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Callus Formation from Protoplasts of a Maize Cell Culture*

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Summary. A finely dispersed cell suspension culture from the friable callus of the 'Black Mexican Sweet' line of maize was obtained. Protoplasts from this cell culture, when grown in a simplified medium described here, showed sustained cell divisions and gave rise to callus.

Key words: Maize – Cell suspension culture – Protoplast culture – Callus

Abbreviations
2,4-D = 2,4-dichlorophenoxyaceticacid
SDS = sodium dodecyl sulfate

Introduction

Important advances have recently occurred in cereal protoplast culture. Regeneration of protoplasts giving rise to callus has been reported with rice (Deka and Sen 1976; Cai et al. 1978) maize (Potrykus et al. 1979a) pearl millet (Vasil and Vasil 1979) and sorghum (Brar et al. 1980). Induction of embryogenic callus from protoplasts is reported in pearl millet by Vasil and Vasil (1980). The first report of maize protoplast culture by Potrykus et al. (1977) from stem sections of a specific inbred line has now been reported as non reproducible (Potrykus et al. 1979b).

In maize tissue culture, the genotype of the original explant is known to critically affect the probability of success. Green (1977) and Chourey (unpublished) observed a significantly higher percentage of regenerating scutellar calli from one line than from others under the same culture conditions. Similarly, Potrykus (1979) reported success in protoplast culture in only one cell line out of hundreds of genotypes tested. Thus, it is important to screen a wide source of genotypes to select promising lines. In this communication we describe callus formation from protoplasts of a cell culture initiated from the genotype 'Black Mexican Sweet' line of maize.

Materials and Methods

Callus culture was initiated from the mesocotyl tissue of germinated seeds of 'Black Mexican Sweet', a line of maize reported by Sheridan (1975) to be well suited for suspension culture. A cell suspension was obtained by gently squashing tiny pieces of the callus culture into liquid medium consisting of MS medium supplemented with 2 mg/liter of 2, 4-D (Green and Philips 1975). This cell line was routinely cultured at 7-day intervals at 27°C on a gyrotory shaker at 150 rpm by inoculating 0.25 g fresh weight of cells in 50 ml medium. Growth curve studies on this cell culture were done during a period of 12 days under the above-described conditions. Cells from two or more flasks were checked for microbial contamination at harvest by plating cells and medium on Difco nutrient agar.

For protoplast isolation, cells were harvested between 4-8 days after inoculation in the liquid medium. Fresh weight of 0.75 g of cells was added to 20 ml of filter-sterilized protoplast isolation medium as defined by Potrykus et al. (1979a) and incubated at 30°C for 4 hours in a shaker bath at 40 oscillations per minute. Protoplasts were separated from undigested cellular mass by filtration. First they were passed through a 53 μm filter and then washed with approximately 20 ml of 0.2 M mannitol washing solution, pH 6.0. The filtrate was then passed through a 44 μm filter and washed in the same manner. Cell wall degradation was monitored at this stage by adding a drop of 20% SDS solution (in 0.2 M mannitol) to a drop of protoplasts and examining under the microscope. Total lysis confirmed the release of protoplasts and the absence of undigested cells. The enzyme solution was removed by washing the filtrate three times with mannitol washing solution followed by centrifugation at 500 rpm for 2 to 4 minutes. This

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was followed by three more washings using culture medium consisting of Green's (1977) liquid culture medium supplemented with 0.2 M mannitol, 20 ml/liter coconut milk (obtained from GIBCO) and 250 mg/liter glucose. Hereafter this will be referred to as protoplast culture medium (PCM). Protoplasts were counted using a hemocytometer and the mean value of three independent counts was used to estimate the final concentration. Protoplast viability was determined using fluorescein diacetate (Widholm 1972). Protoplasts were cultured in the PCM at the final density of 1-5 X 10^5 protoplasts per ml unless stated otherwise. Platings were done either in liquid droplets, with 0.6% agar, or 0.5 ml aliquots were mixed with equal quantity of 1.2% liquid agar and placed on a 4 ml lawn of 0.6% agar, plated in advance, in 50 X 9 mm tight lid petri dishes.

Results and Discussion

Explants of mesocotyl tissue from the 'Black Mexican Sweet' line of maize, when cultured according to Green and Phillips (1975), gave rise to a primary callus. This callus was compact and differentiated (showed aberrant root-like structures) and it retained this morphology for 5-6 subsequent cultures. Callus more appropriate for suspension culture was eventually obtained by selecting bud-like structures of white and friable tissue arising spontaneously on the primary callus. This callus is now about 2 years old and is soft and friable (Fig. 1a). It does not regenerate plants when 2,4-D concentration in the medium is lowered. However, it does undergo certain morphological alterations leading to the formation of root-like structures (Fig. 1b) under prolonged culture in Green and Phillips medium with 0.25 mg/liter 2,4-D.

