TECHNIQUES FOR HIGH-FREQUENCY ISOLATION OF ELM PROTOPLASTS[1]

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ABSTRACT.--Techniques for isolating high frequencies of Ulmus americana, U. pumila, and U. parvifolia protoplasts from cotyledons, cell suspension cultures, and callus cultures are presented. Spherical protoplasts were separated from the cell-wall degradingenzymes, broken protoplasts, intact cells, and debris by a discontinuous density gradient centrifugation technique. The viability of protoplasts was verified using exclusion staining with Evan's blue. Attempts to culture the protoplasts resulted in cell wall formation (verified by calcofluor staining) and apparent cell divisions. However, no further development occurred.

INTRODUCTION

American elm (Ulmus americana L.) has long been one of America's most cherished shade trees. Once planted as the primary shade tree in cities across the United States, the American elm has been taken off most planting lists because of its susceptibility to Dutch elm disease and phloem necrosis. A few American elm trees were found to have good resistance to Dutch elm disease (Sinclair et al. 1974). However, these trees have not proven to be resistant to phloem necrosis (Sinclair et al. 1976).

Siberian elm (Ulmus pumila L.) and Chinese elm (U. parvifolia Jacq.) have excellent resistance to the two diseases. Unfortunately, these Asian elms have smaller leaves, more brittle wood, less urban hardiness, and most importantly, do not have the elegant, vase-shaped crown that is the American elm's trademark. Therefore, they are not acceptable substitutes for the American elm. Hybrid elms are needed to combine the desirable ornamental characteristics of American elm and the disease resistance of the Asian elms.

[1]This research was supported in part by the International Paper Company Foundation.

All attempts to hybridize the tetraploid (2n = 56 chromosomes) American elm with other elm species (all diploid with 2n = 28 chromosomes) have failed. Santamour (1970) has reported on a triploid putative natural hybrid between American and Siberian elms. While the cause of the incompatibility is unknown, the aforementioned chromosome number difference between American elm and all other elm species would cause a "triploid block" for any sexual hybrids which might be obtained.

The capacity to isolate and fuse elm protoplasts has been demonstrated by Redenbaugh (1979) and Redenbaugh et al. (1980). A number of technical problems were encountered, however, to slow the progress in this research. The first problem was that we were not able to consistently isolate sufficient quantities of protoplasts to work with. The second problem was that it is difficult to separate the protoplasts from intact cells, cellular debris, and the cell-wall-degrading enzymes used to isolate the protoplasts. A third problem was that only a few protoplasts developed cell walls and none were actually verified to have formed callus cultures in our early experiments. The purpose of the research presented in this paper was to develop improved techniques for isolating and culturing elm protoplasts.

MATERIALS & METHODS

Plant Materials

Cotyledons from aseptically germinated seedlings of Ulmus americana and U. pumila were used as a source of mesophyll protoplasts. Dewinged seeds of both species (purchased from F.W. Schumacher Co., Sandwich, Mass. 02563) were treated twice at an interval of 24 hours with 10% Clorox and Tween 80 (1 drop/10 ml) for 15 minutes followed by thorough rinsing with sterile distilled water. The seeds were then planted individually in small, sterile jars containing a moist peat: sand (3:1) mix. They were incubated at 26 C beneath two 40 watt Sylvania Gro-lux fluorescent tubes on a 14-hour photoperiod.

Callus and cell suspension cultures of Ulmus americana, U. pumila, and U. parvifolia, incubated at 26 C in the dark, were used as sources of protoplasts lacking chloroplasts. Ulmus americana and U. pumila callus cultures were initiated from 10-day-old seedlings, aseptically germinated as described above. Callus was also initiated from Ulmus americana seedlings by placing them on the root-producing medium of Durzan and Lopushanski (1975) with 0.75% agar (autoclaved). Intact seedlings were placed on the surface of absorbent cotton wetted with Kao's (1977) cell culture medium (filter-sterilized) in which vitamin-free casein had been substituted for vitamin-free casamino acid.

For Ulmus parvifolia, branches were collected from a mature tree in New York City's Central Park during the last week in June and stored in a plastic bag at 4° C for two weeks. Branch sections with leaves were washed in a weak solution of Alconox (5 cc's/liter) and cool water

and then rinsed and dried. Green shoot sections at least 1 cm in length and bearing at least one leaf were excised and two-thirds of the leaf (apex portion) was removed and discarded. Explants were dipped in absolute ethanol and surface sterilized with 20% Clorox for 20 min with occasional shaking. They were then thoroughly rinsed with sterile distilled water and placed on the above-mentioned medium of Durzan and Lopushanski (1975).

Callus of all three species was subcultured three or more times before being used for protoplast isolation. Ulmus americana callus initiated on agar medium was transferred to fresh agar medium and then five drops of cell culture medium (Kao, 1977), modified as above, were added to the callus. All other callus was subcultured to cotton wetted with fresh cell culture medium.

