Tissue culture investigations of bamboo
III. A method for viable protoplast isolation from *Bambusa* cells of liquid suspension culture

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Abstract. High yields of viable protoplasts were obtained by digesting 2.5-g quantities of suspension-cultured cells for 16 h at 12°C and 80 rpm in 10-ml volumes of a solution containing 0.5–2% Cellulysin or Cellulase CEL, 1–2% Driselase, 0.5–1% Pectolyase Y23, and 0.7 M mannitol. Clean preparations resulted from differential sedimention through layers of isotonic mannitol over sucrose solutions, followed by repeated rinsing. Protoplast viability was enhanced by additions of 50 mM arginine HCl, 0.1% BSA, 0.05% malt extract, and certain culture medium ingredients and by performing digestions at low temperature (12°C). Yields averaged 1 to 2×10^6/g cells.

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

Introduction

Protoplasts may provide the parasexual alternative for bamboo cultivar improvement. Traditional breeding has been virtually precluded by rare and unusually late and untimely flowering among the Bambusoideae. In addition to enabling hybridization by their fusion, the protoplasts could be screened for mutants or used for insertion of foreign genes. An early report by Tseng *et al.* (1975) listed bamboo among several Taiwan crops, the mesophyll protoplasts of which were isolable by enzyme treatment; no mention was made of their viability. This report describes the preparation of clean, viable protoplasts, i.e., preparations that were free from cells and debris and contained protoplasts that resume cell proliferation when cultured.

Materials and Methods

Liquid-suspension-cultured cells of *Bambusa multiplex* (Loureiro) Raeuschell and *B. oldhamii* Munro served as protoplast donors. The suspensions contained primarily multicellular aggregates and only a small portion of free cells (Fig. 1). Details of establishing cell cultures can be found elsewhere (Huang *et al.*, 1988). They originated as callus cultures from excised

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[2] **Abbreviations:** BSA, bovine serum albumin; 2,4-D, 2,4-dichlorophenoxyacetic acid; DMSO, dimethylsulfoxide; FDA, fluorescein diacetate; MES, 2-(N-morpholino) ethanesulfonic acid.
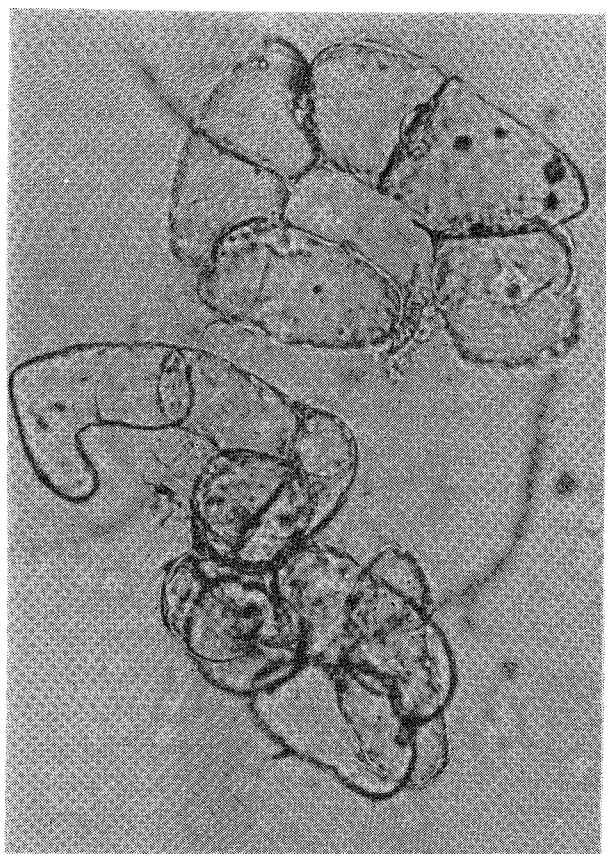
Fig. 1. Suspension-cultured *B. oldhamii* cells employed as source of protoplasts. 250 X.

