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# Isolation and Culture of *In Vitro* Cultured *Populus alba*×*P. grandidentata* Protoplasts[1]

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# *Populus alba*×*P. grandidentata* 組織培養 植物體 원형질의 分離와 培養[1]

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## ABSTRACT

Protoplast-source meterial and enzyme strength had a significant influence on protoplast yield from hybrid poplar, *Populus alba* x *P. grandidentata*. The yield of protoplasts from *in vitro* culture of 1 month-old plantlets was more than that from greenhouse grown 4 month-old stock plant. *In vitro* cultured plantlets regulary produced more viable protoplasts with E-I enzyme solution (0.5% cellulase and 0.1% macerase) than those with E-II enzyme solution (1.0% cellulase and 0.2% macerase) after overnight incubation. The mean yield of protoplasts from *in vitro* cultured plantlets was 4 x $10^6$ with E-I enzyme solution. Cell division was observed in these protoplast cultures after 7-10 days. Protoplast-derived hybrid poplar cells survived over 3 weeks in culture and some continuous cell divisions were evident. Other aspects associated with protoplasts from *in vitro* cultured plantlet are also discussed.

*Key words: protoplast culture; bud culture;* Populus alba x P. grandidentata.

## 要 約

포플러類의 원형질 分離와 培養에 關한 基礎 硏究로서, 北美의 自然雜種 포플러, *P. alba*×*P. grandidentata* 同一 clone의 組織培養 植物體와 溫室에서 生育한 苗木을 이용하여 2種類 酵素處理에 依한 원형질 分離量과 生長을 調査하였다. 組織培養 植物體(1個月生)가 溫室에서 生育한 苗木(4個月生)에서 보다 많은 量의 원형질을 분리, 生産하였다. E-I 酵素容液(0.5% cellulase와 0.1% macerase)이 E-II 酵素容液(1.0 cellulase와 0.2% macerase)보다 組織培養 植物體로부터 원형질을 分離하는데 보다 더 效果的이었으며, 平均 4×$10^6$의 원형질을 分離할 수 있었다. 이들 分離된 원형질을 NAA(2.0mg/l)와 BAP(0.5 mg/l)가 첨가된 MS 액체培地에 培養했을 때 7~10日 後에는 細胞分裂을 관찰할 수 있었으며, 約 3週까지 細胞分裂이 계속되어 6~10個의 細胞群을 관찰할 수 있었다.

## INTRODUCTION

One of the most significant developments in plant tissue culture techniques during recent years has been the isolation, culture and fusion of protoplasts. These techniques have a number of potential uses: (1) Induction of some useful somaclonal

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variation in protoplast cultured plants (Shepard *et al,* 1980; Kemble and Shepard, 1984); (2) The plasma membrane can interact with and take up macromolecules (genetic material) such as foreign organelles and organisms. Thus, thechnique can be applied to plant genetic engineering (Chun, 1985); (3) Adhesion of protoplasts can result in parasexual hybridization by protoplast fusion as with potato and tomato (Gleba and Sytnik, 1984); (4) Alleviation of ploidy level differences or sexual incompartibility problems (Rodenbaugh et al, 1980); (5) Single cells can be used for a mass selection system (Dodds, 1983); And finally, (6) physiological studies (Vasil, 1976).

While there are a few reports describing protoplast isolation and culture with forest tree species, it is not yet possible to regenerate whole plants from protoplast culture of forest tree species (Ahuja, 1984). In the case of *Populus* species, few species have generated succesful protoplast isolation or culture (Ahuja, 1983; Saito, 1980 and Verma and Wann, 1983). Nevertheless, there is no report on continuous cell division and differentiation from poplar protoplasts (Ahuja, 1984).

To fully explore the potentials of protoplast technology with forest tree species, efficient reproduction methods for protoplast isolation and purification must first be established. This paper deals with the isolation and culture of mesophyll protoplast from *in vitro* shoot cultured hybrid poplar plantlets, *Populus alba x P. grandidentata.*

## MATERIALS AND METHODS

Plant materials: *In vitro* cultured Crandon clone of *Populus alba* x *P. grandidentata* was used in this study. The initial establishment and proliferation of *in vitro* culture of this hybrid poplar are previously described in detail (Chun and Hall, 1984). Leaves of greenhouse grown stock plant were also used for comparison of protoplast yield from greenhouse grown 4 month-old stock plants with *in vitro* cultured 1 month-old plantlets. The surface disinfestation method for leaves of greenhouse grown stock plants was tthe same as that described by Chun and Hall (1984).

Protoplast isolation and culture: Protoplasts from leaf and petiole tissue were isolated by incubating transverse sections (1 mm wide) for 15-20 hrs in an enzyme solution containing E-I enzyme combination (0.5 % Cellulysin cellulase and 0.1 % Macerase [both from Calbiochem]) or E-II enzyme combination (1.0 % Cellulysin cellulase and 0.2 % Macerase), and 0.1 % bovine albumin (Sigma) and 0.7 M mannitol in a CPW solution, at PH 5.8. The CPW salts consisted of 250 mg/l magnesium sulphate, 100 mg/l calcium chloride, 170 mg/l potassium dihydrogen phosphate, and 30 mg/l calcium nitrate.

