A Simple and Rapid Procedure to Obtain Nucleated Protoplasts from Plant Material

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Received September 6, 1982

The preparation and culture of plant protoplasts are becoming tools of growing importance in studies on regeneration, fusion and genetic transformation of plant cells (Bajaj 1977, Fowke and Gamborg 1980). In recent years some methods have been published on the isolation of protoplasts from tissue and cell cultures (Dietrich et al. 1980, Röper 1980, Strauss and Potrykus 1980). The resulting protoplast fractions are, however, only poorly characterized. Especially when callus or cells cultured in suspension are used as a source for the isolation of protoplasts, wholly or partly undigested cells may contaminate the fraction of protoplasts.

In this paper we present a method for the isolation of a protoplast fraction highly enriched in nucleated protoplasts from callus cultures of Symphytum officinale L. The use of a high concentration of magnesium sulfate as an osmotic stabilizer leads to a floating protoplast fraction. We also describe some cytological characteristics of these protoplasts. Although the procedure reported here was developed using callus tissue of Symphytum officinale as our starting material, it appeared to be readily applicable to other tissues of Symphytum officinale and to callus tissue of other plant species (see Discussion).

Materials and methods

Preparation and culture of callus tissue

Callus was prepared from leaves of Symphytum officinale L. (2n=24) according to a method which will be described in detail elsewhere (H. J. Huizing, manuscript in preparation). In short, the procedure was as follows: mature leaves of S. officinale were surface sterilized in 70% (v/v) ethanol and subsequently in 5% (w/v) sodium hypochlorite. The leaves were rinsed thoroughly with distilled water, cut into slices of 1 cm², and placed in the dark at 28°C on solid B5-media (Gamborg et al. 1968) supplemented with 2, 4-dichlorophenoxy acetic acid (2 mg/l medium) and 6-benzyl aminopurine (1 mg/l medium).

The primary callus was transferred to fresh solid B5-medium. During the experiments callus was transferred to fresh medium every two weeks.

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Isolation and purification of protoplasts

Protoplasts were prepared from callus one week after transfer of the callus to fresh medium. Callus cells were incubated in a solution containing 0.7 M magnesium sulfate, 5 mM calcium chloride and 1% (w/v) sucrose buffered by 5 mM N-morpholinoethanesulfonic acid (MES, obtained from Serva, Heidelberg, F. R. G.) at pH 5.7.

In addition, this medium contained 2% (w/v) cellulase (Sigma, St. Louis, Mo, U.S. A. cat. no. C-7377) and 17% (w/v) pectinase (Sigma, St. Louis, Mo, U. S. A. cat. no. P-5146).

Previously, glycerol had been removed from the pectinase preparation by dialyzing it overnight against running tap water. The recommended procedure consists of incubation of 1 g of callus in 7 ml of the described enzyme solution under gentle shaking (40 rpm) at 28°C for 6 h. After incubation the digest was filtered through nylon gauze (mesh width 100 μm). The filtrate was centrifuged for 30 min at 1500 g. The resulting floating fraction and the pellet were collected separately and fixed in a mixture of methanol and acetic acid (3:1, v/v), previously diluted with 25% distilled water in order to prevent precipitation of magnesium sulfate. The diluted fixative was then substituted for undiluted one. After two more washings in fixative, the preparations were dropped from a height of 1 m onto dry, clean slides and left for drying at room temperature overnight. Both after the filtration step and after centrifugation, samples of protoplasts were counted in a haemacytometer.

For comparison, protoplasts from leaf cells were prepared in the same way. Prior to incubation with enzymes, the leaves were cut into slices of 1 mm width.

Staining procedures

A stock solution (5 μg/ml in distilled water) of 4’, 6 diamidino-2-phenylindole (DAPI, obtained from Serva, Heidelberg, F. R. G.) was diluted 12.5-fold with Mc Ilvaine’s phosphate-citrate buffer, pH 7.0. Protoplast or cell preparations fixed on slides were incubated in this solution at room temperature for 15 min. The slides were rinsed three times with Mc Ilvaine’s buffer, pH 7.0, transferred to a 0.01% (w/v) solution of Blankophor BA 267% (Bayer, Leverkusen, F. R. G.) in distilled water and incubated at room temperature for 20 min. They were rinsed three times with Mc Ilvaine’s buffer, pH 7.0, and mounted in this buffer. The slides were examined with a Zeiss fluorescence microscope equipped with a Ploem epi-condensor and an HBO 50 W mercury lamp. The filter set used included a UG 1-filter for excitation, a chromatic beam splitter at 450 nm, and a barrier filter at 440 nm.

