NOTES

USE OF DIGITAL IMAGE ANALYSIS, VIABILITY STAINS, AND GERMINATION ASSAYS TO ESTIMATE CONVENTIONAL AND GLYPHOSATE-RESISTANT COTTON POLLEN VIABILITY

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Abstract

Because the success of labor-intensive hand crosses by breeders is dependent upon pollen viability, quick, simple, and inexpensive methods for viability assessment are of interest. Four such cotton pollen viability assays were compared to determine differences in viability estimates, and relative accuracy by correlation to seed set. The methods compared were Brewbaker & Kwack (B & K) medium, B & K medium plus aniline blue, a fluorochromatic reaction method (FCR), and Alexander's stain. Additionally, digital images of germinated pollen grains were analyzed by means of morphometry software to quantify pollen tube area per pollen grain, as a proposed additional method of assessing viability. Pollen from conventional, nontreated glyphosate-resistant (GR) and glyphosate-treated GR cotton (*Gossypium hirsutum* L.) plants was tested by each method. Glyphosate treatments to GR cotton reduced pollen viability and corresponding seed set in all methods tested. Pollen germination measured by the B & K method was most closely related to seed set per boll, while Alexander's stain gave the highest estimates of viability. The FCR method indicated that many pollen grains from glyphosate-treated GR cotton were irregularly shaped and only partially flourescein diacetate (FD) stained. All methods tested showed similar high correlation (0.7–0.8) of pollen viability to seed set. Morphometric analysis of digital images of germinated pollen found the greatest pollen tube area to pollen grain ratio with B & K medium + 30 m*M* sucrose. Because the B & K method most closely predicted the linear magnitude of seed set reduction to reduced pollen viability, allowed the use of morphometry software analysis, and was one of the simplest and least equipment-demanding methods, it may provide broad utility for those assessing cotton pollen viability.

Breeders, as well as growers, are often interested in the pollen viability status of their crops. Hand crosses made by breeders are expensive and time consuming to conduct. Often pollen viability of plants to be used in hand crosses, especially plants resulting from interspecific crosses, or genetically transformed plants, may be reduced. Growers are often interested in how and to what extent environmental factors, such as heat or rain, affect pollen viability and ultimate pollination of their crops. Therefore simple, inexpensive techniques to establish the viability of pollen before crosses are undertaken, or while field pollination is occurring, are of interest to breeders and growers alike.

Numerous methods are used to estimate the viability of cotton pollen and each have biases based on the component of the pollen grain assayed. Assuming no incompatibilities or embryo abortion, the most important indicator of pollen viability and pollination is seed set. Seed set is the true measure of whether pollination is successful. However, fertilization not only depends upon pollen viability, but also on the receptivity of the pistil, and pollen deposition. Poor seed set may be the result of the immature ovules, poor pollen, adverse environments, genotype, lack of pollen vectors, abnormal anatomical development, or any combination of these (Knox, 1986).

Cotton breeders as well as other scientists need a rapid and reliable method to estimate pollen viability. One simple method of measuring pollen viability, is to measure the ability of pollen grains to germinate in vitro. In this method, pollen grains are immersed in a germination solution on a slide containing a drop of liquid media or on a solid agarose media and viewed under a light microscope to view pollen tubes formed from pollen grains (Brewbaker and Kwack, 1963). Percent pollen viability is estimated by counting the pollen grains which have germinated versus those which have not. One drawback to this method is that germination of pollen in a culture media may differ from germination of pollen on the stigma, where interactions with flavonols and other compounds on the stigma may either stimulate or inhibit germination (Baker, 1977; Bewley et al., 2000; Richards, 1986; Stanley, 1970). Also, the dense tissue of a stigma could present more resistance to the growth of a pollen tube than in culture media. Pollen tubes germinating on a slide in a liquid germination media are very susceptible to breakage. Pollen tubes grow almost explosively when they germinate (Barrow, 1981). The force exerted by pollen grains germinating is sufficient to break off the pollen tube of an adjacent pollen grain that has already germinated, thus making the latter appear nonviable when determining percent pollen viability. Also, it is often difficult to distinguish between pollen grains which have formed pollen tubes and pollen grains which have exploded because of the osmotic potential gradient between the germination medium and the pollen. One potential solution to the problem is to include an aniline blue stain in the germination media (Stone et al., 1984). Aniline

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Abbreviations: B & K, Brewbaker and Kwack; EPSPS, 5-enolpyruvylshikimiate 3-phosphatesynthase; FCR, fluorochromatic reaction; FD, fluorescein diacetate; GR, glyphosate-resistant; TIFF, tagged image file format; UV, ultraviolet light.

