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Research Note

An improved protocol for the culture of cassava leaf protoplasts

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Abstract

Viable protoplasts (yield > 1.9×10^7 g^{-1} fresh weight; mean viability 85 ± 2%, n = 5) were isolated from leaves of axenic shoot cultures of *Manihot esculenta* Crantz. cv. M. Thai 8. Protoplasts were cultured for up to 50 days in liquid, ammonium-free MS medium, overlaying agarose-solidified B5 medium with short glass rods embedded perpendicularly within, and protruding from, the agarose layer. Control protoplasts were cultured identically, but without glass rods. Sustained protoplast division was observed only in the presence of glass rods, where the initial plating efficiency was almost 6-fold greater than control ($p < 0.05$). The mean final plating efficiency of treated cultures was 1.0 ± 0.2% while, in contrast, significant colony formation was not observed in controls.

Abbreviations: BA – 6-benzyladenine, CPPU – N-(2-chloro-4-pyridyl)-N′-phenylurea, MES – 2[N-morpholino]ethane sulphonic acid, MS – Murashige & Skoog (1962), NAA – α-naphthaleneacetic acid, IPE – initial plating efficiency, FPE – final plating efficiency

Cassava (*Manihot esculenta* Crantz.), a perennial shrub of the family *Euphorbiaceae*, is cultivated throughout the lowland tropics. It is an important source of calories, ranking fourth following rice, maize and sugarcane. In contrast to other tropical crops, cassava has suffered from a lack of fundamental research at the tissue culture level. However, genetic manipulation technologies should permit the improvement of cassava with respect to useful agronomic traits via genetic recombination involving nominally sexually-incompatible genotypes (Mathews *et al.* 1993). One key pre-requisite for the application of such technologies is the development of an efficient protoplast-to-plant system and, for somatic hybridisation, the subsequent establishment of an effective selection strategy for the recovery of hybrid products. The recalcitrance of cassava as an actively dividing protoplast system *in vitro* is well documented (Byrne 1984), and there is, to date, only one, non-reproducible report on plant regeneration from cassava protoplasts (Shahin & Shepard 1980). Until a reproducible protoplast culture and shoot regeneration protocol has been established, the true potential of protoplast fusion and/or transformation via direct DNA delivery into protoplasts for the genetic improvement of cassava cannot be realised. This paper describes a protocol for inducing sustained division of cassava leaf protoplasts using a novel, simple, glass rod-medium system.

Axenic shoot cultures of *Manihot esculenta* cv. M. Thai 8 were supplied by the International Centre for Tropical Agriculture (CIAT), Cali, Colombia. Shoots of *ca.* 2.0 cm in height were routinely maintained on 50 ml aliquots of MS medium with 87.6 mM sucrose, lacking growth regulators and made semi-solid with 0.8% (w/v) agar (Difco-Bacto, USA; 3 explants per 175 cm^3 glass jar). Cultures were kept under a 12 h photoperiod (19.5 $\mu mol\ m^{-2}\ s^{-1}$, daylight fluorescent tubes, Coolight; Thorn EMI Ltd., UK) at 28 ± 2 °C and sub-cultured at 28–35 day intervals.

The second and third fully expanded leaves (from the apex) of axenic shoot cultures were used as a source of protoplasts. Leaves were sliced transversely into 1.0

Table 1. Mean initial (IPE) and final (FPE) plating efficiencies of cassava (*M. esculenta* cv. M. Thai 8) leaf protoplasts following culture for 50 days in the presence of glass rods compared with controls lacking glass rods.

	IPE (%) (25 days)	FPE (%) (50 days)
Control (no glass rods)	1.5 ± 0.4	No colonies
Glass rods	8.9 ± 0.7*	1.0 ± 0.2

Values are mean ± s.e.m. (n = 5); $^*p < 0.05$

mm strips and plasmolysed by immersion for 1 h in 10 ml CPW salts solution (Frearson *et al.* 1973) containing 0.5 M mannitol (designated CPW9M), pH 5.8. The plasmolysis solution was replaced with an enzyme mixture (10 ml enzyme solution g^{-1} fresh weight of tissue) which consisted of 1.0% (w/v) Hemicellulase (Sigma, UK), 0.4% (w/v) Cellulase RS (Yakult Honsha Co., Japan), 0.1% (w/v) Pectolyase Y23 (Seishim Pharmaceutical, Japan) and 5.0 mM MES in CPW9M solution, pH 5.8. Incubation was carried out in the dark (25 ± 2 °C) on a shaker (40 rpm) for 16 h in 9.0 cm diameter Petri dishes. The digested tissues were filtered through a nylon sieve (30 μm pore size; Wilson Sieves, UK) and the filtrate centrifuged (80 × *g*; 10 min). The protoplast pellet was washed twice in CPW9M solution by repeated resuspension and centrifugation. Protoplast viability was determined by uptake of fluorescein diacetate and the diameter of isolated protoplasts was measured by light microscopy using a stage micrometer.

