ORIGINAL ARTICLE

Microcolony formation from embryogenic callus-derived protoplasts of oil palm

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Abstract

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Microcolony formation from embryogenic callus-derived protoplasts of oil palm
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Embryogenic callus of oil palm induced from young leaves of seedlings DxP was used as initial material for protoplast isolation. Various combinations of cellulase Onozuka RS and macerozyme R-10 were tested. Isolated protoplasts were cultured by various methods in MS medium supplemented with different phytohormones. The results revealed that 2% cellulase RS in combination with 2% macerozyme R-10 (adjusted osmoticum to 0.4 M by manitol) yielded the highest number of viable protoplasts ($1x10^7$ per gram fresh weight). Dicamba at concentration 2 mg/l with 1 mg/l 6-benzyladenin (BA) containing in phytagel semi-solidified MS medium promoted the highest division of 2.3-4.0%. First division of the protoplasts was observed at 4 days after culture. Microcolony formation (8-10 cells) was seen after three weeks of culture. Unfortunately, neither callus formation nor plantlet regeneration were obtained.

Key words : oil palm, embryogenic callus, protoplast, microcolony, dicamba

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บทคัดย่อ

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การสร้างโคโลนีขนาดเล็กจากโปรโตพลาสต์ที่แยกจากเอ็มบริโอเจนิคแคลลัสปาล์มน้ำมัน

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ในการแยกโปรโตพลาสต์ปาล์มน้ำมันในการศึกษานี้ใช้เอ็มบริโอเจนิคแคลลัสที่ชักนำจากการเพาะเลี้ยงใบอ่อนของต้นกล้าปาล์มน้ำมันพันธุ์ DxP มาแยกโปรโตพลาสต์ใช้เซลลูเลสโอโนซูกะอาร์เอสร่วมกับมาเซอร์โรไซม์อาร์ 10 ความเข้มข้นต่าง ๆ นำโปรโตพลาสต์ที่แยกได้ไปเพาะเลี้ยงด้วยวิธีการต่าง ๆ ในอาหารสูตรมูราชิเกะและสกุค (MS) เติมสารควบคุมการเจริญเติบโตหลายชนิด จากการศึกษาพบว่า เซลลูเลสเข้มข้น 2% ร่วมกับมาเซอร์โรไซม์อาร์ 10 เข้มข้น 2% (ปรับความเข้มข้นออสโมติคัม 0.4 โมลาร์ด้วยแมนนิตอล) ให้จำนวนโปรโตพลาสต์ที่มีชีวิตสูงที่สุด ($1x10^7$ ต่อกรัมน้ำหนักสด) การเติมไดแคมบาความเข้มข้น 2% ร่วมกับเบนซิลอะดินิน (BA) ในอาหาร MS กึ่งแข็ง (เติมไฟตาเจล) ส่งเสริมการแบ่งเซลล์ครั้งแรกสูงที่สุด 2.3-4% การแบ่งเซลล์ครั้งแรกปรากฏให้เห็นภายหลังการเพาะเลี้ยง 4 วัน พัฒนาการของโคโลนีขนาดเล็ก (8-10 เซลล์) ตรวจพบหลังการเพาะเลี้ยงเป็นเวลา 3 สัปดาห์ อย่างไรก็ตามไม่พบการสร้างแคลลัส และพัฒนาให้พืชต้นใหม่จากโปรโตพลาสต์

ภาควิชาพืชศาสตร์ คณะทรัพยากรธรรมชาติ มหาวิทยาลัยสงขลานครินทร์ อำเภอหาดใหญ่ จังหวัดสงขลา 90112

Recently, the success in propagation of oil palm through tissue culture techniques has been widely reported using various plant parts, e.g. young leaves (Martin and Rabechault, 1976; Lioret, 1981; Te-chato, 1998b; Te-chato *et al*., 1988; Nualsri *et al*., 1988), roots (Ong 1975; Smith and Thomas, 1973), shoots (Ong, 1975; Martin and Rabechault, 1976; Starisky, 1970) and zygotic embryos (Rabechault and Cas, 1974; Te-chato, 1998a). The ability to propagate oil palm from both young leaves and zygotic embryos through somatic embryogenesis creates a new approach to its propagation and breeding. The development of plant cell technologies, especially protoplast technique, can be used as a tool for producing protoclone and somatic hybrid in oil palm. So far, only few authors have reported isolation and culture of protoplasts of oil palm (Te-chato *et al*., 1989; Sambanthamurthi *et al*., 1996). Many sources of explants were reported to be used for isolation of the protoplasts of oil palm (Sambantha murthi *et al*., 1996). Among them, embryogenic callus yielded the best results both in number and viability of the protoplast. Recently, Aberlenc-Bertossi (1999) was success in induction of embryogenic suspension. Embryogenic cell/mass in suspension culture will be one of the good candidate for isolation and culture of the protoplasts. However, the former reports were very complex in both isolation and culture techniques. In the present study we described the simplification and effectiveness of digestive enzymes to maximize a high yield of protoplasts, culture techniques and phytohormones for its division and microcolony formation.