blue stains callose plugs formed during the elongation of a pollen tube; thus, the true pollen tube would appear blue, while those grains which have exploded and leaked their contents do not stain. Some researchers have also used an agarose medium to provide greater resistance to growing pollen tubes, which may simulate growth in stigma tissue and prevent the explosive growth in culture media (Stanley and Linskens, 1974). Another estimate of pollen viability uses a fluorochromatic procedure where fluorescein diacetate is dissolved in a high sucrose media to prevent pollen germination (Heslop-Harrison and Heslop-Harrison, 1970; Gwyn and Stelly, 1989). The fluorescein diacetate is taken up by pollen grains and then becomes trapped if membranes are intact. Nonspecific esterases then cleave the diacetate portion of the molecule, rendering the fluorescein photoactive under UV light. The advantages of this method are that under ultraviolet (UV) light, the pollen grains can be easily scored as fluorescing (viable), or faintly or not fluorescing (nonviable). The fluorochromatic reaction (FCR) assay measures two features of the pollen grain—the presence of an intact membrane, and the presence of hydrolytic enzymes capable of cleaving the diacetate portion of fluorescein–diacetate (Heslop-Harrison and Heslop-Harrison, 1970). Whether these two features define "a viable pollen grain" is debatable because immature pollen is often considered viable by this method (Kearns and Inouye, 1993). The FCR procedure can also show differences in pollen morphology by varying fluorescence patterns within a pollen grain. Grains with variable or splotchy fluorescence may or may not be viable, and are likely the result of differential compartmentalization of hydrolytic enzymes within membranous organelles.

Alexander's Stain (Alexander, 1969, 1980) has been used to distinguish between viable and nonviable pollen in many species, including cotton (Barrow, 1983). The stain contains malachite green, which stains cellulose in pollen walls and acid fuchsin, which stains the pollen protoplasm. Barrow (1983) reported that Alexander's stain can distinguish pollen grains that aborted early in development (did not develop a protoplasm) from mature pollen grains; however, pollen aborted near maturity may also reduce germination capability and would not be detected.

Glyphosate resistant cotton contains a 5-enolpyruvylshikimiate 3-phosphate synthase (EPSPS) gene [E.C. 2.5.1.19], identified from *Agrobacterium* sp. strain CP4, whose protein product is glyphosate resistant (Barry et al., 1992; Nida et al., 1996; Padgette et al., 1995). Since commercial availability of glyphosate-resistant cotton, there have been indications of decreased performance and yield loss when it was grown in several southeastern states. Performance and yield loss may be due to a widespread but not rigorously documented increase in lower fruiting branch boll abortion and misshapen bolls following glyphosate treatment (Ferreira et al., 1998; Vargas et al., 1998). ^{14}C-Glyphosate has been shown to accumulate in reproductive structures of cotton during development, which may cause increased abscission and reduced fertility (Pline et al., 2001a). Because pollination plays a critical roll in boll retention, the effect of glyphosate on pollen viability has been investigated. Glyphosate treatments to glyphosate resistant (GR) cotton plants caused a reduction in pollen viability as measured by germination in B & K media (Pline et al., 2001b). The current study investigates whether other assays to estimate pollen viability concur with the viability reductions observed using the B & K method, as well as whether seed set per boll can be associated with pollen viability.

The objectives of the current study are to compare four different pollen viability assays to determine their relative accuracy and biases when used on pollen samples that are viable as well as those with reduced viability because of glyphosate treatments. Second, to compare correlation to seed set, ease of use, and equipment requirements for each assay. Finally, to assess the use of digital image analysis of static images of germinated pollen grains as a quantitative method for rapidly assessing pollen germination characteristics such as pollen tube area.

Materials and Methods

Plant Culture and Pollen Collection

Pollen samples were obtained from cotton plants grown in the North Carolina State University Phytotron with a 26/18°C day/night temperature regime. Seeds from two conventional Delta Pine & Land cultivars (DP 50 and DP 90) and two glyphosate-resistant cultivars (DP 5415RR and SureGrow cultivar SG 125RR) were planted in 25.4-cm pots containing a gravel-metro mix combination soil. Plants were thinned to one per pot and were watered with a nutrient mixture two times daily (Downs and Thomas, 1991). Treatments of 1.12 kg ai/ha glyphosate (Roundup Ultra, Monsanto, St. Louis, MO) were applied to some GR cotton plants at the four-leaf stage (foliar application) and at the eight-leaf stage (post-directed application to stem) according to the Roundup Ultra supplemental label for GR cotton (Anonymous, 1999). The growth regulator mepiquat chloride (*N*,*N*-dimethylpiperidinium chloride) was applied to upper leaves at the rate of 0.84 kg ai/ha at the 10-leaf stage to control vegetative growth according to North Carolina Cooperative Extension Service guidelines (Edmisten, 2001). Pollen samples were collected three times weekly, for 4 wk to assay pollen viability. All pollen samples were collected between 0900 and 1100 h. Five to six anthers were removed from each flower using forceps. Pollen from the anthers was then transferred onto microscope slides containing one of the four tested germination media and were immediately covered with a coverslip as described by Barrow (1983). Slides containing pollen were then analyzed for germination or viability 30 min after collection.

