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Comparisons Among Pollen Viability Measurement Methods in Cotton[1]

Jerry R. Barrow[2]

ABSTRACT

Methods for measuring pollen viability were compared to determine their relative value in evaluating cotton (*Gossypium hirsutum* L.) pollen. Pollen was treated at constant temperatures of 30, 33, 35, 37, 40, and 43°C for 15 hours prior to anthesis. Treated and untreated pollen were evaluated using several common techniques. Pollen killed at the highest temperature (43°C) stained readily with a cytoplasmic stain indicating that stains of this type may determine pollen maturity, but not viability. Pollen treated at 33 to 40°C responded positively to vital staining and germinated both on artificial media and on the stigma while pollen treated at 43°C did not. However, pollen fertility (as measured by pollen tubes penetrating the lower style and ovules) was severely reduced at 33°C and absent at 35°C. The best available criterion for pollen fertility appears to be tube penetration into the lower style or ovules. Staining and pollen germination methods are not accurate indicators of pollen fertility in cotton.

Additional index words: **Pollen fertility, Pollen staining, Pollen germination, Pollen tube, Fertilization, Sterility.**

SEVERAL methods are commonly used to evaluate the viability of pollen from cotton (*Gossypium hirsutum* L.) and other species. These include nuclear and vital stains and in vitro germination of pollen. An accurate measure of pollen capability could help determine some of the causes for fruit shedding under stress conditions and could be useful in other fertility studies.

Nuclear dyes, such as acetocarmine and lactophenol, are regularly used to stain pollen in many species. However, dyes of this type are not effective in staining cotton pollen because of its pollen wall characteristics and structure. Alexander (1) developed a differential staining method that stains the cytoplasm of cotton pollen a deep red and the pollen wall a distinctive green. This stain easily distinguishes between mature and immature pollen by their staining patterns.

Vital stains are also frequently used for evaluating pollen. However, some of these stains are excluded by the memoranes of living cells. The tetrazolium stains described by Roberts (9) and Smith (10) are colorless, soluble compounds that are reduced to insoluble red formazan by living tissues, causing the tissues to be stained red to deep purple. Nonliving cells do not stain. Aslam et al. (2) found that 2,3,5-triphenyl tetrazolium chloride (TTC) was effective in staining cotton pollen. They also noted that pollen from so-called semi-sterile translocation heterozygote plants showed high pollen viability and were difficult to distinguish from pollen produced by fully fertile normal plants. Oberle and Watson (8) found that certain fruit pollens, known to be nonfunctional, stained to varying degrees when using TTC.

In vitro germination of pollen is used to evaluate the pollen of many species. The assumption is generally made that pollen capable of germination is fertile, i.e., capable of joining with an egg cell and producing a functional zygote. Pollen from other plant species germinates in water or simple sugar solution. Pollen germination in some plants requires more complex supplements to the media such as calcium nitrate ($Ca(NO_3)_2$), magnesium sulfate ($MgSO_47H_2O$), and boric acid (H_3BO_3).

Bronckers (4) was the first to develop an in vitro technique for cotton pollen. Miravalli (7) popularized this method for researchers working with cotton and noted that the pollen tubes were short and required 24 hours or longer to develop. Taylor (11) surveyed in vitro pollen germination techniques established for other species and developed a dense-agar method supplemented with a relatively high concentration of sucrose with manganous sulfate ($MnSO_4$), $Ca(NO_3)_2$, and H_3BO_3. He observed more rapid germination (2 to 3 hours) and longer and more normal appearing tubes and cytoplasm than did earlier workers. The main deficiency of Taylor's (11) and earlier germination methods was the low percentage of germination obtained. His germination percentages (10 to 64%, averaging 30%) grossly underestimated actual pollen viability or fertility.

A commonly shared concept among pollen researchers is that duplicating the nutritional components of the stigma in an artificial culture system would provide a reliable environment to evaluate pollen viability. Techniques developed for tissue culture research allowed rapid screening for the optimal levels of sucrose and mineral salts used by Taylor (11). Therefore, we were surprised to find that fresh, mature cotton pollen forcefully ejected tube-like structures in less than 5 min in droplets of distilled water supplemented with $Ca(NO_3)_2$ and $MgSO_47H_2O$ (3). The ejection of the tube correlated well with pollen viability and pollen treatments known to affect pollen viability. Over 99% germinability was observed in many cultivars and in all species of cotton tested. The test was simple and rapid and appeared to be the best method to use in measuring pollen viability.

