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Tissue Culture Note

Efficient plant regeneration from protoplasts of eggplant rootstock cultivar and its wild relatives

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Abstract Protoplast culture and shoot regeneration from protoplast-derived calli were compared among different organs of *Solanum integrifolium* and three of its wild relatives (*S. abutiloides*, *S. scabrum*, and *S. toxicarium*). Leaves, cotyledons, and hypocotyls were used as sources of protoplast preparation. After one month of culture, a high frequency of visible colony formation was obtained from cotyledon protoplasts of *S. integrifolium* and *S. scabrum*, hypocotyl protoplasts of *S. integrifolium* and *S. abutiloides*, and leaf protoplasts of *S. integrifolium* and *S. toxicarium*. In addition, when the primary culture was started at a density of $2.5-5\times10^4$ protoplasts ml^{-1}, the highest frequency of colony formation was obtained. Moreover, when the colonies were subcultured for 7 days on solid callus-proliferation medium before being transferred to shoot-induction medium, the plant regeneration frequency improved to between 91.8 and 98.8%.

Key words: Eggplant rootstock, plant regeneration, protoplast culture, wild relatives.

Eggplant (*Solanum melongena*) is widely cultivated in tropical and temperate regions worldwide (Kallo 1993); however, in certain production areas it is susceptible to bacterial wilt caused by *Ralstonia solanacearum*, *Fusarium* wilt due to *Fusarium oxysporum*, and *Verticillium* wilt attributable to *Verticillium dahliae*. In Japan, grafting is the most popular technique for avoiding soil-borne diseases in eggplants. Among the rootstock species, *S. integrifolium*, which is vigorous until the later stages of eggplant production, is resistant to *Fusarium* wilt and is the most common rootstock in Japan (Tachibana 1994). Although this species was reported to be somewhat resistant to bacterial wilt when compared to eggplant cultivars (Sheela et al. 1984, Ozaki and Kimura 1989), scions on this rootstock are severely damaged by the pathogen.

Three wild species (*S. abutiloides*, *S. scabrum*, and *S. toxicarium*) are highly resistant to bacterial wilt and *Verticillium* wilt (Mochizuki and Yamakawa 1979; Sakata 1991), but they are not currently used as rootstocks because of their disadvantages, including graft incompatibility, thin stems, and slow growth (Sakata 1991).

Somatic hybridization is a technique of incorporating desirable genes from related species into cultivated crops (Evans et al. 1981; Guri and Sink 1988; Sihachakr et al. 1989; Puite 1992; Rizza et al. 2002; Tamura et al. 2002). Before such technology can be widely applied to *S. integrifolium*, however, it is necessary to develop an efficient plant regeneration system for protoplasts of *S. integrifolium* and its wild relatives that are resistant to wilt diseases.

In this report, we describe an efficient protocol for plant regeneration via protoplast culture from several explants of *S. integrifolium* and its wild relatives.

In vitro grown seedlings and *in vitro* shoot cultures were obtained and maintained as previously described (Iwamoto 1999). For protoplasts, tissue was taken from hypocotyls and cotyledons of two- to three-week-old seedlings and fully expanded leaves of three-week-old plantlets after subculture.

All media used for protoplast isolation and culture were adjusted to pH 5.6. MS medium (Murashige and Skoog 1962) was modified and used as a basic culture medium. The concentration of the inorganic salts and vitamins was changed to 1/2, and NH_4NO_3 was used at 200 mg l^{-1} (modified 1/2MS). The cotyledons, hypocotyls, and leaves were slightly scarified and then transferred into modified 1/2MS plus a filtrated enzyme solution (0.5 M mannitol, 0.05–0.1% (w/v) Macerozyme R-10 (Yakult Co., Tokyo, Japan), 0.25–0.5% (w/v) Meicelase P-1 (Meiji Seika Co., Tokyo, Japan), and 10 g l^{-1} sucrose). The explants cut into the fragments were incubated overnight at 25°C in the dark. At the end of the digestion period, the mixture was passed through a nylon sieve (50 μm mesh), washed twice with two

