Plant Cell Reports (1992) 10:591–594

Plant Cell Reports

Effect of nurse cultures on the production of macro-calli and fertile plants from maize embryogenic suspension culture protoplasts

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Received July 25, 1991/Revised version received September 30, 1991 – Communicated by I. K. Vasil

Abstract Fertile plants have been obtained from maize (*Zea mays* L.) embryogenic suspension culture protoplasts. Friable, embryogenic callus initiated from an immature embryo from a cross involving the genotypes A188, B73, and Black Mexican sweetcorn was used to establish a rapidly growing embryogenic suspension culture. After nine months in culture, high yields of viable protoplasts (30x 10^6/ gram fresh weight) were obtained following a 1.5 hour enzymatic digestion. Protoplasts cultured with feeder cells divided and formed embryogenic callus, from which male and female fertile plants were regenerated. Protoplast-derived R_1 plants were self-pollinated and immature R_2 embryos isolated for callus initiation. Female fertile plants have also been produced from protoplasts isolated from an R_2-derived suspension culture. Significant interactions between protoplast and feeder-cell lines were observed.

Abbreviations. BC, backcross; BMS, Black Mexican Sweetcorn; 2,4-D, 2,4-dichlorophenoxyacetic acid; PWS, protoplast wash solution (0.2 M mannitol, 80 mM $CaCl_2$); FDA, fluorescein diacetate; ABA, abscisic acid

Introduction

Utilization of somatic cell systems for the genetic improvement of agronomically important cereal crops has been hampered by the lack of efficient methods for plant regeneration from protoplasts. Recent success in fertile plant recovery from maize protoplasts (Mórocz et al. 1990, Prioli and Söndahl 1989, Shillito et al. 1989) has paved the way for genetic improvement of this important crop plant through free DNA uptake or somatic cell hybridization. Unfortunately, despite considerable progress, fertile plant regeneration from maize protoplasts is not yet routine. In this report, we describe fertile plant regeneration from protoplasts of two separate maize cell suspension lines.

The use of nurse cultures and/or conditioned medium has been previously reported to promote the growth of low density platings of BMS cells (Smith et al. 1984), BMS protoplasts (Birnberg et al. 1988, Ludwig et al. 1985, Somers et al. 1987) and protoplasts from embryogenic suspension cultures (Imbrie-Milligan and Hodges 1986, Lyznik et al. 1989, Prioli and Söndahl 1989, Rhodes et al. 1988a, Shillito et al. 1989). We discuss the importance of embryogenic suspension culture development and the use of feeder cells to obtain efficient regeneration from maize protoplasts, and provide evidence for complex interactions between feeder cells and protoplast sources.

Materials and methods

Genotypes A188, Black Mexican Sweetcorn (BMS) and two germplasm sources ("High Type II", B73 BC5 and BC6) specifically developed for superior tissue culture performance were used singly or in crosses for establishing suspension cultures. "High Type II" was derived from an A188 X B73 cross (unpublished; Armstrong et al., Maize Genetics Cooperation Newsletter 65: 92-93). B73 BC5 and BC6 are fifth and sixth backcross generation recoveries of B73, derived from an initial A188 X B73 cross, with selection imposed at each backcross generation for high-frequency Type II callus production (Armstrong et al., in press). See Table 1 for specific genetic backgrounds of each suspension line used in this study.

Table 1 Genotypic background of suspension lines used for protoplast isolation and/or nurse cells.

Source for protoplasts and feeders		
21E3	=	(A188 x BMS) x High Type II
203-12A	=	B73 BC5 S1
M428-3	=	Established from immature R2 embryo from protoplast-derived plant from cell line 21E3
Source for feeders only		
201-23X	=	B73 BC5 S1
153 x 234	=	High Type II x [A1 a2 C C2 R - scm2]
M007 x M003	=	High Type II x (A188 x BMS)
M019-4	=	High Type II
M413-6ED	=	B73 BC6 S2
M512-4	=	High Type II
BMS	=	Black Mexican Sweet

Callus and suspension culture initiation. Immature embryos 1-2 mm in length (9-12 days post-pollination) were placed embryo axis side down onto Phytagel™-solidified, (0.2%; Sigma Chemical Co.) modified N6 (Chu et al 1975) medium containing 1 mg/l 2,4-D, 100 mg/l casamino acids and 25 mM proline (N6 1-100-25; Armstrong and Green, 1985).

