Plant Regeneration from *Ipomoea triloba* L. Protoplasts

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Plants were regenerated from protoplasts of *Ipomoea triloba* L., one of the related species of sweet potato (*I. batatas* (L.) Lam.) at a high frequency. Protoplasts were isolated from *in vitro*-grown plants of *I. triloba* L. and then cultured in a modified liquid Murashige and Skoog (MS) medium containing 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 1.0 mg/l kinetin. First cell division occurred within 3–4 days. After 2 weeks protoplast plating efficiency was up to about 60%. After 12 weeks protoplast-derived calli up to 2 mm in diameter were transferred onto solid MS medium supplemented with 0.2 mg/l 2,4-D for the proliferation. Three weeks after transfer, they were further transferred onto MS medium supplemented with 0–0.1 mg/l 3-indoleacetic acid (IAA) and 1.0–5.0 mg/l 6-benzylaminopurine (BAP) (regeneration medium) and after 2 weeks started to form adventitious roots. Subsequently, the calli were further cultured on MS medium without plant growth regulators and started to regenerate shoots 5 days after transfer. When 2.0 mg/l BAP was added to the regeneration medium, the regeneration frequency of protoplast-derived calli up to 36.7% was achieved. It was found that (0.2 mg/l) 2,4-D included in the proliferation medium played an important role in promoting shoot regeneration. After the regenerated shoots were transferred onto fresh MS medium without plant growth regulators, they developed into whole plants which were grown to maturity in pots with vermiculite.

KEY WORDS: *Ipomoea triloba*, protoplast, callus formation, plant regeneration.

**Introduction**

Plants of *Ipomoea* section *batatas* are classified into two groups based on the sexual compatibility with sweet potato: Group I sexually compatible with sweet potato and Group II sexually incompatible with sweet potato (Teramura 1979). The related species belonging to Group II have not successfully been applied to sweet potato breeding due to the sexual incompatibility. To overcome such sexual incompatibility through protoplast fusion, it is especially important to regenerate plants from *Ipomoea* section *batatas* protoplasts.

Plant regeneration from protoplasts of Group I plants has been obtained (Murata et al. 1987, Shchachkov and Ducreux 1987, Murata et al. 1987, Suga et al. 1990). However, there have been no reports of success at regenerating plants from protoplasts of Group II plants. We have previously developed conditions for plant regeneration in tissue cultures of *I. triloba* L. belonging to Group II (Liu et al. 1990). In the present study we succeeded at regenerating plants from *I. triloba* L. protoplasts at a high frequency.

**Materials and Methods**

*Plant material*

In *vitro*-grown plants of *I. triloba* L. (K121) were prepared as previously described (Liu et al. 1990).
Protoplast isolation

The stems and petioles (approx. 1g fresh weight) of three-week-old plants were cut into thin slices with a surgical blade and then incubated in 10ml enzyme solution consisting of 0.2% (w/v) Macerozyme R-10, 0.4% (w/v) Cellulase ONOZUKA R-10 (Yakult HONSHA Co. Ltd., Japan), 0.6M D-mannitol, 0.5% (w/v) CaCl$_2$·2H$_2$O, and 5.0mM 2-(N-morpholino) ethanesulfonic acid (MES), pH 5.8. The tissues were digested for 16h in the dark at 27°C. The mixture was filtered through a stainless steel sieve of 0.4mm mesh. The filtrate was layered onto 20% sucrose solution and centrifuged at 350xg for 10min. The purified protoplasts were collected with a Pasteur pipette, suspended in W5 salt solution (Negretti et al. 1986, pH 5.8), and centrifuged at 200xg for 4min. This wash sequence was replicated twice. Subsequently, the protoplasts were resuspended in the protoplast medium and centrifuged at 200xg for 4min.

Protoplast culture

Protoplasts were cultured in ø60mm petri dishes containing 2.5ml modified liquid MS medium (mMS), which was composed of 1/2MS inorganic salts, MS vitamins, 0.5mg/l folic acid, 0.05mg/l biotin, 50.0mg/l casein hydrolysate, 0.6M D-mannitol, 1.0% (w/v) sucrose, 0.5mg/l 2,4-D and 1.0mg/l kinetin (pH 5.8), at a density of 1·2·10$^4$ protoplasts/ml. The petri dishes were sealed with parafilm and incubated in the dark at 27°C. After 4 weeks the cultures were continued to incubate in mMS in which D-mannitol was reduced to 0.3M and sucrose was increased to 2.0% (w/v) for an additional 4 weeks in the dark at 27°C. And then the formed colonies/calli (0.7~1.0mm in diameter) were transferred into liquid MS medium containing 0.5mg/l 2,4-D, 1.0mg/l kinetin and 3.0% (w/v) sucrose and cultured for 4 weeks in the dark at 27°C.

