

# Isolation and cell regeneration of protoplasts from sugar pine (*Pinus lambertiana*)

Pramod K. Gupta and Don J. Durzan

Department of Pomology, University of California, Davis, CA 95616, USA

Received June 16, 1986 – Revised version received July 14, 1986 – Communicated by O. L. Gamborg

## ABSTRACT

Protoplasts were isolated at high yields from actively growing callus and cell suspensions of cotyledons and needles of mature trees. The best protoplast growth response was obtained from cell suspensions of cotyledon and needle callus. Lower protoplast yields were obtained directly from young needles of flushing buds on explants from mature shoots (30-year-old trees) growing *in vitro*. In all cases, the first divisions, promoted by dimethyl sulfoxide, were observed in 10-45% of the protoplasts by 7-10 days. After 25-30 days, colonies of 8-10 cells were established. Browning of protoplast-derived cell cultures was observed within 40-45 days (cotyledons) and 20-25 days (mature tree sources).

## ABBREVIATIONS

BA = N<sup>6</sup>-Benzyladenine;  
 DCR = Douglas-fir cotyledon revised medium;  
 2,4-D = 2,4-dichlorophenoxyacetic acid  
 DMSO = dimethyl sulfoxide  
 FDA = Fluorescein diacetate  
 Mes = 2-(N-morpholino)ethanesulfonic acid  
 NAA =  $\alpha$ -naphthaleneacetic acid

## INTRODUCTION

For the genetic improvement of woody perennials, protoplasts from totipotent somatic cells are in some respects equivalent to the egg in genetic potential (Steward and Krikorian 1979). Genetic manipulation of protoplasts may provide a novel way to shorten the life and breeding cycle of long-lived trees. Manipulation now includes hybridization of somatic protoplasts, co-cultivation with vectors bearing foreign genes, microinjection of DNA, and the opportunity to create double haploids from cells of the female gametophyte (Durzan 1980). This approach assumes that the life cycle of a tree can be completed by plantlets regenerated from protoplasts, and that protoplasts from mature trees having useful traits and appropriate genetic markers for these traits are available. Plants have been regenerated from protoplasts of several herbaceous species (Vasil and Vasil 1980). Unfortunately, studies on isolation and culture of protoplasts of forest trees, especially conifers, remain far behind. With the exception of sandalwood (Rao and Atkins 1985), plant regeneration from protoplasts of forestry species has not yet been achieved (Ahuja 1984).

Protoplast culture, particularly from blister-rust resistant explants of mature trees (Kinloch *et al.* 1970), has become increasingly important now that somatic embryogenesis has been achieved with cells of sugar pine and other conifer species (Gupta and Durzan 1986, Hakman and von Arnold 1985).

This paper describes a method of successful isolation and culture of protoplasts from callus, cell suspension cultures, cotyledons, and needles from mature trees of sugar pine. The work differs from earlier studies on conifers in the use of DMSO (dimethyl sulfoxide), and explants from mature trees. All earlier reports have employed tissue such as embryos and cotyledons (David and David 1979, Hakman and von Arnold 1983, Kirby and Cheng 1979, Patel *et al.* 1984).

## MATERIALS AND METHODS

**Callus culture.** Lateral branches and seeds of 30-year-old sugar pine (*Pinus lambertiana* Dougl.) from controlled crosses were provided by the U.S. Forest Service at the Institute of Forest Genetics, Placerville, California (Latitude 38°N 44.2', Longitude 120°W 44.5', elevation 4000'). Callus was isolated from cotyledons of 2- to 3-week-old seedling. Callus was proliferated on a newly formulated DCR basal medium (Gupta and Durzan 1985) with L-glutamine (50 mg/l), 2,4-D (3 mg/l), BA (0.1 mg/l) (DCR-1) in diffuse light at 25°C. Callus was also obtained from needles of 30-year-old trees on DCR-1. Callus was subcultured every 5-6 weeks on DCR-1.

**Suspension culture.** Callus (ca. 2 g) was removed and placed in a 100 ml suspension medium (DCR-1 medium minus agar). Suspension cultures were grown in 1 liter nipple flasks rotating at 1 rpm under continuous light at 22 ± 1°C. After 12 days, cells were screened through 280  $\mu$  mesh and the fine cell suspension resuspended in fresh DCR-1 medium. Suspension cultures were also initiated by the method described above from needle callus of 30-year-old trees. Cell suspensions were maintained by subculture every 10 days.