Pollen Viability Assays

Four different germination–viability assays were used to compare pollen viability. Components of each medium are described in Table 1. Brewbaker and Kwack (B & K), B & K + aniline blue, and FCR media were made fresh daily before use. Alexander's stain was stored in an air-tight dark bottle at room temperature. After pollen samples were placed on slides in each medium, samples were examined with bright-field microscopy (Alexander's stain), phase contrast microscopy to enhance visibility of pollen tubes (B & K and B & K + aniline blue), or fluorescence microscopy using a 470 nm

Table 1. Components of germination–viability media used to estimate pollen viability in cotton.

Brewbaker and Kwack Medium†	Fluorochromatic Reaction‡	Alexander's Stain§
146.1 m*M* sucrose	1.75 *M* sucrose	20 mL Reagent grade ethanol
1.6 m*M* boric acid	3.23 m*M* boric acid	2 mL of 10.8 m*M* malachite green in ethanol
1.2 m*M* calcium nitrate	3.05 m*M* calcium nitrate	50 mL distilled water
0.8 m*M* magnesium sulfate heptahydrate	3.33 m*M* magnesium sulfate heptahydrate	40 mL glycerol
1.0 m*M* potassium nitrate	1.98 m*M* potassium nitrate	10 mL of 17.3 m*M* acid fuchsin + 1 g phenol
+/−0.7 μ*M* aniline blue	7.21 m*M* fluorescein diacetate dissolved in acetone (add 10 drops/10 mL of medium)	2 mL lactic acid

† Brewbaker and Kwack, 1963.
‡ Heslop-Harrison and Heslop-Harrison, 1970; Gwyn and Stelly, 1989.
§ Alexander 1969, 1980.

excitation filter and a 535 nm emission filter (FCR Method). Each slide containing 150 to 400 pollen grains was visually counted, scoring pollen grains as germinated (pollen tube longer than pollen grain diameter) or nongerminated, for B & K and B & K + aniline blue methods (Fig. 1a). For FCR, pollen grains were classified as either viable (bright and uniform fluorescence, Fig. 1c), potentially viable but abnormal (nonuniform, splotchy fluorescence, Fig. 1d), or nonviable (very dim to no fluorescence). Alexander's stain differentially colors viable and nonviable pollen grains, with viable pollen staining a dark purple, and nonviable pollen staining either a pale green or a splotchy dark purple and pale green (Fig. 1b). Bolls (fruit) formed from all flowers where pollen samples were taken were date tagged and harvested at maturity. Bolls were dried at 50°C in a drying oven and the number of seeds per boll were counted. Bolls which had abscised from plants, were not included in the analysis.

Morphometric Analysis of Pollen and Pollen Tubes

Pollen was collected from flowers of DP 50 and DP 90 as described above and placed on slides containing B & K medium containing 0, 30, 146, 290, or 585 m*M* sucrose. Digital images of germinated pollen were made at 40× magnification with a Cohu digital camera (Cohu Incorporated, San Diego, CA). Two fields per slide were imaged, each containing 30 to 70 pollen grains. Images were saved as black and white tagged image file format (tiff) files. Morphometric analysis was conducted on images by the Intergrated Morphometry Analysis option in Metamorph 4.5 (Universal Imaging Corporation, Downington, PA). A software journal (macro) was assembled to threshold and quantify all images according to the same criteria. The sequence of events in the journal were as follows: threshold image so only pollen grains (darkest objects) are included, create a binary image of the thresholded image (binary1), count objects with a size greater than 4100 μm^2, re-threshold the original image so that all pollen tubes and pollen grains are included, create a binary image of this new thresholded image (binary2), subtract binary1 image (pollen grains only) from binary2 image (pollen grains + pollen tubes), and calculate total projected area of pollen tubes in the subtracted image. The sum of all pollen tube areas in the subtracted image was divided by the number of pollen grains from the original thresholded image to obtain the average pollen tube area per pollen grain. The pollen tube area/pollen grain ratio for a slide was calculated by averaging data from the two images captured from each slide.