Protoplasts were cultured in the dark (28 ± 2 °C) at a density of 4.0×10^5 ml^{-1} in 1.0 ml aliquots of liquid, MS medium lacking NH_4NO_3, but supplemented with 87.6 mM sucrose, 25 mM $NaNO_3$, 10.7 μM NAA, 2.22 μM BA and 0.5 M mannitol, pH 5.8 (designated MSP19M-N medium). One ml aliquots of this liquid medium, with protoplasts, was laid over 1.0 ml volumes of B5 medium (designated 1B5CNK; Misawa *et al.* 1982) made semi-solid with 0.6% (w/v) Sea Plaque agarose (FMC Corporation, USA), pH 5.8, in 3.5 cm Petri dishes (Nunc, Denmark). Four autoclaved glass rods (each 6.0 mm diameter × 8.0 mm length) were placed end-on equidistantly within each Petri dish prior to the addition of the semi-solid 1B5CNK medium layer, with the immobilised rods protruding from the agarose medium. Control protoplasts were cultured under identical conditions, but in dishes without glass rods.

Cell wall regeneration was determined after 3 days, by mixing equal volumes (50 μl) of 0.1% (w/v) Calcofluor White (Sigma) in CPW9M solution with cultured protoplasts suspended in MSPI9M-N medium and viewing under UV illumination. Growth responses were assessed in terms of initial (IPE; day 25) and final (FPE; day 50) plating efficiencies. IPE was defined as the number of protoplast-derived cells which had undergone at least one mitotic division; FPE as the number of protoplast-derived cell colonies. The latter (n = 100) were transferred after 50 days to MS medium lacking $NaNO_3$ and mannitol, but supplemented with 87.6 mM sucrose, 20.6 mM NH_4NO_3, 10.7 μM NAA and 2.22 μM BA (designated MSP1) and made semi-solid with 0.4% (w/v) agarose (Sigma Type 1) for further proliferation (20 ml per 9 cm Petri dish). Cultures were maintained under the same growth conditions as for axenic shoots. One-month-old protoplast-derived calli (*ca.* 1.0 cm in diameter) were transferred to MS medium of the same composition as MSP1, but with NAA reduced to 0.107 μM and 4.8 μM CPPU replacing BA. At this stage, protoplast-derived calli were placed on the surface of 10 ml aliquots of medium contained in 5.0 cm Petri dishes (4 calli/dish) and cultured as above.

Means and standard errors (s.e.m.) were used throughout; statistical significance between mean values was assessed using a conventional Student's *t*-test. A probability of $p < 0.05$ was considered significant.

Juvenile leaves, taken from axenic shoot cultures, provided high protoplast yields of 1.95×10^7 g^{-1} fresh weight. Protoplasts were 10–25 μm in diameter with peripherally-aligned chloroplasts, and had a mean viability of 85 ± 2%. Cell wall formation commenced after 1–2 days of culture, with a concomitant increase in the volume and cytoplasmic content of the protoplasts. During the first 25 days of culture, a 6-fold increase ($p < 0.05$) in the number of protoplasts entering their first mitotic division, as assessed by IPE, occurred in dishes with glass rods. In contrast, only 1.5 ± 0.4% of the protoplasts had undergone mitosis in the control cultures (Table 1). In the latter, cell division occurred only where freshly-isolated protoplasts, suspended in the liquid phase, were in contact with the meniscus formed where the culture medium touched the sides of the Petri dishes. In control cultures, mitosis was not sustained and significant numbers of cell colonies were not formed (Fig. 1A). However, when glass rods were introduced into the two-phase culture system, the extent of the liquid meniscus was increased and cell

