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Formation of callus and somatic embryos from protoplasts of a commercial hybrid of maize (*Zea mays* L.)

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Summary. Protoplasts isolated from a totipotent embryogenic cell suspension culture of *Zea mays* L. (cultivar 'Dekalb XL82') underwent sustained cell divisions when cultured in liquid as well as agarose media. Optimal colony formation (5%) occurred in a liquid medium containing 0.5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D). A soft and unorganized callus was formed when the protocolonies were transferred to agar solidified suspension maintenance medium. Compact, organized and yellow to pale green folded structures and somatic embryos were formed upon subsequent transfer of this callus to a low 2,4-D medium. Clusters of somatic embryos germinated precociously but no plants were recovered.

Key words: Cereals – Gramineae – Maize – Protoplasts – Somatic embryogenesis – Suspension culture – *Zea mays*

Introduction

Sustained cell divisions and callus formation have been reported previously in protoplasts isolated from suspension cultures of *Zea mays* (Chourey and Zurawski 1981; Ludwig et al. 1985). So far this has been possible only in a single cultivar of maize, namely 'Black Mexican Sweet'.

Recently, transient expression of foreign genes in, as well as stable transformation of, protoplasts isolated from a 'Black Mexican Sweet' suspension culture of maize has been achieved by electroporation (Fromm et al. 1985, 1986). The cell lines used in each of the above experiments were comprised predominantly of aneuploid and polyploid cells, and showed no competence for morphogenesis or plant regeneration. The use of embryogenic cell suspension cultures has previously been shown to be advantageous for the recovery of somatic embryos and plants from protoplasts of the Gramineae (Vasil and Vasil 1980; Srinivasan and Vasil 1986; IK Vasil and Vasil 1986; Yamada et al. 1986).

In order to further exploit recent successes in the somatic hybridization (Ozias-Akins et al. 1986; Tabaeizadeh et al. 1986) as well as transformation of gramineous species (Fromm et al. 1986), it is important to extend these advances to protoplasts isolated from morphogenically competent cell lines of maize. In a previous report we described the regeneration of plants from an embryogenic cell suspension culture of a commercial hybrid cultivar ('Dekalb XL82') of maize (V Vasil and Vasil 1986). We now report sustained cell divisions in protoplasts, and the formation and precocious germination of somatic embryos in protoplast-derived calli of this morphogenic cell line of maize.

Materials and methods

Isolation and maintenance of cell suspension cultures

An embryogenic cell suspension culture, designated as line M1, was established from Type II friable embryogenic callus obtained from immature embryos of *Zea mays* L. in September-October, 1984 (V Vasil and Vasil 1986). Two separate cell lines, designated as M2 and M4, were isolated from line M1 within nine months after its initial establishment. The suspension cultures were maintained in N_6 medium (Chu et al. 1976) that was supplemented with 1.25 mg/l 2,4-D, 575 mg/l (5 mM) L-proline, 200 mg/l casein hydrolysate, 2% sucrose and 0.02 mg/l abscisic acid (ABA) (= suspension maintenance medium). For weekly subcultures, 8 ml of the suspension was pipetted into 25 ml fresh suspension maintenance medium in a 125 ml Erlenmeyer flask. The cultures were incubated in the dark at 27 °C on a gyratory shaker at 150 rpm. Fresh weights of the suspensions were determined by pipetting 1 ml samples on to pre-weighed filter papers, draining under vacuum, and weighing again.

