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Production of normal, germinable and viable pollen from in vitro-cultured maize tassels

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Summary. Immature tassel meristems (1.0–1.5 cm long) of *Zea mays* L. inbred, Oh43, and single cross hybrid, Se60, cultured on a nutrient liquid medium underwent extensive development through to maturity and produced normal, mature, trinucleate pollen grains. The grains germinated on nutrient agar and on receptive silks and also produced viable kernels. No differences were observed between in vitro-produced pollen and in vivo pollen (pollen from greenhouse-grown plants) in characteristics such as pollen size, in vitro and in situ germination, and pollen tube growth in vitro. The kernels produced with in vitro pollen grew into mature plants (in vitro plants) which were similar to in vivo plants (plants produced with in vivo pollen), with no significant differences for all the morphological characteristics measured, and no phenotypic and cytological abnormalities. Gel electrophoresis of polypeptides revealed no major differences between in vitro and in vivo seedlings. This demonstration of fertilization and production of normal, uniform plants with pollen from cultured tassels has significant potential in basic and applied research studies.

Key words: *Zea mays* – Fertilization – In vitro pollen – In vitro culture – Uniformity

Introduction

The in vitro culture of immature flower primordia allows a number of exploratory and manipulative research opportunities which may not be possible on the intact plant. In addition to the factors underlying flower morphogenesis, the biochemistry and physiology of meiosis, megaand microsporogenesis, and pollen production might be studied in both normal and mutant genotypes. Also, investigations of the nutritional and environmental factors necessary for normal inflorescence growth and differentiation are possible.

Despite many attempts at in vitro culture of floral primordia (see review by Rastogi and Sawhney 1988), reports of normal growth until maturity are rare. The normal development of stamens and ovules, and the differentiation of pollen were documented in cucumber, *Viscaria,* tobacco, *Begonia* and tomato (see Rastogi and Sawhney 1988). In cereals, the culture of immature inflorescences leading to normal development has been documented only recently (Polowick and Greyson 1982; Pareddy and Greyson 1985), though the in vitro culture of inflorescence for callus or plantlets is more common (Vasil 1985). The initial reports of tassel culture of maize, both from cultivars Se60 (Polowick and Greyson 1982) and Oh43 (Pareddy and Greyson 1985), documented that immature tassel primordia undergo exensive normal development from anther initiation through meiosis and microsporogenesis to the production of normal, trinucleate pollen. The ear shoots of maize were also shown to produce mature pollen in vitro under certain conditions (Bommineni and Greyson 1987).

The production of germinable and viable pollen from in vitro-cultured flower primordia (Pareddy et al. 1987) had not been demonstrated in any plant species prior to our preliminary report. Here, we extend our observations to the production of normal, germinable and viable pollen from in vitro-cultured maize tassels and report on the function of in vitro-derived pollen (in vitro pollen) in relation to in vivo pollen, by comparing some pollen characteristics. Also we document that plants produced with in vitro pollen are normal and uniform with no detectable morphological or cytological variability.

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Materials and methods

Plant material

Seeds of *Zea mays*, field corn inbred Oh43 (Su1/Su1) and sweet corn single cross hybrid Se60 (su1/su1) were germinated and grown, 1–2 per pot, in 20 cm plastic pots containing a standard greenhouse soil mixture of loam/sand/peat (8/5/4). The plants were raised in the greenhouse with an 18 h photoperiod supplemented by fluorescent and incandescent illumination. The day/night temperature was maintained at 24/18 ± 2 °C. A soluble fertilizer (20-20-20) was applied at weekly intervals.

Tassel culture

Shoot segments were selected, surface-sterilized and dissected aseptically as described previously (Pareddy and Greyson 1985). The immature tassels of 1.0–1.5 cm size were placed into 125 ml Erlenmeyer flasks containing 40 ml of liquid basal medium. The medium was composed of Murashige and Skoog (1962) major and minor elements, White's (1943) vitamins and glycine, myo-inositol (100 mg/l), sucrose (0.1–0.3 *M*), casein hydrolysate (30 mg/l) and kinetin (10^{-7} *M*). The pH of the medium was adjusted to 5.8 before autoclaving. Flasks were incubated without shaking in growth cabinets at 28 ± 1 °C and with 18 h light (40–50 μE m^{-2} s^{-1})/6 h dark photoperiod.

