Note

Cooperative Effect of Activated Charcoal and Gellan Gum on Grape Protoplast Culture

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Received June 16, 1989

For gene transfer or cell fusion by plant cell technology, it is necessary to establish a method of protoplast culture. In our protoplast culture of grapes, however, cell division failed to occur more than a few times, as previously reported by other workers.1,2 We now report a successful protoplast culture of grapes on Gellan gum containing activated charcoal.

Callus tissues were induced from shoots of Vitis vinifera L. cv. Kosyu on Murashige-Skoog3) (MS) medium supplemented with 2% sucrose, 10 ppm naphthaleneacetic acid (NAA), and 0.6% agar at 28°C in the dark. The calluses were subcultured at one-month intervals on MS medium containing 15 ppm NAA, 3 ppm kinetin, and 0.8% agar.

For cell suspension culture, about 2 g of fresh callus were inoculated in 40 ml of a liquid MS medium containing 10 ppm NAA and 0.01 ppm benzylaminopurine in a 100-ml Erlenmeyer flask. The cultures were agitated on a gyratory shaker (90 rpm/min) at 30°C in the dark and subcultured every 2-3 weeks. For isolation of protoplasts, about 2 g of fresh cell aggregates obtained by the suspension culture were transferred to a petri dish containing 10 ml of a solution containing 1.5% Cellulase Onozuka RS, 0.1% Pectolyase Y-23, and 0.4 M mannitol in a 0.1 M MES buffer (pH 5.5). The mixture was incubated on a gyratory shaker (60 rpm/min) for 2.5-3.0 hr at 30°C in the dark. The released protoplasts were separated from nondigested cell clumps by filtration through Miracloth (Calbiochem-Behring, CA) and washed twice with 0.6 M sucrose solution followed by one washing with a double-strength medium of MS containing 20 ppm NAA.

### Table I. Effects of Activated Charcoal and Various Gelling Agents on the Development of Protoplasts from Grape Cell Culture

<table>
<thead>
<tr>
<th>Condition of medium</th>
<th>Gelling agent (% w/v)</th>
<th>Activated charcoal (% w/v)</th>
<th>Frequency of cell division* after 14 days (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Range</td>
</tr>
<tr>
<td>Agar (0.6%)</td>
<td>0.2</td>
<td>0</td>
<td>0-5</td>
</tr>
<tr>
<td>Purified agar (0.6%)</td>
<td>0.2</td>
<td>0</td>
<td>0-12</td>
</tr>
<tr>
<td>Agarose (0.6%)</td>
<td>0.2</td>
<td>0</td>
<td>0-31</td>
</tr>
<tr>
<td>Na-alginate (0.8%)</td>
<td>0.2</td>
<td>0</td>
<td>0-15</td>
</tr>
<tr>
<td>Gellan gum (0.4%)</td>
<td>0.2</td>
<td>0</td>
<td>47-80</td>
</tr>
</tbody>
</table>

* No. of cells that divided more than once/No. of cells inoculated initially × 100 (%).

### Table II. Diffusion of BPB in Various Gelling Agents

<table>
<thead>
<tr>
<th>Medium conditions</th>
<th>Gelling agent</th>
<th>Activated charcoal (%)</th>
<th>Permeated area of BPB after 60 sec (mm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Agar</td>
<td>0.2</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Purified agar</td>
<td>0.2</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Agarose</td>
<td>0.2</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>Na-alginate</td>
<td>0.2</td>
<td>177</td>
</tr>
<tr>
<td></td>
<td>Gellan gum</td>
<td>0.2</td>
<td>254</td>
</tr>
</tbody>
</table>

Ten μl of BPB solution (0.1%) was centered in a petri dish containing a gelling agent.

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Fig. 1. Callus Formation from Protoplasts of the Grape "Kosyu".

(A) protoplast isolated from cultured cells, $\times$ 400; (B) first division in regenerated cells, $\times$ 200; (C) a cluster of
4-5 cells derived from a single protoplast, $\times$ 200; (D) a colony consisting of more than 10 cells, $\times$ 200; (E)
minute callus (0.4 mm in diameter) derived from protoplast, $\times$ 100; (F) minute callus (2 mm in diameter)
derived from protoplast; (G) nurse culture of minute callus derived from protoplast; (H) further growth of
callus; (I) transfer and culture of the resulting callus on MS medium containing 15 ppm NAA and 3 ppm
kinetin.

0.02 ppm zeatin, 7% mannitol, and 3% sucrose by centrifugation at 150 $\times$ g for 5 min. The washed protoplasts were
again suspended in the latter medium. Two ml of the protoplast suspension, two ml of a gelling agent (agar,
purified agar, agarose, Na-alginate, or Gellan gum) containing 7% mannitol plus 3% sucrose, and 8 mg of activated
charcoal were mixed in a petri dish (60 $\times$ 15 mm), which was then sealed with parafilm and incubated at 27°C in
the dark. The pH of all culture media were adjusted to 5.7-5.8 with 1 n NaOH before autoclaving. The number of
protoplasts in the medium was adjusted to an initial concentration of about $1 \times 10^5$ cells/ml. The frequency of cell
division was counted by microscopic inspection at three arbitrarily chosen points of 0.5 cm$^2$ per dish for 4
replicates.

Effects of activated charcoal and gelling agents on the
development of protoplasts were examined after 14 days of incubation (Table 1). Of ten different conditions tested, a
combination of activated charcoal and Gellan gum was the most satisfactory. When either one of the two additives
was omitted from the medium, the frequency of cell division was greatly reduced. Agar appeared to be unsuit-
able as a gelling agent. About 1.2% of the protoplasts that divided on Gellan gum with activated charcoal developed
to minute calluses (0.2-0.4 mm in diameter) after one month; some of them further developed into calluses of
2-3 mm in diameter after about two months.

However, the calluses eventually turned brown and failed to grow any more on this medium. Even when the
calluses were only transferred to another solid medium prepared from various gelling agents, the callus growth
was not observed. Therefore, a nurse culture was done by
placing a callus (1–2 mm diameter) on a filter paper over a callus culture grown on MS agar medium containing 15 ppm NAA and 3 ppm kinetin. Calluses that developed to 5–7 mm in size after one month of the nurse culture were then transferred to a MS agar medium containing 15 ppm NAA and 3 ppm kinetin. The growth of protoplast-derived cells is shown in Fig. 1.

It has been suggested that impurified agar may contain an inhibitor of cell division, which can be absorbed by activated charcoal.\(^4^\) However, the frequency of cell division in grape cells on an agar medium without activated charcoal could not be improved by using purified agar with activated charcoal. Thus, the inhibition of cell division dose not seem to be caused by any inhibitor present in the agar, but by an inhibitor produced by the cell itself. It has been reported that Gellan gum is more effective in the cultures of plant tissues (tobacco, cabbage, pokeweed, and cucumber) and cabbage protoplasts than agar\(^7^)\) and this difference might be due to the readiness of diffusion of an inhibitor in the former. To examine this possibility, the dye bromophenol blue (BPB) was added to various solid media and the diffusion rate of this pigment was measured. As shown in Table II, the diffusion rate was highest in Gellan gum and was increased by the addition of activated charcoal. These results suggest that any inhibitor formed would diffuse easily in Gellan gum to be rapidly absorbed by the activated charcoal.

Further studies are needed to regenerate plants from the callus cultures of grape.

References