Statistical Analysis

Means from all experiments were subjected to analysis of variance by SAS 8.0 (SAS Institute, Cary, NC). In the pollen viability and germination assay studies, the main effects of cotton cultivar–herbicide treatment and viability assay were

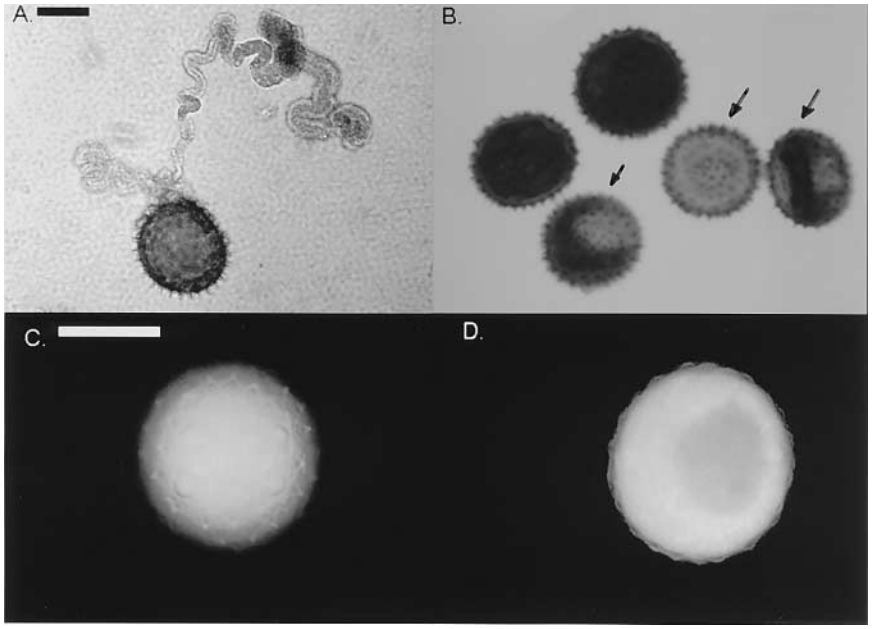

Fig. 1. Comparison of pollen grains using different viability assays. A. Viable, germinated pollen grain assessed by the B & K method and phase contrast microscopy. B. Viable and nonviable pollen (partially stained, arrows) assessed by Alexander's stain method. C. Viable, fluorescing pollen assessed by FCR method. D. Irregularly stained, nonviable pollen grain from glyphosate-treated DP 5415RR plant assessed by FCR method. Scale bars are 50 μm.

both significant at $P \leq 0.05$, as well as their interaction. Means were then separated by cotton cultivar–herbicide treatment and viability assay by means of the Fisher's protected LSD test. Correlations of pollen viability from each method to seed set were computed for all bolls retained on the plant used for pollen viability measures, by Excel 97 (Microsoft Corporation, Redmond, WA). Nonlinear regression of pollen viability to seed set was conducted for each pollen assay method. Data were fit to three-parameter logistic curves by Sigma Plot for Windows 2000, version 6 (SPSS Science, Chicago, IL). For the morphometric image analysis study, the percent sucrose main effect was significant at $P \leq 0.05$ according to ANOVA; however, the cultivar main effect was not significant, so data were combined over the two cultivars. Means of the percent sucrose main effect were then separated by the Fisher's protected LSD test.

Results and Discussion

Comparison of Pollen Germination and Viability Assays

Each assay predicted different pollen viability from common pollen samples (Table 2). Overall, the Alexander's Stain assay gave the highest estimate of viability (82–99%), whereas viability determined by other methods ranged from 34 to 98% (FCR), 57 to 95% (B & K), and 51 to 88% (B & K + aniline blue), primarily depending on the cultivar and glyphosate treatment. Within conventional and nontreated GR cultivars, Alexander's stain gave the highest estimate of viability, whereas B & K + aniline blue provided the lowest estimate (Table 2). Within glyphosate-treated GR cultivars, Alexander's stain again gave the highest estimate of viability while FCR gave the lowest estimate. Alexander's stain, because it assumes that the presence of a protoplasm indicates that a pollen grain is viable, may in fact overestimate pollen viability (Kearns and Inouye, 1993). This assay seemed reliable in distinguishing between mature and immature pollen grains, the latter which had not yet formed a protoplasm. However, the large percentage of glyphosate-treated GR pollen grains that were counted as viable from the Alexander's stain assay, but not by the other assays, suggests that the presence of cytoplasm alone is not sufficient to indicate pollen viability. It may also suggest that the effect of glyphosate on pollen viability occurs later in pollen development, after the protoplasm has formed, or that glyphosate does not affect protoplasm formation in cotton pollen.