shoot apices, and were initiated and maintained in a medium containing Murashige and Skoog salts (1962); modified White (1963) vitamins and glycine (1 mg/l thiamine HCl, 0.5 mg/l each nicotinic acid and pyridoxine HCl, and 2 mg/l glycine); and in mg/l, *i*-inositol, 100; 2,4-D^2, 3; and sucrose, 30,000. Initially, 1-g quantities of cells from stock cultures were subcultured in 25-ml volumes of medium and sampled after designated periods to establish the optimum harvest time of donor cells. The ratio between quantity of cells and volume of digestion solution was determined by examining proto-plast yields from digestions of 0.5-g samples in 0.5- to 5-ml volumes of enzyme solution. Donor cells were prerared by centrifuging suspension cultures at 1,000 g (IEC DPR-6000 refrigerated centrifuge) for 15 min at 25°C. Digestions were performed in 50-ml Erlenmeyer flasks, immersed in a refrigerated gyratory water-bath shaker and incubated for durations and at tempera-tures and at shaking rates as prescribed according to experiments.

The cell digests were filtered through Nitex nylon fabric of 40-μm pore size to obtain filtrates that were essentially confined to protoplasts, small cells and subcellular debris. To remove small cells and debris, pelleted preparations of the filtrates were resuspended in 9-ml volumes of 0.7 M mannitol solution, carefully layered onto 1-ml volumes of isotonic sucrose in 15-ml conical centrifuge tubes, then recentrifuged at 250 g for 5 min.

Fluorescence by isolates after staining with Calcofluor White M2R (1 mg/ml in isotonic mannitol) was considered as indicating presence of cell wall material (Uchimiya and Murashige, 1974).

Viable protoplasts were estimated by adding 20 μl of FDA (Huang *et al.*, 1986) reagent (0.5% in DMSO) to 1-ml samples, waiting 30 sec, then counting fluorescing and non-fluorescing protoplasts with the hemacytometer. Acetone sometimes used in dissolving FDA caused protoplasts to be deformed; thus, the choice of DMSO. A Nikon Fluophot microscope with a B filter was used for observations.

To confirm viability, the resumption of cell division was monitored when protoplasts were cultured. The culture medium contained all ingredients of the rinse solution and 1.6% FMC Sea Prep agarose. Protoplasts were dispersed in the still fluid medium (30°C) and dispensed as 0.5- to 4-ml drops in 15- × 55-mm plastic Petri dishes. They were incubated at 27°C and under 16-h daily illumination with 22.5 μmoles m^{-2} s^{-1} light from Toshiba Fl-40 SBR/38 fluorescent lamps. The proportion of dividing protoplasts was recorded after 3 days and beyond.

In all experiments, 3 samples were employed

per treatment. Reported yields are per g original cells. The data were analyzed for statistical significance by calculating standard errors of means or obtaining their 95% confidence limits from tables of binomials (Snedecor, 1946).

Results

Sampling of Donor Cells

Highest yields of *B. multiplex* protoplasts were obtained from cells harvested 9 to 15 days following transfer of 1-g inocula of cells to fresh nutrient solution (Fig. 2). FDA test also indicated highest proportion of viable protoplasts in the 9-day sample, with progressively lower incidences in younger or older samples. Suspension-cultured cells of *B. oldhamii,* also from 1-g inocula, gave largest protoplast yields 5 days after their transfer from stock to fresh medium. Considerably lower yields were obtained when donor cells were harvested after 9 days. Growth curves of suspension cultures showed substantially faster fresh-weight increase of *B. oldhamii* than of *B. multiplex*. The

Fig. 3. *B. oldhamii* protoplast yield as determined by ratio of cell quantity to digestion solution volume. Cell samples were 0.5 g each.

linear growth period of the former species occurred between 3 and 9 days, and that of the latter between 6 and 15 days. It was possible to advance the harvesting of *B. multiplex* cells from 9 to 5 days, without diminishing protoplast yield, by simply increasing the inoculum for donor cell cultures to 2.5 g per flask.

