Original Paper Plant Tissue Culture Letters, **1** (1), 18–21 (1984)

Protoplast Culture of Potato: Changes in Viability and Initiation of Cell Division

Y. KIKUTA, W. SAITO,* and Y. OKAZAWA

Department of Botany, Faculty of Agriculture, Hokkaido University, Kita-9, Nishi-9, Kitaku, Sapporo 060, Japan

(Received December 26, 1983)
(Accepted February 2, 1984)

A procedure for isolation and culture with high viable protoplast yield from axenic shoot culture of potato (*Solanum tuberosum* L. cv May Queen) has been developed. The isolated protoplasts, cultured in a suitable medium, regenerated cell-wall within 48 hr, initiated DNA synthesis in 76–96 hr, and divided to produce cell colonies. For regeneration of whole plants, cell colonies cultured with IAA and zeatin gave the highest frequency of shoot-bud formation at 20°C under 4,000 lux of irradiance.

Culture and plant regeneration of isolated protoplasts are the two crucial steps in the application of protoplast manipulation for potato improvement. Although there have been several reports demonstrating recovery of plants from protoplasts in *Solanum tuberosum* L.,[1-6] the high viability and consistent development of potato protoplast cultures still remain elusive. The work described here deals with an isolation procedure with high yields of viable protoplasts from mesophyll cells of axenic shoot cultures derived from potato tuber discs. An attempt has also been made to elucidate the changes of viability, cell-well regeneration, and DNA synthesis in the protoplast cultures in the provision for induction of sustained cell division.

Materials and Methods

A potato cultivar, *Solanum tuberosum* L. cv May Queen, was used in the present study. Leaves were collected from an axenic shoot culture regenerated from May Queen potato tuber discs.[7] They were then incubated in an enzyme solution containing 1% Cellulase Onozuka R10 (Yakult, Nishinomiya), 0.04% Pectolyase Y23 (Seishin Seiyaku, Nagareyama), 0.1% bovine serum albumin Fr. V (Boehringer Mannheim GmbH), 0.05 M mannitol, and 5 mM MES, pH 5.6, with 75 rpm shaking at 25°C for 2 hr. The resulting mixture was filtered through a 44 μm mesh stainless steel sieve and layered on Percoll (Pharmacia Fine Chemicals, Uppsala) gradient (6.25% and 25% in 0.55M mannitol), and centrifuged at $1{,}000 \times g$ for 5 min. The mesophyll protoplasts banded at the interface of the two Percoll layers were collected, and then washed in protoplast culture medium (**Table 1**) containing 0.55 M mannitol by centrifugation at $100 \times g$ for 2 min. This was repeated twice.

Protoplasts were cultured at 5×10^3/ml in 6 cm plastic dishes containing 2 ml of protoplast culture medium with 0.4 M mannitol. Cultures were incubated at 25°C in the dark, and the number of divided cells was examined 7 days later. For colony formation, these cultures were further incubated at 22°C under an irradiance of 800 lux. Fresh medium was added to the dishes every two weeks.

---

* Present address; Kikkoman Corporation, Noda.

Table 1. Composition of the protoplast culture medium.

Mineral salts (mg/*l*)			
KNO_3	950.0	$MnSO_4 \cdot 4H_2O$	10.0
$CaCl_2 \cdot 2H_2O$	880.0	$ZnSO_4 \cdot 7H_2O$	4.6
$MgSO_4 \cdot 7H_2O$	185.0	H_3BO_3	3.1
KH_2PO_4	85.0	KI	0.4
$FeSO_4 \cdot 7H_2O$	25.0	$Na_2MoO_4 \cdot 2H_2O$	0.1
Na_2EDTA	35.0	$CoCl_2$	0.1
Organic constituents (mg/*l*)			
Inositol	50.0	Sucrose	500.0
Nicotinic acid	2.5	Casaminoacid	250.0
Glycine	1.0	Zeatin	1.0
Thiamine-HCl	0.25	NAA	0.5
Pyridoxine-HCl	0.25	GA_3	0.1
Folic acid	0.25	MES (pH 6.0)	500.0
Biotin	0.01	Mannitol	72,000.0

