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Callus Formation from Protoplasts of Cultured *Lithospermum erythrorhizon* Cells

Y. Maeda [1] *, Y. Fujita [2], and Y. Yamada [1]

[1] Research Center for Cell and Tissue Culture, Faculty of Agriculture, Kyoto University, Kyoto 606, Japan
[2] Research Center, Mitsui Petrochemical Ind., Ltd., Waki-cho, Kuga-gun, Yamaguchi 740, Japan

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ABSTRACT

Protoplasts isolated from cell cultures of *Lithospermum erythrorhizon* divided repeatedly and formed callus colonies. Factors that affect protoplast division are the use of glucose as osmoticum, a new plating method with twin layers of agar-liquid medium, and the culture of protoplasts under the osmolarity lower than that in the isolation solution. When the sucrose in the protoplast-culture medium was replaced with glucose, and coconut milk was added to the medium, the frequency of colony formation markedly increased. The culture period required for colony formation also was shortened.

INTRODUCTION

Cell cultures of *Lithospermum erythrorhizon* (Boraginaceae) produce particularly large amounts of shikonin derivatives in liquid M-9 medium (Fujita *et al.*, 1981 b). It may be of interest to establish cell lines derived from protoplasts, and to fuse a cell that contains a large amount of shikonin with a cell that shows fast growth.

For many species, however, protoplasts isolated from cultured cells, do not divide easily and form callus colonies. So far, limited success has been reported for the culture of protoplasts mainly isolated from suspension cells of Solanaceous plants: *Nicotiana tabacum* (Uchimiya and Murashige, 1976), *Atropa belladonna* (Gosch *et al.*, 1975), and *Datura innoxia* (Furner *et al.*, 1978). There have been no reports on the Boraginaceae.

We here discuss the isolation and culture conditions which will cause protoplasts to divide and form callus colonies.

MATERIALS AND METHODS

Cell Culture. An *L. erythrorhizon* cell line, M-18, was grown at 25°C in the dark on a rotary shaker (100 rpm, amplitude 1.25 cm) in 300 ml flasks containing 80 ml of liquid medium. We subcultured 6 g fresh wt. of cells every 7 days into a conditioned medium containing 40 ml each of the old and of freshly prepared Linsmaier-Skoog medium (1965). The fresh medium contained 10^{-6} M 3-indoleacetic acid and 10^{-5} M kinetin. These cells divided without a lag phase and showed logarithmic growth for about 5 days. The doubling time was approximately 5 days. We used cells harvested 3-5 days after subculture for protoplast isolation.

Isolation of Protoplasts. We collected cells on a nylon sieve, pore size 40 μ, then added 1 g fresh wt. of cells to 10 ml of filter-sterilized isolation solution containing 0.5% Macerozyme R-10, 1.0% Driselase, 2.5% cellulase Onozuka R-10, 5 mM MES, 5 mM $CaCl_2$, 5 mM $MgCl_2$ and the osmoticum. The pH of the solution was adjusted to 6.0 before it was sterilized. The cell suspension was incubated at 29°C with gentle shaking of 45 strokes per min (amplitude, 2.5 cm). After 2 h of incubation, the protoplast suspension was filtered through a 62 μ nylon sieve to remove any undigested cell aggregates, then it was centrifuged at 70 g for 1 min. The supernatant was removed with a Pasteur pipette and the protoplasts were resuspended in the solution lacking enzymes. This procedure was repeated three times to remove enzymes and cell debris. Staining of these protoplasts with Calcofluor White ST showed them to be devoid of cell wall materials.

Culture of Isolated Protoplasts. The washed protoplasts were resuspended in cell culture medium (pH 6.5 before autoclaving) stabilized with the osmoticum and supplemented with 10^{-4} M 3-indoleacetic acid and 10^{-6} M kinetin. They then were plated in 6 cm Falcon petri-dishes at a density of 1.25×10^5 protoplasts/ml. These dishes were sealed with Parafilm M and kept at 25°C in the dark.

Estimation of Protoplast Viability, Survival and Colony Formation. We first checked the viability of our isolated protoplasts by staining them with Evans blue, or with fluorescein diacetate, after which we observed them for spherical shape, viable colour, and cytoplasmic streaming. The results of these procedures were in good accord, so we could determine viability only by observing shape, colour, and cytoplasmic streaming.

