

Tissue culture investigations of bamboo

II. Liquid suspension cultures of *Bambusa*, *Phyllostachys* and *Sasa* cells¹

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Abstract. A medium containing Murashige and Skoog salts and, in mg/l, sucrose, 30,000; thiamine HCl, 1; nicotinic acid, 0.5; pyridoxine HCl, 0.5; glycine, 2; i-inositol, 100; and 2,4-D, 3; enabled establishment of liquid suspension cultures of *B. multiplex*, *B. oldhamii*, *P. aurea*, and *S. pygmaea*. *B. oldhamii* and *B. multiplex* produced fairly dissociated suspensions, with larger fractions of small, few-celled aggregates and smaller portions of large aggregates. Suspension cultures of *P. aurea* and *S. pygmaea* contained mainly large cell clusters. Stocks were established by tri-weekly subcultures of 500 mg portions of sedimented cells. The suspensions were incubated at 27°C, under 16 h daily exposure to 4.5 nEcm⁻²sec⁻¹ fluorescent light, and with continuous shaking at 150 rpm. Callus growths were reproduced when suspensions were sieved and their filtrates plated in gelled media.

Key words: *B. multiplex*; *B. oldhamii*; Cell dissociation; Cell plating; *P. aurea*; *S. pygmaea*.

Abbreviations: N⁶-benzyladenine; 2,4-Dichlorophenoxyacetic acid; Indole-3-acetic acid; α -Naphthaleneacetic acid; Picloram: 4-Amino-3,5,6-trichloropicolinic acid.

Introduction

The significance of plant tissue culture methods in genetic improvement of bamboos was previously discussed (Huang and Murashige, 1983). Untimely and rare flowering necessitates establishment of parasexual alternatives to traditional breeding methods. That report further described callus cultures of *Bambusa multiplex* (Loureiro) Raeuschell, *B. oldhamii* Munro, *Phyllostachys aurea* A. & C. Riviere, and *Sasa*

pygmaea (Miquel) E. G. Camus. We now present findings with respect to their liquid suspension cultures.

Materials and Methods

Callus in stock culture was used to start the liquid suspensions. In order to establish uniformly small cell aggregates in liquid cultures, a step of cell sieving through stainless steel mesh was required to remove the larger cell clumps after the initial passage in liquid. The nutrient base was comprised yb Murashige and Skoog salts (1962) and, in mg/l, sucrose, 30,000; thiamine HCl, 1; nicotinic acid,

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0.5; pyridoxine HCl, 0.5; glycine, 2; i-inositol, 100; and 2,4-dichlorophenoxyacetic acid (2,4-D). 3. This is the composition standardized for bamboo callus cultures, but without the agar or gelrite. The solutions were dispensed in 25 ml portions into 125 ml flasks, and the flasks capped with Micro Plugs* and sterilized by autoclaving 10 min at 1.05 kg cm^{-2} .

The inocula were prepared by transferring stock cultures to 50 ml conical plastic centrifuge tubes and pelleting at 200 *g* for 10 min. After discarding the supernatant, the tubes were inverted onto dry kimwipes to remove the remaining superfluous liquid. Various quantities (250, 500, 750, and 1,000 mg per culture) of inocula were compared and growth measurements were made at 3-day intervals to determine optimum inoculum size and subculture passage length.

The liquid suspensions were incubated at 27°C and under 16 h daily exposure to $4.5 \text{ nE cm}^{-2} \text{ sec}^{-1}$ fluorescent light. They were shaken constantly at 150 rpm on a gyratory shaker.

For cell dissociation experiments, 500 mg samples of pelleted cells were digested in 5 ml volumes of the strong acids, CrO_3 and HNO_3 , singly and combined and each in concentrations of 1, 5, and 10%, for 12 h at 37°C. The acid-digested cells were rinsed thoroughly with water and subsequently digested an additional 6 h at room temperature in 5 ml of enzyme solution to further reduce the size of cell aggregates. Macerase, driselase and pectolyase Y23 were each tested in a 4% concentration, alone and in combinations. Both digestions were carried out on a gyratory shaker, operating at 80 rpm. After final rinsing of digests, the cells were counted with the hemacytometer.

