Plant regeneration from protoplasts isolated from callus of *Gentiana crassicaulis*

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**Abstract**

Fast growing calli induced from hypocotyl segments of *Gentiana crassicaulis* were used for preparation of protoplasts. High yields of viable protoplasts were produced in an enzyme solution containing 1-2% cellulase, 1% pectinase, and 0.5% Hemicellulase. Protoplasts were cultured in KM8P medium containing 1 mg/l 2,4-D, 0.5 mg/l 6BA, 500 mg/l LH, 0.5 M glucose and 0.1 M mannitol by the solid-liquid dual layer culture method. First division occurred within 4-5 days of culture at a frequency of 17.8%. Sustained divisions led to callus formation. Periodically diluting the cultures with freshly prepared liquid medium containing 2% glucose was critical for colony formation. Protocallons about 2 mm in size were transferred onto MS medium supplemented with 3 mg/l ZT, 2 mg/l 6BA, 1 mg/l GA3, 1 mg/l NAA and 6% sucrose to obtain embryogenic calli. Plantlets were regenerated via somatic embryogenesis at high frequency on hormone-free MS Medium.

**Key words:** Gentiana crassicaulis Duthie ex Burk, protoplasts, callus, plant regeneration

**Abbreviations**

6BA: 6-benzylaminopurine, NAA: naphthaleneacetic acid, 2,4-D: 2,4-dichlorophenoxyacetic acid, ZT: zeatin, GA3: gibberellic acid, LH: lactalbumin hydrolysate, MES: 2-(N-morpholino)-ethane sulfonic acid, MS: Murashige & Skoog's medium(1962)

**Introduction**

*Gentiana crassicaulis* is one of the four Commercial Radix Gentianae Macrophyllae stipulated by the Chinese Pharmacopoeia. The psychopharmacological profiles of major alkaloids in *Gentiana* have been studied. The alkaloids exhibited significant antipsychotic (Bhattacharya et al 1974), antiinflammatory (Sadritdinov 1971), and sedative (Tulyaganov et al 1971) activity. The roots are the source of gentianine and gentianal. Traditionally, the pharmaceutical industry has largely depended on wild sources of *Gentiana*. *G. crassicaulis* is not cultivated in China. Therefore, employing plant tissue culture technique for its rapid clonal propagation, in vitro conservation and genetic manipulation is of great economic value. There is only one report dealing with callus formation from protoplasts of *G. scabra* (Zhou et al 1985). This article describes plant regeneration from protoplasts of *G. crassicaulis*.

**Materials and Methods**

**Callus induction**

Seeds of *Gentiana crassicaulis* Duthie ex Burk were provided by Northwestern Plateau Biology Institute of Academia Sinica. The seeds were surface-sterilized in 0.1% Mercuric chloride(HgCl2) for 10 min and rinsed 4 times (5 min each) in sterile distilled water. Sterilized seeds were placed on hormone-free MS medium plus 0.1% Mercuric chloride(HgCl2) for 10 min and rinsed 4 times (5 min each) in sterile distilled water. Sterilized seeds were placed on hormone-free MS medium plus 2% sucrose and 0.7% agar, pH5.8, and incubated at 25 ± 1°C under fluorescent light (800 Lux). Hypocotyls from four week old seedlings (about 2-3cm in length) were cut into 5 mm segments and cultured on MS medium plus 2 mg/l 2,4-D, 0.5 mg/l 6BA and 0.7% agar to induce callus. Two weeks after culture, calli were transferred to MS medium containing 1 mg/l 2,4-D, 0.5mg/l 6BA, 500 mg/l LH and 4% sucrose, and subcultured every three weeks.
Protoplast isolation and culture

