Determination of Pollen Viability in Tomatoes

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Abstract. A procedure is described for determining pollen viability in tomatoes (*Lycopersicon esculentum* Mill.) by growing pollen in a growth medium containing 0.29 M sucrose, 1.27 mM $\text{Ca}(\text{NO}_3)_2$, 0.16 mM H_3BO_3 , and 1 mM KNO_3 (pH 5.2) to which 0.001% fluorescein diacetate (FDA) was added. Pollen viability can be evaluated within 30 min by determining percent fluorescing pollen in a sample. The procedure further allows the determination of percent germination in vitro and pollen tube growth within 1.5 hours. Neither the germination medium nor FDA has any adverse effects on germination and pollen tube growth. Percent fluorescent pollen and percent total pollen germination were highly correlated, suggesting that fluorescence is a good measure of pollen viability. The combined fluorescence-germination procedure is simple and adapted to routine screening of many samples.

The development of reliable methods for determining the functional quality of pollen would be useful in hybridization programs (Calvert, 1964; Dempsey, 1962) and in evaluating the quality of pollen that develop under environment stresses (Shelby et al., 1978). Heslop-Harrison et al. (1984) and Picken (1984) reviewed three general approaches for evaluating pollen quality: in vivo, in vitro pollen germination and pollen tube growth, and histochemical.

The in vivo approach involves placing the pollen on the stigmas of emasculated flowers and determining the number of pollen tubes in squashed styles (Abdalla and Verkerk, 1968; Dempsey, 1970; van Koot and van Roestijijn, 1963) or the number of seeds in the mature fruit (El Ahmadi and Stevens, 1979; McGuire, 1952; Maisonneuve and Philouze, 1982). These methods are time-consuming and therefore impractical for testing many samples. Furthermore, seed set may depend not only on fertilization, but also on the post-pollination development of the ovary, pistil receptivity, and incompatibility reactions (Berry and Uddin, 1988; Heslop-Harrison et al., 1984).

In vitro germination involves germinating the pollen on artificial media and determining germinability and pollen tube growth (Abdalla and Verkerk, 1968; Charles and Harris, 1972; Hewlett, 1936; Levy et al., 1978; Weaver and Timm, 1989). The procedure requires much less time (about 1.5 h) than the in vivo approach and is adapted to routine screening of many samples. However, its potential ability to predict pollen performance depends heavily on optimization of the germination medium and temperature and on the availability of an adequate amount of pollen in the tested sample (Heslop-Harrison et al., 1984).

The histochemical approach is based either on the ability of the vegetative cell of the pollen grain to stain specific constituents of that cell or on the activity of specific enzymes. Iodine-potassium iodide was applied to stain starch (Charles and Harris, 1972), aniline blue to stain starch and other polysaccharides (Dempsey, 1962; Dionne and Spicer, 1958), phyloxin-methyl green to stain cell wall (Weaver and Timm, 1989; Weaver et al., 1985), and safranin and acetocarmine to stain chromatin and ribonucleic acids (Dionne and Spicer, 1958; Rudich et al., 1977). Enzyme activity most commonly is tested by reduction of the tetrazolium group to yield the colored insoluble formazans (Cook and Stanley, 1960) and the hydrolysis of fluorescein di-

acetate (FDA) by esterase to yield fluorescein (Rotman and Papermaster, 1966).

The histochemical approach for determining pollen viability requires a short time (20 to 30 min), thus making it most suitable for routine screening of many samples. However, the staining media often adversely affect the pollen and interfere with its viability. This may explain, in part, the inconsistent results of some earlier work in which data from histochemical tests and in vitro germination were compared in assessing pollen quality (Calvert, 1964). Therefore, it is essential that the germination and staining media have no adverse effects on terminability and pollen tube growth.

This work was undertaken to develop a fast and reliable method for evaluating pollen viability through the combined use of FDA and in vitro germination approaches. Pollen viability can thus be evaluated in one operation within a short time by first determining fluorescence resulting from the hydrolysis of FDA and then the terminability of the sample.

Materials and Methods

Source of pollen. Sixteen tomato plants of breeding line CL 1131-0-0-43-8-1 were grown in the greenhouse between Apr. and July 1990 under a day/night cycle of $32/26 \pm 3\text{C}$. Sixteen additional plants of 'Long Keeper' and breeding line CL 1131-0-0-13-0-6 were grown in a greenhouse under an optimum regime of $25/22 \pm 3\text{C}$ (day/night). All management operations were similar to those applied for the production of a tomato crop in greenhouses. Pollen from the first breeding line was used to establish time-courses for fluorescence development and germination and to optimize the conditions for viability measurements, using both fluorescence and in vitro germination on the same sample. Pollen from the second breeding line and 'Long Keeper' was used in viability determinations on pollen that had been stored at 6 or 25C for 1, 2, 5, or 10 days.

