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Technology for Automated Analysis of Maize Pollen Used as a Marker for Mutation: 1. Flow-Through Systems

by Harry W. Tyrer*

Maize pollen is used as a monitor for environmental pollutants. Mutant pollen grains (induced by environmental pollutants) are detectable above a background frequency of 5 or less in 10^5. To enumerate a satisfactory number of mutant grains, it is necessary to count 10^6 grains in a sample, a laborious, time-consuming process which should be amenable to automated analysis techniques. High resolution image analysis technology has been used in the morphologic assessment of rare cells in a sample, provided a suitable training set could be devised to instruct the computer on the characteristics of the rare cells. On the other hand, flow cytometry uses primarily cytochemical means for detection and has been shown to detect rare events. Hence, the two technologies, which may be viewed as complementary, are suitable for the task. Alternatively, a hybrid technology employing both cell sorter and image analysis techniques may be extremely desirable for this problem. The potential for archival storage of analyzed samples is very attractive when considering the possibility of an adversary relationship between a putative regulator and polluter.

Introduction

Maize pollen from *Zea mays* has been used as a monitor for environmental pollutants (*1*, *2*). In general, an inbred strain which is homozygous for the *wx-C* allele (referred to here as waxy) is caused to revert to the wild type or *Wx-C* (referred to here as starchy) as a result of environmental pollutants. A linear dose response of mutant frequency resulting from mutagenic agents has been obtained using the forward (starchy to waxy) assay (*3*). The reverse assay (waxy to starchy) has also been calibrated for but for radiation mutation (*4*). Although additional work remains to be done in obtaining a suitable assay for environmental carcinogenesis using maize pollen, it is desirable to consider automated technologies not only for their capacity for work reduction but for the added insight they may provide.

The background frequency of spontaneous mutation is approximately 2-5 in 10^5 grains (*5*). A minimally satisfactory number can be obtained by counting or estimating the number of mutant pollen grains in 10^6 total grains. Investigators of pollen mutation were polled to determine the time required to obtain reliable mutation frequencies either by direct counting or estimation. Typically the response suggested several hours to perform the task. An important consideration here is to express mutant frequency with satisfactory confidence. With the case of the estimation techniques, a sufficiently large number of grains are considered (in some cases substantially more than a million) so that enough redundancy exists to provide the investigator with the accuracy required (*6*).

Instrumentation to enumerate the frequency of a rare event can be based on either image analysis (*7*) or flow cytometry technology (*8-0*). These technologies have been used to obtain sample characteristics and to measure characteristics of individual particles in the sample (*11*, *12*). These technologies must be viewed as complementary and indeed it may be that a hybrid system is the most appropriate for this application (*13*). Here we present data obtained on a flow-through system to assess the possibility of such a system to differentiate be-

* Bioengineering and Biophysics Department, Cancer Research Center, Columbia, Missouri 65205, and Department of Electrical Engineering, University of Missouri, Columbia, Missouri 65212.

tween waxy and starchy corn pollen, pointing the way to enumerate the frequency of mutant pollen grains. The choice of flow systems over high resolution or television scanning systems was predicated by convenience.

Materials and Method

Maize pollen in 70% ethanol was obtained from Dr. W. R. Lower. Two vials were received: one contained pollen from a wild type plant (starchy), and the other contained pollen from a plant that expressed the waxy phenotype. A portion of the pollen was crushed for spectral analysis as follows. A small aliquot of the pollen was withdrawn from its container with a Pasteur pipet, 20 drops of either starchy or waxy pollen were crushed using a mortar and pestle then the pollen material was resuspended in 3 ml deionized water. After further crushing and swirling of the sample, it was allowed to settle momentarily before gently decanting to a test tube. Spectra were obtained from these samples or from samples stained by iodine: for the unstained samples, one drop of pollen material was suspended in 3 ml of deionized water; for iodine stained samples, 1 drop of iodine potassium iodide was additionally placed into the solution.

