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Research note

Plant regeneration from embryogenic cell suspension-derived protoplasts of rubber

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Abstract

Embryogenic cell suspensions of rubber derived from immature inflorescences and inner integuments of immature fruits released $3.1 \pm 0.2 \times 10^7$ protoplasts g^{-1} f. wt. (mean ± s.e.m. $n = 10$) and $3.2 \pm 0.2 \times 10^7$ protoplasts g^{-1} f. wt., with mean viabilities of 83 ± 2% and 77 ± 8%, respectively. Sustained mitotic division was observed only when protoplasts were cultured in KPR liquid medium on nitrocellulose membranes overlying the same semi-solid medium containing *Lolium multiflorum* nurse cells. Protoplast-derived cell colonies were produced within 2 months of culture. Protoplast-derived cell colonies proliferated, upon subculture to MS-based regeneration medium, with 40% of the protoplast-derived calli developing somatic embryos. The latter germinated into plants on the same medium after 3 months of culture.

Abbreviations: BA – 6-benzyladenine; 2,4-D – 2,4-dichlorophenoxyacetic acid; FDA – fluorescein diacetate; FPE – final protoplast plating efficiency; MES – 2-(N-morpholino)ethanesulphonic acid; MS – Murashige and Skoog (1962); NAA – α-naphthaleneacetic acid; PCV – packed cell volume

Natural rubber can be found in many species representing 300 genera and 79 families, but the main source is *Hevea brasiliensis* (Euphorbiaceae). The latter is a heterozygous perennial tree crop with a long reproductive cycle and, hence, improvement via conventional breeding is lengthy. The ever-increasing commercially-driven demand for natural rubber has made it imperative to develop *in vitro* techniques for rapid propagation of elite *H. brasiliensis* germplasms and to facilitate plant improvement through somatic cell approaches. Somatic embryogenesis has been described for a range of *H. brasiliensis* explants, including plant production by anther culture or from unpollinated ovules (Guo et al., 1982).

Somatic hybridisation or cybridisation, mediated by protoplast fusion, provides a means of circumventing the sexual incompatibility barriers encountered in traditional plant breeding approaches and may be especially beneficial for rubber, since agronomically-important traits are cytoplasmically controlled. Direct gene transfer, via protoplasts, will also be a future option for the introgression of agronomically useful genes, such as those coding for key enzymes in rubber biosynthesis.

Protoplast culture has been attempted for *H. brasiliensis* and *H. pauciflore* (Cailloux and Lleras, 1979; Wilson and Power, 1989), but sustained mitotic division was not reported in these cases. Subsequently, Cazaux and d'Auzac (1994) obtained microcalli from embryogenic callus-derived protoplasts of *H. brasiliensis*, but without plant regeneration. This paper reports, for the first time, the efficient regeneration of plants, via somatic embryogenesis, from protoplasts of *H. brasiliensis*.

Immature inflorescences and inner integuments of immature fruits, taken 6–8 weeks after anthesis from

15 year-old mature trees of *H. brasiliensis* (clone RRII 105), were supplied by the Rubber Research Institute of India (Kerala, India). Newly excised explants were surface sterilised in 0.1% (w/v) mercuric chloride (3 min) and washed 4 times in sterile reverse-osmosis water. Four, undissected inflorescence buds (each 2–3 mm in length) or inner integument tissues were each placed into individual culture tubes (2.5 × 15 cm) containing 20 ml of MS-based medium supplemented with 0.90 μM 2,4-D, 2.68 μM NAA, 0.93 μM kinetin and 3.0% (w/v) sucrose and semi-solidified with 0.2% (w/v) Phytagel (Sigma, Poole, UK), pH 5.6 (MSC medium). Cultures were maintained in the dark at 24 ± 2 °C for 40–50 days. Developing calli were transferred individually to 20 ml aliquots of a differentiation medium (designated MSD) which was MS-based but contained 370 mg l^{-1} KH_2PO_4, 120 mg l^{-1} $MgSO_4.7H_2O$, 1.33 μM BA, 1.07 μM NAA and 3.0% (w/v) sucrose and was semi-solidified with 0.2% (w/v) Phytagel at pH 5.6. Callus was maintained in the dark at 24 ± 2 °C with transfer every 21 days until it became embryogenic, irrespective of origin, with differentiation of somatic embryos after 84–112 days of culture.

