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Isolated microspore culture of maize: effects of isolation technique, reduced temperature, and sucrose level

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ABSTRACT

Improvements in ab initio microspore culture of maize are presented using a modified isolation technique, reduced temperature during early stages of culture, and an elevated sucrose level in the culture medium. Blending-isolation, using excised anthers, was less stressful on microspores than pressing anthers against a stainless steel sieve and resulted in a 3-fold increase in the yield of embryo-like structures (ELS). Exposure to reduced temperature (15°C) during the first 4 days of culture improved microspore viability and increased by 2-fold the number of ELS produced. Higher levels of sucrose (8.0-9.5%) also resulted in improved response. Maximum yield in the present study was 92 ELS per 100 anther equivalents, exceeding previously reported values of 15 ELS per 100 anther equivalents for ab initio microspore culture of maize. The increase in the total number ELS produced had no observable effect on their quality as evidenced by the frequency of formation of callus capable of regenerating plants.

INTRODUCTION

Plant regeneration from isolated microspores, mechanically removed or allowed to dehisce from anthers floating in liquid medium, has been reported for various cereal species including barley (Kohler and Wenzel 1985; Wei et al 1986), rice (Chen 1986), and wheat (Wei 1982; Datta and Wenzel 1987). More recently, plants have been successfully recovered from isolated microspores of maize (Coumans et al 1989; Pescitelli et al 1989). Although the reported frequency of androgenesis was relatively high in maize microspores precultured within the anther (Pescitelli et al 1989), plating efficiencies for microspores isolated prior to culture initiation (ab initio) were considerably lower (Coumans et al 1989; Pescitelli et al 1989).

The early stages of microspore development may offer an attractive target for genetic transformation (Neuhaus et al 1987, Petolino 1989). However, successful application of an isolated microspore system to genetic modification will require plating efficiencies above those currently reported for maize, particularly for ab initio cultures. Important factors determining plating efficiency in isolated microspore culture include, isolation procedure (Swanson et al 1987), culture temperature (Chuong and Beversdorf 1985), and composition or conditioning of the culture medium (Kasha et al 1989). In studies with maize microspore cultures, Coumans et al (1989) applied a blending technique, temperature treatment, and an induction medium designed originally for Brassica. Pescitelli et al (1989) used float culture (Sunderland and Roberts 1977) and homogenization (Lichter 1982) for microspore isolation together with medium developed for maize anther culture (Petolino and Jones 1986).

In the present study, a modified isolation technique, reduced temperature during early stages of culture, and an elevated sucrose level in the culture medium resulted in significant improvements in embryo-like structure (ELS) formation from ab initio microspore cultures of maize.

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Table 1. Effects of isolation technique on density, viability at 1 and 7 days post-isolation, induction at 7 days post isolation, number of embryo-like structures (ELS) per 100 anther equivalents, and regenerable callus per 100 ELS; 420 anthers per treatment (n=8, ± SE).

Isolation Technique	Microspore Density/mL	---Percent Viability--- 1-Day	7-day	Percent Induction	ELS per 100 Anthers	RC Per 100 ELS
Homogenization	8203 ± 652	20.3 ± 2.9	7.3 ± 1.4	4.7 ± 1.0	22.4 ± 8.6	4.3 ± 1.5
Blending	6109 ± 367	36.3 ± 7.6	10.7 ± 1.2	7.8 ± 1.0	73.7 ± 31.9	4.1 ± 3.5