The low estimate of pollen viability determined by the FCR method on glyphosate-treated GR pollen is a result of the high number of pollen grains with either abnormal morphology or splotchy FD staining (Fig. 1d, Table 2). The percentage of these pollen grains ranged from 46 to 55% in glyphosate-treated GR cultivars, and only 2 to 4% in conventional and nontreated GR cotton cultivars. Gwyn and Stelly (1989) counted such pollen grains as nonviable; however, when viewing the percentage of these pollen grains forming pollen tubes in the B & K method from the current study, it appears that 10 to 20% of these abnormal pollen grains are capable of germinating (Table 2). The FCR method was the only method where these abnormalities were observed. Conventional brightfield microscopy only assays the surface features of these pollen grains, whereas the FCR method gives some description of the internal structure of the pollen grain. The presence and localization of esterase enzymes capable of hydrolyzing the FD ion into a photoactive compound allows some visualization of internal features. Because the hydrolyzed FD ion is restricted by membranes (Heslop-Harrison and Heslop-Harrison, 1970; Heslop-Harrison et al., 1984; Shivanna and Heslop-Harrison, 1981), differences in fluorescence patterns may suggest differing levels or locations of esterase enzymes within internal membranous organelles in the pollen grain. Further studies on this pollen using electron microscopy have reported that pollen from glyphosate-treated GR cotton plants was aborted at the vacuolate microspore and vacuolate microgamete stages of microgametogenesis, resulting in immature pollen at anthesis (Pline et al. 2002a). In both of these stages, a large or several smaller vacuoles are present in the pollen grain. These vacuoles would normally shrink, eventually filling with storage material by anthesis if development had proceeded normally (Wetzel and Jensen 1992, Frankel and Galun 1977). The vacuoles in pollen grains from glyphosate-treated plants correspond to the nonfluorescing regions of pollen grains from glyphosate-treated GR plants assayed by the FCR method (Fig. 1d).

The B & K assay of pollen germination tests the potential of a pollen grain to germinate. In this method, pollen grains rapidly and almost explosively germinate within minutes of immersion in the medium (Barrow,

Table 2. Pollen Viability, abnormal pollen, and seeds per boll from conventional, GR, and glyphosate-treated GR cotton plants. Percent pollen viability was determined using Alexander's stain method, fluorochromatic reaction method, Brewbaker & Kwack (B & K), or B & K + aniline blue germination solutions. Abnormal pollen was the measured as the percent of pollen grains that had splotchy, or nonuniform fluorescence. Seeds per boll were counted at the completion of the study.

Cultivar	Alexander's stain method	Fluorochromatic reaction method	B & K method	B & K method + aniline blue	Abnormal pollen (FCR)	Seeds per boll
	% Pollen Viability				% of Total	
DP 50	98.2a†(a)‡	97.7a(a)	93.6a(a)	85.3a(b)	1.8c†	36.6a
DP 90	99.0a(a)	95.4a(b)	94.0a(b)	88.3a(c)	2.6c	33.1ab
DP 5415RR	98.1a(a)	96.8a(a)	95.3a(a)	87.2a(b)	1.7c	37.4a
DP 5415RR (Treated)	85.7b(a)	34.7c(b)	57.4b(b)	55.9b(b)	54.7a	21.6d
SG 125RR	98.2a(a)	94.9a(ab)	91.6a(bc)	85.9a(c)	3.5c	29.5bc
SG 125RR (Treated)	82.1b(a)	49.4b(b)	60.4b(ab)	51.2b(b)	45.8b	25.6cd

† Means within a row followed by the same letter in parentheses are not significantly different at the 0.05 probability level.
‡ Means within a column followed by the same letters not in parentheses are not significantly different at the 0.05 probability level.

1983). This method, as well as all other methods tested, clearly distinguished pollen from conventional or nontreated GR cultivars (92–95% germination) from that of glyphosate-treated GR cultivars (57–64% germination). Inclusion of aniline blue in the medium was useful for distinguishing between true pollen tubes (containing callose) and pollen grains which simply burst. However, the addition of aniline blue to the B & K media appeared to cause lower pollen germination than in B & K media alone (Table 2), and the pollen tubes that formed were observed to be shorter than those forming in B & K media alone. This lower pollen germination may suggest that the addition of aniline blue to B & K germination media was inhibitory to pollen germination.