* Foot Note: Rubber stopper with nylon filter to facilitate gas exchange while excluding air-borne infectious agents, made and distributed by Microwave Enterprise, Inc., Taiwan, ROC)

The optimum inoculum density for cell plating was established by simply mixing and dispersing prescribed quantities of sieved (40-mesh screen) cells in 10 ml volumes of nutrient gel (still fluid by holding at 39°C in water bath) and pouring the mixtures into sterile petri dishes. The plates were incubated at 27°C on culture shelves that were illuminated 16 h daily with $4.5 \text{ nE cm}^{-2} \text{ sec}^{-1}$ fluorescent light.

All data were evaluated with the aid of standard errors of means, calculated according to Snedecor (1946).

Results and Discussion

Our earlier study (Huang and Murashige, 1983) established that for callus cultures 2,4-D in a 3 mg/l concentration was superior to other auxins, i. e., indole-3-acetic acid (IAA), α -naphthaleneacetic acid (NAA) and picloram, in any concentration. This study revealed that 2,4-D in 3 mg/l was also satisfactory for liquid suspension culture of *B. oldhamii*. Cell yields for *B. oldhamii* were virtually the same in 2,4-D concentrations of 1 to 6 mg/l (Fig. 1); a 0.3 mg/l level was too low and 10 mg/l, too high. For *B. multiplex* and *P. aurea*, the optimum 2,4-D level was also 3 mg/l. A 1 mg/l concentration gave the highest cell yield of *S. pygmaea*.

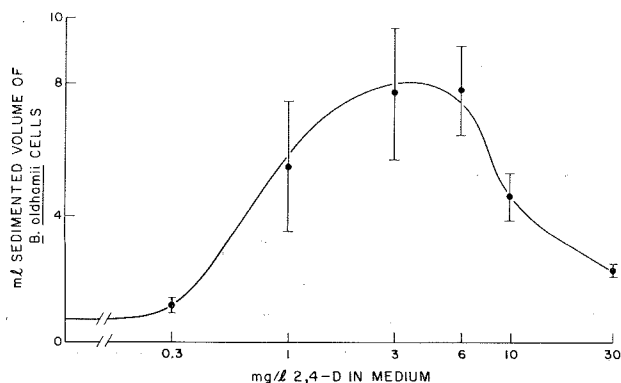


Fig. 1. Yield of *B. oldhamii* cells as influenced by 2,4-D level of nutrient solution.

but not substantially more than 3 mg/l; hence, a 3 mg/l level of 2,4-D has been chosen for all bamboo suspension cultures.

Picloram was 10 times more potent than 2,4-D. The maximum yield of *B. oldhamii* cells was obtained with this auxin at a level as low as 0.3 mg/l (Fig. 2). It also enabled more rapid growth and a higher peak yield of cells than 2,4-D (Fig. 3). With both auxins, the logarithmic growth occurred from the sixth to twelfth day of the passage. Beyond the exponential growth period, however, picloram caused the yield to decline very sharply, rather than to plateau as

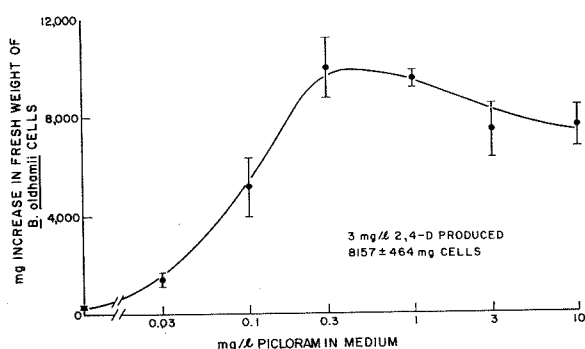


Fig. 2. Yield of *B. oldhamii* cells as influenced by picloram level of nutrient solution.

