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Plant Regeneration from Mesophyll Protoplasts Culture of *Solanum sisymbriifolium*

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Abstract

The optimal culture conditions were studied for plant regeneration from mesophyll protoplasts of *Solanum sisymbriifolium*. Axenic seedlings of *S. sisymbriifolium* were used as a explant for protoplast culture. Many viable protoplasts were isolated by incubating leaf slices in an enzyme solution containing 0.25% Meicerase and 0.05% Macerozyme for 16 hr at 25℃ without shaking. Protoplast density of 5.0 × 10^4 ml^{-1} in Kao medium containing 5.0 mg/L NAA, 1.0 mg/L 2,4-D and 1.0 mg/L BA was optimal for colony formation. Most colonies were formed when protoplasts were cultured at 25℃ after initial culture at 30℃ for one week. On the MS agar medium with 1.0 mg/L zeatin, 38.4% of protoplast-derived calli differentiated shoots. These shoots rooted on 1/2MS medium with 5.0 g/L sucrose and 2.5 g/L gellan gum, and developed into whole plants.

Key words: *Solanum sisymbriifolium*, temperature, protoplast-derived calli

Introduction

The wild South American species *Solanum sisymbriifolium* has resistance to root-knot nematodes (Fassuliotis and Bhatt 1982), carmine spider mites (Schalk et al. 1975) and a few race of bacterial wilt (Ali et al. 1990). In addition, this species is not susceptible to low temperature, and fruit yield of eggplant grafted on this species is higher than that on eggplant (Ali 1991), However, it is not easy to prepare this species as a rootstock because of existing spines on its stems and leaf veines.

The protoplast culture is an attractive way of rearing a new breed, which is useful to obtain the cybrid from the regeneration of a plant and fusion of protoplast of wide-related or sexually non-affinitive plants and a somatic hybrid, and which can be used to regenerate a plant having a new character by inserting the external genetic materials (e.g., exogenous DNA, virus, bacteria, nitrogen fixing bacterium, nucleus, chloroplast, and mitochondria) into the protoplast. Since the success in isolating the protoplast from the tomatos root tip tissue using cellulase in 1960 (Cocking 1960), many researches have been conducted targeting *Nicotiana* (Nagata and Takebe 1970), *Petunia* (Binding 1974; Seon et al. 1985), *Datura* (Krumbiegel and Shieder 1979), *Daucus* (Grambow et al. 1972), *Brassica* (Gleba and Hoffmann 1980; Jee and Chung 1985), etc. and the scope of applicable plants is expanding as many reports on the redifferentiation from protoplast and somatic hybrid in various plants are released.

This study surveyed the protoplast culturing media and its method, and the media for the plant redifferentiation from callus, so as to establish a system of the plant redifferentiation from the protoplast of *S. sisymbriifolium*.

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Materials and Methods

Plant material

Seeds of *Solanum sisymbriifolium* were sterilized with sodium hypochlorite solution (2% active chlorine) for 20 min and washed three times with sterilized distilled water. They were aseptically sown in 100 mL conical flasks containing 30 mL of MS medium (Murashige and Skoog 1962)

supplemented with 1% sucrose and 0.25% gellan gum, and were incubated in a growth cabinet (light intensity was 30 μE m^{-2} sec^{-1} for 16 hr day^{-1} and the temperature was kept at 25℃ both in the dark and the light). Proliferated leaves of 3-4 week-old plants were used for protoplast production.

Protoplast isolation

The sterile leaves were cut into stripes 1~2 mm in width and placed in 80 × 15 mm petri dishes containing 20 mL enzyme solution which contained 0.4 M mannitol, 0.03 M sucrose, 0.05-0.20% (w/v) Macerozyme and 0.25-1.0% (w/v) Meicerase and adjusted to pH 5.5. The dishes were sealed with parafilm and kept still at 25℃ in the dark. After 4-16 hr incubation, protoplasts were separated from undigested tissues and cells by successive passage through 50 μm nylon filter. They were then washed three times in culture medium by centrifugation (100 × g, 3min). Medium for the protoplast culture was Kao medium (Kao 1997) modified by the addition of 0.4 M mannitol, 0.03 M sucrose, 5.0 mg/L 1-naphthaleneacetic acid (NAA), 1.0 mg/L 6-benzylaminopurine (BA).

The protoplasts were cultured as a thin layer (2 mL/dish) in 60 × 15 mm petri dishes at densities adjusting to 5.0 × 10^4 protoplasts per mL. The dishes were sealed with Parafilm and kept at 30℃ for the first 7 days and thereafter at 25℃ in the dark. Two mL of fresh medium was added to each of culture dishes at 14 day intervals. Protoplast- derived colonies of 0.5-1.0 mm in diameter (5 weeks old) were counted.