Removal of anthers from flowers where seed set is to be assessed could potentially confound seed set results in species where pollen or anther production is limited. However, Gore (1935) reported that cotton flowers contain between 50 to 125 anthers, or more. In the current study, only five anthers, (therefore 4–10%, or less, of total anthers) were removed from each flower. Further, the anthers selected were from the lowest portion of the staminal column, and were the least likely to contribute to natural, noninsect aided pollen deposition on the stigma, because of their geographical distance from the receptive stigma. Tsyganov (1953) reported that cotton produces over 45 000 pollen grains per flower. Therefore, each anther in a cotton flower would theoretically produce between 360 to 900 pollen grains. By removing five anthers, 1800 to 4500 pollen grains would have been removed. Ter Avanesyan (1952) showed that 600 to 1000 pollen grains on the stigma was the minimal "norm" quantity of pollen required to obtain complete pollination. Therefore, in this study, 40 500 to 43 200 pollen grains would have remained in the flower, 40.5 to 72 times the number of pollen grains that the plant requires for adequate pollination. Thus, in cotton, removal of the pollen from the same flower where seed set was subsequently assessed likely does not affect seed set, because more than adequate pollen remains in the flower to ensure pollination.

Seed set in bolls developing from flowers whose pollen had been analyzed was highly dependent on the cultivar–herbicide treatment main effect (Table 2). Conventional and nontreated GR cultivars had a similar number of seeds per boll with the exception of SG 125RR. Seeds per boll ranged from 30 to 37 in conventional and nontreated GR cultivars and 22 to 26 in glyphosate-treated GR cultivars. Glyphosate-treated DP 5415RR plants averaged 15 seeds (42%) less per boll than nontreated DP 5415RR plants. The magnitude of this decline was not as striking in glyphosate-treated SG 125RR versus nontreated SG 125RR, with only a four seeds per boll (13%) decline. These data suggest that the decline in pollen viability observed with glyphosate-treated GR plants corresponds to a decline in seeds per boll, perhaps because there was not enough viable pollen, capable of pollen tube formation, on the stigma. Further research with GR cotton has shown that even with hand pollinations, which ensure adequate pollen coverage on the stigma, the seed number per boll was reduced if the pollen donor parent in a cross was treated with glyphosate (Pline et al. 2002b). This work also demonstrated that no reduction in seed set occurred if only the female parent in a cross was glyphosate treated, suggesting that ovary and pistil development were not affected by glyphosate treatments in the same manner as anther and pollen development (Pline et al. 2002b).

Seeds set per boll, under nonstressful growing conditions, is a direct measure of reproductive efficiency. The efficiency of fertilization depends on many factors including the condition and receptivity of the stigma, maturity of ovules, movement of pollen to the stigmal surface, pollen viability, pollen tube growth, as well as environmental factors (Knox, 1986). The portion of the variability in seed set that pollen viability may comprise likely varies as a function of all other factors. Because glyphosate has been shown not to affect female reproductive organs in GR cotton (Pline et al. 2002b; Mery, 2002), and the current studies were conducted in a controlled phytotron environment where the impact of environmental stress is minimized, a majority of the variability in seed set likely lies in the state of the pollen. The current study, conducted in a phytotron greenhouse, was free of insect-aided pollination. Pollen deposition on the stigma was achieved only by anther–stigma contact, or by gravity. When comparing the decline in pollen viability seen in glyphosate-treated GR cotton plants with the decline in seed set from these same flowers, certain pollen viability assays predicted the magnitude of the decline better than others. The Alexander's stain assay found a 13 and 16% reduction in pollen viability of glyphosate-treated DP 5415RR and SG 125RR plants, respectively, compared with their nontreated controls (Table 2). Because the actual reduction in seed set was 42 and 13% for DP 5415RR and SG 125RR, respectively, this method may overestimate pollen viability, especially in the case of DP 5415RR. The FCR assays found a 64 and 48% reduction in pollen viability of glyphosate-treated DP 5415RR and SG 125RR plants, respectively, compared with their nontreated controls, suggesting that it may underestimate pollen viability (Table 2). The B & K and B & K + aniline blue methods gave 40% and 34% and 36% and 40% declines in pollen viability, respectively, for DP 5415RR and SG 125RR. Pollen viability as measured by the B & K method was a good predictor of seeds per boll for DP 5415RR (42% decrease in pollen viability versus 40% decrease in seed set), but was not as good for SG 125RR (34% decrease in pollen viability versus 13% decrease in seed set).

Correlation of pollen viability to seeds per boll was high (r = 0.74–0.82) for all methods and differences between methods were negligible (Table 3). However, differences in correlation between glyphosate-treated and nontreated plants were evident. For pollen with reduced viability due to glyphosate treatments, correlation ranged from 0.72 to 0.8, suggesting that as viability decreased, seed set decreased in a similar manner (Table 3). In contrast, pollen viability from nonglyphosate treated GR or conventional plants had low correlation to seed set (0.04–0.26), suggesting that seed set was not affected by changes in pollen viability, when viability

Table 3. Correlation of pollen viability to seed set as estimated by four methods of assessing pollen viability.

