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Tissue culture investigations of bamboo
V. Recovery of callus from protoplasts of suspension-cultured *Bambusa* cells

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Abstract. The primary determinants of sustained protoplast division were feeder-cell provision and timely reduction of osmoticum. The protocol that emerged involved supplementing the cell culture medium with BSA, arginine HCl, MES, and mannitol (initially 0.6 M); embedding in *ca*. 1-ml drops of Sea Prep agarose; flooding with weekly refreshed suspensions of feeder cells the first 4 weeks; and lowering the mannitol in 0.2 M increments after 2, 6 and 7 weeks. Sustained division was observed among as high as 60% of *Bambusa multiplex* and 40% of *B. oldhamii* protoplasts from suspension-cultured cells.

Key words: *Bambusa multiplex*; *Bambusa oldhamii*; Cell culture; Protoplast culture.

Introduction

The preparation of viable protoplasts from suspension-cultured cells of *Bambusa multiplex* (Loureiro) Raeuschell and *B. oldhamii* Munro was described previously (Huang *et al.*, 1989). When placed in culture, the protoplasts divided and produced clusters of 8 or more cells, but the division was unsustainable to produce isolable callus. A multitude of methods and media employed successfully with other plant groups was ineffective. We now report of recoverable callus growths eventually achieved with the aid of feeder cells and a sequence of osmotic lowering.

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[2]**Abbreviations:** BSA, bovine serum albumin; 2,4-D, 2,4-dichlorophenoxyacetic acid; FDA, fluorescein diacetate; MES, 2-(N-morpholino) ethanesulfonic acid.

Materials and Methods

The protoplasts were prepared by the procedure described for suspension-cultured *Bambusa* cells (Huang *et al.*, 1989). Principal experiments were performed with *B. multiplex*, and confirmatory tests with *B. oldhamii* or cells were subcultured following the procedure for liquid suspension cultures (Huang *et al.*, 1988), using inocula of 2.5 g/25 ml and 1 g/25 ml, respectively, for *B. multiplex* and *B. oldhamii*, at 7-d intervals to ensure active division. *B. multiplex* cells were used for protoplasts 6 d and *B. oldhamii* 5 d following transfer to fresh medium.

Enzyme preparations used were Cellulysin (1%), Driselase (2%) and Pectolyase Y23 (1%), and addenda included 0.7 M mannitol, Murashige and Skoog (1962) nutrient salts, 100 mg/l *i*-inositol, 1 mg/l thiamine HCl, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine HCl, 2 mg/l glycine, 3 mg/l 2,4-D, 10 mM

MES, 50 mM arginine HCl, 0.1% BSA, and 0.05% Difco Bacto malt extract. Quantities of 2.5 g cells, pelleted from suspensions at 1000 g for 10 min, and 10 ml enzyme solution were used throughout. Cells were digested 16 h at 12°C and constant shaking (80 rpm).

Digests were filtered through 40-μm Nytex fabric; pelleted at 250 g for 5 min; resuspended in the above solution, minus enzymes and containing lower, or 0.6 M mannitol; layered onto a similar solution prepared with isotonic sucrose; and centrifuged at 250 g 5 min. The interface fraction was removed and rinsed twice by resuspending in enzyme-less solution containing 0.6 M mannitol and pelleting at 250 g. All solutions were used and centrifugations carried out at 12°C. The final pellet was resuspended at a protoplast concentration of 1.5 x 10^6/ml in nutrient solution containing 0.6 M mannitol.

The basal medium for protoplast culture contained all ingredients for cell culture (Huang *et al.*, 1988) plus the following: 0.1% BSA, 0.05% malt extract, 10 mM MES, 50 mM arginine HCl, and 0.6 M mannitol. Difco Bacto purified agar in a 0.8% concentration was used as gelling agent in early experiments. The agar was subsequently replaced by 1.2% FMC Sea Plaque agarose and eventually by 1.6% Sea Prep agarose. Sea Prep agarose was chosen because of its low gelling temperature, *ca.* 17°C, and therefore possibly affecting the embedded protoplasts less adversely.