Feulgen-Schiff staining consisted of hydrolysis of preparations in 4 N HCl at 30°C for 45 min and staining with a 1% (w/v) solution of pararosaniline (Chroma, Stuttgart, F. R. G.) in a 22.5 mM potassium disulfite buffer, pH 4.5, at room temperature in the dark for 2 h (Graumann 1953). After staining, the preparations were rinsed three times in freshly prepared sulfite water, and once in distilled water. Subsequently, they were incubated in Mc Ilvaine’s buffer, pH 4.5, for 30 min, rinsed with distilled water, and dried with a stream of compressed air. The preparations
were sealed in Euparal (Roth, Karlsruhe, F. R. G.) and stored at 4°C in the dark to prevent bleaching. For microdensitometry, the absorbance of the nuclei was measured with a Zeiss MPM 01 microscope photometer with a scanning stage according to Tempelaar (1980).

**Results**

**Plasmolysis and cell wall degradation using magnesium sulfate as an osmotic stabilizer**

The application of 0.7 M magnesium sulfate as an osmotic stabilizer during the degradation of cell walls of *Symphytum officinale* yielded several protoplasts per cell (Fig. 1). For 150 randomly chosen non-aggregated cells the number of protoplasts per cell was determined to be on average 2.3±0.05. A steady increase in the total number of protoplasts in the incubation mixture was observed during the first six hours (Fig. 2). Prolonged incubation caused no further increase of the total number of protoplasts. Per gram fresh weight of callus a total number of protoplasts of about $7 \times 10^5$ was found after 6 h of digestion.

**Purification and characterization of protoplasts**

After digestion of cell walls and filtration of the incubation mixture in order to remove large cell clumps, the filtrate was centrifuged as described. By this centrifugation the mixture of cells and protoplasts was separated into a pelleting fraction and a fraction that floated on top of the medium in the centrifuge tube (Fig. 2). In the floating fraction 7-9.8% of the total number of protoplasts was found (range from three experiments). Due to aggregation of protoplasts and cells it was not possible to quantitate the number of protoplasts in the pellet fraction. The floating protoplast fraction was very pure: we never found less than 90% of protoplasts in this fraction (Fig. 4).

In preliminary attempts with 0.7 M sorbitol or 0.7 M mannitol as osmotic stabilizers, no floating fraction was obtained. Instead, the protoplasts pelleted on the remaining undigested cells during centrifugation.

Samples of pellet and floating fraction were fixed and stained with the AT-specific DNA fluorochrome DAPI and the β 1-6 glucan-specific fluorochrome Blankophor BA 267% in order to determine the proportion of nucleus-containing protoplasts in these fractions. In this double staining intact cells showed a clear fluorescence of their walls, besides the bright DAPI-fluorescence of their nuclei. Protoplasts lacked the peripheral fluorescence. Presence of a nucleus was immediately evident form the intense DAPI-fluorescence. Quantitation of the composition of the pellet and the floating fraction with the use of these staining characteristics showed, that the pellet contained 6–10% nucleated protoplasts, while the floating fraction contained 75–80% nucleated protoplasts (range determined in three experiments).

**Determination of the degree of ploidy of the protoplast fraction**

Microdensitometry of fixed, Feulgen-stained nuclei of *Symphytum officinale* protoplasts, prepared from callus cells, revealed a bimodal distribution representing G1
and S+G2 phase cells, respectively, and moreover a higher ploidy level in 4 out of 100 nuclei (Fig. 5).

Because the latter nuclei showed an absorbance approximately four times greater than the G1-phase nuclei and twice the absorbance of S+G2-phase nuclei,

Figs. 1–4. 1, microphotograph of the plasmolysis of a cell of *Symphytum officinale* in a solution of 0.7 M magnesium sulfate. Characteristically, the plasmalemma invaginates on several places, eventually resulting in more than one protoplast per cell. Magnification 400×. 2, time course of the release of protoplasts from an enzymatic digest of callus cells of *Symphytum officinale*, with magnesium sulfate as an osmotic stabilizer. 3, separation of the digestion mixture in a floating fraction and a pelleting fraction through centrifugation for 30 min at 1500×g. 4, microphotograph of an aliquot from the floating fraction. Magnification 160×.
we concluded that these nuclei represent a tetraploid population in our callus culture. The same procedure of protoplast isolation was also successfully applied to leaf material. Here, also a bimodal distribution but only one ploidy level was detected (Fig. 6). The preparations for Figures 5 and 6 were stained with different batches of pararosaniline, which explains the difference in staining intensity of the nuclei in these figures.

