Plant Cell, Tissue and Organ Culture **46**: 35–41, 1996.

Oil palm (*Elaeis guineensis*) protoplasts: isolation, culture and microcallus formation

Ravigadevi Sambanthamurthi, Siti Hasnah Parman & Mohd. Roslan Md. Noor
Plant Science and Biotechnology Unit, Palm Oil Research Institute of Malaysia, P.O. Box 10620, 50720 Kuala Lumpur, Malaysia

Received 31 October 1995; accepted in revised form 10 June 1996

Key words: Elaeis guineensis, oil palm, protoplasts

Abstract

Procedures are described for the efficient isolation of protoplasts from a variety of oil palm (*Elaeis guineensis* Jacq.) tissues. Various factors including donor source, composition of enzyme mixture and culture medium affected the yield and viability of the protoplasts Polyembryogenic cultures of oil palm were the most suitable starting material in terms of yield, viability and metabolic competence. Pectolyase Y-23 in association with cellulase and hemicellulase was required for the efficient release of protoplasts from the oil palm tissues. Limited cell division to form microcallus was observed at very low frequency (<0.01%) when glutathione and catalase were incorporated in the culture medium.

Abbreviations: 2,4-D – dichlorophenoxyacetic acid; DTT – dithiothreitol; MES – 2[N-morpholino] ethanesulphonic acid; NAA – 1-naphthalene acetic acid; PVP – polyvinylpyrrolidone

Introduction

The oil palm is an important source of edible oil accounting for about 16.5% of total world production of oils and fats (Mielke, 1995). Genetic engineering by transformation offers potential for improvement of the crop. Since the oil palm cannot be readily transformed by *Agrobacterium*, protoplasts offer an alternative transformation system. Several reports have described transgenic plants from maize (Rhodes et al., 1989) rice (Toriyama et al., 1988) and barley (Zhang et al., 1995) protoplasts. Despite the economic importance of the oil palm there has been very little information on oil palm protoplasts. The oil palm being a monocotyledon and a woody plant has been recalciterant to protoplast isolation and culture. Bass and Hughes (1984) reported the isolation and regeneration of viable protoplasts of oil palm and there have been no further reports since. In this paper, we report the isolation of highly viable protoplasts from various oil palm tissues and the formation of microcalluses from protoplasts of polyembryogenic cultures.

Materials and methods

Plant material

Polyembryogenic cultures (derived from leaf explants) and clonal ramets of *Elaeis guineensis* var. tenera, clone P9 were kindly supplied by Dr. K. Paranjothy, PORIM Headquarters. *E. guineensis* seed (germinating and non-germinating) and inflorescence material were obtained from PORIM Research Station, Serdang.

The polyembryogenic cultures were maintained on modified Murashige and Skoog (1962) basal medium (solid) and subcultured at three month intervals as described by Paranjothy et al. (1989). At the time of protoplast isolation, cultures were two months old.

Protoplast isolation

Protoplasts were isolated from

- polyembryogenic cultures (solid medium),
- vegetative apices of clonal ramets,
- seed embryos,

– embryonic axes of germinating seeds, and

– young inflorescences.

Surface sterilization was carried out on all tissues except polyembryogenic cultures as follows: soaking in a solution containing 8% Clorox (v/v) and 0.1% (v/v) Tween 20 for 5 min followed by rinsing in three changes of sterile distilled water. The tissues were then treated with 70% ethanol for 1 min and rinsed three times with sterile distilled water. The tissues were sliced finely and plasmolysed (30 min) in medium A (Table 1). This medium was a modification of those of Gamborg et al. (1968) and Kao (1982). Medium A was removed and replaced with enzyme solution containing various combinations of 2% (v/v) Celluclast (Novo Industri A/S Novo Allè DK-2880 Bagsvaerd, Denmark), 1% (v/v) Pectinex 3 XL (Novo Ferment Ltd. Vogesenstrasses 132, CH-4013 Basel Switzerland), 0.25% (w/v) Pectolyase Y-23 (Seishin Pharmaceutical, 4-13, Koami cho, Nihombashi, Tokyo, Japan), 1% (w/v) Macerozyme R-10 (Yakult Pharmaceutical Ltd., 1-1-19, Higashi Shinbashi, Minato-ku, Tokyo) and 0.5% (w/v) hemicellulase (Amano Pharmaceuticals Ltd., Nagoya, Japan) and 0.01% (w/v) trypsin inhibitor (Sigma, USA). Trypsin inhibitor was included in all the enzyme mixtures to arrest possible protease action by contaminants present in the enzyme solutions.