A ratio of 1:4, g donor cells : ml digestion solution, was optimum (Fig. 3). Higher or lower ratios gave significantly lower yields. From these data, samples of 2.5 g cells and 10 ml of digestion solution were chosen as standard.

Composition of Digestion Solution

Enzymes. The reference digestion solution contained 10% Cellulysin (Calbiochem-Behring), 5% Driselase (Kyowa Hakko Kogyo), 4% Macerase (Calbiochem-Behring), and 4% Pectolyase Y23 (Seishin Pharmaceutical). The concentrations were determined through digestions at 27 to 37°C for 4 to 8 h. Exclusion of Macerase did not reduce protoplast yield. But yields
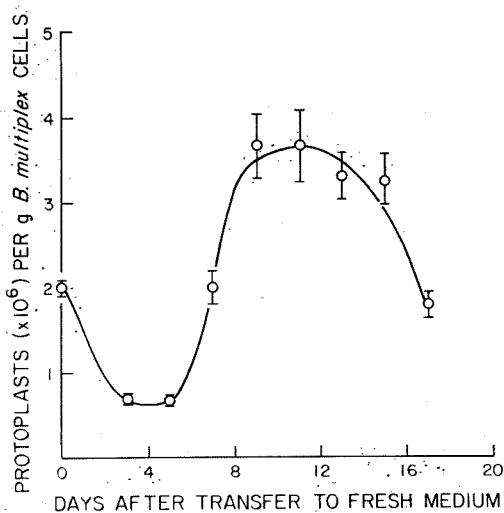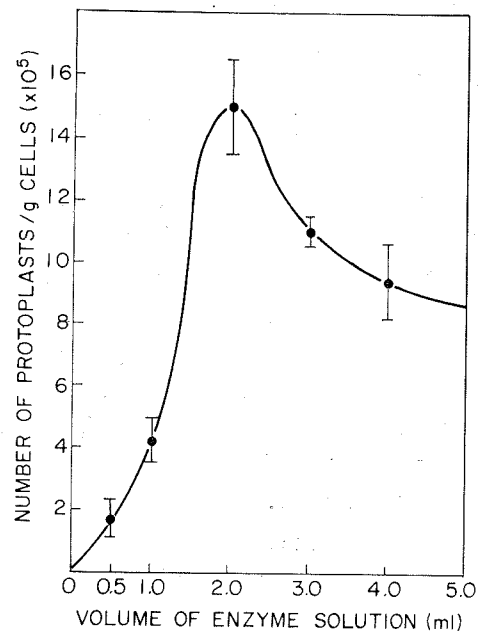
Fig. 2. Dependence of *B. multiplex* protoplast yield on time after transfer of donor cells to fresh cell culture solution. Inoculum in preparing donor cells was 1 g cells/25 ml medium.

were drastically reduced by deleting Driselase or Pectolyase Y23. Other experiments showed lowere yields when Cellulysin was also omitted.

Cellulase CEL (Worthington Diagnostics), a more highly purified cellulase preparation, effectively replaced Cellulysin. Hence, Cellulase CEL was preferred over Cellulysin when the protoplasts were intended for culture. But a highly purified pectinase preparation, Pectinase PASE (Worthington Diagnostics), was unable to substitute for Pectolyase Y23. When employed in the range 0.001 to 1%, Pectinase PASE only suppressed the combined activities of the other enzymes.

Substantial reductions in enzyme concentrations were possible by lowering the digestion temperature to 12°C and extending the digesting time to 16 h. For *B. multiplex,* the optima became 2%, 2% and 1%, respectively, of Cellulysin, Driselase and Pectolyase Y23. For *B. oldhamii* cells, they were 0.5%, 1% and 0.5% (Fig. 4). The differences between species have been confirmed through repeated tests.