Table 1. Growth, yield of protoplasts and spontaneous fusion, and efficiency of colony formation from protoplasts in lines M1, M2 and M4 (data based on two separate experiments, each with three replicates)

Suspension line (age in months)	Fresh weight (mg/l) after 7 days	Protoplast yield/g fresh wt	Spontaneous fusion (%)	Colony formation (%)
M1 (15)	572	2×10^6	5–10	3–5
M2 (6)	688	3×10^6	10–15	3
M4 (6)	812	5×10^6	20–30	4

Protoplast isolation and culture

The suspension cultures were subcultured 2–3 days prior to protoplast isolation. Four to 5 ml of the settled cells (1 gm fresh weight) were mixed with 50 ml filter-sterilized enzyme solution and distributed equally in three 100×15 mm Petri dishes. Protoplasts were released after 5–6 h at room temperature on a gyratory shaker at 50–60 rpm. The enzyme solution was prepared by dissolving cellulase Onozuka RS (3%) and pectinase Serva (1%) in a buffer solution consisting of 7 mM $CaCl_2 \cdot 2\,H_2O$, 0.7 mM $NaH_2PO_4 \cdot H_2O$, 0.5 M mannitol and 3 mM MES at pH 5.6. The $CaCl_2 \cdot 2\,H_2O$ tended to cause crystal formation and since its presence was not found to be critical, it was eliminated from the solution in later experiments.

The protoplast-enzyme mixture was filtered first through a double layer of Miracloth and then successively through 100, 50 and 25 μm stainless steel filters to remove undigested cells and larger spontaneous fusion products. The protoplasts were collected by centrifugation ($100\times g$, 3 min) and washed three times with the buffer solution. The washed protoplasts were counted in a Batch Counting Chamber (Hausser Scientific, PA). Calcofluor White ST (0.1%) was used to determine the presence or absence of cell wall in the protoplasts (Nagata and Takebe 1970). Kao and Michayluk's (1975) modified KM medium (Vasil and Vasil 1980), adjusted to pH 5.6 and filter sterilised, was used for the culture of protoplasts. The KM medium was supplemented with 0.45 M glucose, and 2,4-D (0.1–2 mg/l) alone or in combination with zeatin (0.25 mg/l). The osmolarity of the enzyme solution, buffer, and the culture medium was adjusted to 650 m osm · kg/l H_2O. The protoplasts were cultured at a density of $1–3\times10^5$/ml in 1–1.5 ml liquid medium or in different concentrations (0.3 to 1.2%) of low-melting-point Seaplaque agarose (FMC Corp., Rockland, ME) in CoStar dishes (60×15 mm). The cultures were sealed with Parafilm and incubated in the dark at 27 °C in growth chambers.

Fresh medium (0.5 ml) with 0.3 M glucose was added to the cultures after 10–18 d. After another week, the protoplast-derived colonies were pipetted (0.75–1 ml) on agar (0.8%) solidified suspension maintenance medium with 2,4-D (0–1.25 mg/l), either alone or in combination with zeatin or benzylaminopurine (0.1–1 mg/l). For growth and organization, small pieces (ca. 5 mm^2) of the resulting 3–4 week old calli were transferred to various media.