Plant regeneration

Twelve weeks after initiation of suspension culture, protoplast-derived calli up to about 2mm in diameter were transferred to ø60mm petri dishes containing MS medium supplemented with either only 0.2mg/l 2,4-D or 0.5mg/l 2,4-D+1.0mg/l kinetin, 3.0% (w/v) sucrose and 0.8% (w/v) agar, pH5.8, for the proliferation. The petri dishes were kept for 3 weeks in the dark at 27±1°C. The obtained calli (6~8mm in diameter) were then transferred onto MS medium supplemented with IAA (0, 0.1; mg/l), BAP (1.0, 2.0, 5.0; mg/l), 3.0% (w/v) sucrose and 0.8% (w/v) agar, pH5.8 (regeneration medium) and cultured under 13h day-light at 3000lux and 27±1°C. All calli cultured on the regeneration medium for 6 weeks were further transferred onto MS medium without plant growth regulators under 13h day-light at 3000lux and 27±1°C. The shoots were regenerated from protoplast-derived calli. Regenerated shoots were transferred onto fresh MS medium without plant growth regulators to promote root development. Regenerated plantlets were then transplanted in pots with vermiculite at a high humidity and grew to maturity.

Results and Discussion

Protoplast culture and callus formation
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Protoplasts were obtained from *in vitro*-grown plants of *I. triloba* L. and cultured in mMS (Fig. 1A). In general, the culture conditions used in this study were effective to cell divisions and callus formation. First cell division occurred within 3-4 days (Fig. 1B). Two weeks after initiation of suspension culture, protoplast plating efficiencies up to about 60% were obtained. Colonies derived from protoplasts were easily seen with the unaided eye and up to 0.3–0.5 mm in diameter after 4 weeks (Fig. 1C), and further developed into small calli (Fig. 1D). In this study, a high callus formation frequency was achieved (Fig. 1E). After twelve weeks, protoplast-derived calli were up to about 2 mm in diameter.

*Callus proliferation and shoot regeneration*

Twelve weeks after initiation of suspension culture, the transference of protoplast-derived calli onto MS medium containing either only 0.2 mg/l 2,4-D or 0.5 mg/l 2,4-D+1.0 mg/l kinetin resulted in rapid callus proliferation. The similar results were obtained on the two proliferation media. Three weeks after transference, the calli were 6–8 mm in diameter, white in colour and friable in texture (Fig. 1F). These protoplast-derived calli were similar to those obtained from tissue cultures of *I. triloba* L. in our earlier study (Lu et al. 1990).

Subsequently, the calli were transferred onto the regeneration medium supplemented with IAA and BAP, and after 2 weeks started to form adventitious roots. After 6 weeks the formation frequency of adventitious roots reached 100% on all regeneration media used.

The calli with adventitious roots were further transferred onto MS medium without plant growth regulators and after 5 days started to regenerate shoots (Fig. 1G). Five weeks after transference, shoot regeneration from protoplast-derived calli was shown in Table 1. It was obvious that the regeneration efficiencies were markedly different due to the differences of the concentrations of 2,4-D and kinetin included in the proliferation medium. When the proliferation medium only contained 0.2 mg/l 2,4-D, the regeneration frequencies of protoplast-derived calli ranged from 27.5% to 36.7%, the highest of which (36.7%) was obtained from the calli that had been cultured on the regeneration medium supplemented with 2.0 mg/l BAP; the regeneration frequencies of shoots exceeded 60.0% (from 60.0% to 96.2%).

The much lower regeneration efficiencies were obtained from the calli which had been cultured on the proliferation medium contained 0.5 mg/l 2,4-D and 1.0 mg/l kinetin. Therefore, 2,4-D and its concentrations included in the proliferation medium played a very important role in promoting shoot regeneration. The concentrations of IAA and BAP included in the regeneration medium have also effects on the shoot regeneration.

Murata *et al.* (1987) and both Shachakr and Ducieux (1987) obtained plant regeneration from sweet potato protoplasts at a very low frequency. They emphasized the role of abscisic acid (ABA) and the role of zeatin in the plant regeneration, respectively. And in this study, using the culture media and conditions described as above, the calli were efficiently derived from *I. triloba* L. protoplasts and then plants were easily regenerated from these protoplast-derived calli at a high frequency. Therefore, it is possible that genotypes significantly influence plant regeneration in protoplast cultures of sweet potato and its related species. The culture media and conditions have also effects on the plant regeneration.
Fig. 1. Plant regeneration from *I. triloba* L. protoplasts. A: Freshly isolated protoplasts (about 27 μm in diameter). B: First cell division within 3–4 days. C: Colonies derived from protoplasts after four weeks (0.3–0.5 mm in diameter). D: Small callus derived from a single protoplast after seven weeks (about 1 mm in diameter). E: Calli obtained from protoplasts at a high frequency (1.0–2.0 mm in diameter). F: Protoplast-derived calli rapidly proliferating on the proliferation medium (6–8 mm in diameter). G: Shoot regenerated from a protoplast-derived callus. H: Plantlet developed from the regenerated shoots. I: Maturing plants regenerated from a protoplast-derived callus in a pot with vermiculite.
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<th>Table 1. Shoot regeneration from protoplast-derived calli of <em>I. triloba</em> L. on MS medium without plant growth regulators.</th>
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<td>Proliferation medium</td>
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Development and domestication of regenerated plants

