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Isolation and Culture of Petiole Protoplasts of Sweet Potato, *Ipomoea batatas* (L.) Lam. and its Related Species

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Introduction

Based on the normal mode of sexual reproduction and the chromosome number, sweet potato and its related species in the section *Batatas* can be tentatively divided into A-, B- and X-groups[7]. In the A group[3], four species, *Ipomoea triloba* (2X), *I. lacunosa* (2X), *I. ramoni* (2X), and *I. trichocarpa* (2X) are included, while in the B group, *I. batatas* (6X) and three other species, *I. leucantha* (2X), *I. littoralis* (4X) and *I. trifida* (6X) are included. Two species, *I. tiliacia* (4X) and *I. gracilis* (4X) belong to the X group. The B group is cross-incompatible with the A and the X groups[7].

The development of the technique of isolation and culture of plant protoplasts probably will offer a promising method to take the isolation barriers away between sweet potato and its related species. Wu and Ma[11] reported successful isolation of protoplasts from stem callus of *I. batatas*. Using the cell layer-reservoir system[8], Bidney and Shepard[1] cultured sweet potato petiole protoplasts. Important factors cited for the cell layer-reservoir method were low auxin concentration, gradual reduction of sucrose in the cell layer, and influx of mannitol from the reservoir.

Nishimaki and Nozue[6] investigated optimum conditions for the isolation and culture of protoplasts from the high antocyanin-producing callus of sweet potato. Murata *et al.*[5] reported successful plant regeneration from callus derived from petiole protoplasts of sweet potato cultivar, Chugoku No. 25. Shimada *et al.*[10] reported the root formation from callus derived from protoplasts of sweet potato cultivar, Narutokintoki and Kokei No. 14. The present paper describes the results of the examinations for the isolation and culture conditions of petiole protoplasts of sweet potato and its related species.

Materials and Methods

Plant materials: Plant materials used were sweet potato cultivar, Kokei No. 14, Okinawa No. 100 and Minamiyutaka, and its related species, *I. triloba, I. gracilis* and *I. tiliacea*. The stems of these species were grown in pots in a green house where the temperature was kept at about 15°C to 30°C. The plants were fertilized weekly with dilute solution of "Hyponex" (Murakami-bussan Co., Tokyo), 2 ml/l.

Protoplast isolation: Young petioles were excised from the plants. They were sterilized first in 70% ethanol for 10 seconds and then in 1% sodium hypochlorite solution for 15 minutes. And they were rinsed away with sterile distilled water. The sterile petioles were split by using a

surgical knife. These petiole pieces, measuring 0.5 g in fresh weight each, were placed in 6 cm petri dishes containing 5 ml of the enzyme solution. The enzyme solution used at the beginning of this experiment was the same one as used by Bidney and Shepard[1]; 0.2% Macerozyme R-10 and 0.4% Cellulase ONOZUKA R-10 (Kinki Yakult Co. Ltd.), 1/4 strength of Murashige and Skoog major elements[4] minus ammonium nitrate (NH_4NO_3), 0.3 M sucrose, 10 mg/l casein hydrolysate and 5 mM 2-N-Morpholino-ethanesulfonic acid (MES). But, by using the above enzyme solution, higher yields of petiole protoplasts were not gained. Therefore, the effects of the constituent of this enzyme solution on protoplast isolation were investigated in this experiment.

The petri dish was evacuated for about 5 minutes in a vacum desicator and then it was shaken on a reciprocating shaker set 45 strokes/minute for 3 to 4 hours at 28°C. The effects of evacuation and shaking of the dish during the enzyme treatment on the protoplast isolation were also examined in this experiment. Following the enzyme treatment, the crude protoplast suspension was filtered through a nylon mesh (pore size 60 μm) and the filtrate was centrifuged at 150 xg for 2 minutes. Then the precipitate was washed twice in the digestion solution minus the enzymes and MES used by Bidney and Shepard[1]. This washing solution, however, was not necessarily efficient, so the effect of the constituents of the washing solution was examined in the present experiment and the modified constituents of washing solution were described in the results.

The washed protoplasts were collected by floatation in Babcock bottles as described by Shepard and Totten[9]. The collected protoplasts were resuspended in the washing solution to give a concentration of 10^5 protoplasts/ml.

Protoplast culture: A cell layer reservoir medium[1] was used as the initial plating medium in the beginning of the present experiment. However, the concentrations of sucrose and mannitol of this plating medium were modified because they were unsuccessful in the protoplast proliferation in our experiment. All protoplasts were cultured in the thin layer of liquid medium or the solid one at a density of $10^4-2.5\times10^4$/ml. Culture dishes (30 mm or 60 mm in diameter) were sealed with parafilm and placed in a incubator set at 28°C under dark condition.