Embryogenic calli were transferred to 50 ml aliquots of shoot regeneration medium (designated MSR) in 175 ml screw-capped glass jars (Beatson Clarke and Co. Ltd., Rotherham, UK). The formulation of MSR medium was as for MSD medium, but with 5.0% (v/v) liquid coconut endosperm and 5.7 μM IAA replacing both BA and NAA. Later, cultures were incubated in the light (20–30 μmol m^{-2} sec^{-1}, Cool-White fluorescent tubes; Thorn EMI Ltd., Hayes, UK) with a 16-h photoperiod (25 ± 2 °C), for germination of somatic embryos (within 28 days). For immature inflorescence protoplast-derived calli, MSR medium was modified by the inclusion of glutamine at 50, 100, 200, 400 or 500 mg l^{-1}, since Montoro et al. (1995) have shown that free amino acids and, in particular glutamine, are implicated in the development of rubber somatic embryos.

For protoplast isolation, friable embryogenic calli (Figure 1a) from immature inflorescence and/or inner integuments (84–112 days old; 1.0 g f.wt.) on MSD medium were used to initiate cell suspensions by transfer from semi-solid MSD medium to 20 ml volumes of the same liquid medium in 100 ml Erlenmeyer flasks. Cultures were incubated in the dark on a horizontal platform shaker (90 rpm) at 25 ± 2 °C. Suspensions were maintained by fully replacing the spent MSD medium with new medium every 7 days. When the input PCV had increased about 3-fold, suspensions were transferred to 50 ml aliquots of MSD medium, contained in 250 ml Erlenmeyer flasks and subcultured, as before, every 7 days. Such cell suspensions were used for protoplast isolation 56 days after initiation. Aliquots consisting of 1.0 g f. wt. of embryogenic cells were transferred to 20 ml of an enzyme mixture, in a 9.0 cm Petri dish. The enzyme mixture consisted of 1.0% (w/v) Cellulase RS (Yakult Honsha Co., Nishinomiya Hyogo, Japan), 0.1% (w/v) Pectolyase Y23 (Seishin Pharmaceutical, Tokyo, Japan) and 5 mM MES in CPW13M solution (Frearson et al., 1973) at pH 5.8. CPW13M solution was modified from the published formulation by increasing $CaCl_2.2H_2O$ to 1.48 g l^{-1}. The mixture was incubated with constant agitation of 40 rpm, in the dark for 17 h, at 28 ± 2 °C. The enzyme-protoplast mixture was filtered through a 30 μm pore size nylon sieve to remove undigested cells. The filtered protoplasts were subsequently transferred to 16 ml screw-capped tubes and pelleted by centrifugation (80 × *g*; 8 min). Protoplasts were washed twice by resuspension and centrifugation in modified CPW13M solution prior to counting using a haemocytometer. Protoplast viability was assessed using FDA (Widholm, 1972).

Protoplasts were cultured in 1.0 ml aliquots of liquid KPR medium (Abdullah et al., 1986) in 3.5 cm diameter Petri dishes (Nunc, Roskilde, Denmark). Cultures were maintained in the dark at 25 ± 2 °C. Protoplasts were also embedded at 1.0, 2.0 or 6.0 × 10^5 ml^{-1} in KPR medium semi-solidified with 0.8% (w/v) Sea Plaque agarose (FMC Bioproducts, Rockland, USA). KPR agarose medium containing suspended protoplasts, was dispensed as 5.0 ml semi-solid thin layers contained in 5.5 cm diameter Petri dishes, or as 100 μl semi-solid droplets (6 droplets per 5.5 cm Petri dish) overlaid with 10 ml of liquid KPR medium. KPR medium was chosen since it contained a relatively high concentration of 2,4-D known to promote embryogenic development of rice protoplast-derived calli (Abdullah et al., 1986), the division of rubber protoplasts (Cazaux and d'Auzac, 1994) and would also support both the growth of the *Lolium multiflorum* and *Moricandia arvensis* nurse culture cells.

