Vitis **14**, 177—180 (1975)

CSIRO Division of Horticultural Research, Adelaide, Australia

Production of callus from protoplasts of cultured grape pericarp

by

K. G. M. Skene

Kallusbildung aus Protoplasten von kultiviertem Traubenperikarp

Zusammenfassung. — Eine Nährlösung wird beschrieben, in der Protoplasten, die aus Kalluskulturen von Traubenperikarp isoliert worden waren, neue Zellwände bildeten und Zellteilungen eingingen. Spätere Übertragung auf einen festen Nährboden ergab aktives Kalluswachstum.

Introduction

Despite significant progress recently made in the culture of hybrid cells derived from intergeneric fusion of protoplasts (Kao *et al.* 1974), basic data on the culture of protoplasts from many economic plants is still lacking. In particular, information on the performance of protoplasts from woody perennial species is remarkably sparse.

In an earlier report (Skene 1974) I described the initial stages of cell regeneration and division of protoplasts isolated from callus cultures of grape vine pericarp. Although division proceeded rapidly during the first week of culture, it was not sustained. Protoplasts embedded in agar rarely passed through more than two division cycles before cytoplasmic extrusion prevented further development; in liquid culture protoplasts did not divide more than once.

Repeated attempts (unpublished) to prevent the ultimate extrusion of cytoplasm in agar-containing media have so far failed. However, I now report on the successful production of callus from grape vine protoplasts cultured in liquid media.

Materials and Methods

Culture of source material and preparation of protoplasts has been described elsewhere (Skene 1974). In brief, callus from immature pericarp tissue of grape vines (*Vitis vinifera* cv. Sultana) was cultured in the dark (26 °C) on a medium based on that of Gamborg and Eveleigh (1968), supplemented with casein hydrolysate 2 g/l, kinetin 0.2 mg/l, naphthaleneacetic acid (NAA) 0.1 mg/l, and agar 10 g/l (Hawker *et al.* 1973).

Protoplasts were prepared by incubating 2 g fresh weight of callus per 10 ml of filter-sterilized Cellulase Onozuka SS (2%) and Macerozyme (1%) dissolved in a mixture of 0.14 molal KCl and 0.10 molal $CaCl_2$, at pH 5.5. Incubation generally proceeded for 16 h in the dark at 26 °C. Exposure to the enzymes for shorter periods did not appear to improve protoplast viability.

The incubate was passed through 150 µm and 61 µm stainless steel sieves in series, and protoplasts were sedimented by centrifuging at 100 *g* for 3 minutes. After washing three times with KCl-$CaCl_2$ (0.14 and 0.10 molal), followed by centrifugation (50 *g* for 3 min), protoplasts were suspended in the appropriate culture media, and 3 ml aliquots containing approximately 10^5 protoplasts per ml were transferred to 6 cm Petri dishes sealed with Parafilm. Dishes were placed in the dark at 26 °C.

