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Isolation of Protoplasts from Mesophyll Cells of *Paulownia* and *Populus*

By

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Summary : Expanding young leaves of *Paulownia* and *Populus* seedlings grown in the open air were cut into pieces. Fresh pieces were incubated in the renewed enzyme solution containing pectinase, hemicellulase and cellulase every 20 min for *Paulownia* and 30 min for *Populus* at 30°C with shaking. The intact cells contained in each liquid after the 3rd period of shaking were resuspended in fresh enzyme solution in a water-bath at 30°C without shaking for 30 min for the former and 60 min for the latter. The protoplasts of 700×10^4 pieces of 40 μ in diameter from the former and $560\sim1,200\times10^4$ pieces of 13 μ in diameter from the latter were thus isolated from 2 g in fresh weight of excised leaves.

Introduction

Active protoplasts have been isolated enzymatically from mesophyll cells of higher plants[3)7)9)]. Whole plant regeneration from *in vitro* cultured tissues originated from mesophyll cells has been demonstrated by several authors[4)5)6)]. Moreover, production of a somatic hybrid of amphidiploid nature is expected by fusion of protoplasts isolated from 2 different species. CARLSON et al. (1972) have succeeded in producing *in vitro* somatic hybrid plant with biochemical and morphological characteristics identical to those of the sexual amphidiploid *Nicotiana*[1)].

Since success in isolating protoplasts from somatic cells of higher plant in large quantities using cellulase has been reported by COCKING[2)] in 1960, protoplasts have been used as materials in studies of cytophysiology, cytogenetics and cytobiochemistry of higher plants.

It has been suggested that the naked protoplasts without cell wall may be usefull for hybridizing sexually incompatible species, for introducing foreign nucleic acids or beneficial microorganisms into plant cells, and for genetic manipulation in general[8)]. However, a few workers have isolated tree protoplasts up to the present[7)].

This paper reports the successive isolation from *Populus* and *Paulownia* leaves of morphologically intact mesophyll protoplasts on a sufficiently large scale to permit various biochemical experimentation.

Materials and Methods

Newly expanded young leaves excised from 4-year-old seedlings of *Paulownia taiwaniana* HU et CHANG, sp. nov. and 6-year-old *Populus* × *euramericana* cv I-45/51 grown in a nursery were used for the isolation of cells.

With the aid of a forceps, the midrib was removed from excised leaves and then leaf blade was cut into pieces of approximately 2 mm×15 mm. Two grams (fresh weight) of leaf

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Table 1. Enzyme solution used for isolation of protoplasts

	for *Paulownia*	for *Populus*
Macerozyme R 10 (Pectinase)*	0.3%	1.0%
Hemicellulase**	0.6%	2.0%
Cellulase "Onozuka" R 10*	0.6%	2.0%
D-Mannitol	0.6M	0.6M
Potassium Dextran Sulfate***	1.0%	1.0%
$CaCl_2$	6mM	6mM
NaH_2PO_4	0.7mM	0.7mM
Sodium Citrate ($2H_2O$ salt)	10mM	5mM
Dithiothreitol (DTT)**	2mM	2mM
pH value	5.6	5.6

* KINKI YAKURUTO CO. Ltd.
** SIGMA CHEMICAL CO. Ltd.
*** MEITO SANGYO CO. Ltd.

pieces were used in every treatments.

The isolation of protoplasts was practiced by a single-step procedure that cell separation and wall degradation occur simultaneously by employing a mixture of cellulase and pectinase.

The leaf tissues were incubated in flat-bottomed triangular flask containing 15 m*l* of enzyme solution (Table 1) and then shaken in a water bath at 30°C by a reciprocal shaker (stroke 4.5 cm) at a frequency of 60 excursions per min.

After 15 min of preincubation, during which broken cells were released from cut surface of leaf pieces, the enzyme solution was removed by decantation and was replaced by 20 m*l* of fresh enzyme solution. Thereafter, the solution was renewed in the same way at 20 min intervals in *Paulownia* and 30 min intervals in *Populus*.