We determined the survival of protoplasts by counting the number of protoplasts that were viable and colony formation by counting the number of calluses that contained more than 17 cells, in known volumes of the culture. The optimum conditions obtained were used as the standard in the next experiment.

RESULTS AND DISCUSSION

Concentration of Glucose at Protoplast Isolation. We isolated protoplasts from cells with glucose at various concentrations as osmoticum (Fig. 1). The yields of viable protoplasts increased with an increase in the concentration of glucose up to 0.6 M, but decreased above 0.8 M. Therefore, we chose 0.6 M

* Present address: Research Center, Mitsui Petrochemical Ind., Ltd., Waki-cho, Kuga-gun, Yamaguchi 740, Japan

as the best concentration for isolation. Yamada and Morimoto also have obtained viable protoplasts from *Coptis japonica* cells at the same concentration of glucose (unpublished).

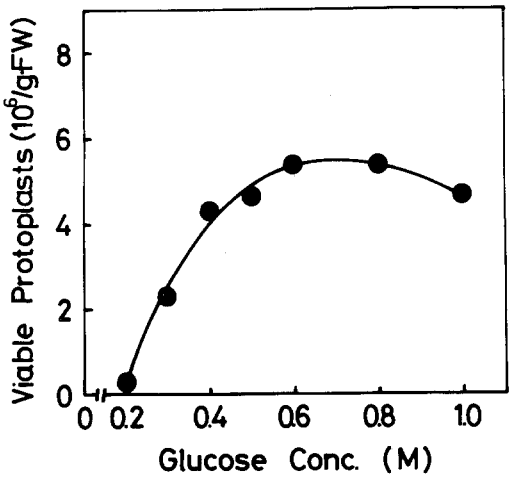

Fig. 1. Effect of Varying the Concentration of Glucose on Protoplast Isolation.

Effect of Various Osmotica. We investigated the effect of sugar-alcohols and sugars on protoplast culture (Table 1). A 0.45 M concentration of each was added to the medium. Thus the osmolarity in the medium was approximately equal to that in the isolation solution. When cultured in medium stabilized with mannitol or sorbitol, used as the osmoticum in many studies, no protoplasts survived after 2 weeks of culture. As with the cultured *Coptis japonica* cells of Yamada and Morimoto (unpublished), about 15% of the protoplasts survived when cultured in medium stabilized with sucrose or glucose. Fructose and galactose produced fewer survivers than glucose or sucrose, so we selected glucose as the best osmoticum for protoplast culture.

Table 1. Effect of Various Osmotica on the Survival of Protoplasts in Culture Medium. Protoplasts were plated in liquid medium and cultured for 2 weeks.

Osmoticum	Survival[a] (%)
Mannitol	0
Sorbitol	0
Cellobiose	0
Lactose	0
Maltose	0
Sucrose	15.2
Fructose	4.4
Galactose	8.9
Glucose	16.2

[a] $\frac{\text{surviving protoplasts}}{\text{inoculated protoplasts}} \times 100$

Culture Medium for Protoplasts. To establish a culture medium that supports continuous protoplast growth, we tested nine media under the same hormonal and osmotic conditions (Table 2). The Linsmaier-Skoog (1965), M-9 medium (1981) has been used for cell growth and production of shikonin derivatives from *L. erythrorhizon* cells, and the seven other media have been widely used for protoplast culture. Arnold and Eriksson's medium (1977) was best for the survival of protoplasts, and Linsmaier-Skoog and Gamborg *et al.* B-5 (1968) were next best. None of the other media supported good growth.

Table 2. Effect of the Type of Media on the Survival of Protoplasts and Regeneration of the Cell Wall. Protoplasts were plated in liquid medium and cultured for 10 days.