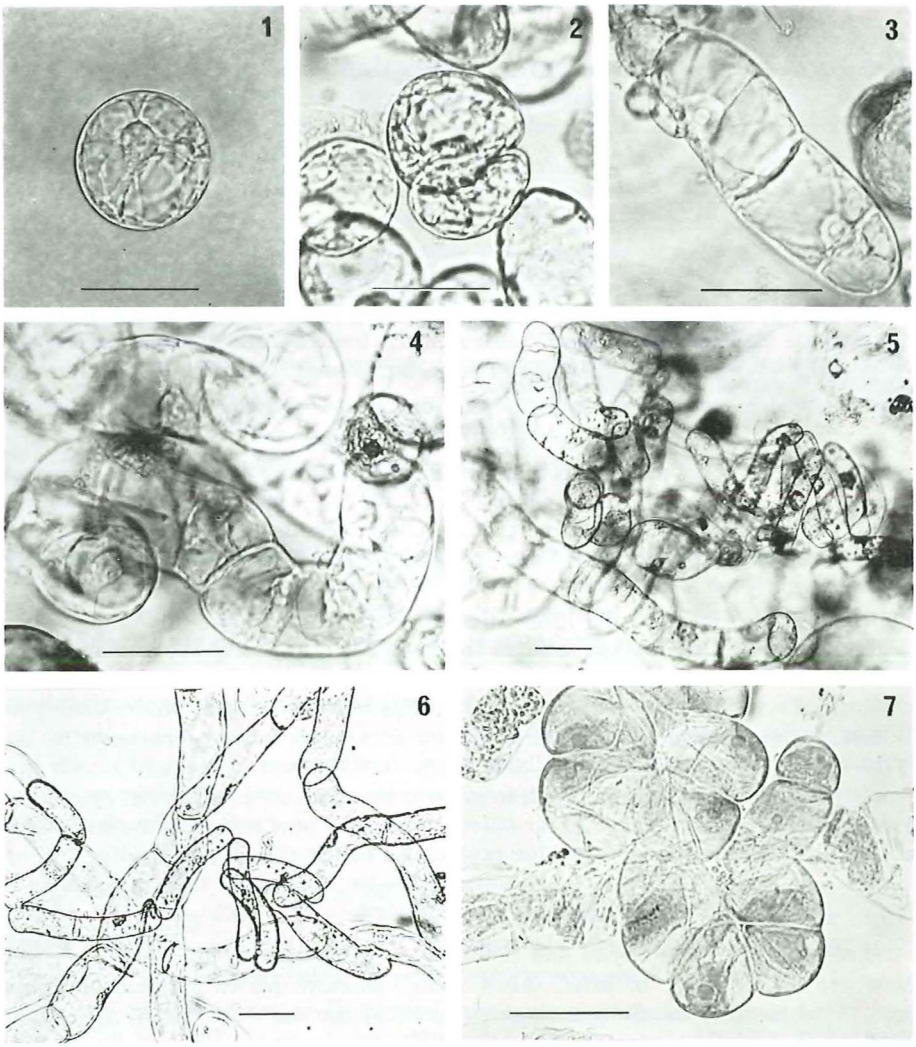

Fig. 1: Freshly isolated protoplast from grape pericarp callus.
Fig. 2: First cell division; 6th day of culture.
Figs. 3 and 4: Multiple divisions after 2 weeks' culture. Sorbitol osmoticum, NAA 1 mg/l.
Fig. 5: Cell groups during 5th week of culture. Sucrose osmoticum, 2,4-D 0.1 mg/l.
Fig. 6: Sub-sample at 10 weeks; before transfer to agar-containing medium. Sucrose osmoticum, 2,4-D 0.1 mg/l.
Fig. 7: Dividing callus cells produced from protoplasts. Two weeks after transfer from protoplast medium to pericarp medium with agar. Aceto-carmine stain.
———— = 50 μm.

Abb. 1: Frisch isolierter Protoplast aus Kallus von Traubenperikarp.
Abb. 2: Erste Zellteilung; 6. Kulturtag.
Abb. 3 und 4: Mehrfache Teilung nach 2wöchiger Kultur. Sorbit als Osmoticum und 1 mg NAA/l.
Abb. 5: Zellgruppen in der 5. Kulturwoche. Saccharose als Osmoticum und 0,1 mg 2,4-D/l.

The following components of the culture medium remained unchanged from the previous report (SKENE 1974): macroelements, microelements and iron of OHYAMA and NITSCH (1972), vitamins of GAMBORG and EVELEIGH (1968), casein hydrolysate 2 g/l, glutamine 2 g/l, sucrose 10 g/l, ribose 250 mg/l, glucose 250 mg/l, xylose 150 mg/l, arabinose 150 mg/l, glycine 4 mg/l and benzyladenine (BAP) 1 mg/l. In the present study, however, auxin was added either as 2,4-dichlorophenoxyacetic acid (2,4-D, 0.1 mg/l) or NAA (1—4 mg/l), the concentration of inositol was raised to 1 g/l, and serine (100 mg/l) was included. The osmotic strength of the medium was controlled with either 0.24 M sorbitol or 0.23 M sucrose, to give a total osmolality between 0.4 and 0.5 osmolal, as measured by a Knauer osmometer. Glutamine and serine were filter-sterilized; all other components were autoclaved.

