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Plant Regeneration from Mesophyll Protoplasts of Gentiana (*Gentiana scabra* BUNGEI)

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Protoplasts were isolated enzymatically from leaves of gentiana propagated *in vitro*. Cell division started after 4-5 days of culture in a modified MS or B 5 liquid medium, and after 6-8 weeks small calli were formed by the addition of fresh medium. Shoots were differentiated from the protoplast-derived callus on a MS agar medium containing 1.0 mg/*l* IAA and 6.0 mg/*l* BA. Plantlets were regenerated by rooting from the shoots on a hormone-free MS medium.

Plant regeneration from protoplasts is one of the essential techniques for plant breeding through genetic transformation and somatic hybridization. Gentiana (*Gentiana* spp.) is an economically important flower and ornamental plant in cool regions in Japan. *In vitro* techniques have been recently reported on gentiana[1-3]. However, little is known about the protoplast culture of this plant. In this paper, we describe the regeneration of plantlets from mesophyll protoplasts of gentiana.

Materials and Methods

Plant material. The axenic plants of gentiana (*G. scabra* BUNGEI) TO strain were obtained from the shoot-tip culture (Jomori and Takahata, unpublished). They were propagated *in vitro* on a MS medium without hormones.

Protoplast isolation and culture. The leaves were cut into 2-3 mm wide strips and soaked in an enzyme solution containing 2.0% Cellulase Onozuka R-10, 0.2% Macerozyme R-10, 0.1% $CaCl_2 \cdot 2H_2O$ and 10% mannitol pH 5.5. After overnight incubation at 25℃, the enzyme solution was reciprocally shaken with 60 strokes/min for 30 min at room temperature. The isolated protoplasts were filtered through a nylon sieve of 82 μm pore size and washed three times with a washing solution containing 0.1% $CaCl_2 \cdot 2H_2O$ and 10% mannitol by centrifugation at $100 \times g$ for 3 min. Then they were overlied on a 20% sucrose solution and centrifuged at $100 \times g$ for 3 min. The protoplasts floating on the sucrose solution were collected and washed with the culture medium.

Protoplasts were cultured at the density of $0.5\text{-}1.0 \times 10^5$/ml in a 60 mm plastic petri dish with 2 ml liquid media. The culture media consisted of the basal medium of modified MS[4] (400 mg/*l* NH_4NO_3) or B 5,[5] 1% sucrose, 9% mannitol, 2.0 mg/*l* NAA and 1.0 mg/*l* BA. The dishes were placed in a plastic box and incubated at 25℃ in 16-hour photoperiod. The same fresh medium was added at one week intervals and after three weeks the culture medium containing 4% mannitol was added two to three times at the equivalent intervals.

Protoplast-derived calli were transferred to different MS agar solidified media to obtain regeneration. The calli were transferred to the new media after a culture of 4-5 weeks. The conditions of the callus culture were the same as the protoplast culture.

Results and Discussion

The average yield of protoplasts was 3.0×10^5/g fw after the whole washing procedure (**Fig. 1**-A).