However, when we measure pollen viability, we are interested primarily in the capacity of the pollen to fertilize egg cells and induce the development of mature fruit and viable seed, not in whether the pollen cells are living or dead per se. Iyengar (5) analyzed the deficiencies of previously developed techniques and used microtechnique methods to determine cotton pollen germination in situ and to follow the development of pollen tubes in the stigma, style, and ovary. Because of the laborious and time-consuming methods required, his techniques did not find widespread favor among investigators evaluating pollen viability. Mr. Gia Ju Pan (personal communication, 1980), a recent visiting scientist from the People's Republic of China, described to me a simpler method than Iyengar's (5) for observing cotton pollen tubes in the stigma and style. Briefly, the stigmata and styles are collected 24 h after pollination and stored in Carnoy's solution overnight in a refrigerator. They are then hand-sectioned by cutting cross sections of the tissue approximately 0.25 mm thick. The cross sections are stained with acetocarmine, and the pollen tubes can be observed as holes in the conductive portion of the stigmatic and stylar tissue, providing a relatively simple method for determining the capacity of pollen to germinate and penetrate the stylar tissue.

Different methods used to measure pollen viability actually measure different potentials of the pollen grain. The objectives of this work were to compare commonly used

[1] USDA-ARS, in cooperation with the New Mexico Agric. Exp. Stn., Las Cruces, NM 88003. Received 30 Aug. 1982.

[2] Research geneticist, USDA-ARS, Cotton Breeding, Las Cruces, NM 88003.

methods for evaluating pollen capability, to determine what aspects of viability or fertility each measured, and to decide which is the best method for evaluating pollen fertility in cotton.

MATERIALS AND METHODS

Pollen was treated at several temperatures known to have critical effects on function. Flower buds were collected 15 h prior to anthesis and placed in a dry 100-ml beaker which in turn was placed inside a 250-ml beaker containing 20 ml of tap water. A petri dish cover was placed over the larger beaker to form a chamber with nearly 100% relative humidity. Those beakers were then placed in chambers where temperatures were controlled at 30 ± 0.5, 33 ± 0.5, 35 ± 0.5, 37 ± 0.5, 40 ± 0.5, and 43 ± 0.5°C. In all treatments the anthers dehisced normally. Fully fertile control pollen was collected from greenhouse-grown plants. All pollen was collected from a double haploid, cotton breeding line designated as DHNE.

Pollen Stainability. Pollen, collected after the 15-h temperature treatments, was placed in a cytoplasmic stain prepared according to Alexander (1) and observed at 200× after 2 h for degree of pollen staining.

Pollen Viability. This measurement was made by staining heat-treated pollen with the vital dye, tetrazolium red (2% tetrazolium red in distilled water with 60% sucrose to prevent the pollen from bursting in solution). The degree of pollen staining was observed after 6 h at 200×.

Pollen Germinability in Vitro. Two methods were used to evaluate germinability. Taylor's (11) method germinates pollen grains on a supplemented agar base. Heat-treated pollen was dusted on the agar plates which were kept at 100% relative humidity for 3 to 4 h. The number of grains germinating, as determined by pollen tube growth, was recorded. Treated pollen was dispersed in droplets of distilled water supplemented with $Ca(NO_3)_2$ and $MgSO_4 7H_2O$ acording to Barrow's PMDA method (3). The number of pollen grains ejecting tube-like structures was recorded approximately 5 min after dispersal in the droplets.

Pollen Germinability in Situ. Receptive stigmata from flowers emasculated 15 h prior to anthesis were excised, pollinated with heat-treated pollen, placed on moistened filter paper in a 60 mm petri dish for 2 h at room temperature, and stained with Alexander's (1) stain. Within 5 min the emerging pollen tubes stained a deep red, and the degree of germination could be determined by manipulating the pollen on the stigma with a dissecting needle and detecting the existence of pollen tubes through a stereo microscope.