Preparing the pollen sample. In preliminary tests, we established that pollen samples taken from flowers born on inflorescences one (lowest), two, and three of the same plant exhibited equal viability levels. Therefore, all subsequent determinations on pollen viability presented in this study were performed on pollen taken from flowers borne on the lowest three inflorescences. Flowers were harvested every other day, brought to the laboratory, and held at room temperature for 1 h. Pollen from 30 flowers (70 flowers for the storage experiment) was collected in a 35-mm petri dish by touching the anthers for 5 sec with a

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Abbreviation: FDA, fluorescein diacetate.

Fig. 1. Pollen viability expressed as percent normal (circles) and percent total (abnormal plus normal-appearing pollen) (squares) germination in the control sample (without FDA) and percent fluorescence (triangles) in medium with FDA. Individual points plus SE bars represent means of eight replicates.

Table 1. Fluorescence and total germination of pollen after storage at 6 or 25C.

Cultivar/line	Storage		Fluorescence ^z (%)	Total ^z germination (%)
	Temp (°C)	Duration (days)		
Long Keeper	25	0	83.2 ^y	85.3 ^y
	25	1	65.7	63.8
	25	2	24.7	33.0
	25	5	22.8	11.5
	25	10	0	0
	6	0	83.2	85.3
	6	1	74.8	79.1
	6	2	64.6	73.6
	6	5	24.8	30.3
	6	10	2.7	4.0
CL1131-0-0-13-0-6	25	0	71.9	83.8
	25	1	55.9	46.2
	25	2	10.0	11.0
	25	5	7.1	6.3
	25	10	0	0
	6	0	71.9	83.8
	6	1	55.0	61.2
	6	2	37.6	29.0
	6	5	7.2	11.5
	6	10	2.8	2.7

^yFluorescence at 20 to 30 min and germination at 90 min on the same sample in a growth medium containing 0.001% FDA.

^zAverage of two determinations.

mechanical vibrator (Dremel, Racine, Wis.). After mixing, the pollen was divided into 0.3-mg portions, each representing a sample for one determination. Fluorescence and germination tests were performed within 4 h of harvesting the flowers. For determining the effect of storage temperature and duration on pollen viability, eight pollen samples (0.3 mg each) of each line or cultivar were held at 25C; another eight samples were held at 6C; and two samples were tested immediately (zero time).

Table 2. Analysis of variance on data shown in Table 1.

Source of variation	df	F value	Significance
Cultivar (Cult)	1	140.45	**
Temperature (Temp)	1	87.09	**
Cult × temp	1	8.80	**
Storage duration (days)	4	720.20	**
Cult × duration	4	16.91	**
Temp × duration	4	25.20	**
Cult × temp × duration	4	1.91	**
Type (germination vs. fluorescence)	1	2.96	NS
Cult × type	1	0.09	NS
Temp × type	1	3.18	NS
Cult × temp × type	1	0.57	NS
Duration × type	4	2.01	NS
Cult × duration × type	4	2.57	NS
Temp × duration × type	4	1.93	NS
Cult × temp × duration × type	4	0.76	NS

NS, **Nonsignificant or significant at $P = 0.05$.

Duplicate samples from each storage condition were tested after storing for 1, 2, 5, or 10 days.

Viability determinations. Viability determinations were made by two criteria on 0.3-mg samples of pollen: 1) fluorescence in the presence of FDA and 2) in vitro germination. The pollen samples were germinated in 35-mm petri dishes, each containing 1.4 ml freshly prepared Murashige and Skoog's (1962) growth medium (a liquid culture without agar) consisting of 0.29 M sucrose, 1.27 mM $\text{Ca}(\text{NO}_3)_2$, 0.16 mM H_3BO_3 , and 1 mM KNO_3 (pH 5.2) for the control and the same medium containing 0.001% FDA (Sigma, St. Louis) for the fluorescence treatment. They were incubated at 27C for durations specified in the Results. In vitro germination was determined by scoring percent normal germination and total (includes the abnormal) germination using a Diaphot-TMD inverted microscope (Nikon, Image Systems, Columbia, Md.) and a light source from a halogen lamp. A pollen grain was considered to be germinated when the length of the pollen tube was equal to or longer than the diameter of the grain. Fluorescence in FDA was determined by scoring percent fluorescing pollen grains under an ultraviolet (UV) source provided by a mercury lamp. The principle is based on the uptake of nonfluorescing FDA by the vegetative cells of a viable pollen grain and subsequent hydrolysis by esterase to release fluorescein, which fluoresces under UV (excitation filter = 485 nm and barrier filter = 520 rim). In contrast, nonviable cells are incapable of hydrolyzing FDA and, therefore, do not fluoresce (Heslop-Harrison and Heslop-Harrison, 1970; Heslop-Harrison et al., 1984; Rotmand and Papermaster, 1966). Each determination consisted of counting 1000 to 1200 pollen grains by examining eight to 10 locations in a series of parallel traverses across the petri dish that contained the sample under test.

Analysis of variance was conducted to determine if there were differences in percent total germination and percent fluorescence. The analysis was conducted as a $2 \times 2 \times 2 \times 5$ factorial of cultivar, temperature, type (germination vs. fluorescence), and storage duration.

