In order to induce embryoid development 182 calli of the above have been subcultured on an auxin-rich medium. It was found that after one week of subculture callus growth was almost completely stopped. Up to the present, no embryoid initiations were definitely identified. However, it is hoped that some of them will develop into plantlets before long. Previously it took 4-6 weeks for maize callus to differentiate into plantlets.

Nineteen of the calli were maintained by subculturing them on a 2, 4-D-containing medium.

It was also found that it did not make any difference in callus initiation and growth in the first four to six weeks of culture whether they were kept in dark or in light. Nevertheless, the calli were pale green if they were grown in light, while they were pale if they were grown in dark.

If this experiment will eventually lead to the production of plantlets, either haploid, diploid or aneuploid, it might become a new and useful technique in maize genetics, breeding, development and molecular biology.

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Methods of maize pollen germination in vitro, collection, storage, and treatment with toxic chemicals; recovery of resistant mutants

One vigorous corn plant sheds over 10^7 haploid, trinucleate pollen grains. Waxy and alcohol dehydrogenase-1 are known to be expressed after meiotic anaphase II, and the many correlations of duplication-deficient gametes with pollen abortion suggest that much of the pollen phenotype is encoded by its haploid genotype rather than the genotype of its pollen mother cell. For this reason, Nelson (commencing in 1958, Science 130: 794 with wx) and later, Freeling (1976, Genetics, in press, with Adh) were able to study intracistronic recombination and reversion, and Schwartz (1975: lecture at the International Maize Symposium, Urbana) was able to select Adh-deficient mutants among allyl alcohol-resistant pollen grains. Over the last two years, this laboratory has perfected numerous procedures involving maize pollen. Following Schwartz's lead, we have also recovered mutants via pollen selection. The methods and recipes we use follow. We hope they prove generally useful.

In vitro germination using "David's Bread Loaf": We have revised the pollen germination medium and conditions reported by Cook and Walden (1965, Can. J. Bot. 43: 779). Our concoction--called the Cook and Walden Revised Medium (CWRM)--is composed of 17% w/v sucrose, 300 mg/l $CaCl_2 \cdot 2H_2O$, 100 mg/l H_3BO_3 and 0.7% w/v Difco Bactoagar, to a pH of 6.4 after the addition of agar but before heating. After heating until just clear (120 C for 8 min), this medium is gelled in a Griffin beaker of the desired diameter and stored at 4 C for at least four days without change. This column of gel is called "David's bread loaf", from which 2 mm slices are cut and immediately used for pollen germination at 25 C and low (uncontrolled) humidity within an unsealed petri dish. Our methods differ from Cook and Walden's in sucrose concentration, pH and humidity requirement; the major difference is that our pollen grains germinate on a newly-cut solid surface. We achieve 75-95% germination of healthy pollen after 30 min for all of the seven different lines and inbreds we have tried. Before we devised the "bread loaf" technique, we experienced dramatic genotype-specific fluctuations; pH and sucrose concentration had to be continuously adjusted. The "bread loaf" technique affords the reproducibility necessary for determining kill curves for pollen pretreatments, and slices with gametophytes are easily moved to other dishes for staining, counting, fixing and the like.

Pollen collection and storage: The majority of our pollen viability studies utilized a 23± 2 C greenhouse, low humidity and natural January to March lighting. We find that our typical plant sheds over a five-day period. First-day pollen usually germinates poorly; second and third day collections 2-4 hrs after dawn are

optimal; we avoid afternoon collections. Tassels should be stripped of anthers and pollen the evening prior to collection. Pollen is collected as shed in glassine bags, desiccated ($CaSO_4$) and stored at 4 C for 30 min to 4 hr. This cold storage-desiccation consistently elevates percent germination 5-10% to our modal 85%.

Berkeley's cool, dry summer permits routine collection of viable pollen from the field. Temperatures above 35 C or any discernible humidity greatly reduced the pollen's germinating ability. We typically collect 8 x 10^5 grains per plant in the late afternoon (2-5 p.m. PDT), subject them to chemical selective treatment, and pollinate at dusk. Our field pollen is from 50 to 90% viable. We suspect that the heat and humidity characterizing a corn belt summer might necessitate using cooled greenhouses for the male parents.

Pollen counting: Pollen samples were suspended in 40% technical glycerin in a 250 ml graduate cylinder to a final concentration of 4-6 x 10^3 pollen grains per ml by visual estimation. A 1.0 ml sample of homogeneous suspension was further diluted and the sample was layered evenly over a gridded Gelman filter (GA-6, 0.45 µm, 47 mm diam.) and quickly deposited by evacuation; circular currents were avoided in the layering process. The number of particles in four radial strips, each containing 5 squares (each square is one percent of the total area of the filter) were counted under incident light at a total magnification of 16X. In the rare instances where these numbers were significantly different from a 1:1:1:1: (by χ^2), the entire sampling process was repeated. For each original pollen sample, two filters were prepared, counted and preserved for further reference; this gave eight statistically equivalent numbers on which to base our estimates of total pollen grains per ml of original suspension. In general, 1 mg of our desiccated pollen contains about 2,000 grains.