Protoplast density

The protoplasts isolated from lamina stripes were incubated for 16 hr at 25℃ in the dark in an enzyme solution containing 0.4 M mannitol, 0.03 M sucrose, 0.05% (w/v) Macerozyme and 0.25% (w/v) Meicerase (pH 5.5). Protoplast density ranged from 0.5 × 10^4 mL^{-1} to 50 × 10^4 mL^{-1}. Protoplast culture and colony count were carried out using the same methods as described in protoplast isolation.

Effect of media

The protoplasts were cultured in the MS and Kao media, which were modified by the additions of 0.4 M mannitol, 0.03 M sucrose, 0-5.0 mg/L NAA, 0-1.0 mg/L 2,4-D and 0-1.0 mg/L BA (Table 1). Protoplast culture and colony count were done following the same methods in protoplast isolation.

Effect of temperature

Protoplasts were cultured at 25 or 30℃ for the first 7 days and thereafter at 25 or 30℃ in the dark. Protoplast culture and colony count were held in the same manners as described in protoplast isolation.

Plant regeneration

Protoplast-derived calli of 1-3 mm in diameter (after 8 weeks of culture) were transferred to 100 mL conical flasks containing 30 mL of regeneration medium ; MS medium supplemented with 30 g/L sucrose, 7.0 g/L agar, 0.1 mg/L indole-3-acetic acid (IAA), and BA, kinetin or zeatin of various concentrations (1.0, 3.0, or 5.0 mg/L). The pH was adjusted to. 5.5. The calli were incubated in the growth cabinet at 25℃ with 16 hr day^{-1} illumination (14.2 μE m^{-2} sec^{-1}). After 4 weeks of culture, regenerated plants were transferred for rooting on MS medium supplemented with 5.0 g/L sucrose and 2.5 g/L gellan gum without any plant hormones.

Table 1. Components of media used for protoplast culture.

Medium	Mineral salts	Organic components	NAA (mg/L)	2,4-D (mg/L)	BA (mg/L)
A 1-18	1/2 MS	MS	0.1, 0.5, 1.0	0, 0.1	0.1, 0.5, 1.0
B 1-3	1/2 MS	MS	1.0, 3.0, 5.0	1.0	1.0
C 1-18	Kao	Kao	0.1, 0.5, 1.0	0, 0.1	0.1, 0.5, 1.0
D	Kao	Kao	1.0	1.0	1.0
E	Kao	Kao	3.0	1.0	1.0
F	Kao	Kao	5.0	1.0	1.0

Results

Protoplast isolation

The results of enzyme treatment for protoplast isolation of *S. sisymbriifolium* are summarized in Table 2. Cell division was not observed in high concentration enzymes, long period incubation and shaking. Incubation in enzyme solution composed of 0.25% Meiserase and 0.05% Macerozyme for 16 hr gave the best yields of viable protoplasts (Fig. 1). Protoplast division often started after 2 days of culture (Fig. 2) and colony formation was also observed (Fig. 3).

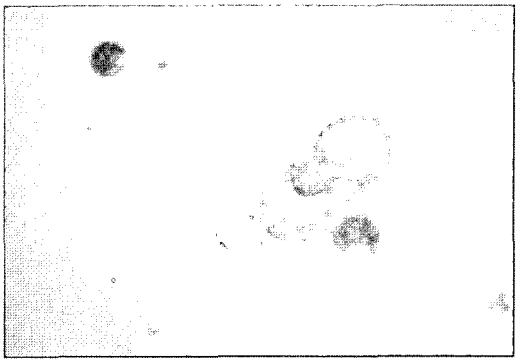

Figure 2. Cell division of *S. sisymbriifolium* (2 days after culture).

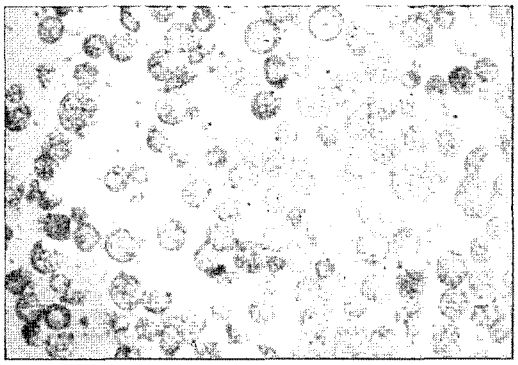

Figure 1. Mesophyll protoplasts of *S. sisymbriifolium*.