Pollen collection and measurements

The cultured tassels were harvested after 16–25 days of culture when the spikelets extruded anthers. Freshly extruded anthers were excised, dried on either Whatman no. 1 or no. 3 filter paper in a desiccator containing a mixture of dehydrated silica gel and $CaCl_2$ for 0.5–5 h while the pollen was shed. Unextruded anthers were also excised from spikelets and dissected to release pollen in either liquid pollen-germinating medium or paraffin oil (Cook and Walden 1965). For controls, pollen was collected from greenhouse-grown plants between 10.00 and 12.00 o'clock. In each case, pollen was used for germination or fertilization within 15 min of collection. For pollen measurements, freshly collected pollen was stained in propiocarmine and photographed. Pollen dimensions were measured from photomicrographs or projected negative images.

In vitro germination of pollen

In vitro germination of pollen was conducted on the medium containing 12% sucrose, 0.03% $CaCl_2 \cdot 2\,H_2O$ and 0.01% H_3BO_3 (Cook and Walden 1965) modified by increasing the agar content from 0.7% (Noble agar) to 1.5% (DIFCO no. 0140-01 agar) (modified Cook and Walden basal medium, MCWBM). Pollen grains from (1) extruded anthers; (2) unextruded anthers, or (3) greenhouse-grown plants were applied uniformly to the medium in petri dishes (Falcon no. 3002) in sealed containers with a relative humidity in approximately 90%–100% and were incubated at 26 °C. Samples were collected after 1.5–2 h of inoculation, fixed in FPA and stored at 4 °C (Pfahler 1967), with the germination response measured directly with a compound microscope at a magnification of 40 × (10 × 4). Pollen was classified as: (1) germinated, (2) ungerminated, or (3) ungerminated and burst (Cook and Walden 1967). Five fields were choosen randomly from each of 4–8 replicate petri dishes and photographed at the magnification of 100 × (10 × 10) on 35 mm Plus-X film (Kodak). Tube lengths were measured from the projected images of the negatives.

In situ germination of pollen

Greenhouse-grown plants were detasseled and ear shoots were bagged prior to silk emergence. After extrusion, the silks were trimmed to within 2–3 cm of the tip of the ear husks 16–18 h prior to pollination. Pollinations were carried out in the laboratory by carefully spreading pollen on the silks with a clean, dry, artist's camel's hair brush. Samples of silk, 2–3 cm long, were removed at 1 h intervals and fixed in absolute ethanol and glacial acetic acid (3:1) for 12–24 h, cleared in 5 *N* NaOH for approximately 18–24 h (Reger and James 1982) and stained in decolorized aniline blue (pH 10). Pollen tubes were detected by the fluorescence of the callosic inner layer of the tube wall (Linskens and Esser 1957).

Pollination

Controlled pollinations were carried out according to the procedure of Neuffer (1982). Greenhouse-grown plants, prepared as described above including prior removal of the tassel, were taken to the laboratory, where all pollinations were performed. Pollinations were made with the pollen from three sources: (1) extruded anthers of cultured tassels; (2) unextruded anthers of cultured tassels, or (3) greenhouse-grown plants. With unextruded anthers, the pollen suspension, either in liquid germinating medium or paraffin oil (Coe et al. 1966), was spread on the silks. After pollination, ears were bagged and the plants were returned to the greenhouse for approximately 40 days before the ears were harvested. The ears were air-dried at room temperature for several days before the numer of kernels and ovules per ear were counted and *P* (fertilization ability) values were computed (Walden and Everett 1961).

Morphological analysis

Samples of seeds from each progeny produced with in vitro pollen (R_1) and their selfed progeny (R_2) and controls, F_1 and F_2, produced by sibbing and subsequent selfing, were grown in the greenhouse in 1985 and 1986 for preliminary observation. Larger plantings were observed in the field nursery during the 1985 and 1986 seasons. The following characteristics were recorded: (1) plant height, (2) number of leaves per plant, (3) leaf width: average width of top ten leaves measured at tassel emergence, (4) tassel length, (5) number of tassel branches, (6) pollen fertility, (7) fertilization ability (*P*) and (8) seed weight × 100.

Pollen fertility was determined by staining freshly collected pollen betwen 10.00 and 11.00 o'clock, with iodine potassium iodide (I_2KI) solution. Mean values and standard deviations of all measurements were calculated. Count data and percent data were transformed by using square root and arc sine transformation, respectively (Zar 1974). An analysis of variance was performed and mean differences were tested by Scheffe's multiple range test ($P = 0.05$) to study the significance of morphological variation.