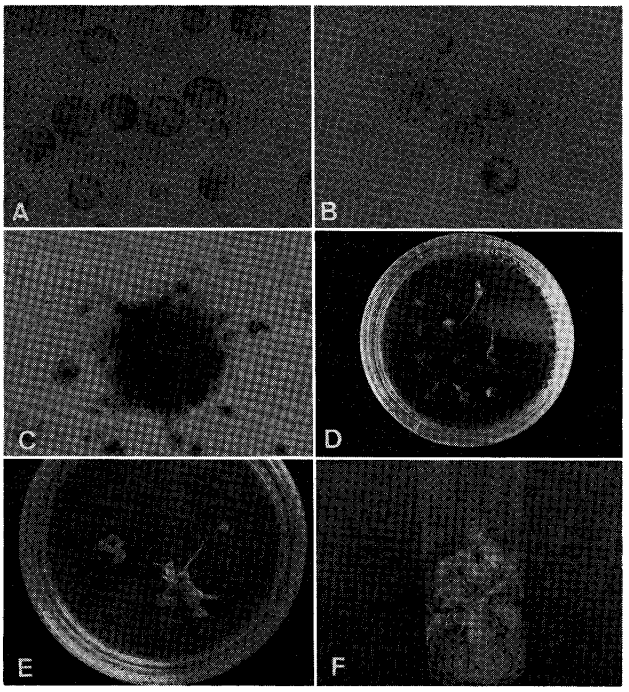

Fig. 1. Plant regeneration from mesophyll protoplasts of gentiana. A. Freshly isolated mesophyll protoplasts; B. Cell division after 4 days of culture; C. Small callus after 6 weeks of culture; D. Root differentiation from protoplast-derived calli; E. Shoot differentiation from callus; F. Plantlet regeneration.

The initiation of cell division was observed after 4–5 days of culture (**Fig. 1**-B). However, after 10–14 days, browning of cell clusters occurred when they were left in the initial medium. Addition of the fresh medium was essential to prevent browning of gentiana protoplast-derived cell clusters. Visible calli appeared within 4 weeks (**Fig. 1**-C). Plating efficiency after 6 weeks of culture varied from 0.1% to 0%. This fluctuation seemed to be due to the physiological condition of donor plants. The difference between basal, modified MS and B 5 media was not found in plating efficiency.

After 6–8 weeks, calli 0.5–1.0 mm in diameter were transferred to various MS agar media. Roots differentiated from small calli on the media containing 0.2–2.0 mg/*l* IAA or NAA with a frequency of 1.3–7.2% (**Fig. 1**-D), but shoots could not be initiated. Many calli further developed on the media containing 0.5–2.0 mg/*l* 2, 4-D and 1.0–4.0 mg/*l* BA or 0.2 mg/*l* IAA or NAA and 4.0 mg/*l* BA. The greenish calli were formed by the high concentration of cytokinin.

For shoot regeneration from protoplast-derived calli, we tested many combinations of auxin (IAA, NAA, 2, 4-D) and cytokinin (BA, kinetin) at various concentrations (auxin 0–2.0 mg/*l*, cytokinin 1.0–10.0 mg/*l*). Shoot differentiation was induced from greenish calli only on the MS medium containing 1.0 mg/*l* IAA and 6.0 mg/*l* BA at low frequency of 1.0% (**Fig. 1**-E). On the other hand, root formation was also observed in many calli on the media containing IAA and NAA. The shoots developed roots and plant regeneration was achieved when transferred to a MS medium without hormones (**Fig. 1**-F).

This report describes plant regeneration from protoplasts of gentiana for the first time. However, the yield of protoplasts and the frequencies of plating efficiency and plant regeneration are low at present. Further work is necessary to establish the efficient culture procedure of gentiana protoplast.

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≪和文要約≫

リンドウ (*Gentiana scabra* Bungei) 葉肉プロトプラストからの植物体再生

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リンドウの葉肉プロトプラストの単離と植物体再生について検討した．茎頂由来の無菌植物の葉を2.0% セルラーゼ“オノズカ”R-10 0.2% マセロザイム R-10，0.1% $CaCl_2 \cdot 2H_2O$，10% マンニトールの酵素液で一晩処理し，プロトプラストを単離した．精製したプロトプラストを 2.0 mg/*l* NAA，1.0 mg/*l* BA，9% マンニトール，1% 蔗糖を含む改変 MS (400 mg/*l* NH_4NO_3) および B5 培地で培養した．プロトプラストは培養後 4〜5 日で第一分裂が起こり，新鮮培地を一週間ごとに添加していくことで 6〜8 週間後に直径 0.5〜1 mm の小カルスを形成した．プロトプラスト由来のカルスから植物体再生をはかるため種々の再分化培地（オーキシンとサイトカイニンの種類と濃度を変えた MS 寒天培地）を検討したところ，1.0 mg/*l* IAA，6.0 mg/*l* BA 添加の MS 培地で 1.0% の頻度で芽が分化し，さらに植物ホルモン無添加の MS 培地で発根し植物体が再生した．