When simply counting for percent germination, the slice of germination medium is placed over a grid of any desired color, or over a transparent grid if underlighting is desired.

Treatment with toxic vapor and recovery of resistant mutants: We have selected pollen grains resistant to various toxic vapors. Only allyl acohol (CH_2=CH-CH_2OH) resistance (rationale after Megnet, 1967, Arch. Biochem. Biophys. 121: 194, in yeast)--as it affects alcohol dehydrogenase activity--has been biochemically characterized: Megnet's scheme selectively kills ADH^+ cells owing to their capacity to oxidize relatively innocuous allyl alcohol to deadly acrolein. Megnet's selection is now being used in Drosophila and, as previously cited, Schwartz has reported success with maize ADH in pollen. Neither the selection scheme nor the strategy of using the male gametophyte are original to this laboratory, but the methods and results which follow are.

Pollen grains from a plant heterozygous for a gamma-induced ADH-deficient mutant (Adh-Fγ25, Freeling, unpublished), Adh^+/Adh^- were collected, stored, and dispensed in 20 mg lots (40,000 grains) onto glassine paper for vapor treatment in a 500 ml Mason jar with sealed lid containing 20 ml of $CaSO_4$ dessicant. In this experiment, methanol was used as an inert carrier. 0 - 50 µl 100% allyl alcohol was pipetted into the jar before sealing (1 µl allyl alcohol vapor/500 ml is ca. 7.4 x 10^{-5} M). After 40 min of treatment, the pollen was evenly dusted with a camel hair brush over a newly cut slice of CWRM and germinated as described. After the germination was complete, the slice was frozen at -23 C for 3 hr and defrosted for 30 min. ADH-specific stain was carefully layered over the gametophytes, left for 30 min, and replaced three times using methods of Freeling and Brown (1975, MGCNL 49:19). The 50% of the pollen grains that were ADH^+ stained blue and opaque; the other 50%, ADH^-, stained yellow and translucent. Thus the four phenotypes: blue with pollen tube, blue without tube, yellow with tube, and yellow without tube. The data tabulated below compare the allyl alcohol kill curves for sibling Adh^+ and Adh^- male gametophytes.

Allyl Alcohol µl/500 ml	Allyl Alcohol mM	Mean Relative Percent Germination* ADH^+ (blue)	Mean Relative Percent Germination* ADH^- (yellow)
0	0	100 (78.5%)	100 (80.7%)
1	0.07	40	98
2	0.15	8.5	99
4	0.29	Zero	86
10	0.74	Zero	77.5
20	1.47	Zero	55.8
30	2.21	Zero	36.8
40	2.94	Zero	30.5
50	3.68	Zero	8.3

*The results of four independent experiments are averaged for each data point; mean absolute control was 79%; allyl alcohol was diluted with methanol such that 50 µl of foreign vapor were present; treated for 40 min.

According to the data, treatment under these conditions with about 0.3 mM allyl alcohol should let just a few ADH^+ gametophytes germinate. Does a positive germination assay indicate ability to fertilize an ovum successfully? Pollen samples treated exactly as above were brushed onto silks which had been cut back the previous day. Routine electrophoretic methods were used to ascertain whether $\underline{Adh^+}$ or $\underline{Adh^-}$ sperm participated in the fertilization. The results of these progeny tests were:

Allyl Alcohol (mM)		Seed Set	No. Seeds Receiving Allele* $\underline{Adh^+}$	No. Seeds Receiving Allele* $\underline{Adh^-}$
0.30		100%	0	72
0.30		100	0	70
0.22		100	3	65
0	Control	100	42	38

*Less than 25% of the seed set was genotyped.

We conclude that ability to germinate $\underline{in\ vitro}$ at least approximates ability to fertilize an ovum. However, when ADH^+ pollen is treated with higher concentrations of allyl alcohol, permitting fewer than 10^{-5} germinations, the vast majority of these survivors remain ADH^+. As might be expected, there are alternative ways to be resistant to any toxin.

$\underline{\text{Pollen Progaganda}}$: Since the recent dawn of "plant somatic cell genetics" much has been said of selecting mutants in haploid, totipotent suspension cultures. We submit that the tricks of "plant germ cell genetics" are not yet exhausted. Pollen is available in huge numbers; is haploid; mutants may be recovered in informationally normal cells; and pollen expresses many $\underline{\text{differentiated functions}}$, possibly permitting selection of agronomic traits. We see the pollen grain as a powerful genetic resource. We hereby grant permission to cite these methods.

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