Sucrose solution was prepared as follows. Into 100 ml deionized water approximately 100 g of sugar was placed. The water was first heated to 70°C and the sugar added while stirring without heat. This results in a 73% sucrose solution. After stirring overnight, the sample was filtered using a 20 μm Nalgene filter system. To measure the settling times, fixed amounts of intact maize pollen were dispensed into test tubes containing equal amounts of fluid, but the 73% sucrose diluted with water as follows: no dilution; 1/1; ½; ¼; no sucrose. After stoppering and mild shaking by inverting the test tube, the tubes were allowed to sit overnight for settling. The samples were carefully introduced into a Bausch and Lomb Spect-20 spectrometer, set at 500 nm, maximum transmission was measured and adjusted to 100%. The sample was then gently inverted several times and measured again so that minimum transmission could be measured (0% transmission). The sample was shaken again and placed into the spectrometer to determine the time for transmission to go to ½ and then ¾ of the range of value.

Fluorescence spectra of maize pollen was obtained with a fluorescence activated cell sorter (FACS-III, B-D Division, Sunnyvale, California) after some modification. The main requirement was to increase the inner diameter of the sample tubing and the use of a large orifice nozzle (200 μm). Pollen was suspended in 73% sucrose for introduction in the machine. The sheath consisted of the standard isoton saline solution. Scatter was measured using the wide angle aperture as previously described (*14*), and fluorescence was measured by using two 522 nm blocking filters to eliminate the 488 nm exitation light of 200 mW. No effort was made to clean up the sample prior to its introduction to the flow cytometer. The scatter distribution of the pollen was obtained by gating on the autofluorescence of the pollen. This substantially eliminated all of the non-pollen debris and the pollen husks. Pollen stained with iodine potassium iodide was run on the sorter under the same conditions and prepared as follows. Approximately 60,000 grains of pollen were suspended in ½ ml of 70% ethanol and 1 drop of iodine was added. The solution was allowed to stand 20 min, after which the staining solution was decanted off, 3 ml of 73% sucrose added, the sample shaken, and introduced into the sorter.

Results

Maize pollen in a water solution settles in essentially two minutes. With such rapid settling, pollen falls out of solution in the transporting medium, preventing satisfactory flow in the flow cytometer. To mitigate this effect, pollen was suspended in a 73% sucrose solution; further, the tubing in the flow system had to be increased to permit this viscous fluid to flow. Sequential dilutions of this sucrose system showed decreasing settling times for the pollen (Table 1). The 73% solution was satisfactory and required no stirring to maintain the pollen in suspension. An objective figure for the settling times of the pollen was obtained with the spectrometer by measuring the times required for 50% and 75% light transmission due to reduction in opacity by settling. These values are shown in Table 1.

Unstained maize pollen in a fluorescence microscope showed a substantial amount of autofluorescence in the intact pollen grains with no fluores-

Table 1. Corn pollen settling times.[a]

	Settling time, sec				
		Sucrose: water			
Light transmission, %	No dilution	1:1	1:2	1:4	No sucrose
50	2160	250	154	104	78
25	3180	293	185	137	102

[a]After overnight settling of the sample, light transmission was measured and adjusted to 100%. 0% transmission was set by measuring immediately after sample stirring.

cence from pollen husks or non-pollen debris. To determine the characteristics of this autofluorescence, pollen was crushed and the spectrum of the resulting solution obtained. This spectrum is shown in Figure 1 and the choice of emission wavelength (522 nm) and exitation wavelengths (300 nm-00 nm) was determined by suitability to flow cytometry. The four spectra shown in Figure 1 were from unstained starchy and waxy pollen material, demonstrating the autofluorescence spectrum, and from stained starchy and waxy pollen material, demonstrating fluorescence reduction by iodine potassium iodide. The spectrum of the unstained pollen material shows a maximum at 370 nm and the minimum at 425 nm with little or no difference between the shape of the waxy or starchy spectra. However, there is a difference in fluorescence intensity at a given wavelength due to the difference in amount of material in the samples (this was set purposely to distinguish between the two curves). The lower two curves show the collapse of the fluorescence as a result of iodine staining. By the differential staining property of the intact pollen for iodine, it should be possible selectively to collapse the fluorescence of the starchy, the more intensely stained pollen of the two.