Suspension-cultured *B. multiplex* cells were used for feeder. They were no older than 7 days since subculturing in fresh medium. They were suspended in cell culture solution (Huang *et al.*, 1988) with variations in osmotic addendum and employed at a rate of 1 g cells/10 ml.

Conditioned nutrient solution was prepared by culturing high densities, 5 g/50 ml medium, of *B. multiplex* cells in liquid suspension for 10 days, then removing the cells by centrifugation and filtration through 2-4 layers of kimwipes. Osmoticum was added, the pH adjusted, and the solution membrane -filtered for use in protoplast culture.

Cultures were incubated at 25°C and under low intensity illumination (16 h/d with 22.5 μmol $\cdot$ m^{-2} $\cdot$ s^{-1} from Toshiba F1-40 SBR/38 fluorescent lamps).

Proportions of protoplasts that had divided or lysed were recorded at intervals of 3-7 d until callus had developed or cultures displayed total cytolysis. The 95% confidence limits of percentages were obtained from tables for binomials in Steel and Torrie (1960).

Results and Discussion

Initial experiments compared the behavior of protoplasts embedded in gelled media (purified agar or Sea Plaque agarose) with those suspended in liquid. The gels were plated or distributed in 1-4 ml drops in culture dishes. The latter enabled flooding with nutrient solutions of varied osmotic concentrations at desired intervals during culture. The liquid suspensions were placed in culture dishes in 1-4 ml quantities and shaken gently (50 rpm) continuously or in 0.10-0.15 ml droplets and held stationary. The osmotic concentration of the larger suspensions was alterable by pelleting the protoplasts and replacing with new nutrient solutions at desired intervals. More than 30% of protoplasts embedded in agarose-gelled medium, and whether plated or as drops, had undergone division in 3 d. None in liquid suspensions had divided. Nevertheless, dividing protoplasts diminished rapidly to near 10% after 7 d and 5% in 10 d; both divided and undivided protoplasts had undergone cytolysis progressively. The agarose drops disclosed that an inoculum density of 1.5 x 10^5 protoplasts/ml was about optimum.

Replacement of the inorganic nitrogen with an amino acid mixture (Thompson *et al.*, 1986) and of the vitamins with mixtures recommended by Kao and Michayluk (1975) and Negrutiu and Mousseau (1980) failed to improve protoplast division or survival. Sucrose, glucose, sorbitol, and inositol were experimented as osmoticum; none was superior to mannitol. However, as carbon source, glucose was as effective as sucrose.

Viability as indicated by FDA tests was prolonged when cultures were initiated at a low temperature, then gradually transferred to standard incubation temperature, e.g., 5°C the first day, followed by 15° for 2 d and 20° for 3d, then finally 25°C for the remainder of culture. But this procedure also did not result in sustained division of protoplasts or prevention of their cytolysis.

Sustained division and reduced cytolysis were eventually achieved by employing feeder cells. The feeders were most effective when used as suspensions flooding agarose drops of embedded protoplasts, simi-

lar to the method developed for rice by Kyozuka *et al.* (1987). The feeder was replaced weekly for the first 4 wk with fresh suspensions of cells. Results of representative experiments with the two *Bambusa* species can be seen in Figs. 1 and 2. More than 50% of protoplasts had divided after 42 d, and 10% had produced clusters of 20-100 cells and isolable callus (Figs. 3). The incidence of divided protoplasts was also very high when feeder cells were plated in nutrient agar or agarose and with agarose drops of protoplasts placed on the feeder plates. However, most of the cell clusters did not enlarge beyond the 10-celled size. Like suspensions, feeder plates were refreshed and reduced in osmoticum during the course of protoplast culture, although not as conveniently. The double filter paper technique of Horsch and Jones (1980) was ineffective as a method of furnishing feeder cells. Also, conditioned nutrient solution was unable to replace feeder cells.

