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# Fusion of Protoplasts Isolated from Somatic Cells of Tree Species

By

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**Summary** : Induction of fusion between protoplasts isolated from expanding young leaves of *Populus* and *Paulownia* seedlings grown in the open air was developed. It was found that polyethylene glycol (PEG) had the effect of causing aggregation of high frequency at 1 by 1, in the case of mixed protoplasts of 2 different species, even between cells of different individuals or strains within same species. Consequently, complete fusions of cells between different strains in *Populus* or different individuals in *Paulownia* were observed. However, a specific phenomenon was found out in aggregation of cells between *Populus* and *Paulownia*. Cytoplasm within protoplast of *Populus* were transferred to the inside of protoplast of *Paulownia* in a moment, but cell membrane of *Populus* was left behind for ever.

## Introduction

Much has been written concerning the significance of induced fusion of isolated protoplasts in higher plant[3)4)8)9)]. In order to develop the somatic cell hybridization successfully, it is natural that various conditions suitable to induce protoplast aggregation should be satisfied. Conditions to induce the aggregation of cells in higher plant have been looked for since the example was shown by CARLSON et al. (1972)[1)] on hybridization of cells between *Nicotiana glauca* and *N. longsdorffii*.

In examining the influence of various substances on protoplasts of various species, KAMEYA (1975) found out that high molecules of dextran sulfate had the effect of causing protoplast aggregation like gelatin. He also stained the vacuoles of the protoplasts with neutral red, in order to distinguish one protoplast from another. This procedure was useful for staining the protoplasts isolated from somatic cells of *Populus* and *Paulownia*.

Exposure of protoplasts to solutions containing a high concentration of Ca at a high pH, sodium dextran sulfate or PEG has been attempted to induce fusion.

This paper reports the effect of PEG which is high molecules on cell aggregation of *Populus* and *Paulownia*. Fused cells were not cultured under the aseptic condition.

## Materials and Methods

Leaves excised from 3-year-old seedlings of *Populus* × *euramericana* cv, I-45/51 and cv, I-214 grown in the open air and 2-year-old seedlings of *Paulownia taiwaniana* HU et CHANG grown in a growth chamber (2,700 lux, 25℃, 14 hr illumination per day) were as materials for protoplast isolation. Leaves of *Populus* were cut into small pieces and incubated in a solution containing 0.3% macerozyme (Onozuka R 10), 0.6% hemicellulase, 0.6% cellulase (Onozuka R 10), 0.6 M D-mannitol, 1.0% potassium dextran sulfate, 6 mM $CaCl_2$, 0.7 mM $NaH_2PO_4$, 5 mM sodium

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citrate and 2 mM dithiothreitol (DTT) at pH 5.6 for 3 hr at 25℃ according to the procedure previously described[7].

In the case of *Paulownia* leaves were exposed in a solution containing 0.3% macerozyme, 0.6% hemicellulase, 0.6% cellulase, 0.4 M D-mannitol, 1.0% potassium dextran sulfate, 10 mM $CaCl_2$, 2 mM DTT, 10 mM potassium citrate, 53 mM L-glycine, 0.1 mM potassium fluoride, 6.5 mM ammonium chloride, 0.07 mM phenylhydrazine hydrochloride, 0.2% bovine serum albumine and 0.15 M HCl at pH 5.6 for 2 hr at 26℃.

After isolation of protoplasts, each protoplasts were washed free of enzymes in a solution containing 0.8 M D-mannitol at pH 5.2 and resuspended in a solution containing 0.8 M D-mannitol, 6 mM $CaCl_2$, 0.7 mM $NaH_2PO_4$, 5 mM sodium citrate, 10 mM KCl and 10 mM $MgCl_2$ at pH 5.2 keeping about 10 protoplasts/m*l*.

Vacuoles of isolated protoplasts were stained with neutral red (100 ppm) solution containing 0.6 M D-mannitol in M/15 phosphate buffer (pH 7.7) for 10 min. The stained protoplasts were washed 3 times in 0.6 M D-mannitol solution (pH 5.2) and used to distinguish protoplast of different origin (Photo. 1).