Suspension cultures were initiated by placing approximately 1 gm of actively growing callus in a 50 ml flask containing 10 ml of Kao's (1977) cell culture medium, modified as above. Flasks were closed with aluminum foil and incubated in the dark at 26° C on a gyratory shaker (100 rpm). Stocks were subcultured at two-week intervals by 1:1 dilution.

Protoplast Isolation

Cotyledons of Ulmus americana and U. pumila were harvested when they had reached full expansion of approximately 0.5 cm^2 at 10 to 20 days of age. Four intact cotyledons were placed in a petri dish (3.5 x 1 cm plastic petri dishes were used throughout the study) containing six layers of paper toweling wetted with 4 ml of Kao's (1977) protoplast medium (filter-sterilized) modified to contain 0.1 M $CaCl_2$ and vitamin-free casein. The cotyledons were preincubated for 1/2 hr and then sliced into strips 0.5 mm or less in width. An angled dissecting needle was used to hold the cotyledon in place while thin strips were cut from the cotyledon with a scalpel.

The cotyledon strips (total area approximately 1 cm^2) were transferred to another petri dish containing 2 ml of an enzyme solution consisting of 0.25% Onozuka cellulase R 10 (Kinki Yakult Mfg. Co., Ltd., Nishinomiya, Japan), 0.25% Rhozyme HP 150 (Rohm and Haas Co. Canada, Ltd., West Hill, Ontario) and 0.125% Sigma pectinase (Sigma Chemical Co., St. Louis, Mo. USA) in the above-mentioned protoplast medium. The enzyme solution was adjusted to pH 5.7 and filter-sterilized. The dish was sealed with parafilm and incubated in the dark at 26° C for 4 to 48 hours with occasional gentle swirling.

Suspension cultures of all three species were used 10 days after they were subcultured. Suspension cultures were centrifuged at 400 x g for 5 min. The supernatant was withdrawn with a Pasteur pipette and the cells were resuspended in the above-mentioned enzyme solution (2 ml for each 0.5 ml of packed cells). Two ml of cell-enzyme mixture was incu-

bated per petri dish as above.

Actively growing callus was used 14 days after subculture. Callus (0.2 g) was added to 2 ml of enzyme solution in a petri dish and gently separated into small pieces. The dish was sealed with parafilm and incubated as above.

After incubation, protoplast-containing enzyme solutions were passed through a 43 um mesh stainless steel filter (TETKO Inc., Elmsford, N.Y.).

Protoplast Purification

Protoplasts were separated from the enzyme solution, cells, and debris by a modification of the discontinuous density gradient method of Piwowarczyk (1979). The gradient was prepared in a 15 ml transparent, thin-wall polyallomer tube (Fisher Scientific Co., Springfield, N.J.). Concentrations of sucrose and glucose (glucose was substituted for sorbitol) were proportionally adjusted to conform to the concentration of glucose (0.38 M) in the protoplast medium and enzyme solutions being used.

The bottom layer of the gradient consisted of 0.63 M sucrose dissolved in Kao's (1977) protoplast medium modified to contain 0.1 M CaCl2 and no glucose. Four ml of this solution was added to the tube and the height marked on the outside of the tube. To prevent mixing of the layers, the tube was tipped at an angle of 10° and 4 ml of 0.46 M glucose and 0.18 M sucrose dissolved in the above-mentioned protoplast medium was slowly pipetted onto the edge of the solution surface. Finally, 2 to 4 ml of the protoplast-enzyme filtrate (0.38 M glucose) was added. The tube was capped with sterile aluminum foil and

Protoplasts were removed from the gradient with a syringe with a #16 stainless steel needle attached. The outside of the tube was swabbed with 70% ethanol and the needle was inserted, bevel down, to the center of the tube at the top of the 0.63 M sucrose layer above which a band of protoplasts had accumulated. The needle was rotated 180° so that the bevel was facing up and 1 ml of solution was slowly withdrawn. The syringe was disconnected from the needle and its contents were emptied into a petri dish.

Protoplast Culture

Protoplast medium (Kao, 1977) containing 0.38 M glucose and 0.1 M CaC12 was added (1:1) to the protoplast suspension removed from the gradient. One ml of diluted protoplasts was incubated in a thin layer spread over the bottom of a petri dish. Dishes were sealed with parafilm and incubated in dim light at 26° C in a closed plastic box lined

with moist paper toweling.

Protoplast viability was determined by exclusion staining with 0.1% Evan's blue (Berliner et al., 1978) dissolved in the above mentioned protoplast medium. The occurrence of cytoplasmic streaming was also considered as evidence of protoplast viability. Cell wall regeneration was monitored by observing the change in shape of the protoplasts from spherical to oblong. Cell wall regeneration was also verified with calcofluor staining (Berliner et al., 1978).

