APPENDIX - II

Progress in Plant Protoplast Research

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Attempts at isolation and culture of mesophyll protoplasts of tree species have revealed problems of low yields (1,2) coupled with poor dispersibility which has necessitated lengthy exposure times to enzymes. There have also been problems of leaf necrotisation (3). Only recently, a case of successful mesophyll protoplast culture has been reported (4). Some researchers have overcome these problems by isolating protoplasts from callus cultures derived from mature trees (5,6) and have reported the complete protoplast system utilizing such tissues for Salix and Ulmus respectively. It has now been possible to induce embryogenic cell suspension of Populus xilin'ta. The somatic embryos produced by this cell suspension developed into complete plants which have been transferred to field conditions (7,8). This study was undertaken to determine the suitability of totipotent cell suspensions for isolation and culture of protoplasts.

MATERIALS AND METHODS

Development of embryogenic cell suspension cultures

The cell suspension culture used for isolation and culture of protoplasts were derived from embryogenic callus obtained from long-term leaf cultures of mature trees of Himalayan Poplar (P. xilin'ta wall ex.Roylie) (for details see Refs. 7,8). The callus tissue gave rise to finely dispersed and highly friable suspension cultures, which almost consisted entirely of unorganised small groups of cells. The cells were small, richly cytoplasmic and contained many starch grains. The suspensions were subcultured at 1 week intervals with a dilution of 1:2 or 1:3 with fresh medium. The cell suspensions were maintained on a gyrotory shaker at 120 rpm at 23±2°C in diffuse light. Seven to 14 days old cell suspensions have been used for isolation and culture of protoplasts.

Protoplast isolation and culture

The requisite cell suspensions were centrifuged at 500 rpm for 5-7 min. The pellet of cells was plasmolysed in CPW salts with 13%(w/v) mannitol (CPW 13M). The plasmolysed cells were digested in CPW 13M or 0.33M mannitol solution containing enzymes: 1%(w/v) Cellulose R-10, 0.3%(w/v) Macerozyme R-10 and 0.5%(w/v) Drieslase, pH 3.8. The cells were incubated overnight in the enzyme mixture at 25°C on a gyrotory shaker set at 50 rpm. After the completion of digestion the mixture was filtered through 30p stainless steel mesh, followed by two washes in CPW 13M solution. The pellet was resuspended in CPW 13M solution and layered on a CPW salt solution containing 18%(w/v) sucrose (CPW 18S) and centrifuged at 500 rpm for 10 min. The band of protoplasts, floating at the top, was removed and resuspended in fresh CPW 18S solution for further purification and concentration. This procedure resulted in debris free protoplasts. Protoplasts were counted and diluted to approximately 10⁶ protoplasts per ml. Protoplasts were cultured in 5 ml of liquid
medium at densities of 1x10° protoplasts/ml; 1x10² protoplasts/ml and 8x10⁵ protoplasts/ml, in each sterile dish. Murashige and Skoog (1962) (MS) medium with the following hormone concentration was used for protoplast culture. MS+2,4-D (0.2 or 0.5 mg/l) +6% (w/v) sucrose, MS+2,4-D (0.2 mg/l) +6% (w/v) mannitol. Protoplasts were incubated in dark at 23°C. Cultures were observed regularly for development. Fresh culture medium was added to each dish at 1 wk intervals. Cultures were brought to diffuse light conditions after 10 days.

RESULTS AND DISCUSSION

Cell suspensions grown for 7 days after subculture yielded good, stable protoplasts capable of division. Older cell suspensions (10-15 days after subculture) generally yielded highly vacuolated protoplasts which though remained viable for considerably long time, did not divide. The concentration of Murashige and Skoog (1962) (MS) medium had a positive effect on viability and stability of protoplasts: as manifested in early division. Preliminary experiments revealed several factors having important, inhibitory or beneficial effects. The accumulation of exudates in the culture medium is aggravated by using aged cultures, high light intensity and agar base of the culture medium. Protoplasts cultured in liquid medium in dark exhibited cell wall formation and division within 3-4 days culture. Fresh culture medium had to be added at weekly intervals to produce multicellular colonies. However, for yet unknown reason, cell growth became stagnant and signalled senescence after 6-8 days. The problem of browning and division arrest could be sorted out to some extent by embedding the protoplast in 0.7% agarose.

This study showed that protoplasts isolated from rapidly dividing cell suspension usually divided within 3-4 days of isolation. The plating efficiency calculated for dividing protoplast was 13-14%. This contrasts with very low (1-2%) plating efficiency and slow growth of mesophyll protoplasts reported for poplar (9). The important parameters that need to be taken care for successful protoplast system are (a) the nature and conditions of suspension used, (b) the duration of enzyme treatment and (c) culture conditions. An culture induced from P. ciliata can regenerate at high frequencies on MS+BAP (2mg/l) medium (9), it should be possible to regenerate plants from the protoplast-derived colonies.

REFERENCES