Basal Medium	Survival (%)	Cell Wall Regeneration[a] (%)
White (1954)	25.6	19.9
Linsmaier & Skoog (1965)	61.7	50.9
Gamborg *et al.* B-5 (1968)	58.0	46.0
Nagata & Takebe (1971)	29.0	18.7
Schenk & Hildebrandt (1972)	42.0	32.0
Kao medium 2 (1974)	43.9	36.2
Arnold & Eriksson (1977)	76.6	46.1
Potrykus P-2 (1977)	26.9	11.1
M-9 (1981)	28.9	21.8

[a] $\frac{\text{cell-wall regenerated protoplasts}}{\text{inoculated protoplasts}} \times 100$

This result was in good accordance with results from the cell culture of *L. erythrorhizon* for which Linsmaier-Skoog medium was best for cell growth and B-5 was next best, but neither White's nor M-9 medium supported good cell growth (Fujita *et al.*, 1981 a). Fewer protoplasts regenerated their cell walls, in Arnold and Eriksson's medium than in the Linsmaier-Skoog and B-5 media.

We retained the Linsmaier-Skoog medium for protoplast culture because we believe that the nutritional requirements for protoplast growth are basically the same as those of the cultured cells.

Plating Method. We first cultured protoplasts in liquid or agar medium. After 2 weeks of culture, about 32-45% of the inoculated protoplasts had survived, except in the 0.5 ml liquid medium in which protoplasts had dried up due to the evaporation of water. No protoplast, however, survived after 4 weeks of culture under these two conditions. Therefore, we adopted a new plating method with each petri-dish having twin layers of agar-liquid medium. The liquid medium was about 0.35 mm deep on top of the agar. With this method, about 11% of the inoculated protoplasts survived after 4 weeks of culture (Table 3).

Table 3. Relation between the Plating Method and the Survival of Protoplasts.

Plating Method	Survival (%)	
	After 2 weeks	After 4 weeks
Liquid Method[a]		
0.5 ml	0	0
1.0 ml	44.9	0
2.0 ml	34.4	0
Agar Method[b]	32.3	0
Agar-liquid Method[c]	42.7	11.7

[a] Liquid medium containing protoplasts was poured into petri-dishes.

[b] Liquid medium containing protoplasts was mixed with an equal volume of melted agar medium (1.2% agar) that had been kept at 40°C, then 1-ml protoplast samples were poured into petri-dishes.

[c] The agar medium first was poured into a petri-dish and solidified, then 1.0 ml of liquid medium containing protoplasts was poured on to the agar layer.

This agar-liquid method was able to support viability of protoplasts for over 4 weeks probably because of the availabilities of more oxygen and moisture as compared with only liquid or agar medium.

Reduction of Glucose Concentration in the Culture Medium. No cell division was observed when protoplasts were incubated in Linsmaier-Skoog medium stabilized with 0.45 M glucose. We thought that the high osmolarity required to prevent protoplasts from bursting inhibited division, thus we reduced the glucose concentration in the culture medium from 0.45 M to 0.35, 0.25, 0.15, or 0.05 M at plating (Fig. 2). Protoplasts survived best in medium stabilized with 0.25 M glucose. Their survival rate decreased with higher concentrations, as well as with concentrations lower than 0.25 M.

Callus colonies formed only with the reduced concentration, except at 0.05 M which was too low to sustain the protoplasts. Colonies formed best with 0.25 M glucose, at the rate of 0.08% of the inoculated protoplasts.

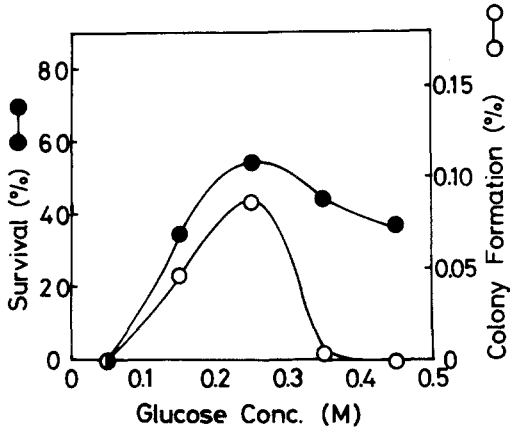

Fig. 2. Effect of Reducing the Glucose Concentration in the Culture Medium on the Survival of Protoplasts and Colony Formation. The survival of protoplasts was determined after 2 weeks of culture and colony formation was checked after 6 weeks of culture.

