1. INTRODUCTION

Procedures for the routine regeneration of large numbers of plants from isolated microspore cultures of maize have been recently developed (1,2). Haploid microspores are competent to undergo abnormal cell divisions culminating in the formation of embryo-like structures from which plants can be recovered. These procedures have now been adapted for the production of suspension and protoplast cultures capable of plant regeneration. The present paper describes the establishment of regenerable, haploid cell suspensions directly from cultured maize microspores. The recovery of plants from protoplasts isolated from these microspore-derived suspension cultures is also discussed.

2. PROCEDURE

2.1 Plant Material

The genotype used was a highly anther culturable S₂ line (139/39-02) developed by inter-mating microspore-derived doubled haploids descended from a three-way cross, (H99xFR16)xPa91. Donor plants were field-grown during April-August 1988 and 1989 in Champaign, IL.

2.2 Anther Culture and Microspore Isolation

Anthers containing predominantly early binucleate microspores, as determined by mithramycin-fluorescent staining, were excised and placed in 20x60 mm Petri dishes (60 anthers per dish) containing 10 mL of induction medium. The induction medium consisted of YP major salts, N6 minor salts, 60 g/L sucrose, 27.9 mg/L FeSO₄·7H₂O, and 37.3 mg/L Na₂EDTA adjusted to pH 5.8. Dishes were sealed with Parafilm and placed in plastic boxes covered with aluminum foil. Cultures were maintained in the dark at 28°C for 10 days after which microspores were isolated. Anthers were placed onto a 380 micron mesh and gently squashed using a glass stirring rod. Microspores passed
through the screen while the majority of the anther wall debris was left behind.

2.3 Suspension Initiation and Maintenance
The entire contents of 4 dishes (induction medium, released microspores from 240 anthers, and some anther wall debris) were placed into a 125 mL Erlenmeyer flask along with 10 mL of initiation medium for a final volume of 50 mL. The initiation medium consisted of MS salts and vitamins, 30 g/L sucrose, 100 mg/L myo-inositol, and 2 mg/L 2,4-D adjusted to pH 5.8. The cultures were then placed onto an orbital shaker (150 rpm) and maintained in the dark at 28°C. After approximately 10-14 days, the cultures were allowed to settle for a few seconds and 15 mL of the supernatant was removed and replaced with fresh initiation medium. After approximately 4-8 weeks of this replenishment procedure, the cultures became thick and mucilaginous. At this point, they were centrifuged (AccuSpin, Beckman Instruments, Inc., Palo Alto, CA) at 2500 rpm for 3 min and the upper portion of the sediment along with some of the supernatant was transferred to a maintenance medium. The maintenance medium consisted of LM salts (3), MS vitamins, 60 g/L sucrose, 0.7 g/L L-proline, 8.0 mg/L dicamba, and 0.8 mg/L 2,4-D adjusted to pH 5.8. Once established, sub-culturing was performed every 7-10 days by allowing the culture to settle, selecting 20 mL from the sediment-supernatant interface, and placing it into 30 mL of fresh maintenance medium.

2.4 Protoplast Isolation and Culture
Three days after subculture, 1 mL of suspension was transferred to a 60x15 mm Petri dish along with 2 mL of BH3 medium (4) and 2 mL of filter sterilized enzyme solution. The enzyme solution contained 128 g/L mannitol, 3.6 g/L CaCl₂, 1.2 g/L MES buffer, 0.1 g/L NaH₂PO₄, 1% (w/v) Onozuka RS cellulase, 1% (w/v) macerozyme R10, and 0.2% (w/v) pectolyase Y-23 adjusted to pH 5.6. All enzymes were obtained from Karlan Chemical Corporation (Torrance, CA). After 16 hours, the partially digested preparation was passed through a 45 micron stainless steel screen to remove the debris and large cell clusters and then centrifuged at 700 rpm for 5 minutes. The supernatant was removed and the pellet was re-suspended in 5 mL of 25% sucrose solution containing 250 mg/L MgSO₄, 150 mg/L MgSO₄, 100 mg/L KNO₃, 27.2 mg/L KH₂PO₄, 2.5 mg/L Fe(SO₄)₃.6H₂O, 0.16 mg/L KI, and 0.0003 mg/L CuSO₄. Before centrifuging at 700 rpm for 5 minutes, 2 mL of a 13% mannitol solution was carefully over-layered. Protoplasts were removed from the sucrose-mannitol interface and washed twice in BH3 medium (4) before diluting to a final density of approximately 1 x 10⁵