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Plant regeneration from protoplasts of *Gentiana straminea* Maxim

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Abstract: A protocol is described for plant regeneration from protoplasts of *Gentiana straminea* Maxim. via somatic embryogenesis. Protoplasts were isolated from embryogenic calli in an enzyme solution composed of 2% Cellulase Onozuka R-10, 0.5% Macerozyme R-10, 0.5% Hemicellulase, and 0.5 M sorbitol with a yield of 3.0×10^6 protoplasts per gram of fresh weight. Liquid, solid-liquid double layer (sLD) and agar-pool (aPL) culture systems were used for protoplast culture. The aPL culture was the only method that produced embryogenic, regenerative calli. With aPL culture, the highest frequencies of protoplast cell division and colony formation were 39.6% and 16.9%, respectively, on MS medium supplemented with 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg/L N^6-benzylaminopurine (BA). Microcalli were transferred to solid MS medium containing a reduced concentration of 2,4-D (0.5 mg/L) to promote the formation of embryogenic calli. Somatic embryos developed into plantlets on MS medium supplemented with 2 mg/L BA at a rate of 43.7%.

Keywords: *Gentiana straminea* Maxim., Protoplast culture, Plant regeneration, Agar-pool method

Abbreviations

aPL - Agar-pool culture;
BA - N^6-Benzylaminopurine;
CH - Casein hydrolysate;
2,4-D - 2,4-Dichlorophenoxy acetic acid;
FDA - Fluorescein diacetate;
MES - 2-(*N*-morpholino) ethanesulfonic acid;
MS - Murashige and Skoog (1962) medium;
sLD - Solid-liquid double layer culture

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1 Introduction

Gentiana straminea Maxim., a member of the Gentianaceae family, is an important medicinal plant in China [1]. It is distributed mainly in the alpine and subalpine belts of the Qinghai-Tibet Plateau at altitudes from 2 500 to 4 700 m [1]. Its roots and flowers are widely used as *Qin-jiao* in traditional Chinese medicine and as *Jie-ji* in traditional Tibetan medicine, respectively, to treat many diseases, including rheumatism, osteoarthritis, hepatitis, gastritis and cholecystitis [2]. In the pharmaceutical industry, *a major source* of *G. straminea* is *wild harvest*. Due to uncontrolled collection from their natural habitats and lack of organized cultivation, wild *G. straminea* plants are rapidly disappearing [2, 3]. Moreover, *G. straminea* is propagated by seed, but its germination rate is extremely low and seedlings grow slowly in natural conditions [3]. Therefore, *in vitro* propagation should be a feasible alternative for rapid multiplication and maintenance of this germplasm. Recently, *in vitro* technology for this species has been developed for potential use in the production of high quality, consistent plant material as well as for genetic improvement [3-5].

Protoplast-based technologies, such as somatic hybridization, cytoplasmic recombination, micronucleus transfer, direct DNA uptake, transformation and mutation selection, may also provide useful means of genetic improvement [6-9]. A basic requirement for achieving this goal is the successful regeneration of plants from isolated protoplasts. In the genus *Gentiana*, plant regeneration from protoplasts has been achieved from leaf mesophyll cells of *G. scabra* [10], *G. triflora* × *G. scabra* [11], *G. decumbens* [12], callus tissues of *G. triflora*, *G. acaulis* [13], *G. crassicaulis* [14] and suspension cells of *G. kurroo* [15] and *G. macrophylla* [16]. However, there have been no reports on protoplast isolation and culture of *G. straminea*, until now. In the present investigation, we have developed an efficient protocol that allows plant regeneration

from protoplasts isolated from embryogenic calli of *G. straminea*, an endangered medicinal plant.

2 Experimental Procedures

2.1 Plant material

Mature *G. straminea* seeds were provided by Northwest Plateau Institute of Biology, Chinese Academy of Sciences. They were collected during the months of September and October from healthy *G. straminea* plants growing in the northeastern Qinghai-Tibet Plateau in China. Seeds were surface sterilized with 70% (*v/v*) ethanol for 45 seconds, then with 0.1% mercuric chloride ($HgCl_2$; *w/v*) for 12 minutes and rinsed five times with sterile distilled water. The seeds were germinated on half-strength Murashige and Skoog (MS) medium [17] supplemented with 3% (*w/v*) sucrose and 0.85% (*w/v*) agar (Jing-Hai, Qingdao, China) without growth regulators. Leaves excised from 3-month-old seedlings were used as explants.