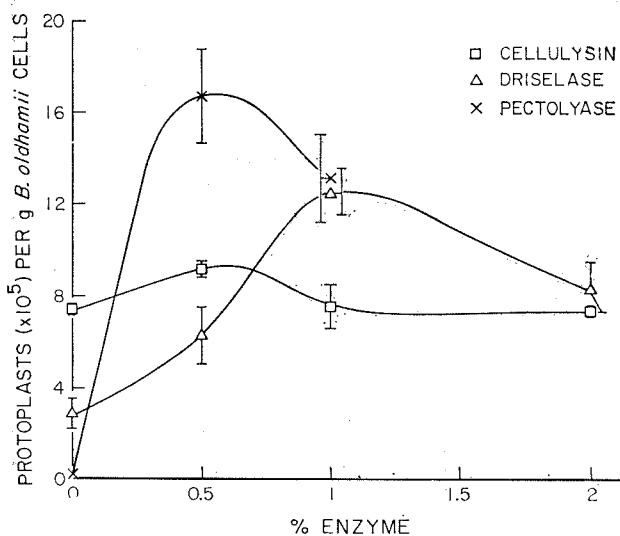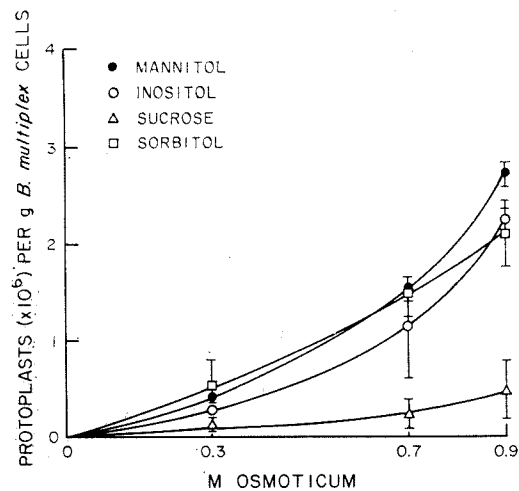*Osmoticum.* Inositol and sorbitol were nearly

Fig. 5. Effectiveness of mannitol, sorbitol. sucrose, and inositol as osmotica in release of *B. multiplex* protoplasts.

as effective as mannitol, but sucrose was not (Fig. 5). At the 0.7 M level, mannitol was clearly the most effective, yielding 2.8×10^6 protoplasts/g cells, followed by sorbitol with 2.6×10^6, inositol with 1.4×10^6, and sucrose with 8×10^5/g cells.

Other addenda. Preliminary experiments suggested improved yields by additions to the digestion solution of the polyamine, putrescine, and the basic amino acid, L-arginine. The polyamine, spermidine, and amides, L-asparagine and L-glutamine, were ineffective. Further study established a 50 mM optimum for L-arginine HCl, the associated yield being nearly twice that of arginine-less solution (Table 1). FDA test also indicated extended viability of protoplasts when cells were digested in arginine-containing solution. A similar study with putrescine disclosed only negative effects.

Improved yields were also obtained with additions of Difco Bacto malt extract and BSA (Sigma A-7638), the optima being 0.05% and 0.1%, respectively.

Ascorbic acid at 3 g/l increased yield markedly. However, this addendum hastened browning of protoplasts.

Inclusion. of all ingredients of the bamboo

Fig. 4. Enzyme concentions required for release of protoplasts from cultured *B. oldhamii* cells. Digestion was performed at 12 °C and 80 rpm for 16 h.

Table 1. *Yield of B. oldhamii protoplasts as influenced by arginine HCl addenda to digestion solution.*

Digestion solution contained 0.5% Cellulysin, 1% Driselase, 0.5% Pectolyase Y23, 0.7 M mannitol, and all supplemental addenda. Digestion was performed at 12°C and 80 rpm for 16 h.