Cytological analysis

Chromosome counts from slides prepared from fixed root tips from 45 plants were made according to Chen (1969). For meiotic analysis, immature tassels were removed, fixed in Farmer's solution for 24–28 h at room temperature and then transferred to 70% ethanol and stored at 4 °C. Bivalents and meiotic structures were observed in microsporocytes from 40 plants produced with in vitro pollen in addition to 10 controls.

Gel electrophoresis

The terminal 1.0 cm tips of plumules and 2.0 cm, tips of radicles from 5-day-old seedlings of Oh43 and Se60 (both in vitro pollen-derived and controls) were excised, labelled with ^{35}S-methionine and extracted in cold plant extraction buffer containing 200 m*M* TRIS-HCl (pH 7.5), 5% SDS, 7.5% 2-mercaptoethanol and 1 m*M* PMSF, as described by Baszczynski et al. (1982). Protein concentrations were determined by a turbidimetric assay (Comings and Tack 1972), and radioactivity was measured as described in Baszczysnki et al. (1982).

One-dimensional gel electrophoresis in the presence of SDS was carried out according to the method of Laemmli (1970), except that the separating gel consisted of a 7.5%–17.5% polyacrylamide linear gradient. Two-dimensional gel electrophoresis was performed as described by O'Farrel (1975) with some modifications: Noniodet P-45 and ampholines (consisting of a mixture of 80% pH range 5–8 and 20% pH range 3.5–10) were added to the samples prior to isoelectric focussing. The former was added to yield a final NP-40 : SDS ratio of 8 : 1 and the latter were added to yield a final concentration of 2% ampholines. The second-dimension slab gel consisted of 7.5%–17.5% polyacrylamide with a 3% polyacrylamide stacking gel. Fluorography of Coomassie brilliant blue G-250 stained gels was done according to Lasky and Mills (1975).

Results

Tassel maturation and pollen production

Immature tassel meristems of Se60 and Oh43 explanted to a nutrient medium grew to maturity after 16–25 days of culture and produced 100–200 'normal' spikelets per tassel. Frequently, 20%–50% of normal spikelets yielded extruded anthers with elongated filaments. Freshly extruded anthers, each containing 1,000–2,000 pollen grains, dehisced and liberated pollen after 0.5–5 h in a desiccator. The pollen was normal, trinucleate with considerable starch. The diameter of in vitro-produced pollen (in vitro pollen) was similar to that of in vivo pollen (greenhouse pollen) for both cultivars, Se60 and Oh43 (Table 1). However, Oh43 pollen was consistently larger in diameter than Se60 pollen (ca. 96 μm compared to 90 μm).

Pollen germination

The results of in vitro germination of pollen from three different sources are summarized in Table 2. Pollen from extruded anthers germinated well on the surface of an agar medium. For Se60 and Oh43, approximately 70% and 50% of the pollen germinated, respectively. The rest of the pollen either remained intact or burst without germinating. Germination of in vitro and in vivo pollen from both the genotypes was not different (Table 2). Surprisingly, pollen from unextruded anthers also germinated (8%–11%), but at a reduced rate for both cultivars. In these, a higher percent (10%–15%) of "pollen burst" was observed (Table 2). The pollen tube growth responses of in vitro and in vivo pollen were also similar, with no significant differences in the growth rates for either genotypes (data not shown).

Table 1. A summary of diameter of in vitro- and in vivo-derived pollen from two genotypes, Se60 and Oh43 ($N=100$), from two different experiments

Genotype	System	Pollen diameter (μm) $\bar{x}\pm$S.E.
Se60	in vitro	88.3±0.5
	in vivo	90.6±0.4
Oh43	in vitro	96.5±0.5
	in vivo	96.9±0.4

Table 2. A summary of pooled data from three different experiments on germination on MCWBM of pollen from (1) cultured tassels (in vitro) and (2) greenhouse-grown plants (in vivo) from two genotypes Oh43 and Se60