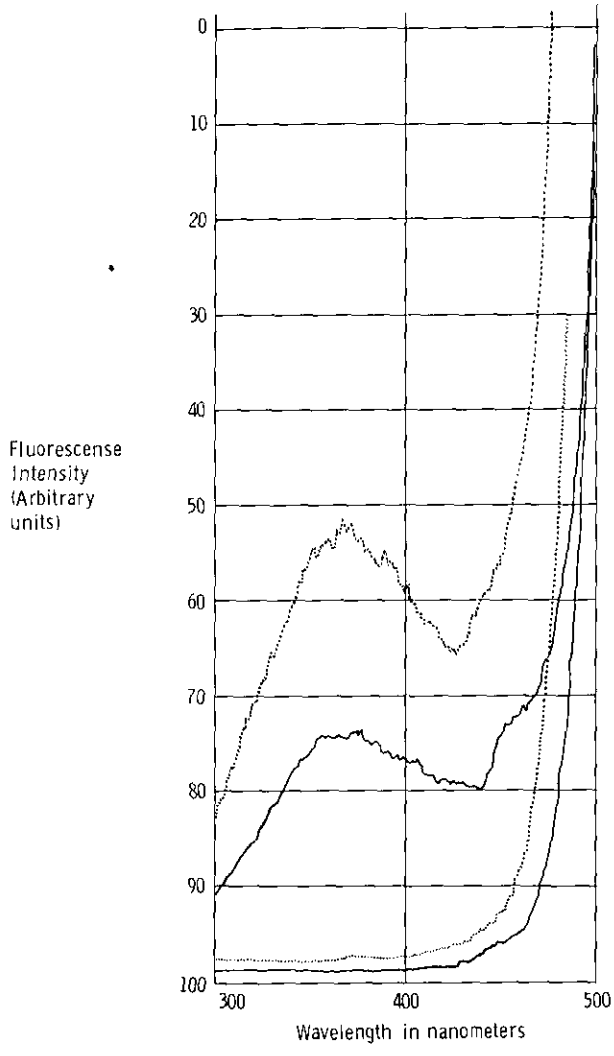

FIGURE 1. Spectra obtained from crushed pollen: (——) autofluorescence spectrum of the starchy pollen (. . .) autofluorescence spectrum of the waxy pollen. The lower spectra are from crushed pollen, stained with iodine potassium iodide for both (. . .) waxy and (——) starchy pollen. The difference in the spectrum of waxy and starchy for both stained and unstained is due to the amount of crushed pollen material.

On our flow cytometer, size is measured by the intensity of laser light scattered by the particle. The conditions for measurement are such that size is proportional to the diameter squared, hence, it is a surface area measure. Figures 2 and 3 show the size distributions of the pollen grains which had autofluorescence. In essence, the machine was adjusted so that only those particles which fluoresced (above a certain low threshold) contributed to the distribution. From this data, it appears that pollen from starchy maize (Fig. 2) is slightly larger than pollen from the waxy maize (Fig. 3). It may be that this difference in size is due to factors which could not be controlled in this set of experiments. A more definitive answer will come when the F_1 hybrid of these two inbred strains can be measured. It is remarkable that the waxy population shows a substantial number of pollen grains which are approximately half the mode of the major peak. Such a population exists in the starchy pollen, but it is not as prominant.

We define autofluorescence as the self-fluorescence of a particle to distinguish from fluorescence induced by staining. The autofluorescence of this pollen is interesting because of its highly similar intensity in both the starchy and waxy, in spite of the substantially reduced amounts of amylose in the waxy pollen. For both the starchy (Fig. 4) and waxy (Fig. 5), a major peak with a mode at channel 200 is present. The main difference between the two is the broadness of the peak for the starchy compared to the waxy; also, as in the scatter distributions (Figs. 2 and 3) the waxy contains a substantial population with a mode approximately half the value of the maximum fluorescence mode, and in the starchy there is a small population with a mode approximately one third the value of the maximum fluorescence mode.

Maize pollen incubated in iodine potassium iodide, has a substantially altered autofluorescence in the intact pollen. As expected, the starchy, which stains dark blue with iodine, is substantially less

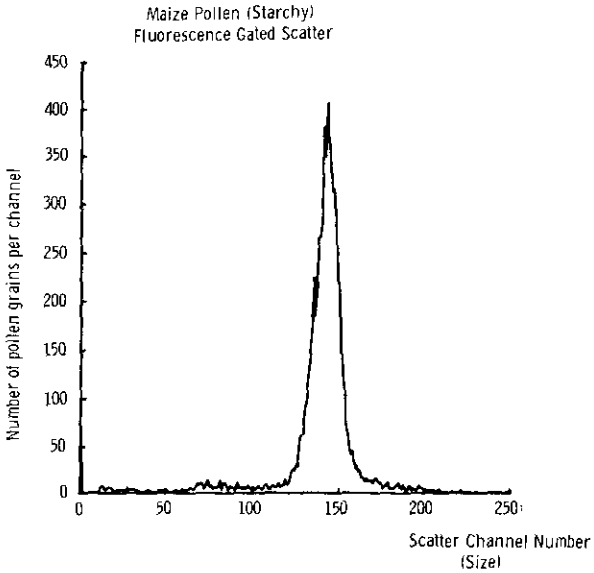

FIGURE 2. Size distribution of starchy pollen grains determined by light scatter. These distributions were obtained from particles with autofluorescence.

