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Anther culture of maize and the visualization of embryogenic microspores by fluorescent microscopy

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Summary. Three maize genotypes previously shown in the literature to respond to anther culture were tested under various conditions. Studies indicated that embryogenic response ranged from 0 to 100 embryos per 1,000 anthers plated and was significantly lower without cold pretreatment of the anthers. Culture in liquid media tended to produce more embryos than in semi-solid as did the addition of activated charcoal to either liquid or solid culture media. Most results were confounded by plant-to-plant variation which tended to obscure significant differences. In one study, germination rate of androgenetic embryos averaged about 20%, but only 26% of those embryos that germinated completed their reproductive cycle and formed seed albeit through sib-pollination since plants could not be selfed. Chromosome counts using root tip squashes indicated that regenerated plants were either haploid or diploid but plants scored as non-diploid yielded as much seed as scored diploids. This suggests that progeny can be recovered even from putative haploids, presumably as a result of "sectoring" in the developing ear. A DNA-specific fluorescent dye was used to visualize the presence of putative embryogenic microspores (PEMs) during the culture period. PEM counts were a function of time in culture and were apparently greater than the number of embryos obtained for a given treatment. The data indicate that, as previously reported for other species, both induction and survival phases also exist in maize anther culture.

Key words: Androgenesis – Anther culture – *Zea mays* L. – Maize – Fluorescent microscopy

Introduction

Anther culture of maize offers many opportunities for the augmentation of breeding programs, primarily through the rapid production of homozygous lines. In order to use this technique efficiently, however, it is imperative that the response frequencies be as high as possible since the culture protocol is labor and resource intensive. Hence this study was conducted to determine the efficiency of the presently available protocols and to evaluate selected procedures that might enhance the response, using three genotypes that have been shown previously to respond to anther culture.

Materials and methods

Donor plants

Maize (*Zea mays*, L.) was grown in a greenhouse using supplemental lighting (16 h photoperiod), a potting mixture containing one-third (by volume) sterilized loam and daily watering with Hoagland's solution (Hoagland and Arnon 1950). Three genotypes previously shown in the literature to respond to anther culture were used in this study: 'Seneca 60' ('Sen60'), a North American sweet corn hybrid (Brettell et al. 1981), and two genotypes from the People's Republic of China, 'Da-Bai-Tou-Shuang' ('DBTS') and 'Ching-Huang 13' ('CH13') which is the F_1 of 'DBTS' × 'Yellow 204' (Genovesi and Collins 1982). Tassels were harvested from the plants just prior to their emergence from the whorl.

Culture technique

Harvested tassels were slightly moistened, wrapped in plastic bags and pretreated at 8 °C for 14 days. Some experiments tested variations of this pretreatment scheme and are noted in the text. After pretreament, segments of the axillary spikes of the tassel having microspores that were predominantly in the mid to late uninucleate stage of development were surface sterilized for 15 min with a 10% commercial bleach solution, followed by four rinses with sterile distilled water. Anthers were removed

from the florets and anthers placed at various densities (see text) per 60×15 mm plate and containing the specific media under investigation. Three media were evaluated; Yu-Pei (Genovesi and Collins 1982), Zheng-14 (Ting et al. 1981), and 79-10 (Gu Ming-guang, pers. commun.). The media 79-10 consists of: KNO_3 (35 mM), $CaCl_2$ (1.4 mM), KH_2PO_4 (0.85 mM), $MgSO_4$ (3.0 mM), $(NH_4)_2SO_4$ (1.1 mM), Yu-Pei micronutrients, Fe-Sequestrene 330 (43 mg Fe/l), Zheng-14 organic amendments, sucrose (15%), activated charcoal (6 g/l), adjusted to pH 5.8 before autoclaving. Plates were wrapped with Parafilm and incubated in the dark at 28 °C. Plates were scored for androgenic embryos weekly for eight weeks beginning three weeks after plating and normalized to a "per 1,000 anthers" basis.

Unless otherwise stated, individual plants were used as replicates, with anthers from each plant partitioned equally across each treatment in an experiment. Results were analyzed by analysis of variance. Neither square root nor arcsine square root transformation of the data altered the conclusions made on the basis of analyzing the raw data directly.