MATERIALS AND METHODS

The genotype used in this study (139/39-02) was a highly anther culturable S_2 line developed by intermating microspore-derived doubled haploids (Petolino et al 1988). Donor plants were either field- or greenhouse-grown during March - August 1989 in Champaign, IL. Tassel harvest and pretreatment followed previously published procedures (Petolino and Jones 1986). The induction medium (MC) consisted of YP major nutrients (Ku et al 1978), N6 minor nutrients (Chu et al 1975), 27.9 mg/L $FeSO_4 \cdot 7H_2O$, 37.3 mg/L Na_2EDTA, and 5 g/L activated charcoal, which was filtered out after autoclaving. With the exception of the sucrose experiment, all media contained 60 g/L sucrose. Harvest of embryo-like structures (ELS) and initiation of regenerable cultures (RC) from ELS have been previously described (Pescitelli et al 1989). Results from the present study are expressed on the basis of ELS per 100 anther equivalents, assuming 2,500 microspores per anther. Viability estimates were made using fluoracein diacetate staining (Heslop-Harrison and Heslop-Harrison 1970). Microspores displaying abnormal divisions (inductions), were counted using the nuclear stain mithramycin (Pace et al 1987; Pescitelli and Petolino 1989). A minimum of 1,500 microspores per treatment were counted for viability and induction estimates. Microspore density was 7,500-10,000 per mL (unless otherwise indicated) and was determined using a Speirs-Levy Eosinophil hemocytometer (C.A. Hausser and Son, Philadelphia, PA). A randomized complete block design was used in all experiments with individual tassels or the combination of two tassels as replicates.

In the first experiment, mechanical isolation by homogenization (Lichter 1982) was compared to isolation via blending (Swanson et al 1987). Isolations were performed on anthers after excision from the florets and prior to culture initiation. Homogenization involved gently pressing 60 anthers against a 113 micron stainless steel sieve using a glass rod. The microspores were then washed through the mesh which retained the anther wall material. For the blending procedure, 60 anthers were placed into a 110 mL stainless steel blender attachment (Waring MC-2) along with 40 mL of MC culture medium and blended at low speed for 10 sec. The slurry was then passed through a 113 micron sieve and the microspores were collected by centrifugation at 1,000 RPM for 5 min. Microspores from both isolation techniques were cultured in 10 mL of MC medium. Densities were estimated for both procedures immediately following isolation. Viability counts were made at 1 and 7 days post-isolation. Inductions were counted at 7 days after isolation.

In the second experiment, the effects of a reduced temperature treatment on cultural response were evaluated. Isolations were made using the blending technique described above. Immediately following isolation, microspores were cultured at 15°C for 1, 4 and 7 days, and then transferred to 28°C. A density of 100,000 microspores per mL in 3 mL total volume was used in this experiment, therefore, 2 mL of the media was replenished weekly (Marsolais and Kasha 1985). Microspores in each replicate were collected en masse after isolation by centrifugation and distributed to each treatment. Samples for the 0 day viability counts were taken before distribution. Subsequent samples were taken directly from the plates and averages were obtained for all replicates.

In a third experiment, the effect of different sucrose concentrations on microspores isolated via blending was examined. A total of 6 levels, ranging from 4.5 to 18.0% (Table 3) were evaluated. Microspores were cultured in a total volume of 10 mL at 28°C. In addition to ELS and RC counts, viability estimates were made at 1 day post-isolation and inductions were counted at day 7.

RESULTS AND DISCUSSION

Both homogenization and blending liberated a high percentage of microspores (>80 %), although blending provided more consistent isolation densities as indicated by the lower SE (Table 1). Moreover, the increased viability of microspores at 1 day post-blending indicated that this process was less stressful than homogenization (Table 1). By day 7, the difference in viability was diminished somewhat, however, the number of inductions was higher in the blending treatment. After 21 days, the total number of ELS produced per 100 anther equivalents was much greater for the blending procedure (Table 1). This represents a more than 3-fold increase in yield over the homogenization technique with no apparent effect on the quality of the ELS as evidenced by the number of RC produced per 100 ELS.

In addition to the increase in ELS production, the blending technique also improved the consistency of response for isolated cultures. Similar results have been reported using micro-blending for the isolation of Brassica napus microspores (Swanson et al 1987). Coumans et al (1989) applied the Brassica system directly to maize using whole tassel segments for blending isolations. In the present study, it was found that blending of excised anthers yielded higher responses than when tassel segments were used (unpublished results). This may reflect the importance of selecting only those anthers at the appropriate developmental stage. Reduced contamination, particularly when using field grown donor plants, was also observed when excised anthers were blended.

