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Cell Division and Differentiation in Protoplasts from Cell Cultures of *Glycine* Species and Leaf Tissue of Soybean

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ABSTRACT

Protoplasts were isolated from cell cultures of G. soja and G. tabacina, respectively. The isolation procedure employed Percoll for the separation and concentration of protoplasts. The cultured protoplasts formed cells which developed into embryo-like structures. Protoplasts also were isolated from leaf tissue of soybean cv. Williams 82. Upon culture, the protoplasts regenerated cell walls and divided to form cell cultures.

ABBREVIATIONS:

2,4-D	2,4-Dichlorophenoxyacetic acid
BA	Benzyladenine

INTRODUCTION

An extensive literature exists on the isolation and successful culture of plant protoplasts (Gamborg and Bottino, 1981). Soybean (Glycine max) and related species of this Genus have been amongst the more difficult plant tissues from which to obtain protoplasts and achieve regeneration of cells and plants. The exception is suspension cultured cells which were amongst the first to be cultured successfully (Kao et al. 1971). Only recently have reports appeared on isolation of protoplasts from soybean leaf tissues (Schwenk et al. 1981) and the culture of protoplasts from pods (Zieg and Outka, 1980) and root tissues (Xu et al. 1982). These studies were performed with Glycine max (soybean) cultivars.

In this publication we report the successful isolation and cell regeneration of protoplasts from cell cultures with embryogenic capability and from leaf tissues of soybean.

MATERIALS AND METHODS

Protoplasts from cell cultures - The cell materials were suspension cultures of Glycine tabacina PI 193232 (SB-11) and of Glycine soja Sieb and Zucc. PI 407065 (SB-26). For details on the development and growth of the cell cultures see Gamborg et al. (1983). The cells of the SB-26 but not the SB-11 line have embryogenic capability which is expressed in liquid media (Gamborg et al. 1983).

The cell cultures were subcultured every 7 days and were grown in the SL medium of Collins and Phillips (1982) modified as described previously (Gamborg et al. 1983).

The optimum age of cells for protoplast isolation was 3-6 days after subculture. Five ml samples of the cultures consisting of a fine suspension of single cells and small aggregates were used as the source of protoplasts. The cells were collected by centrifugation at 1000 rpm for 10 min The pellet was resuspended in 10 ml IS-1 medium consisting of 0.7 mM KH_2PO_4, 6.0 mM $CaCl_2.2H_2O$, 0.15 M sorbitol, 0.15 M mannitol, 0.1 M glucose and 3 mM MES buffer (2-(N-Morpholino) ethanesulfonic acid) at pH 5.6 and centrifuged. After one washing the pellet was dispersed in IS-1 solution in such a manner that the packed cell was 10-20% of the total volume.

Three ml of the cell suspension in IS-1 was then pipetted into a 15 x 60 mm petri dish. Two ml of the enzyme solution in IS-1 was added. The enzyme solution consisted of: 1.6% W/V Cellulysin (Calbiochem - Behring, California), 0.8% Pectolyase Y23 (Seishin Pharmaceutical Company, Japan) and 0.2% Macerase (Yakult Housha Company, Japan). The enzymes were used without desalting.

The dishes were sealed with Parafilm^R and placed in light at 200 lux at 27-28°C. The time required for incubation was 3-4 hrs for SB-11 and 6-8 hrs for SB-26, respectively.

After incubation the protoplast-enzyme preparation was filtered through four layers of cheese cloth followed by a stainless steel filter mesh of 65 um into a conical centrifuge tube. The tubes were centrifuged at 100 x g for 5 min, after which the protoplast pellet was resuspended in 5 ml or less of IS-1 in a 40 ml graduated conical glass tube. Five ml of a 20% Percoll^R solution was pipetted to the bottom of the centrifuge tube. The 20% Percoll solution (Pertoft et al. 1979) was made up of 2 ml Percoll^R, 3 ml distilled water and 5 ml of culture medium at 2 x normal concentration. After centrifugation at 1,000 rpm for 10 min the protoplasts appeared as a narrow band at the interphase between the two solutions.