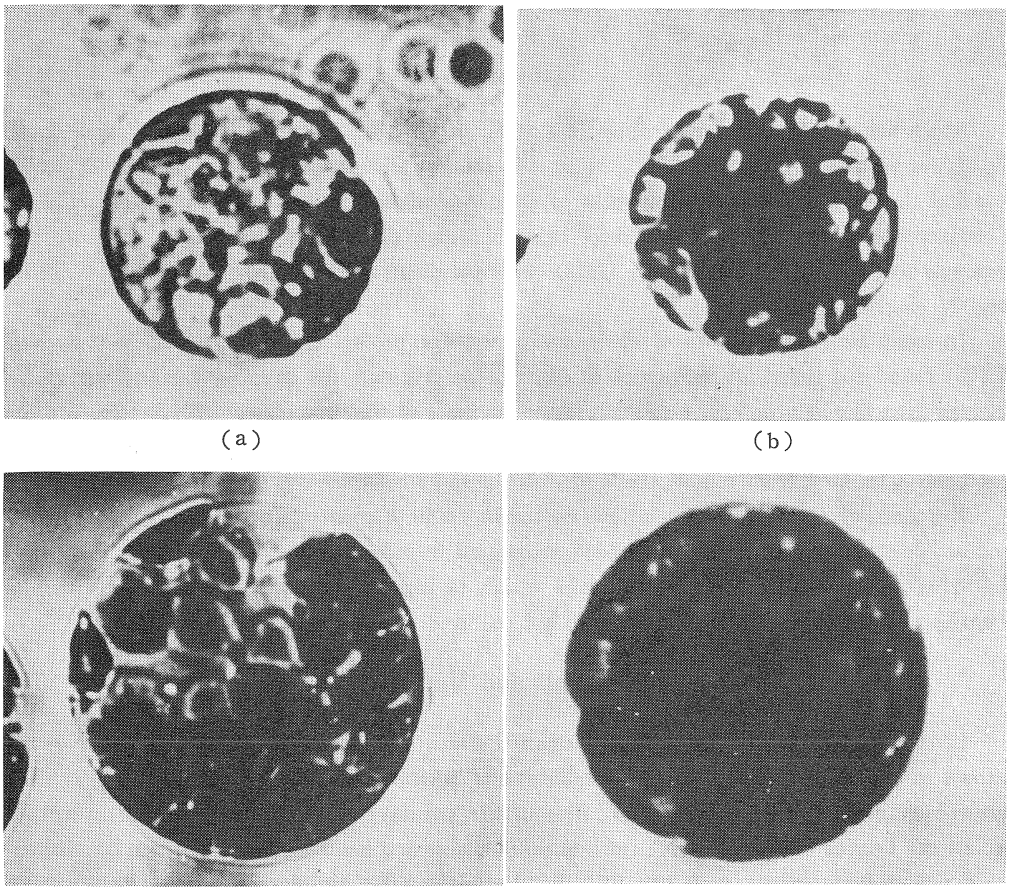


Photo. 1　Isolated protoplasts; No stained (a) and stained (b) protoplasts of *Populus*, and no stained (c) and stained (d) protoplasts of *Paulownia*.

The 2 kinds of protoplasts were finally mixed in 1 by 1 proportion (V ; V) and placed in droplets of 0.7 M D-mannitol solution containing various concentrations of dextran sulfate, PEG, $NaNO_3$, KCl and $CaCl_2$ on a slide with a depression (18 mm in diameter and 0.25 mm in depth), covered with glass. The slide was incubated at 25°C. After 30 min incubation, influence of various kinds of aggregation media was investigated with a light microscope.

## Results and Discussions

Among various aggregation media, 0.3 M D-mannitol solution containing 29% PEG and 40 ppm $NaNO_3$ (solution named D in Table 1) was effective for protoplast aggregation. Some amount of $NaNO_3$ and osmotic pressure were essential to protoplast aggregation. Moreover, PEG had the effect of causing aggregation of high frequency with each other at 1 by 1, between protoplasts of same and different species.