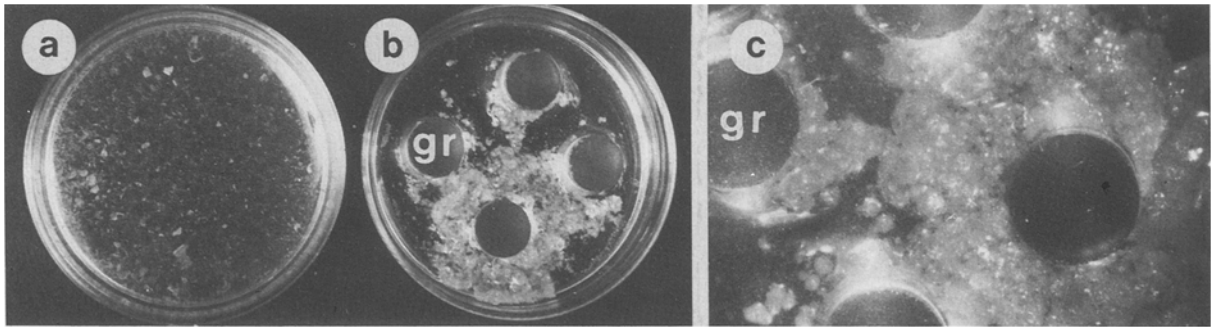

Fig. 1. Petri dishes (3.5 cm diameter) in which leaf protoplasts of *M. esculenta* cv. M. Thai 8 were cultured for 40 days in (a) liquid medium over agarose-solidified medium (control), (b) liquid over agarose-solidified medium with glass rods (gr) protruding from the underlying agarose layer, and (c) higher magnification of part of (b) showing glass rods surrounded by protoplast-derived cell colonies.

colonies developed in close association with the glass rods. Protoplasts cultured with glass rods proliferated into dense colony aggregates by day 40 (Figs. 1B, 1C).

Proliferating multicellular colonies, when transferred to semi-solid MSP1 medium after 50 days of culture, developed rapidly to produce friable callus (2–3 cm diameter) by day 90. Such callus typically developed 1–2 roots per tissue portion after a further 21 days of culture. Calli transferred to semi-solid MS medium containing CPPU, developed a nodular appearance within 30 days. Such nodular callus, when sectioned, displayed an epidermal layer and distinguishable cortex and vascular tissues, with an internal organisation similar to that of cassava tubers. Meristematic regions were absent.

The present experiments demonstrate that high yields of cassava protoplasts can be routinely obtained from leaves of cultured shoots. These isolated protoplasts can be induced to undergo sustained division to form multicellular colonies when partially embedded glass rods are included in the two-phase culture system. Such use of glass rods, to increase the number of liquid:glass/plastic contact points, provides a simple method for ensuring a sustainable plating efficiency of cassava leaf protoplasts which, hitherto, has not been obtained with other approaches (Byrne 1984).

The novel system described here probably facilitates a greater gaseous exchange between the liquid phase, containing the protoplasts, and the immediate atmosphere above the medium in the dish. In this regard, previous studies have shown that oxygen-enriched atmospheres also enhance the plating efficiency of cultured protoplasts of jute, rice and tomato (d'Utra Vaz *et al.* 1992). Furthermore, the division of protoplasts from cell suspensions of albino *Petunia hybrida* is stimulated by culture of the protoplasts at the interface between oxygenated perfluorocarbon and an overlaying aqueous culture medium (Anthony *et al.* 1994). Consequently, it will be of interest, in future work, to assess whether combinations of these physical and chemical approaches to gaseous manipulation can further enhance protoplast division and cell colony formation in cassava, including protoplasts which have been modified genetically. In cassava, such studies should also involve the use of a broader range of growth regulators in the medium ultimately to promote plant regeneration from protoplast-derived tissues. Additionally, it may be feasible to regenerate plants directly from protoplasts through somatic embryogenesis without an intervening callus phase. In this respect, embryogenic cell suspensions may be useful source material for the isolation of protoplasts.

To date, studies have shown adenine-type cytokinins to be ineffective growth regulators for inducing differentiation of protoplast-derived callus (Anthony *et al.* unpublished). In the present study, the substituted pyridyl phenylurea compound, CPPU, was evaluated, since this synthetic cytokinin is effective at very low concentrations on a wide range of species (Fellman *et al.* 1987) and induces somatic embryogenesis in grape (Matsuta & Hirabayashi 1989). The induction, by CPPU, of nodular and tuber-like structures in protoplast-derived callus of cassava, indicates that the use of this phenylurea derivative may, ultimately, result in the differentiation of shoots in the cv. M. Thai 8 tested in these experiments and in other cassava genotypes previously considered to be recalcitrant to differen-

tiation when exposed to adenine-type cytokinins. A focus of future studies to further improve protoplast growth and differentiation in this biotechnologically-important plant will be to optimise not only gas supply, but also growth regulator-induced differentiation.

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