2.2 Embryogenic callus induction

Somatic embryogenesis of *G. straminea* was induced from leaf explants using the method described by He et al. [4], with minor modifications. Leaf segments were cultured on MS medium supplemented with 2 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5 mg/L N^6-benzylaminopurine (BA), 3% (*w/v*) sucrose and 0.85% (*w/v*) agar. Cultures were incubated at 20 ± 2°C with a 16-hour photoperiod. A light intensity of 30 μmol m^{-2} s^{-1} was provided by cool white fluorescent lamps.

Embryogenic calli were cultured on MS medium containing 0.5 mg/L 2,4-D for subculture. Calli were subcultured at 2 week intervals.

2.3 Protoplast isolation

Embryogenic calli that had been subcultured for 1-21 days were used for protoplast isolation. Approximately 1 g of fresh calli were mixed with 10 mL enzyme solution containing 2% (*w/v*) Cellulase Onozuka R-10 (Yakult Pharmaceutical Co. Ltd., Japan), 0.5% (*w/v*) Macerozyme R-10 (Yakult Pharmaceutical Co. Ltd., Japan), 0.5% (*w/v*) Hemicellulase H2125 (Sigma, St. Louis, Mo.), 50 mM $CaCl_2 \cdot 2H_2O$, 0.05% (*w/v*) 2-(*N*-morpholino) ethanesulfonic acid (MES), 0.5 M sorbitol with the pH 5.8 and filter sterilized with 0.45 μm pore size membranes. The mixture was incubated on a shaker (30 rpm) at 25 ± 2°C in the dark. After 14-16 hours incubation, the protoplast enzyme mixture was filtered through a nylon mesh (45 μm). The filtrate was centrifuged at 70 *g* for 5 minutes. Protoplasts were purified using a gradient centrifugation method described by Hou and Jia [18]. The yield of protoplasts was determined by counting the number of protoplasts using a haemocytometer. The viability of the protoplasts was assessed with fluoresceindiacetate (FDA) at a final concentration of 0.01% [19].

2.4 Protoplast culture

Purified protoplasts were cultured in 3.5 cm Petri dishes at a density of 3-5 × 10^5 protoplasts per milliliter. Three culture systems were used in this study:

1. Liquid culture. Protoplasts were suspended in 1.5 mL of liquid medium P1 (Table 1) in a Petri dish.
2. Solid-liquid double layer culture (sLD). 1.5 mL melted solid medium P1 (Table 1) was poured into a Petri dish. After solidification, 1.5 mL of liquid medium P1 with suspended protoplasts was layered onto the solid medium.
3. Agar-pool culture (aPL). Preparation was based on the method described by Hu et al. [16]. Briefly, 1.5 mL melted solid medium P1 (Table 1) was poured into a Petri dish. After solidification, a bottle cap (1.5-2.5 cm in diameter) was placed upside-down on the solid medium. Then 1.5 mL melted solid medium P1 (Table 1) was poured into the space between the bottle cap and Petri dish walls. After solidification, hot sterile water was added into the bottle cap. When the solid medium around the bottle cap melted slightly, the bottle cap with sterile water was carefully removed from the solid medium, which resulted in an agar-pool. Finally, 0.5-1 mL of liquid medium P1 with suspended protoplasts was added into the agar-pool.

Media were solidified using 0.85% (*w/v*) agar. All cultures were maintained at 20±2°C in the dark. After 7 days of culture, half of the old medium was replaced with fresh liquid medium P2 containing half the amount of mannitol (Table 1). Every 1 week thereafter, the old medium was 50% renewed with fresh liquid medium P3 without mannitol (Table 1). Division frequency of cultured protoplasts was recorded on day 14 after culture and colony formation frequency on day 28.

Table 1: Media used for protoplast culture.