Results and Discussion

Protoplasts (Fig. 1) formed a new wall within the first few days of culture, and 0.3—0.5% of the total population had divided once by the sixth day (Fig. 2). At this stage, there was little difference between the response to 2,4-D and NAA at the lower levels tested, or to sorbitol and sucrose. No divisions occurred if the osmolality of the medium exceeded 0.5 m.

In the complete medium containing sorbitol and 1 mg NAA/l, cell division in approximately 1% of the population continued during the second week of culture. Half of these cells divided more than once, and groups or filaments consisting of 3—4 cells (Fig. 3) or greater (Fig. 4) were observed. Further medium was added at periodic intervals until cell density became too high for effective observation. Cell groups also occurred at higher levels of NAA (2 and 4 mg/l), but the frequency of division and rate of cell development was slower, especially at 4 mg NAA/l.

Development also tended to be slower if sucrose was included as the osmotic stabilizer in the complete medium containing 0.1 mg 2,4-D/l. Although the first cell divisions took place during the initial week of culture, cell groups or filaments did not appear in discernible numbers until the end of the third week. Thereafter, the number of multi-cellular groups increased steadily (Fig. 5). After ten weeks (Fig. 6; sub-sample diluted for photography) the culture was transferred to the surface of basal pericarp callus support medium (HAWKER *et al.* 1973) solidified with 10 g agar/l. Some initial browning occurred, but clumps of callus with actively dividing cells (Fig. 7) appeared within two weeks. Callus growth has now continued through several passages.

Attempts are currently in progress to arrive at a simpler protoplast culture medium, as it is unlikely that all the components listed are essential. At the same time, the initially low percentage of protoplasts that yield dividing cells needs improvement.

It is hoped that the results will assist in further studies on protoplasts from other tissues of the grape vine, such as haploid callus derived from the culture of grape anthers (GRESSHOFF and DOY 1974).

Abb. 6: Subkultur nach der 10. Woche, vor der Übertragung auf das Agarmedium. Saccharose als Osmoticum und 0,1 mg 2,4-D/l.

Abb. 7: Sich teilende Kalluszellen aus Protoplasten 2 Wochen nach der Übertragung aus dem Protoplastenmedium auf das agarhaltige Perikarpmedium. Carminessigsäure-Färbung.

——— = 50 µm.

Summary

Protoplasts were isolated from callus cultures of grape vine pericarp after an overnight incubation at 26 °C in 2% Cellulase Onozuka SS plus 1% Macerozyme dissolved in a mixture of 0.14 molal KCl and 0.10 molal $CaCl_2$ at pH 5.5. When cultured in a liquid medium with either 0.24 M sorbitol or 0.23 M sucrose as osmotic stabilizer, new cell walls were regenerated within the first few days. Cell division commenced by the sixth day of culture, and approximately 0.5% of the population continued dividing. Eventual transfer of the culture to the surface of medium solidified with agar resulted in callus formation.

Acknowledgement

Mr. A. W. Billitzer provided help with the cultures and preparation of the photographs.

Literature Cited

Gamborg, O. L. and Eveleigh, D. E., 1968: Culture methods and detection of glucanases in suspension cultures of wheat and barley. Canad. J. Biochem. **46**, 417—421.

Gresshoff, P. M. and Doy, C. H., 1974: Derivation of a haploid cell line from *Vitis vinifera* and the importance of the stage of meiotic development of anthers for haploid culture of this and other genera. Z. Pflanzenphysiol. **73**, 132—141.

Hawker, J. S., Downton, W. J. S., Wiskich, D. and Mullins, M. G., 1973: Callus and cell culture from grape berries. HortScience **8**, 398—399.

Kao, K. N., Constabel, F., Michayluk, M. R. and Gamborg, O. L., 1974: Plant protoplast fusion and growth of intergeneric hybrid cells. Planta **120**, 215—227.

Ohyama, K. and Nitsch, J. P., 1972: Flowering haploid plants obtained from protoplasts of tobacco leaves. Plant Cell Physiol. **13**, 229—236.

Skene, K. G. M., 1974: Culture of protoplasts from grape vine pericarp callus. Austral. J. Plant Physiol. **1**, 371—376.

Eingegangen am 12. 5. 1975

Dr. K. G. M. Skene
CSIRO
Division of Horticultural Research
G.P.O. Box 350, Adelaide
South Australia, 5001
Australia