Growth and reproduction of Ho plants

In some experiments embryos were recovered in order to gather germination and ploidy information. Embryos were recovered from culture within two weeks of their first appearance and transferred to regeneration media described for embryos from Yu-Pei media (Genovesi and Collins 1982), or as described for embryos recovered from Zheng-14 (Ting et al. 1981), both without activated charcoal. Germination was defined as forming a viable plantlet having both a root and shoot. Germinated seedlings were transferred to rooting medium consisting of the major and minor salts of Shenk and Hildebrandt at half-strength, 25 μM gibberellin A_3, 0.25 μM NAA and 0.8% agar (Stuart and Strickland 1984), and grown until large enough to be transferred to potting mix. Potted plants were hardened-off in high humidity growth chambers before being transplanted to the glasshouse. All plants were sib-pollinated using seed-derived DBTS as the pollen parent due to consistent problems with fertility and synchronization of the reproductive organs.

Cytology

Microspores were staged using the DNA-specific fluorescent dye mithramycin (Sigma M7393). Sessile florets were taken from sequential locations along the length of the tassel, the three major anthers dissected out and chopped on a glass slide in the presence of a few drops of "chopping buffer" (CB: 45 mM $MgCl_2$ * 6 H_2O, 30 mM Trisodium citrate, 20 mM MOPS, 1.0 mg/ml Triton X-100, pH 7.0; Galbraith et al. 1983). Five microliters of a mithramycin stock (0.5 mg/ml in water) were added to the slide, mixed gently and covered with a glass slip. The stain reaction could be enhanced by heating on a slide warmer at 40 °C for a few minutes.

Chromosome counts were determined from root tip squashes at approximately 30 days after germination of the anther-derived embryos and again at transfer of the plantlets to the glasshouse using the procedure outlined by Kindiger and Beckett (Kindiger and Beckett 1983). Chromosomes were also visualized using mithramycin. Root tips were pretreated and fixed as described above, then treated with a few drops of CB, 10 μl of mithramycin stock and squashed on a slide. Root tips prepared in this fashion were difficult to spread since there was no hydrolysis step (acid hydrolysis quenches mithramycin fluorescence).

Assay for embryogenic microspores

Mithramycin was also used to visualize the development of multicellular structures within the plated anthers during the incubation period. Tassels from each of the three genotypes were pretreated at 8 °C for 14 days and the anthers plated onto semi-solid Zheng-14 medium containing activated charcoal. Four plants of each genotype were used. One plate was sampled at weekly intervals for each plant during the culture period.

Groups of 10 anthers from a single plate were infused with cryoprotectant (0.5 M DMSO, 0.5 M glycerol, 1.0 M sucrose and 0.1% Triton X-100), frozen rapidly and stored in liquid nitrogen until analyzed. Anthers were then thawed at room temperature and the cryoprotectant was replaced with a HEPES/Ficoll buffer (0.25 M sucrose, 50 mM HEPES, 1.0% Ficoll, pH 7.0). Anthers were placed on glass slides, cut into halves or quarters and the contents were gently teased from the anther wall tissue and collected in a microcentrifuge tube. Collected microspores were centrifuged at low speed for 5 min and the HEPES/Ficoll buffer was replaced by 25 μl of CB. Five μl of mithramycin were added, then the entire 30 μl volume was transferred to a glass slide and examined using fluorescent microscopy. Treated anthers could be stored overnight in the HEPES/Ficoll buffer, but the extraction could be done in CB if samples were completely processed the same day. This extraction procedure collected more than 98% of the contents of the anthers, based on random examination of the debris remaining after extraction.

Two classes of structures were defined, putative embryogenic microspores (PEMs; multinuclear, but not yet broken out of the exine, Fig. 1 A) and embryogenic microspores (EMs; multicellular structures broken out of the exine and clearly larger than the remaining pollen grains, Fig. 1 B). The data is presented as the average of two groups of 10 anthers and normalized to a 1,000 anthers basis for ease of comparison with culture response data. The resolution of the assay was therefore 100 structures per 1,000 anthers plated (i.e. 1 per 10 anthers).