Suspension cells of *Lolium* or *Moricandia* were used as feeder layers for nurse culture of rubber protoplasts. Nurse cells (5 ml PCV) harvested 4 days after sub-culture, were mixed with 100 ml of KPR medium semi-solidified with 0.8% (w/v) Sea Plaque agarose. KPR medium was prepared at double final strength and mixed with an equal volume of molten 1.6% (w/v)

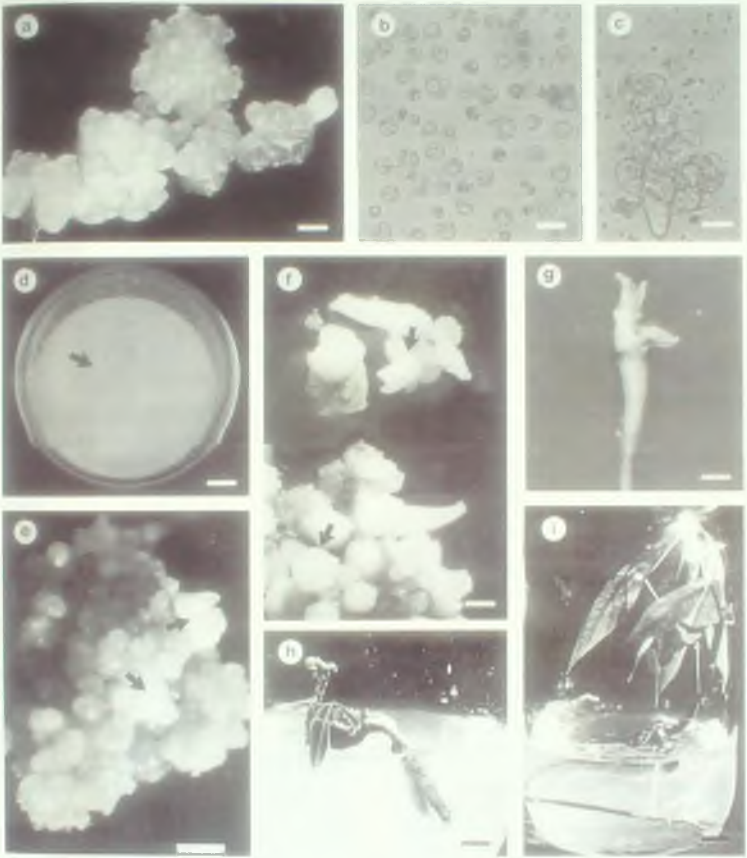

Figure 1. (*a*) Inflorescence derived embryogenic callus of rubber used to initiate cell suspensions. Bar = 0.8 mm. (*b*) Protoplasts immediately after isolation from cell suspensions initiated from inflorescence-derived embryogenic callus. Bar = 27 μm. (*c*) A protoplast-derived cell colony. Bar = 52 μm. (*d*) Protoplast-derived cell colonies [illegible] on a membrane overlaying semi solidified KPR medium containing nurse cells of *Datura* [illegible]. Bar = 0.7 cm. (*e*, *f*) Protoplast-derived tissues showing the presence of somatic embryos (arrowed) at different stages of development. Bars = 3.3 mm; 1.4 mm. (*g*, *h*) Germinating protoplast-derived somatic embryos. Bars = 2.2 mm, 0.5 cm. (*i*) A protoplast-derived rubber plant of clone RRII 105. Bar = 0.8 cm.

Table 1. Plating efficiency and plant regeneration of rubber protoplasts isolated from cell suspensions derived from immature inflorescences and integuments and cultured on membranes overlaying KPR medium in the presence of *Lolium* nurse cells

Protoplast source/ initial plating density	PE%	Protoplast-derived colonies giving one or more somatic embryos[a,b] (%)	Protoplast-derived colonies regenerating one or more plants[b] (%)
Inflorescence-derived cell suspensions			
3.0×10^5 ml^{-1}	32 ± 9	43 ± 1	11 ± 6
1.5×10^6 ml^{-1}	23 ± 6	34 ± 2	3 ± 2
Integument-derived cell suspensions			
3.0×10^5 ml^{-1}	18 ± 8	32 ± 2	5 ± 1
1.5×10^6 ml^{-1}	12 ± 5	23 ± 2	4 ± 3

[a]Mean ± s.e.m.; $n = 3$ Petri dishes each containing 50 colonies selected at random.
[b]Colonies cultured on MSR medium without glutamine.