RESULTS

Protoplast Isolation

Protoplasts were released from cotyledon strips of Ulmus and U. americana after 4 hr of incubation in the enzyme solution. The highest protoplast frequencies from cotyledon strips ranged from 50% to 90% for Ulmus pumila and 40% to 80% for U. americana after they had been incubated in the enzyme for 48 hr. Although protoplast frequencies were relatively high for both species, as few as one-half of the total number of protoplasts isolated were present in the enzyme solution after it had been passed through the 43 $_{}$am filter. The other protoplasts could not pass through the filter since they remained trapped within the cotyledon strips. Agitation or repeated pipetting of the culture dislodged some of these protoplasts but was ineffective because it also caused breakage of many cells and protoplasts.

Protoplast release from ten-day-old subcultures of suspension cultured Ulmus americana, U. pumila, and U. parvifolia cells also began after 4 hr of incubation in the enzyme solution. However, the highest protoplast frequencies (40-60% for Ulmus americana, 70% for U. pumila, and 80% for U. parvifolia) were attained after the suspension cultured cells had been incubated in the enzyme solution for 24 hr. Similar results were obtained with 14-day-old subcultured callus except for Ulmus americana which yielded only 30-50% protoplasts. Also, incubation time in the enzyme solution had to be increased to 48 hr to isolate protoplasts at high frequencies from callus cultures.

Protoplast Purification

The enzyme filtrate placed at the top of the discontinuous density gradient contained viable protoplasts, broken protoplasts, intact cells, and debris. After centrifugation, spherical, viable protoplasts were concentrated in a visible band located just above the 0.63 M sucrose layer while the enzyme solution remained at the top of the gradient. Broken protoplasts, intact cells, and debris were sedimented at the bottom of the tube.

The majority of protoplasts in the enzyme filtrate were recovered in

purified form from the central band of the centrifuged gradient. All protoplast types (i.e., those isolated from cotyledons, suspension cultures, and callus cultures) of the three Ulmus species tested were equally recoverable in purified form by the discontinuous density gradient centrifugation technique employed.

Protoplast Culture

The viability of cultured protoplasts, which settled to the bottom of the liquid medium, was indicated by the pronounced cytoplasmic streaming between cellular organelles, generally located in the center of the protoplasts, and the protoplast surface. Cell wall regeneration occurred for many protoplasts by the fifth day of culture as evidenced by their change in shape from spherical to oblong. Calcofluor staining verified the presence of newly formed cell walls and revealed. that many protoplasts which were still spherical in shape had also regenerated cell walls. Frequencies of cell regeneration from protoplasts approached 80%.

After two weeks in culture most regenerated cells exhibited yeast-like budding. In some cases, chains of successively smaller buds, not separated by cell walls and devoid of organelles and cytoplasmic streaming, were seen protruding from cells. This has been described as pathological budding (Nagata and Takebe, 1970). A few regenerated cells (approximately 1%) appeared to have divided by bud formation, as noted by Herth and Meyer (1978), where both the bud and the cell showed active cytoplasmic streaming and contained many organelles. We did not confirm the presence of a nucleus in each structure, however. After further incubation (a total of 4 weeks), cultures contained several regenerated cells which appeared to have divided two or three times. No further development occurred.

DISCUSSION

Although Redenbaugh (1979) demonstrated the ability to isolate elm protoplasts, his methods produced low frequencies (usually 10%) of protoplasts from cotyledons, callus, or suspension cultures. This hampered attempts to somatically hybridize Ulmus americana with U. pumila or U. parvifolia. We therefore sought to develop techniques to produce consistently large numbers of viable protoplasts from cotyledons, callus, and suspension cultures of the three Ulmus species involved.

Protoplast isolation frequency and the viability of the resultant cultured protoplasts is affected by the condition of source tissues and cells (Constabel, 1975). In an attempt to obtain readily reproducible quantities of protoplasts, we standardized culture conditions for callus and suspension cultures and seedling material to be used in isolating protoplasts. Nevertheless, we noted considerable variation in protoplast isolation frequencies for a given source within a species, especially between pairs of cotyledons and between callus cultures.

Genotypic as well as physiological differences between seedlings (unequal ages) may have contributed to the variation of protoplast isolation frequencies observed between pairs of cotyledons. Different protoplast isolation frequencies between subcultures of a callus (initiated from a whole seedling and therefore containing a mixture of cell types) probably resulted from the different characteristics of the predominating cell type in the subculture.