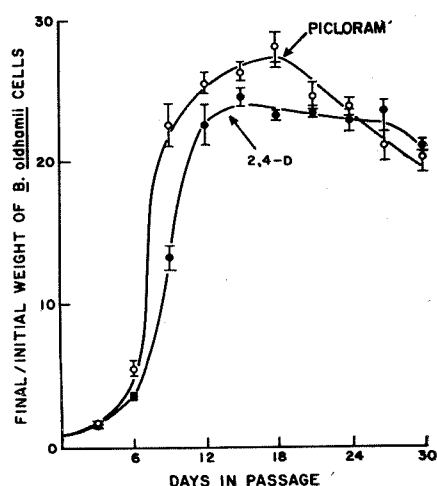


Fig. 3. Growth curves of *B. oldhamii* cells in nutrient media supplemented with 3 mg/l 2,4-D and 0.3 mg/l picloram.

was the case with 2,4-D. Because of this declining yield and other observations that suggested possibly irreversible inhibitions, picloram was not selected as the standard for bamboo cell cultures, even though it is the preferred auxin for many other grasses (Collins *et al.*, 1978). Indeed, death of *B. multiplex* cells resulted when subcultured successively in picloram medium, even in the relatively low concentration. Also based on these data, a 3-week culture period was selected for routine subculturing of bamboo cells in liquid suspension.

Suspension cultures of both *Bambusa* species were comprised predominantly by small cell-aggregates and a minor fraction of large aggregates. Those of *P. aurea* and *S. pygmaea*, on the other hand, contained higher proportions of large aggregates; their dissociation could not be improved by employing higher levels of 2,4-D. *P. aurea* cells also developed an intensely purplish-red coloration, perhaps as result of being exposed to light. An earlier observation disclosed the same pigmentation in illuminated *P. aurea* callus cultures. Lowering the 2,4-D level decreased pigment intensity as well as cell yield. Reducing the sucrose did not decrease coloration, indicating that the pigment was probably not anthocyanin. The other bamboo species produced creamy white suspensions, even under illumination.

No exogenous cytokinin was required by bamboo suspension cultures. Continuous culture of the four species in cytokinin-free, but auxin-containing, nutrient solutions did not diminish cell yield or quality.

Thiamine was clearly essential. Nevertheless, depletion of its residual supply was necessary to observe the requirement or to establish the optimum concentration. Inoculum cells, freshly obtained from stock culture, were unresponsive to removal of the thiamine supplement; the final yields were the same in media with and without thiamine HCl (Fig. 4). Howe-

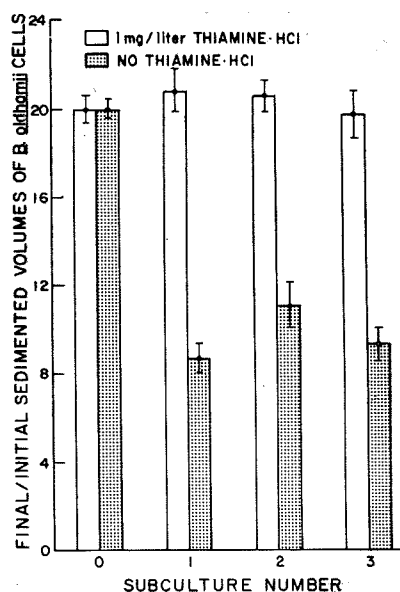


Fig. 4. Dependence of the thiamine response of *B. oldhamii* cells on depletion of residual thiamine from inoculum cells.

ver, subcultures displayed marked growth differences, with yields in the thiamine-less medium being reduced to half of that in the thiamine-containing medium. The cultured bamboo cells evidently biosynthesized some thiamine, since growth continued in all subcultures, albeit at a lower rate than when supplied with the vitamin.

Several retests were needed to establish the optimum concentration of thiamine HCl. In one experiment, progressively higher yields were obtained by increasing the thiamine HCl concentration to as high as 1,000 mg/l. The more consistently observed optimum was 1 mg/l; this level was therefore retained as standard.

