Cell Divisions in Cells regenerated from Protoplasts of Soybean and *Haplopappus gracilis*

THERE have been several reports of plant cell wall regeneration[1-4] and two reports of division following protoplast formation[3,4] but only one of sustained cell division[3].

Since our initial report of the culture of soybean protoplasts[3] we have been able to eliminate the use of "conditioned" medium by increasing the protoplast concentration to about 10^4 live protoplasts/ml. and by using 50 µl. drops of the suspension. Nagata and Takebe[4] also achieved wall regeneration and some division of tobacco protoplasts in similar culture conditions. It was not possible to form a thin liquid layer when 500 µl. was placed on a plastic dish (Falcon), and so aeration by diffusion may not have been adequate. The use of 50 µl. drops increases aeration by increasing the surface to volume ratio. Attempts to increase aeration by various methods of agitation resulted in the death of protoplasts. Ten 50 µl. drops containing protoplasts in fresh B5 medium[5] plus 0.22 M sorbitol and 1 mM monocalcium phosphate were placed in 10 × 35 mm plastic Petri dishes and sealed with Parafilm. Because the dishes lost moisture during lengthy storage at low humidity, they were placed inside 15 × 150 mm glass Petri dishes containing wetted 425 mm Whatman No. 1 paper disks.

During the first 3 or 4 days of culture about 50% of the soybean protoplasts divided, compared with the 10% previously reported[3]. By this time the cell walls were well established and the cells adhered to the plastic sufficiently so that the liquid could be spread in a thin layer. The suspension was diluted with 5–10% by volume of B5 medium. Further dilutions were made at 2 or 3 day intervals. Lowering of the osmolarity is not essential but gradual increase in culture volume by the addition of fresh medium is necessary to complement the increase in cell number.

When the culture in a dish had been increased to about 1 ml. (approximately 3 weeks) the contents were washed into a 50 ml. Erlenmeyer flask with 10 ml. of B5 medium. The cells were then grown as a suspension culture on a gyrator shaker. Cytological examination revealed the presence of nuclear divisions in polyaneuploid as well as aneuploid cells, both in the first few days of culture and at the time of transfer to the shake culture. (All the original cells were aneuploids containing 37 chromosomes[6].) No detailed study has yet been made of the chromosome complements of the cells nor the relative distribution of aneuploids and polyaneuploids.

The methods reported earlier for the production and washing of soybean protoplasts[3] were slightly modified for use with *H. gracilis*. The enzyme mixture used for the production of *H. gracilis* protoplasts contained the following: 4% w/v cellulase (desalted 'Onozuka P1500', All Japan Co. Ltd), 4% w/v hemicellulase (desalted 'Rhozyme HP150', Rohm and Haas Co. of Canada), 2% w/v pectinase (Sigma), 0.3 M sorbitol, 0.3 M mannitol and 2 mM monocalcium phosphate. The *p*H was adjusted to 5.5 using KOH. 'Onozuka P1500' and 'Rhozyme HP150' were desalted using a column of 'Biogel P6' (Calbiochem). The enzymes were dissolved in dilute NaCl, passed on to the column and eluted with water. The enzyme was collected until the presence of chloride was detected. The enzymes were then freeze-dried and stored at −20° C. When washing the protoplasts free of enzyme an 8 µm 'Millipore' filter was placed over the 47 mm absorbent filter pad[3]. (The 8 µm filter retains the protoplasts but allows filtration without the use of vacuum.)

Attempts to culture *H. gracilis* protoplasts, using B5 medium plus 0.22 M sorbitol and 1 mM monocalcium phosphate, were unsuccessful and no divisions were observed. When the concentration of sorbitol was increased to a range of 0.24 to 0.30 M a few protoplasts underwent one or two divisions. The addition of 25 mM kinetin increased the proportion of dividing protoplasts to approximately 2%, but division was not sustained.

Further modification of the medium by replacing part of the sorbitol with mannitol enhanced division so that usually more than 10% of *H. gracilis* protoplasts divided within the first 3 or 4 days. The medium contained 120 mM sorbitol, 100 mM mannitol, 1 mM $CaH_4(PO_4)_2.H_2O$, 25 µM 2,4-D and 25 µM kinetin in 1.2 × normal strength B5 medium (2,4-D free).

When the first divisions were observed the drops were joined to form a thin liquid layer. The medium was then diluted by adding 5–10% by volume of B5 medium and similar dilutions then made at weekly intervals. A more rapid dilution rate resulted in cell death. After 8 weeks some colonies were established and the cells had distinct walls which could not be broken by a dilution shock using distilled water. Also, labelling with Calcofluor White[4] (American Cyanamid Company, Bound Brook, New Jersey), as well as plasmolysis, indicated that substantial cell walls had been formed.

This study shows that wall formation and sustained division can be achieved by both soybean and *H. gracilis* protoplasts using a simple, defined medium. Complex organic supplements and conditioning of the medium are not essential. It should be pointed out that although we have not made absolute osmolarity measurements, it is recognized that control of osmolarity is critical. This is especially true in the initial stage of new cell wall development. Although both types of protoplasts used were successfully cultured in defined, unconditioned medium, it is apparent from this study that the conditions suitable for culturing one species of protoplasts may not be satisfactory for culturing another species.

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K. N. KAO
O. L. GAMBORG
R. A. MILLER

National Research Council of Canada,
Prairie Regional Laboratory,
Saskatoon,
Saskatchewan

W. A. KELLER

Crop Science Department,
University of Saskatchewan,
Saskatoon,
Saskatchewan

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Further Observations on Acetylcholine Noise

IN a recent communication we reported that the end-plate depolarization produced by a steady dose of acetylcholine (ACh) is accompanied by a significant increase in voltage noise across the membrane[1]. We have continued to analyse this phenomenon on the assumption that the average depolarization as well as the superimposed voltage fluctuations arise from the same molecular "shot effects", that is, from summation and statistical variation in the number of ACh-operated ionic membrane gates which open and shut during any given time interval.