Arginine HCl (mM)	Protoplasts (No. /g cells)	FDA staining (%)
0	683,110±175,798	95
3	732,444±168,525	96
10	602,222± 56,381	93
30	632,592±145,037	97
50	1,044,888±141,882	97
100	449,481± 83,432	95

cell culture medium doubled protoplast yield. Beneficial components were not specifically identified, although experiments with sole additions of 3 mM $CaCl_2$ the calcium salt and at the level found in the Murashige and Skoog medium, increased yield only slightly. A 10 mM level of this salt was depressive. Exclusions of vitamins and inositol were without effects.

MES was added to the digestion solution to maintain pH (Slavik and Widholm, 1978). MES

Table 2. *Yield of B. multiplex protoptasts as infuluenced by digestion temperature*

Data show ratios of yields at prescribed temperatures, T°C, to those at 27°C. Digestions were performed at 80 rpm for 16 h, using solutions containing 2% each of Cellulysin, Driselase and Pectolyase Y23; 0.7 M mannitol; and 50 mM arginine HCl.

Temperature (°C)	Yield ratio	FDA staining (%)
2	1.58	92
7	1.28	87
12	1.96	89
17	1.24	88
22	1.24	82
27	1.00	87
32	0.90	71
37	0.57	89

concentrations below 10 mM failed to prevent drifts; hence, a 10 mM level was chosen as standard.

Digestion Conditions

Superior yields and proportions of FDA-staining protoplasts were attained by digesting at relatively low temperatures. Table 2 shows yields at various temperatures relative to the yield at 27°C. Higher yield ratios were obtained from all digestions at 22°C and lower, with the maximum resultant at 12°C. Digestions carried out above 27°C gave considerably lower ratios. FDA tests also disclosed lower percentages of viable protoplasts at higher temperatures.

Shaking incubation was critical. Protoplast yields from digestions at shaker speeds of 80 to 130 rpm were 4 times greater than those from stationary conditions (Fig. 6). The frequencies of protoplasts that fluoresced with FDA were the same for all speeds.

Temperature during Purification of Protoplasts

Temperature during rinsing of protoplasts was also important. The rinse solution was the digestion solution with enzymes omitted.
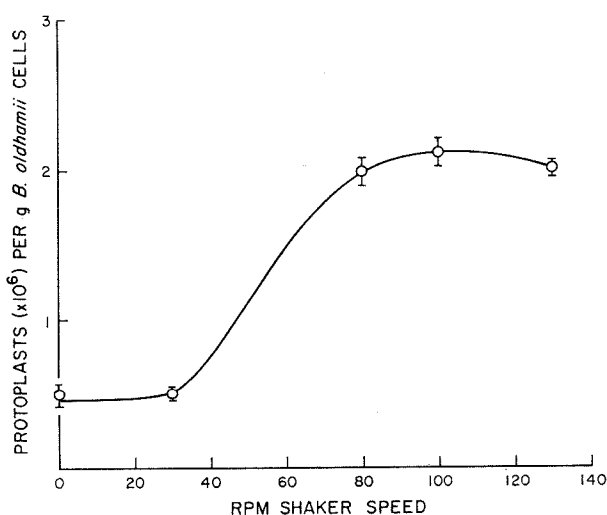

Fig. 6. Yield of *B. oldhamii* protoplasts as related to shaking speed during digestion.

Rinsing at 10°C and below gave consistently higher protoplast yields than at room temperature. FDA-staining protoplasts were also higher with the cold rinse, e.g., 82% at 10°C vs. 66% at room temperature.

Protoplast Yields and Confirmation of Viability

The final procedure gave protoplast yields that averaged 1 to 2×10^6 protoplasts/g donor cells. Cell counts revealed 1.2×10^7 cells per g donor sample. The average protoplast yield was, thus, 10% of the bamboo cells. The protoplasts ranged from 22 to 27 μm in diameter. They did not fluoresce with Calcofluor White and confirmed complete removal of cell wall. FDA tests indicated usually better than 90% of protoplasts to be alive (Fig. 7). Over 30% had divided after 3 days in agarose medium. Clusters of 8 cells and more were observed within 10 days (Fig. 8).