The protoplasts were purified by a combination of filtration, centrifugation and washing. The protoplasts were filtered through 100 $\mu$ mesh and pelleted in 0.7 M mannitol + CPW solution by centrifugation at 200 r/min for 10 minutes. The pellet was resuspended in a 20% sucrose + CPW solution and centrifuged at 100 r/min for 10 minutes. Floating protoplasts were washed in 10% mannitol + CPW solution and plated in 2 ml of protoplast culture medium at a density of 10 to 5 x $10^4$ /ml.

Protoplast cultures were grown on Murashige and Skoog basal medium (Murashige and Skoog, 1962) supplemented with naphthaleneacetic acid (2.Omg/1), benzyladenine (0.5 mg/1), sucrose (30 g/1) and mannitol (90 g/1). The protoplasts were plated either in a liquid medium or embedded near the surface of a semi-solid agar medium by placing 2.0 ml of protoplast solution on 2.0 ml of melted agar medium (0.7 % Difco Bacto Agar) prior to solidification.

Cultures were maintained at a daytime temperature of 25-28 °C, with a 16-hr photoperiod and photosynthetically active radiation (PAR) level of 50-60 $\mu$mol $s^{-1}$ $m^{-2}$ from cool-white fluorescent tubes. The nighttime temperature was controlled at 20-23 °C with 8 hours darkness.

## RESULTS AND DISCUSSION

**Table 1.** Effect of different enzyme solutions and material sources on protoplast yield per gram fresh weight and mitotic activity

| Enzyme solution | Source of Material | Yield* | Mitotic activity** |
|---|---|---|---|
| E-I enzyme | Greenhouse grown stock plant | trace | no division |
| 0.5% cellulase<br>0.1% macerase | | | |
| | *In vitro* cultured plantlet | $4x10^6$ | second and third cell division |
| E-II enzyme | Greenhouse grown stock plant | trace | no division |
| 1.0% cellulase<br>0.2% macerase | *In vitro* cultured plantlet | $0.5x10^6$ | first cell division |

* Mean of 5 replicate experiments.
** Observation of 3 weeks after culture.

*In vitro* cultured *Populus alba x P. grandidentata* regularly produced more viable protoplasts when treated with E-I enzyme solution than those with E-II enzyme solution after overnight incubation (Table 1). By overnight incubation with E-I and E-II enzyme solution, mean yields of protoplasts from *in vitro* cultured plantlets were $4x10^6$ and $0.5x10^6$, respectively. Thus, the strength of enzyme solution influenced the yield of protoplasts. In some poplar species, a strong enzyme solution should be used to isolate protoplasts (Verma and Wann, 1983); however, other poplar species only require a relatively weak enzyme solution to isolate protoplasts (Ahuja, 1983; Smith and McCown, 1983).

The yield of protoplasts from *in vitro* cultured plantlets was higher than that from greenhouse grown plants. Generally, leaves from *in vitro* cultured juvenile plantlets and greenhouse grown stock plants provide a reasonably good protoplast-source material. In this experiment, however, greenhouse grown stock plants generated an insufficient number of protoplasts for plating. Similar results have been reported earlier which demonstrated that more protoplasts derived from *in vitro* cultured *Betula* and *Rhododendron* plants in comparison with those produced from green-house grown stock plants (Smith and McCown, 1983). Inadequate yield and cell division have been shown to be partly due to inappropriate enzyme concentrations during incubation, and also partly due to the physiological growth condition of the greenhouse grown stock plating density also resulted in no cell division (Table 1). Optimal growth and differentiation of plant protoplast requires a plating density of a limited range (Evans and Cocking, 1977).

Protoplasts obtained from *in vitro* cultured plantlets were spherical with well-distributed cell contents. There was, however, variability in the size of leaf mesophyll protoplasts derived from *in vitro* cultured hybrid poplar (Fig. 1). This size difference was also reported by Ahuja (1983) who grouped *Populus tremula* and *P. tremuloides* protoplsts into two categories; normal and mega protoplasts. The origin of mega protoplasts is not known.

Cell wall regeneration of these protoplasts usually occurred within 48 hrs. Initiation of budding from protoplasts in agar-solidified medium was observed after 5-7 days in culture (Fig. 2). After 7-10 days, the first cell divisions were apparent in the protoplast culture (Fig. 3 and 4). Cells usually enlarged and become less cytoplasmically dense during this period. Protoplast-derived hybrid poplar cells survived over three weeks in culture and some continuous cell divisions were evident (Fig. 5). Nevertheless, cell division did not continue beyond the 6-10 cell stages (Fig. 6).

