REGENERATION OF YELLOW-POPLAR FROM PROTOPLAST CULTURE

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Abstract .--Protoplasts were isolated from suspension cultures of two embryongenic lines of yellow-poplar (Liriodendron tulipifera) by incubation in a medium containing 2 percent cellulase and 1 percent pectinase, and cultured in droplets of a regeneration medium supplemented with 1 mg/1 2,4-D and 0.25 mg/1 6BA and solidified with agarose. The protoplasts reformed cell walls within 3 days, and 75 percent of them divided at least once within 1 week in culture. Embryogenic suspension or callus cultures were regenerated by placing agarose-solidifed droplets containing protoplast-derived microcalli directly into liquid conditioning medium or onto plates of conditioning medium solidified with agar. Embryogenesis was obtained by transferring the callus to a hormone-free induction medium, and protoplast-derived plantlets were obtained following transfer of somatic embryos to a germination medium. We believe this to be the first report of regeneration of a North American tree species from protoplast culture via somatic embryogenesis.

Additional keywords: Somatic embryogenesis, tissue culture, Liriodendron tulipifera.

Since the first reported enzymatic isolation of plant protoplasts by Cocking (1960), protoplasts have been isolated and cultured from a wide range of higher plant species. With the appropriate techniques, cultures of plant protoplasts have achieved cell wall formation, cell division, colony and callus formation and plantlet regeneration. Protoplasts isolated from several herbaceous plant species have been fused to yield somatic hybrids (Evans et al. 1983). Protoplasts also open possibilities for the uptake of nuclei, organelles, chromosomes and plasmids (Ahuja 1984). Finally, protoplast cultures have been cited as a source of novel genetic variation, which might provide a method for plant improvement (Shepard et al. 1980).

Protoplasts have been isolated from several woody plant species including both gymnosperms and angiosperms (Ahuja 1984). Sources of protoplasts have included leaf mesophyll, cotyledonary tissue, callus cultures and suspension cultures. However, there are very few reports of the successful regeneration of whole plants from protoplast cultures of woody species, although the list has recently begun to expand more rapidly. Among those woody species successfully cultured from protoplasts are Citrus sp. (Vardi et al. 1982), Santalum album (Rao and Ozias-Akins 1985), Pyrus communis (Ochatt and Caso

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1986), Ulmus sp. (Sticklen et al. 1985, 1986), Broussonetia kazinoki (Oka and Ohyama 1985) and Populus sp. (Russell and McCown 1986). In this paper, we will describe the isolation and culture of protoplasts of yellow-poplar and the regeneration of plantlets from these cultures via somatic embryogenesis.

MATERIALS AND METHODS

Aggregates of immature yellow-poplar samaras were collected at weekly intervals from July through September from 12 trees located on the University of Georgia campus and the University's Whitehall Forest. Samaras were dissected from the aggregates and surface sterilized using a 70 percent ethanol dip, followed by a 10 percent Roccal dip, a full strength Clorox soak for 5 minutes, a sterile water rinse, a 0.01 N HC1 rinse and three additional sterile water rinses. Following surface sterilization, the embryo and endosperm were removed and placed on Merkle and Sommer's (1986) conditioning medium, supplemented with 2 mg/1 2,4-D, 0.25 mg/1 6BA and 1000 mg/1 casein hydrolysate, and solidified with 0.8% agar. Cultures were incubated in the dark at 22 C and transferred every 3 weeks to fresh conditioning medium.

Within 2 months after being placed in culture, an embryogenic callus was initiated from a percentage of the cultures, which varied with both the mother tree and the date of seed collection. The embryogenic callus could easily be distinguished from nonembryogenic callus by its nodular, friable structure, fast growth and pale yellow color. Suspension cultures were established by subculturing this callus into liquid conditioning medium. Suspension cultures were maintained in 125 ml erlenmeyer flasks on a gyratory shaker at 90 rpm. As long as the callus or suspension was maintained on the 2,4-D-supplemented conditioning medium, no differentiation of somatic embryos occurred.

Prior to protoplast isolation, embryogenic suspension cultures were put on an accelerated transfer schedule, so that about 5 g of embryogenic material was transferred to fresh medium every 6 days. These accelerated subcultures were carried out at least 4 times prior to protoplast isolation, and resulted in very rapid growth of the suspension. Cultures to be used for protoplast isolation were divided into a dark pretreatment and a light pretreatment (14 hours of light per day).

To isolate protoplasts, approximately 1 g of suspension was placed in 10 ml of filter-sterilized digestion medium containing major salts, minor salts and iron as in Merkle and Sommer's (1986) conditioning medium, 500 mg/1 $CaCl_2*2H_2O$, 100 mg/1 MES, 0.5 M mannitol, 2 percent Cellulysin, 1 percent Macerase and 100 mg/1 bovine albumin. The tissue was incubated in the digestion medium for *12 hours* at 30°C with gentle agitation on a gyratory shaker at 50 rpm. Following digestion, the protoplasts were purified by dripping the suspension, which contained protoplasts, undigested cells and debris, through 2 layers of Miracloth, then through a 25 pm pore size stainless steel sieve. The filtrate was centrifuged at 100 X g for 5 minutes. The supernatant was discarded and the pellet resuspended in 5 ml of filter-sterilized wash medium, which was of the same composition as the digestion medium, minus the enzymes and bovine albumin. This washing step of centrifugation and resuspension in wash medium was repeated twice more.

