Chapter 2

COTTON FLOWERS: POLLEN AND PETAL HUMIDITY SENSITIVITIES DETERMINE REPRODUCTIVE COMPETITIVENESS IN DIVERSE ENVIRONMENTS

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INTRODUCTION

Crop species grown throughout the world experience environmental stresses that limit their growth, development, and full expression of their genetic potential for agronomic yield. Comparison of average crop yields with reported record yields has shown that the major crops grown in the U.S. exhibit annual average yields three- to seven-fold lower than record yields due to unfavorable environmental conditions (Boyer, 1982). Analysis of yields from corn (*Zea mays* L.), wheat (*Triticum aestivum* L.), soybean (*Glycine max* L.), sorghum (*Sorghum vulgare* L.), oat (*Avena sativa* L.), barley (*Hordeum vulgare* L.), potato (*Solanum tubersosum* L.), and sugar beet (*Beta vulgaris* L.) revealed that the average yield represented only 22% of the mean record yield. Crops with economically valuable reproductive structures showed the greatest discrepancy between average and record yields. Those crops having marketable vegetative structures exhibited approximately three-fold reductions in yield (Boyer, 1982). These data suggest that plants have high productivity potential, but are operating well below their genetic potential.

Yield loss might be lessened by identifying and optimizing those plant protective mechanisms that could be used to improve stress-resistant germplasm stocks. One such protective mechanism is acquired thermotolerance, a process postulated to be closely linked to the heat shock response. Plants are frequently exposed to elevated soil and air temperatures resulting in a reduction in their growth, development and ultimately productivity. Subjecting them to a period of sub-lethal elevated temperatures induces a transient state of thermotolerance, which raises the injury threshold and protects the plants from subsequent, otherwise lethal, high temperatures (Vierling, 1991). This acquisition of thermotolerance is a complex physiological phenomenon that has been shown to involve at least some heat shock proteins (HSPs). Although varying in magnitude among plant cultivars, most vegetative tissues exhibit an inducible heat shock response. Germinating pollen, however, has not been found to exhibit the heat shock protein induction pattern upon exposure to elevated sub-lethal temperatures and concomitantly exhibits rapid losses in viability upon heat exposure (Hopf *et al*., 1992). This may explain Boyer's observation that crops with economically valuable reproductive structures show the greatest discrepancy between average and record yields (Boyer, 1982).

Modest progress has been achieved in selecting cotton cultivars with improved heat tolerance by heat treatment of pollen prior to pollination allowing only the more heat tolerant pollen to be effective in subsequent crosses (Rodriguez-Garay and Barrow, 1988). The process of selecting pollen with improved heat tolerance could be accelerated with a rapid and reliable method of germinating cotton pollen to measure viability across a range of environmental stresses. Current pollen germination techniques include "hanging drop culture", "sitting drop suspension culture", "suspension culture" and "surface culture" (Shivanna and Rangaswamy, 1992). The hanging drop and sitting drop cultures use only small volumes of germination media and small amounts of pollen, and are therefore of limited usefulness in physiological and biochemical studies.

Cotton pollen has proved to be recalcitrant to traditional in vitro germination and pollen tube growth protocols. Kearney and Harrison (1932) described the failure of in vitro techniques and went so far as to use the percentage of pollen grains that burst when placed in weak sugar solutions as a measure of viability. Failures to germinate cotton pollen in vitro drove Iyenger to dissect cotton pollen tubes from in situ germinated pollen (Iyenger, 1938). Bronkers (1961) first described a reliable technique for in vitro cotton pollen germination. Miravalle (1965) has since reported that the pollen tubes grown in this media were short, the cytoplasm was cloudy and granular, and the process required 24 h or longer. Taylor (1972) described a medium that overcame many of the limitations outlined by Miravalle (1965). Taylor reported rapid pollen germination (2 to 3 h), more normal appearing cytoplasm, and longer pollen tubes. Wauford (1979) further improved upon Taylor's medium and averaged 47% germination and 2.6 mm pollen tube lengths. Although Wauford's protocol was an improvement upon Taylor's medium, the 2.6 mm pollen tube length achieved in vitro does not compare with the 20 to 40 mm tube lengths reported in vivo. The most recent in vitro cotton pollen germination report by Barrow (1981) described the use of a hanging drop technique to forcefully eject pollen tube-like structures. Recent findings in our laboratory revealed that the pollen tube-like structures were not tubes but were pollen cytoplasm ejected from the pollen as it osmotically ruptured in a way similar to that reported by Kearney and Harrison (1932).