Results and discussion

An initial study was conducted using published protocols for maize anther culture. Three genotypes previously reported to respond to culture were pretreated with cold temperature following either Genovesi and Collins (Genovesi and Collins 1982), or Nitsch et al. (Nitsch et al. 1982), on either Yu-Pei or Zheng-14 solidified with agar. Both media types gave similar responses (data not shown) and so were pooled within genotypes. Mean frequencies of embryogenesis ranged from 0 to 43 embryos/1,000 anthers and exhibited a significant genotype-by-pretreatment interaction (Table 1 A). Cold pretreatment (less than 8 °C) for more than 5 days was clearly necessary for a response in both CH13 and DBTS and confirms previous observations with maize (Genovesi and Collins 1982; Nitsch et al. 1982; Sheridan 1984). 'Sen60' did not respond well at any pretreatment tested in this experiment. The most responsive genotype on average was 'DBTS'; followed by 'CH13' and 'Sen60' (Table 1 A). Another Chinese inbred line, 'Yellow 204', was also evaluated but it responded no

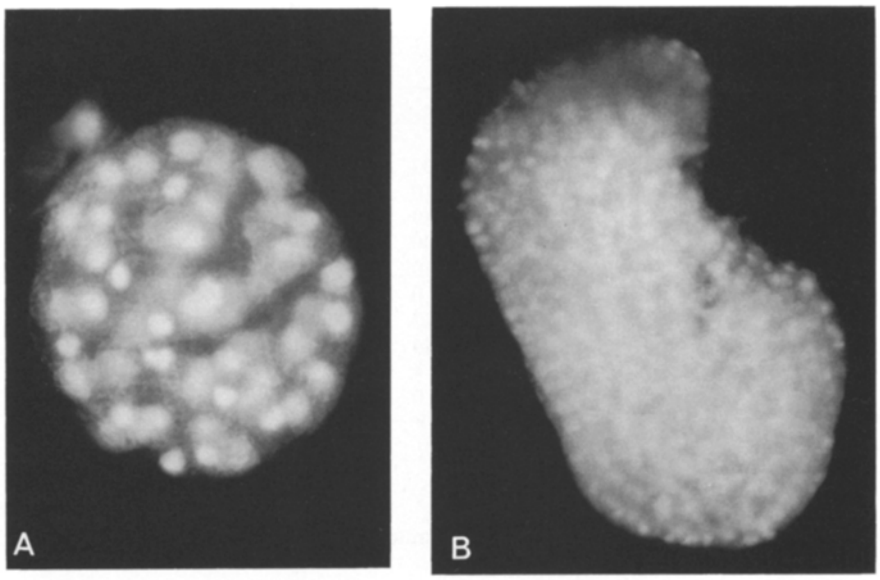

Fig. 1 A, B. Fluorescent photomicrographs of multicellular structures induced in maize anther culture, using mithramycin for visualization. A putative embryogenic microspore (PEM) is depicted in **A** and an embryogenic microspore (EM) is depicted in **B**. Both figures are magnified 250×. The bright spots in the objects are stained nuclei, which appear yellow on a green background under epifluorescent microscopy

Table 1. Response of three genotypes to different temperature pretreatments and the use of either liquid or solid media. Results are presented as the combination of several studies. A range between 700 and 2,000 anthers were plated for each replicated treatment depending on the study. In some experiments data was pooled since anther plating density and media composition were tested and found to be non-significant

A Effects of pretreatment temperature:

	Embryos/1,000 anthers			
	8 °C–14 days	4 °C–7 days + 8 °C–7 days	4 °C, 13 °C, 20 °C at 5 days each	No pretreatment
'Da-Bai-Tou-Shuang'	43	12	1	1
'Ching-Huang 13'	19	20	5	3
'Seneca 60'	4	1	2	0
$LSD_{0.05} = 6$				

B Effects of media type:

	Media	Embryos/1,000 anthers				
		Liquid with AC	Liquid without AC	Solid with AC	Solid without AC	$LSD_{0.05}$
'DBTS'	Zheng-14	93	35	28	13	NS[a]
'CH13'	Yu-Pei	73	NT[b]	6	NT	37
'Sen60'	Yu-Pei	41	24	NT	NT	13

[a] NS = non-significant; [b] NT = Not tested

better than 'Sen60' (data not shown). The relative ranking of the three Chinese lines is noteworthy since 'CH13' is the F_1 hybrid of 'DBTS'×'Yellow 204' and seemed to respond in an intermediate fashion, suggesting an incomplete dominance genetic response.