Results and Discussion

At the beginning of the present experiment the enzyme solution used by Bidney and Shepard[1] was applied for the digestion of petioles, but with the above solution, sufficient yields of protoplasts were not isolated. Therefore, the effects of the constituents of this enzyme solution on the yield of protoplasts were evaluated (Table 1.). When mannitol instead of sucrose was added to the enzyme solution as osmoticum, higher yields of protoplasts were obtained, showing the highest one at 0.6 M mannitol among the concentrations tested. The addition of 1/4 strength of Murashige and Skoog major elements (minus NH_4NO_3) did not show satisfactorily good yield of protoplasts.

When the petri dish containing the petiole pieces was shaken during the digestion, the protoplast yield showed the highest one in 3~5 hours after the beginning of enzyme treatment, and thereafter the isolated protoplasts from petiole pieces were destroyed and reduced in number. When the dishes were left unshaken, the protoplast yield gradually increased with the time of the enzyme treatment. Therefore, the destruction of the protoplasts brought out in the longer enzyme treatment was probably attributable to collisions of isolated protoplasts against undigested petiole pieces owing to shaking, but not to injurious effects of the enzyme solution. The protoplast yields at 18 hours after the beginning of enzyme treatments without shaking are

Table 1. Effect of the constituents added to the enzyme solution on protoplast isolation

Plot	Constituent added			No. of protoplasts $\times 10^3$/ml		
	Sucrose	Mannitol	MS major element*	*I. gracilis*	*I. tiliacea*	Mean
1	0.3 M		addition	0.0	0.0	0.0
2		0.5 M	〃	9.3	0.2	4.7
3		0.6 M	〃	11.9	4.5	8.2
4		0.7 M	〃	2.7	1.0	1.8
5	0.3 M			0.6	0.0	0.3
6		0.5 M		10.5	1.9	6.2
7		0.6 M		17.5	4.9	11.2
8		0.7 M		8.8	2.1	5.5

* 1/4 strength of Murashige and Skoog major elements (minus NH_4NO_3)

Table 2. Protoplast yields at 18 hours after the beginning of enzyme treatments

Cultivar	No. of protoplasts ($\times 10^5$/ml sol.)
Kokei No. 14	3.0
Okinawa No. 100	1.0
Minamiyutaka	1.5

shown in Table 2.

The effect of the constituent of the solution used for washing the enzyme solution was also tested. As a result of testing it was noticed that the protoplast yield was higher in 0.6 M mannitol solution containing 10 ml/l casein hydrolysate and 5 mM MES than in o.6 M mannitol solution without above additions.

Finally, the enzyme solution containing 0.2% Macerozyme R-10, 0.4% cellulase ONOZUKA R-10, 0.6 M mannitol, 0.5% $CaCl_2 \cdot 2H_2O$ and 5 mM MES (pH 5.6) was used for the isolation of petiole protoplasts and the above digestion solution minus the enzymes was used for washing the enzymes.

The centrifugal conditions for collecting the protoplasts from its suspensions were investigated in three steps, 100 *xg*, 150 *xg* and 200 *xg* for two minutes, respectively. The centrifugal condition with 150 *xg* for two minutes presented the highest yield of protoplasts among the tested ones. Another centrifugal condition for purifying the crude protoplasts pored on 20% sucrose solution in a Babcock bottle was also tested and the successful floatation of pure protoplasts was obtained under the condition with 350 *xg* for 4 minutes.

As the initial plating medium of plotoplasts, the cell layer and reservoir medium used by Bidney and Shepard[1] was examined. However, the frequency of cell divisions of plotoplasts was not observed to be high in this medium. Nishimaki and Nozue[5] reported that the frequency of cell division was the highest when 0.7 M mannitol and 0.025 M sucrose were added to modified PRL-4-C medium[2]. It was ascertained in this experiment that the modified cell layer medium containing both 0.025 M sucrose and 0.7 M mannitol as osmoticum instead of 0.3 M sucrose alone, and also 1 mg/l 2-4-dichlorophenoxy-acetic acid (2, 4-D) and 0.3 mg/l kinetin as

phytohormone in the cell layer medium was efficient to induce higher cell division of protoplasts.

