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https://doi.org/10.5109/4711

出版情報：九州大学大学院農学研究院紀要. 51 (1), pp.63-66, 2006-02-01. Faculty of Agriculture, Kyushu University
バージョン：
権利関係：

J. Fac. Agr., Kyushu Univ., **51** (1), 63–66 (2006)

Establishment of Protoplast Culture of *Solanum sisymbriifolium*

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(Received October 28, 2005 and accepted November 16, 2005)

The optimal culture conditions were studied for plant regeneration from mesophyll protoplasts of *Solanum sisymbriifolium*. Aseptic seedlings of *S. sisymbriifolium* were used as a material for protoplast culture. Many viable protoplasts were isolated by incubating leaf slices in an enzyme solution containing 0.25% Meiserase and 0.05% Macerozyme for 16 h at 25 °C without shaking. Protoplast density of 5.0×10^4/ml 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 formed when protoplasts were cultured at 25 °C after initial culture at 30 °C for one week. On the MS agar medium with 1.0 mg/l zeatin, 38.5% of protoplast–derived calli differentiated shoots. These shoots rooted on 1/2 MS medium with 5.0 mg/l sucrose and 2.5 g/l gellan gum, and developed into whole plants.

INTRODUCTION

Eggplant (*Solanum melongena* L.) has a wide range of genetic diversity including many wild relatives. The center of its origin is considered to be Indo–Myanmar region (Vavilov, 1926). Eggplant is a very important and popular crop in Asia, and it is easy to grow it in the tropics because of the hardiness to high temperature (Yamaguchi, 1983). Its cultivation has been known for the last 1,500 years in China (Khan, 1979) and for more than 1,200 years in Japan (Asahira, 1989), consequently, East Asia is said to be the secondary center of origin.

Eggplant often suffers from some soil–borne diseases like bacterial wilt (*Pseudomonas solanacearum* E. F. Smith), *Fusarium* wilt (*Fusarium oxysporum, F. melongenae*), *Verticillium* wilt (*Verticillium albo–atrum*) and root–knot nematodes (*Meloidogyne* spp.). Several desirable agronomic traits, such as resistance to these diseases, have been discovered in some wild *Solanum* species (Mochizuki and Yamakawa, 1979; Yamakawa and Mochizuki, 1979; McCammon and Honma, 1983; Ali *et al.*, 1990; Ali *et al.*, 1992). They have been, therefore, used for grafting as a stock plant in eggplant cultivation in order to prevent from these various diseases.

A wild South American species *S. sisymbriifolium* Lam. has resistance to root–knot nematodes (Fassuliotis and Bhatt, 1982), carmine spider mites (Schalk *et al.*, 1975) and a few races of bacterial wilt (Ali *et al.*, 1990). It is, however, not easy to prepare this species as a rootstock because of existing hard and pointed spines on its stems and leaf veins. If the favorable characters of the species can be introduced to eggplant or other relative species currently used as rootstocks in eggplant cultivation, it is possible to produce promising rootstocks. As the cross compatibility between *S. sisymbriifolium* and other *Solanum* species is low, the optimal rootstock has not been produced until now (Nishio *et al.*, 1984; Ali and Fujieda, 1990; Ali, 1991).

Somatic hybridization is a technique that allows the manipulation of genomes by protoplast fusion. This may serve as a method of transferring desirable characters, even if the combination between species is sexually incompatible. This investigation was carried out to find out the optimal conditions for isolation and culture of protoplast and regeneration from protoplasts to establish the basis of somatic hybridization in *S. sisymbriifolium*.

MATERIALS AND METHODS

Plant material

Seeds of *S. 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 where the light intensity was 30 μE/m^2·s for 16 h/day and the temperature was kept at 25 °C. Proliferated leaves of 3–4 week–old plants were used for protoplast production.

Protoplast isolation

The sterile leaves were cut into strips of 1–2 mm in width and placed in 80×15 mm petri dishes containing 20 ml enzyme solution consisted of 0.4 M mannitol, 0.03 M sucrose, 0.05–0.20% (w/v) Macerozyme and

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0.25–1.0% (w/v) Meicerase (pH 5.5). The dishes were sealed with Parafilm® and kept on a reciprocating shaker (30 strokes/min) or kept still at 25 °C in the dark. After 4–16 h incubation, protoplasts were separated from undigested tissues and cells by successive passage through 50 μm mesh nylon filter. They were then washed three times in culture medium by centrifugation (100×g, 3 min). Medium for the protoplast culture was Kao medium (Kao, 1977) 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 2,4–dichlorophenoxyacetic acid (2,4–D) and 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/ml. The dishes were sealed with Parafilm® and kept at 30 °C for the first seven days and at 25 °C thereafter in the dark. Two ml of fresh medium were added to each of culture dishes with 14 days intervals. Protoplast–derived colonies of 0.5–1.0 mm in diameter (five weeks old) were counted.

Protoplast density

The protoplasts isolated from lamina strips were incubated for 16 h at 25 °C 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 to 50×10^4 /ml. Protoplast culture and colony count were carried out using the same methods as described in the previous section.