Supplements of 30 to 1,000 mg/l of i-inositol generally increased cell yields. The difference in yield enhancement by widely differing concentrations were, however, not substantial. Thus, the original 100 mg/l level of inositol was adopted as standard for the liquid suspension cultures.

Yields of bamboo cells were improved slightly by a 1-3 g/l addendum of casein hydrolysate (Enzymatic digest, ICN Nutritional Biochemicals). However, in view of its chemically undefined and complex quality, the casein hydrolysate has been left as an optional ingredient.

Inoculum densities of 250 to 750 mg cells per culture gave similar final yields, averaging about 1,200 mg. An initial density of 1,000 mg cells per culture gave a yield of 3,000 mg after 3 wk; but the growth value (final/initial cell weight ratio), or efficiency, was lowest at this high density. The growth efficiencies were the same when 250-750 mg of cells were employed as inocula. After repeating the experiment, a starting density of 500 mg cells per culture was established as standard for all bamboo suspension cultures.

The cell dissociation experiments disclosed

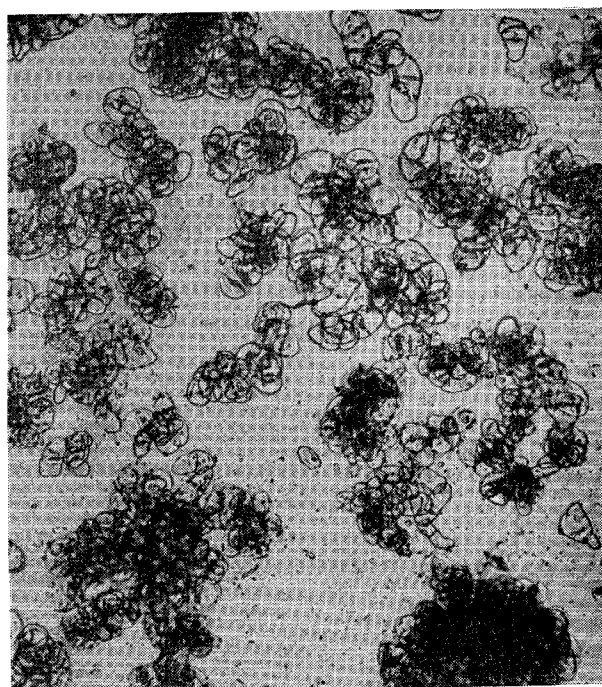


Fig. 5. *B. oldhamii* cells and cell aggregates in liquid suspension culture, before acid/enzyme digestions. 400 X.

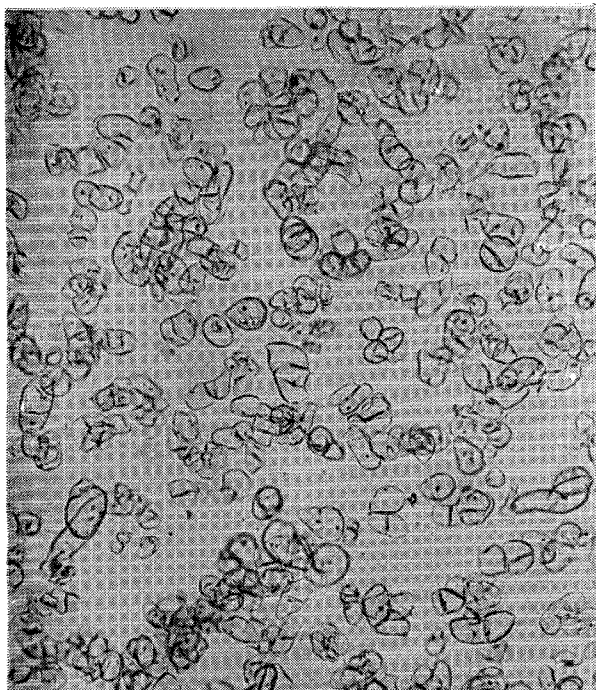


Fig. 6. Dissociated cells and cell aggregates following treatments with chromic-nitric acid and driselase-pectolyase solutions. 400 X.