Lowering the mannitol concentration of the flooding solution further extended protoplast division. Several variations in lowering sequence were examined and results of one experiment are shown in Table 1. The division of protoplasts in the high mannitol concentration, 0.6 M, was not sustainable much beyond the 2-celled stage; nearly 30% had divided, but none produced clusters larger than 10-celled. Lowering the mannitol to 0.4 M after 2 wk enabled 45% to undergo division and nearly 20% to proliferate into 26- to 200-celled clusters after 8 wk. Although the total frequency of dividing protoplasts was only 31.7%, virtually all had attained the 26-200 celled size by further reducing the mannitol to 0.2 M after 6 wk. Lowering the mannitol level to 0.2 M prematurely, i.e., after only 2wk in 0.4 M, resulted in fewer dividing protoplasts and a diminishing effect on sustained division, i.e., only 23.7% had divided, and 17.2% had enlarged into large clusters. A subsequent closer study disclosed that the most suitable schedule for osmotic lowering was as follows: 0.6 M for the first 2wk, 0.4 M the subsequent 4 wk, 0.2 M during the 7th wk, and finally exclusion of mannitol.

Sustained division among bamboo protoplasts leading to recoverable callus requires feeder cells and timely lowering of osmoticum concentration. The agarose drop method readily allows the use of fresh feeder cells and a coordinated reduction of osmoticum. The method involves placing a *ca.* 1-ml suspension of protoplasts in agarose nutrient medium, 1.5×10^5 protoplasts/ml, at the center of a 1.5- × 5.5-cm plastic Petri dish. After the drop solidifies, the dish is flooded with 7 ml of feeder-cell suspension, 1-g cells/10 ml. The schedule of modifications in composition of the flooding solution is as follows: feeder cells and 0.6 M mannitol the first 2 wk; feeder cells and 0.4 M mannitol the next 2 wk; withdrawal of feeder cells, but retention of osmoticum at 0.4 M an additional 2 wk; mannitol reduced to 0.2 M one wk; and finally, no osmoticum.

The feeder cells should be refreshed for effectiveness and to minimize their proliferation. Cultures should be rinsed thoroughly to remove all feeder cells when replacing flooding solutions. With experience, divided protoplasts and cell clusters derived from protoplasts are easily distinguishable by their morphology from feeder cells.

The protocol resultant from this investigation is yet another step in cell culture and protoplast manipulations for parasexual genetic improvement of bamboo

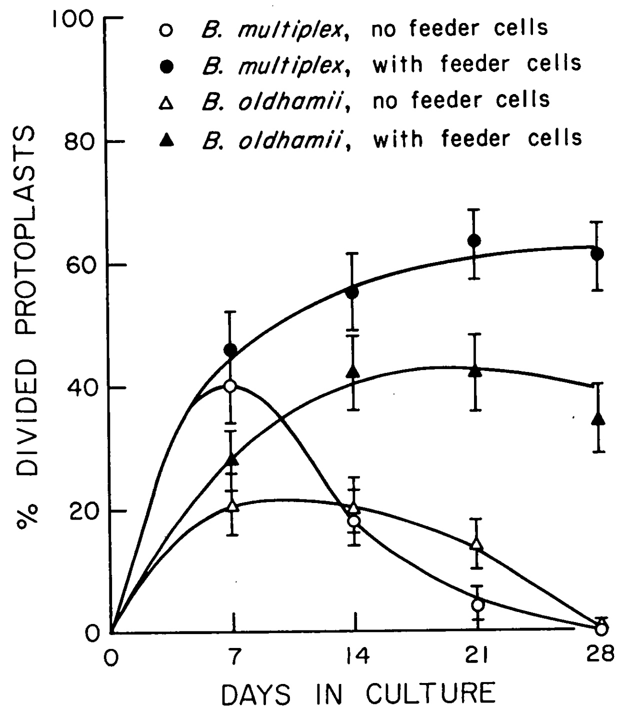

Fig. 1. Influence of feeder cells on sustained division of protoplasts prepared from suspension-cultured cells of *B. multiplex* and *B. oldhamii*.