Effect of media

The protoplasts were cultured in the 1/2MS and Kao media, both modified by the additions of 0.4 M mannitol, 0.03 M sucrose, 0.1–5.0 mg/l NAA, 0–1.0 mg/l 2,4–D and 0.1–1.0 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 °C for the first seven days and at 25 or 30 °C thereafter 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 eight 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 °C with 16 h/day illumination (14.2 μE/m^2·s). After four 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 hormone.

RESULTS

Protoplast isolation

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

Protoplast density

Number of colonies formed after five weeks of culture is shown in Fig. 1. Cell division was not observed in the protoplasts with low density (1.0×10^4/ml). In high density (5.0×10^5 /ml), protoplasts started to divide after two days of culture but did not form any colony. Optimal culture density was found to be 5.0×10^4 protoplasts/ml.

Effect of culture conditions

A variety of media (A–F, Table 1) was compared for their ability to promote cell division and colony formation (Table 3). When the protoplasts were cultured in media A, B and C, cell division started within four days but colony formation was not observed. In media D, E and F, cell division started after two days and thereafter colony formation was observed. The medium to optimize the protoplast division and colony formation was found to be the medium F.

Effect of temperature

Number of colonies formed after five weeks of culture is presented in Table 4. Cell division of the proto-

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

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)	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 Table 1)
[z] Volume of protoplast suspension=6.0 ml, protoplast density=5.0×10^4/ml, +: Cell division, –: No cell division.
[y] Days to first cell division.

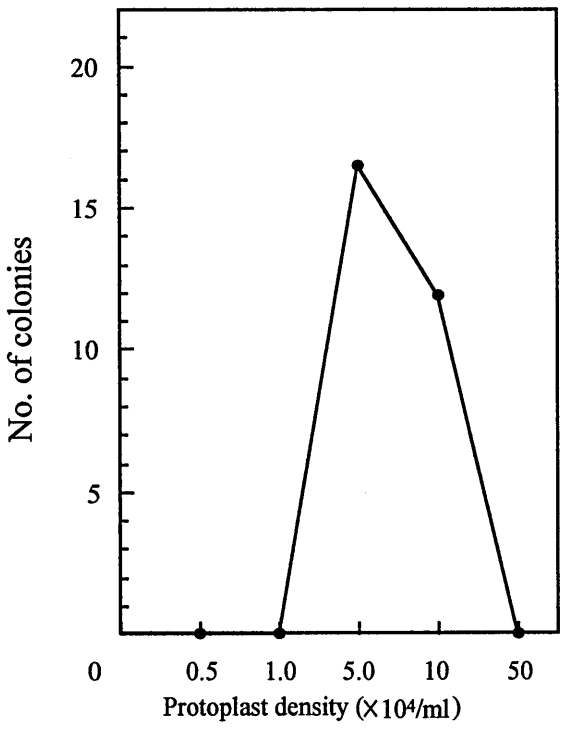

Fig. 1. Effect of protoplast density on colony formation in protoplast culture of *S. sisymbriifolium*.

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

Medium[z]	Cell division[y]	No. of colonies
A 1–18	+(4)[x]	0
B 1–3	+(4)	0
C 1–18	+(2)	0
D	+(2)	3
E	+(2)	8
F	+(2)	17

[z] See Table 1.
[y] Volume of protoplast suspension=6.0 ml, protoplast density=5.0×10^4/ml, +: Cell division.
[x] Days to first cell division.

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

Temperature (°C)		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=5.0×10^4/ml, +: 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.

plasts was faster at 30 °C than that at 25 °C for the first seven days. The protoplasts did not form colonies at 30 °C but at 25 °C after seven days until five weeks of culture. The temperature for the optimal protoplast division and colony formation was judged to be 30 °C for the first seven days and then at 25 °C.

Plant regeneration

A variety of cytokinins was compared for their ability to regenerate shoots from protoplast-derived calli (Table 5). Shoot formation was observed in calli transferred to the medium containing kinetin or zeatin after four weeks of culture. BA was less effective on shoot

formation than other cytokinins, kinetin and zeatin. 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 ten days after placing them on rooting medium. They were then 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. The division frequency was comparable with that from protoplasts derived from cell suspensions allowing a high capacity for further cell development (Gleddie *et al.*, 1985).

Maintaining the protoplasts of *S. sisymbriifolium* at 30°C for more than one week resulted in decrease and loss of cell totipotency. It seems to be a common occurrence during a prolonged period of maintenance at high temperature in protoplast culture, for similar phenomenon was observed in protoplasts of *S. integrifolium* and *S. toxicarium* (Sadohara, 1993)

In the current study, zeatin was found to be more effective than BA for regenerating shoots from protoplast-derived calli as also reported in other *Solanum* species (Nishio *et al.*, 1987; Asao *et al.*, 1989; Sadohara, 1993). The possible cause that zeatin was the most effective cytokinin is considered that only zeatin is a natural cytokinin and the others are synthetic.

The results of the present investigation showed that protoplast culture system of *S. sisymbriifolium* was established. The simple and efficient protoplast culture system is suggested to be useful to produce somatic hybrids between *S. sisymbriifolium* and other *Solanum* species.

ACKNOWLEDGMENTS

The authors wish to express our sincere appreciation to Prof. Emeritus Dr. Kunimitsu Fujieda, Kyushu University for the valuable advice during this experiment.

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