Genotype	System	Anther type	Pollen burst $\bar{x}\pm$S.E. (%)	Pollen germinated $\bar{x}\pm$S.E. (%)	*N*
Oh43	in vitro	unextruded	14.9±0.7	7.5±1.2	3,261
	in vitro	extruded and dehisced	1.3±0.2	50.0±1.7	2,920
	in vivo	extruded and dehisced	4.1±0.5	52.7±0.5	2,237
Se60	in vitro	unextruded	10.2±1.5	10.6±1.5	2,300
	in vitro	extruded and dehisced	1.8±0.3	68.8±2.2	3,244
	in vivo	extruded and dehisced	3.9±0.4	72.4±4.5	2,832

The germinability of pollen collected after increasing the interval of drying of extruded anthers is shown in Fig. 1 A. For both genotypes, pollen germination decreased with increased drying time until 5 h. No growth of pollen was observed after drying the anthers for 5 h. Overall, germination of Se60 pollen was higher than Oh43 pollen.

In situ germination of in vitro pollen was also observed and compared to that of in vivo pollen. Although the percentage of germination on the silks and lengths of pollen tubes was not measured, no obvious differences were detected between the pollen from cultured tassels and that produced on the tassels of greenhouse-grown plants. Generally, pollen germinated through trichomes and, occasionally, on the body of the silk directly. The tubes grew for a short distance between the cells of the cortex and traversed into the transmitting tract similar to that observed with in vivo pollen.

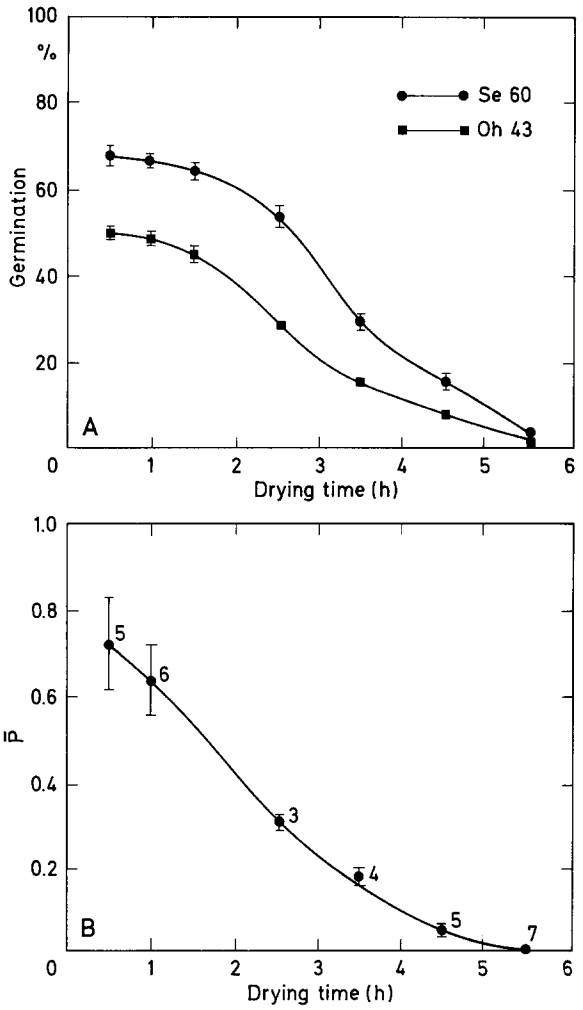

Fig. 1 A and B. Germination and fertilization response of pollen from extruded anthers dried for different times in a desiccator. **A** Germination response. Each point represents a mean value ± S.E. counted from 20 microscopic fields from 4–5 replicate petri dishes ($N \geqq 1{,}500$). **B** Fertilization response (P). Each point represents a mean value ± S.E. of P (P = number of kernels per ear/number of ovules per ear). The data from Oh43 and Se60 were pooled. The number above each point shows the number of ears used to calculate P value

Fertilization

The in vitro-produced pollen, after pollination, not only germinated on silks but also fertilized ovules to produce well-developed, viable kernels. Kernels were produced on all ears that were pollinated with fresh pollen, obtained within 5 h from the start of desiccation. Approximately 100–200 kernels were produced per ear with pollen collected from 500–1,000 extruded anthers from about 2–5 tassels, depending on the cultivar and percent extrusion of anthers.

The fertilization ability of pollen in relation to the drying time of anthers is plotted in Fig. 1 B. As observed with in vitro germination, the fertilization ability of pollen also declined with the increased drying time of anthers. Nevertheless, a considerable number of kernels was produced with pollen collected within 1.0 h. However, no kernels were produced with the pollen collected from extruded anthers which had dried for more than 5 h. Also, pollinations with pollen from unextruded anthers did not result in fertilization.