Following the final washing centrifugation, the supernatant was removed and the pellet of protoplasts was resuspended in a filter-sterilized protoplast culture medium, which was of the same composition as Merkle and Sommer's (1986) conditioning medium, but supplemented with 500 mg/1 $CaCl_2*2H_2O$, 250 mg/1 xylose, 250 mg/1 fructose and only 250 mg/1 sucrose, instead of the 40 g/1 used in the conditioning medium. The concentration of 2,4-D was also cut from 2 mg/1 to 1 mg/1 in the protoplast culture medium. The osmoticum for the medium was *0.5 M* glucose. Since centrifugation resulted In clumping of the protoplasts, the resuspended protoplasts were dripped once more through a 25 um sieve to disperse the protoplasts from the clumps. The protoplasts were counted using a hemacytometer and cell wall digestion was confirmed by Calcofluor staining.

Although other plating techniques were attempted, including thin liquid layer and hanging droplet methods, the high density plating technique of Binding and Kollman (1985) proved to be the most successful. Following this method, the protoplasts were diluted to 2-4 X 10^5 protoplasts per ml, pipetted into 0.2 ml drops in plastic petri dishes and mixed with an equal amount of protoplast culture medium containing 2.5% low melting point agarose, which had been autoclaved and cooled to 38° C. The mixture was immediately taken up with a pipette and redistributed as 0.05 ml droplets around the perimeter of the plate. Following solidification of the droplets, the plates were flooded with 2 ml of liquid protoplast culture medium. Thus, while the density of the protoplasts in the agarose droplets was 1-2 *X* 10 per ml, the actual plating density per dish was much lower.

Petri dishes containing the agarose droplet cultures were placed in inverted Magenta boxes to maintain humidity and incubated in darkness at 30° C for approximately 1 month. Then the culture medium was diluted by an equal volume of liquid yellow-poplar conditioning medium containing no osmoticum. One month following this dilution, agarose droplets containing calla were either transferred to 40 ml of liquid conditioning medium in 125 ml erlenmeyer flasks and maintained at 90 rpm on a gyratory shaker, or placed directly onto agar-solidifed conditioning medium in plastic petri dishes.

RESULTS AND DISCUSSION

Embryogenic suspension cultures grown under light cgnditions and transferred to fresh media every 6 days yielded 1-2 X 10 protoplasts per gram of tissue. Significant numbers of protoplasts could not be isolated from dark-grown suspension cultures. Protoplasts isolated from light-grown cultures (Figure 1) were very dense and filled with large starch granules. Because of their high density, protoplasts could not be separated from undigested cells by flotation, as can protoplasts derived from other tissues, such as leaf mesophyll. Because of this problem, purification was accomplished by the relatively long (12 hours) digestion time, during which virtually all free cells were converted to protoplasts, and by the sieving described earlier.

Once in culture, cell wall regeneration, as determined by Calcofluor staining and loss of spherical shape, occurred within three days. The first cell divisions were observed within 4 days and within one week of culture,