In order to distinguish one protoplast from the other, the vacuoles of the protoplasts were stained with neutral red. The stained and no stained protoplasts were mixed with the others in a medium containing 29% PEG, 0.35 M D-mannitol and 40 ppm $NaNO_3$.

In the case of mixed protoplasts of 2 different individuals or strains within same species, they aggregated successfully with each other (Photo. 2). However, a specific phenomenon was found out in aggregation of cells between different species (Photo. 2). Cytoplasm within protoplast of *Populus* were transferred to the inside of protoplast of *Paulownia* in a moment, but cell membrane of *Populus* was left behind forever.

Protoplast fusion offers exciting possibility for augmenting current tree improvement programs, because somatic cells of tree species has the totipotency being characteristics of plant[8].

Haploid cells can be used for protoplast production, so that the fused hybrid cells will reconstitute the normal diploid condition found in somatic cells. However, one of the most important practical uses of protoplast cultures is for somatic hybridization. This could be especially important in sexually incompatible plants, and in cases where conventional methods of breeding fail to operate. The successive example of fusion between different species within same genera was shown by Carlson et al. in 1972[1]. Hybrid of cells between *Nicotiana glauca* and *N. longsdorffii* was grown. Much attention is being denoted to somatic cell hybridization in higher plants since the 1st example was shown by Carson et al.[1] However, it is unknown if the nuclei of the heterokaryons induced from cells between different individuals belonging to different families or genera are induced to fuse probably during the simultaneously mitotic divisions, to give a true hybrid nucleus.

Table 1. D-Mannitol solution for protoplast fusion

| | A | B | C | D |
|---|---|---|---|---|
| Potassium dextran sulfate | 20% | 20% | | |
| PEG | | | 29% | 29% |
| D-mannitol | 0.35M | 0.35M | 0.35M | 0.35M |
| $NaNO_3$ | 5 ppm | 40 ppm | 5 ppm | 40 ppm |
| KCl | 20 ppm | | 20 ppm | |
| $CaCl_2$ | 5 ppm | | 5 ppm | |
| pH value | 5.4 | 5.4 | 5.4 | 5.4 |

