formation occured.

Development, Maturation, and Germination of Somatic Embryos. To achieve somatic embryo development and maturation, clumps of callus with somatic embryos in early developmental stage were transferred to MS medium supplemented with 30 g/l sucrose and 2 g/l AC (EDM1) and MS medium supplemented with 1 µM BA (EDM2). All media were also supplemented with 100 mg/l glutamine and 500 mg/l casein hydrolysate. Some of somatic embryos were germinated on germination medium (GM) containing MS salts, 30 g/l sucrose, 2.2 M µM BA, 1.44 µM gibberelic acid (GA3) and 4.9 µM indolebutyric acid (IBA).

Media Preparation and Culture Conditions. All cultures for primary callus induction were incubated on 30 ml agar media (in 350 ml culture bottles capped with aluminum foil) in a dark culture room at 25 ± 2 °C. Whereas all cultures for embryogenic callus induction, somatic embryo initiation, development and germination were incubated at 25 ± 2 °C under 1500 lux white-fluorescent light.

RESULTS

Primary Callus Induction. Data from the first experiment showed that formation of callus was observed after 2 months of incubation. After 7 months of incubation, it was shown that increasing of 2,4-D concentrations from 5 to 15 µM led to the increasing percentage of callus formation, but higher concentration of 2,4-D up to 50 µM resulting decreasing percentage of callus formation (Table 1). The highest percentage of callus formation (34.2%) was obtained by the treatment of 15 µM 2,4-D, followed by 20 µM 2,4-D (32.9%), and 10 µM 2,4-D (26.7%). Morphology of Callus was compact or slimy and white in color (Figure 1).

Table 1. Effects of 2,4-D concentrations on the percentage of callus formation in tissue culture of oil palm using immature leaf segments as explants

2,4-D (µM)	Number of surviving explants	Number of explants producing callus	Percentage of explant producing callus
5	80	14	17.5
10	90	24	26.7
15	76	26	34.2
20	76	25	32.9
30	94	14	14.9
50	82	7	8.5

No activated charcoal was added in media.

Figure 1. Callus formed on immature leaf segments in tissue culture of oil palm. a and b were callus formed on leaf explants treated with MS medium without activated charcoal containing 10 and 15 µM 2,4-D, respectively.

Figure 2. Callus formed on immature leaf segments in MS medium containing 2,4-D 450 µM and 2 g/l activated charcoal.

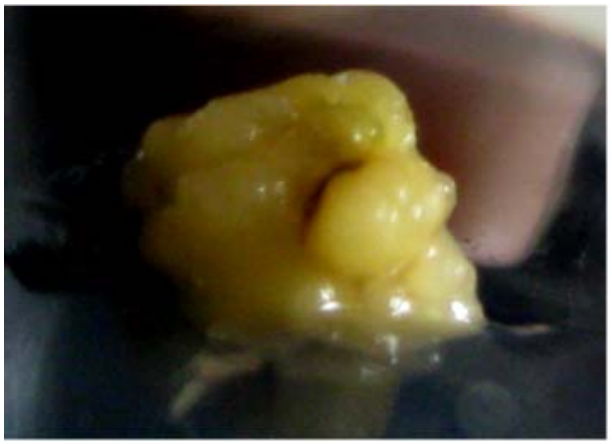

Figure 3. Clumps of callus on embryogenic callus induction medium (ECIM).

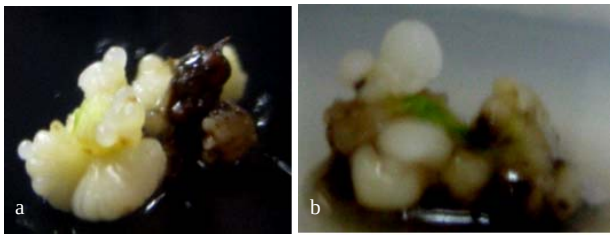

Figure 4. Clusters of oil palm somatic embryos in various stages (globular, torpedo, and heart-shaped) developed on: a) EDM1 (MS medium free of growth regulator containing 2 g/l activated charcoal) and b) EDM2 (MS medium containing BA 1 µM without activated charcoal).