Packed cell volume was measured after centrifugation of cell suspension of each flask at 250x g for 10 min. Suspension cultures were maintained by subculturing of about 5 ml packed cells to 100 ml fresh medium.

### Protoplast preparation media:

- Enzyme solution ( $E_1$ ) contained 2% (w/v) cellulysin (Calbiochem), 1% pectinase (Sigma), 1% macerase (Calbiochem), 0.5% potassium dextran sulphate, 12% mannitol, DCR mineral salts at pH 5.8. Undissolved material was removed by centrifugation at 20,000x g for 5 min at 4°C and was filter-sterilized through a 0.45 micron disposable filter.
- Enzyme solution ( $E_2$ ) was composed of  $E_1$  and 50 mM Mes.
- Washing medium contained DCR mineral salts, 12% mannitol, 0.5% potassium dextran sulphate, pH 5.8, and was autoclaved (1.1 Kg cm<sup>-2</sup> at 121°C for 20 min).
- The regeneration medium for protoplasts contained DCR basal medium, sucrose (1%), glucose (2%), mannitol (6%), L-glutamine (50 mg/l), CH (casein hydrolysate) (200 mg/l), 2,4-D (1 mg/l), NAA (0.5 mg/l), BA (0.2 mg/l), DMSO (1%) and 5 mM Mes, pH 6.0, at 1063 mM/kg osmolality and was filter-sterilized as above.

Isolation and culture of protoplasts. Cells in 6-day-old suspension cultures were centrifuged at 100x g for 10 min. Cells (2 ml packed cell volume) were resuspended in 5 ml of  $E_1$  enzyme solution. The mixture was placed in a sterile 50 ml conical flask and incubated on a rotary shaker (60 rpm at 23°C in darkness). For protoplast isolation from cotyledon callus, 500 mg was placed in 50 ml sterile conical flask with 5 ml  $E_1$ . For mature tree callus  $E_2$  was substituted for  $E_1$ .

For protoplasts isolated from needles of mature trees, young needles were excised from sprouting apical and axillary buds on shoots of growing in vitro on DCR basal medium. Excised needles were cut into 2 mm pieces. These pieces were preconditioned with 10% sterile mannitol for 1-2 hr. After the mannitol solution was decanted, 5 ml of  $E_2$  was added in a 50 ml sterile conical flask and incubated as above.

After incubation for 3-5 hr for callus and suspension culture and 16-18 hr for needles, the mixture of enzymes, protoplasts, and debris was filtered through a 50  $\mu$  nylon filter to remove undigested materials. Crude protoplast preparations were centrifuged at 100x g for 5 min. The supernatant was removed and the protoplast pellet resuspended in 5 ml washing medium and recentrifuged at 100x g for 5 min. Protoplasts were then resuspended in washing medium.

Purification. Ficoll density gradient centrifugation was applied to crude protoplast preparations according to Boller and Kende (1976). One volume of 20% (w/v) Ficoll 400 in 10% mannitol was added to one volume of washed protoplast preparation of sugar pine. It was then overlaid with 5 ml each of 10, 5, and 0% (w/v) Ficoll in 10% mannitol. This step gradient was centrifuged at 100x g for 10 min. After centrifugation two bands were formed, one between 0-5% Ficoll and the other between 5-8% Ficoll. Bands were collected by a Pasteur pipette and examined under the microscope for purity. Clean, intact protoplasts from the 5-8% Ficoll band were suspended in regeneration medium and centrifuged at 100x g for 3 min. Protoplasts were again suspended in regeneration medium. Viability of protoplasts was examined by staining with 0.01% FDA. A portion (0.5 ml) of the protoplasts suspension (density 10<sup>4</sup>-10<sup>5</sup>/ml) was

transferred to 2 ml well of a 24-well multiwell plate (Falcon #3047). Plates were sealed with parafilm and incubated at 22°C in diffuse light, ca. 60% relative humidity. After 4-5 days 0.5 ml fresh regeneration medium was added with a low osmotic level (500 mM/kg). Cell-wall regeneration was tested by calcofluor white ST (0.1% solution in washing medium). The presence of phenolics was tested using nitroso reaction as described by Jensen (1962).