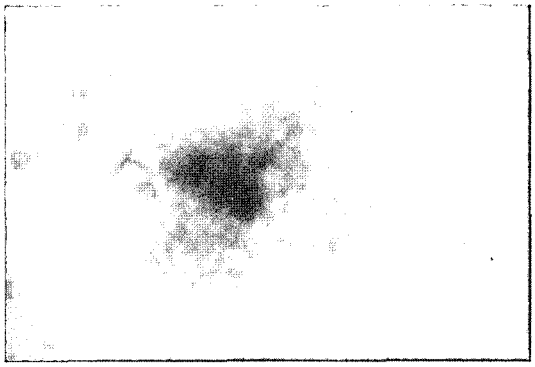

Figure 3. Cell colony of *S. sisymbriifolium* (5 weeks after culture)

Table 2. Effects of enzyme treatment on protoplast yield and colony formation in protoplast culture of *S. sisymbriifolium*.

Meicerase (%)	Macerozyme (%)	Treatment	Period (hr)	Yield ($\times 10^5$ ml^{-1})	Cell division[z]	No. of colonies
0.25	0.05	No shaking	8	0.2	+ (2)[y]	0
			16	7.2	+ (2)	20
			24	7.5	-	-
		Shaking	4	0.2	-	-
			8	0	-	-
1.00	0.20	No shaking	8	1.1	-	-
			16	7.7	-	-
			24	7.7	-	-
		Shaking	4	0.2	-	-
			8	0	-	-

Medium: F (see to Table 1)

[z]Volume of protoplast suspension = 6.0 ml, protoplast density = $5.0 \times 10^4 \cdot$ ml^{-1}, + : cell division, -: no cell division.

[y]Days to first cell division.

Protoplast density

Number of colonies formed after 5 weeks of culture is shown Fig. 4. Cell division was not observed in the protoplasts with low density (1.0×10^4 mL^{-1}). In high density (5.0×10^4 mL^{-1}), protoplasts started to divide after 2 days of culture but didn't form any colonies. Optimal culture density was found to be 5.0×10^4 protoplasts per mL (Fig. 4).

Effect of culture conditions

A variety of media (A-F) were compared for their ability to promote cell division and colony formation (Table 3). When the protoplasts were cultured in medium A, B and C, cell division started within 4 days but colony formation was not observed. In media D, E and F, cell division started after 2 days and thereafter colony formation was observed. The medium to optimize the protoplast division and colony formation was found to be medium F.

Effect of temperature

Number of colonies formed after 5 weeks of culture was indicated in Table 4. Cell division of the protoplasts was more rapid at 30℃ than at 25℃ for the first 7 days. The protoplasts did not form colonies at 30℃ but at 25℃ after 7 days until 5 weeks of culture (Table 4). The temperature for the optimal protoplast division and colony formation was judged to be 30℃ for the first 7 days and then at 25℃.

Plant regeneration

A variety of cytokinins were compared for their ability to

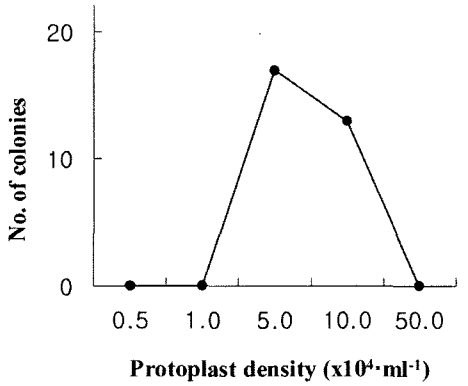

Figure 4. Effect of protoplast density on colony formation in protoplast culture of *Solanum sisymbriilium*.

Table 3. Effect of media on colony formation in protoplast culture of *S. sisymbriifolium*.

Medium	Cell division[z]	No. of colonies
A	+ (4)[y]	0
B	+ (4)	0
C	+ (2)	0
D	+ (2)	3
E	+ (2)	8
F	+ (2)	17

A-F; see Table 1.
[z]Volume of protoplast suspension = 6.0 ml, protoplast density = $5.0 \times 10^4 \cdot ml^{-1}$, +: cell division.
[y]Days to first cell division.

Table 4. Effect of temperature on colony formation in protoplast culture of *S. sisymbriifolium*.

Temperature (℃)		Cell division[z]	No. of colonies
0-1 weeks	1-5 weeks		
25	25	+ (2-3)[y]	9
25	30	+ (2-3)	0
30	25	+ (2)	22
30	30	+ (2)	0

Medium: F (see Table 1)
[z]Volume of protoplast suspension = 6.0 ml, protoplast density = $5.0 \times 10^4 \cdot ml^{-1}$, +: cell division.
[y]Days to first cell division.