Cell-wall regeneration and DNA synthesis were determined by ^{14}C-glucose-1-phosphate (specific activity of 5.5 GBq/mmol) and (methyl-^{14}C)-thymidine (specific activity of 2.0 GBq/mmol) incorporation into the perchloric acid insoluble fraction of protoplast cultures. Changes in radioactivity were the indication of cellulose and DNA synthesis in protoplast cultures. Protoplast viability was determined by hydrolysis of fluorescein diacetate (FDA).[8]

Cell colonies were transferred after 8 weeks to a modified White's nutrient medium[7] and incubated at 20°C under an irradiance of 4,000 lux (16 hr day/8 hr night) for shoot-bud regeneration.

Results and Discussion

Freshly isolated protoplasts showing over 90% viability subsequently decreased to 50% after 24 hr incubation. At this stage, the cellulose synthesis was found to occur in protoplasts (**Fig. 1**). The failure of cell-wall regeneration was the first indication of decreased viability. Light was not required. Protoplasts cultured without calcium, NAA, and zeatin died within 96 hr. In our culture medium, protoplasts generated cell-wall in 48 hr in the presence of 14.6 mM sucrose or 13.9 mM glucose. However, higher concentrations of sucrose and glucose were inhibitory for the induction of the first cell division of protoplasts.

The importance of osmotic conditions in potato protoplast culture has been documented.[1] Mannitol played a significant role in initiation of the first cell division at a critical concentration. Equal concentrations of inositol and sorbitol were less effective than mannitol. $CaCl_2$ was ineffective. Sucrose and glucose could not replace mannitol as osmoticum for potato protoplast cultures, probably because mannitol was not metabolized by potato cells and could act as an inert osmoticum.

After 72–96 hr, the DNA synthesis was initiated in potato protoplast cultures followed by the first cell division. At this stage, the viability further decreased to 25% under our optimal conditions. The decrease was coincident with the failure of DNA synthesis.

The initiation of the first cell division was stimulated by the simultaneous application of auxin (0.5 mg/*l* NAA) and cytokinin (1.0 mg/*l* zeatin), whereas a marked deterioration of protoplasts was observed when they were subjected to higher concentrations of auxin. Zeatin plus NAA proved to be the only hormone combination that was effective in initiating the first

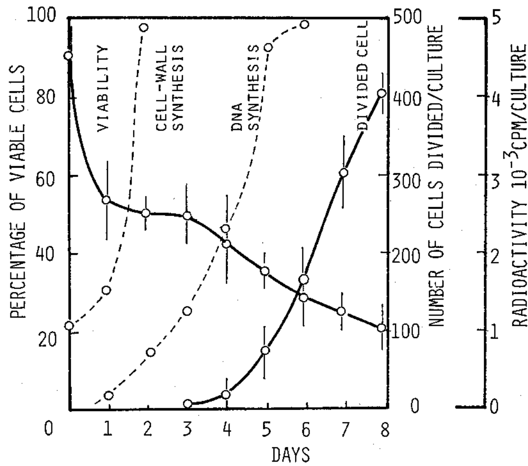

Fig. 1. Changes of viability and initiation of cell division in potato protoplast culture.
Protoplasts were cultured in the medium listed in Table 1 at 25°C in the dark. Viability was determined by FDA staining; cell-wall and DNA syntheses were measured by radioactive glucose-1-phosphate and thymidine incorporation into acid insoluble fraction.