Penetration of Pollen Tubes into the Stigma and Style. Flowers were emasculated 15 h prior to anthesis, and the stigmata were examined with a hand lens to ensure that they were free of pollen. Stigmata were pollinated with heat-treated pollen on the day of anthesis and left on the plant. After 24 h, the styles were excised at the junction of the ovary; and the entire stigma and style were placed in Carnoy's solution (30 ml absolute ethylalcohol : 15 ml chloroform : 5 ml glacial acetic acid) and stored at least 24 h in the refrigerator. The stigmata and styles remained well preserved for at least 6 weeks in Carnoy's solution. Thin cross sections (approximately 0.25 mm) were cut by hand from the ovary end of the style and from the central portion of the stigma with a razor blade. The cross sections were immersed in Alexander's (1) stain for about 10 min before observations were made at 300×. The number of pollen-tube holes per stigma or stylar cross section was recorded. The ovary (with its fertilized ovules) was left on the plant to mature. At maturity, the number of mature seed per boll was recorded. For each of the above measurements, four flowers were used per treatment with five rplications.

Number of Ovules Penetrated by Pollen Tubes per Boll. Because the penetrated-ovule method destroys the boll and fertilized ovules, an additional four flowers were pollinated with the heat-treated pollen and replicated five times. Twenty-four h after pollination, the ovules were excised; and the ovary wall was carefully cut away to expose the ovules. The ovules were carefully lifted from the placenta with sharp pointed dissecting forceps under a stereo microscope. After some practice, it was relatively simple to observe pollen tubes penetrating the micropyle. The number of ovules penetrated with pollen tubes in each ovary was also recorded.

Table 1. Comparisons among pollen viability measurement methods in cotton as affected by temperature.

	Pollen treatment						
Pollen character	Untreated	30°C	33°C	35°C	37°C	40°C	Above 43°C
Pollen stainability†	Yes	Yes	Yes	Yes	Yes	Yes	Yes
Pollen viability‡	Yes	Yes	Yes	Yes	Yes	Yes	No
Pollen germinability§	Yes	Yes	Yes	Yes	Yes	Yes	No
Pollen germinability on stigma¶	Yes	Yes	Yes	Yes	Yes	Yes	No
Tube penetration per stigma#	53.6	54.9	26.6††	4.3††	0	0	0
Tube penetration per style#	30.3	29.8	15.2††	0	0	0	0
Number of ovules penetrated per boll#	29.7	28.9	14.5††	0	0	0	0
Seed per boll#	28.9	23.1	14.8††	0	0	0	0

† As determined by Alexander's stain (1).
‡ As determined by tetrazolium red vital stain (9, 10).
§ As determined by the PMDA and agar germination methods (3, 11).
¶ In situ germination on the stigma.
# Mean numbers of tube penetrations, pollen tubes, or seed per boll.
†† Means were significantly less than those at 30°C at the 0.05 probability level.

RESULTS AND DISCUSSION

Table 1 summarizes how viability and fertility of cotton pollen were affected by the temperature treatments. Untreated pollen was considered fully fertile. Pollen treated 15 h at 30°C was as fertile as untreated pollen, indicating that handling the flowers had no detrimental effect on pollen fertility. However, temperature treatments from 33 to 40°C had increasingly severe effects on the viability and fertility of pollen. Above 43°C, all pollen was killed. However, all pollen samples, treated or untreated, stained equally well with Alexander's (1) cytoplasmic stain. Therefore, this stain does not differentiate between living and nonliving pollen. It does distinguish between aborted vs. nonaborted or mature vs. immature pollen. The cytoplasmic stain does give a measure of pollen maturity, but not viability, at the time of anthesis.

Pollen treated with the vital stain tetrazolium red stained after exposures of up to 40°C. Above 43°C, pollen was killed. Pollen also germinated up to 40°C with the PMDA method, the in vitro agar method, and the in situ germination on the stigma; but the degree of germination was reduced at temperatures 33°C and above.

The degree of penetration of pollen tubes into the stigmatic and stylar tissue was significantly affected by temperature. Pollen heat-treated at 30°C was also equal to fully fertile untreated pollen in its ability to set mature seed and fruit. Pollen treated at 33°C was reduced in fertility by approximately 50% as determined by penetrations into the stigma, style, and ovules and by the ultimate set of mature seed. At 35°C, the ability of pollen to penetrate the stigma was even more severely restricted; and the tubes did not penetrate into the style.

From these results, the different methods of pollen measurement appear to actually measure different aspects of pollen capability. Nuclear and cytoplasmic stains measure the degree of pollen maturity. Pollen that has developed normally to anthesis will stain with Alexander's (1) stain and can be distinguished from aborted grains that failed to mature for whatever reasons. Stresses applied to pollen after maturity that would kill or impair pollen capability would not be detected using such stains. This was evident by the almost normal stainability of pollen treated above 43°C for 15 hours.