The decantation of liquid was carried out after centrifugation at 1,000 rpm for 2 min. The pellet obtained at the end of each periods of decantation was resuspended in a small amount of fresh liquid and looked at cell shapes through a microscope. Most of the cells in the enzyme solution isolated at the end from 3rd to 6th period in *Paulownia* and from 3rd to 8th period in *Populus* became spherical indicating wall removal. Each spherical cells in the enzyme solution isolated during these periods were reincubated in flask containing 5 m*l* of fresh enzyme solution and kept statically in a water bath at 30°C until removal of cell wall was practically complete.

The released protoplasts were collected as a pellet by gentle centrifugation (700 rpm), and the enzyme supernatant containing fine debris is discarded. The pelleted protoplasts were resuspended in a washing solution shown in Table 2 containing mannitol as plasmolyticum and centrifuged gently. The isolated protoplasts were washed several times with washing solution.

Table 2. Washing solution of protoplasts

D-Mannitol	0.8M
$CaCl_2$	6mM
NaH_2PO_4	0.7mM
Sodium Citrate ($2H_2O$ salt)	5mM
KCl	10mM
$MgCl_2$	10mM
pH value	5.6

This study was carried out during from the middle of August to the middle of September.

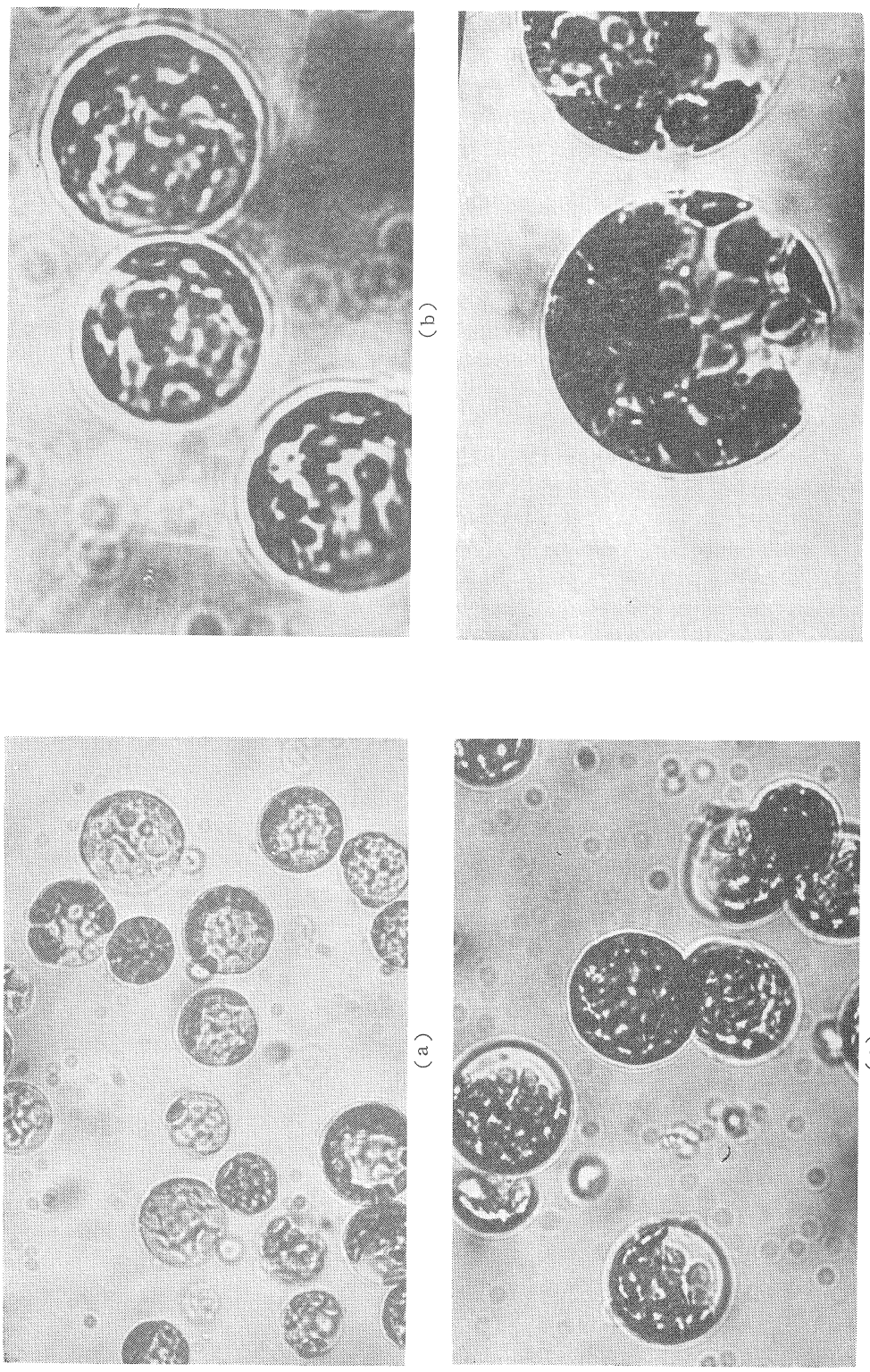

Photo. 1 Protoplasts isolated from leaves of *Populus* (a, b) and *Paulownia* (c, d).

Results and Discussions

When *Paulownia* and *Populus* leaf pieces were treated according to the above mentioned procedure, mesophyll of the leaf pieces released separated protoplasts into suspension, as shown by examples in Photo 1. Shape of protoplasts was spherical. The protoplasts of *Paulownia* were about 40μ in diameter and larger than those of *Moraceae*[7]. The protoplasts isolated from *Populus* were about 13μ in diameter and smaller than cells of *Paulownia*.

The protoplasts contained to the total suspension per 1 g of fresh leaf materials corresponded to 700×10^4 pieces in *Paulownia* and $560\sim1,200\times10^4$ pieces in *Populus*. The calculation was carried out with Thoma Hemacytometer.

Mannitol gave the best results at concentrations of 0.6 M. However, the mannitol of 0.8 M which stabilized protoplasts was used in washing solution.