Sea Plaque agarose medium, prior to addition of the respective nurse cells. Such nurse cultures were immediately dispensed as 10 ml aliquots in 5.5 cm diameter Petri dishes, 24 h prior to protoplast culture. A 47 mm diameter membrane filter (0.2 μm pore size; type GVWP; Millipore, Maidstone, UK) was placed on the surface of each nurse culture. Two hundred μl of the rubber protoplast suspension, in KPR medium, was spread over the surface of the membrane filter using a sterile plastic bacterial inoculation loop (Elkay Laboratory Products Ltd., Basingstoke, UK). Two plating densities (3×10^5 or 1.5×10^6 protoplasts ml^{-1}) were used. Protoplasts were further cultured on the surface of membranes overlaying KPR medium, but lacking *Lolium* cells, in order to assess any beneficial effects of nurse culture. Cultures were maintained in the dark at 25 ± 2 °C. Growth was assessed at 40 days in terms of the FPE, defined as the percentage of protoplasts which had formed cell colonies compared with the total population of protoplasts initially plated on the membranes. Discrete protoplast-derived cell colonies, which developed on membranes overlaying *Lolium* nurse cultures, were transferred after 50 days of culture to 25 ml aliquots of MSR medium, semi-solidified with 0.8% (w/v) agar (Sigma; 50 colonies per 9 cm Petri dish). Cultures were maintained for 28 days on MSR medium under the same growth conditions as used for the germination of somatic embryos.

Means and standard errors (s.e.m.) were used throughout. Statistical significance between mean plating efficiencies was determined using a Mann-Whitney U test. A probability of $p < 0.05$ was considered significant.

Embryogenic cell suspensions from immature inflorescences gave a mean yield of $3.1 \pm 0.2 \times 10^7$ protoplasts g^{-1} f. wt. (Figure 1b) with a mean viability of 83 ± 2%. Similarly, the mean yield and viability of protoplasts from immature integument-derived cell suspensions was $3.2 \pm 0.2 \times 10^7$ g^{-1} f. wt. and 77 ± 8%, respectively ($n = 10$ throughout). There were no significant differences between protoplast yields and viabilities from the two cell suspensions of different explant origin. Individual protoplast populations from immature inflorescence-derived cell suspensions, underwent sustained division to give cell colonies (Figure 1c,d) when cultured at 3×10^5 ml^{-1} in KPR medium on membranes overlaying *Lolium* nurse cells. Such protoplasts sometimes gave a significantly ($p < 0.05$) greater mean PPE (38 ± 5%, $n = 12$) than protoplasts at a density of 1.5×10^6 with the same nurse cells (20 ± 2%), but this was not always significantly different for all individual protoplast isolations. However, the mean PPE for a given protoplast population cultured on membranes in the absence of *Lolium* nurse cells, was always significantly ($p < 0.05$) lower at both 3.0×10^5 ml^{-1} (4 ± 2%) or 1.5×10^6 ml^{-1} (14 ± 6%) compared to protoplasts cultured in the presence of *Lolium* nurse cells (cf. data in Table 1). The trend in these data for the PPE of protoplasts cultured without *Lolium* nurse cells was the reverse of the PPE for protoplasts cultured with nurse cells (i.e. the value was higher at 1.5×10^6 ml^{-1} in the former case). Sus-

tained mitotic division was not observed when rubber protoplasts were cultured on membranes overlaying KPR medium either alone or containing *Moricandia* nurse cells. Additionally, division was not sustained when protoplasts were cultured in KPR liquid medium, or in KPR medium semi-solidified as agarose droplets or as thin layers.