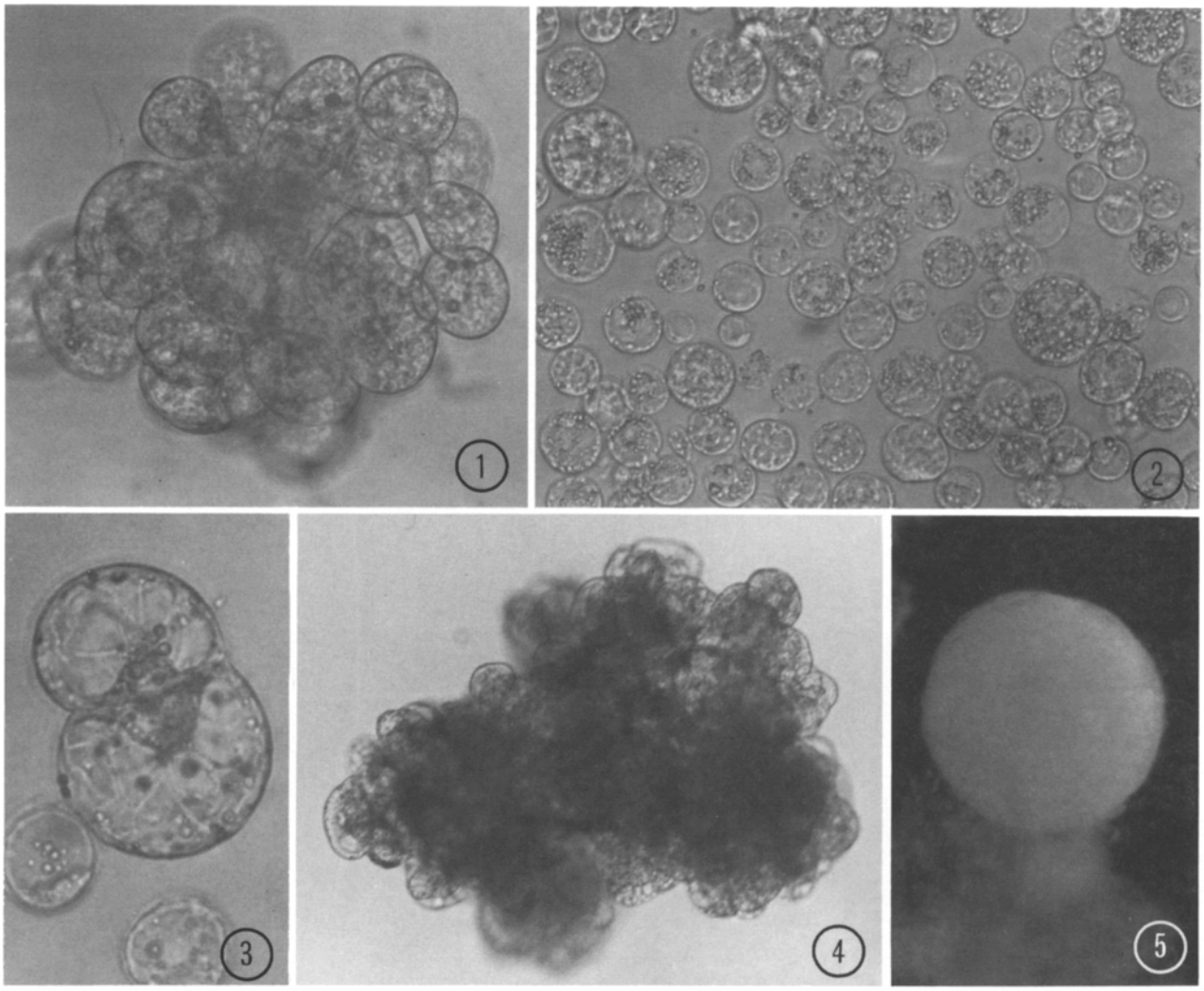
Results

Formation of protocolonies

The original suspension culture line M1 was embryogenic and, as previously described (V Vasil and Vasil 1986), produced somatic embryos and plants upon plating on agar solidified medium. The suspension culture maintained its capacity to produce somatic embryos 15 months after isolation. The suspension was comprised of small groups of thin-walled, highly cytoplasmic and starch-containing embryogenic cells (Fig. 1) and some (10–15%) elongated, thick-walled cells. The thick-walled cells neither divided nor formed protoplasts during enzymatic digestion. Almost all the thick-walled cells were removed effectively during washing and sieving and were seen only rarely in washed protoplast preparations. The freshly isolated protoplasts (Fig. 2) were devoid of any remaining cell wall material as demonstrated by staining with Calcofluor White. Protoplast yields ranged from 4×10^5 to 6×10^6 protoplasts/gm fresh weight of the suspension culture. Protoplast yield and the degree of spontaneous fusion varied in the different experiments even though identical procedures were used each time both for the maintenance of the suspension culture as well as for the isolation of protoplasts.

The protoplasts were 15–35 μm in diameter, highly cytoplasmic and contained many small starch grains (Fig. 2). A majority of the protoplasts formed cell walls within two days and became oval in shape. Two-celled structures were first seen around the fifth day after culture (Fig. 3). Further divisions then followed in quick succession. The resulting protocolonies resembled the embryogenic cell groups present in the suspension cultures (Fig. 4). Frequently, single file chains of 4–8 cells also were formed. A large number of protocolonies were produced within the first two weeks of culture.