Cell divisions of sweet potato cultivar protoplasts in this modified cell layer liquid medium are shown in Table 3. The cell divisions of protoplasts were observed two days after plating and the frequency of cell divisions increased four days after. The frequency of cell divisions of protoplasts two and four days after plating did not differ both in the medium supplemented with 2, 4-D and kinetin and in the one not supplemented with above phytohormones. But the number of cells in a colony four days after plating showed larger values in the medium supplemented with phytohormones than in those without them.

One week after plating, the colonies could be seen with the naked eye. The initial protoplast division was a little delayed in the solid medium supplemented with 0.5% agar, compared with

Table 3. Cell divisions of sweet potato cultivar protoplasts in the modified cell layer liquid medium

Cultivar	Medium	2 days after		4 days after	
		Frequency*3 of cell divisions	No. of cells in a colony	Frequency of cell divisions	No. of cells in a colony
Kokei	H*1	33.3	2–8	71.7	2–20
No. 14	C*2	42.9	2	83.8	3– 4
Okinawa	H	43.5	2–8	88.0	30–40
No. 100	C	27.2	2	67.2	2– 8
Minami-	H	38.3	10–20	60.7	30–40
yutaka	C	30.9	2–8	55.6	2– 8

*1 : medium supplemented with 1 mg/l 2, 4–D and 0.3 mg/l kinetin.
*2 : medium supplemented with no phytohormone.
*3 : No. of cell division/No. of planting protoplasts (%).

Table 4. Effect of constituent of solid medium on the cell divisions of *I. gracilis* protoplasts

No.*1	Medium constituent					Frequency of cell divisions*7 of protoplasts Days after plating				
	Suc.*2 M	Mann.*3 M	2, 4-D*4 mg/l	Kin.*5 mg/l	NAA*6 mg/l	3	5	7	9	10
1	0.3				0.05	−	−	−	−	−
2	0.025	0.7	0.3	0.1		±	+	++	+++	+++
3	0.025	0.7	1.0	0.1		+	++	++	+++	+++
4	0.025	0.7	3.0	0.1		±	+	++	++	+++

*1 1: cell layer medium, 2–4: modified cell layer medium. Other constituents are the same as cell layer medium.
*2 sucrose, *3 mannitol, *4 2, 4-dichlorophenoxyacetic acid, *5 kinetin, *6 1-naphthaleneacetic acid,
*7 −: shrinkage of protoplasts,
±: no cell division,
+: two cells,
++: three and four cells,
+++: colony.

the one in the liquid medium.

Modified cell layer midium supplemented with phytohormones was efficient to the cell divisions of the related species protoplasts. Effects of the constituents of solid medium on the cell divisions of *I. gracilis* protoplasts are shown in Table 4. Although protoplasts plated on the cell layer medium shrank and showed no cell division, those plated on modified cell layer medium exhibited cell divisions 3 days after and the mumber of cells of the colonies increased with the days after plating.

The cell divisions of *I. triloba* protoplasts in the modified cell layer solid medium were shown in Table 5. The frequencies of cell divisions and the number of cells of colonies increased gradually with the days after plating.

Table 5. Plating efficiency*[1] of the protoplasts of *I. triloba* in the modified cell layer solid medium*[2]

No. of cells of colony	Days after plating			
	3	10	15	20
1- 3	2.3	4.3	5.8	4.6
4- 6		2.0	3.0	5.1
7- 8		0.5	0.7	1.9
10-12		0.1	0.1	1.2
13-15		0.2	0.1	0.5
16		0.1	0.1	0.6
Total	2.3	7.2	9.8	13.9

*[1] Percentage of No. of colony/No. of plating protoplasts.
*[2] 2, 4-D 1 mg/l, kinetin 0.3 mg/l.

Summary

1. Isolation and culture procedure of petiole protoplasts of sweet potato and its related species which are uncrossable to sweet potato were investigated.

2. The enzyme solution containing 0.2% Macerozyme R-10, 0.4% Cellulase Onozuka R-10, 0.6 M mannitol, 0.5% $CaCl_2 \cdot 2H_2O$ and 5 mM MES (pH 5.6) was efficient to isolate physiologically active protoplasts from petiole and also the digestion solution minus enzymes was efficient as the washing solution.

3. The centrifugal condition with 150 *xg* for 2 minutes was efficient to collect the protoplasts from its suspention and also the one with 350 *xg* for 4 minutes was efficient to get successful floatation of pure protoplasts on 20% sucrose in a Babcock bottle.

