Isolation and culture of suspension-derived protoplasts of *Beta vulgaris* L.

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Abstract

Sugar beet protoplasts (*Beta vulgaris* L.) were isolated from hypocotyl-derived suspension cells and cultured on modified Murashige and Skoog medium supplemented with 5 μM naphthaleneacetic acid (NAA) and 2 μM 6-benzyl-aminopurine (BAP). Protoplasts were plated at a density 1.0 - 1.5 × 10^5 cm^{-3} and incubated in either liquid medium or in medium solidified by 1.2 % agarose, at 25 °C in the dark. Comparison of two methods of culture unequivocally showed the second to be superior. Immobilizing the protoplast in agarose proved to be essential for obtaining sustained protoplast division and reproducible colony formation. The plating efficiency after two weeks of culture, expressed as the percentage of protoplasts which developed to form colonies, reached 40 %. Subsequent subcultures of protoplast-derived callus to regeneration media with different concentrations of BAP (5 μM, 10 μM, 20 μM, 30 μM) resulted in very good callus proliferation at the three lowest concentrations, although organogenesis was not achieved.

Introduction

Sugar beet (*Beta vulgaris* L.), an important agricultural crop, is receiving much attention with regard to the production and improvement of new cultivars. This requires new methods of genetic manipulation; protoplasts are a suitable material to achieve significant progress in sugar beet breeding. Currently, the following problems need solution: (*1*) the introduction of new male sterility traits from *Beta maritima* to *Beta vulgaris* (Hall 1989, Krens *et al.* 1990), (*2*) the transfer of cytoplasmic male sterility to fertile sugar beet (Schlangstedt *et al.* 1991), (*3*) obtaining transformants resistant to diseases and herbicides.

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A prerequisite for the successful application of protoplast techniques in breeding programmes is the development of efficient systems of protoplast isolation, transformation, culture and regeneration into plants (Schlangstedt *et al.* 1992). Considering the great genotype-dependent variability of the sugar beet with regard to its response to *in vitro* conditions (Bhat *et al.* 1985), it would seem extremely important to establish and optimize specialized protocols of protoplast culture and regeneration suitable for selected genotypes of agronomic interest. Our paper presents the results of experiments on the isolation and culture of suspension-derived protoplasts from diploid line PL 1, which was chosen as one of the best lines in Polish agriculture for further genetical manipulation (Majewska-Sawka *et al.* 1990).

Materials and methods

Callus initiation and establishment of suspension cultures: Mature embryos of sugar beet (diploid line PL 1) were isolated from seeds and germinated on half-strength modified Murashige and Skoog medium (MS) with no hormones. Pieces of hypocotyl from one week-old seedlings were placed on MS medium supplemented with 5 μM NAA and 2 μM BAP to induce callus formation. Suspension cultures were initiated from white friable callus, and were maintained in 100 cm^3 flasks containing 20 cm^3 of modified MS medium with the same hormone content as was used for callus induction. The cultures were kept on a rotary shaker (80 rpm) in the dark at 25 °C, and were subcultured every 7 d by transferring approximately 1.5 - 2.0 cm^3 of settled suspension cells to fresh medium. During 1 - 2 months, the size of cell aggregates gradually decreased giving rise to smaller cell clumps suitable for protoplast isolation.

Isolation and culture of protoplasts: Protoplasts were isolated two days after subculture. Routinely, about 1.5 - 2.0 g of suspension cells were placed in 20 cm^3 of enzyme solution containing 2 % *Cellulase Onozuka RS* (*Yakult Honsha Co.*, Tokyo, Japan) and 0.5 % *Macerozyme R-10* (*Yakult Honsha Co.*, Tokyo, Japan) dissolved in salt solution according to Freason *et al.* (1973) with 0.6 M mannitol. Incubation was performed in the dark, at 27 °C for 4 h, without shaking. The protoplast-enzyme mixture was then filtered successively through 60 μm and 20 μm nylon mesh, and protoplasts were collected by centrifugation for 10 min at 75 *g*. The protoplasts were purified by washing twice in salt solution (Freason *et al.* 1973) with 0.6 M mannitol, followed by centrifugation. After the yield was determined by hemocytometer, protoplasts were plated at a density of 1.0 - 1.5 × 10^5 cm^{-3} and cultured in modified MS medium containing 5 μM NAA and 2 μM BAP. Two methods of culture were used: (*1*) liquid MS medium in 60 mm Petri dishes, with a 1 cm^3 supplement of fresh medium once a week, (*2*) MS medium solidified by 1.2 % agarose (*Sea Plaque, FMC Bioproducts,* Rockland, USA).