In comparisons of the FPE of protoplasts isolated from cell suspensions derived from immature inflorescences or inner integuments and cultured in KPR medium with *Lolium* nurse cells at either density, there were no significant differences in the respective FPE and the mean percentage of colonies regenerating into plants (Table 1). If protoplast-derived colonies/calli reached approximately 3 mm in diameter in KPR medium, this permitted their direct transfer to MSR medium, thus eliminating the need for a proliferation/induction period on MSD medium. The majority of protoplast-derived colonies/calli reached this size without further sub-culture on KPR medium. Protoplasts isolated from both inflorescence-derived and integument-derived cell suspensions and cultured at 3×10^5 ml^{-1} in KPR medium gave a significantly greater ($p < 0.05$) percentage of colonies capable of producing somatic embryos (Figure 1e, f) on MSR medium than when initially cultured at the higher density of 1.5×10^6 ml^{-1} (Table 1). Furthermore, protoplasts isolated from inflorescence-derived cell suspensions gave a significantly greater ($p < 0.05$) percentage of colonies producing somatic embryos than protoplast-derived colonies from integument-derived cell suspensions, irrespective of the initial protoplast plating density. Thus, the choice of initial explant for the initiation of cell suspensions can significantly influence plant regeneration capacity via protoplasts, with inflorescence-derived cell suspensions for this clone (RRII 105) of rubber being the preferred option for the isolation of totipotent protoplasts.

In the present study, the conversion of protoplast-derived somatic embryos to plants contrasted with the report of Veisseire et al. (1994) in which the development of somatic embryos required a period of growth on medium containing adenine and cytokinins, followed by transfer to a medium lacking growth regulators. Thus, in the rubber protoplast system reported in the present paper, plants could be recovered directly from somatic embryos (Table 1) maintained on medium containing liquid coconut endosperm and IAA. Montoro et al. (1995) have demonstrated that glutamine can promote somatic embryogenesis from callus of rubber. Indeed, this observation has been confirmed in the present study, in which the addition of glutamine at 400 mg l^{-1} to MSR medium increased the percentage of protoplast-derived tissues giving rise to somatic embryos from 40.7% (control) to 59.3%. There was also a corresponding increase in the number of protoplast-derived tissues regenerating plants (Figure 1 g,h,i) from 11.3% (control) to 28.7%.

References

Abdullah R, Cocking EC & Thompson JA (1986) Efficient plant regeneration from rice protoplasts through somatic embryogenesis. Bio/Technol. 12: 1087–1090

Cailloux M & Lleras E (1979) Fusão de protoplastos de *Hevea brasiliensis* e *Hevea pauciflora*. Estabelecimento de tecnica. Acta Amazonica 9: 5–13

Caraux E & d'Auzac J (1994) Microcallus formation from *Hevea brasiliensis* protoplasts isolated from embryogenic callus. Plant Cell Rep. 13: 272–276

Chen Z (1984) Rubber (*Hevea*). In: Sharp WR, Evans DA, Ammirato PV & Y Yamada (eds) Handbook of Plant Cell Culture, Vol 2, Crop Species (pp 546–571). MacMillan, New York

Frearson EM, Power JB & Cocking EC (1973) The isolation, culture and regeneration of *Petunia* leaf protoplasts. Dev. Biol. 33: 130–137

Gilmour DM, Golds TJ & Davey MR (1989) *Medicago* protoplasts, fusion, culture and plant regeneration. In: Bajaj YPS (ed) Plant Protoplasts and Genetic Engineering I. Biotechnology in Agriculture and Forestry, Vol 8 (pp 370–388). Springer-Verlag, Berlin, Heidelberg, New York

Guo G, Jia X & Chen L (1982) Induction of plantlets from ovules *in vitro* of *Hevea brasiliensis*. Hereditas 4: 27–28

Montoro P, Etienne H & Carron MP (1995) Relation between nitrogen uptake, amino-acid contents, and embryogenic intensity of rubber tree calli. J. Plant Nutrition 18: 1693–1704

Murashige T & Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 56: 473–497

Veisseire P, Linossier L & Coudret A (1994) Effect of abscissic acid and cytokinins on the development of somatic embryos in *Hevea brasiliensis*. Plant Cell Tiss. Org. Cult. 38: 219–223

Widholm J (1972) The use of FDA and phenosafranine for determining viability of cultured plant cells. Stain Tech. 47: 186–194

Wilson ZA & Power JB (1989) Elimination of systemic contamination in explant and protoplast cultures of rubber (*Hevea brasiliensis* Muell. Arg.). Plant Cell Rep. 7: 622–625