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Cultures were incubated in the dark at 28°C. Type-II embryogenic callus was selected and transferred to fresh N6 1-100-25 medium every 14 days. Suspension cultures were initiated by adding ~1 gram of highly friable embryogenic callus to 50 mls liquid N6 1-100-25 in a 250 ml Erlenmeyer flask. Suspension cultures were incubated on an orbital shaker at 150 rpms, in the dark, at 28°C. Suspensions were subcultured every 7 days by transferring 3 mls packed cell volume into 50 mls fresh N6 1-100-25 medium. Midway through each subculture cycle, liquid media was removed by pipetting, and replaced with fresh N6 1-100-25.

Protoplast isolation. Suspension cultures were selected for protoplast isolation on the basis of rapid growth rate and an abundance of small, densely cytoplasmic cells in clumps of ~20 to 400 cells. Suspension culture cells were collected on a 74μ screen 1 day post-subculture and weighed. One gram (fresh weight) of cells was placed in 20 mls of filter-sterilized enzyme solution in a 100x25 mm Petri dish, and incubated for 1.5 hours on an orbital shaker, 50 rpms, at 28°C, in the dark. The enzyme solution consisted of 2% cellulase (CEL, Worthington Biochemical Company) 0.1% pectolyase Y-23 (Seishin Pharmaceutical), and 0.2% Bovine serum albumin (Sigma Chemical Company) dissolved in 0.2 M mannitol, 80 mM calcium chloride ("protoplast wash solution" = PWS). The pH of the enzyme solution was in the range of 4.0 - 4.5. Protoplasts were separated from undigested cell clumps by filtering through a 46μ mesh then transferring to 16x125 mm round-bottom test-tubes and centrifuging at 100x g for 8 minutes. The supernatant was removed, and the protoplast pellet re-suspended in 10 mls of PWS. Protoplasts suspended in 10 mls of PWS were further purified by centrifugation over 2 mls of 20% sucrose at 100x g for 10 minutes and the protoplasts collected from the PWS:sucrose interface. Protoplast sample volume was adjusted to 10 mls with PWS and protoplast yield determined by hemacytometer count.

Protoplast culture. Protoplasts in PWS were pelleted by centrifugation (100x g, 8 minutes), the supernatant removed, and protoplasts re-suspended in N6 1-100-25 medium with 8% mannitol at a density 2x the desired plating density (generally 5 x 10^5 protoplasts/ml). An equal volume of N6 1-100-25 8% mannitol, 2.4% Seaplaque agarose (FMC) was added to the protoplasts, which were then plated as a modified bead culture (Shillito et al. 1983). One ml aliquots of the protoplast mixture were plated as a strip across the bottom of a 20x60 mm dish (Nunc). After solidification, 4 mls liquid N6 1-100-25 8% mannitol +/- nurse culture (1 volume suspension culture:1volume N6 1-100-25 16% mannitol) was added. The plates were wrapped with parafilm and incubated at 28°C in the dark on an orbital shaker at 50 rpm. After 7d, the liquid overlay media and ~80% of the feeder cells were removed and replaced with 4 mls of N6 1-100-25 4% mannitol liquid medium. At 14 days the overlay medium was removed, the plates were rinsed with 5 mls sterile water (up to 5 times) to remove all feeder cells, and 4 mls of N6 1-100-25 was added. About 14 days later, the agarose-embedded protoplasts were transferred to Phytagel-solidified N6 1-100-25. Macro-calli were visible approximately 7-14 days later, and were individually transfered to fresh N6 1-100-25 medium.

