# Sugarbeet Pollen Viability Indicators[1]

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## ABSTRACT

**Ten fluorochromes and seven stains were evaluated as indicators of viability of sugarbeet (*Beta vulgaris*) pollen. The reactions of these materials were compared with in vitro germination of pollen in a liquid medium. Among these 17 materials, fluorescein diacetate (FDA), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and isatin exhibited viability reactions that were positively related to in vitro pollen germination. However, only the FDA fluorescence reaction was related consistently to germination in all pollen sources. The linear regression model of germination (Y) and FDA reaction (X) was Y = 0.6X - 12. The fluorochromes 4',6-diamidino-2-phenylindole (DAPI), ethidium bromide, and aniline blue each exhibited some potential to detect and display the presence of DNA, sperm cells, and in vivo pollen tubes, respectively.**

**Additional Key Words:** *Beta vulgaris*, vital stain, fluorochrome, pollen germination.

Accurate and expeditious determination of pollen viability is necessary for the potential use of sugarbeet (*Beta vulgaris* L.) pollen as an in vitro tissue for genetic assay and selection. Viability determination methods generally are based on (a) fertilization or seed set, (b) pollen germination, or (c) pollen staining, fluorescence, or enzyme activity. Ability of pollen to deliver male gametes to the embryo sac should be the ultimate functional test for pollen viability. In practice this is very difficult to assess because of dependence on unidentifiable and uncontrollable factors such as postpollination environment, pistil receptivity, and incompatibility. Pollen germination in artificial media generally is considered a good measure of pollen viability (10). Hecker and McClintock (8) recently described techniques for germination of sugarbeet pollen in a liquid medium, and reviewed many factors affecting in vitro germination of this tricellular pol-

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len. Earlier, Hecker (6) reported a general relationship between seed set and in vitro pollen germination, but precise comparisons never have been made. However, germinability provides the best standard against which to assess other pollen viability indicator methods, and it has been used as such in the research being reported here.

Numerous pollen viability stains, fluorescence, and enzyme reactions have been reported (18), but the reagents tested on sugarbeet are few, namely, the tetrazolium salts (6, 7) and aniline blue (2). We tested various stains and fluorochromes for reaction with sugarbeet pollen, and compared these reactions with pollen germinability in an attempt to develop a rapid, reliable method to measure the viability of sugarbeet pollen.

## MATERIALS AND METHODS

The pollen viability indicators (Table 1), stains as well as fluorochromes, were tested individually or in combinations for their interaction with sugarbeet pollen. Those that showed little promise as viability indicators were not studied further. Those with positive reactions were studied more extensively and compared with in vitro germination.

**Table 1.** Fluorochromes and stains tested as viability indicators of sugarbeet pollen.

| Indicator | Optimum conc. % | Optimum reaction time (min) | Reference |
|---|---|---|---|
| **Fluorochrome** | | | |
| Fluorescein diacetate (FDA) | 0.01 | 30 | (10) |
| 4',6-diamidino-2-phenylindole (DAPI) | 0.01 | 90 | (3) |
| Phenosafranin | 0.01 | 30 | (4) |
| Aniline blue lactophenol | 0.01 | 30 | (14) |
| Aniline blue | 0.01 | 30 | (2) |
| Merocyanin 540 | 0.08 | 60 | (5) |
| Mithramycin | 0.005 | 60-1440 | (3) |
| Ethidium bromide (EB) | 0.002 | 30 | (12) |
| Acridine orange | 0.005 | 90 | (17) |
| Hoechst 33258 | 0.0025 | 90 | (17) |
| **Stain** | | | |
| 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyl-2H-tetrazolium bromide (MTT) | 0.5 | 30 | (6) |
| Isatin | see ref. | 5 | (15) |
| Alexander's stain | see ref. | 1 | (1) |
| Neutral red | 0.1 | 20 | (4) |
| Iodine (IKI) | 1.3 | 5 | (16) |
| Evan's blue | 0.5 | 30 | (19) |
| 2,3,5-triphenyl-2H-tetrazolium chloride | 1.0 | 60 | (6) |

Pollen was collected at Fort Collins, Colorado, from various heterozygous sugarbeets that were photothermally induced,

then brought to flower in the greenhouse under a 12-hour, or longer, photoperiod, regardless of season. Techniques of pollen collection, humidification, incubation in liquid medium, and germination counts were described by Hecker and McClintock (8).

