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RESEARCH ARTICLES

IN VITRO CULTURE OF EMBRYOS AND CALLUS OF OIL PALM (*ELAEIS GUINEENSIS* JACQ.)

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ABSTRACT

*Surface sterilization of oilpalm (*Elaeis guineensis *Jacq.) embryos with 40% aqueous solution of Clorox for 20 minutes produceda high percentage of uncontaminated embryos suitable for callus initiation. Shoots with well developed roots were produced when embryos were cultured on 1/2 MS (Murashige and Skoog,1962), MS and Y3 (Eeuwens 1976) media supplemented with 0.05% activated charcoal (AC). It was found that 1/2 MS and Y3 media gave better growth than MS medium. The inclusion of auxins and cytokinins in media containing AC resulted in improved growth of embryos. Balanced shoot and root development was obtained by supplementing media with NAA and BAP, with the optimal concentration being 10 mg/l. Callus was produced in the presence of 1-10 mg/l 2,4-D and 30-70 mg/lNAA on 1/2 MS medium supplemented with 0.05% AC. The presence of an auxin was found to be essential for culture of oil palm callus and 2,4-D was superior to NAA.*

INTRODUCTION

Oil palm (*Elaeis guineensis* Jacq.) is an important source of vegetable oil. It is one of the major oil producing crop in theworld and accounts for about 15% of the world's total production of vegetable oil[1]. In Thailand, oil palm is produced mainly in plantations in the south. The average rate of increase in plantation area is 75% annually. Such a high increase rate causes several problems, in particular, the lack of good quality seedlings. This palm is generally propagated by seeds hence wide variation in the field is commonly due to the heterozygosity of the seedlings. Moreover, seed germination of some genotypes e.g. *pisifera* and *tenera* has been reported to be poor. Oil palm cannot be propagated vegetatively unlike date palm[3] and sago palm[4] that can be propagated from suckers and give rise to a more uniform planting.

Vegetative propagation using *in vitro* techniques could play an important role in this regard. Embryo culture, which involves excising an embryo aseptically from the seed and transferring it to a sterile nutrient medium, has proved useful to increase the number of

seedlings. Embryo culture of palm holds promise for shortening germination time and it is also an important prerequiste for cryopreservation of germplasm. The use of embryo culture is interesting not only for an embryo rescue program but also for its use as a convenient source of explant material for callus initiation.

In this paper we report the culture of embryos and callus of oil palm. This study is part of a larger program designed to investigate the strategies for reliable, efficient methods for clonal multiplication of oil palm. Attempts will be made to develop the somatic embryogenesis process from callus culture for plant regeneration.

MATERIAL AND METHODS

Plant material

The most commonly used seedling in oil palm plantation is the *tenera* hybrid obtained from crosses between *dura* and *pisifera*. Mature tenera nuts (seed + endocarp) were collected from a garden in Changwat Krabi, Southern Thailand and dehusked manually. The exposed hard endocarp (or shell) was cracked open with a hammer. The kernels were recovered with a pair of clean forcep and placed in a conical flask for surface sterilization. They were immersed in 70% ethanol and then cut into small cubes. These cubes were surface sterilized in 10-50% Clorox solution containing 1-2 drops of Tween-20 per 100 ml solution for 20 minutes, followed by several washings with sterile distilled water. The sterile cubes containing intact embryos were then transferred to sterile distilled water and kept for 24 hours prior to excision.

Embryo culture media

Embryos within kernels were lifted out a septically on ascalpel blade and transferred immediately to a number of different media including :

(1) Murashige and Skoog (1962)[5] medium (MS)
(2) 1/2 MS medium
(3) 1/2 MS medium + auxins (0.1, 1, 5, 10, 15 mg/l 2,4-D/NAA)
(4) 1/2 MS medium + cytokinins (0.1, 1, 5, 10, 15 mg/l kinetin/BAP)
(5) Eeuwens (1976)[6] medium (Y3)

Callus induction media

In order to induce callus production, embryos were cultured on different media as follows :

(1) 1/2 MS medium + 2,4-D (1, 3, 5, 7, 10 mg/l) + 3% sucrose + 0.05% activated charcoal (AC), pH 5.6
(2) 1/2 MS medium + 3 mg/l 2,4-D + 3% sucrose, pH 4.6, 5.6, 6.6
(3) MS medium + 3 mg/l 2,4-D + 3% sucrose, pH 4.6, 5.6, 6.6
(4) 1/2 MS medium + 3 mg/l 2,4-D, pH 5.6 + sucrose (3, 4.5, 6%)
(5) MS medium + 3 mg/l 2,4-D, pH 5.6 + sucrose (3, 4.5, 6%)

(6) 1/2 MS medium + NAA (30, 50, 70 mg/l) + 3% sucrose + 0.05% AC, pH 5.6

All media were solidified with 0.2% Gelrite with or without AC. The concentration of sucrose was 3% and the pH of the media was adjusted to 5.6 unless otherwise stated. The media were autoclaved at 121° C for 15 minutes.