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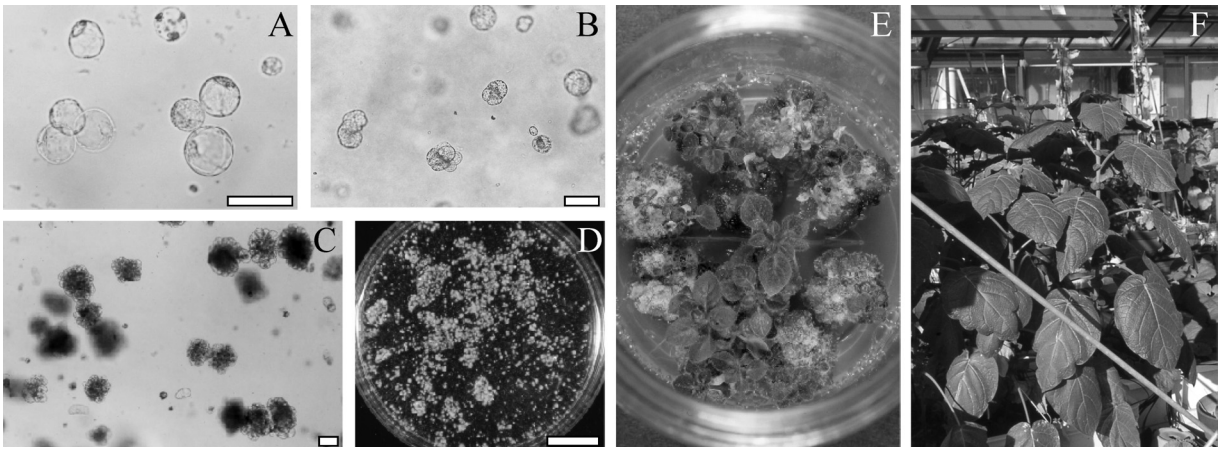

Figure 1. Plant regeneration from protoplasts of *Solanum abutiloides*. Protoplasts were cultured in medium A. (A) Protoplasts isolated from hypocotyls. Bar=50 μm. (B) Protoplast division after 5 days of culture. Bar=100 μm. (C) Colony formation after 2 weeks of culture. Bar=100 μm. (D) Protoplasts derived callus after one month of culture. Bar=2 cm. (E) Shoots regenerated from protoplast-derived callus on medium F. (F) A regenerated whole plant grown in a green house.

Table 1. Composition of culture media for protoplasts of *Solanum* species.

Contents	Culture media						
	A[1]	B[2]	C[3]	D[4]	E[5]	F[5]	G[6]
Basal medium	1/2 MS	1/2 MS	1/2 MS	1/2 MS	MS	MS	1/2 MS
(NH_4NO_3 mg l^{-1})	200	200	1650	1650	1650	1650	825
Mannitol (M)	0.4	0.2	–	–	–	–	–
2,4-D (mg l^{-1})	1.0	0.1	–	0.1	–	–	–
NAA (mg l^{-1})	1.0	0.1	–	0.1	–	–	–
Kinetin (mg l^{-1})	1.0	1.0	1.0	1.0	–	–	–
IAA (mg l^{-1})	–	–	–	–	0.1	–	–
Zeatin (mg l^{-1})	–	–	–	–	2.0	0.2	–
Sucrose (%)	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Gerlite (%)	0.2	–	–	0.2	–	–	–
Agar (%)	–	–	–	–	0.7	0.7	0.7

All kinds of media were autoclaved at 121°C for 15 min.

[1] The protoplast cultures had an initial density of 10×10^5 protoplasts ml^{-1} in solid medium A.

[2] Two ml of liquid medium B was added after one week of culture.

[3] After two weeks of culture, four ml of liquid medium C was added, and colonies and media transferred to new Petri dish (90×15 mm).

[4] Medium D for calli proliferation was used experiment of Table 4 before transfer to medium E or F.

[5] After 1 month of culture, visible calli were transferred to either medium E (*S. integrifolium*, *S. scabrum*, and *S. toxicarium*) or medium F (*S. abutiloides*).

[6] Regenerated shoots excised from calli were transferred to medium G for rooting.

volumes of W5 solution (Menczel et al. 1981), and recovered by centrifugation at 100 *g* for 3 min (As shown in Figure 1A).

The protoplast cultures had an initial density of 1×10^5 protoplasts ml^{-1} in medium A (Table 1). Plastic Petri dishes (60×15 mm) containing 2 ml of medium each were used for protoplast culture, and were incubated 16 h per day under dim light (10 $\mu E\,m^{-2}\,s^{-1}$) at 25°C for 7 days.