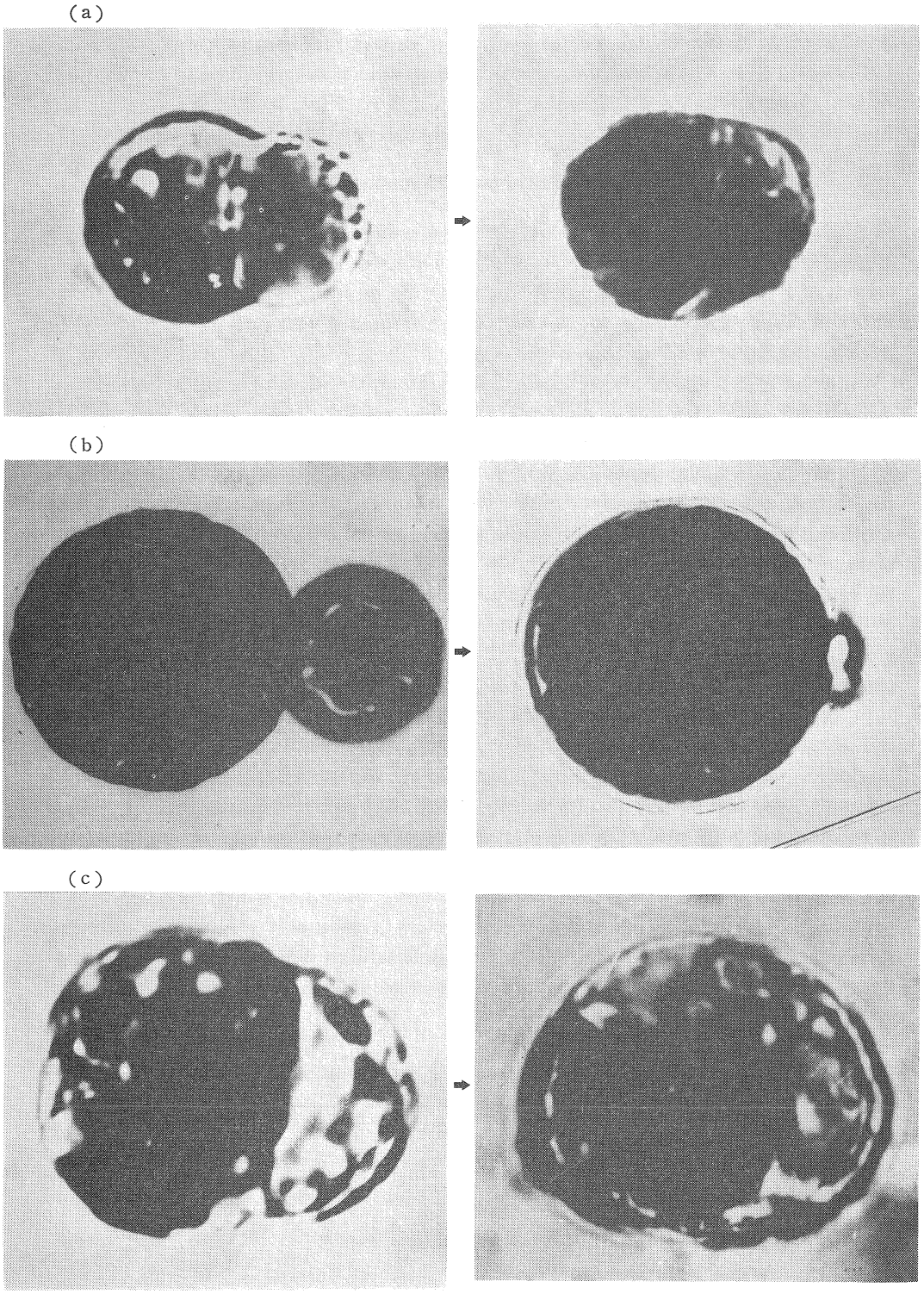


Photo. 2　Somatic cell fusion; *Populus* to *Populus* (a), *Paulownia* to *Populus* (b) and *Paulownia* to *Paulownia* (c).

Power et al. have recently described the selective growth of heterokaryons formed as a result of the sodium nitrate induced fusion of *Petunia* leaf mesophyll protoplasts and protoplasts isolated from cultured cells of *Parthenocissus* (Boston Ivy) crown gall. The study resulted in the growth of a cell line which possessed only the nuclei of *Parthenocissus*, but which showed iso-enzyme patterns specific both for *Parthenocissus* and *Petunia*. Cocking (1976) first suggested these results probably indicate that selective chromosome elimination has taken place with the formation of a cybrid[2]. He indicated that it is possible to take place the formations of a cybrid with selective chromosome elimination and a true hybrid with nuclei fusion of the heterokaryons. However, iso-enzyme pattern specific for *Petunia* disappeared gradually during the one year of culture. It seemed likely that cell lines resemble to the cybrid can be produced, when nucleus only is left in the cell membrane of *Populus* and cytoplasm only is transferred into cytoplasm of *Paulownia* cell, at the aggregation test of protoplasts isolated from *Populus* and *Paulownia*. However, it seems most reasonable to conclude that heterokaryon undergo nuclear fusion and somatic cell hybridization develop successfully.

The next stage in the development of procedures for the somatic hybridization of plants must be the study of cell wall development and regeneration after protoplast fusion.

## Acknowledgement

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# 樹木体細胞から単離したプロトプラストの融合

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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から単離したプロトプラストの融合の誘起を試みた。その結果，PEG 処理が1対1の融合を高頻度に誘起できることがわかった。さらにポプラの異系統間とキリの個体間の融合は容易であったが，キリとポプラ間の融合では特異な現象が観察された。すなわち，ある時点でポプラのプロトプラストの細胞質が瞬間的にキリのプロトプラスト内に移行するが，ポプラの細胞膜は決してキリの細胞膜と合体しないでいつまでもとり残された。

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