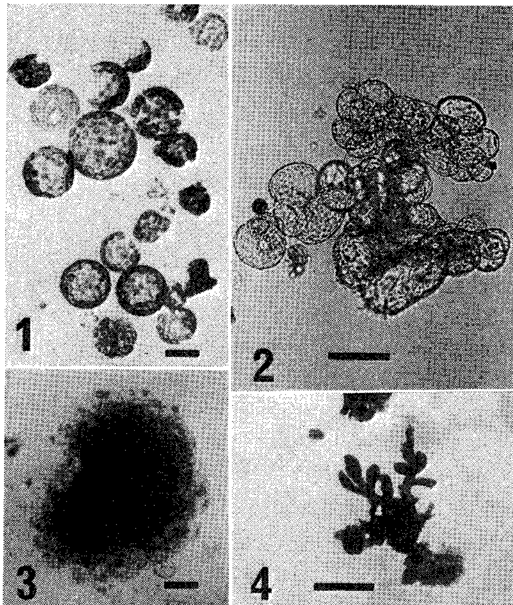

Fig. 2. Shoot-bud formation from protoplast culture of potato, *Solanum tuberosum* L. cv May Queen.
1: Protoplast culture; 2 days after isolation. A bar represents 20 μm. 2: Initiation of cell colony; 14 days after isolation. A bar represents 40 μm. 3: A cell colony, a small callus, transferred to an agar medium suitable for shoot-bud formation; A bar indicates 80 μm. 4: A callus produced shoot-buds; A bar represents 5 mm.

cell division in potato protoplasts. Among other combinations, benzyladenine plus NAA and isopentenyladenine plus NAA were effective to a certain extent for maintaining the viability of protoplasts. Kinetin was completely ineffective in inducing the first cell division.

For plant regeneration, the best results were obtained when small calluses derived from protoplasts were cultured in White's basal medium containing 0.25 M mannitol, 8.8 mM sucrose, 1.0 mg/*l* IAA, and 1.0 mg/*l* zeatin, pH 5.8, solidified with 0.7% Bacto-agar, at 20°C under an irradiance of 4,000 lux (16 hr day/8 hr night). However, the present experiments demonstrate that it is possible, using simple techniques and defined media, to obtain approximately 500 cell colonies, 100 calluses, and 10–25 plants in an experiment involving 10^4 protoplasts isolated from axenic shoot cultures of May Queen potato. The results also suggest that further study of the whole process of development of isolated protoplasts will help to achieve crop improvement through protoplast manipulation in this important crop.

This work was in part supported by a Grant-in-Aid for General Scientific Research (B) 00548026 and in part by a Special Research Project (A) 411910 from the Ministry of Education, Science and Culture of Japan. The results were in part presented in the 6th International Protoplast Symposium, Basel, and the 8th Symposium for Plant Tissue Culture, Toyama.

References

1) Shepard, J. F., R. E. Totten, 1977. Plant Physiol., **60**: 313–316.
2) Binding, H., R. Nehls, O. Scheider, S. K. Sopory, G. Wenzel, 1978. Physiol. Plant., **43**: 52–54.
3) Gunn, R. E., J. F. Shepard, 1981. Plant Sci. Lett., **22**: 97–101.
4) Thomas, E., 1981. Plant Sci. Lett., **23**: 81–88.
5) O'Hara, J. F., G. G. Henshaw, 1982. In "Plant Tissue Culture 1982" (ed. by Fujiwara, A.), p. 591–592, Maruzen, Tokyo.
6) Saito, W., K. Masuda, Y. Kikuta, Y. Okazawa, 1982. Jpn. J. Crop Sci., **51** (Suppl. 2): 169–170.
7) Kikuta, Y., Y. Okazawa, 1982. J. Fac. Agric. Hokkaido Univ., **61**: 167–179.
8) Larkin, R. J., 1976. Planta, **128**: 213–216.

《和文要約》

ジャガイモのプロトプラスト培養における生存率の変動と分裂の開始

喜久田嘉郎・*斉藤　渉・岡澤養三
北海道大学農学部作物生理学研究室
*(現在)　(株)キッコーマン

ジャガイモ(品種メイクイン薯)を用いて，高い生存率と分裂活性をもつ葉肉プロトプラストを無菌茎葉培養より単離・培養する方法を開発した。単離したプロトプラストは適当な培地で暗所 25°C に培養すると，48 時間以内に細胞壁を再生し，72〜96 時間後に DNA 合成を開始した。その後，細胞分裂を開始しカルスを形成した。細胞分裂の開始誘導には 0.5 mg/*l* の NAA と 1.0 mg/*l* のゼアチンが最も効果的であった。形成したカルスを 0.1 mg/*l* IAA と 1.0 mg/*l* のゼアチンを含む適当な培地に照度 4,000 lux, 20°C で培養すると茎葉分化が認められた。