Results and Discussion

Pollen viability is expressed as percent normal and percent total germination (abnormal and normal-appearing grains) in the control samples as compared with that in FDA (expressed as percent fluorescence) over 120 min (Fig. 1). Although pollen tubes in some grains began to grow ≈ 25 min after being placed

Fig. 2. Pollen viability as fluorescing pollen in a germination medium containing 0.001% fluorescein diacetate. (A) Fluorescing pollen 4 min after being placed in growth medium containing FDA. (B) Fluorescing pollen showing normal pollen tube growth after 140 min of germination and nonfluorescing, nonviable pollen. The same sample germinated for 90 min in the presence of FDA with (C) subjected to UV alone and (D) lighted by both UV and a halogen lamp. Bar length = 110 μ .

Fig. 3. Control sample (A) after 60 min germination, (B) after 100 min germination. Bar length = 110 μ .

in the growth medium, maximum germination of normal pollen was reached" after 90 min. At that time, percent total germination was still increasing slightly due to the delayed germination of some abnormal pollen grains in the sample. In the FDA treatment, the pollen grains began to show some fluorescence within 2 to 4 min of being placed in the growth medium (Fig. 1). All viable pollen grains (intact and abnormal) fluoresced. However,

after 30 min in the growth medium, fluorescence in abnormal grains began to fade away, presumably due to the leaching of fluorescein from the pollen grain into the medium and the inability of abnormal pollen to continue to hydrolyze FDA. This presumed response explains the slight decline in percent fluorescence after 30 min (Fig. 1). The data suggest that maximum fluorescence in FDA at 30 min represents percent total viability

and appears comparable to percent total germination at 90 min in the control sample.

No significant difference was found between percent in vitro pollen germination at 90 min and percent pollen fluorescence at 20 to 30 min (Tables 1 and 2). Likewise, there were no significant interactions between viability tests and other factors (temperature and storage duration). Pollen viability decreased significantly over storage time, and pollen stored better at 6 than at 25°C. The correlation between the FDA method and the in vitro germination method in assessing viability of tomato pollen was strong ($r = 0.97$) and highly significant ($P < 0.001$). The results are in agreement with the findings of Heslop-Harrison and Heslop-Harrison (1970) and Heslop-Harrison et al. (1984). However, their microfluorimetry method focused on membrane integrity and involved the measurement of fluorescence in individual pollen grains and the leaching of fluorescein into the incubation medium. Their procedure requires considerable time to determine fluorescence in several hundred grains per sample. Furthermore, prolonged exposure to *W* light during scoring reduces the intensity of fluorescence and viability. In addition, Heslop-Harrison et al. (1984) used a medium that was high in sucrose, lacking in boron, and the FDA concentration was 40 times higher than that used in the present study.

The use of FDA to assess pollen viability by determining percent fluorescing pollen in a sample within 30 min of incubating in a growth medium offers a simple and reliable method that is adapted to routine screening of many samples. Neither the growth medium nor FDA, at the concentration used, had any adverse effects on germination and pollen tube growth (compare Figs. 2C and 3A and Figs. 2B and 3B). Several stains (phenosafranin, lacmoid, trypan blue, and 2,3,5-triphenyl-tetrazolium chloride) were also evaluated in preliminary experiments and proven to damage pollen membranes severely and inhibit germination at the concentrations recommended for staining.

Two drawbacks arise from the use of FDA in pollen viability determinations. The first is difficulty in recognizing some of the nonfluorescing grains that appear almost as dark as the background (Fig. 2 B and C). Underscoring nonfluorescing grains tends to overestimate the fluorescing grains in the sample. The second is the inability to distinguish between fluorescing pollen grains that will germinate normally and those that will give rise to abnormal germination, because both fluoresce. The first problem can easily be overcome by subjecting the sample during scoring to both *W* and low-intensity light from a halogen lamp (Fig. 2 C and D). Under dual illumination, fluorescing grains can still be recognized by their yellow fluorescence, and non-fluorescing grains become clearly visible (compare Fig. 2C with 2D). However, resolving the fluorescing pollen in a sample into those that will germinate normally and those that will give rise to abnormal germination requires extending the germination time to 90 min and examining the sample under a light source from the halogen lamp. Under these conditions, pollen grains with damaged membranes as well as abnormal pollen (such as those with two pollen tubes) can easily be distinguished from intact pollen that exhibit normal germination. Damaged and abnormal pollen eventually release their content into the growth medium, shrink, and lose their fluorescence.

Because of the increase in pollen abnormalities under stressful conditions, it is recommended that the fluorescence measurement be made at 30 min followed by a second measurement at 90 min under dual light to gain specific information about the integrity of the germinating pollen and the frequency of abnormalities in the tested sample.

The use of FDA in the Murashige-Skoog growth medium for evaluating pollen viability provides a fast and reliable technique in which viability can be determined both by fluorescence within 30 min and by the actual in vitro germination after 90 min.

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