All experiments were carried out in five replicates and repeated at least twice. Results were expressed as a percentage which was determined according to method described earlier (David et al. 1984).

Cultures were incubated in diffuse white light. Light intensity less than 0.5  $\mu$ W cm<sup>-2</sup> nm<sup>-1</sup> (blue and red) and nil in far-red.

### RESULTS AND DISCUSSION

Protoplasts could not be easily isolated from old needles of in vitro grown shoots of 30-year-old trees. Better yields of protoplasts (0.1 x 10<sup>4</sup>/g fresh wt) were obtained from sprouting needles from buds, directly placed in enzyme mixture. Low yields may be due to phenolic compounds which were leached from cut ends of needles and inhibited enzyme activity.

An improved yield of protoplasts (0.5 x 10<sup>4</sup>/g fresh wt) was obtained when cut pieces of sprouting needles were placed in 10% mannitol in DCR basal medium for 1-2 hr before protoplast isolation. Viability of these protoplasts was 20  $\pm$  1.5% with  $E_1$  enzyme mixture (without Mes). Viability of protoplasts increased to 75  $\pm$  6.5%, when 50 mM Mes was included in enzyme mixture ( $E_2$ ) (Figs. 1 and 2).

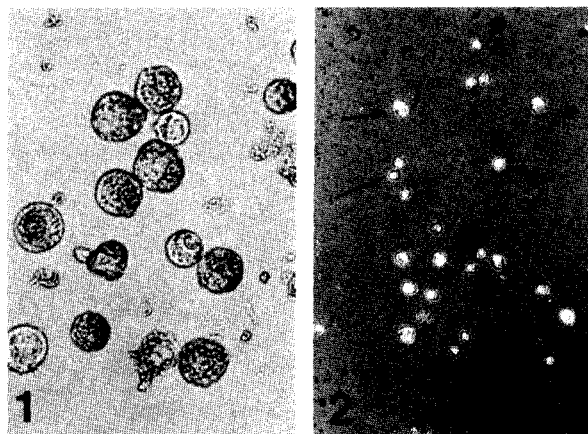


Fig. 1. Freshly isolated protoplasts from needles of 30-year-old trees. x20.

Fig. 2. Double photographic exposure to reveal viable and moribund protoplasts in a crude preparation from needles of 30-year-old trees. Viable protoplasts show a bright fluorescence (arrow) with FDA staining. x8.

Highest yields of viable protoplast (90  $\pm$  7.5%) were obtained from cell suspension cultures of cotyledons and needle (mature tree) callus.

For 6-day-old suspension cultures (cotyledon and needle callus),  $65 \pm 4.0\%$  of the protoplasts regenerated cell walls within 2 to 3 days. For 3-to-5 or 8-to-10 day old suspension cultures, wall regeneration was reduced to  $30 \pm 2.0\%$ .

After 4-5 days of protoplast culture, a fresh regeneration medium with a lower concentration of mannitol (3%) and glucose (1%) was added to cultures. After 1 day  $5 \pm 0.6\%$  of protoplasts exhibited first cell division from suspension culture of cotyledons and needle callus. This was increased to  $45 \pm 3.0\%$ , when 1% DMSO was supplemented in regeneration medium (Fig. 3). At 14-15 days, fresh medium was added again with sucrose (2%) but without mannitol and glucose. By 25-30 days, small colonies of 6-8 cells were observed (Fig. 4).

Cell clusters were obtained only from protoplasts isolated from suspension cultures of cotyledons and needle callus, and not from needles of mature trees. Nevertheless,  $10 \pm 1.0\%$  of proto-

plasts isolated from sprouting needles of mature trees exhibited first cell division within 10-12 days. Afterwards the browning of cultures was observed. Within 40-45 days browning of cultures from cotyledons was also observed (Fig. 5). No further growth of cell cultures was obtained.

Comparable results have been reported with *Pinus contorta* (Hakman and von Arnold 1983) and *Pinus coulteri* (Patel et al. 1984). In our study, DMSO encouraged the division of protoplasts. Apparently DMSO can initiate cell division in protoplasts by promoting cortical microtubule assembly (Hahne and Hoffman 1984).