Out of the 195 embryos obtained from this study, 38 germinated for an average of 20%. This frequency is virtually identical with other reports with maize (Brettell et al. 1982; Genovesi and Collins 1982; Petolino and Jones 1986), suggesting that low germinability is a major obstacle to the use of this technique. There was little difference in germination frequency across the various treatments and there was no evidence of albinism among the germinated seedlings. Only 11 of 38 plantlets were established in the greenhouse with 10 of these producing seed. Nine of these 10 were 'DBTS' (Table 2). None of the plants could be selfed primarily due to a long time period between pollen shed and silk emer-

Table 2. Ploidy, pedigree and seed yield of plants regenerated from anther culture

Ho ploidy	Pedigree	No. of kernels				
		Ear 1	Ear 2	Ear 3	Ear 4	Seed total
Haploid	'DBTS'-Ho × 'DBTS'	13	2	2	16	33
NRC	'DBTS'-Ho × 'DBTS'	8	2	–	–	10
NRC	'DBTS'-Ho × 'DBTS'	4	15	–	–	19
Haploid	'CH13'-Ho × 'DBTS'	14	21	5	–	40
Diploid	'DBTS'-Ho × 'DBTS'	8	2	–	–	10
Diploid	'DBTS'-Ho × 'DBTS'	0	0	–	–	0
Haploid	'DBTS'-Ho × 'DBTS'	9	18	–	–	27
NRC	'DBTS'-Ho × 'DBTS'	44	–	–	–	44
NRC	'DBTS'-Ho × 'DBTS'	4	–	–	–	4
Diploid	'DBTS'-Ho × 'DBTS'	0	–	–	–	0

NRC – Not reliable count; two separate determinations provided different results

Table 3. The response of maize anthers to different liquid media compositions. A minimum of 1,500 anthers were plated for each replicated treatment

	Media volume ml	Activated charcoal	Media composition Embryos/1,000 anthers		
			Yu-Pei	Zheng-14	79-10
'DBTS'	4	(–)	66 $LSD_{0.05} = 48$	52	10
'Sen 60'[a]	6	(+)	30	NT[b]	2
	5[c]	(+)	15	NT	0
	4	(+)	10 $LSD_{0.05} = 16$	NT	1

[a] Plating density varied but was non-significant; treatment data was pooled
[b] NT – Not tested
[c] One replicate lost to contamination, LSD corrected

gence, a problem with maize encountered by others (Brettell et al. 1982; Genovesi and Collins 1982; Petolino and Jones 1986). Therefore, all fertilizations were made using pollen from seed-derived 'DBTS'. Both haploidy and diploidy were observed (determined on two separate occasions at the seedling stage) in the regenerated plants. The sample of plants was too small to accurately determine a frequency for spontaneous doubling of the regenerates, but it appears to be in accordance with previous estimates of 30–50% (Brettell et al. 1981; Genovesi and Collins 1982). Seed count was independent of the ploidy score (Table 2), probably due to sectoring in the ear (cf. Genovesi and Collins 1982).

Given the relatively poor response of these genotypes using the existing protocols several experiments were undertaken to evaluate alterations in the established procedures. First, experiments were conducted comparing liquid media (4 ml per plate) with that solidified with 8 g/l agar (volume not controlled). These studies suggested that liquid media was superior, although good statistical separation was lacking despite the large number of anthers plated (Table 1 B). The effect of activated charcoal was also investigated, and it too was suggested to be beneficial in enhancing response (Table 1 B).

These observations are in accord with previous studies with maize indicating that both the use of liquified media (Kuo Chung-shen 1982), and the addition of activated charcoal (Brettell et al. 1981; Genovesi and Collins 1982; Kuo Chung-shen 1982; Sheridan 1984), can enhance the response to culture.