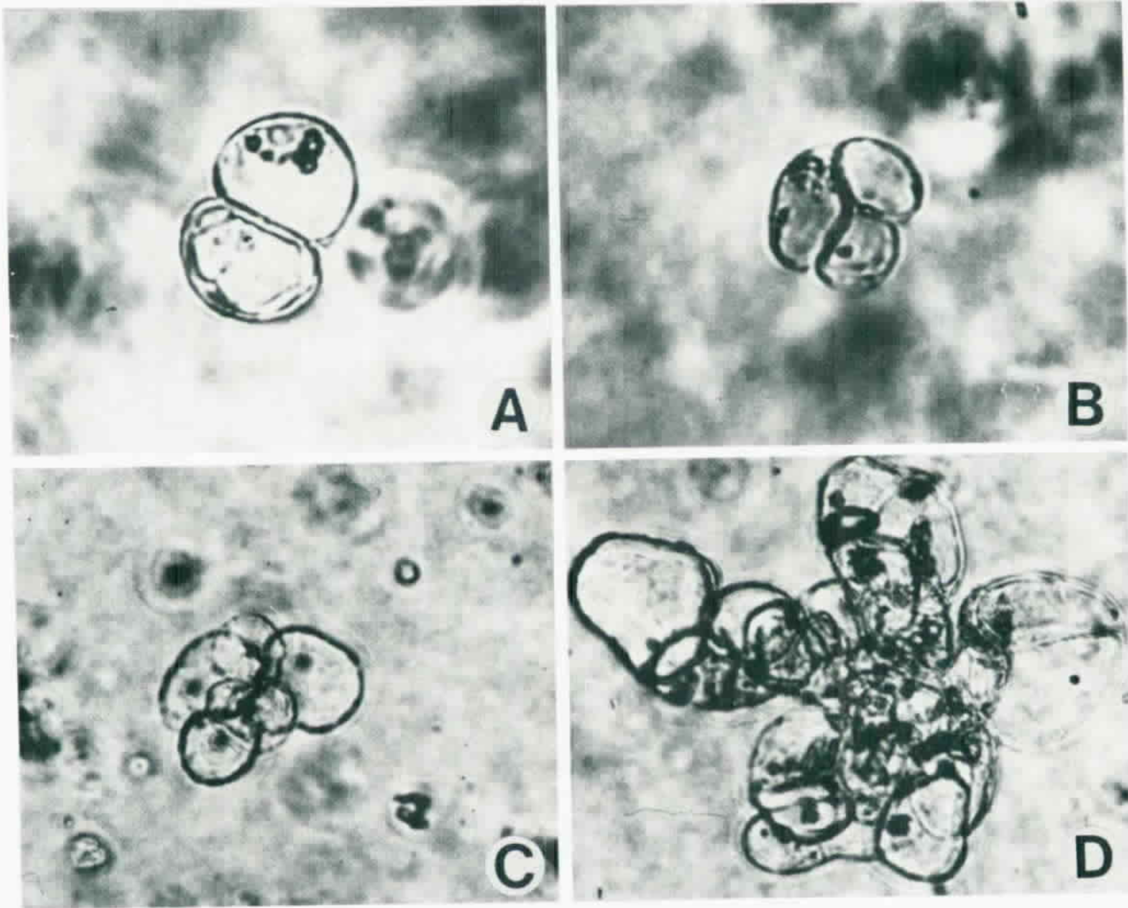

Fig. 2. Sequences of *B. multiplex* protoplast divisions during culture process. A-B, Protoplasts divided to two and four cells after one wk in feeder cell suspension with 0.6 M mannitol. C-D, Continuous cell divisions led ten- to 50-cell formations with mannitol being reduced to 0.4 M after two to four wk.