Protoplasts cultured in liquid media tended to aggregate at the periphery of the culture dish which made it difficult to accurately determine plating efficiencies. Therefore, the total number of apparently independent protocolonies that were produced during the first two weeks of culture were counted. The efficiency of colony formation was expressed as the percentage of total plated protoplasts which formed colonies. In basal KM medium the protoplasts remained round and neither regenerated a cell wall nor divided. When 0.1 mg/l 2,4-D was added to the medium, a majority of the protoplasts formed cell walls, became oval in shape and occasionally divided (0.01% colony formation) to form 2- to 8-celled groups which failed to develop further. The



Figs. 1–5. Formation of colonies from protoplasts isolated from embryogenic cell suspension cultures of *Zea mays* L. **1** Groups of highly cytoplasmic embryogenic cells from the suspension culture (×510). **2** Freshly isolated protoplasts. Note absence of undigested cells (×410). **3** A 2-celled group following the first division of the protoplasts (cultured in medium with 0.6% agarose) (×465). **4** Protocolony (×130). **5** An organized, globular somatic embryo (×610)

highest frequency of colony formation (4–5%) was obtained with 0.5 mg/l 2,4-D. Higher concentrations of 2,4-D (1–2 mg/l) supported initial divisions in protoplasts (0.1–3% colony formation) but were inhibitory for continued cell divisions and growth. Zeatin (0.2 mg/l) did not generally prove useful, and it caused browning of the protocolonies when used with 0.5 mg/l 2,4-D.

The frequency of colony formation was not improved by culture of protoplasts in media gelled with various concentrations of agarose. Optimum colony formation (3.5–4%) was obtained with 0.3% agarose; concentrations above 0.3% inhibited both the initial divisions as well as further growth of the protocolonies.

The three suspension culture lines (M1, M2 and M4) showed differences in growth as measured by fresh weight increase, the yield of protoplasts, and the degree of apparent spontaneous fusion during protoplast isolation (Table 1). Protoplast yields from lines M2 and M4, which showed higher fresh weight values, were also higher than those from M1. Although protoplast yields were considerably higher in M4, this line also showed a higher frequency of spontaneous fusions. The three cell lines did not differ significantly in the frequency of colony formation.

Sustained divisions in cultured protoplasts were obtained in each of the 14 separate isolations, with a maximum plating efficiency of 5%.