#### ACKNOWLEDGMENTS

This work was supported in part by Cooperative Agreements (PSW-83-0038CA and PSW-84-0011CA) with the Institute of Forest Genetics, Pacific Southwest Forest and Range Experiment Station, USDA Forest Service, Berkeley, California. We thank Dr. Tom Ledig for his interest and help in obtaining access to forest trees at Placerville, California.

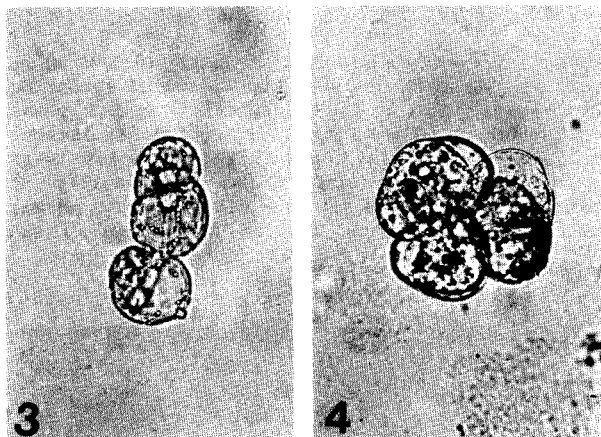


Fig. 3. Protoplast showing first cell division from suspension culture of cotyledon callus after 6 days. x32

Fig. 4. Cluster of cells from protoplasts from suspension cultures of cotyledon callus after 30 days. x32.

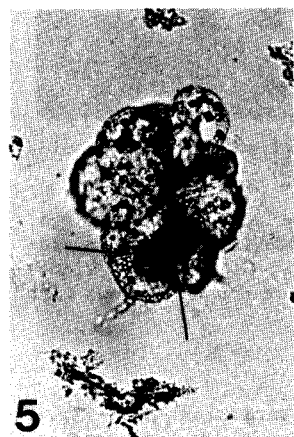


Fig. 5. Cluster of cells from protoplasts from suspension culture of cotyledon callus showing the accumulation of exogenous phenolics (arrow) after 45 days. x32.

#### REFERENCES

- Ahuja MR (1984) *Silvae Genetica* 33:32-37.
- Boller T, Kende T (1976) *Plant Physiol.* 63:1123-1132.
- David A, David H (1979) *Z. Pflanzenphysiol* 94:1652-1657
- David H, Jarlet E, David A (1984) *Physiol. Plant.* 61:477-482.
- Durzan DJ (1980) *In* 50th Anniversary, May 8-10, 1979, The Institute of Paper Chemistry, Appleton, WI, pp. 31-60.
- Gupta PK, Durzan DJ (1986) *Bio/Technology*, 4:643-645.
- Gupta PK, Durzan DJ (1985) *Plant Cell Rept.* 4:177-179.
- Hahne G, Hoffman F (1984) *Proc. Nat. Acad. Sci. USA* 81:5449-5453.
- Hakman, IC, von Arnold S (1983) *Plant Cell Rept.* 2:92-96.
- Hakman I, von Arnold S (1985) *J. Plant Physiol.* 121:149-158.
- Jensen WA (1962) *In* Botanical histochemistry, principles and practice. W.A. Freeman, San Francisco.
- Kinloch BB Jr., Parks GK, Fowler CW (1970) *Science* 167:193-195.
- Kirby EG, Cheng TY (1979) *Plant Sci. Lett.* 14:145-154.
- Patel KR, Shekhawat RS, Berlyn AP, Thorpe TA (1984) *Plant Cell Tiss. Organ Cult.* 3:85-90.
- Rao PS, Atkins PO (1985) *Protoplasma* 124:80-86.
- Steward FC, Krikorian AD (1970) *In* WR Sharp, PO Larsen, EF Paddock, V Raghavan (eds.). *Plant cell and tissue culture, principles and application.* Ohio State Univ. Press, Columbus, pp. 221-262.
- Vasil IK, Vasil V (1980) *In* IK Vasil (ed.), *Perspectives in plant cell and tissue culture.* Intl. Rev. Cytol., Academic Press, New York, pp. 1-19.