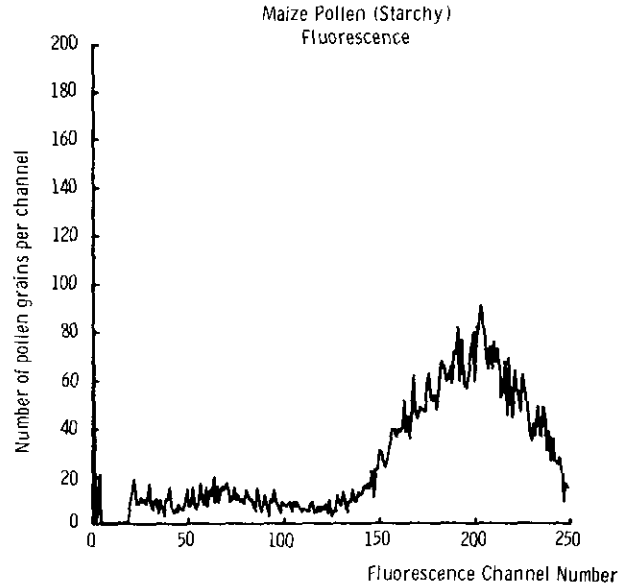

FIGURE 4. Distribution of the autofluorescence of starchy pollen grains.

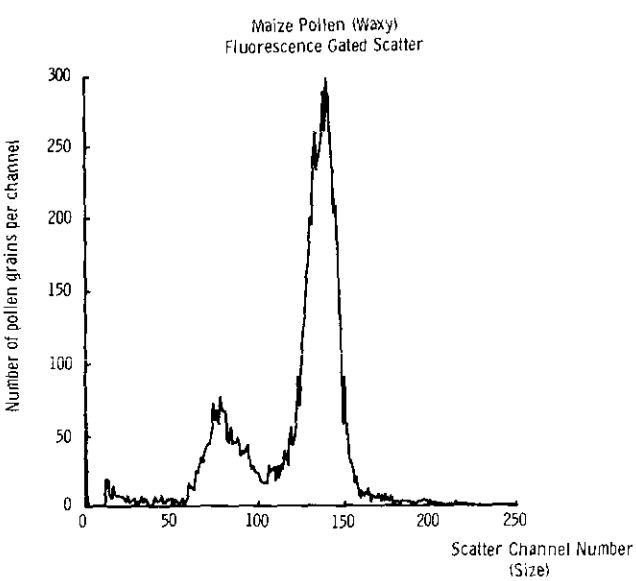

FIGURE 3. Size distribution of waxy pollen grains. Pollen is discriminated from debris by the use of the autofluorescence of the pollen.

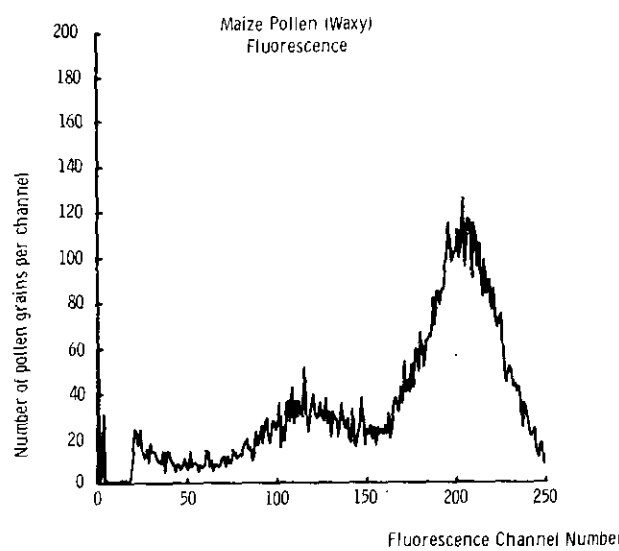

FIGURE 5. Distribution of autofluorescence of waxy pollen grains.