Cultivar–Treatment	Alexander's stain method	Fluorochromatic reaction method	B & K method	B & K method + aniline blue
	Correlation			
Overall	0.74	0.80	0.76	0.82
Conventional & nontreated GR only	0.26	0.04	0.13	0.17
Glyphosate-treated GR only	0.76	0.72	0.74	0.80

exceeded 85% (Tables 2, 3). The differences in correlation from pollen samples with high viability and reduced viability suggest that seed set is not dependent on pollen viability if viability exceeds some threshold level (Fig. 2). In order for a boll to have yielded 30 or more seeds (the lowest mean seed set of bolls from nonglyphosate treated plants), pollen viability must have exceeded 82, 95, 76, and 84% as measured by the B & K, Alexander's stain, B & K + aniline blue, and FCR methods, respectively (Fig. 2). Additional increases in viability above these levels correlated poorly with increases in seed set, likely because of the limited number of ovaries in each boll. However, if pollen viability was below these levels, then reductions in seed set more closely paralleled reductions in pollen viability for all methods (Fig. 2, Table 3). Because these studies were conducted without insect-aided pollen deposition, the estimates of pollen viability needed to yield 30 or more seeds in the presence of pollinators may actually be less, because of increased pollen deposition.

Barrow (1983) found that the best criterion for pollen fertility was tube penetration into the lower style and ovules. Mery et al. (2002) reported that the growth of pollen tubes and ovule penetration when viable pollen from nontreated plants was placed on stigmas of glyphosate treated plants, was not different from that of pollen placed on stigmas of nontreated plants. Research analyzing the effect of pollen tube growth and ovule penetration of pollen from glyphosate-treated plants placed onto nontreated stigmas is ongoing (R. Mery, personal communication, 2002). It would seem probable that pollen from glyphosate-treated plants has reduced pollen tube growth and ovule penetration, causing the reduction in seed set observed in the current study.

Morphometric Analysis of Pollen and Pollen Tubes

The area of pollen tube per germinated pollen grains was affected by the concentration of sucrose in B & K germination media (Table 4). The greatest area of pollen tube per germinated pollen grain was achieved with 30 m*M* sucrose in B & K media. Pollen tube area per germinated pollen grain was significantly lower in B & K media with 146 or 290 m*M* sucrose than 30 m*M*. This reduction in pollen tube area might be due to differences in osmotic potentials caused by different sucrose concentrations in the media. Although differences in pollen tube area per germinated pollen grain were evident, the percentage of pollen grains that germinated (formed a pollen tube longer than the diameter of the pollen grain) did not differ among any of the sucrose concentrations (data not shown). This lack of influence on pollen grain germination would suggest that the sucrose concentrations might affect pollen tube growth beyond simply