Even though sustained callus formation and differentiation from cultured protoplasts was

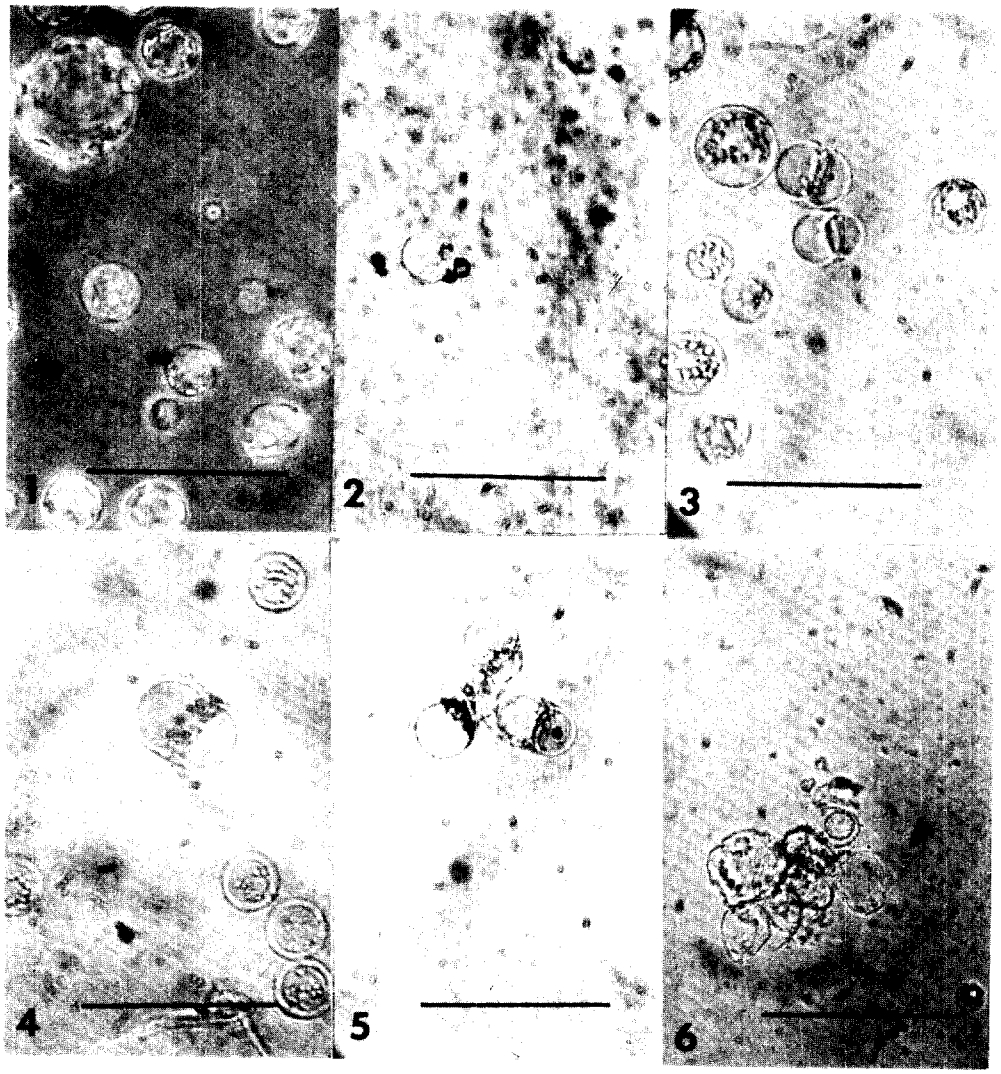


**Fig. 1.** Freshly isolated mesophyll protoplasts of *P. alba* x *P. grandidentata*
**Fig. 2.** Initiatic. of budding in solid-agar medium after 1 week
**Fig. 3.** First cell division in liquid medium after 1 week
**Fig. 4.** First cell division from mega-protoplast after 1 week
**Fig. 5.** Two week-old cell clusters
**Fig. 6.** Three week-old cell clusters
The scale for figures 1-6 is 100 $\mu$m.

not obtained in this study, it was shown that *in vitro* cultured hybrid poplar provides a very useful protoplast-source material. There are also other advantages when *in vitro* cultured plantlets are used for protoplast isolation and culture: 1) *in vitro* bud (or shoot) culture provides stable genotype source (Lawrence, 1981), 2) juvenile growth condition are maintained under *in vitro* conditions (Chun and Hall, 1984), 3) it provides high morphogenic potentials for embryogenesis or organogenesis, and 4) it can also provide year-round plant source with a cyclical growth phase in a small space.

This study clearly demonstrates that protoplast

source material and enzyme strength have a significant influence on yield of protoplasts from hybrid poplar, *Populus alba* x *P. grandidentata*. At the time of this writing, only limited attempts have been made to culture these protoplasts. More intensive studies are required on their growth and differentiation.

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