Plant Regeneration. Independent protoplast-derived calli were transferred from N6 1-100-25 to MS (Murashige and Skoog 1962) medium with 2% sucrose, 0.1 mg/l 2,4-D and 10^{-7} M ABA for 10-14 days, and then transferred to N6 medium with 6% sucrose (no plant growth regulators or proline) for 10 to 14 days. For both of these regeneration steps, the cultures were maintained at 28°C in the dark. Mature somatic embryos were moved to MS 0-D medium (2% sucrose, no growth regulators) for conversion into plants (25°C, 16:8 hour light:dark cycle). Rooted plants were transferred to 25x200 mm glass test-tubes containing 15 mls MS 0-D solidified with 0.6% agarose (Sigma type I, Low EEO) for further development. Well developed plants were transferred to soil (Metro Mix 350) in 3 inch pots and hardened-off. Plants at approximately the 4-leaf stage were transplanted to 12" diameter pots and grown under greenhouse conditions.

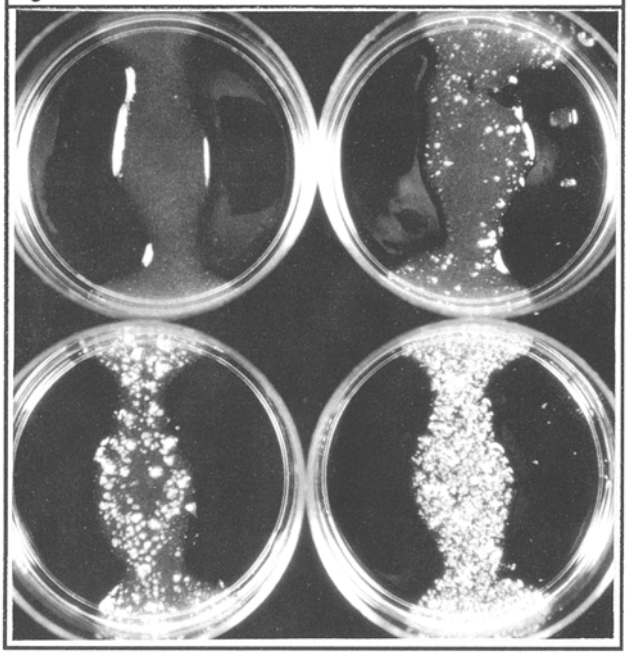
Results

A 2.5 month old embryogenic suspension culture, 21E3, was used in initial experiments to determine optimal protoplast isolation and culture conditions. Many combinations of suspension cell pre-treatments, enzyme formulations, digestion times, plating techniques, media formulations and nurse culture parameters were tested in 32 experiments over a 5 month time period before the first sustained divisions were observed. Concurrently, the 21E3 culture was visually selected for small clumps of densely cytoplasmic cells. At 7 months (9 months from explant), the suspension culture consisted almost entirely of small, densely cytoplasmic cells. Protoplast yields were as high as 30x 10^6 per gram fresh weight. Purity of the preparation (96%) was determined by the cell wall fluorescence stain, calcofluor white (Galbraith 1981) (Polysciences). Viability staining (FDA, Widholm 1972) indicated that the contaminants were dead cell debris. Protoplasts cultured with either 21E3 or BMS nurse cells formed micro-colonies after five weeks. A total of 173 plants were regenerated from protoplast-derived embryogenic calli in one experiment. Complete fertility was observed for many of the plants and progeny were obtained from both self and sib pollinations. Line M428-3, used in subsequent experiments, was developed from a 21E3 protoplast derived R_2 immature embryo.

Additional experiments studied the plating efficiencies of 21E3 protoplasts with 5 different nurse cell lines (21E3, 203-12A, 201-23X, 153x234 and a mixture of 21E3 nurse cells and BMS conditioned medium). The number of macroscopic colonies after 3 weeks of culture was noted for each of the feeder treatments. The least effective feeder line was 21E3 itself (0.000015% plating efficiency) and the most effective feeder was line 203-12A (0.02 % plating efficiency). These experiments were not continued on to plant production.