Many investigators have kept the same osmolarity for the isolation and for the culture of protoplasts. In contrast, Wallin and Eriksson (1973) and Diettrich *et al.* (1980) reported that the optimum osmolarity for protoplast culture was lower than that for protoplast isolation. This was also true for *L. erythrorhizon* protoplasts.

The relation between protoplast survival and glucose concentration was similar to that between colony formation and glucose concentration. This result indicates that conditions favoring the survival of protoplasts are a helpful guide to colony formation.

Sucrose Concentration in the Culture Medium. To increase the rate of colony formation, we reduced the concentration of sucrose in the protoplast-culture medium and instead added glucose at the same molarity (Fig. 3). The rate of colony formation increased to 0.14% of the inoculated protoplasts when sucrose was replaced fully with glucose, but it was independent of the concentration of sucrose at more than 29 mM. Protoplasts survived well in medium stabilized with glucose. This use of glucose as both the osmoticum and carbon source favors protoplast growth and division.

Addition of Coconut Milk. We added natural organic

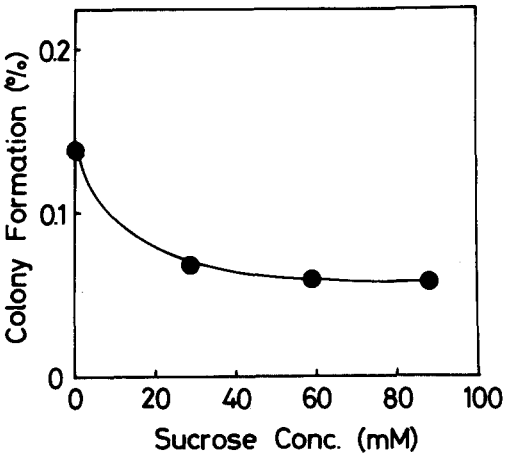

Fig. 3. Relation between the Sucrose Concentration in the Culture Medium and Colony Formation. Colony formation was checked after 6 weeks of culture. Base-level of sugars was 0.338 M (0.25 M + 0.088 M).

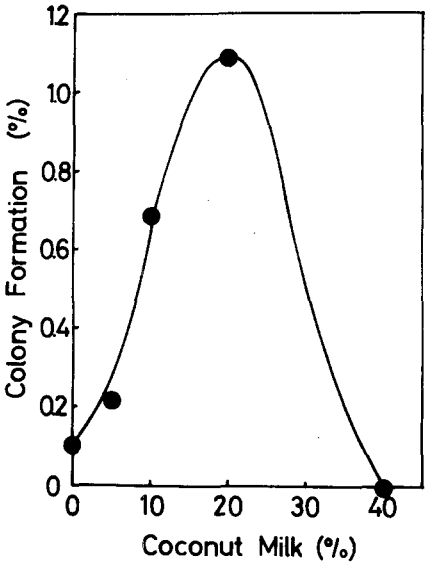

Fig. 4. Effect of the Addition of Coconut Milk to the Medium on Colony Formation. Colony formation was checked after 3 weeks of culture.

substances to the culture medium (casein hydrolyzate and coconut milk) which included well-balanced vitamins and amino acids. When 0.1% casein hydrolyzate was added to the medium, the rate of colony formation was approximately doubled. Marked increase in colony formation was observed when coconut milk was added (Fig. 4). The optimum concentration of coconut milk for colony formation was 20%. The rate of colony formation at this concentration was about 1.10% of the inoculated protoplasts, 11-fold the rate without coconut milk. The incubation time required for colony formation also was shortened from 6 to 3 weeks.