The enzyme mixture was adjusted to pH 5.7. Sucrose was added to obtain an osmolality of 800 mOsm (kg $H_2O)^{-1}$. Osmolality measurements were carried out with a Wescor osmometer. The enzyme solution was filter sterilized. Digestion was carried out for 5 h in the dark and the mixture filtered through a 120 μm followed by 69 μm pore size nylon mesh to remove cell clumps and vascular tissue. The filtrate was centrifuged (100 *g*; 5 min). Under these conditions, protoplasts from polyembryogenic cultures formed a floating band which was removed and purified by resuspension in medium A (800 mOsm) and centrifugation (100 *g*; 5 min). The floating protoplast layer was pipetted off. Protoplasts from the other tissues formed a pellet which was washed in medium A and then layered onto 25% (w/v) sucrose solution. The floating layer, which contained the protoplasts, was washed in medium A. Protoplasts isolated from the vegetative apices of clonal ramets were very dense and did not float even in 25% (w/v) sucrose so the pellet was washed in medium A and filtered through a 30 μm pore size nylon mesh.

Determination of protoplast yield, viability and metabolic integrity

Protoplast yield was estimated with a haemocytometer.

Evans Blue, which is a stain that is excluded by viable cells was used to measure protoplast viability (Nagata and Takebe, 1970). Protoplasts were kept in medium A before viability determinations.

The metabolic integrity of the oil palm protoplast preparations was gauged by their ability to respire $^{14}CO_2$ and synthesize [^{14}C] lipids when incubated with [1-^{14}C] acetate. The determinations were carried out on freshly isolated protoplasts. Two ml of protoplast suspension (10^4 - 10^5 cells in medium A) were incubated with 15 μCi of [1-^{14}C] acetic acid (263 nmol, Amersham International, England) for 5 h without shaking, in a conical flask fitted with a Kontes rubber stopper and a plastic centre well. The well contained fluted filter paper moistened with 0.2 ml of 0.2M KOH to trap $^{14}CO_2$. The incubation was stopped with 0.2 ml 4M H_2 $S0_4$. The moist filter paper was transferred directly into a scintillation vial for counting. The suspension was extracted with 3 × 15 ml chloroform/methanol (2:1; v/v). The extract was washed with 0.2 vol 0.8% (w/v) NaCl solution. The chloroform layer was carefully removed and an aliquot transferred to a scintillation vial and evaporated to dryness under a stream of nitrogen. Ten ml of scintillation fluid were added to each sample and counting carried out on a LKB Wallac Rackbeta 1217 liquid scintillation counter.

The fatty acid composition of the radioactive lipids was analysed by radio gas chromatography as described by Sambanthamurthi et al. (1987).

Protoplast culture

Density was adjusted to 1×10^5 protoplasts/ml in medium A with 1.2 μM NAA (800 mOsm). Aliquots (2.5 ml) were dispensed in 60 × 15 mm plastic culture plates. Aspirin (10-50 mg l^{-1}) silver nitrate (20 mg l^{-1} 2,4-D (0.5 mg l^{-1}) and zeatin (1.5 μM) were also added to the culture media either individually or in various combinations in some experiments. The culture plates were sealed with Parafilm and incubated (dark) at 25 °C. During culture, the osmolality of the medium was progressively reduced by the addition of 0.25 ml medium A containing 100 mM sucrose every 5 days until day 25. For solid culture, 1.2% Sea Plaque agarose (FMC Bio Products, Rockland, U.S.A.) was added. To prevent the plates from drying up and nutrients from being depleted, 0.5 ml solution A containing

Table 1. Composition of Medium A.