Inoculations of tiny pieces of white and friable callus to the liquid medium led to a cell suspension which initially consisted of large aggregates of cells. Repeated filtrations through 210 μm nylon filter sieves during 3 months of subculturing resulted in a finely dispersed cell suspension, similar to the well-established tobacco and carrot cell suspensions. At this stage, the cell culture was used to determine the growth curve shown in Figure 2. During the log phase (4 to 8 days after inoculation) of growth, the majority of cells in the suspension culture appeared to divide once every 2 days as determined from the linear rate of doubling of fresh weight of cell mass. Most of the cell population at this stage consisted of 2-20 celled colonies (Fig. 3a); however, the presence of single cells which were elongated and vacuolated was also recorded. Protoplasts were readily obtained from the cells in the log phase when treated with the enzyme solution (Fig. 3b). More than 80% of the cells yielded protoplasts during a 4-hour treatment in the enzyme solution. Cells grown beyond the log phase seemed resistant to enzymatic digestion of the cell wall because poor yields of protoplasts were recovered even after overnight incubation in the enzyme solution. We conclude that the log phase of the cell cycle represents a stage of optimum physiological competence for the release of protoplasts from this cell culture of maize. Routine checks of protoplast viability before plating showed that about 95% of the total protoplasts were viable. Potrykus et al. (1979a) also noted that the 'quality of the cell suspension' was an important factor in the release of protoplasts in their maize cell line.

Observations on the initial progress of growth were conducted on protoplasts cultured in liquid medium. At the end of a 48 hour incubation period, nearly half of the total protoplasts became elliptical in shape (Fig. 3c) presumably due to cell wall formation. The remaining protoplasts were either still spherical and without any signs of cell wall formation or had turned brown and even-

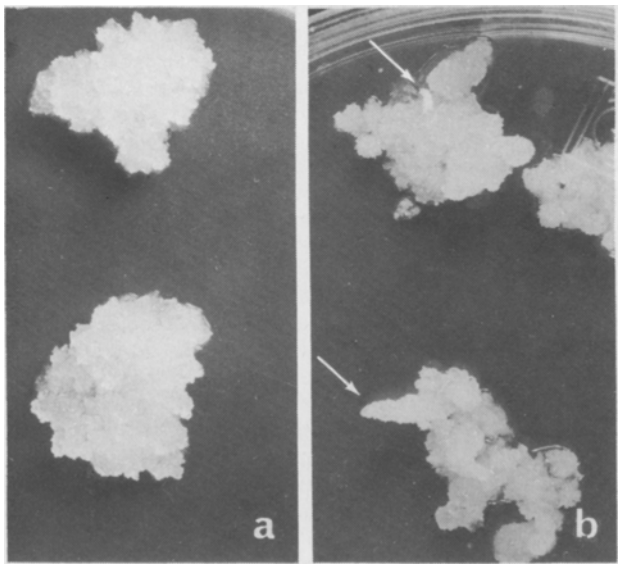

Fig. 1a. and b. Mass of callus tissue on Green's medium a with 2-4 mg/l 2, 4-D and b with 0.25 mg/l 2, 4-D: arrows point to the root-like structures

