Plant Cell, Tissue and Organ Culture15: 59–65(1988)

Culture of droplets containing asparagus cells and protoplasts on polypropylene membrane

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Received 26 August 1987; accepted in revised form 28 April 1988

Key words: polypropylene membrane, protoplast, cell culture

Abstract. A method of using a buoyant polypropylene membrane floated on liquid medium to culture asparagus cells and protoplasts was examined. Compared with direct culture in lquid medium the method using a polypropylene membrane was found to be superior for small-volume culture of cells at low density as well as for the culture of protoplasts.

Introduction

Growth of cell culture is often affected by cell density. Most cells grow well at a relatively high density, e.g. 10^4 to 10^5 cells per ml. Growth is reduced at lower density and there may even be no growth if the density is too low. A number of approaches have been used to overcome the low cell density problem, including conditioned medium [1, 2], complex medium enriched with various amino acids, vitamins, and organic acids [3], feeder layer techniques [4], and microdroplet culture [5]. In microdroplet culture, desiccation of the culture is a problem, due to the small volumes and large evaporation rates. Recently, Hamilton et al. [6] developed a technique to culture plant cells on buoyant porous polypropylene membranes floated on liquid medium. This technique allows the culture to be continuously supplied with medium from a reservoir without diluting the density of the culture. In the study of Hamilton et al., cells of relatively high density were cultured. The purpose of this investigation was to examine whether this technique could be adopted for the culture of asparagus cells and protoplasts at relatively low density.

Materials and methods

Cell culture

Callus culture of *Asparagus officinalis* L. was initiated from segments of shoot tissue of two-week-old seedlings. Asparagus seeds were sterilized with 0.5% household bleach for 15 min, followed by 4 rinses with autoclaved distilled water. Seeds were germinated on 2 sheets of sterilized Whatman No. 1 filter paper moistened with a thin film of distilled water in 100 × 10 mm petri dishes in the dark at 26 ± 2°C. Shoot segments of 1 to 2 mm in thickness wee placed on Murashige & Skoog (MS) medium [7] containing 3% sucrose, 1 mg l^{-1} NAA, 1 mg l^{-1} BA, 1000 mg l^{-1} glutamine and 0.6% agar. Cell suspension culture was established by transferring the callus to 50 ml of the same medium minus agar in 125 ml flasks. The flasks were kept on a gyrotary shaker at 160 rpm diffused light (4 μmol m^{-2} sec). The cultures were maintained by transferring 10 ml of culture to 40 ml fresh medium every 7 days. Consistent cell culture was obtained after 4 to 6 subcultures.

To obtain cells of a certain density, cells from 7-day-old culture were sieved and then collected by centrifugation at 100 × *g*. The sieve used was constructed with a Nalgene polymethylpentene (top i.d. 100 mm, stem o.d. 20 mm) with a 38 μm mesh stainless steel screen glued to the stem end with silicone glue. Sieving effectively screened out cell colonies and produced essentially all single cells. The cells were resuspended in the same medium, counted with a hemacytometer (Bright Line), and then diluted to the desired density.

Protoplast isolation

Protoplasts were isolated from 5 to 6-day-old cell cultures. Cells were collected by centrifugation at 100 × *g* and incubated in an enzyme mixture consisting of 1% cellulase Onozuka RS, 1% Pectolyase Y-23, 3 mM 2-[N-morpholine] ethanesulfonic acid (MES), 7 mM $CaCl_2$ and 0.6 M glucose in MS medium. The digestion mixture was kept on a gyrotary shaker at 60 rpm under diffused light at 25 to 28 °C. Complete digestion was obtained in approximately 4 h. The protoplasts were sieved through a 38 μm mesh stainless screen and collected by centrifugation at 100 × *g*. The protoplasts were then washed 3 times with MS medium containing 0.6 M glucose, 1 mg l^{-1} 2, 4-D, 1 mg l^{-1} NAA, 1 mg l^{-1} BA and 1000 mg l^{-1} glutamine. After the final wash, the protoplasts were resuspended in the same medium containing 3% sucrose and 0.3% agarose.

Protoplast culture

Fifty μl of protoplasts at a density of 10^5 per ml were placed in petri dish and several water droplets were placed around the culture droplet. The culture was then kept in the dark at 26 $\pm$ 2 °C.

Division of cells and protoplasts

Divisions of cells and protoplasts were examined and counted using an inverted microscope. Presence of cell clusters of 2 or more cells was used as indication of cell division.

Cell growth

Increase in fresh weight was used as one measurement of cell growth. Cells were transferred to a preweighed plastic petri dish, carefuily blotted dry with Whatman No. 1 filter paper and weighed with an analytical balance.