A second set of experiments was conducted with a two year old suspension line with a B73 genetic background (line 203-12A). Protoplast yields averaged 28 million per gram of cells digested (five independent experiments). While 203-12A was the most effective feeder of the lines tested for 21E3 protoplasts, the reciprocal treatment of 203-12A protoplasts and 21E3 nurse cells did not promote colony formation. The 203-12A protoplasts divided quite well however, in response to several other feeders (Figure 1). BMS also proved to be a very effective feeder for 203-12A protoplasts (data not shown). Up to 10% of the protoplasts divided after 4 days of culture and 0.25% formed macroscopic colonies. One hundred colonies from these experiments were further cultured in an attempt to regenerate plants. Two colonies produced small, green leaf-like structures, but no plants were obtained.

Figure 1



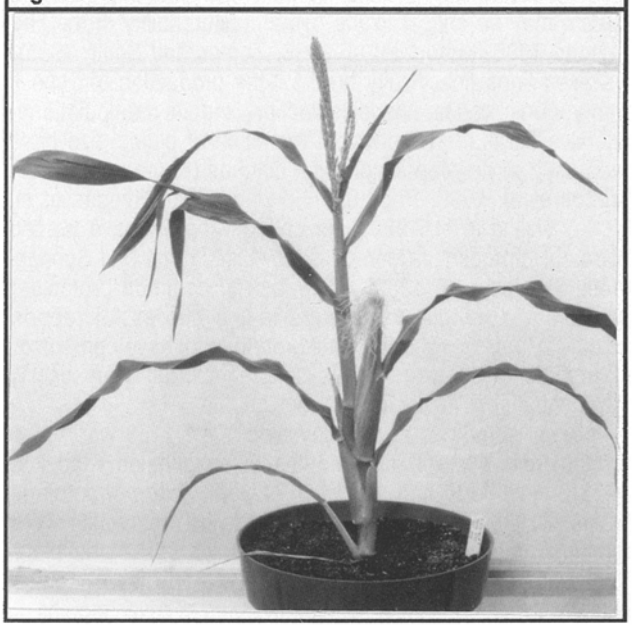
Colony formation from 5 x10^5 203-12A protoplasts 4 weeks post-isolation.

Key to Nurse Culture Treatments:

Plate	Nurse Culture	Colonies
Upper-left	21E3	0
Upper-right	203-12A	220
Lower-left	201-23X	435
Lower-right	(M007xM003)-1E3	1215

A third set of experiments was conducted with M428-3, a four month old suspension cell line. Protoplast yields in five early experiments ranged from less than 1x10^6 to 35x10^6 protoplasts per gram and only a few non-embryogenic colonies were recovered. After 7 months in culture, protoplast yields stabilized and embryogenic calli were obtained. In an experiment using non-regenerable BMS feeder cells to eliminate possible contamination of the protoplast culture with another totipotent cell line, 83 colonies were obtained from 7.5 million protoplasts. After further culture, 65 of the 83 colonies (81%) developed into embryogenic callus and produced plants (Figure 2). All plants were male sterile, therefore pollen from non-cultured control plants was used to pollinate plants representing 42 independent lines. Progeny were produced from 16 of these lines.

Figure 2



Female fertile plant from M428-3 protoplasts

Additional experiments demonstrated that other nurse cultures were more effective than BMS for M428-3 protoplasts (Table 2). The largest number of colonies were obtained when using cell lines M413-6 and M512-4 as feeders.

Table 2 Influence of feeder cell source on colony formation from M428-3 protoplasts.

	Number of Colonies per Plate*			
Feeder Line	Rep 1	Rep 2	Rep 3	ave
No Feeder	0	0	0	0
BMS	0	2	0	0.6
M428-3	0	0	0	0
203-12A	0	0	0	0
M019-4	0	0	0	0
M413-6E.D	285	524	315	375
M512-4	212	121	333	222

* Protoplasts plated in N6 1-100-25 8% mannitol, 1.2 % low melting point agarose at 5x 105 protoplasts per ml, 1 ml per 20x60 mm plate.