It has been found from the results of this experiment that protoplasts were isolated enzymatically from mesophyll cells of *Paulownia* and *Populus*. However, *Populus* leaves were resistant to enzyme digestion and required much macerozyme R 10, hemicellulase and cellulase "Onozuka" R 10 for their complete digestion.

The following phenomena were generally observed in the enzymatic isolation of protoplasts by employing 2 step procedure from mesophyll cells of the tree species in comparison with herbaceous plant. (1) Upon longer incubation in enzyme solution cells quickly showed indications of damage such as loss of green color. (2) Digestion capacity of enzyme reduced quickly by shaking leaf materials. (3) Yield of intact cells was poor. (4) Separation of intact cells from debris and undigested cells was very difficult.

Conditions described above were best in yield of intact cells. The high yield of intact cells seems to depend principally on the following 6 factors, i. e., the quick renewal of enzyme solution, the use of a single-step procedure employing a mixture of cellulase and pectinase (mixed-enzyme treatment), the presence of dithiothreitol (DTT, SH protector) excepting other reducing agents from enzyme solution, the presence of reagents to prevent the activity of proteinase inhibitory to intact cells because it is accelerated by DTT and the presence of sodium citrate which facilitates classification between intact cells and wound cells or debris and the use of ion and osmolality effective to stabilization of protoplasts.

Most of damaged cells burst and released quickly chloroplasts and other cellular particles when incubated in media containing sodium citrate of 5 mM in *Populus* and 10 mM in *Paulownia*. Thus, the classification between intact cells and others became easy.

A procedure using enzyme was developed to rapidly release mesophyll cells from *Paulownia* and *Populus* leaves in this paper. The protoplasts were not cultured.

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キリとポプラの葉肉細胞からのプロトプラストの分離

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摘　　要

屋外で育てられたキリとポプラの苗木の十分に展葉した比較的若い葉を材料としてプロトプラストの分離を試みた。葉を細断し，ペクチナーゼ，ヘミセルラーゼ，セルラーゼを含む酵素液にとりこみ，30℃のもとで振盪した。酵素液はキリでは20分ごとに，ポプラでは30分ごとにとりかえられた。健全な細胞がキリでは40分後に，ポプラでは60分後に解離しはじめた。この細胞を遠心分離によって回収し，その細胞壁を完全に溶かしこむために再び新しい酵素液に懸濁し，30℃のもとで静置した。すると，球状のプロトプラストが得られた。キリのプロトプラストは直径40μで生重1gあたり700×10^4粒，ポプラのプロトプラストは直径13μで生重1gあたり$560\sim1,200\times10^4$粒を単離できた。

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