Discussion

At the outset, we applied the method of Tseng *et al.* (1975) to the suspension-cultured cells of *B. oldhamii* and *B. multiplex* and obtained no protoplasts. The protocol of Uchimiya and Murashige (1974) for suspension-cultured *Nicotiana tabacum* cells also yielded no bamboo protoplasts. A study with factorial combinations of 1 to 5% Cellulysin and 0.2 to 1% Macerase, employing digestion periods of 2 to 24 h, similarly gave negative results. Some protoplasts were eventually obtained by increasing the Cellulysin level to 10% and the Macerase to 4% and by adding Driselase and Pectolyase Y23 at 5 and 4%, respectively.

Having obtained a few protoplasts, the priority was directed at sampling of donor cells. Protoplast yield from bamboo cells was highest when cultures were in the linear phase of fresh weight increase. With cell cultures of
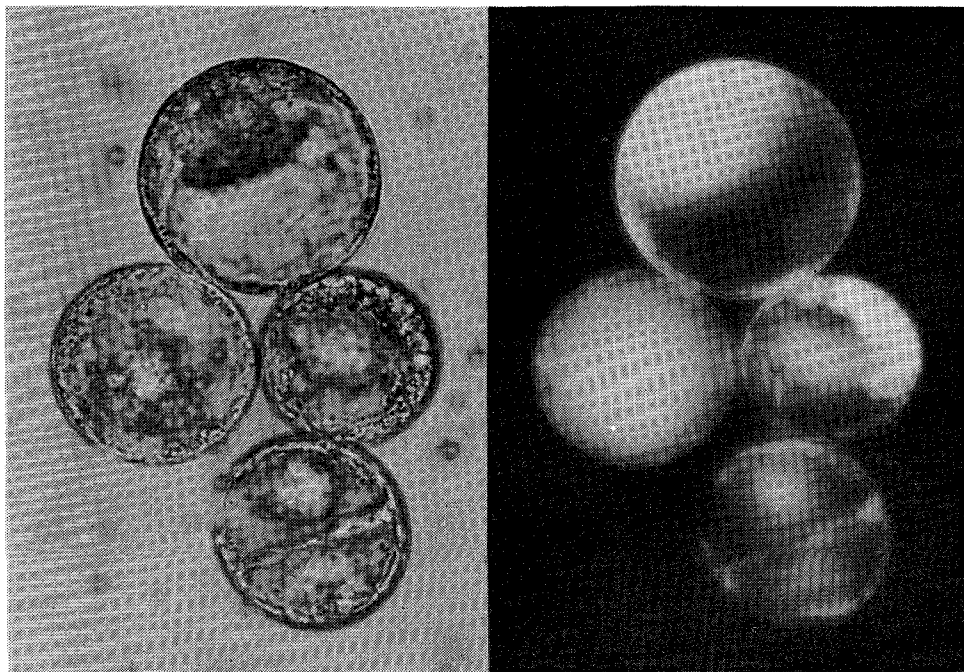