Table 2. Effects of 2,4-D in high concentrations on the percentage of callus formation in tissue culture of oil palm using immature leaf segment as explants

2,4-D (µM)	Duration of culture (Months)	Number of surviving explants	Number of explants producing callus	Percentage of explant producing callus
400	7	93	7	7.5
450	7	96	19	19.8

Activated charcoal (2 g/l) was added in the culture media.

Table 3. Effects of low concentrations of 2,4-D in combination with 1 µM of picloram on the percentage of callus formation in tissue culture of oil palm using immature leaf segment as explants

2,4-D (µM)	Number of surviving explants	Number of explants producing callus	Percentage of explant producing callus
1	112	11	9.8
2	96	10	10.4
3	98	16	16.3
4	102	28	27.5
5	95	32	33.7

No activated charcoal was added in the culture media.

The first callus formed in the second experiment was observed from 2 months of incubation. After 7 months incubation, percentage of callus formation was higher on the media containing 450 µM 2,4-D (19.8%) than that of media containing 400 µM 2,4-D (7.5 %) (Table 2). Generally most callus formed was compact, white to yellowish in color (Figure 2).

Results of third experiment showed that increasing concentration of 2,4-D from 1 to 5 µM led to increasing percentage of callus formation. Combination of 1 µM of picloram and 5 µM of 2,4-D performed the highest percentage (33.7%) of callus formation (Table 3), however most of callus produced in this experiment were slimy in morphology.

Embryogenic Callus Induction and Somatic Embryo Development. After 12-18 weeks with subculture interval of 6-weeks, for about 10.8% (27 out of 250 clump callus) became embryogenic (Figure 3). When clumps of the embryogenic callus were transferred to medium for somatic embryo development (EDM1 or EDM2), various stages of somatic embryos (SE) i.e. globular, torpedo, and heart-shaped were observed within 6-8 weeks (Figure 4). Apparently, EDM1 was better than EDM2 for SE development indicated by more somatic embryos were formed on EDM1 with clear meristematic center on the tip. About 3% of the embryo clusters showed early stage of embryo germination after 4 weeks incubation on germination medium.

DISCUSSION

Somatic embryos can either differentiate directly from explants or indirectly after callus formation (Von Arnold 2008). In oil palm, somatic embryogenesis was reported mostly by indirect embryogenesis (Teixeira *et al*. 1999; Hilae & Te Chato 2005). Development process of somatic embryogenesis in oil palm occurred both on stage of requiring auxin and no/lower auxin (Rival & Parveez 2005). As in other plants such as peanut (Yusnita *et al*. 2006), sugarcane (Khan & Khatri 2006), rice (Meneses *et al*. 2005), or *Gentiana straminea* Maxim (Cai *et al*. 2009), formation of callus and embryogenic cell clusters in palm oil requires auxin, while their development and maturation do not need auxin or need lower auxin concentrations. Furthermore, Rival and Parveez (2005) described somatic embryogenesis in oil palm as a process consisting of several steps, i.e induction, embryogenesis, somatic embryo development and maturation, shoot development and rooting. In the induction stage, explants are induced to form primary callus in auxin-containing medium under dark condition. In the embryogenesis stage, the callus is also transferred to auxin-containing medium, but usually in lower concentrations and generally under light condition. In this step proliferated callus is promoted to develop into embryogenic cell clusters. In the development and maturation stage, embryogenic cell clusters are cultured on plant growth-free medium to allow them to develop to be embryos and to maturation process. Von Arnold (2008) also stated that plant regeneration via somatic embryogenesis consists of several steps; firstly initiation of embryogenic cultures by culturing primary explants on medium supplemented with plant growth regulator, mainly auxin or auxin + cytokinin, secondly proliferation of embryogenic cultures in medium supplemented with plant growth regulator, thirdly pre-maturation of SE in medium lack of plant growth regulator, then SE maturation in medium with ABA, and finally plant regeneration from SE in medium lack of plant growth hormone.