Agarose disks were cut into segments and floated on 5 cm^3 of liquid medium (replaced by fresh medium every 7 - 10 d), and rotated on a shaker at 80 rpm. All

culture dishes were incubated in growth chambers in the dark at 25 °C. Plating efficiency was determined after two weeks, and was expressed as the percentage of protoplasts which developed to form microcolonies.

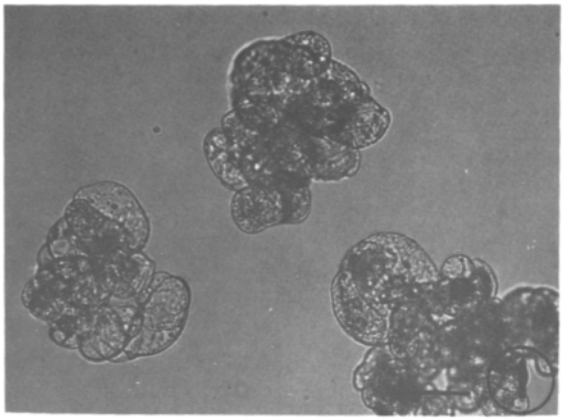
After 2 - 3 weeks, agarose segments with visible colonies were transferred onto 0.25 % soft agarose medium (*Sigma Chemical Co.*, St. Louis. USA) and cultured for the following 2 weeks under light (16/8 h photoperiod, irradiance 90 μmol m^{-2} s^{-1}). The microcalli were then picked up and placed individually on the same medium with a higher agarose concentration (0.5 %). Colonies of about 2 mm in diameter were transferred to regeneration media with 1 % agarose and one of the following concentration of BAP: 5 , 10 , 20 and 30 μM.

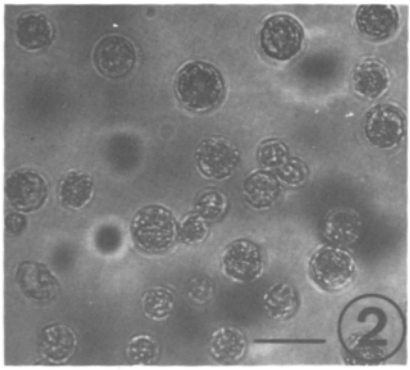
Results and discussion

The ability of protoplasts to undergo division, form calli and regenerate plants depends on many factors, among which the genotype plays a major role. This makes it necessary to develop and optimize procedures for protoplast isolation and culture, suitable for specific genotypes of interest. In our studies of *Beta vulgaris* protoplasts we have used some agronomically important lines cultivated in Poland and Japan, as well as some *Beta maritima* accessions, selected as excellent material for further genetic manipulation (Majewska-Sawka *et al.* 1990). The present paper concentrates on diploid line PL 1, which has shown the greatest capacity to form embryogenic calli and suspension cells (Fig. 6).

The isolation of protoplasts from hypocotyl-derived suspensions of PL 1 presents some advantages over isolation from mesophyll tissue (Majewska-Sawka *et al.* 1990), *e.g.* higher protoplast yield, much better reproducibility of the procedure, and relatively smaller amounts of impurities. Established suspensions consisted of small colonies of round or oval cells rich in cytoplasm (Fig. 1). Digestion of cell clumps with an enzyme solution containing *Cellulase Onozuka RS* and *Maceroenzyme R-10* resulted in the release of many protoplasts ranging in size between 15 and 60 μm. After filtration and purification (Fig. 2), the yield obtained ranged from 0.85 to 1.04 $\times$ 10^8 protoplasts per gram of fresh mass. The protoplasts were plated at a density 1.0 - 1.5 $\times$ 10^5 cm^{-3}, which was previously reported as optimal for several sugar beet genotypes (Szabados and Gaggero 1985, Bhat *et al.* 1985, Lindsey and Jones 1989).