Pollen was mixed and humidified before staining or treating with a fluorochrome. In general, stains were prepared as described in the references and were used at the concentrations shown (Table 1). For stains, pollen was mixed into a drop of stain solution on a microscope slide and a cover slip was applied, except for isatin (15). The reaction time is shown in Table 1. For fluorochromes, where pollen density and fluorochrome concentration were critical, about 0.15 mg of pollen was added to 0.3 ml of solution; the sample vial was capped, shaken well, and left at room temperature for the appropriate time. Then a drop of this suspension was placed on a slide, and a cover slip was added. For fluorescein diacetate (FDA), a stock solution in acetone, 20 mg $ml^{-1}$, was added to germination media immediately before use.

Epifluorescent illumination was used for all the fluorescence microscopy procedures. The filter combinations are listed in Table 2. In most cases at least 300 pollen grains were counted from random fields in each of three samples. The same pollen collection was used for both viability testing and germination counts. Pollen was germinated in liquid medium for 20 hours at 23°C (8).

**Table 2.** Filters used for epifluorescent viewing of sugarbeet pollen treated with various fluorochromes; Olympus (Vanox) microscope filters, xenon XBO lamp, and fluorescence illuminator (A-RFL).

| | Exciter filter | | Barrier filter | |
|---|---|---|---|---|
| Fluorochrome | Built-in | External | Built-in dichroic mirror | External |
| FDA | none | IF-490 & BG-12 | O-515 | none |
| DAPI | UG-1 | UG-1 or none | O-515 or Y-455 | Y-475 |
| Phenosafranin | BG-12 | none | O-515 | none |
| Aniline blue | BG-3 | BG-12 | O-515 | Y-495 or O-530 |
| Merocyanin 540 | IF-545 & BG-36 | none | O-590 | none |
| Mithramycin | none | IF-490 | O-590 | O-570 or O-590 |
| EB | BG-12 | BG-12 | O-515 | none |
| Hoechst 33258 & Acridine orange | UG-1 | none | O-590 | none |

## RESULTS AND DISCUSSION

Fluorescein diacetate (FDA) and 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were the only materials that had potential as pollen viability indicators in our experiments.

FDA, as described by Heslop-Harrison et al. (9,10), produces fluorescence that results from the presence of active esterases

and an intact plasmalemma, both necessary for viability. FDA is a nonpolar molecule, hence, able to pass through the intact pollen plasmalemma, where esterases cleave the acetate and fluorescein moieties. The fluorescein becomes a polar molecule, unable to pass through the plasmalemma, hence, it accumulates in the pollen grain.

Hydration of pollen (8) was essential for uniform and maximum fluorescence; according to Hoekstra (11), hydration rate affects membrane integrity. Thorough mixing of pollen into the FDA-amended medium was necessary to improve contact of pollen and FDA. The green fluorescence of the entire pollen grain was maximal for intensity and frequency at incubations between 15 and 105 min. (Fig. 1); fluorescence diminished thereafter. Our standard reaction time was 30 min. The surfactant Tween 20 in the medium reduced fluorescence. As pollen germination proceeded in the FDA-amended medium, fluorescence in the pollen diminished or disappeared, and appeared in the pollen tube, especially at the tip. This indicated that the accumulated fluorescein molecules migrated with the cytoplasm in germinating pollen. The relationship of in vitro germination with FDA fluorescence was the most consistent of all the indicators tested. Correlation coefficients from five separate experiments ranged from 0.88 to 0.52. The closest relationship occurred in a germination and FDA test of 20 pollen sources that ranged from 0 to 51% germination and 0 to 94% FDA fluorescence, when the medium was amended with 0.1 RVU of a pectic enzyme mixture, Pectinol HS (Genencor, Inc., 180 Kimball Way, S. San Francisco, CA 94080).