Burke *et al*. (2004) described the development of a pollen germination media and technique that provides high pollen germination levels and improved pollen tube growth. In developing the media it was necessary to evaluate the following variables: temperature, humidity, pH, and carbon source. The temperature effect on pollen germination and pollen tube elongation was evaluated over a range of temperatures from 20 to 43˚C. Pollen germination was high across the range of temperatures from 20 to 37˚C. The percent pollen germination declined from a mean of 71% at 37˚C to 23% at 40˚C, with little germination occurring at 43˚C. Pollen tube elongation rate was low at 20˚C and increased with increasing temperature up to 28˚C. The 28 and 31˚C samples exhibited similar pollen tube lengths with significant (0.05 level) declines in tube length observed at 34˚C and above. Kakani *et al.* (2005) showed optimal pollen germination and elongation at 30˚C when evaluating pollen responses to temperature in five-degree increments.

The effect of humidity levels on pollen germination and pollen tube elongation was evaluated at 35, 50, 80, and 100% relative humidity (RH) (Burke *et al*., 2004). Pollen that germinated on media in 35% RH had short pollen tubes located at the interface between the pollen grain and

the germination medium. The 50% RH resulted in increased pollen tube length, while the best elongation occurred at 80% RH. Although germination levels were high, most pollen tubes remained short as they ruptured when incubated under 100% RH. A range of humidity (50 to 80%) can be used during pollen germination; however, if humidity levels are too low (35% or less), germination occurs, but only short tubes are observed. If the humidity level is too high (100%), germination occurs and tubes rupture shortly thereafter.

The present study investigated genetic variability in the abiotic stress tolerance of mature pollen. Heat stress was imposed on pollen in situ and evaluated in vitro for germination and pollen tube development responses. The importance of humidity levels on pollen viability and germination was also investigated. Laboratory-based tests permitting rapid evaluation of the overall abiotic stress tolerance of the pollen were developed. Our findings provide breeders with a previously unexplored reservoir of genetic diversity associated with reproductive abiotic stress tolerance.

HEAT SENSITIVITY

Cotton seeds were planted into 5 gallon pots containing 900 g of Sunshine Mix #1 soil (Sun Gro Horticulture Distributors Inc., Bellevue, WA). Three seeds were planted per pot pots were placed on benches in a greenhouse set to provide a 30/25°C day/night cycle. Plants were grown throughout the year and 430 W high-pressure sodium lights (P. L. Light Systems, Canada) were used to maintain a 16/8 h photoperiod. Nutrients were maintained by daily application with Peters Excel fertilizer (Scotts-Sierra Horticultural Products Company, Marysville, OH) through the automated watering system. Flowers were harvested between 0930 and 1030 h from the greenhouse plants and were placed on moistened Model 583 Gel Dryer Filter Paper (Bio-Rad Laboratories, Hercules, CA) in a Pyrex baking dish. The flowers and filter paper were covered with CO_2 permeable Glad ClingWrap (The Glad Products Company, Oakland, CA). Temperature incubations were performed in the presence of high humidity from the wet filter paper in an attempt to separate temperature stress from humidity responses. Replicate samples were placed in the dark in VWR Model 2005 incubators (Sheldon Manufacturing, Inc., Cornelius, OR) set to 39 or 28°C. The trays containing the flowers were incubated for 5 h, the flowers were then removed from the trays and the pollen collected by gently tapping the inverted flower. The pollen was germinated in vitro at 28°C according to the procedure of Burke *et al*. (2004). The pollen was incubated on the media for 1 h prior to analysis. Pollen germination was determined microscopically using a Leica MZ6 modular stereomicroscope (Leica Microsystems Inc., Bannockburn, IL). The percent germination was determined for 16 replicate samples harvested over a two-month period.

Temperature incubations (39°C 5h) under high humidity in an attempt to separate temperature stress from humidity responses showed no significant difference in the heat induced decline in pollen germination among the Suregrow 248, Stoneville 474, Deltapine 565, NM67, Acala Maxxa and Phytogen 72 cotton lines (Fig. 1). All of the lines exhibited a 55 to 65% decline in pollen germination following the heat treatment.

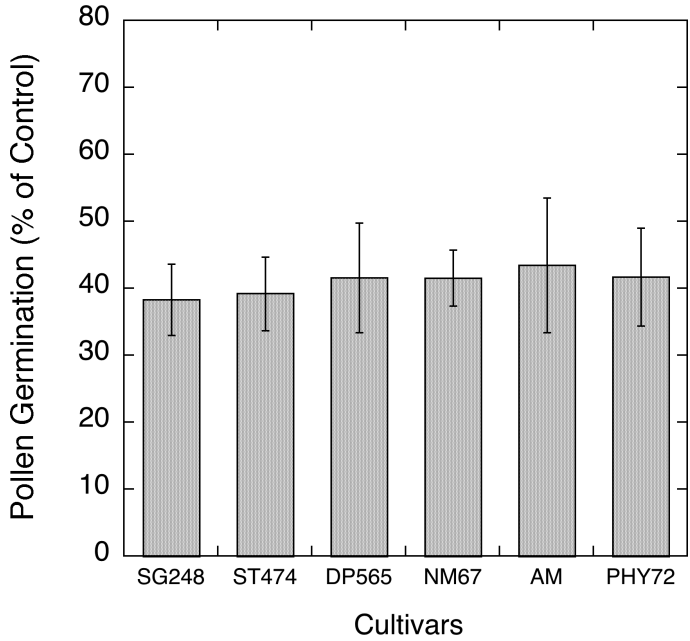

Figure 1. High temperature sensitivity of cotton pollen from Suregrow 248, Stoneville 474, Deltapine 565, NM67, Acala Maxxa, and Phytogen 72 cotton cultivars. Flowers were incubated at 28 or 39°C in high relative humidity chambers for 5 h, the pollen removed, and in vitro germination evaluated at 28°C and 80% relative humidity. Error bars represent the standard error of twelve replications.