Table 2. Effects of cold temperature (15°C) treatment on the number of embryo-like structure (ELS) per 100 anther equivalents and regenerable cultures (RC) per 100 ELS for ab initio microspore cultures of maize (n=6, ± SE).

Days in Cold	Total No. Anthers	ELS per 100 Anthers	RC per 100 ELS
0	720	36.8 ± 3.2	11.6 ± 1.6
1	720	44.5 ± 3.7	10.9 ± 1.3
4	600	68.9 ± 6.0	8.8 ± 2.1
7	840	26.1 ± 2.2	7.9 ± 3.8

Viability counts indicated that the 15°C cold treatments were effective in reducing microspore mortality following isolation. After only one day in culture, viability in the 28°C control decreased from 21.9% to 6.5%. Viability in all of the cold treatments remained unchanged from levels observed immediately following isolation. These effects were mitigated by day 7 when viability in the 1 and 4 day cold treatments was comparable to the controls. However, ELS production in the 4 day treatment was nearly 2-fold greater than the control (Table 2). The effect of reduced temperature on RC was minimal, although there appeared to be a slight decline in ELS quality with increased time of low temperature exposure. Reduced temperature treatments following culture initiation have been used in barley anther cultures (Kao 1981) with effects similar to those observed in the present study. Split treatments using higher (32°C) initial temperature have also been successful in Brassica microspore cultures (Chuong and Beversdorf 1985).

Effects of sucrose concentration on microspore culture are presented in Table 3. Generally, there was little effect on viability at day 1 except at the 18% sucrose level. No effect on the number of induced microspores was observed after 7 days in culture. The number of ELS was highest in the 8.0 and 9.5% sucrose concentrations. This is in contrast to dehisced microspore culture in which 6.0% sucrose produced the highest number of ELS (unpublished results). With the exception of the 4.5% sucrose treatment, no effects on RC were observed. The level of sucrose has typically been a major factor in anther and isolated microspore cultures of various species (Dunwell 1985, Lichter 1982). Although the level of sucrose did not affect microspore induction in this study, survival to the ELS stage appeared to be enhanced at the higher concentrations.

Table 3. Effects of sucrose levels on percent viability at 1 day post isolation, percent induction at 7 day post isolation, embryo-like structures (ELS) per 100 anther equivalents and regenerable cultures (RC) per 100 ELS for ab initio microspore cultures of maize; 300 anthers per treatment (n=10, ± SE).

Sucrose Conc(%)	Percent Viability	Percent Induction	ELS per 100 Anther	RC per 100 ELS
4.5	16.6 ± 9.9	1.5 ± 1.3	18.5 ± 6.1	1.6 ± 1.6
6.0	25.5 ± 7.0	1.5 ± 0.4	38.5 ± 15.1	9.3 ± 5.4
7.9	26.9 ± 8.2	1.0 ± 0.4	92.3 ± 42.8	9.0 ± 3.8
9.6	25.6 ± 9.6	1.4 ± 0.5	85.7 ± 41.8	7.1 ± 3.8
13.0	18.6 ± 11.8	1.0 ± 0.5	71.0 ± 27.1	6.5 ± 2.2
18.0	3.7 ± 2.3	0.6 ± 0.2	20.0 ± 8.2	7.8 ± 3.5

Significant improvements in the ab initio microspore culture of maize have been achieved by manipulating various cultural parameters. Previous studies had reported production of 15 ELS per 100 anther equivalents with 6% producing RC upon transfer to callus induction medium (Pescitelli et al 1989). In the present study, a maximum of 92 ELS per 100 anther equivalents was obtained. Moreover, the increase in the total number of ELS had no apparent effect on their relative quality as indicated by the yield of RC (which ranged from 4 to 10% in the more productive treatments). These frequencies approach those observed for intact anther cultures which range from 100-500 ELS per 100 anthers for this particular genotype (Pescitelli et al 1989).

The combination of the blending-isolation technique with reduced temperature during early stages of culture and an elevated sucrose level in the culture medium allows for the reliable production of large numbers of plants from ab initio microspore culture. This effectively provides a single, haploid cell regeneration system for maize which may be useful for genetic and development studies as well as for doubled haploid line production.

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