Polypropylene membrane

Polypropylene membrane Celgard 3500 supplied by Questar Corp., Charlotte, NC was used. The Celgard 3500 membrane is buoyant with a density of 0.45, autoclavable, and has a pore size of 0.04 μm. Membrane was cut into approximate 2.5 or 7 cm square, placed on distilled water in 100 $\times$ 15 cm glass petri dish and autoclaved for 15 min at 121 °C before use.

Results

Culture of asparagus cells

To study the effects of cell density on growth, 0.2 ml of cells were plated in a 35 $\times$ 10 mm petri dish. Growth as judged by the cell division frequency was found to correlate with density. No cell division was observed when the original density was 10^2 cells per ml as seen in Fig. 1. As the cell density increased, the frequency of cell division also increased. The highest division frequency was observed with a density of 10^5 cells per ml.

When 0.2 ml of cells were placed on polypropylene membrane floating on 2 ml of medium, cell division was observed at 10^2 cells per ml. In general, cells on the membrane were found to divide at higher frequency than cells directly plated on petri dish, particularly at the lower densities.

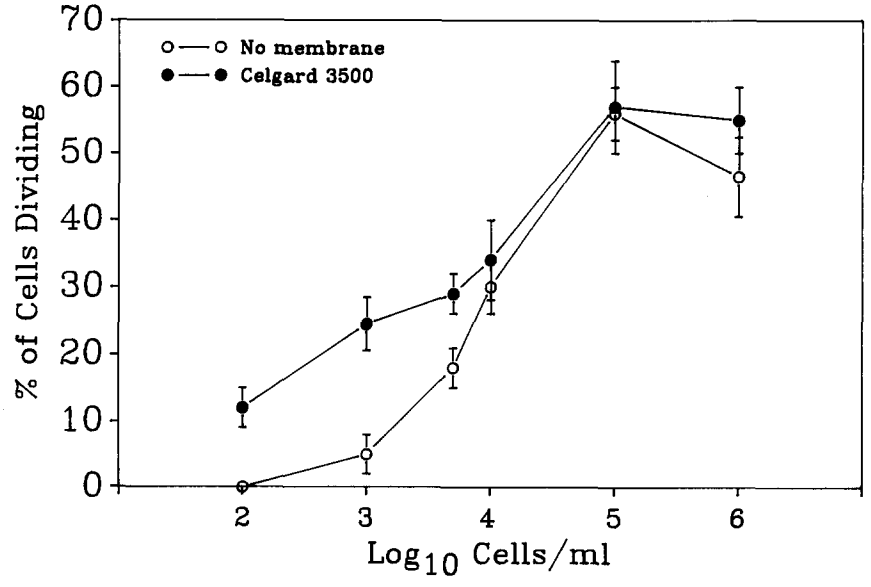

Fig. 1. The effect of cell density on division frequency of asparagus cells. The data represent the means of 3 experiments, each with 5 replicates.

A small number of asparagus cells (i.e. ± 50 cells) can be cultured in a 50 μl droplet of medium. To reduce the evaporation leading to drying, several water droplets were placed around each droplet of culture. Even so, it was necessary to replenish the culture with fresh medium every 10 to 14 days to compensate for the loss of medium to evaporation. Such droplet cultures grow very slowly and only limited growth was obtained in 80 days as demonstrated in Fig. 2.

To examine the use of polypropylene membranes for low density asparagus cell culture, a single 50 μl droplet of culture containing approxi mately 50 cells was placed on a membrane floating on 5 ml of culture medium in a 100 × 10 mm petri dish. Culture droplets maintained in this way did not suffer any visible loss in volume and therefore did not need periodic replenishment of medium. Growth of the culture on the membrane was also much higher than that without the membrane. The doubling times were about 10 to 12 days and 15 to 20 days for the membrane droplet and free droplet cultures, respectively. Fig. 2 is a summary of the results of two techniques.