Culture conditions and data treatment

All the cultures were incubated at 25 ± 1° C in a temperature-controlled room with a 16-hours photoperiod provided by Gro-lux light. The initial cultures and subcultures were incubated for eight weeks, data being recorded at these intervals. One embryo was planted per culture. All experiments were carried out at least three times with twenty cultures per treatment. Morphogenetic responses such as germination were recorded as percentage. Average score of callus formation was calculated from the following score levels

Score level	Meaning
0	dead tissue / no callus formation
1	size of callus clump (C) < 5 mm
2	5 < C < 8 mm
3	8 < C < 11 mm
4	11 < C < 14 mm
5	C > 14 mm

RESULTS

1. Surface sterilization of explants

When the hard endocarp was cracked open, the endosperm, in which embryo was embedded, was cut into small cubes and surface sterilized in 10-50% Clorox. Embryos were then excised aseptically and cultured on MS medium. It was found that 40% and 50% Clorox gave 95% of uncontaminated tissues and 40% Clorox produced the best callus growth (Table 1). Therefore we routinely used 40% Clorox for 20 minutes for the sterilization of seeds.

2. Effects of activated charcoal

Within 7-10 days after excision, enlargement of the embryo was observed. Germination usually occurred during the first 4-8 weeks but some embryos did not germinate completely although they were recultured to fresh medium. Embryos germinated in all media with or without AC. However, te best growth of seedlings was achieved on medium containing AC which resulted in improved germination and produced balanced growth of shoot and root (Fig. 1a), while the shoots in media lacking AC did not root (Fig. 1b). The root systems of seedlings were charcaterized by single, slender primary roots and usually grew vigorously. Moreover, in the presence of AC, the growth of seedlings grown on 1/2 MS and Y3 media was better than that on MS medium (Table 2). Hence we used either 1/2MS orY3 media in combination with AC for further experiments.

TABLE 1 Sterilization of embryos using various concentrations of commercial Clorox for 20 minutes.

Concentration of Clorox (%)	Uncontaminated tissue (%)	Contaminated tissue (%)	Dead tissue (%)	Embryo-derived callus (%)
10	65	35	0	65
20	75	25	0	75
30	85	15	5	80
40	95	5	0	95
50	95	5	10	85

TABLE 2. The effects of AC on growth of embryos cultured on 1/2 MS, MS and Y3 media.

Experimental units		Germination	Average (cm)	
Medium	AC	(%)	Shootheight	Root length
1/2 MS	+	90	2.60	1.71
1/2 MS	-	85	1.10	0.00
MS	+	85	2.20	1.60
MS	-	75	1.10	0.00
Y3	+	90	2.88	5.54
Y3	-	80	1.66	0.02