Analysis of variability

The kernels produced with pollen from cultured tassels had 90%–100% germinability upon planting in the greenhouse or the field and yielded mature, healthy, fertile plants. No phenotypic abnormalities were detected among the progeny of the F1 ($N=290$) or F2 ($N=80$) grown in the field and greenhouse. The statistical analysis of morphological characters showed that both the plants derived from in vitro pollen (R_1) and their selfed progeny (R_2) were not significantly different from the control plants.

Cytological analyses of root tip cells from 45 in vitro pollen-derived plants (in vitro plants) showed that all plants were diploid ($2n=20$). Analysis of microsporocytes from 40 plants consistently showed 10 tetrads at diakinesis and metaphase I, no bridges or fragments at anaphase I and II, and no other abnormalities at the quartet stage as seen in the control plants ($N=10$). In all plants tested, mature, fertile pollen was observed.

The analysis of polypeptide patterns by 1-D gel electrophoresis in plumules and radicles of Oh43 and Se60 produced with in vitro and in vivo pollen showed neither qualitative nor quantitative differences between the in vitro and in vivo plants. The analysis by 2-D gel electrophoresis showed no qualitative differences but minor quantitative differences between in vitro and in vivo seedlings for a few polypeptides. However, apparent qualitative and quantitative differences were observed between plumules and radicles, and between Oh43 and Se60.

Discussion

The present study demonstrates that pollen produced from cultured tassels is normal, germinable and viable, and yield normal, fertile plants with no morphological or cytological abnormalities. The in vitro pollen and in vivo pollen, based on characteristics such as pollen size, in vitro and in situ pollen germination, and pollen tube growth in vitro were very similar (Tables 1 and 2). The similarity of in vitro and in vivo pollen was consistent, despite the differences observed between two genotypes (Oh43 and Se60) for pollen size and pollen germination. The genotypic differences were noted previously for pollen size (Rambaugh and Wahlen 1973) and pollen germination (Herrero and Johnson 1980) in maize, and the

pollen germination values obtained here are similar to those reported previously for these genotypes (Cook and Walden 1965, 1967; Herrero and Johnson 1980). The pollen tube lengths, however, were similar for these genotypes containing Su1 (Oh43) and su1 (Se60) allele as described in earlier studies (Pfahler and Linskens 1972).

The percentage of germination and fertilization ability (P) of in vitro pollen decreased with the increased drying time of anthers in a desiccator (Fig. 1 A and B). In maize and other grasses, pollen has a water content as high as 60% (Knox et al. 1986); consequently, it remains viable for only a short period even under optimum conditions (Johri and Vasil 1961; Walden 1967). Further, it has been shown that loss of water content generally results in a decrease in the percentage of pollen viability (Dumas et al. 1983). In the present study, the rapid decline of pollen viability may be due to the water loss of pollen at a faster rate in the desiccator.

The seed produced with in vitro pollen yielded normal, fertile and uniform plants which were apparently similar to in vivo plants (controls), with no significant differences for all the morphological characters measured and no detectable chromosomal or phenotypic abnormalities. The electrophoretic data also supported the view that in vitro plants are similar to the controls, as no observable major differences between polypeptides of these plants were detected. The tissue culture-induced variability, somaclonal variation (Larkin and Scowcroft 1981), may be useful for some breeding purposes but it is an undesirable complication, especially where genetic modifications of plant cells or tissues in vitro are expected. Since no major changes were observed in the progeny produced with in vitro pollen, the tassel culture technique might be conveniently used for genetic modifications of pollen.

This and our earlier report (Pareddy et al. 1987) are, to our knowledge, the first reports that demonstrate that the pollen from cultured flower/inflorescence primordia germinate and fertilize ovules to produce seed. With this demonstration that the in vitro-derived pollen is normal, germinable and viable, and produce normal, uniform plants, a number of basic research studies and genetic manipulations such as transformation, mutagenesis and selection of pollen can be contemplated as can attempts on haploid production and chemical reversion of genic male steriles. These possibilities can be anticipated in other cereals when inflorescence culture is successfully demonstrated.