For *S. abutiloides*, *S. integrifolium*, and *S. scabrum*, the protoplasts isolated from all three organs began to divide after 4–7 days of culture (As shown in Figure 1B); however, in *S. toxicarium*, only the leaf protoplasts formed colonies, because its seedlings grew terrifically slow. The protoplasts sustained cell division, and cell proliferation was promoted by the addition of 2 ml of fresh medium B (after 1 week of culture) and 4 ml of medium C (after 2 weeks of culture) (Table 1, As shown in Figure 1C). After 2 weeks of culture, colonies and media were transferred to new Petri dishes (90×15 mm). After 1 month of culture, a relatively high frequency of visible calli formation was observed in cotyledon-derived protoplasts of *S. integrifolium* and *S. scabrum*, hypocotyl-derived protoplasts of *S. integrifolium* and *S. abutiloides*, and leaf-derived protoplasts of *S. integrifolium* and *S. toxicarium* (Table 2). Visible calli (ca. 0.5–1.0 mm in diameter; As shown in Figure 1D) were transferred to either medium E (*S. integrifolium*, *S. scabrum*, and *S. toxicarium*) or medium F (*S. abutiloides*) (Table 1) for shoot regeneration under

Table 2. Differences in colony formation and shoot regeneration among different sources for protoplast isolation in four *Solanum* species.

Solanum species	Protoplast source	Colony(%)[1]	Shoot regeneration(%)[3]
S. abutiloides	Leaves	1.12±0.29[b2]	0.5±0.5[c]
	Hypocotyls	2.38±0.22[a]	34.8±3.8[a]
	Cotyledons	1.39±0.31[b]	8.6±1.1[b]
S. integrifolium	Leaves	3.13±0.94[a]	19.4±4.3[b]
	Hypocotyls	2.97±0.32[a]	30.4±4.9[a]
	Cotyledons	3.29±0.42[a]	34.2±6.1[a]
S. scabrum	Leaves	0.05±0.02[c]	0.0±0.0[b]
	Hypocotyls	0.37±0.14[b]	0.6±0.9[b]
	Cotyledons	0.89±0.25[a]	8.4±3.0[a]
S. toxicarium	Leaves	1.37±0.28	45.2±5.7

[1] For examining frequencies of colony formation, each experiments consisted of five culture dishes in which 10×10^4 protoplasts ml^{-1} were plated. Percentage of plated protoplasts which developed into visible calli (ca. 0.5–1 mm in diameter) after 1 month of culture.

[2] Within the same species, mean±S.D. followed by different letters (a, b and c) indicate significant differences at 5% level by Tukey test.

[3] Percentage of protoplast-derived calli which regenerated shoots by 2 months after transfer to medium E or F. Values represent the mean±S.D. of five independent experiments each of which consisted of at least 100 protoplast-derived calli produced after 1 month of culture.

Table 3. Effect of plating concentrations on colony formation and shoot regeneration of four *Solanum* species.

Solanum species	Protoplast source	Plating Conc. ($\times10^4$)	Colony[1] (%)	Shoot regeneration[3] (%)
S. abutiloides	Hypocotyls	2.5	4.07±0.36[a2]	57.8±14.4[a]
		5.0	4.99±0.80[a]	59.0± 6.7[a]
		10.0	2.38±0.22[b]	34.8± 3.8[b]
S. integrifolium	Cotyledons	2.5	5.44±0.76[a]	40.6± 8.1[a]
		5.0	5.63±0.35[a]	44.8± 5.0[a]
		10.0	3.29±0.42[b]	37.0± 4.5[a]
S. scabrum	Cotyledons	2.5	1.18±0.21[ab]	28.6± 6.3[a]
		5.0	1.40±0.37[a]	34.6±13.6[a]
		10.0	0.89±0.25[b]	8.4± 3.0[b]
S. toxicarium	Leaves	2.5	4.35±0.80[a]	73.2±13.1[a]
		5.0	4.30±0.56[a]	75.6±14.0[a]
		10.0	1.37±0.28[b]	45.2± 5.7[b]

[1] For examining frequencies of colony formation, each experiments consisted of five culture dishes in which $2.5–10\times10^4$ protoplasts ml^{-1} were plated. Percentage of plated protoplasts which developed into visible calli (ca.0.5–1 mm in diameter) after 1 month of culture.

[2] Within the same species, mean±S.D. followed by different letters (a, b and c) indicate significant differences at 5% level by Tukey test.

[3] Percentage of protoplast-derived calli which regenerated shoots by 2 months after transfer to medium E or F. Values represent the mean±S.D. of five independent experiments each of which consisted of at least 100 protoplast-derived calli produced after 1 month of culture.

fluorescent light ($60\,\mu E\,m^{-2}\,s^{-1}$) for 16 h per day at 25°C. After 8 weeks, adventitious shoots were regenerated from green calli (As shown in Figure 1E).