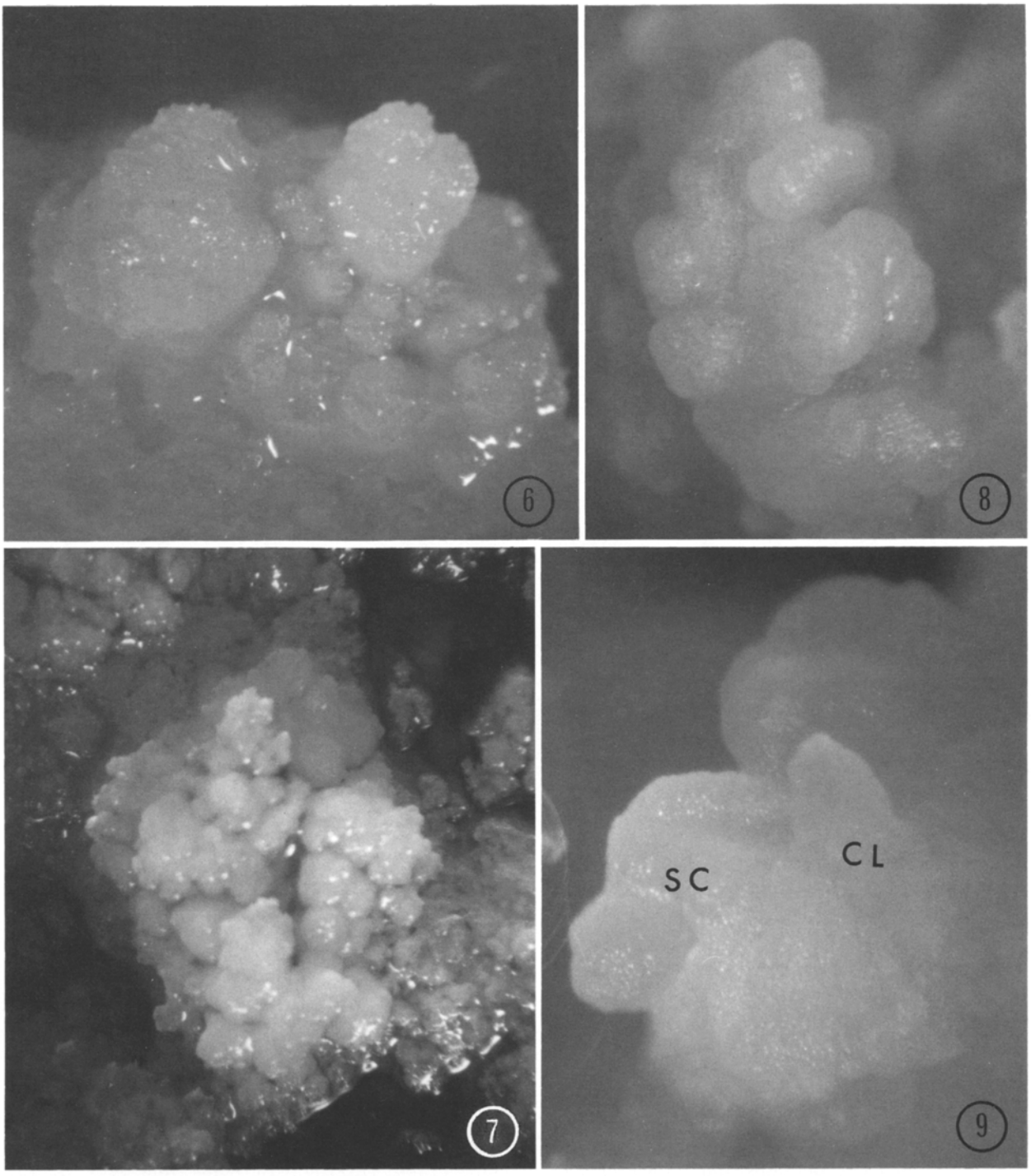


Fig. 6–9. Morphogenesis in protoplast-derived calli of *Zea mays* L. **6** and **7** Compact and organized sectors of protoplast-derived callus (×200, ×75). **8** Organised and folded structures formed in the protocalli (×540). **9** Somatic embryo with scutellum (SC) and coleoptile (CL) (×770)

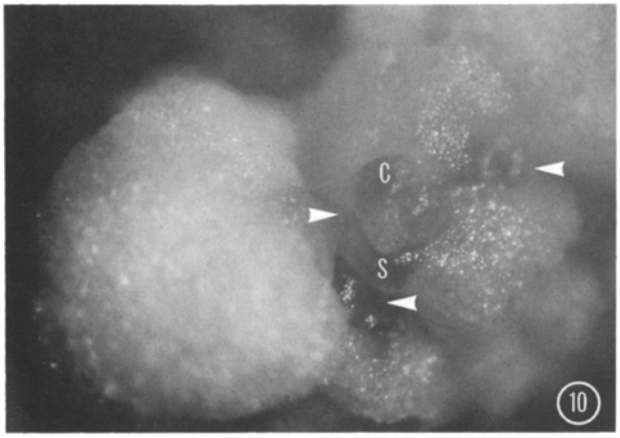


Fig. 10. A cluster (arrow heads) of precociously germinating somatic embryos (S scutellum, C coleoptile)

Growth, organization and formation of somatic embryos in protoplast-derived callus