Fig. 7. *B. oldhamii* protoplasts showing fluorescence when stained with FDA and viewed under UV illumination. Left=unstained; right=stained. 2000 X.
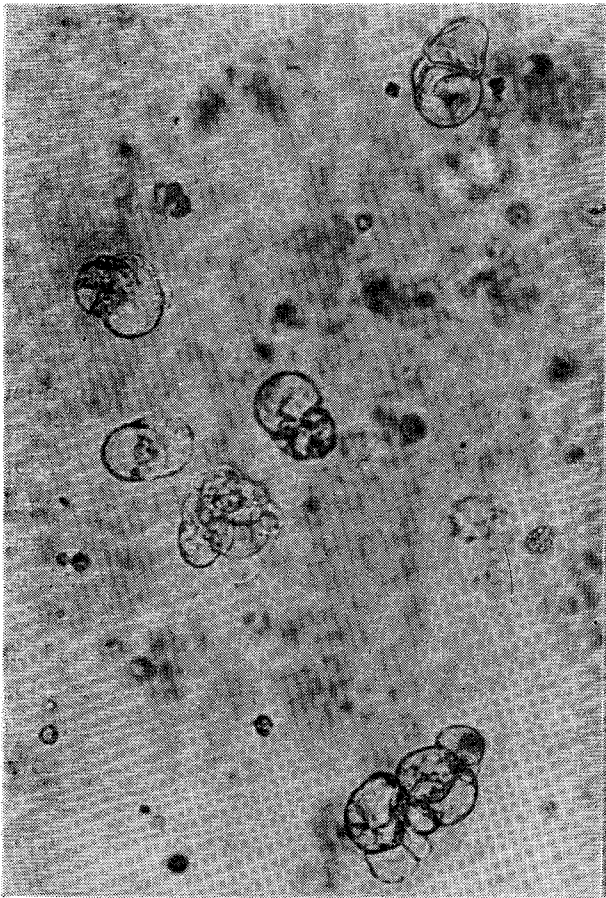
Fig. 8. Dividing *B. oldhamii* protoplasts after 10 days in agarose medium. 1000 X.

other plants, greatest yields have been correlated with periods of active cell division (Uchimiya and Murshige, 1974). Whether cell division was a major contributor to rapid fresh-weight increase of bamboo cell cultures has not been explored. Nevertheless, for *B. oldhamii* and *B multiplex,* high yields of viable protoplasts are obtainable 5 days after donor cells have been placed in fresh nutrient solution. But to compensate for differences in cell proliferation rates of the two species, the inocula in establishing donor cells needed to be standardized differently. That of the more rapidly proliferating *B. oldhamii* has set at 1 g cells per 25 ml culture medium and, for the slower growing *B. multipex,* 2.5 g per 25 ml.

The importance of balance between quantity of donor cells and volume of digestion solution was clearly evident and confirmed an earlier observation of tabacco (Uchimiya and Murashige. 1974). A ratio of 1:10, g cells:ml digestion solution, was optimum when preparing tobacco protoplasts. The optimum ratio for bamboo is 1:4.

The increased protoplast yields at colder tempratures were unexpected, in spite of a rare instance of superior yield of mesophyll protoplasts when tobacco leaves were digested at 3 to 5°C (Dorokov and Aleksandrova, 1981). Dorokov and Aleksandrova noted that the digestion at near-freezing temperatures produced intact and viable protoplasts, whereas that at room temperature or higher (37°C) gave only collapsed and non-viable protoplasts. The superior yield and quality of protoplasts at the lower temperatures were probably due to reduced cytolysis by endogenous enzymes and other destabilizing and senescence-promoting substances that were released by injured cells and protoplasts.

Rapid loss of viability is a serious problem when preparing bamboo protoplasts. Thus, a variety of substances with reported senescence retarding or viability prolonging effects were tested as addenda to the enzyme solution. The basic amino acid, arginine (Altman *et al.,* 1977; Galston *et al.,* 1978), was the most beneficial. But the polyamines, putrescine and spermidine (Altman *et al.,* 1977; Galston *et al.,* 1978), were ineffective. Gibberellin (Landgren, 1981) showed no effects. We included 2,4-D only because it is a standard constituent of the bamboo cell culture medium. The advantage of BSA (Dorokov and Aleksandrova, 1981) is probably due to its protection against proteases, but that of malt extract is unclear. Nutrients of the cell culture medium have been beneficial for protoplast yields and subsequent culturability (Saxena *et al.,* 1981). Their effects could not be

attributed solely to calcium (Rose, 1980) or vitamins (Saxena *et al.*, 1981).