Compound *a.* Mineral salts	Concentration mM	Compound *b.* Vitamins	Concentration mM
K_2HPO_4	7.5	Thiamine HCl	30.0
$(NH4)_2SO_4$	7.5	Pyridoxine HCl	5.0
NH4-acetate	10.0	O-Calcium pantothenate	1.0
KH_2PO_4	7.3	p-Amino benzoic acid	0.1
K_2SO_4	10.0	Biotin	0.2
	μM	Vitamin D3	0.1
KI	4.5	Folic acid	0.5
H_3BO_3	50.0	Choline-chloride	7.2
$MnSO_4.H_2O$	60.0	Myoinositol	555.0
$ZnSO_4.7H_2O$	7.0	Nicotinic acid	5.0
$Na_2Mo0_4.2H_2O$	1.0		
$CuSO_4.5H_2O$	0.1		
Compound *c.* Sugars and organic acids	Concentration μM	Compound *d.* Others	Concentration mM
Sucrose	400.0	MES	5.0
Ribose	2.0	DTT	1.0
Ascorbate (Ascorbic acid)	1.0	PVP	0.1(%)
Citrate (Citric acid)	2.0		
Malate (Maleic acid)	1.0	pH adjusted to 5.7 with KOH	

100 mM sucrose was added to the solid medium every 7 days from day 14 onwards.

Cell wall regeneration was determined every day from day 1 onwards using 0.01 % Calcofluor White ST according to the method of Nagata and Takebe (1970). Assessments were carried out in triplicate for every experiment.

Results and discussion

Composition of protoplast isolation mixture

A combination of cellulase (Celluclast), pectinase (Pectinex III/Pectolyase Y-23) and hemicellulase (Hemicellulase Amano 10) was required for efficient maceration of the oil palm tissues (Table 2). The substitution of Pectinex III with Pectolyase Y-23 increased maceration and was essential for polyembryogenic cultures. A combination of Celluclast, Pectolyase Y-23, Pectinex III and Hemicellulase Amano 10 gave the highest protoplast yield for all donor sources and this enzyme mixture was used for subsequent isolation procedures.

Viability and metabolic integrity

The viability of protoplasts obtained from the various donor tissues is given in Table 3. Protoplasts from polyembryogenic cultures were highly viable (> 99%). The metabolic competence of the protoplasts is also indicated in Table 3. Protoplasts from polyembryogenic cultures and vegetative apices were metabolically the most active (in terms of lipid synthesis and CO_2 production.). Analysis of the radioactive lipids synthesised by protoplasts (both from polyembryogenic cultures and vegetative apices) indicated that the lipid metabolism had been altered. The protoplasts synthesised high levels of palmitoleic acid (up to 27%). This fatty acid is normally produced only in trace amounts by oil palm tissues (<0.1%). The altered metabolism was probably induced as a result of osmotic stress.

Protoplast source

Tissues that are themselves easily regenerable i.e. amenable to tissue culture are more likely to be amenable to protoplast culture and regeneration. The various tissues used for protoplast isolation in this study have previously been successfully used for tissue

Table 2. Protoplast yield with different enzymatic mixtures.

Enzyme mixture	Protoplast yield (g f.wt.)$^{-1}$				
	Embryogenic culture	Vegetative apex	Seed embryo	Inflorescence	Embryonic axis
Celluclast	$< 10^2$	$< 10^3$	$< 10^2$	$< 10^3$	$< 10^3$
Macerozyme - R10	$< 10^2$	$< 10^3$	$< 10^2$	$< 10^2$	$< 10^3$
Celluclast, Macerozyme - R10	$< 10^2$	$1\pm0.2\times10^4$	$< 10^3$	$2\pm0.5\times10^4$	$< 10^3$
Celluclast, Pectinex, Hemicellulase	$< 10^2$	$2\pm0.3\times10^4$	$6\pm0.3\times10^3$	$1\pm0.1\ times10^5$	$1\pm0.2\times10^4$
Celluclast, Pectolyase Y-23, Hemicellulase	$5\pm0.4\times10^5$	$2\pm0.3\times10^5$	$5\pm0.6\times 10^4$	$5\pm0.8\times10^5$	$7\pm1.2\times10^4$
Celluclast, Pectolyase Y-23, Pectinex 3XL, Hemicellulase	$1.5\pm0.3\times10^6$	$1.0\pm0.1\times10^6$	0.5×10^6	$1.0\pm0.2\times106$	$5\pm0.3\times10^5$

Values represent the mean of 5 independent experiments. Each experiment was carried out in triplicate.