Materials and Methods

Induction of primary and embryogenic callus

Young leaves of oil palm seedling were cultured on Murashige and Skoog (MS) medium containing 3% sucrose, 200 mg/l ascorbic acid and 1-4 mg/l dicamba (Di) (designated as MS-P). Primary calli initiated from cut surface and veinlet of the explant after 6 weeks of culture were subcultured montly to fresh medium of the same components (except for the concentration of Di was decreased to 0.1-1 mg/l) (designated as MS-E) for 6 times until embryogenic callus appeared. Embryogenic callus was maintained in MS-E by regularly subculturing at 6-8 weeks intervals under 1500 lux illumination, 14 h photoperiod and

26±4ºC. After culture of the callus for 6 months (Figure 1) they were subjected to protoplast isolation procedure

Enzyme preparation and incubation conditions

Various concentrations of wall-digesting enzyme, cellulase Onozuka RS and macerated enzyme, macerozyme R-10 were compared to optimize a high yield of viable protoplasts. Each combination of the enzyme was dissolved in a solution of 0.4 M mannitol, the pH adjusted to 5.7 and the solution sterilized by passing through a Millipore filter of pore size 0.45µm. Ten ml of the enzyme solution was used to incubate 1 gram fresh weight of embryogenic callus. The incubation took place in 6 cm sterile plastic Petri-dishes. The mixture of cell or tissue and enzyme solution was incubated on a gyratory shaker at 70 rpm under 500 lux illumination at 26±4ºC.

Protoplast isolation and culture

After incubation for 2 hours the resulting solution was then passed through a 30-µm nylon mesh to separate protoplasts from cell debris and aggregates. The filtrate containing intact protoplasts was centrifuged at ca. 800 rpm for 3 min. The supernatant was discarded and the pellet was resuspended in 10 ml of washing solution consisting of 0.4 M mannitol. This washing sequence was repeated twice. The final protoplast suspension was purified by floating on 21% sucrose. The complete protoplasts at mid-phase were collected and brought to a known volume and aliquots were taken to measure protoplast yield and viability. In the case of culture, the protoplasts were washed twice with washing solution and once with culture medium, then embeded in 0.2% Phytagel semi-solidified MS medium supplemented with 0.4M mannitol, 3% sucrose, 1-3 mg/l NAA or 2,4-D or dicamba and 1 mg/l BA. In the other cases the protoplasts were cultured as thin layers (3-5 ml) or embeded in Seaplaque agarose (low melting point agarose) in 6 cm sterile plastic Petri-dishes.

Yield and viability determination

Yield of the protoplasts was determined by counting with hemacytometer. Viability was assessed using fluorescein diacetate (FDA) as a test of membrane integrity and internal diesterase activity. After 10-15 min in 0.01% (w/v) FDA in culture medium, protoplasts were observed under ultraviolet or green light using fluorescence microscope. The percent viability was calculated as the number of protoplast fluorescing green per total number of intact protoplasts existing x 100.

Determination of optimum enzymes

In this study, two-week-old embryogenic callus after subculture was chopped into small pieces with razor blade and exposed to a mixture of enzyme solution consisted of 1 or 2% cellulase Onozuka RS in combination with 1 or 2% mcerozyme R-10. An osmoticum of all the enzymes combinations was adjusted to 0.4 M by mannitol. The calli were incubated in the same way as described in enzyme preparation and incubation conditions for 2 hours. Then the number and viability of protoplasts at different period after subculture were determined.

Determination of phytohormones and culture methods

Three types of auxin, 2,4-D, NAA and dicamba at concentration of 2 mg/l were tested in combination with 1 mg/l BA. Culture medium used in this present study was MS supplemented with 3% sucrose and adjusted osmoticum to 0.4 M by mannitol. Protoplasts at the density of $5x10^5$ /ml were cultured by 3 different methods, thin layer, embedding and algenate bead. Plating efficiency, percentage of isolated protoplasts undergoing division, was estimated after one week in culture. At the time of plating, random fields of protoplasts in agar were marked by etching a circle 1.2 mm in diameter around the area of interest on the plastic petri-dish. The number of protoplasts in each field was counted. After three weeks, the number of dividing protoplasts in each field was determined. The plating efficiency was calculated as the number of dividing protoplasts by the number of protoplasts plated, and multiplying by 100.