Medium	Basal medium [1]	CH (mg/L)	Mannitol (M)	Sucrose (%)	Glucose (%)	Growth regulators (mg/L)	
						2,4-D	BA
P1	MS (without NH_4NO_3) + 3 g/L L-glutamine	500	0.4	2.0	1.0	2.0	0.5
P2	MS (without NH_4NO_3) + 3 g/L L-glutamine	500	0.2	2.0	1.0	2.0	0.5
P3	MS (without NH_4NO_3) + 3 g/L L-glutamine	500	0	2.0	1.0	2.0	0.5

[1] Based on Fiuk and Rybczyński [15].

2.5 Plantlet regeneration

Microcalli (0.5-1 mm in diameter) derived from protoplasts were transferred to a solidified MS medium supplemented with 2 mg/L 2,4-D and 0.5 mg/L BA for further proliferation. The proliferated calli (0.5-1 cm in diameter) were transferred to MS medium containing 0.5 mg/L 2,4-D for the production of embryogenic calli. The calli produced were subcultured at 2 week intervals. After 4 weeks of subculture, the embryogenic calli were transferred to MS medium supplemented with 2 mg/L BA for plantlet regeneration. The regenerated plantlets (1-2 cm in height) were initially maintained on half-strength MS medium without growth regulators for rooting. All cultures were supplemented with 500 mg/L casein hydrolysate (CH), 3% (*w/v*) sucrose and 0.85% (*w/v*) agar, pH 5.8, and incubated at 20 ± 2°C with a 16-hour photoperiod.

2.6 Statistical analysis

Each experiment was repeated three times. Data were analyzed statistically using Duncan's multiple range test [20]. Least significant differences were calculated at the 5% level of probability.

3 Results and discussion

3.1 Protoplast isolation

Protoplasts were successfully isolated from friable, yellow-green embryogenic calli of *G. straminea*. In order to obtain large quantities of viable protoplasts, the embryogenic calli of various ages were tested for viability of protoplast isolation. The results showed that 9 to 14 day-old subcultured calli, which were just in the exponential growth phase, offered enough good quality protoplasts. Under these conditions, protoplast yield could reach more than 3.0×10^6 protoplasts per gram of fresh weight after incubation in 2% Cellulase Onozuka R-10, 0.5% Macerozyme R-10, 0.5% Hemicellulase, and 0.5 M sorbitol. The freshly isolated protoplasts were highly cytoplasmic and relatively uniform in size following purification (Fig. 1a). The viability of protoplasts was found to exceed 90% as detected by FDA staining (Fig. 1b).

3.2 Protoplast culture

No visible changes in the shape of the cultured protoplasts were evident during the first day. After 2 days of culture, most protoplasts became larger and oval in shape, suggesting new cell wall regeneration. The first division of 10% protoplasts occurred after 3 days (Fig. 1c). Five days later, the second and third divisions were observed (Fig. 1d, e). Further division then followed in quick succession.

As reported for other species, the culture system itself is an important factor for protoplast development [21]. In *Solanum virginianum*, the protoplasts were unable to regenerate the cell wall and divide when cultured directly in liquid medium [21]. Yang et al. [22] indicated that a double-layer culture system, with liquid over solid medium was the best method for protoplast culture of *Gossypium davidsonii*. In *Oenothera hookeri*, embedding protoplasts in thin alginate layers was considered one of the most crucial parameters for cell division and colony formation [23]. In *Gentiana kurroo*, the plating efficiency of protoplasts reached 68.7% using agarose bead culture [15]. We also tested the effect of culture methods on protoplast division and colony formation. In this study, liquid culture, solid-liquid double layer culture (sLD) and agar-pool culture (aPL) were used for protoplast culture.

As shown in Table 2, the frequencies of protoplast cell division and colony formation in liquid culture were similar to those in sLD culture. The division and colony formation frequencies were 31.7 and 7.9, 34.4 and 11.3 in liquid and sLD cultures, respectively. However, the frequencies of cell division and colony formation in aPL culture were significantly (p<0.05) higher than those in liquid and sLD cultures. In aPL culture, the division frequency reached 39.6%, and the colony formation frequency was 16.9% (Table 2). The result is similar to the one obtained in *G. macrophylla* [16]. Hu et al. [16] assumed that these differences might be related to the cell browning. We found that the cells or cell-groups near the Petri dish walls were more likely to turn brown in liquid and sLD culture systems. In aPL culture system, however, the cultured cells and the wall of Petri dish were separated by solid medium, which resulted in lower oxidation.