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Protoplast culture - The protoplasts were collected by a wide bore pasteur pipette and suspended in 0.5 ml culture medium which had the following composition: The SL mineral salts (Collins and Phillips 1982), the vitamins as in B5 (Gamborg 1975) and calcium and multivitamins as in Gamborg et al. (1979). Also added were the following compounds in mg/l: Casamino Acids, 125; L-glutamine, 100; sodium pyruvate, 5; sodium citrate, 10; ribose, 125; sorbitol, 27300; sucrose, 150; m-inositol, 150; glucose, 36000; 2,4-D, 0.22; picloram, .06; BA, 0.1. The medium was adjusted to pH 5.7 and filter sterilized. The final concentration of protoplasts was about 10^5 per ml (Fig. 1). The suspension was dispersed as 0.150 ml liquid droplets in 15 x 60 mm petri dishes, which were sealed with Parafilm[R] and incubated in plastic containers at 23-25°C in diffused light at 200 lux.

SOYBEAN LEAF PROTOPLAST ISOLATION AND CULTURE

The leaf material was taken from seedlings of Glycine max cv. Williams 82, grown aseptically on low salt nutrient agar (See Collins and Phillips, 1982) in jars. The seeds were sterilized as described previously (Gamborg et al. 1983) and germinated in glass jars covered with aluminum foil and incubated in a chamber in 18 hr light at 1,500 lux at 30° and 22° in dark.

The first and second set of nearly expanded leaflets of 10-12 day old seedlings were used. They were cut into 1-2 mm strips immediately after removal from the plants. The sections of 6-10 leaflets were placed in 15 x 100 mm petri dishes with 15 ml of enzyme solution. The enzymes were the same as those used by Schwenk et al. (1981), and were dissolved in IS-1 medium. The mixture was incubated overnight on a gyrotory shaker at 30 rpm at 28-29°C. The isolation and washing procedure was nearly the same as was used for the cell culture protoplasts. The exception was a change in Percoll[R] concentration to 35%. The optimum concentration, to achieve the best separation, varied with the protoplast source but was usually in the range from 20-40%.

The isolated protoplasts were cultured in liquid droplets as described above, except that the culture temperature was 28°C. The petri dishes were placed in opague plastic, containers which permitted diffused light at 200 lux to penetrate (Gamborg, et al. 1981).

RESULTS AND DISCUSSION

The protoplasts from the cell cultures regenerated cell walls after 24-36 hrs. and divisions were observed within 4-5 days (Fig. 2). After four weeks the regular cell culture medium (Gamborg et al. 1983) was added at weekly intervals to the drops at the rate of 1:1 (v/v). After 3-4 weeks the regenerated cell suspensions in the dishes were placed on a shaker. When the cells had become established usually after 2-3 weeks, most of the medium was removed and replaced with liquid El-CA medium (Gamborg et al. 1983) for induction of embryo-genesis. Embryo-like structures as shown in Fig. 3 were observed after incubation for a further 2-3 weeks.

With leaf protoplasts cell wall regeneration occurred after 2-3 days followed by division. After appropriate dilution with liquid media as described for cell culture protoplast a suspension culture was ultimately obtained. During the early stages of cell regeneration and division the inclusion of L-glutamine was critical. The concentration used was 730 mg/per liter.

The described procedures have been successfully used for isolation and culture of protoplasts from cells of Glycine species including soybean. The division of the regenerated cells occurred consistently in the protoplasts in all experiments.

In the initial experiments the purification was achieved by using the Babcock[R] flasks and washing by centrifugation to remove enzymes (Gamborg et al. 1981). This method did not provide good separation and the yields were variable. The Percoll[R] approach gave more reliable results and had no apparent deleterious effect on viability. Experiments also were performed on plating of protoplasts from the cell cultures. Successful cell regeneration and division was obtained when the protoplast population was 10^5 per ml. When the protoplasts were cultured in drops of agar, the apparent survival rate and divisions were very reliable. This is a qualitative assessment but the method may be a valuable approach. The embryo-like structures were produced from cell suspension cultures derived from protoplasts. They were observed in protoplast-derived cell cultures of both SB-11 (G. tabacina) and SB-26 (G. soja).