Regenerated shoots produced white roots and developed into whole plantlets after they were transferred onto fresh MS medium without plant growth regulators (Fig. 1H). Regenerated plantlets with roots were transplanted in pots with vermiculite at a high humidity and vigorously grew to maturity (Fig. 1I).

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Literature Cited


Ipomoea triflora L. のプロトプラストからの植物体再生

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Ipomoea 属 batatas 節植物はサツマイモ (I. batatas (L.) LAM.) との交雑親和性によって二つに分けられる。第II群に属する近縁野生種はサツマイモと交雑不可能なためサツマイモ育種に利用されていない。この交雑不親和性を克服するために体細胞融合法を利用する場合、プロトプラストからの植物体再生条件を確立しておくことは極めて重要である。これまで第I群植物のプロトプラストからの植物体再生は報告されているが、第II群植物のプロトプラストからの植物体再生はまだ報告されていない。本研究では、第II群に属する2倍体の近縁野生種 I. triflora L. のプロトプラストからの植物体再生に成功した。

I. triflora L.の無菌植物を供試材料とした。培養3週目の無菌植物の若い茎及び葉柄（約12生重）を細切し、0.2%マセロライム R-10、0.4%セルラーゼオカリナ R-10、0.6 M D-マンニトール、0.5%CaCl2・2 H2O 及び 5.0 mM MES を含む 10 ml 酵素溶液 (pH 5.8) で、27℃、暗黙下で 16 時間処理した。処理後 0.4 mm 網目のステンレス製で通過し、20%ジオ糖液に懸濁し、300 xg、10 分間遠心をかけた。懸濁したプロトプラストを W2 液で 2 回、その後プロトプラスト培養培地で 1 回。200 xg、4 分間遠心をかけて洗浄した。 (Fig. 1 A)

プロトプラストの培養は、1×10^4 個/ml の密度で、50.0 mg/l カゼイン、0.6 M D-マンニトール、1.0%ジオ糖、0.5 mg/l 2,4-D 及び 1.0 mg/l kinetin を含む修正 MS 液体培地 (pH 5.8) で、27℃、暗黙下で行った。培養 3-4 日目に最初の細胞分裂が見られた (Fig. 1 B)

培養 2 週後に観察効率は約 60% であった。培養 4 週後に形成したコロニー(Fig. 1 C)を 50.0 mg/l カゼイン、0.3 M D-マンニトール、2.0%ジオ糖、0.5 mg/l 2,4-D 及び 1.0 mg/l kinetin を含む修正 MS 液体培地で、その後 0.5 mg/l 2,4-D、1.0 mg/l kinetin 及び 3.0%ジオ糖を含む MS 液体培地で、27℃、暗黙下で、それぞれ 4 週間培養した。カルスは高効率で形成された (Fig. 1 D, E).

培養 12 週後にカルスを 0.2 mg/l 2,4-D 及び 0.5 mg/l 2,4-D+1.0 mg/l kinetin を添加した MS 固体培地に移殖し、27±1℃、暗黙下で 3 週間培養した結果、カルスは急速に増殖し、6-8 mm となった (Fig. 1 F)。カルスをさらに 0-0.1 mg/l IAA 及び 1.0-5.0 mg/l BAP を添加した MS 培地に移植し、27±1℃、3000 lux、13 時間照明下で 6 週間培養した。これから全ての培地中で 100% のカルスから不定根の形成が認められた。不定根を形成したカルスをさらに植物生長調節物質を含まない MS 培地に移植し、27±1℃、3000 lux、13 時間照明下で培養した結果、カルスから多数の苗条再生が見られた (Table 1, Fig. 1 G). 増殖培地に添加した 2,4-D 及び kinetin の濃度は再生効率に著しく影響を与え、0.2 mg/l 2,4-D を添加した場合、カルスの最高再生率は 36.7% となった (Table 1). 再生培地に添加した IAA 及び BAP の濃度も再生効率に影響があった。

再生した苗条を植物生長調節物質を含まない新鮮な MS 培地に再移植し、苗条は発根し健全な幼植物体となった (Fig. 1 H). これらの幼植物体をバーミキュライトを入れた鉢に移植し、乾燥個体を得た (Fig. 1 I).