In comparison with liquid and sLD culture methods, the aPL culture system developed recently in our laboratory presents several advantages, e.g. (1) the protoplasts are kept off the Petri dish walls, which is beneficial to cell development; (2) the diameter of the agar-pool may be adjusted according to the yield of protoplasts isolated, which allows culture of fewer protoplasts [16].

In aPL culture system, microcolonies appeared after 3 weeks (Fig. 1f). After 4 weeks of culture, microcalli were visible to the naked eye (Fig. 1g).

3.3 Plantlet regeneration

Microcalli were transferred to a solidified MS medium supplemented with 2 mg/L 2,4-D and 0.5 mg/L BA for further proliferation. After 4 weeks of culture, two types of calli were recognized. One type consisted of soft, watery, white non-embryogenic calli; the other consisted of compact, friable, yellow-green embryogenic calli. The calli derived from sLD and liquid culture systems were non-embryogenic, while those derived from aPL culture system showed both embryogenic and non-embryogenic characteristics. The effects of various culture systems on callus formation from protoplasts have also been reported in other plants [7, 8, 15, 24]. In *Gentiana kurroo*, globular somatic embryos were detected one week after the microcolonies obtained in the thin layer culture and bead culture had been transferred to induction medium, but the cell aggregates obtained in the liquid culture died when transferred to the same medium [15]. In *Ulmus minor*, all the proliferating calli derived from the agarose droplet culture acquired the embryogenic characteristics 15 weeks after culture initiation [24]. Previous research suggested that the regeneration potential of the protoplasts in different culture systems might be influenced through stimulating cell wall formation, stabilizing the plasmalemma, supply of nutrients, the dilution of inhibitory substances, etc [7, 8]. In this study, the aPL method gave compact colonies with small homogenous cells, which readily turned into embryogenic calli, once transplanted onto subculture medium. This was consistent with our previous study in *G. macrophylla* protoplast culture [16].

These non-embryogenic calli grew very slowly on subculture medium, and no plant regeneration was successful in further culture.

On subculture medium, most embryogenic calli became more compact. Numerous proembryos appeared on the surface of calli within 2 weeks (Fig. 1h). The proembryos grew and matured when they were transferred to MS regeneration media supplemented with 2 mg/L BA. Organized somatic embryos on the surface of calli were evident within 2 weeks (Fig. 1 i-k) and approximately 43.7% of somatic embryos developed into plantlets. From three experiments, a total of 87 plantlets were regenerated from protoplasts (Table 2). Our results indicated that the aPL culture system was the only successful method for protoplast regeneration of *G. straminea*.

When green plantlets with or without primary roots (Fig. 1l) were transferred to the half-strength MS medium without growth regulators, thick white roots developed

Table 2: Effect of culture methods on cell division and colony formation of cultured protoplasts of *Gentiana straminea* Maxim.

Culture systems	First division time (d) [1]	Division frequency (%) after 14 days of culture	Colony formation frequency (%) after 28 days of culture	Number of regenerated plants
Liquid culture	3	31.7 ± 4.4[b]	7.9 ± 2.5[b]	0
Solid-liquid double layer culture	3	34.4 ± 3.8[b]	11.3 ± 2.6[b]	0
Agar-pool culture	3	39.6 ± 2.1[a]	16.9 ± 1.8[a]	87

[1] First division time means the time that there were 10% protoplasts dividing at the same visual field of microscope.
Values represent mean ± standard error. Values followed by different letters in a column are significantly different at $P < 0.05$ according to Duncan's multiple range test.