Table 3. Viability and metabolic integrity of protoplasts isolated from various oil palm tissues.

Protoplast source	Viability				Incorporation of [1-^{14}C] into labelled products per 10^5 freshly isolated protoplasts (counts/min $\times 10^{-7}$)	
	Freshly isolated	24h	48h	72h	CO^2	Lipid
Embryogenic culture	>99	>99	95±3	95±2	8.2±1.1	4.5±0.8
Vegetative apex	>99	72±8	65±4	50±5	7.8±0.8	3.2±0.9
Seed embryo	42±10	10±8	1±0.5	0	0.1±0.03	0.02±0.001
Inflorescence	83±9	65±7	52±6	45±7	1.0±0.1	0.1±0.04
Embryonic axis	70±12	62±6	45±8	33±6	2.0±0.3	0.5±0.03

Values represent the mean of 3-5 independent experiments. Each experiment was carried out in triplicate.

culture of oil palm (Paranjothy, 1982; Brackpool et al., 1986) and were thus identified as good donor sources for protoplast isolation and culture. Of the various tissues used, protoplasts from polyembryogenic cultures and vegetative apices appeared to be the most promising material in terms of yield and metabolic competence. However, protoplasts from vegetative apices were full of starch granules and often burst, releasing the starch granules. They were thus difficult to work with. Furthermore, the viability of these protoplasts quickly declined during incubation. Protoplasts from polyembryogenic cultures were thus the material of choice. The protoplast preparations from polyembryogenic cultures were very clean. Contamination by cell debris and undigested cells could not be detected microscopically. Furthermore, Calcofluor White staining confirmed the absence of undigested cell walls in the protoplast preparations. A drawback of the polyembryogenic cultures used in our study however, was that many of the cells were vacuolate. It is generally known that dense cytoplasmic cells undergo cell division better than vacuolate cells.

Reproducible methods of plant regeneration have been reported for protoplasts from rice (Abdullah et al., 1986; Toriyama et al., 1988, maize (Rhodes et al., 1989; Donn et al., 1990), barley (Wang and Lorz, 1994; Zhang et al., 1995) and wheat (Li et al., 1992; Ahmad and Sagi, 1993). These successes were accomplished using suspension cultures as the protoplast source. In our experiments however, oil palm suspension cultures were not available. The polyembryogenic cultures used were from solid medium. This may be the main reason for our limited success as embryogenic suspension cultures would be a better source of totipotent cells. Another reason for the limited success in culturing the protoplasts may be the fact that the metabolism of the protoplasts had been altered. The altered metabolism may have in some way altered mitotic competence.

Co-culturing of recalcitrant protoplasts especially those from monocotyledons, with nurse cultures has been reported to induce cell division and increase plating efficiencies (Jain et al., 1995; Ghosh Biswas et al., 1994). Nurse cultures generally assist in maintaining a critical density of active cells, producing sufficient levels of "growth promoting factors" for sustained cell division. Such feeder cells have been used successfully to promote cell division and callus formation in protoplasts from monocotyledons such as banana (Megia et al., 1992), rice (Jain et al., 1995) and maize (Shillito et al., 1989). Bass and Hughes (1984) reported the regeneration of oil palm protoplasts using nurse culture which comprised oil palm cell suspension as the feeder layer and oil palm protoplasts on a filter paper disc. In the absence of suspension cultures in our laboratory, attempts were made to use polyembryogenic cultures from solid medium as a feeder layer. However, the experiments were not successful.