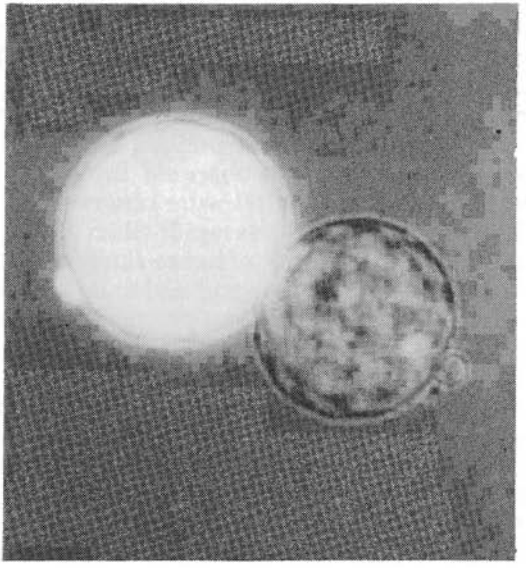

**Figure 1.** Sugarbeet pollen in liquid germination medium; presence and absence of FDA induced fluorescence.

MTT stains cells when it is reduced to an insoluble purple formazan by the action of respiratory dehydrogenases. In our experiments the relationship of the MTT reaction and pollen germination in vitro was inconsistent. The simple correlations of germination with MTT stain in three experiments ranged from 0.1 to 0.7.

Isatin was the only other indicator that was significantly correlated with in vitro germination. Isatin, reported as a viability stain for maize pollen (15), reacts specifically with proline in pollen to produce a blue-black color. The isatin technique (15) was not satisfactory on sugarbeet pollen due to the volatility of the acetic acid-acetone-isatin mixture and the extreme variability in color development across a slide. The isatin reaction and in vitro germination were correlated ($r = 0.56^{*}$). The correspondence of percent stained pollen with in vitro germination was closer than that for any other stain: e.g., 16 pollen sources ranged in germination from 8 to 54% and their isatin staining range was 3 to 42%. However, isatin consistently underestimated in vitro germination. We conclude that the somewhat arbitrary color classification, the great variability in color development, and rigid timing requirements make the stain impractical to use on sugarbeet pollen.

Other indicators tested showed little potential as viability indicators for sugarbeet pollen, but some had potential for other purposes.

DAPI (4',6-diamidino-2-phenylindole), which binds specifically to double stranded DNA (3), is an indicator of a normal DNA complement, but not necessarily of pollen viability. DAPI caused a blue-green fluorescence in sugarbeet pollen nuclei when pollen was incubated in darkness in the standard germination medium amended with 100$\mu$g DAPI $ml^{-1}$ for 1 hour. The fluorescence usually appeared as a bright point, probably indicating the commencement of a germ tube with the sperm nuclei concentrated at that point. After longer periods of incubation, autofluorescence in the pollen wall obscured the nuclei, however, under ideal conditions the nuclei were observed in the pollen tube. DAPI fluorescence faded less rapidly than FDA. Surfactants and vacuum infiltration did not enhance fluorescence. All solutions of DAPI were light sensitive and had to be kept in the dark. A mixture of DAPI and FDA was not practical because each had a different optimum reaction time and different fluorescence filters were required.

Phenosafranin, a fluorochrome that has been used for viability studies on plant cells and protoplasts in culture (4), works on the dye exclusion principle, and thus, fluoresces only in dead cells. Freshly collected sugarbeet pollen in a germination medium with 0.01% phenosafranin for 15 min. resulted in pink fluorescence in 11 to 19% of pollen grains. Higher concentrations produced no fluorescence, and more than 15 min. incubation resulted in a cloudy solution. In the 0.01% solution, bright, faint,

and no fluorescence was not definitive.

Alexander's stain (1), a mixture of malachite green, acid fuchsin, and organic solvents, fixes and stains pollen of many species (1). We found that aborted sugarbeet pollen was stained green, whereas normal mature pollen was red. Since aborted sugarbeet pollen also was readily distinguishable by size and shape, Alexander's stain was of little value because it did not differentiate between viable and nonviable mature pollen.

Aniline blue lactophenol (14) stained nonaborted sugarbeet pollen blue, but it did not distinguish viable and nonviable pollen. Aniline blue in 0.67M $K_2HPO_4$ was found to be useful as a fluorochrome to show pollen germ tubes in vitro and in vivo. A 1:5 v/v mix of 0.1% neutral red and 0.05% aniline blue, prepared the day of use, was useful to observe or count germinated pollen in vivo. The pollen tubes fluoresced a brilliant green and were easily distinguishable from the yellow fluorescing stigma cells. Under a mixture of bright field and epifluorescent illumination, the pollen on the stigma were red and the green fluorescing tubes could be traced to specific pollen grains (Fig. 2). Hence, aniline blue may be useful for in vivo germination studies. Pollen tube observations involved several steps. Horizontal sections were cut free-hand from stigma surfaces 1 to 2 hours after pollen was blown onto flowers that had been open for about 3 days. The sections were boiled in sodium sulfite 15 min. (13), then the liquid was decanted. Specimens were hand sectioned longitudinally to appropriate size, put into a drop of neutral red and aniline blue on a slide, covered, squashed gently, and observed. The addition of calcofluor to enhance fluorescence (13) was not useful in sugarbeet because increased fluorescence of stigmatic tissue tended to obscure the pollen tubes.