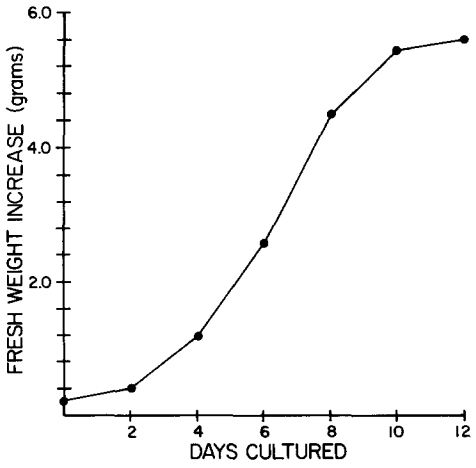

Fig. 2. Growth pattern of the maize cell suspension culture

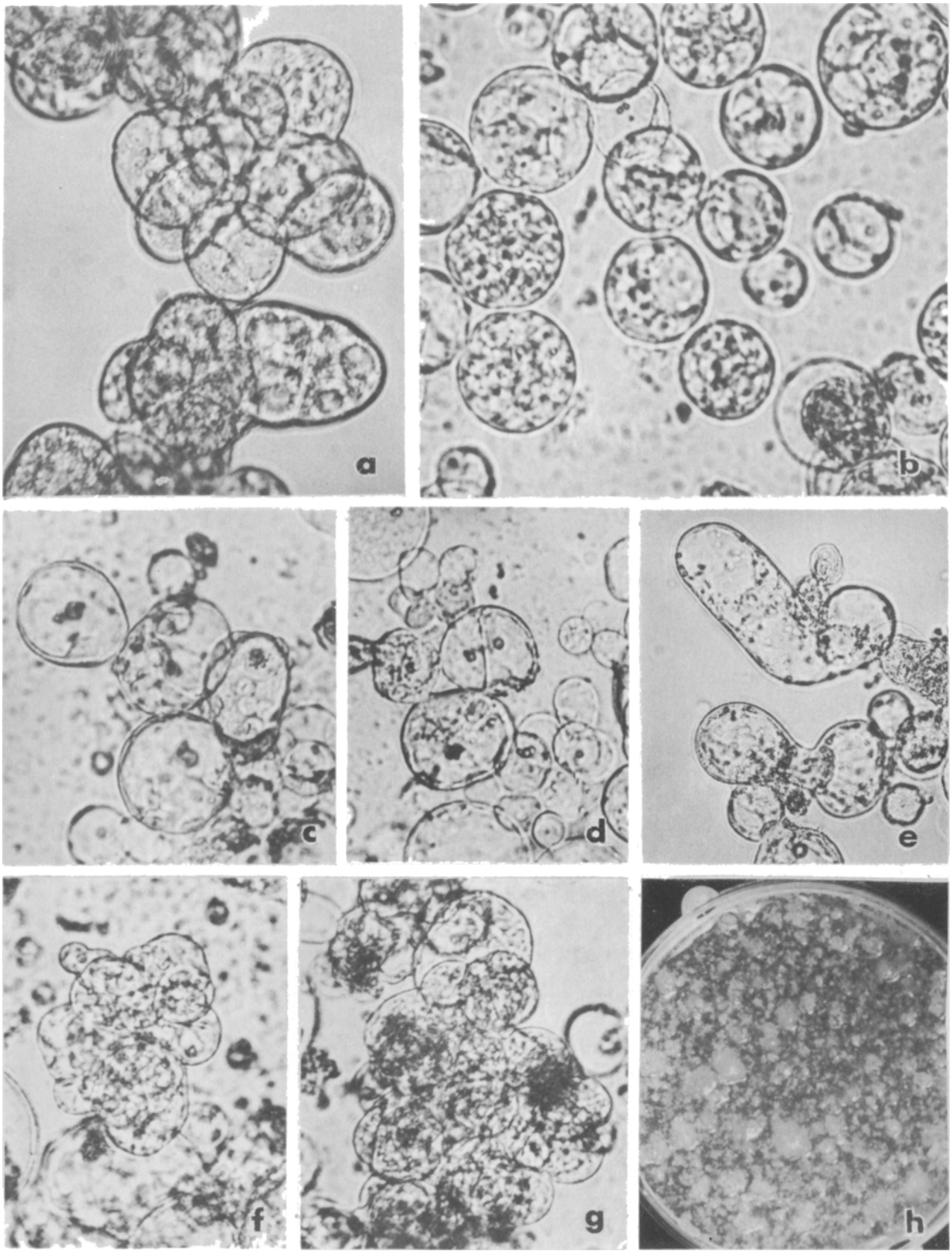

Fig. 3a.-h. Various stages of maize callus formation from protoplasts: **a** cell aggregates in the suspension culture before enzyme treatment (1200 X); **b** freshly isolated protoplasts (640 X); **c** elliptical cells after 2 days of culture (640 X); **d** a dividing cell and **e** a budding cell after 4 days (640 X); **f** and **g** cells clumps after 10 and 15 days of culture, respectively (640 X) **h** numerous cell colonies after 21 days

tually died. By the 4th day (Fig. 3d), more than 30% of the cells showed the presence of a cell plate within the confines of the cell, indicating that a cell division had been completed. The presence of a cell plate, however, was not restricted to the equatorial region of the original cell. Peanut-like structures showing two enlarged cells joined by a constriction in the center (Fig. 3e) were also present. Presumably, these cells arose through budding (Vasil and Vasil 1974; Potrykus 1979) and did not show any subsequent cell divisions. Presence of long and elongated cells was also recorded (Fig. 3e). Again, these cells showed no cell divisions. Sustained cell divisions were restricted to the class of cells which developed cell plates without any apparent increase in size (Fig. 3f and g). Approximately 25% of the plated protoplasts gave rise to cell colonies. In about 3 weeks, abundant cell colonies were visible to the unaided eye (Fig. 3h). These colonies were then transferred to fresh medium where they gave rise to larger colonies similar to the one shown in Figure 1a, in about 4 weeks time.

Protoplasts of this cell line of maize have now been reproducibly cultured in numerous experiments. No significant differences in the plating efficiency have been observed between solid and liquid media. A minimum plating density of 5×10^4 protoplasts per ml seems to be a critical requirement to obtain sustained cell divisions. Platings of lower densities have failed to show cell divisions. Attempts are now in progress to initiate liquid cell cultures from calli obtained through protoplast culture, and to attain differentiation and regeneration of plants.

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