We found a significant difference between the two culture methods tested. Protoplasts maintained in liquid medium underwent few divisions, which were frequently accompanied by budding and the gradual loss of regular shape. Subsequently, these protoplasts aggregated and adhered to the bottom of the dish, and finally turned brown. The addition of 1.2 % agarose to the medium proved to be essential for obtaining sustained divisions and reproducible colony formation. The first divisions were observed on the 3rd day of incubation (Fig. 3), and the second between the 4th and 7th days (Fig. 4). The plating efficiency calculated after two weeks of culture was as high as 40 % (in ten individual experiments from 35.9 to 44.4 %). Similarly, Lindsey and Jones (1989) reported a significant increase in

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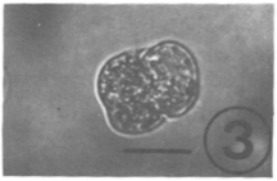
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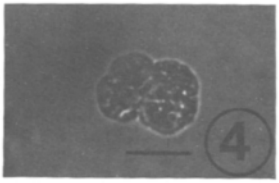
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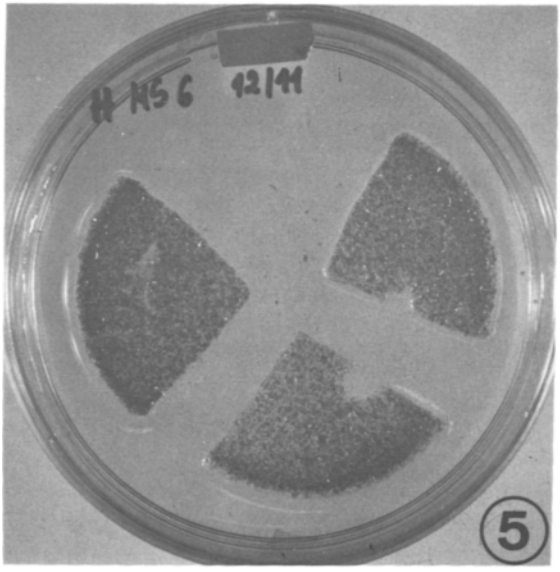
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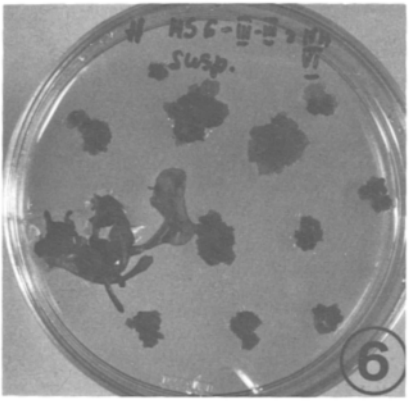
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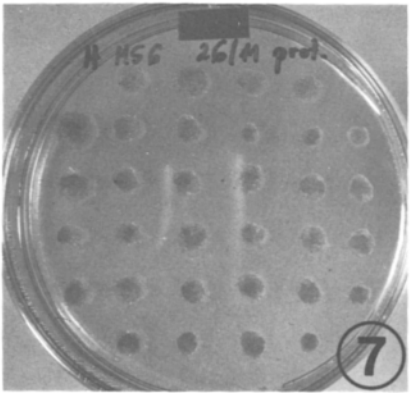
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division efficiency of sugar beet protoplasts when they used the agarose method. Moreover, the immobilization of protoplasts in agarose led to successful culture on simple MS medium, without organic or inorganic additives. On the contrary, previously described attempts to culture protoplasts in liquid MS or PGo media resulted in a relatively low plating efficiency, although in some cases it could be significantly improved by using nutritionally rich media, *e.g.* KM8p (Bhat *et al.* 1985, Bhat *et al.* 1986), conditioned media, or by adding casein hydrolysate and/or yeast extract (Szabados and Gaggero 1985).

Microcolonies, which developed in agarose segments (Fig. 5), were actively growing after subsequent subcultures, and produced a white, friable callus (Fig. 7). Transfer of the callus to regeneration media with a higher agarose concentration (1 %) resulted in the gradual loss of water and rapid proliferation of white, yellow or pale green tissues. The percentage of initially transferred pieces of callus which proliferated successfully was 100 % in medium with 5 μM BAP, 94 % in medium with 10 μM BAP, 86 % in medium with 20 μM BAP, and 7 % in medium with 30 μM BAP. Although organogenesis was not obtained in these experiments, the method described is simple and highly efficient with respect to protoplast division and callus formation. We therefore expect, that screening of *Beta* genotypes for their regeneration ability, together with suspension initiation from highly totipotent tissues, may lead to whole plant regeneration.

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