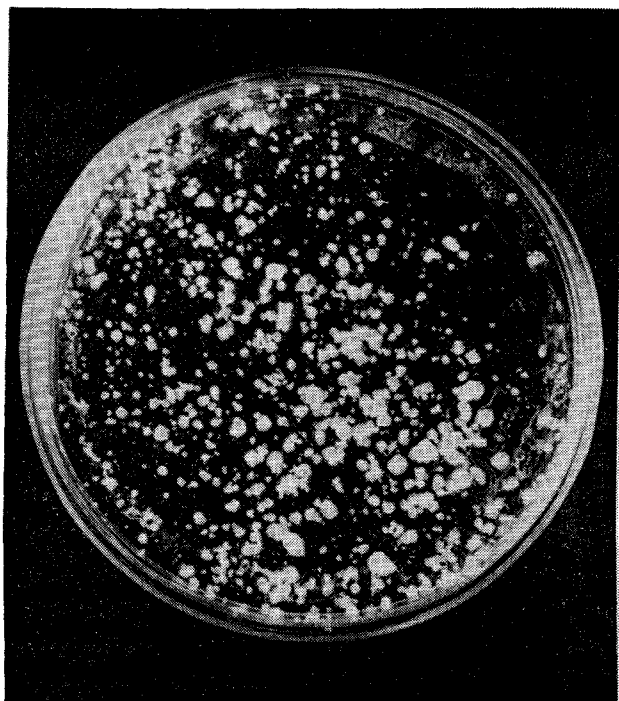


Fig. 7. Recovery of callus growths following plating of liquid suspensions of *B. multiplex* cells.

that digestion in CrO_3 or HNO_3 , used alone in concentrations of 1, 5, and 10%, was ineffective. But the acids caused significant cell separation when used together. Among 9 combinations examined, the mixture of 1% CrO_3 and 10% HNO_3 produced the smallest cell clusters. Subsequent treatment with enzymes showed macerascase to be ineffective, alone or in combination with the other enzymes. The combination of pectolyase Y23 and driselase was the most effective as post-acid treatment, dissociating the bamboo cell aggregates into 2- to 3-celled units. Countable dimensions attained by the sequence of treatments with strong acids and cell-wall degrading enzymes can be seen by comparing Figs. 5 and 6. Counts made following the dissociation procedure revealed a content of 1.2×10^7 cells in each gram of the sedimented bamboo suspension culture.

Callus growths redeveloped when cells and aggregates of liquid suspension cultures that passed 40-mesh sieves were dispersed in nutrient gel (Fig. 7). The plating efficiency was relatively low, averaging below 5% for initial densities of 100,000 to 300,000 cells per ml nutrient medium. Nevertheless, plating of bamboo suspensions is currently applicable in isolating agronomically desirable somaclonal variants.

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竹類植物組織培養之探討

II. *Bambusa*, *Phyllostachys*, 及 *Sasa* 之細胞液體培養

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固體培養基上生長之癒合組織的細胞移置於液體培養基中含 Murashige and Skoog 鹽類及每升 1 毫克之 Thiamine HCl, 0.5 毫克之 Nicotinic acid, 0.5 毫克之 Pyridoxine HCl, 100 毫克之 i-Inositol, 2 毫克之 Glycine, 3 毫克之 2,4-D, 及 30 克之 Sucrose, 並置於 27°C 溫度, 16 小時光照, $4.5 \text{ nEcm}^{-2} \text{ sec}^{-1}$ 之光照, 及 150 rpm 振盪器上培養可建立綠竹 (*Bambusa oldhamii*), 蓬萊竹 (*B. multiplex*), 布袋竹 (*Phyllostachys aurea*), 及箬竹 (*Sasa pygmaea*) 之細胞液體培養, 由實驗得知於培養基中培養 500 毫克的細胞, 經 3 週後再取 500 毫克的細胞於新的培養基中繼續培養, 可維持最佳的生長條件。