We tested iodine (IKI), which stains dark blue any starch present in pollen (16). Iodine did not stain mature sugarbeet pollen; hence, it was not found to be useful as a viability or maturity indicator.

Evan's blue has been used to assess cell survival after salt stress (19). Viable cells exclude the stain, and thus, Evan's blue tests for membrane integrity. On germinated sugarbeet pollen, the tubes were stained but the pollen grains were not. Evan's blue may have some application for observation of pollen tubes in sugarbeet.

Merocyanin 540 is a fluorochrome that has been used as a viability bioassay of plant cells (5). Up to 55% of the pollen grains exhibited a relatively bright red-orange fluorescence in sugarbeet pollen sources, but germination and fluorescence were not related.

Mithramycin is a DNA-specific fluorochrome reported as useful in pollen biology research (3). However, with sugarbeet pollen no fluorescence was seen with the filter combinations listed in Table 2 and various concentrations, reaction times, and pH.

Ethidium bromide (EB), a fluorescent DNA probe, was used

by Hough et al. (12) in pollen biology studies. With sugarbeet pollen, EB produced a bright orange fluorescence in the sperm and vegetative nuclei that was sometimes obscured by autofluorescence of pollen walls. The two sperm nuclei were most distinct in pollen germ tubes. EB should be useful for studying the biology of sugarbeet pollen germination.

**Figure 2.** Sugarbeet pollen on the stigma, mixed illumination; bright field shows neutral red stained pollen and epifluorescent light shows aniline blue fluorescence in germ tubes.

Acridine orange is a fluorochrome that has been reported as a viability indicator of human cells (17). On sugarbeet pollen the best treatment, 50$\mu$g/ml for 90 min., produced green fluorescence in 16 to 56% of the pollen grains of different sources, but there was no correlation with in vitro germination.

Hoechst 33258 is a fluorochrome that is excluded from living human cells (17). It causes blue fluorescence in dead cells. On sugarbeet pollen in tests with various concentrations and reaction times, variable amounts of fluorescence was observed in different pollen sources, but there was no correlation with in vitro germination. A combination of Hoechst 33258 and acridine orange on sugarbeet pollen did not give the positive viability detection results reported for human lymphocytes (17).

For sugarbeet pollen, 2,3,5-triphenyl-2H-tetrazolium chloride was reported to be of little value as a viability indicator (6). Our results were similar, in that the frequency and intensity of pink stained pollen was variable and not related to germina-

tion.

The results and potential uses of seven indicators are summarized in Table 3. FDA was the only relatively consistent indicator of pollen germinability. The regression equation of percentage pollen germination and fluorescent pollen was $Y = 0.6X - 12$. It appeared that variability occurred in both germination and the FDA test. Also, a low frequency of fluorescence occurred in heat-killed pollen.

**Table 3.** Fluorochrome and stain reactions on sugarbeet pollen, and potential uses.

| Indicator | Reaction with pollen and potential use |
|---|---|
| FDA | Green fluorescence; related to pollen viability; over estimates in vitro germination. |
| MTT | Purple stain; inconsistent as a viability indicator. |
| Isatin | Blue-black stain; impractical for pollen viability; tends to correspond to germination. |
| DAPI | Blue-green fluorescent spots revealed nuclei; walls autofluoresce; potential to determine DNA location and quantity. |
| Alexander's stain | Red stained mature pollen not necessarily viable; green aborted pollen. |
| Aniline blue & neutral red | Red pollen and green tubes with mixed light; useful for in vitro germination study. |
| EB | Orange sperm nuclei; may be useful to study germination process. |

FDA testing of sugarbeet pollen is simple to use and rapid, but it requires fluorescence microscopy. It should be used with caution as a pollen germinability or viability predictor because some unidentified factors seem to differentially affect both germinability and the FDA reaction.

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