In somatic embryogenesis of oil palm, callus induction from various types of explant such as zygotic embryos, young infloresens, or young leaf segments occurs in the presence of auxin (s), either on full strength or half-strength MS basal medium (Rival & Parveez 2005). Various types and concentrations of auxins are employed to induce callus formation from different primary explants. In general, high auxin concentrations are always used in the presence of AC, whereas low auxin concentrations are used in the presence of ascorbic acid as an anti oxidant. The use of AC or ascorbic acid is to overcome browning of the explants, which is a major and common problem in palm tissue culture (Teixeira *et al*. 1994; Patcharapisutsin & Kanchanapoom 1996). Since AC is a non-selective adsorbent and could adsorb up to 99% of plant growth regulators amended in the media (Rival & Parveez 2005), the use of AC to prevent browning must be accompanied by the use of high concentration of auxin. AC can absorb toxic substances, which may be present in media ingredient, produced as a result of autoclaving, or exuded by cultured tissues (Moshkov *et al*. 2008). Teixeira *et al*. (1994) reported 6-13% of callus formation from young female oil palm infloresence explants using half strength MS medium containing 475-550 µM 2,4-D+3 g/l AC after 30 weeks (or 7 months) of culture. Patcharapisutsin and Kanchanapoom (1996) reported callus formation from oil palm mature zygotic embryo explants using half strength

of MS medium containing 0.5 g/l AC and 30 mg/l naphthaleneacetic acid (NAA) or 3 mg/l 2,4-D, however numerical data or percentage of callus formation were not presented. Using immature zygotic embryo as explants, Teixeira *et al*. (1999) established callus cultures on Y3 or MS medium containing 475-500 µM 2,4-D or 250 µM picloram with addition of 3 g/l AC. There was also no numerical percentage data presented on the reports. Hilae and Te Chato (2005) obtained primary callus formation from oil palm leaf explants on MS medium supplemented with 5 mg/l dicamba and 200 mg/l ascorbic acid.

This experiment was tried to achieve stages in oil palm somatic embryogenesis. The induction stage of primary callus formation was achieved after 7 months of culture on MS medium supplemented with 15 µM 2,4-D, 5 µM 2,4-D and 1 µM picloram without AC, or on MS medium containing 2 g/l AC and 450 µM 2,4-D. The percentage of callus formation was 34.2, 33.7, and 19.8%, respectively. Wong *et al*. (1996) reported that 20% of callus formation was achieved after 12 months of culture on MS medium with 2,4-D and NAA. Te-chato *et al.* (2004) reported 10% of callus formation and Corley and Tinker as cited by Low *et al*. (2006) reported 19% of callus formation.

To get into the embryogenesis step, the primary callus should be transferred to another medium formulation to enable them to become embryogenic and get proliferated. In our experiment this step was achieved when primary callus was put on embryogenic callus induction medium (ECIM) (Figure 3) even though they exhibited slow proliferation during the first 12 weeks of culture. Embryogenicity of the callus was indicated by development of embryo when they were transferred to embryo development medium (EDM) as indicated by the appearance of globular and heart-shaped embryos (Figure 4). This step of somatic embryogenesis was in the development and maturation stage.

When cultured on germination medium for 4 weeks, some of these embryos showed early stage of germination. They showed a meristem-like structure, green in color, and looked like an initiation of shoot growth.

Obtaining embryogenic callus is a key to success in micropropagation of oil palm through somatic embryogenesis. Therefore, identification of embryogenic callus is important. Rajesh *et al.* (2003) reported that embryogenic callus of oil palm was characterized by its whitish yellow in color and friable, while non-embryogenic one looked translucent and slimy. Our experiment showed that all of 2,4-D treatments or mixture of 2,4-D and picloram led to two different callus, i.e., compact or translucent and slimy callus. Therefore, the compact callus was used for embryogenesis experiment step. Convertion of 10.8% was achieved from primary callus to develop into embryogenic callus and somatic embryos after 18 weeks of cultures. Corley and Tinker (2003) as cited by Low *et al*. (2006), reported convertion of 6% and Wong *et al*. (1996) reported convertion of 3%.

In this study, the induction stage was established with 19.8 to 34.2% of primary callus induction. In the embryogenesis stage, the callus exhibited slow proliferation during the first 12 weeks of cultures. Therefore, further research is needed to find medium allowing fast proliferation of embryogenic callus as well as to find the optimum medium for embryo development, maturation and germination.

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