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References

Baszczynski CL, Walden DB, Atkinson BG (1982) Regulation of gene expression in corn (*Zea mays* L.) by heat shock. Can J Biochem 60:569–579

Bommineni VR, Greyson RI (1987) In vitro culture of ear shoots of *Zea mays* and the effect of kinetin on sex expression. Am J Bot 74:883–890

Cheng CC (1969) The somatic chromosomes of maize. Can J Genet Cyt 11:752–754

Coe EH, Cook FS, Walden DB (1966) Pollinations with liquid suspensions. Maize Genet Coop Newslett 40:108

Comings DE, Tack LO (1972) Similarities in the cytoplasmic proteins of different organs examined by SDS gel electrophoresis. Exp Cell Res 75:73–78

Cook FS, Walden DB (1965) The male gametophyte of *Zea mays* L. II. In vitro germination. Can J Bot 43:779–786

Cook FS, Walden DB (1967) The male gametophyte of *Zea mays* L. III. The influence of temperature and calcium on pollen germination and tube growth. Can J Bot 45:605–613

Dumas C, Duplan JC, Said C, Soulier JP (1983) ^{1}H Nuclear magnetic resonance to correlate water content and pollen viability. In: Mulcahy DL, Ottaviano E (eds) Pollen: Biology and implications for plant breeding. Elsevier Biochemical, New York, pp 15–20

Herrero MP, Johnson RR (1980) High temperature stress and pollen viability of maize. Crop Sci 20:796–800

Johri BM, Vasil IK (1961) Physiology of pollen. Bot Rev 27:325–381

Knox RB, Williams EL, Dumas C (1986) Pollen, pistil and reproductive function in crop plants. Plant Breed Rev 4:9–79

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680–685

Larkin PJ, Scowcroft WR (1981) Somaclonal variation – a novel source of variability from cell cultures for plant improvement. Theor Appl Genet 60:197–214

Laskey RA, Mills AD (1975) Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. Eur J Biochem 56:335–341

Linskens HF, Esser K (1957) Über eine spezifische Anfärbung der Pollenschläuche im Griffel und die Zahl der Kallosepropfen nach Selbstung und Fremdung. Naturwissenschaften 44:1–2

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Neuffer MG (1982) Growing maize for genetic purposes. In: Sheridan WF (ed) Maize for biological research. University of North Dakota, Grand Forks, pp 19–30

O'Farrell PH (1975) High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250:4007–4021

Pareddy DR, Greyson RI (1985) In vitro culture of immature tassels of an inbred field variety of *Zea mays* cv. Oh43. Plant Cell Tissue Organ Cult 5:119–128

Pareddy DR, Greyson RI, Walden DB (1987) Fertilization and seed production with pollen from in vitro cultured maize tassels. Planta 170:141–143

Pfahler PL (1967) In vitro germination and pollen tube growth of maize (*Zea mays* L.) pollen. I. Calcium and boron effects. Can J Bot 45:839–845

Pfahler PL, Linskens HF (1972) In vitro germination and pollen tube growth of maize (*Zea mays* L.) pollen. VI. Combined effects of storage and alleles of the waxy (wx), sugary (su1) and shrunken (sh2) loci. Theor Appl Genet 42:136–140

Polowick PL, Greyson RI (1982) Anther development, meiosis and pollen formation in *Zea* tassels cultured in defined liquid medium. Plant Sci Lett 26:139–145

Rambaugh MD, Whalen RH (1973) Measurement of pollen grain size in the diallele. Maize Genet Coop Newslett 47:170–171

Rastogi R, Sawhney VK (1988) In vitro development of angiosperm floral buds and organs: a review. Plant Cell Tissue Organ Cult (in press)

Reger BJ, James J (1982) Pollen germination and pollen tube growth of sorghum when crossed to maize and pearl millet. Crop Sci 22:140–144

Vasil IK (1985) Somatic embryogenesis and its consequences in the gramineae. In: Henke RR, Hughes KW, Constantin MP, Hollaender A (eds) Tissue culture in forestry and agriculture. Plenum, New York, pp 31–47

Walden DB (1967) Male gametophyte of *Zea mays* L. I. some factors influencing fertilization. Crop Sci 7:441–444

Walden DB, Everett HL (1961) A quantitative method for the in vivo measurement of the viability of corn pollen. Crop Sci 1:21–25

White PR (1943) A hand book of plant tissue culture. Jacques Cattell Press, Lancaster

Zar JH (1974) Biostatistical analysis. Prentice-Hall, Englewood Cliffs/NJ