Table 5. Effect of cytokinins on shoot formation of *S. sisymbriifolium*.

Cytokinin	Concentration (mg/L)	No. of calli	
		Cultured	Forming shoots
BA	1.0	52	0 (0)[z]
	3.0	52	0 (0)
	5.0	52	0 (0)
Kinetin	1.0	52	8 (15.4)
	3.0	52	15 (28.8)
	5.0	52	10 (19.2)
Zeatin	1.0	52	20 (38.5)
	3.0	52	17 (32.7)
	5.0	52	7 (13.5)

[z]Percent calli forming shoots.

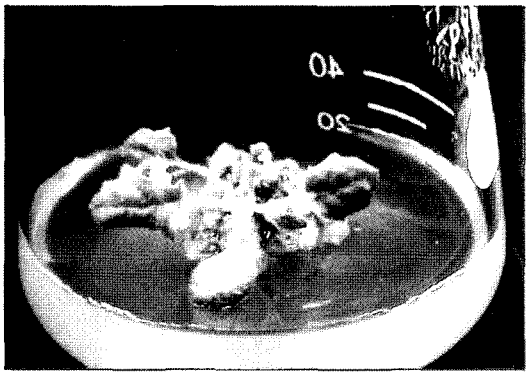

Figure 5. Shoot regeneration from protoplast-derived callus of S. sisymbriifolium 4 weeks after transfer.

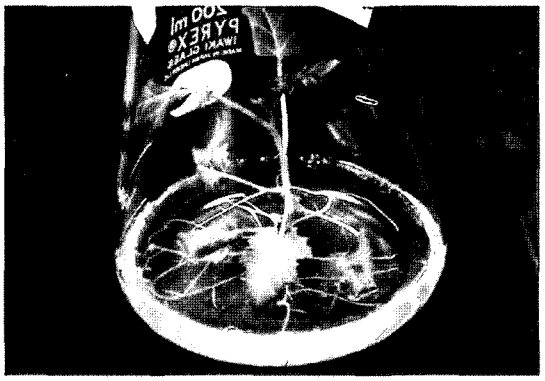

Figure 6. Regenerated plantlet of S. sisymbriifolium.

regenerate shoots from protoplast-derived calli (Table 5). After 4 weeks of culture, shoot formation was observed in calli transferred to medium containing kinetin or zeatin (Fig. 5, Table 5). BA was less effective on shoot formation than other cytokinins, kinetin and zeatin (Table 5). The optimal medium for shoot formation from the protoplast-derived calli was considered to be MS medium supplemented with 0.1 mg/L IAA and 1.0 mg/L zeatin.

Regenerated shoots formed roots, 10 days after placing on rooting medium (Fig. 6), and were transferred to pots containing vermiculite and grown in a greenhouse. Almost all the plants except a few showed normal phenotypes, flowering and good seed set.

Discussion

Protoplast sources are the most important factor to improve culture success in favorable culture environments. *In vitro* conditions from which the best results were obtained provided juvenile and homogeneous materials (Saxena et al. 1981; Sihachakr and Ducreux 1987). In most cases, mesophyll tissue was selected as the source of protoplast culture because of being homogeneous material (leaf age, etc.) and great possibility for high yield of protoplasts. In this study, high yield and division frequency of protoplasts obtained from mesophyll tissues were observed. This division frequency was comparable with that from protoplasts derived from cell suspensions allowing a high capacity for further cell development (Gleddie et al. 1985). The temperature had great influence on cell division and colony formation during initial protoplast culture. Maintaining protoplasts of *S. sisymbriifolium* at 30℃ for more than one week resulted in decrease and loss of cell totipotency. Similar phenomenon was observed in protoplasts of *Solanum* species (Sadohara 1993) and *Licopersicon* species (Zapata et al. 1977). I consider this phenomenon to be a common occurrence during a prolonged period of maintenance at high temperature in *Solanaceae* protoplast culture, though the optimal tem-

perature in protoplast culture varies in each species (Zapata et al. 1977). In this study, zeatin was found to be more effective than BA or kinetin for regenerating shoots from protoplast-derived calli. This result was also reported in other *Solanum* species (Nishio et al. 1987; Asao et al. 1989; Sadohara 1993).

In the present investigation, protoplast culture system of *S. sisymbriifolium* was established. This simple and efficient protoplast culture system is useful to produce somatic hybrids between *S. sisymbriifolium* and other *Solanum* species.

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