fluorescent than is the waxy. For this experiment, it is required that the ratio of iodine concentration to the number of pollen grains be a constant. The main peak of the iodine stained waxy pollen (Fig. 7) has a mode at approximately channel 180, a reduction from the mode of the unstained waxy pollen (Fig. 5). The mode of the iodine stained starchy pollen (Fig. 6) is at approximately 140 compared to 200 for the unstained (Fig. 4). Furthermore, the mode of iodine stained starchy pollen (Fig. 6) compared to the iodinated waxy pollen (Fig. 7) is also reduced. It is possible to optimize the system by further reducing the starchy pollen fluorescence; this may result in a system able to distinguish the few brightly fluorescing waxy maize pollen grains in the presence of a large number of low intensity fluorescence starchy maize pollen grains.

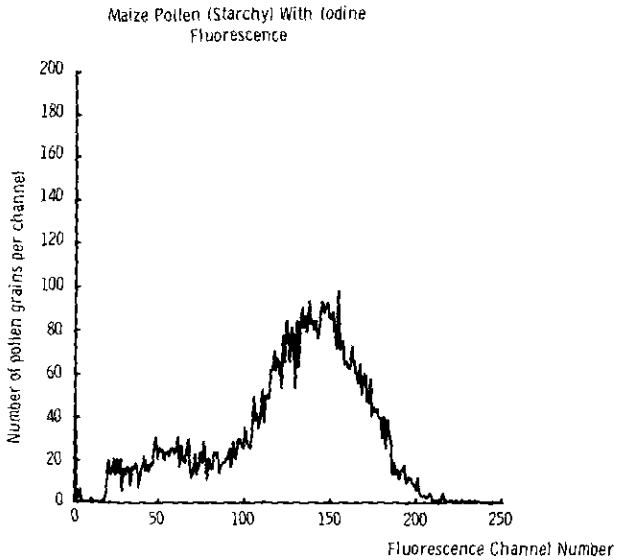

FIGURE 6. Distribution of autofluorescence of starchy pollen grains stained with iodine.

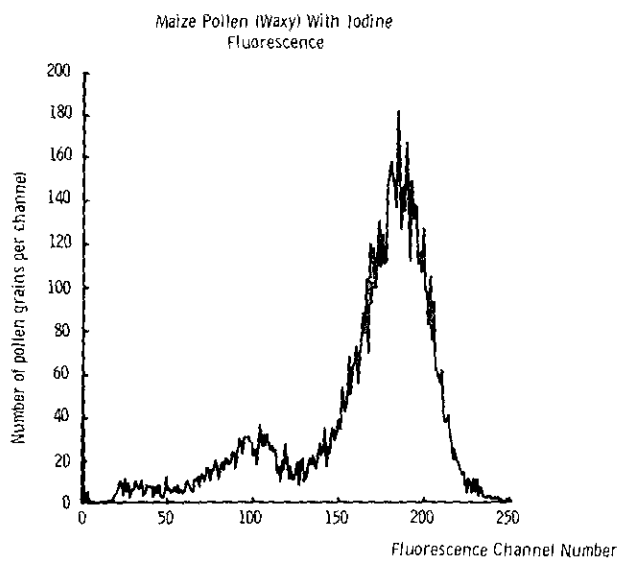

FIGURE 7. Autofluorescence of waxy pollen grains stained with iodine.

Conclusions

We have used cytometric means to distinguish between starchy and waxy maize pollen. There are differences between the two systems in both size and reduction of autofluorescence by staining; furthermore, these differences can be optimized to decrease the overlap between these distributions. In addition, there are iodine based fluorescent stains that may be used as positive markers to distinguish waxy in a field of starchy. This can lead to the enumeration of the number of waxy pollen grains in the presence of a large number of starchy pollen grains. Further work remains to be done on this to test these fluorescent compounds and to elucidate the nature of the bimodal distributions shown here.

The need for automation and its utility can only be determined by the individual laboratory. Automation can be used in research laboratories, for compound screening, and in pollution monitoring. However, it is in this latter area that automation may have its greatest usefulness. Indeed it may be that pollution monitoring with pollen systems may have such a labor intensive component for data analysis that it will be prohibitive to use such systems without an instrumented means of enumerating the mutant frequency of the pollen grains.

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Fruitful discussions with Drs. M. Plewa and W. Lower served to keep an engineer from being completely overwhelmed by the maze of maize genetics.

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