About 1 g calli of 10-15 day old was placed in 10 ml enzyme solution containing different combinations of cellulase R-10, macerozyme R-10 (Kinki Yakult Manufacturing Co. Ltd. Mishinomiya, Japan), pectinase (Serva Co. USA), hemicellulase (Sigma Chemical Co., St. Louis, MO, USA) (Table 1). After 8-10 hours incubation on a shaker (50rpm) at 25 ± 1 °C in the dark, the released protoplasts were filtered through two stainless steel sieves (75 μm and 40 μm) and centrifuged at 500 rpm for 5 min. The pellets were resuspended in wash solution consisting of 0.18 M Calcium chloride (CaCl₂) and 0.1% (w/v) MES, and layered on a volume 21% (w/v) sucrose solution. After centrifugation at 80 rpm for 10 min, the protoplasts were concentrated at the interface of the two solutions, while the debris sank to the bottom of sucrose solution. Collected protoplasts were washed three times with wash solution, and once with culture medium. Protoplasts were resuspended in KM8P medium (Kao and Michayluk 1975) supplemented with 1 mg/l 2,4-D, 0.5 mg/l 6BA, 500 mg/l LH, 0.5 M glucose, and 0.1 M mannitol, and adjusted to a density of 1 × 10⁵ protoplasts/ml. Liquid thin layer culture method and liquid-solid dual layer culture method were used for protoplast culture. The dual layer culture method involved suspending protoplasts in liquid KM8P medium and pouring 2 ml protoplast suspension into a 60 × 15mm petri dish in which 0.4% agarose containing KM8P medium were placed previously. All cultures were incubated at 25 °C in the dark. Protoplast viability was estimated by staining with 0.1% phenosafranine dissolvable in 0.4 M mannitol solution. After first division occurred, the osmotic pressure of the liquid medium was gradually reduced by weekly adding supplemental medium containing 1% (w/v) glucose. The components of supplemental medium the same as the initial culture medium, but the concentration of glucose was lowered to 1% (w/v). Plating efficiency (PE) (numbers of protocolonies visible by naked eye/ the total number of inoculated protoplasts × 100%) was evaluated after 30 days. The protocolonies (2 mm in diameter) were transferred to MS medium supplemented with 1 mg/l 2,4-D, 0.5 mg/l 6BA, 500 mg/l LH and 4% (w/v) sucrose for further proliferation.

Plant regeneration

Protoplast-derived calli were transferred onto MS medium containing 2 mg/l 6BA, 3 mg/l ZT, 1 mg/l NAA, 1 mg/l GA₃, 500 mg/l LH and 6% (w/v) sucrose. Three weeks later, embryogenic calli were picked up and placed on hormone-free MS medium with 1200 lux illumination at 25 ± 2 °C to promote plantlet development.

Results and discussion

Protoplast isolation

Different combinations of enzyme solution were tested for protoplast isolation. As shown in Table 1, the combination of 1-2% cellulase R-10, 1% pectinase and 0.5% hemicellulase was more suitable for efficient protoplast isolation. When the concentration of cellulase R-10 exceeded 2%, the viability of protoplasts dropped significantly. Hemicellulase was beneficial for protoplast isolation. Using macerozyme R-10 instead of pectinase gave lower protoplast yield and viability. Moreover, it is advantageous to reduce the incubation time of callus-enzyme mixture so that the viability of protoplasts could be improved. In our experiments, 8-10h was optimal.

Table 1. The effect of enzyme combination on protoplast yield and viability*

<table>
<thead>
<tr>
<th>Cellulase</th>
<th>Pectinase</th>
<th>Macerozyme</th>
<th>Hemicellulase</th>
<th>Yield of</th>
<th>Viability of</th>
</tr>
</thead>
<tbody>
<tr>
<td>OnozukaR-10</td>
<td>Serve</td>
<td>OnozukaR-10</td>
<td>Sigma</td>
<td>Protoplasts</td>
<td>protoplasts</td>
</tr>
<tr>
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<td>%</td>
<td>%</td>
<td>%</td>
<td>prot/gFW</td>
<td>%</td>
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<td>0</td>
<td>0</td>
<td>2 × 10⁵</td>
<td>90</td>
</tr>
<tr>
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<td>1</td>
<td>0</td>
<td>0.5</td>
<td>1-2 × 10⁶</td>
<td>90</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1-2 × 10⁶</td>
<td>85</td>
</tr>
<tr>
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<td>0.5</td>
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<td>90</td>
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<tr>
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<td>1</td>
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<td>70</td>
</tr>
<tr>
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<td>0</td>
<td>1</td>
<td>1</td>
<td>5 × 10⁵</td>
<td>70</td>
</tr>
</tbody>
</table>

*Enzymes were dissolved in the solution containing KM8P inorganic components, 4.5 mM CaCl₂, 0.5 M glucose, 0.1 M mannitol, 0.1% MES (pH5.8).