In another experiment, various concentra-

tions of dicamba (1, 2 and 3 mg/l) were combined with two concentrations of BA (0.5 and 1 mg/l) in order to find out an optimum concentration of the two enzymes. Density of the protoplasts was adjusted to $5x10^5$/ml and cultured in phytagel semi-solidified medium. Plating efficiency was determined as described above.

Result and Discussion

Determination of optimum enzymes

Increasing concentration of cellulase from 1 to 2 % yielded higher number of released protoplasts. Similar result was obtained when concentration of macerozyme increased to 2%. A 2% of cellulase in combination with 2% macerozyme gave the best result in the number of isolated protoplasts. In addition, the highest viability of the protoplasts was obtained in this combination of the enzymes. Generally, 1% of macerozyme is sufficient for maceration the tissue to be single celled (Te-chato, 1989). For oil palm, embryogenic callus was very tough and some produced phenolic compounds. These might hamper maceration action of the enzyme or decreasing activity of the enzyme during incubation. Accordingly, a high concentration of the enzyme was required. However, 2% cellulase Onozuka RS in combination with 2 % macerozyme gave an optimum results in yield and viability of protoplasts (Table 1). Pectolyase Y-23 is an alternative macerated enzyme that can enhance the efficiency of isolation of protoplasts in many plant species (Ishii, 1989). This enzyme should be used in combination with macerozyme at a low concentration 0.1-0.5% (Te-chato, 1989). Unfortunately, this experiment did not used pectolyase Y-23. However, it is recommended this enzyme be tried for optimization of yield and viability of protoplast. In this experiment, it was clear that both yield and viability are high enough for further investigation. Size of protoplasts was heterogeneous, ranging from 20 to 80 μm (Figure 2A). This might be due to the different sizes of cells in embryogenic callus. Some were from embryogenic tissues while some were from non-embryogenic ones or fast growing callus (FGC) before developing to embryogenic ones. However, all sizes of the protoplasts were viable under fluorescent microscopy of green light/ excitation (Figure 2B). Moreover, the size of more than 80 μm occurred from fusion of the smaller protoplasts. This evidence was markedly observed in zygotic embryo-derived protoplasts (data not shown).

Determination of phytohormones and culture methods

The protoplasts were plated in semi-solid MS medium supplemented with various concentrations of Di and BA. The result showed that Di at concentration of 2 mg/l in combination with 1 mg/l BA gave the highest division of protoplasts (Table 2). In order to test the other auxin in comparison with Di, 2 mg/l of each auxin in combination with 1 mg/l BA were selected. Types of culture were also designed to optimize division of protoplasts. Among auxins tested, Di showed the best performance for divison of the protoplasts, followed by NAA, while 2,4-D gave the poorest result (Table 3). In the case of types of culture, embedd-

Table 1. Effect of kinds and concentrations of digestive enzyme on yield and viability of protoplasts.

Concentration of enzyme (%)		No. of protoplasts ($x10^6$/g. fr. wt.)	Viability (%)
Cellulase RS	Macerozyme R10		
1	1	4.86±0.34	71.39±4.72
1	2	7.99±0.91	70.65±6.51
2	1	10.03±2.65	73.83±2.31
2	2	10.06±0.08	81.67±3.14

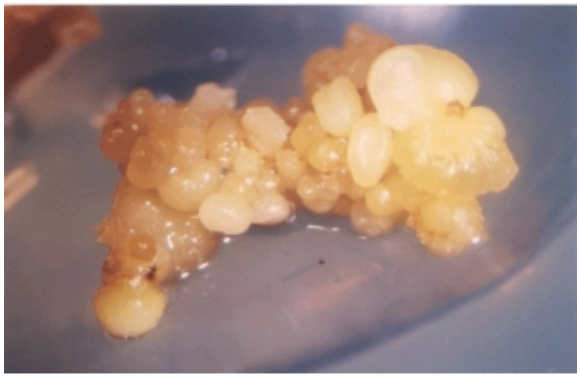
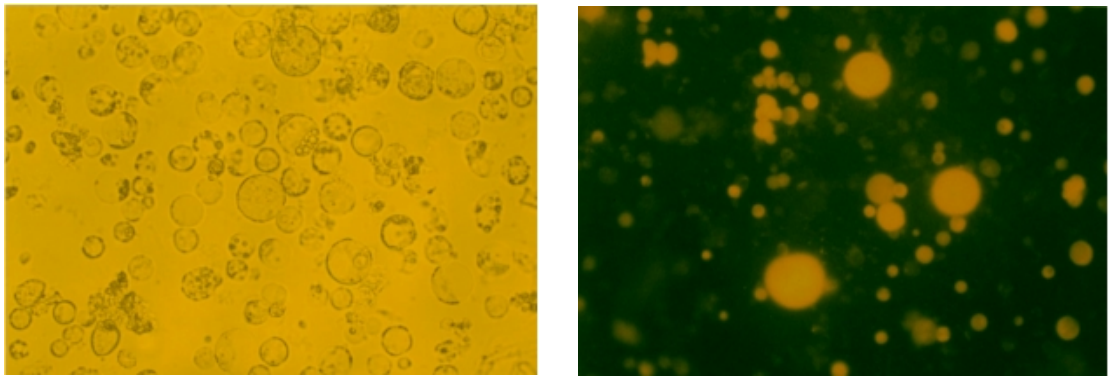


Figure 1. Embryogenic callus derived from young leaf culture used as plant material for isolation of the protoplasts.