As observed when isolating tobacco protoplasts (Uchimiya and Murashige, 1974), shaking during digestion of bamboo cells is critical.

By serving as sources of endogenous enzymes and other cytolytic agents, cellular and subcellular debris could hasten deterioration of protoplasts. Their removal may not be achieved by simply filtering the digests and rinsing the pelleted protoplasts. They are excludable by differentially sedimentation through layers of isotonic solutions of differing in densities. Bamboo protoplasts gather as a clean band at the interface when mannitol is used as the upper layer and sucrose (Hughes *et al.*, 1978) or Percol (Thomas, 1981) is the lower layer. Percol gives sharper protoplast bands, but is more expensive than sucrose. Cytolysis might also be reduced by rinsing with reagents held at a low temperature (Dorokov and Aleksandrova, 1981).

The investigation showed clearly the need to evaluate key parameters systematically for each bamboo species. Whereas most requirements were the same, the quantities of cells employed in preparing donor cells and the required levels of enzymes differed significantly between *B. oldhamii* and *B. multiplex*. The procedure finally settled on is as follows: donor cells are prepared by transferring 1 g *B. oldhamii* or 2.5 g *B. multiplex* cells from stock suspension culture to fresh nutrient solution. The cells are harvested for digestion after 5 days. Each sample of 2.5 g cells is placed in 10 ml of digestion solution. The solution contains Cellulysin, 0.5% for *B. oldhamii* and 2% for *B. multiplex* cells; Driselase, 1% for *B. oldhamii* and 2% for *B. multiplex*; and Pectolyase Y 23, 0.5% for *B. oldhamii* and 1% for *B. multiplex*. For osmoticum, 0.7 M mannitol is employed. Other additions to the enzyme solution are 50 mM arginine HCl, 0.1% BSA, 0.05% Difco Bacto malt

extract, Murashige and Skoog salts, modified White vitamins and glycine (1 mg/l thiamine HCl, 0.5 mg/l each nicotinic acid and pyridoxine HCl, and 2 mg/l glycine), 100 mg/l inositol, 3 mg/l 2,4-D, and 10 mM MES, The cells are digested in 50-ml Erlenmeyer flasks, with the flasks immersed in a water-bath shaker set at 12°C and operating at 80 rpm. After 16 h, the digests are filtered through 40-μm nylon cloth and the filtrates centrifuged at 250 g for 5 min. The pelleted fraction fraction is resuspended in 9 ml of the above solution without enzymes, layered onto 1 ml of the enzyme-less solution prepared with 0.7 M sucrose, and centrifuged at 250 g for 5 min. The protoplasts that band at the interface are collected and rinsed by resuspending in 10 ml of the enzyme-less solution and recentrifuging. Rinsing is performed at 10°C and repeated twice, the pellet fraction being retained each time. The final pellet is resuspended in a smaller volume of rinse solution for examination and culture. The requirements for culture remain under investigation and will be the subject of a future report.

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竹 類 植 物 組 織 培 養 之 探 討

III. 綠竹及蓬萊竹液體培養細胞分離高活力原生質體之基本條件

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2.5 克液體培養的細胞置於 10 ml 酵素溶液中，於 12°C 及 80 rpm 之振盪下處理16小時，可分離 1~2×10⁶ 之原生質體，酵素溶液包括 Murashige 和 Skoog 無機塩類，Modified White 維生素，inositol，及 0.5-2% Cellulysin 或 Cellulase CEL, 1-2% Driselase, 0.5-1% Pectolyase Y23, 0.7 M Mannitol，分離液中加入 50 mM arginine. HCl, 0.1% BSA, 0.05% Malt extract 可增進原生質體產量 1-2 倍，低溫處理不僅提高原生質體產量，更顯著增進及維持原生質體活力，分離後之原生質體經等濃度之 Mannitol/sucrose 之雙層法淨化清洗，可導致原生質體成活，及細胞分裂。