To increase the isolation frequency and viability of protoplasts, we stimulated the growth rate of callus and suspension cultures with favorable medium and cultural conditions. This involved a change from the defined medium of Durzan and Lopushanski (1975) to the modified cell culture medium of Kao (1977), a highly enriched medium containing a range of vitamins, organic acids, sugars and sugar alcohols, coconut water, and casein. The growth rate of Ulmus callus and suspension cultures was approximately doubled using Kao's medium and regular subculturing.

In all phases of the experiment, our methods were aimed at maintaining the vigor of the plant material by minimizing injury to the cells and protoplasts. Aseptically germinated seedlings were used as a source of sterile cotyledons. We therefore avoided damage from harsh surface-sterilizing treatments normally employed to disinfect the tissues. Cotyledon cells were plasmolyzed during preincubation to minimize protoplast breakage during dissection. The preincubation solution and all subsequent solutions that came in contact with protoplasts contained the nutrients of Kao (1977) and were fortified with 0.1 M CaC12 to increase the mechanical resistance of the plasmalemma.

Cotyledons were dissected in a relatively large volume of preincubation solution (4 m1:1 cm^2 cotyledon) rather than in the enzyme solution used to break down the cell walls. Thus, harmful materials released from broken cells, such as lytic enzymes, phenols, and tannins were effectively diluted and there was little carry-over of toxic substances into the enzyme solution caused by the transfer of tissue pieces. Therefore, the possibility of inactivation of cell-wall-degrading-enzymes and damage to cells and protoplasts by exposure to these harmful substances for extended periods (4 to 48 hr) was virtually eliminated.

We also developed a cotyledon dissection technique which minimized damage to cells and increased the surface area of the cells exposed to the enzyme solution. Our technique involved the use of a compressable support (several layers of paper toweling) to hold the cotyledon in place with an angled dissecting needle. The heel of the needle was gently pressed onto the support next to the cotyledon. Thus, no direct pressure was applied to the cotyledon. The tissues were not bruised and there was no need to puncture or grasp the cotyledon to hold it in place. Very thin cotyledon sections, 0.5 mm or less in width, were made by guiding a scalpel blade along the angled dissecting needle. The cell

wall-degrading-enzymes could easily penetrate to the cells located in the center of the thin tissue sections, thus speeding the protoplast isolation process.

One of the problems encountered in the elm protoplast fusion work has been the purification of viable protoplasts from broken protoplasts, cells, debris, and cell-wall-degrading enzymes in the enzyme-filtrate solution. Several methods (Constable, 1975; Kao, 1975; Harms and Potrykus, 1978; Power et al., 1975) including filtration, repeated centrifugation and washing, and floatation of protoplasts on a sugar solution were tested and gave poor results (Lange and Karnosky, unpublished). However, the discontinuous density gradient centrifugation technique of Piwowarczyk (1979), with several modifications, did an excellent job of separating viable protoplasts from broken protoplasts, cells, debris, and enzymes. Even when enzyme solutions contained very few protoplasts (<10%), these could be retrieved in pure and concentrated form from the gradient.

Piwowarczyk (1979) determined that bacteria were separated from protoplasts by his discontinuous density gradient technique and that they were located in fractions overlying the protoplast band. However, he did not mention a method for removal of the protoplasts from the gradient without recontamination. We solved this problem by preparing the gradient in a transparent, thin-wall polyallomer centrifuge tube. This enabled us to aseptically remove protoplasts from the side of the tube with a needle and syringe.

Various protoplast-containing solutions may be purified on the gradient of Piwowarczyk (1979) if, as he suggests, a certain volume of the protoplast solution is first diluted with several volumes of 0.3 M sorbitol and 0.1 M $CaCl_2$. We, however, found it more practical to tailor the gradient to the protoplast medium and enzyme solutions which had given us good results. This way protoplasts were not subjected to unnecessary osmotic changes.

Several aspects of Ulmus protoplast isolation and culture need further work. 1. Protoplast isolation frequencies from Ulmus pumila and U. americana cotyledons were effectively halved because protoplasts were trapped in the tissues. Inclusion of higher concentrations of pectinase in the enzyme solution may help to isolate cells prior to protoplast isolation. Alternatively, a two-step isolation procedure (Nagata and Takebe, 1970) may be needed. 2. We were able to isolate large numbers of Ulmus protoplasts; however, the highest protoplast isolation frequencies were not reached until 24 to 48 hr of incubation in the enzyme solution. Although most protoplasts reformed cell walls, few regenerated cells divided. Possibly the prolonged enzyme treatment had an adverse affect on this aspect of growth. Different enzyme combinations and concentrations as well as modified incubation conditions (i.e., elevated temperatures, agitation, and/or vacuum infiltration) might shorten the time needed to produce protoplasts and thereby increase regenerated cell division frequency. 3. Many regenerated cells

exhibited pathological budding, indicating a need for different culture conditions. After these problems have been addressed, work on the somatic hybridization of Ulmus species can proceed.

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