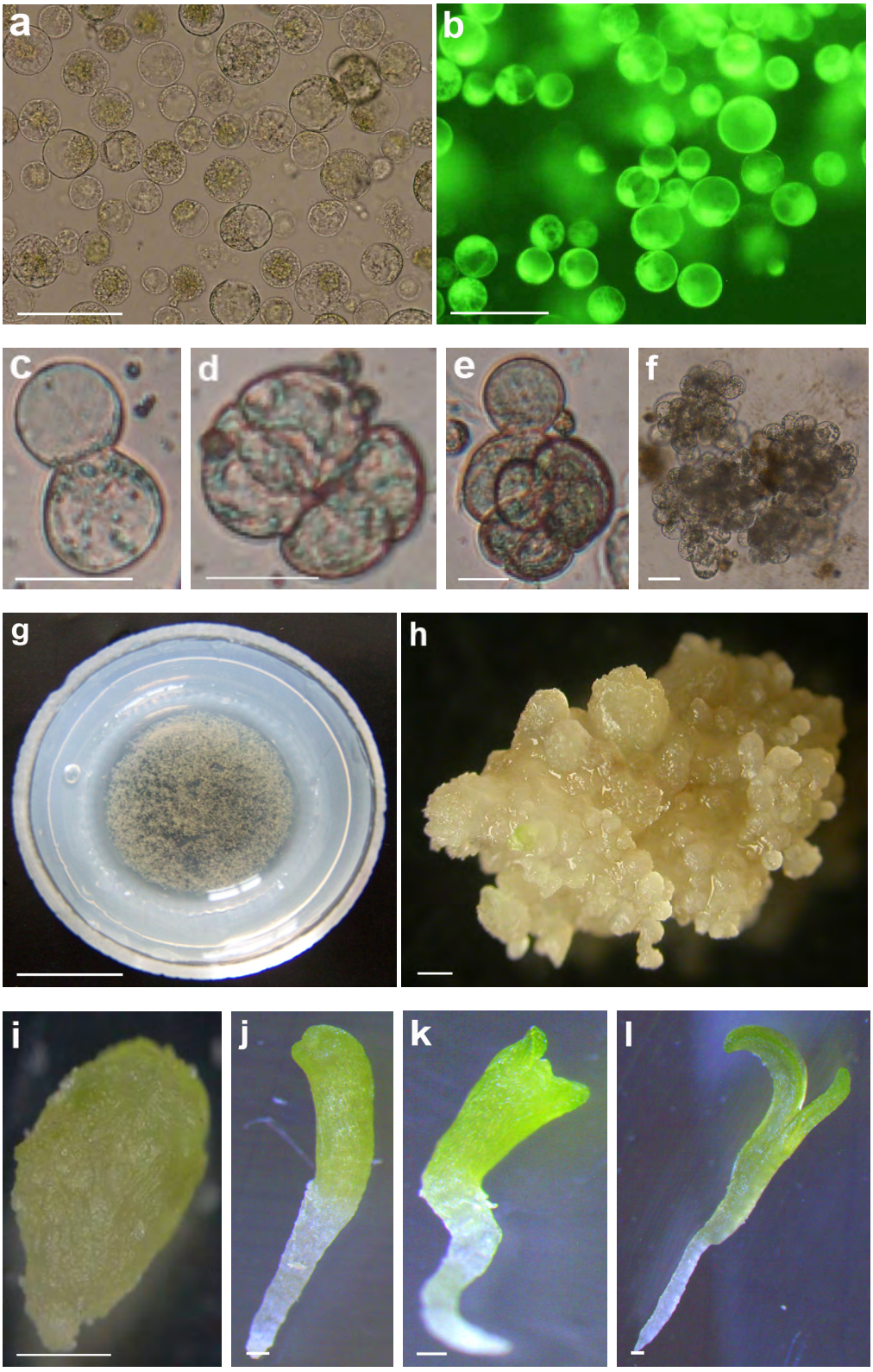

Figure 1: Plant regeneration from protoplasts of *Gentiana straminea* Maxim. using agar-pool culture. Freshly isolated protoplasts from embryogenic calli (a); Viability of freshly isolated protoplasts stained by FDA (b); First cell division of protoplast (c); Second cell division of protoplasts (d); Third cell division of protoplasts (e); Microcolony formation (f); Microcalli formation (g); Embryogenic callus formation from protoplast-derived cell colony (h); Somatic embryos at different development stages (i-k); Regenerated plantlet (l). (a-f: *bar* = 50 μm; g: *bar* = 1 cm; h-l: *bar* = 1 mm).

in about 4 weeks. Regenerated plants showed no visible morphological differences under *in vitro* conditions.

In conclusion, an efficient and reproducible protocol for plant regeneration from protoplasts isolated from embryogenic calli of *G. straminea* has been established using aPL culture system. By utilizing this method, we are now conducting somatic hybridization studies of *G. straminea* with a cultivar of *G. macrophylla*.

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Conflict of interest: The authors declared that they have no conflicts of interest to this work.

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