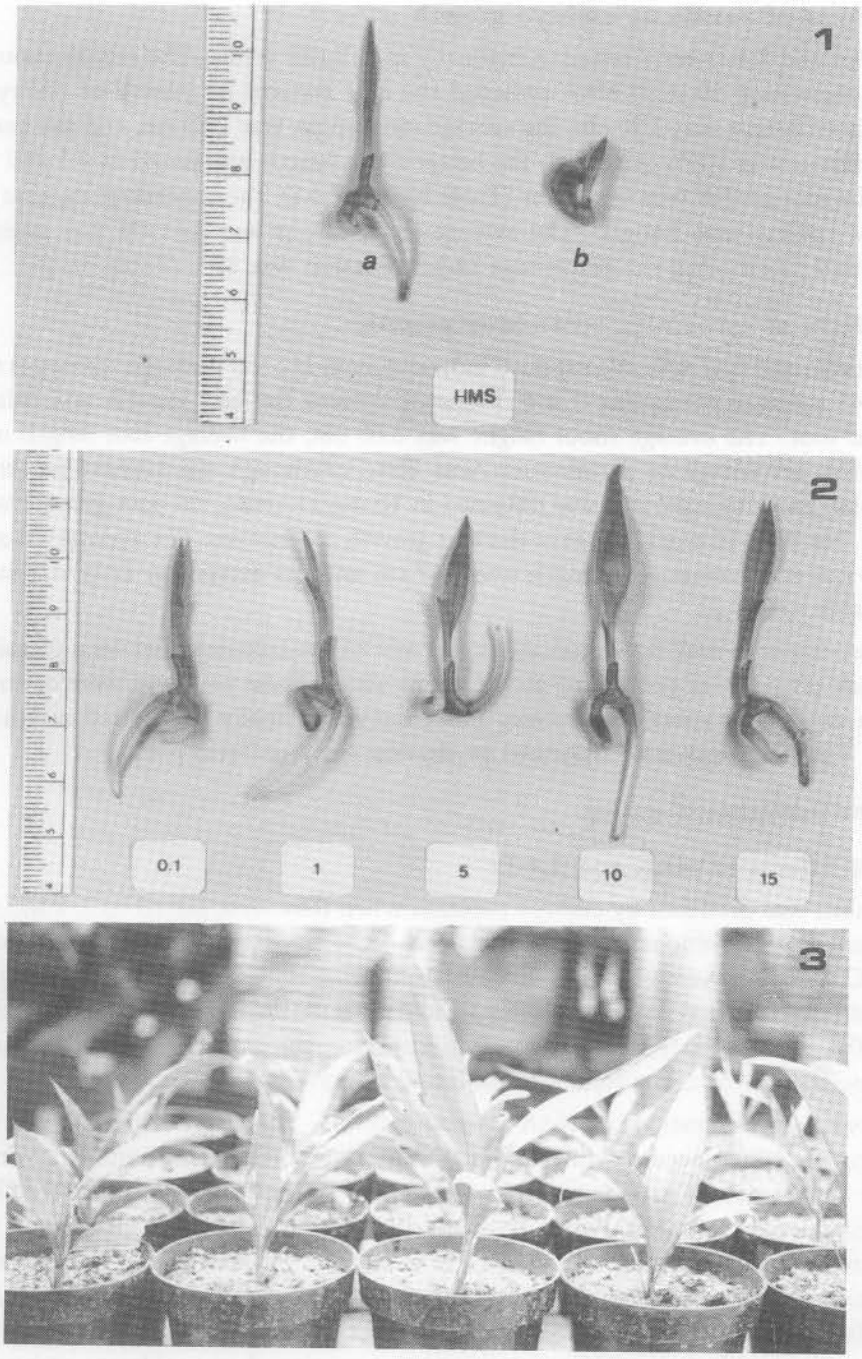

Fig.1 Seedlings cultured on 1/2 MS medium showing shoot with root (a, plus AC) and shoot only (b, minus AC).

Fig.2 The effects of BAP on growth of embryos.

Fig.3 Seedlings cultured on potting soil.

3. Effects of auxins on embryo growth

NAA and 2,4-D were tested individually in 1/2 MS media. The results showed that medium containing 10 mg/l NAA provided the best support of growth of embryos. The average shoot height was 2.98 cm, the average root length was 2.43 cm, and the percentage of germination was 100% Although the better shoot length was found in 0.1 and 1.0 mg/l NAA, the root length was very poor (Table 3). For 2,4-D, the concentration that favored growth of embryo was 1 mg/l. The average shoot height was 3.41 cm, the average root length was 2.52 cm, and the percentage of germination was 85% (Table 3).

4. Effects of cytokinins on embryo growth

Kinetin and BAP were also applied individually in 1/2 MS medium. Embryos cultured on 1/2 MS medium containing 1 mg /l kinetin showed the best growth and balanced of shoot and root. The average shoot height was 3.05 cm, the average root length was 2.50 cm and the percentage of germination was 85%. Although the highest percentage of germination and shoot growth was obtained in 15 mg/l kinetin, the root growth was poor (Table 4). For BAP, 10 mg/l BAP gave the best growth of embryos. The average shoot height was 3.98 cm, the average root length was 2.47 cm and the percentage of germination was 100% (Table 4, Fig. 2).

Seedling with well developed shoot and root in the culture vessel were transferred to small black plastic bags containing sterile vermiculite. These seedlings were covered with a transparent plastic sheet and watered twice daily and finally transferred to potting soil (Fig. 3). They subsequently continued to develop fairly uniform plants.

5. The initiation of callus

5.1 Effects of NAA and 2,4-D

Callus was produced by embryo explants when they were placed on 1/2 MS medium supplemented with various concentrations of 2,4-D (1, 3, 5, 7, 10 mg/l), 3% sucrose, and 0.05% AC. The calli were visible 45-60 days after initial culture. The primary callus in each treatment was creamy yellow, more compact and grew more slowly than the original explant (about 50% increase in fresh weight in 2 months compared to the original explant). The highest percentage of callus formation in 3 and 5 mg/l 2,4-D was 85% (Table 5).