Culture medium

In addition to medium A, attempts were made to isolate and culture protoplasts in other basal media such as Murashige and Skoog (1962) medium, KM medium (Kao and Michayluk, 1975) and Woody Plant medium (WPM) reported by Russell and McCown (1986). However, medium A consistently performed better. Glutathione and catalase were important components of the culture medium. Various levels of glutathione and catalase were tested (Table 4). At the time of isolation and 24h later, all the protoplasts had more than 99% viability. In the absence of these two components, viability decreased significantly after 1 week (Table 4). The best level to maintain cell viability was 1000 units catalase in combination with 2 mM glutathione and these were therefore included routinely in the culture media. The marked improvement in protoplast viability in the presence of both these components suggested that oxygen damage by lipid peroxides may be one of the factors contributing to the poor response of oil palm protoplasts to culture. Similar results were reported by Ishii (1989) who found that in rice cells, the enzyme treatment used for protoplast isolation caused the cells to generate the superoxide radical. Addition of catalase or superoxide dismutase to the medium resulted in significant improvement in protoplast viability. Aspirin (O-acetyl-salicylic acid), silver nitrate, 2,4-D and zeatin did not have an effect on viability or cell division at the concentrations used. Reports have shown the beneficial effect of aspirin (Carswell et al., 1989) and silver salts (Perl et al., 1988) on protoplast division and colony formation. These compounds are believed to exert their effect by inhibition of ethylene production (Perl et al., 1988 and Carswell et al., 1989). However, inclusion of these chemicals in the culture medium individually and in combination did not improve viability or division of oil palm protoplasts.

In liquid medium, Calcofluor staining showed that cell wall formation occurred after two days. However, protoplast division was not observed and the cells aggregated after two weeks in culture medium. On solid medium, however, both cell wall regeneration and

Table 4. Effect of catalase and glutathione on viability of protoplasts isolated from embryogenic cultures of oil palm.

Compound		Viability (%)		
Catalase units/ml	Glutathione mM	72h	1 Wk	2 Wk
0	0	95±2	45±6	5±3
0	1	95±3	75±4	10±5
0	2	97±1	80±8	27±5
0	3	96±2	73±7	28±8
500	0	96±1	78±8	55±3
500	1	97±1	70±3	53±5
500	2	96±3	75±6	60±6
500	3	95±2	80±10	60±10
1000	0	96±2	83±5	67±5
1000	1	96±3	89±3	69±6
1000	2	99±1	99±1	75±6
1000	3	98±1	85±8	68±7
1500	0	95±1	79±6	60±5
1500	1	96±2	80±2	59±3
1500	2	97±1	80±6	57±5
1500	3	96±1	75±8	53±5

Value represent the mean of 3-5 independent experiments. Each experiment was carried out in triplicate.

limited cell division were observed. First cell division was observed after three days of culture and colonies of up to 12 cells could be observed after 10 days. Further division to form microcalluses (300-500 cells) occurred at a very low frequency (<0.01% of protoplasts plated) after three to four weeks. Further growth of the microcalluses could not be sustained on the same medium.

Efforts are in progress to improve the culture system in order to obtain sustained cell division. Regenerable embryogenic suspension cultures have been reported for the oil palm in other laboratories (Touchet et al., 1991; Teixeira et al., 1995). Efforts are also in progress to obtain embryogenic suspension cultures as a source material for protoplasts.

Acknowledgement

The authors thank the Director-General of PORIM, Dr. Yusof Basiron for permission to publish these results.

References

Abdullah R, Cocking EC & Thompson JA (1986) Efficient plant regeneration from rice protoplasts through somatic embryogenesis. Bio/technology 4: 1087–1090

Ahmad KZ & Sagi F (1993) Culture of and fertile plant regeneration from regenerable embryogenic suspension cell-derived protoplasts of wheat (*Triticum aestivum* L.). Plant Cell Rep. 12: 175–179

Bass A & Hughes W (1984) Conditions for the isolation and regeneration of viable protoplasts of oil palm. Plant Cell Rep. 3: 169–171

Brackpool AL, Branton RL & Blake J (1986) Regeneration in palms. In: Vasil IK (ed) Cell Culture and Somatic Cell Genetics of Plants, Vol 3 (pp 207–222). Academic Press, Orlando

Carswell GK, Johnson CM, Shillito RD & Harms CT (1989) O-acetyl-salicylic acid promotes colony formation from protoplasts of an elite maize inbred. Plant Cell Rep. 8: 282–284

Donn G, Nilges M & Morocz S (1990) Stable transformation of maize with a chimaeric, modified phosphinotrycin-acyltransferase gene from *Streptomyces viridochromogenes*. In: Nijkamp HJJ, Van der Plas LHW & Aartrijk J (ed) Progress in Plant Cellular and Molecular Biology, Abstr. A2–38 (p. 53). Kluwer Publicatians, Dordrecht

Gamborg OL, Miller RA & Ojima K (1968) Plant cell cultures I. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50: 151–158