Several other observations should also be noted. First, anther density was investigated for 'Sen60' in liquid media, ranging from 6 to 90 anthers per plate. No significant effects were observed across this range (data not shown), and the treatments were pooled to give the numbers quoted for this genotype in Tables 1 B and 3. Second, the experiment on 'Sen60' described in Table 1 B was composed of 13 plants for each of the two activated charcoal levels. The response on an individual plant basis is depicted in Fig. 2, which shows the extreme variation typically seen throughout the course of

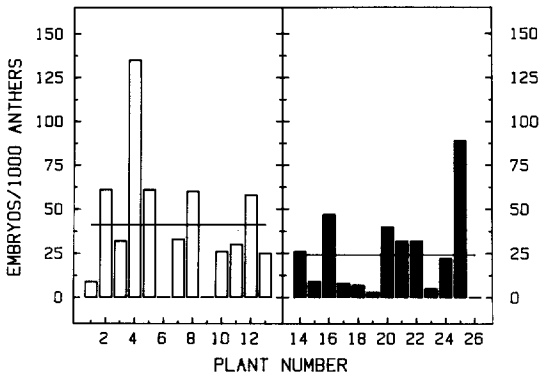

Fig. 2. Response of 'Seneca 60' to anther culture on an individual plant basis. Data taken from the experiment described in Table 1 B. The open columns represent the (+) activated charcoal treatment; the solid columns represent the treatment without charcoal added. The horizontal line superimposed on the graph represents the average value for the treatment. Note that plants numbered 6, 9 and 26 had zero response

this study. Statistical significance was often confounded due to this extremely wide variation in response. The biological nature of this phenomenon is unknown. Third, the experiment described in Table 1 A used tassels that were cold treated, whereas those described in Table 1 B used cold treatment of the plated anthers. A comparison of the data indicates only slight differences resulting from the method of pretreatment, as suggested by Nitsch et al. (Nitsch et al. 1982).

Media tests showed that Yu-Pei and Zheng-14 were not greatly different in effecting a response (Table 3 and pooled results noted in Table 1 A) while the media designated 79-10 performed poorly using both 'Sen60' and 'DBTS' (Table 3). This is striking since 79-10 shares several components with both Yu-Pei and Zheng-14. Furthermore, Yu-Pei and Zheng-14 themselves are distinct in composition, notably in their complement of growth regulators. Additional experimentation comparing these three media may reveal one or two components primarily involved in inducing androgenesis in maize anther culture.

The response frequencies presented here cannot be compared to those studies which reported only the percentage of anthers responding (Genovesi and Collins 1982; Petolino and Jones 1986). The presence of multiple embryos per responding anther prevent direct conversion of embryos per 1,000 anthers to percent anthers responding. Furthermore, percent response cannot be determined accurately in liquid culture because the embryos frequently become detached and float free in the media. The frequency of 1.5 embryos per 1,000 anthers reported by Brettell et al. (Brettell et al. 1981) for 'Sen60' is considerably lower than the best responses observed here (Table 1 B and 3). Ting et al. (Ting et al. 1981), reported a response of 136 embryos per 1,000 anthers for the Chinese hybrid 'Dan-San 91', which is comparable to the best "per plant" responses in the present study of 135 for 'Sen60' (Fig. 2), 169 for 'DBTS' (from the experiment in Table 3) and 125 for 'CH13' (from the experiment in Table 1 B).

Investigators working in anther culture have frequently observed multicellular structures during the culture period for both monocotyledonous and dicotyledonous species. These multicellular structures have been quantitated and used to determine the success or failure of various treatments, most notably in tobacco (Heberle-Bors 1983), barley (Shannon et al. 1985), and *Brassica napus* (Dunwell and Thurling 1985; Thurling and Chay 1984). All these attempts have used classical stains such as acetocarmine for visualization. With the advent of the use of DNA-specific fluorescent dyes in pollen biology (Coleman and Goff 1985) and their recent use to visualize multicellular structures in cultured microspores of *Nicotiana rustica* (Kyo and Harada 1985), it was thought that a similar procedure could be used to observe and quantify these structures in maize anthers.