It can be deduced from Fig. 5 that at least 4% of the nuclei in the protoplast population isolated from callus were tetraploid. No higher ploidy levels than this were observed.

Figs. 5-6. 5, frequency distribution of the relative DNA-content of nuclei in the floating protoplast fraction, prepared from callus cells of *Symphytum officinale*. 6, frequency distribution of the relative DNA-content of nuclei in the floating protoplast fraction, originating from leaf material of *Symphytum officinale*.

**Discussion**

Quite a number of methods for releasing protoplasts from plant cells have been published in recent years. A qualitative and quantitative comparison of these methods is very difficult in view of the many factors that influence plasmolysis and the yield of protoplasts (Scott, *et al.* 1978, for reviews see Bajaj 1977, Ruesink 1980).

Magnesium sulfate, though only at low concentrations and in combination with potassium chloride, has been used as an osmotic stabilizer in a few previous reports (see Bajaj 1977). Its effect on protoplast release from mold cells has been described in detail by De Vries and Wessels (1975). It appeared that high concentrations of magnesium sulfate (0.5–0.8 M) were very effective in releasing protoplasts from the cells of *Schizopyllum commune*. A floating fraction of protoplasts emerged, which has a high capability to regenerate a cell wall. Our results demonstrate that a high concentration of magnesium sulfate can also be very effectively used to prepare protoplasts from cells of higher plants i.e. from callus cultures and leaves of *Symphytum officinale*. The described method yields a very pure fraction of protoplasts that
is highly enriched in nucleated protoplasts. This same method has also been applied with success to callus cultures of *Mucuna pruriens*, *Phaseolus vulgaris* (Wichers and Huizing, unpublished results), *Nicotiana tabacum* and *Solanum tuberosum* (E. Jacobsen, personal communication).

Among the impurities of the protoplast fraction there were still intact cells. Where characteristics of protoplast fractions are not always given in reports on culture of protoplasts, the possibility cannot be excluded that occasionally whole cells present in a protoplast preparation gave rise to observed cell growth instead of presumed regenerating protoplasts (Dietrich *et al.* 1980, Röper 1980). The occurrence of genetic instability in tissue and cell cultures of plant species renders serious problems in regeneration experiments of plants and in all experiments where a genetically well defined or stable cell population is required (Sunderland 1973, Sheridan 1974, Bayliss 1975, Kibler, *et al.* 1980). The presence of 2, 4-D in the culture media might be responsible for this genetic instability, although its presence does not give a completely satisfactory explanation for the observed phenomena. Bayliss (1975) gives evidence that rather the lack of tissue organization in callus and suspension cultures than the presence of 2, 4-D or any other medium component accounts for the presence of mitotic abnormalities. In our protoplast fraction two different ploidy levels were found. This heterogeneity might originate from the nature of the callus tissue used for protoplast preparation. Kibler *et al.* (1980), using callus and cell suspensions of *Datura innoxia* Mill, showed a correlation between cell division activity and the distribution of ploidy levels. Slowly dividing cells showed a wide range of ploidy levels, while high cell division activity resulted in a population of cells in which the lowest possible ploidy level predominated, while only little polyplody occurred. Since we used rapidly dividing, light brown cells from the outer areas of the callus clumps for the preparation of the protoplasts, our results might reflect a similar effect.

The described procedure represents a rapid method for the preparation of a fraction of nucleated protoplasts not only from callus tissue of *Symphytum officinale* L., but also from other tissue and from other plants. So far we only examined interphase nuclei. The methods described here are now being applied to metaphase cells in order to characterize our cell cultures at the chromosome level.

**Summary**

A simple procedure is described for the isolation of protoplasts from plant cells using a high concentration of magnesium sulfate as an osmotic stabilizer. This new procedure was developed with callus cells of *Symphytum officinale* as a starting material, but was also successfully applied to other tissues and other plants. The floating protoplast fraction obtained had a purity of more than 90% and was highly enriched in nucleated protoplasts (about 80%). Microdensitometry of Feulgen-stained fixed protoplasts from callus cells of *Symphytum officinale* revealed two ploidy levels (diploid and tetraploid), in contrast to protoplasts from leaf material that appeared to be diploid only.
References