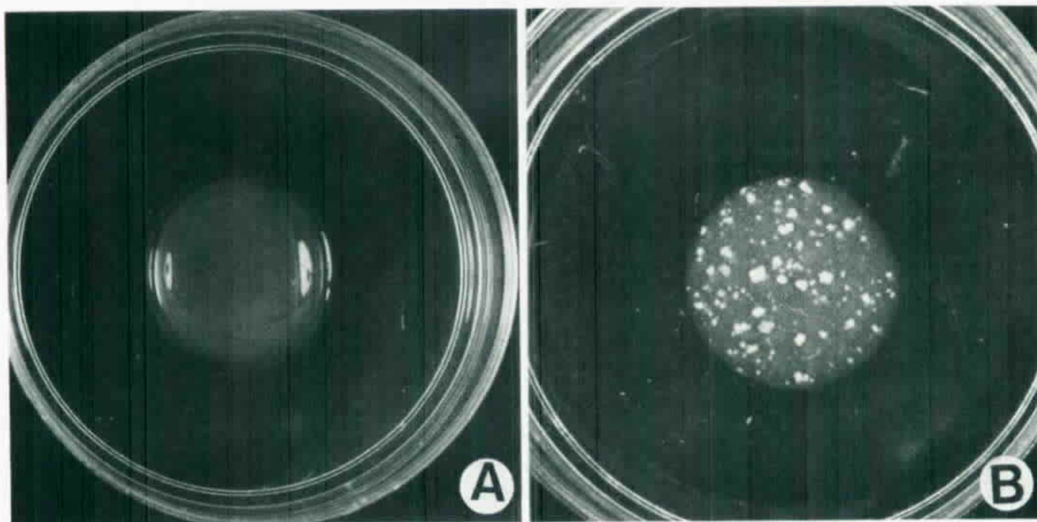

Fig. 3. Effects of provision of feeder cells on protoplast culturability. A. Faildre of callus development from *B. multiplex* protoplasts embedded in Sea Prep agarose drop, but without feeder cells. B. Reculturable callus growth provided with weekly-refreshed feeder-cell suspension the first 4 wk. Culture period = 8 wk.

Table 1. *Sustained division of **B. multiplex** protoplasts as influenced by lowering of osmotic concentration during culture.* Culture dishes with agarose drops of embedded protoplasts were flooded with weekly-refreshed nutrient solution containing mannitol and feeder cells. Feeder cells were withdrawn after 4 wk. Data are from observations of 300 original protoplasts per treatment after 8 wk. Numbers in parentheses are 95% confidence limits. Mannitol provisions were: A= 0.6 M throughout; B= 0.6 M 2 wk and 0.4 M thereafter; C= 0.6 M 2 wk, 0.4 M next 4 wk, and 0.2 M thereafter; and D= 0.6 M 2 wk, 0.4 M the next 2 wk, and 0.2 M thereafter.

	% Clusters with Cell Numbers as Follows						Total Divided Protoplasts, %
	2	6-10	11-15	16-20	20-25	26-200	
A	24.9 (20.2-30.3)	3.9 (2.2-6.8)	0	0	0	0	28.8 (23.4-34.5)
B	13.3 (9.4-17.3)	7.9 (5.2-11.6)	2.3 (0.9-4.2)	1.7 (0.9-4.2)	0.6 (0.2-2.9)	19.4 (14.7-23.9)	45.0 (39.3-50.8)
C	1.1 (0.2-2.9)	0.6 (0.2-2.9)	0	0	0	30.0 (24.9-35.5)	31.7 (26.8-37.6)
D	2.2 (0.9-4.2)	4.3 (2.2-6.8)	0	0	0	17.2 (12.9-21.7)	23.7 (19.3-21.2)

cultivars. The task now is the regeneration of plants from protoplast-derived callus.

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竹類植物組織培養之探討

V.蓬萊竹屬懸浮細胞之原生質體之培養

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促進竹類細胞之原生質體成活及進行細胞分裂之基本條件爲哺育細胞之輔助培養及滲透壓之定時遞降，培養基之成份爲細胞培養基中加入 BSA, arpinine HCl, MES，及 mannitol（始自0.6M），原生質體嵌於1 ml 大之 Sea Prep Aparose 點滴中，點滴外供以懸浮的細胞以資哺育，四週後則無須哺育，原生質體可順利成活，液體培養的細胞須每週更新，滲透壓以0.2 M 之比率於培養後第2、6、7週遞降之，蓬萊竹屬液體培養的細胞蓬萊竹之成活率爲60%，綠竹爲40%。