Samples of anthers were taken at weekly intervals and both PEMs and EMs were counted. The results are presented in Table 4. There was considerable variation in the PEM count from replicate to replicate (i.e., plant-to-plant), which was punctuated with values of 100 or below (i.e., at or below the resolution of the assay). This type of variation was also seen in the culture experiments described above, where some plants were essentially non-responsive. The results tended to show higher PEM counts for the first two weeks of culture with a decline towards zero over time. This suggests that some plants have a significant potential to form androgenetic embryos but that this high induction is quickly mitigated by poor survivability. One of the highest PEM counts, 5,100 per 1,000 anthers, was observed for CH13 one week after plating (Table 4). This represents only 0.2% of the microspore population (assuming 2,500 microspores per anther). This contrasts sharply with similarly obtained values for other crop species, such as 2.2% for *Triticum aestivum* (Marsolais et al. 1984), 21.7% for *Hordeum vulgare* (Shannon et al. 1985) and 56.5% for *Brassica napus* (Thurling and Chay 1984). In general, this comparison suggests that induction is a less frequent event for maize than for some other species, including other grasses.

In addition, the PEM counts were significantly larger than the 10 to 100 macroscopic embryos per 1,000 anthers observed in the culture protocol experiments described above. Embryogenic microspores appeared at about three to four weeks after plating in some anthers and at frequencies closer to those for macroscopic embryos, which were routinely seen at about four to five weeks in other experiments. Embryogenic microspores were never observed when the PEM count was less than approximately 1,000 per 1,000 anthers (i.e., average of one per anther) and were always seen when it was above this level, suggesting that this may be a usable definition for "non-responsive". There did not appear to be a correlation, however, between the quantity of PEMs at week one and the number of EMs appearing later during culture (Table 4). This observation suggests that induction and survivability may be independent processes.

Table 4. Quantification of putative embryogenic microspores (PEMs) and embryogenic microspores (EMs) in maize anthers during culture. PEMs and EMs have been defined in the text. Each number is the mean of two counts of ten anthers each; a replicate is one plant

	Weeks after plating PEMs (EMs) per 1,000 anthers					
G'type/rep	1	2	3	4	5	6
'DBTS'/ 1	ND[a]	ND	1,275	250	100	50
2	1,150	4,900	2,050	800	5,350 (3,600)	2,200 (550)
3	1,500	700	100 (50)	100 (50)	100	0 (50)
4	50	100	0	100	150	50
'Sen 60'/1	750	550	400	0	0	0
2	100	200	100	50	0	0
3	850	1,700	350	350 (50)	0	0
4	2,300	3,850	2,050	2,350 (250)	1,600	200 (150)
'CH13'/ 1	5,100	850	850	550	250 (50)	450
2	150	200	50	350	150	50
3	50	50	100	150	0	ND
4	1,250	0	0	299 (50)	150	ND

[a] ND = Not determined

The phenomenon of high induction and low survivability has been demonstrated in anther culture of other species (e.g., *Brassica napus;* Dunwell et al. 1983) and it is generally recognized that abortion of induced microspores represents a serious limitation to the achievement of high culture responses (e.g., Wenzel et al. 1975). Since it is not unusual for the culture protocol to influence the induction frequency (e.g., Shannon et al. 1985) as well as the survival frequency (e.g., Dunwell and Thurling 1985), it should be possible to increase the frequency of both processes for maize.

In conclusion, the embryogenic response of three maize genotypes was shown to be influenced by a variety of cultural conditions. The lack of a cold pre-treatment step as well as the use of the media designated 79-10 essentially produced no androgenic response. The effect of liquid media and the addition of activated charcoal were less clear although the trend was towards an enhanced response. The highest rates observed here, about 100 embryos per 1,000 anthers plated, are comparable to other literature reports. Both haploid and diploid plants were regenerated from culture. The overall efficiency of the process was, however, low. In the initial study (Table 1 A), 195 embryos were obtained from over 23,000 anthers plated. Only 10 of those completed their reproductive cycle. A further inefficiency is that a significant proportion of the plants that are cultured do not respond at all, regardless of the treatment applied. Improvements in culture conditions, as demonstrated here and elsewhere, appear to provide only small, incremental increases in the response frequency. The observation that the induction potential of anther culture for maize, as demonstrated by PEM counts, exceeds the average rate of recovery of embryos by 5 to 10 fold strongly implies that more improvements can be made. Furthermore, the induction potential for maize appears to be lower than that for other species amenable to anther culture. Future research should focus on, 1) identifying the factors involved in plant-to-plant variation, 2) increasing germination frequency, 3) overcoming the selfing problem, 4) increasing the induction potential, and 5) enhancing the survivability of induced microspores.

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