A **B**

Figure 2. Fresh protoplasts just isolated from the callus (A) and its fluorescien staining (B).

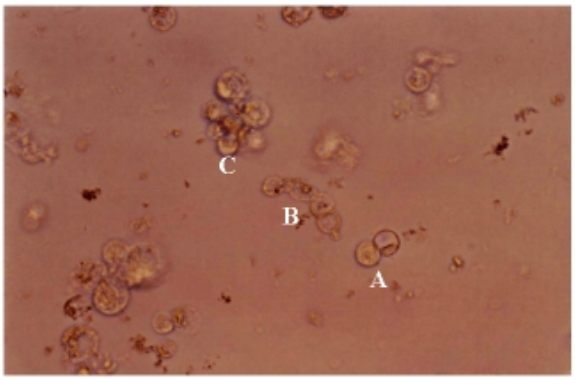


Figure 3. Development of the protoplasts in MS medium supplemented with 3% sucrose, 0.4 M mannitol, 2 mg/l Di and 1 mg/l BA.
A: first protoplast division
B: second protoplast division (asymmetry)
C: microcolony formation

Table 2. Effect phytohormone containing in MS medium on growth of protoplasts.

Phytohormone (mg/l)		Division of the protoplasts (%)
Dicamba	BA	
1	0.5	0.61
1	1	0
2	0.5	0.96
2	1	2.33
3	0.5	1.38
3	1	0

Table 3. Effect of culture techniques and phytohormones containing MS medium on development of the protoplasts.

Culture Technique	Phytohormone	Time required for 1st division (day)	1st division (%)	Agglutination (%)	No. of cell adhesion
Thin layer	2,4-D	-	0	100	-
	Di	-	0	100	-
	NAA	-	0	100	-
Semi-solid	2,4-D	4	2.33	34.25	3.14
	Di	4	4.00	28.20	3.60
	NAA	4	3.54	34.37	3.16
Agarose Bead	2,4-D	-	0	27.86	4
	Di	-	0	38.09	4.25
	NAA	-	0	20.28	4.42

ing the protoplasts in 0.2% phytagel supplemented MS medium (semi-solid MS medium) promoted division of protoplasts in all kinds of auxin, whereas the other two types of culture never promoted division of the protoplasts (Table 3). Agglutination of the protoplasts was found in the thin layers and when embeded in the agarose matrix. Even phytagel could cause agglutination of the protoplasts, first division and microcolony formation were obtained. Sambanthamurthi et al. (1996) reported division of the protoplasts in thin layer liquid medium A. However, many steps must be applied to induce microcallus formation. First division of protoplasts was obtained in liquid medium supplemented with 1.2 µM NAA then addition of a mixture solution consisting of 10-15 mg/l aspirin, 20 mg/l silver nitrate, 0.5 mg/l 2,4-D and 1.5 µM zeatin were required. The osmoticum of the medium must be reduced by addition 0.25 ml liquid medium A with 100 mM sucrose every 5 days for 25 days. Time required for first division of protoplasts was 3 days, which was similar to the present study that took 4 days for the first division. The differences between the two experiments might be due to the component of culture medium. In the presence of silver nitrate activity of ethylene is suppressed causing the rapid division of perennial cells or protoplasts (Cazaux and d'Auzac, 1995). However, this study did not add silver nitrate in culture medium. For further division and microcolony formation in this study, it took about 3 weeks of culture (Figure 3), which was similar to that reported by Sambanthamurthi *et al*. (1996) but without reduction of the osmoticum. So that very

simple technique of culture is reported in this present study whereas the former reports needed many steps of culture. Unfortunately, microcallus and plantlet regeneration from protoplast-derived callus was not obtained. Reduction of osmoticum and plant growth regulators should be surveyed after 3 weeks of culture. Moreover, density of the protoplasts for culture should be taken into account, like those reported in rubber protoplast culture (Te-chato *et al*., 2002). The culture density might be too low for induction of further development to microcallus or callus formation. So far, there have been no reports on plantlet regeneration from culturing protoplasts of oil palm. Many attempts have been made to isolate and culture oil palm protoplasts but success in plantlet regeneration is limited. This study should provide basic data for research on the next step in propagation and improvement of oil palm through protoplast technology.

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