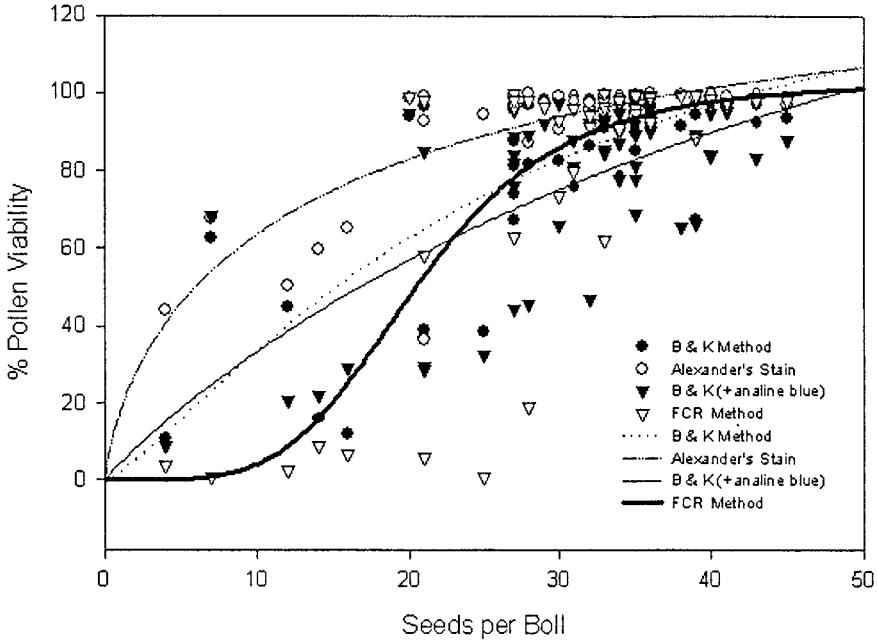

Fig. 2. Regression of percent pollen viability to seeds per boll for four different pollen viability assays. Three-parameter logistic curves were fit to data. Each data point represents the pollen viability and corresponding seed set per boll for one of the four methods. Equations of curves and R^2 are as follows: B & K method, $y = 155.9/(1 + (x/27.2)^{-1.29})$, $R^2 = 0.57$; Alexander stain, $y = 150.9/[1 + (x/15.3)^{-0.74}]$, $R^2 = 0.61$; B & K + aniline blue method, $y = 237.8/[1 + (x/68)^{-0.95}]$, $R^2 = 0.47$; and FCR method, $y = 103.3/[1 + (x/20.8)^{-4.3}]$, $R^2 = 0.63$.

Table 4. Pollen tube area (μm^2) per pollen grain in Brewbaker and Kwack (B & K) germination media with different sucrose concentrations.

m*M* sucrose (in B & K media)	μm^2 Pollen tube per pollen grain
0	27 658ab†
30	29 930a
146	25 568b
290	25 520b
585	26 863ab

† Means within a column followed by the same letter in parentheses are not significantly different at the 0.05 probability level.

stimulating the pollen grain to germinate. In agreement with our data, Barrow (1981) reported that pollen germination was similar at all sucrose concentration less than 1.7 to 2.0 *M* sucrose, but that the length of pollen tubes formed decreased with increasing sucrose concentrations. Therefore, sucrose concentrations of 0 to 585 m*M* are all conducive to pollen germination, with greatest pollen tube area per pollen grain occurring at 30 m*M*.

The number of pollen grains as counted by the morphometry software were within 5% of the total number of pollen grains on every image examined visually. Because pollen tubes rarely grow in straight lines and are much more commonly serpentine like tubes, conventional measurements of length may not be as accurate as those provided by morphometrical image analysis. The effect of different treatments on germination and pollen tube growth could also be assessed by this technique. By thresholding differences in intensity using morphometry software, one can easily distinguish pollen tubes from pollen grains and can obtain an accurate estimate of pollen tube area. The ability to create a "journal" of thresholding levels and counting events, which can be applied to all similar images in a study, greatly speeds the number of samples that can be analyzed. This technique also gives a more quantitative assessment of pollen viability than germination counts alone, because pollen tubes, in addition to pollen germination are measured. It is crucial, however, that all images analyzed by the same "journal" be similar in nature, i.e., taken under the same microscope with the same light intensity. Another benefit of the morphometric analysis is that the images can be saved permanently, and reanalyzed using new thresholding and counting criteria. The archiving of these images may allow the researcher who lacks time to measure viability immediately, to gather data from these slides by capturing images, and to analyze them at leisure. Because of the simplicity and rapidity of data collection by morphometrical analyses, the ability of the software to quantify subtle differences between samples, and the time saving benefits provided, these techniques could be of value to those studying numerous pollen samples.

In summary, our data have shown that pollen viability assays differ in their estimates of pollen viability and pollen germination of pollen samples taken from the same anthers. These differences are likely due to the specific characteristics of the pollen grain assayed by each method. The FCR method appeared to underestimate, while Alexander's stain seemed to overestimate pollen viability. The addition of aniline blue to B & K media appeared to inhibit pollen germination and tube growth. Glyphosate treatments to GR cotton significantly reduced pollen viability in all methods tested. The magnitude of reductions in pollen viability due to glyphosate treatment were most closely reflected in the magnitude of seed per boll decline by the B & K method in DP 5415RR. There were essentially no differences between correlation of pollen viability to seed set between the four methods; however, differences in correlation of viability to seed set between glyphosate-treated and nontreated plants were evident. To achieve normal seed set (mimimum of 30 seeds per boll), pollen viability typically must have ranged between 76 and 95% as measured by the four assays. Sucrose concentrations in B & K media significantly affected pollen tube area, but did not affect pollen germination. Morphometric analysis of digital images of pollen grains germinated in B & K media allowed measurement of germination factors, such as the pollen tube area per pollen grain, which cannot be accurately computed by traditional methodology. Additionally, this method creates an archive of image data which can be analyzed later, if pollen viability cannot be assessed immediately after pollen harvest. Because the components used in the B & K assay are relatively nontoxic compared with those used in the Alexander's stain assay and because there is no need for a microscope equipped with a UV lamp, the B & K method may be the safest and least equipment demanding method of those compared. The B & K assay also allows for the use of morphometry software analysis. As a whole, the B & K method provides broad utility for those assessing cotton pollen viability, and can easily be adapted for use in the field by breeders who question the viability of pollen to be used in crosses or growers who are interested in pollination potential.

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