Compact protocolonies were formed both in liquid and agarose media approximately three weeks after culture. Some of the colonies were highly organized and spherical with a distinctly uniform and continuous surface layer reminiscent of globular embryos (Fig. 5). The protocolonies continued to grow and produced a soft, beige-colored, and mucilaginous callus 3–4 weeks after transfer to agar-solidified suspension maintenance medium. The callus was unorganised initially but differentiated deep yellow to pale green sectors upon transfer to the same medium containing 0.1–0.2 mg/l 2,4-D and 0.2 mg/l ABA (Fig. 6). Parts of these sectors became further organized and somewhat white in color (Fig. 7). Leafy appendages, organised sectors with an outer epidermal layer, and highly organised folded scutellar structures (Fig. 8) developed in some of the protoplast-derived calli. Somatic embryos with an identifiable scutellum and coleoptile were formed (Fig. 9). These were not fully organised and did not reach maturity. Clusters of somatic embryos germinated precociously and parts of the scutellum became green and leafy in light (Fig. 10). An elongated green coleoptile was formed in the germinating somatic embryos.

The addition of L-proline (10–160 mM) substantially increased the growth of the callus but was not helpful in increasing the degree of organization or embryo development.

Discussion

This study has extended the success previously reported in the culture of protoplasts of 'Black Mexican Sweet' (Chourey and Zurawski 1981; Ludwig et al. 1985) to a commercial hybrid cultivar of maize.

Plating efficiency of 4–5% obtained in the present experiments, although low in comparison to the 20–30% reported in similar embryogenic protoplasts of other gramineous species (Vasil et al. 1983; Srinivasan and Vasil 1986), nevertheless compared well with earlier reports on maize (Chourey and Zurawski 1981; Ludwig et al. 1985). Further improvements can perhaps be obtained by using the cell suspension as a nurse or feeder layer.

Ludwig et al. (1985) did not find any significant variations in the callus forming ability of 'Black Mexican Sweet' protoplasts. In our experiments protoplast yields, frequency of spontaneous fusions, and the rate of division of protoplasts varied from experiment to experiment. This is similar to the results obtained in this laboratory with protoplasts isolated from embryogenic suspension cultures of several other species of the Gramineae. The presence of 2,4-D was found to be essential for the induction of division in cultured protoplasts of maize, unlike those of *Panicum maximum* (Lu et al. 1981) which divided in the absence of 2,4-D and showed a vigorous capacity for colony formation when 0.1–1 mg/l 2,4-D was added. Optimal colony formation from embryogenic protoplasts in the present study was obtained in a liquid medium containing 0.5 mg/l 2,4-D, in comparison to 2 mg/l reported for protoplasts from non-morphogenic suspensions (Chourey and Zurawski 1981; Ludwig et al. 1985).

The frequency of colony formation at 0.3% agarose was about the same as that in liquid medium. Higher concentrations of agarose inhibited colony formation. These results are consistent with our earlier experience with protoplasts of *Pennisetum purpureum* (Vasil et al. 1983).

A second and perhaps more important objective of this study, namely the establishment of a reliable protoplast culture system from a morphogenically competent cell line of maize has been also achieved. In all previous studies on the culture of maize protoplasts, only non-morphogenic cell lines were used. The suspension culture used in the present experiments, when initially established, formed somatic embryos and plants which could be grown to maturity (V Vasil and Vasil 1986). At the time of protoplast culture, about 15 months later, the cell lines were still embryogenic as evidenced by the formation of distinct somatic embryos.

The cell colonies recovered from suspension-derived protoplasts resembled the groups of embryogenic cells found in the suspension cultures as well as protocolonies of *Saccharum officinarum* (Srinivasan and Vasil 1986) from which somatic embryos and mature plants were obtained. The formation of somatic embryos in protoplast derived calli of maize in low 2,4-D media is similar to the results obtained in *Panicum maximum* (Lu et al. 1981) and *Pennisetum purpureum* (Vasil et al. 1983). Recovery of mature plants from these calli, as reported recently from embryogenic protoplasts of sugarcane (Srinivasan and Vasil 1986) and rice (Yamada et al. 1986), would be especially meaningful in light of the stable transformation of protoplasts isolated from non-morphogenic maize (Fromm et al. 1986) and embryogenic *Panicum maximum* (Hauptmann et al. unpubl. results) suspension cultures.

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