The embryo-like structures did not show continued development, but their formation could be important in the steps toward plant regeneration which has not yet been observed in Glycine protoplasts. The present results indicate that the protoplasts retain the embryogenic capability observed in the original cell cultures (SB-26).

With respect to SB-11, the original cell culture had not exhibited embryogenesis. The production of embryo-like structures from the protoplast derived cell culture of this species was unexpected, but indicates that embryogenesis may soon be achieved readily also in cultured soybean cells.

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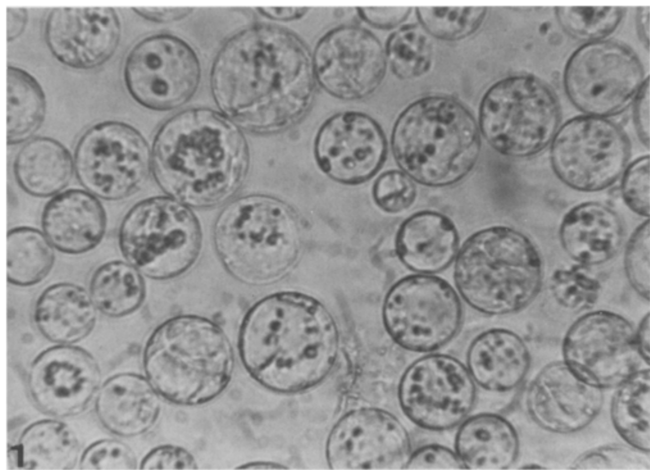

Fig. 1 Protoplasts from cell cultures of G. soja (SB-26).

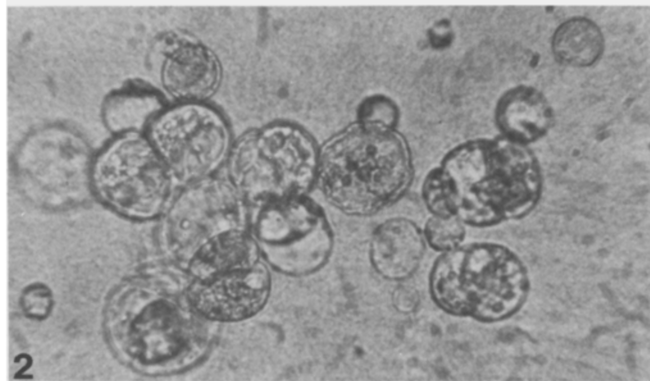

Fig. 2 Divisions in cells regenerated from protoplasts.

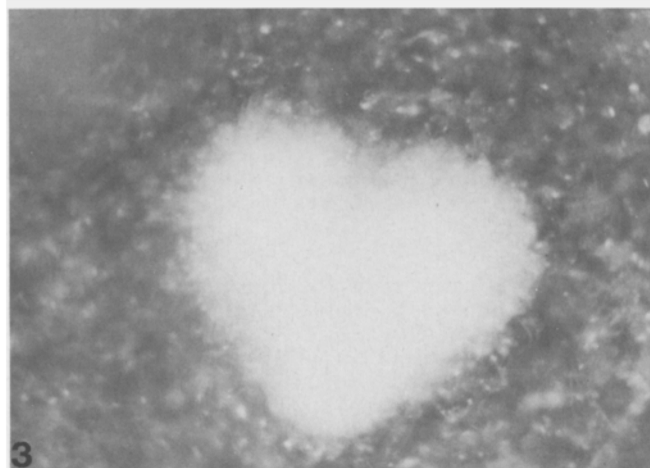

Fig. 3 Embryo-like structure from protoplast-derived cell cultures.