Culture of asparagus protoplasts

The effect of polypropylene membranes on the culture of asparagus proto-

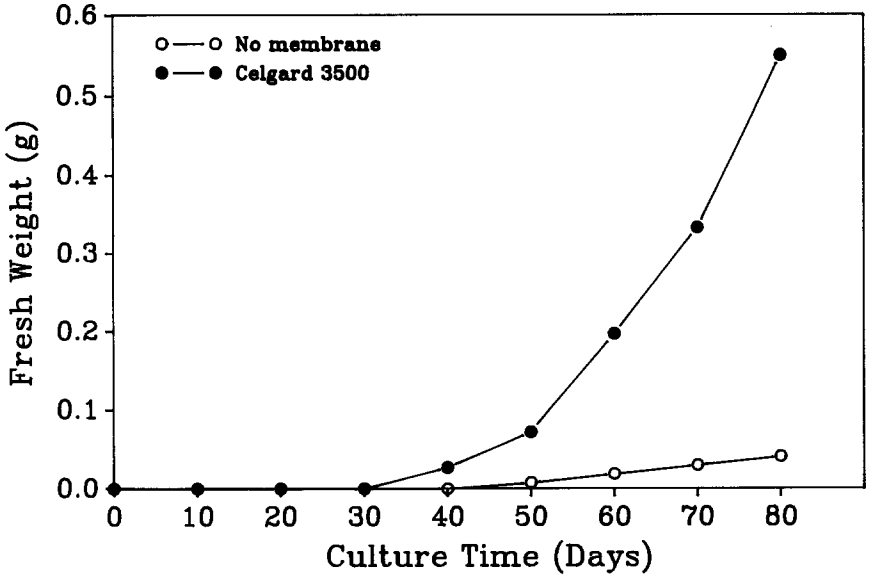

Fig. 2. Growth of low-density cell cultures of asparagus. At intervals, cell cultures were transferred to preweighed petri dishes, carefully blotted dry and weighed. The data represent the means of 3 experiments, each with 5 replicates.

plasts was studied by comparing the division frequency of 500 protoplasts in 50 µl droplets and in 50 µl droplets on polypropylene membrane floated on 5 ml of medium. After 20 days, approximately 1% of the cells in the culture without the membrane were dividing. By comparison in the same period more than 10% of the cells in the culture with the membrane were found to be dividing.

Just as in the culture of cells in droplets, it was necessary to replenish the protoplast droplet culture with fresh medium periodically but not the droplet on the membrane with fresh medium (minus agarose and osmoticum glucose) periodically.

In three experiments, each with at least four replicates, calli visible to the naked eye were observed on the membrane in 30 to 40 days, whereas calli of comparable size were observed in liquid droplet culture in 50 to 70 days.

Discussion

The results from this investigation show that the use of a polypropylene membrane promoted growth of asparagus cells at relatively low density and also growth of asparagus protoplasts. It is believed that at low cell desnity,

metabolites lost to the surrounding medium were diluted to the degree that they were not adequate to support good growth. One approach to overcome the problem is to maintain a high enough density by reducing the volume of the medium. To avoid desiccation of the small volume of culture, the periodic replenishment of medium is necessary. This is cumbersome and further increases the probability of contamination. In addition, it appears that the rather large proportion of the amount of water evaporated to the small culture volume reduced the growth of cells and protoplasts. The results in this study show that these problems can be avoided by the use of a simple polypropylene membrane floated on a reservoir of medium. A number of polypropylene membranes varying in structure, pore size and density are available. The polypropylene membrane used in this study had a pore size of 0.04 μm. In preliminary studies, a membrane with a pore size of 0.02 μm was tested and found to be less effective in supporting growth of asparagus cells and protoplasts. Polypropylene membrane is hydrophobic and does not transfer water. However, the polypropylene membrane used here is treated with a non-ionic surfactant by the manufacturer, rendering it partially hydrophilic. Polypropylene membrane not treated with surfactant had been tested by us and found not able to support asparagus cell and protoplast growth (data not shown). It is believed that both the pore size and partial hydrophobicity function together to control the supply of nutrients, probably by capillary action, to the culture and minimize water losses. Hence, on the membrane the culture appeared moist but not overly wet whereas the membrane appeared dry at all times.

Recently, Gilmour et al. [8] reported the use of cellulose nitrate membrane as a partition for a device consisting of an outside chamber for protoplasts at high density and an inside chamber for protoplasts at low density. Such a device allowed the growth of forage legume protoplast at a low plating density which would not grow in direct liquid culture. They observed that membranes of larger pore size (0.45, 5 and 12 μm) supported better growth of *Onobrychis viciifolia* cell suspension derived calli than membranes of smaller pore size (0.22 μm). In contrast, in this study we obtained good growth of asparagus cells and protoplasts on a membrane with a much smaller pore size (0.04 μm). The cellulose nitrate membrane is hydrophilic and therefore facilitates evaporation. The membrane used in this study was only partially hydrophilic and appeared to have less evaporation problem. This difference may explain why asparagus cells and protoplasts maintained good growth on a polypropylene membrane with such a small pore size.

A problem found with using the buoyant membrane was that if it was not carefully handled the culture could fall off the membrane into the medium reservoir. Also, when the culture grew and became heavy, eventually the

membrane would sink. These two problems limit the membrane for small-volume culture.

Acknowledgement

New Jersey Agricultural Experiment Station, Publication No. D-12151-14-88, supported by state funds and US Hatch Act funds.

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