4. Modified cell layer medium containing both 0.025 M sucrose and 0.7 M mannitol instead of 0.3 M sucrose alone as osmoticum and 1 mg/l 2, 4-D and 0.3 mg/l kinetin as phytohormone in cell layer medium was efficient to induce higher frequencies of cell divisions of protoplasts.

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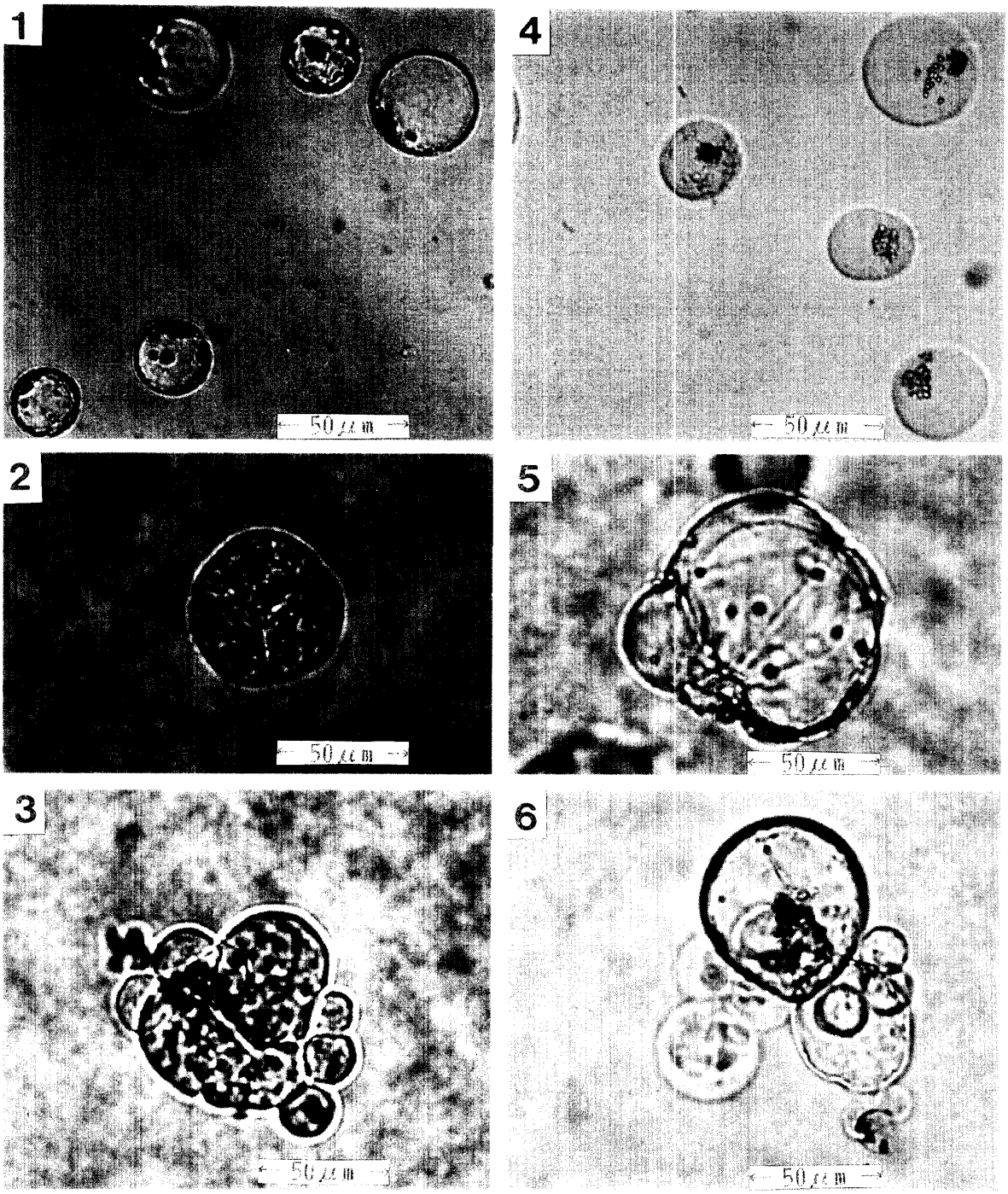

Fig. 1. Protoplasts isolated from petiole of sweet potato.
Fig. 2. Cells derived from a single protoplast of sweet potato 3 days after plating.
Fig. 3. Colony of sweet potato 8 days after plating.
Fig. 4. Protoplasts isolated from petioles of *Ipomoea triloba*.
Fig. 5. Cells derived from a single protoplast of *I. triloba* 3 days after plating.
Fig. 6. Colony of *I. triloba* 8 days after plating.