Time Course of Protoplast Culture. All the surviving protoplasts regenerated cell walls after 3 days of culture, and some began to divide (Fig. 5C). The second cell division took place 5 days after culture (Fig. 5D), thereafter, successive cell division resulted in the formation of callus colonies (Fig. 5E). No new colonies could be counted after 3 weeks of culture (Fig. 5F). At that time, the value for protoplasts that divided at least once was about 2.1%, and that for colony formation was about 1.3% of the

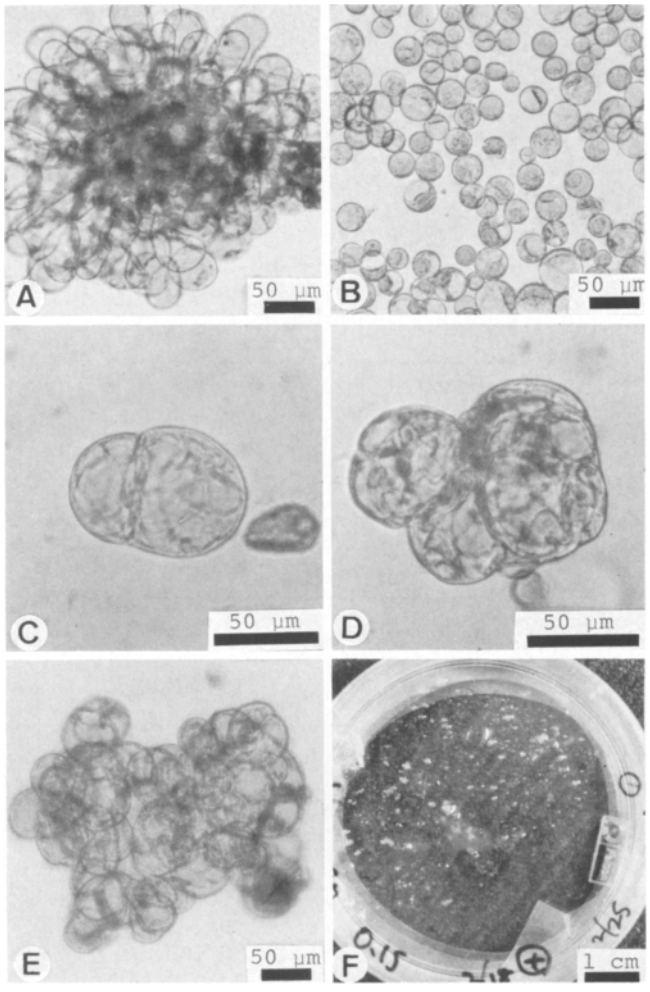

Fig. 5. Time Course of Protoplast Culture. (A) Cultured cells of *L. erythrorhizon*. (B) Freshly isolated protoplasts. (C) First cell division which began after 3 days of culture. (D) Second cell division which began after 5 days of culture. (E) Callus colonies after 2 weeks of culture. (F) Callus colonies visible in the petri-dish after 3 weeks of culture.

inoculated protoplasts.

We are now culturing callus colonies to establish protoplast-derived cell lines. As the cell lines probably will differ in their characteristics, we will investigate the yield of shikonin derivatives and cell growth of each line.

REFERENCES

Arnold SV, Eriksson T (1977) Physiol. Plant. 39: 257-260

Diettrich B, Neumann D, Luckner M (1980) Planta medica 38: 375-382

Fujita Y, Hara Y, Ogino T, Suga C (1981 a) Plant Cell Reports 1: 59-60

Fujita Y, Hara Y, Suga C, Morimoto T (1981 b) Plant Cell Reports 1: 61-63

Furner IJ, King J, Gamborg OL (1978) Plant Science Letters 11: 169-176

Gamborg OL, Miller RA, Ojima K (1968) Exp. Cell Res. 50: 151-158

Gosch G, Bajaj YPS, Reinert J (1975) Protoplasma 86: 405-410

Kao KN, Constabel F, Michayluk MR, Gamborg OL (1974) Planta 120: 215-227

Linsmaier EF, Skoog F (1965) Physiol. Plant. 18: 100-127

Nagata T, Takebe I (1971) Planta 99: 12-20

Potrykus I, Harms CT, Lorz H, Thomas E (1977) Molec. gen. Genet. 156: 347-350

Schenk RU, Hildebrandt AC (1972) Can. J. Bot. 50: 199-204

Uchimiya H, Murashige T (1976) Plant Physiol. 57: 424-429

Wallin A, Eriksson T (1973) Physiol. Plant. 28: 33-39

White RP (1954) The Cultivation of Animal and Plant Cells, The Ronald Press Co., New York.