The shoot regeneration frequency in cotyledon protoplasts of *S. integrifolium* and *S. scabrum* was higher than that of leaf or hypocotyl protoplasts. However, in *S. abutiloides*, the shoot regeneration frequency of hypocotyl protoplasts was higher than that of leaves or cotyledons. In *S. toxicarium*, only leaf protoplasts were tested and they showed a high shoot regeneration frequency. Shoot regeneration frequency was significantly different among the organs used as sources of protoplast preparation in the four species (Table 2).

In *S. scabrum* and *S. abutiloides*, 0–0.5% of calli derived from leaf protoplasts regenerated shoots up to 2 months after transfer to medium E or medium F. However, a relatively high frequency of shoot regeneration was obtained from the other two protoplast sources of the four *Solanum* species (Table 2).

In addition, when the primary culture was begun at a density of $2.5–5\times10^4$ protoplasts ml^{-1}, the highest frequencies of colony formation and shoot regeneration were obtained from protoplasts separated from optimum explants (Table 3).

Moreover, duration of callus proliferation on medium D was very important for shoot regeneration. When visible colonies (ca. 0.5–1.0 mm in diameter) were subcultured for 7 days on medium D for callus proliferation before being transferred to medium E or medium F, callus color changed slightly yellow green, and the regeneration frequency improved to between 91.8 and 98.8% (Table 4). However, when visible colonies of *S. abutiloides*, *S. integrifolium*, and *S. scabrum* were subcultured for 14 days on medium D, the

Table 4. Effect of callus proliferation period on shoot regeneration from protoplast derived calli of four *Solanum* species.

Solanum species	Protoplast source	callus proliferation period (day)		
		0	7	14
S. abutiloides	Hypocotyls	59.0±6.7[b123]	98.0±2.3[a]	35.2±10.0[c]
S. integrifolium	Cotyledons	44.8±5.0[c]	92.0±6.9[a]	65.4±12.6[b]
S. scabrum	Cotyledons	34.6±13.6[c]	91.8±7.9[a]	73.4±10.0[b]
S. toxicarium	Leaves	75.6±14.0[b]	98.8±1.8[a]	96.8±5.2[a]

[1] For examining frequencies of regeneration, calli which was begun to culture at a density of 5×10^4 protoplasts ml^{-1}, were used.
[2] Within the same species, mean±S.D. followed by different letters (a, b and c) indicate of significant differences at 5% level by Tukey test.
[3] Percentage of protoplast-derived calli which regenerated shoots by 2 months after transfer to medium E or F. Values represent the mean ±S.D. of five independent experiments each of which consisited of at least 100 protoplast-derived calli.

callus proliferated friable, and the regeneration frequency improved only 35.2–73.4%.

The first successful regeneration of plants from *S. melongena* protoplasts was reported by Bhatt and Fassuliotis (1981) and Saxena et al. (1981). Subsequently, several successful examples were reported in eggplant rootstock cultivars and their wild relatives, including *S. torvum* (Guri et al. 1987), *S. integrifolium* (Asao et al. 1989), *S. sanitwongsei* (Asao et al. 1989), *S. toxicarium* (Sadohara et al. 1993), and *S. abutiloides* (Kondo 2002); however, the frequency of plant regeneration was low. A previous report on *S. toxicarium* (Sadohara et al. 1993) showed a plant regeneration frequency per protoplast of 0.04%. Kondo (2002) reported that both *in vitro* shoot culture conditions and enzymatic treatment conditions affected the frequency of plant regeneration from protoplasts in three *Solanum* species, and their frequencies per protoplast of *S. sanitwongsei*, *S. abutiloides*, and *S. toxicarium* were 0.09, 0.14, and 0.07%, respectively.

The results of this study indicate that the explants used as the protoplast source, the density of the protoplasts in the primary culture, and the duration of callus proliferation significantly affect the frequency of plant regeneration in four *Solanum* species. The regeneration frequencies of *S. abutiloides*, *S. integrifolium*, *S. scabrum*, and *S. toxicarium* were improved to 4.89, 5.18, 1.29 and 4.25%, respectively. Furthermore, this is the first report to describe the regeneration of plants from protoplasts of *S. scabrum*.

Most of the regenerated shoots developed roots following excision from the calli and transfer to plant growth-regulator-free medium G. No abnormal shoots were observed in the protoplast-derived plantlets from our four *Solanum* species, and the plantlets were successfully transferred to the greenhouse after acclimatization (As shown in Figure 1F).

In conclusion, we have developed an efficient protocol for protoplast regeneration using *S. abutiloides*, *S. integrifolium*, *S. scabrum*, and *S. toxicarium*. The efficient plant regeneration system established here should prove useful in the production of somatic hybrids through cell fusion and gene transfer.

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