Several concentrations of NAA were also tested. The callus initiated from medium containing NAA produced a white, more friable callus unlike those initiated from 2,4-D. The best callus growth was obtained in 30, 50 and 70 mg/l NAA, respectively (Table 6). Embryonal shoot was not inhibited by 30 mg/l NAA thus callus was apparent at the base of the green shoot. An increase of NAA to a level of 50 and 70 mg/l decreased shoot development. Morphological studies of callus formation are insufficient to ascertain the origin of cells that gave rise to callus. However, it seemed that calli were visible on both the blunt and the tapered end of the cultured embryos and continued to proliferate and eventually covered the zygotic embryo.

TABLE 3. The effects of auxins on growth of embryos cultured on 1/2 MS media.

Auxins (mg/l)		Germination (%)	Average (cm)	
			Shoot height	Root length
NAA	0.1	90	4.07	1.88
	1	85	4.25	1.46
	5	100	2.71	1.86
	10	100	2.98	2.43
	15	100	2.73	1.32
2,4-D	0.1	80	3.17	1.51
	1	85	3.41	2.52
	5	75	2.07	1.28
	10	90	0.60	0.00
	15	90	0.00	0.00

TABLE 4. The effects of cytokinins on growth of embryos cultured on 1/2 MS media.

Cytokinins (mg/l)		Germination (%)	Average (cm)	
			Shoot height	Root length
Kinetin	0.1	90	3.07	2.36
	1	85	3.05	2.50
	5	80	2.77	2.50
	10	90	2.80	2.36
	15	100	3.18	1.07
BAP	0.1	90	2.65	2.05
	1	95	2.79	2.34
	5	100	3.27	1.90
	10	100	3.98	2.47
	15	95	3.31	1.92

TABLE 5. The effects of 2,4-D at various concentrations on callus formation of embryos cultured on 1/2 MS media.

2,4-D (mg/l)	Callus formation (%)	Average score of callus
1	70	1.97
3	85	2.10
5	85	2.00
7	70	2.06
10	70	2.10

TABLE 6. The effects of NAA at various concentrations on callus formation of embryos cultured on 1/2 MS media.

NAA (mg/l)	Callus formation (%)	Average score of callus
30	91	2.51
50	89	2.37
70	80	2.32

TABLE 7. The effects of pH on callus formation of embryos cultured on 1/2 MS and MS media.

Media	pH	Callus formation (%)	Average score of callus
1/2 MS	4.6	75	1.80
	5.6	80	1.90
	6.6	55	1.54
MS	4.6	50	1.90
	5.6	55	2.00
	6.6	45	1.66

TABLE 8. The effects of sucrose on callus formation of embryos cultured on 1/2 MS and MS media.

Media	Sucrose conc.(%)	Callus formation (%)	Average score of callus
1/2 MS	3	80	1.90
	4.5	65	1.84
	6	90	2.36
MS	3	55	2.00
	4.5	45	2.11
	6	75	1.80

5.2 Effect of pH

Excised embryos were cultured on both 1/2 MS and MS media supplemented with 3 mg/l 2,4-D and 3% sucrose. The pH was adjusted to 4.6, 5.6, 6.6 before autoclaving. The results revealed that pH 5.6 in both media gave the highest percentage of callus formation at 80% and 55%, respectively (Table 7).

5.3 Effect of sucrose

The media used in this experiment was the same as that in 5.2 except for that sucrose concentrations were 3%, 4.5% and 6%. Inboth media, 6% sucrose showed the highest percentage of callus formation. However, 3% sucrose appeared to be adequate for callus growth (Table 8).

DISCUSSION

Contaminants have been found to be a considerable problem in oil palm cultures since the seeds generally contain large amount of fungi and bacteria, thus surface sterilization is a time-consuming process. The use of 40% Clorox for 20 minutes could overcome this problem and was found to be satisfactory.

Another problem encountered during *in vitro* culture of embryo was the difficulty of excising uninjured embryos since they were embedded in hard endosperm. Imbibition of embryo within the endosperm for 24 hours in sterile water seemed to increase the numbers of undamaged embryos. Rehydrated embryos swelled and could be easily isolated from the haustorial region of the embryos. Rabechault *et al.*[7] found that embryos developed satisfactorily *in vitro* if the moisture content of the seeds was raised to 20-22% before removal of the embryos. In addition, storage of rehydrated seeds for 10-15 days further improved subsequent growth of the embryo indicating that rehydration of embryos within seeds was superior to their rehydration on nutrient media.