Discussion

The totipotency of maize suspension culture protoplasts has been previously demonstrated (Mórocz et al. 1990, Prioli and Söndahl 1989, Shillito et al. 1989). Further evidence of the usefulness of maize suspension cultures for the isolation and culture of totipotent protoplasts is illustrated here with the recovery of fertile plants from two different suspension cultures. In common to these reports is the use of suspension cultures typically characterized as having large numbers of small, densely cytoplasmic cells in small aggregates.

The parameters for development of such suspension cultures have not been clearly defined. Genetic background of the initial explant may be critical to the overall cultureability of the line (Lupotto 1986, Mórocz et al. 1990, Tomes and Smith 1985). We used lines specifically bred for the production of Type II embryogenic callus. Visual selection, media manipulations, selective filtration and physical reduction of clump size have been used to develop suspension cultures (Kamo et al. 1987, Mórocz et al. 1990, Prioli and Söndahl 1989, Rhodes et al. 1988a, Shillito et al. 1989) yet only a very few have yielded totipotent protoplasts (Mórocz et al. 1990, Prioli and Söndahl 1989, Shillito et al. 1989). The lack of common protoplast isolation and culture parameters in the successful reports indicates that the genetic background and development of appropriate suspension cultures is more important than specific enzyme, buffer or media formulations.

A time factor may also be involved. Line 21E3 was seven months from explant (five months in suspension) and line M428-3 was four months from explant (three months in suspension) before the first high yields of protoplasts were obtained. Successful divisions, callus formation and plant regeneration did not occur for another two and three months respectively. In previous reports, a minimum of seven months of culture passed before successful isolation of totipotent protoplasts (Prioli and Söndahl 1989, Shillito et al. 1989). Recently, Mórocz et al. (1990) reported successful isolation and culture of protoplasts from a three week old suspension (70 days from embryo explant). The embryo's parentage, however, included long-term culture (30 and 10-month) R_0 plants, indicating a possible "extended culture period" effect necessary for efficient release of totipotent protoplasts. The "window" for isolating totipotent protoplasts from suspension cultures may be of short duration and highly variable from line to line. With continued culture of suspensions, protoplast yields and division rates increase, but recovery of completely fertile plants becomes increasingly difficult (for example, lines 203-12A, M428-3).

Nurse cultures were necessary for protoplast division in our experiments. Nurse cultures have previously been reported as beneficial for maize protoplast division (Imbre-Milligan and Hodges 1986, Ludwig et al. 1985, Lyznik et al. 1989, Prioli and Söndahl 1989, Rhodes et al. 1988a, Shillito et al. 1989). We observed a strong interaction between the sources of protoplasts and nurse cultures. BMS suspension cells were the optimum feeder for 203-12A protoplasts, but only marginally increased colony formation from M428-3 protoplasts. Birnberg et al. (1988) reported on a partially characterized Conditioned Medium Factor(s) (CMF). The differences in the feeder response of M428-3 and 203-12 protoplasts may be due to the lack of receptiveness of the M428-3 protoplasts to the CMF(s) produced by the BMS feeder cells. Because the M428-3 and 203-12A protoplasts were not compared in the same experiment, the results could also be explained by differences in the amount of CMF(s) produced in the BMS cells used for each protoplast line. Our data (table 2) does indicate that the amount and/or composition of CMF(s) produced by embryogenic cells may vary from one suspension culture to the next. The optimum nurse culture needs to be determined empirically for each protoplast source.

The routine conversion of maize protoplasts to fertile plants is a key component of maize genetic engineering technology. Rhodes et al. (1988b) produced sterile transgenic corn plants by the introduction of foreign DNA into protoplasts. We have recovered transgenic embryogenic calli through PEG-mediated transformation of suspension-derived protoplasts (unpublished results), confirming the potential utility of protoplasts for the genetic improvement of maize. Additional research is needed on routine development of young embryogenic suspension cultures capable of releasing large numbers of totipotent protoplasts and on protoplast:nurse-culture interactions for improved colony development before this potential can be fully realized.

Acknowledgements

The authors thank Debra Salomone and Briana Dennehey for their technical assistance.

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