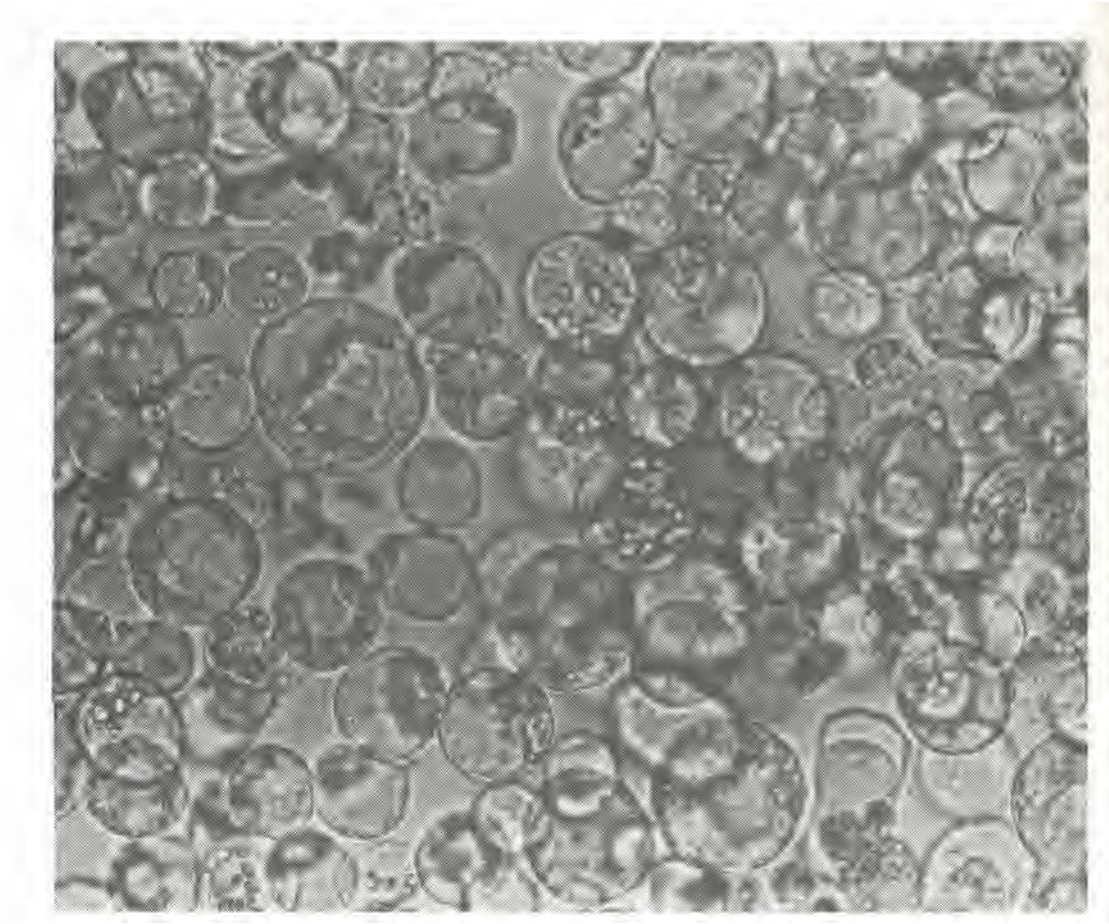

Figure 1. Protoplasts recently isolated from embryogenic yellow-poplar suspension culture (300X).

over 75 percent of the protoplast-derived cells had divided at least once. Many divisions were asymmetrical and budding was frequently observed. Cell colonies of 8 or more cells could be found within 2 weeks and microcalli large enough to be seen without a microscope appeared within 3 weeks. These regeneration times are quite rapid when compared to those reported for protoplasts isolated from shoot cultures of Populus, which took 6 weeks for the first cell division (Russell and McCown 1986) and protoplasts isolated from callus cultures of Ulmus x 'Pioneer,' which required 7 days (Sticklen et al. 1986). However, the regeneration timetable for yellow-poplar protoplast cultures was very similar to that reported for Santalum (Rao and Ozias-Akins 1985).

Suspension cultures could readily be regenerated from microcalli by transferring the agarose droplets containing the microcalli into 20 ml of liquid yellow-poplar conditioning medium in 50 ml flasks on a gyratory shaker. Within a few weeks, the microcalli expanded out of the agarose and proliferated rapidly in suspension. Agarose droplets containing microcalli were also placed directly onto conditioning medium solidified with agar, where

the microcalli grew out of the agarose and onto the conditioning medium. The callus and suspensions regenerated from protoplast cultures were identical in appearance to embryogenic cultures described by Merkle and Sommer (1986).

Embryogenesis was induced by transferring the callus to Merkle and Sommer's (1986) induction medium, which is of the same composition as the conditioning medium, except for the absence of hormones. Within 1 month following transfer of callus to the hormone-free medium, differentiation of somatic embryos could be observed.

Germination of the somatic embryos was accomplished as described previously (Merkle and Sommer 1986). Briefly, somatic embryos at the late torpedo stage were transferred to a Risser and White's (1964) medium in test tubes. Root growth was usually observed within one week, followed by development of 2-3 heart-shaped leaves. At this stage, plantlets could be transferred from the tubes to a peat-perlite potting mixture and placed in the humidifying chamber for acclimatization. During the following 6 weeks in the humidifying chamber with 14 hour daylengths and weekly fertilization with commercial plant food, the humidity was gradually lowered to ambient. Currently we have a few dozen plantlets of protoplast-somatic embryo origin growing in the greenhouse.

The availability of a system to regenerate yellow-poplar from protoplast culture via somatic embryogenesis opens many possibilities for basic and applied research using this tool. An obvious application of this system is the testing of techniques for the genetic engineering of forest species by Agrobacterium Ti plasmid-mediated DNA transformation or by direct DNA transfer enhanced by electroporation. The fact that the yellow-poplar system is an embryogenic one makes it an excellent candidate for studies into the commercial scale up of transformation-regeneration systems for forest trees, for example using bioreactor technology. An additional area for potential research using the products of protoplast research is the investigation of somaclonal variation among the regenerants. Although we have begun to look at within-clone variation at the DNA level among plantlets regenerated from yellow-poplar somatic embryos (Chou et al. 1986), a similar study of protoplast-derived plantlets will have to wait until a larger number of plantlets have been regenerated from the protoplast system.

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