In this study oil palm embryos germinated *in vitro* and most of them required 4-8 weeks to do so. Successful cultures of embryos were obtained only in media containing 0.05% AC. In fact, the inclusion of AC seems to be significant on culturability of several

plant species e.g. moss protonema[8], *Phaelaenopsis* and *Dendrobium*[9], datepalm[10, 11], and oil palm[12]. The effect of AC is attributed to adsorption by AC of toxic metabolites released by the plant tissues[9]. Browning of cultured embryos was evident if the intact embryos were injured and this apparently inhibited the growth of the cultured tissues.

The requirements for germination of excised embryos have been worked out. A balanced hormone supply was required to maintain normal root and shoot development. The effects of NAA, 2,4-D, kinetin and BAP have been compared and results showed that BAP was comparable or superior to the others in oil palm embryo culture. The level of response to a given treatment was variable but embryos could be readily induced by these plant growth regulators. Slow growth and poor root development of embryo *in vitro* was also reported in sago plm[4], coconut[13], and date[14]. The incorporation of AC in a culture medium appeared to promote root development. Therefore, improved vigor in medium containing AC may be an indirect consequence of the development of the root system. This is in agreement with makapuno coconut[15]and date[3].

It is well understood that vegetative propagation of palms proceeds from a callus phase, through a process of somatic embryogenesis, to the production of plantlets. To obtain plantlets from these processes, callus formation must be achieved. Seed embryos and aseptically grown seedlings developed from embryo culture have been used as a source of explants[16,17].

Calli from all explants were amenable to subculture, but were slow growing in comparison with other species. Apparent doubling times of 40 or 50 days were usual. Attempts were made to induce maximum callus production through the addition of auxins and cytokinins to nutrient media. An auxin was clearly essential for callus initiation while cytokinin tended to inhibit callus formation[18]. Likewise, in our study 2,4-D was found to be superior to NAA for promoting callus production. This is in contrast to Alang[4] who reported that no callus was produced by sago embryos cultured on medium containing AC. Inaddition, Smith and Thomas[18] concluded that initiation of callus required a more vigorous auxin than its subsequent maintenance.

Growth of callus was observed over a wide pH range, from 4.6 to 6.6, and this is in concurrence with Smith and Thomas[18] who reported that no apparent optimum pH for growth of callus between pH 3.7 and 7.3. Sucrose appeared to be the best source of carbon and energy, giving vigorous callus gowth in media containing 3% to 6% sucrose.

In conclusion, the use of embryo culture technique to produce desirable oil palm suitable for breeding program and extension work has been accomplished. The technique can shorten the length of germination time required to obtain a seedling. The promising results obtained could therefore feasibly be extended to other palm species.

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บทคัดย่อ

การฟอกฆ่าเชื้อที่ผิวเอ็มบริโอปาล์มน้ำมันด้วยคลอรอกซ์เข้มข้น 40% เป็นเวลา 20 นาที ให้เปอร์เซนต์ของเนื้อเยื่อที่ไม่ปนเปื้อนและการเกิดแคลลัสสูงสุด เมื่อเพาะเลี้ยงเอ็มบริโอบนอาหารสูตร 1/2 MS (Murashige และ Skoog 1962), MS และ Y3 (Eeuwens 1976) ที่มีผงถ่าน 0.05% พบว่าเจริญเป็นต้นกล้าพร้อมรากที่สมบูรณ์ อาหารสูตร 1/2 MS และ Y3 จะให้การเจริญเติบโตของเอ็มบริโอดีกว่าอาหารสูตร MS การเติมสารควบคุมการเจริญเติบโตของพืชกลุ่มออกซินและไซโทไคนินในอาหารที่มีผงถ่านจะยิ่งช่วยการเจริญเติบโตของเอ็มบริโอ ในอาหารที่มี NAA และ BAP ความเข้มข้นอย่างละ 10 มิลลิกรัมต่อลิตร จะช่วยให้ต้นและรากเจริญอย่างสมดุล แคลลัสจะเกิดขึ้นเมื่อเลี้ยงเอ็มบริโอบนอาหารสูตร 1/2 MS ที่มี 2,4-D ความเข้มข้น 1-10 มิลลิกรัมต่อลิตร และ NAA 30-70 มิลลิกรัมต่อลิตร ร่วมกับผงถ่าน 0.05% การมีออกซินนั้นมีความจำเป็นต่อการเพาะเลี้ยงแคลลัสปาล์มน้ำมัน และ 2,4-D จะให้ผลดีกว่า NAA