The use of vital stains, as compared to the PMDA method or to in situ germination on the stigma, responded similarly to the range of temperature treatments. Pollen cells were essentially alive and germinable through temperature treatments of 40°C. At 40°C, there were severe reductions in viability. The in vitro germination of cotton pollen followed this same pattern. However, because of the low initial in vitro germination percentage estimates of cotton pollen, even with fully fertile pollen, the level of germinability is seriously underestimated. The vital staining and germination methods only measure at best whether the pollen cells are living or dead. This in some sense could be called a measure of viability. Many think of the term viable to mean the pollen is fully fertile. It is evident from these studies that living pollen cells or germinable pollen grains are not always fertile, i.e., capable of functioning with an ovule. As the temperatures increased to 35 and to 37 and 40°C, decreased ability of the pollen to function was observed. It is possible that the pollen stained with TTC by Oberle and Watson (8) could have been living or viable, but was not functional. The apparently high level of stained pollen from heterozygous translocation stocks in cotton by Aslam et al. (2) also indicated that genetically deficient pollen grains may be viable or stainable with TTC, but that fertility could be questionable because of the genetic deficiencies.

Pollen fertility, or the capacity of a pollen grain to fertilize an egg cell and produce a healthy zygote, is the best measurement to make in evaluating pollen. Currently, the surest way to do this is to examine the lower style for tube penetration or the ovules for penetration of the tube into the micropyle. However, it is conceivable at this point that even if the pollen tube can penetrate the micropyle, slight injury previously induced could prevent the intricate fertilization procedure described by Jensen and Fisher (6). Seed set is also a good indication of pollen fertility, but care must be used with this measurement. Pollen could be completely fertile and form a healthy zygote, but stress conditions imposed upon the maternal plant after fertilization may cause abortion of the developing embryos or fruit. Fruit abortion can be caused by factors other than defective pollen. Also, pollen fertility could be variable and low on the average. Yet, if large amounts of pollen are used, complete seed set may be attained.

Considerable effort has gone into developing pollen germination techniques. Many attempts have been made to develop a nutritional media equal to that provided by the stigma. However, the microenvironment and the water relationships between the stigma and pollen are probably as important as the nutritional aspects in pollen germination and tube growh and probably would be extremely difficult to artificially duplicate. Even if germinability of cotton pollen could be accurately assessed, it would not provide an accurate assessment of pollen fertility.

REFERENCES

1. Alexander, M.P. 1969. Differential staining of aborted and non-aborted pollen. Stain Technol. 44:117–122.
2. Aslam, M., M.S. Brown, and R.J. Kohel. 1964. Evaluation of seven tetrazolium salts as vital pollen stains in cotton *Gossypium hirsutum* L. Crop Sci. 4:508–510.
3. Barrow, J.R. 1981. A new concept in assessing cotton pollen germinability. Crop Sci. 21:441–443.
4. Broncckers, F. 1961. Une technique simple pour la germination du pollen de contonnier. Meded. Zittingen K. Acad. Overzeese Wet. Brussels. VII:601–603.
5. Iyengar, N.K. 1938. Pollen-tube studies in *Gossypium*. J. Genet. 37:69–106.
6. Jensen, W.A., and D.B. Fisher. 1968. Cotton embryogenesis: The entrance and discharge of the pollen tube in the embryo sac. Planta (Berl.) 78:158–183.
7. Miravalle, R.J. 1965. Germination of cotton pollen *in vitro*. Emp. Cott. Grow. Rev. 42:287–289.
8. Oberle, G.D., and R. Watson. 1953. The use of 2,3,5-triphenyl tetrazolium chloride in viability tests of fruit pollens. Am. Soc. Hort Sci. Proc. 61:299–303.
9. Roberts, L.W. 1950. A survey of tissues that reduce 2,3,5-triphenyltetrazolium chloride in vascular plants. Bull. Torrey Bot. Club 77:372–381.
10. Smith, F.E. 1951. Tetrazolium salt. Science 113:751–754.
11. Taylor, R.M. 1972. Germination of cotton (*Gossypium hirsutum* L.) pollen on an artificial medium. Crop Sci. 12:243–244.