Ghosh Biswas GC, Burkhardt PK, Wann J, Kloti A & Potrykus I (1994) Fertile Indica rice plants regenerated from protaplasts isolated from scutellar tissue of immature embryos. Plant Cell Rep. 13: 528–532

Ishii S (1989) Factors influencing protoplast viability of suspension cultured rice cells during isolation process. Plant Physiol. 88: 26–29

Jain RK, Khehra GS, Lee SH, Blackhall NW, Marchant R, Davey MR, Power JB & Cocking EC (1995) An improved procedure for plant regeneration from indica and japonica rice protoplast. Plant Cell Rep. 14: 515–519

Kao KN (1982) Plant protaplast fusion and isolation of heterokaryocytes. In: Wetter LR & Constable F (ed) Plant Tissue Culture Methods (2nd edition, pp 49–52). National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan. NRCC 19876.

Kao KN & Michayluk MK (1975) Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at a very low population density in liquid media. Planta 126: 105–110

Li ZY, Xia GM, Chen HM & Guo GQ (1992) Plant regeneration from protoplasts derived from embryogenesis suspension cultures of wheat (*Triticum aestivum* L.). Plant Physiol. 139: 714–718

Megia R, Haicour R, Rossignol L & Sihachakr D (1992) Callus formation from cultured protoplasts of banana (*Musa* sp). Plant Sci. 85: 91–98

Mielke T (1995) Oil World Statistics Update. In: Mielke T (ed). Oil World (pp 55–56). ISTA Mielke GmbH, Hamburg

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioyassays with tobacco tissue cultures. Physiol Plant 15: 473–497

Nagata & Takebe I (1970) Cell wall regeneration by isolated tomato fruit protoplasts. Protoplasma 64: 460–480

Paranjothy K, Rohani O, Tarmizi AH, Tan CS & Tan CC (1989) Current status and strategies of oil palm tissue culture research. Proceedings of the 1989 PORIM International Palm Oil Development Conference (Agriculture) (pp 109–121)

Paranjothy K (1982) A review of tissue culture of oil palm and other palms. PORIM Occasional Paper 3: 1–22

Perl A, Aviv D & Galun E (1988) Ethylene and *in vitro* culture of potato: suppression of ethylene generation vastly improves protoplast yield, plating efficiency and transient expression of an alien gene. Plant Cell Rep. 7: 403–406

Rhodes CA, Pierce DA, Mettler IJ, Mascarenhas D & Detmer JJ (1989) Genetically transformed maize plants from protoplasts. Science 240: 204–207

Russell JA & McCown BH (1986) Culture and regeneration of populus leaf protoplasts isolated from non-seedling tissue. Plant Science 46: 133–142

Sambanthamurthi R, Oo KC & Ong ASH (1987) Lipid metabolism in oil palm (*Elaeis guineensis* and *Elaeis oleifera*) protoplasts. Plant Sci. 51: 97–103

Shillito RD, Carswell GK, Johnson CM, DiMaio JJ & Harms CT (1989) Regeneration of fertile plants from protoplasts of elite inbred maize. Bio/Technol. 7: 581–587

Teixeira JB, Sondahl MR, Nakamura T & Kirby EG (1995) Establishment of oil palm cell suspensions and plant regeneration. Plant Cell Tiss. Org. Cult. 40: 105–111

Toriyama K, Arimoto Y, Uchimiyia H & Hinata K (1988) Transgenic rice plants after direct gene transfer into protoplasts. Bio/Technol. 6: 1072–1074

Touchet BD, Duval Y & Pannetier C (1991) Plant regeneration from embryogenic suspension cultures of oil palm (*Elaeis guineensis* Jacq.). Plant Cell Rep. 10: 529–532

Wang XH & Lorz H (1994) Plant regeneration from protoplasts of wild barley (*Hordeum murinum* L.). Plant Cell Rep. 13 :139–144

Zhang J, Tiwari VK, Golds TJ, Blackhall NW, Cocking EC, Mulligen BJ, Power JB & Davey MR (1995) Parameters influencing transient and stable transformation of barley (*Hordeum vulgare* L.) protoplasts. Plant Cell Tiss. Org. Cult. 41: 125–138