IN SITU POLLEN DEHYDRATION

Pollen drying was evaluated in a laboratory with an ambient 25% RH environment. Humidity level was monitored using a Model H08-004-02 HOBO RH and temperature sensor (Onset Corporation; Bourne, MA) and found to be 25% RH throughout the experiment. Flowers were harvested between 0930 and 1030 h from the greenhouse plants and placed in Ziploc plastic bags for transport into the laboratory. Upon returning to the laboratory, the petals of the flowers were removed and the flowers with exposed pollen were placed on a bench top for 6.5 h. Following the treatment, the pollen was germinated in vitro at 28°C according to the procedure of Burke *et al*. (2004). The pollen was incubated on the media for 1 h prior to analysis. Pollen germination was determined microscopically using a Leica MZ6 modular stereomicroscope (Leica Microsystems Inc., Bannockburn, IL). The percent germination was determined for 17 replicate samples harvested over a two-month period. The Stoneville 474 pollen showed a 44% reduction in pollen germination, the Suregrow 248 pollen showed a 31% reduction, Deltapine 565 pollen showed a 20% reduction, NM67 pollen showed a 33% increase, Acala Maxxa showed a 45% reduction and Phytogen 72 showed a 42% reduction in pollen germination (Fig. 2).

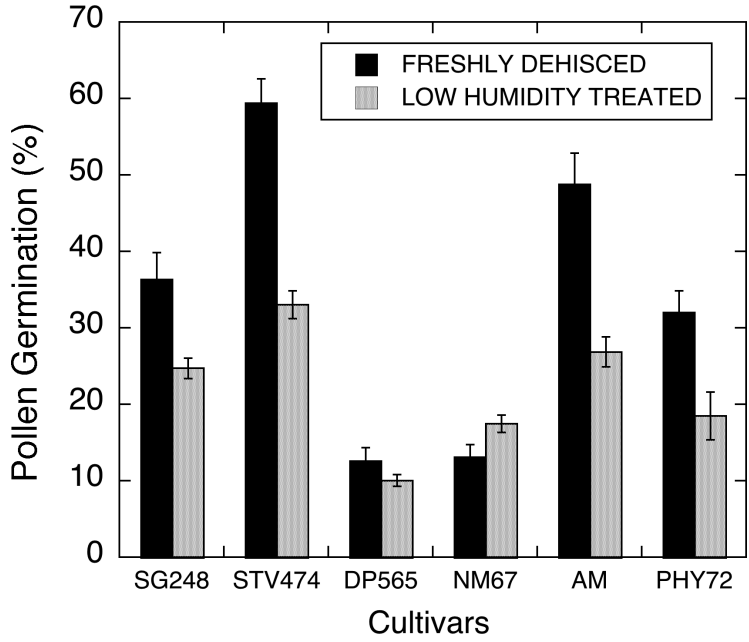

Figure 2. In situ pollen dehydration of cotton pollen from Suregrow 248, Stoneville 474, Deltapine 565, NM67, Acala Maxxa, and Phytogen 72 cotton cultivars. Flower petals were removed and the exposed anthers and pollen were incubated for 6.5 h in a 25% relative humidity. In vitro pollen germination was evaluated at 28°C and 80% relative humidity. The percent pollen germination of the low humidity treated pollen (grey bar) was compared with the germination of freshly dehisced pollen (black bar). Error bars represent the standard error of seventeen replications.

It is interesting to note that the germination percentages of the NM67 and DP565, the two lines showing the greatest dehydration resistance were also the two lines showing the lowest percent germination prior to the dehydration treatment. These results suggested the possibility of genetic differences in the pollen's ability to retain internal moisture or in their ability to take up moisture from the in vitro pollen germination medium. Before testing this hypothesis further it was necessary to determine if the pollen from these lines had similar moisture contents at the beginning of the study.

POLLEN WATER CONTENT

The water content of the pollen grains was evaluated according to the procedure of Nepi *et al*. (2001). Fresh pollen was weighed, dried in an oven at 104°C, and reweighed to determine the amount of water loss. Drying was continued until no further change in pollen weights was observed. The percent pollen water content was determined for 5 replicate samples per line. Replicate experiments showed no significant differences in water contents among the pollen from all lines immediately following dehiscence. The lines exhibited water contents of 51% (Fig. 3).

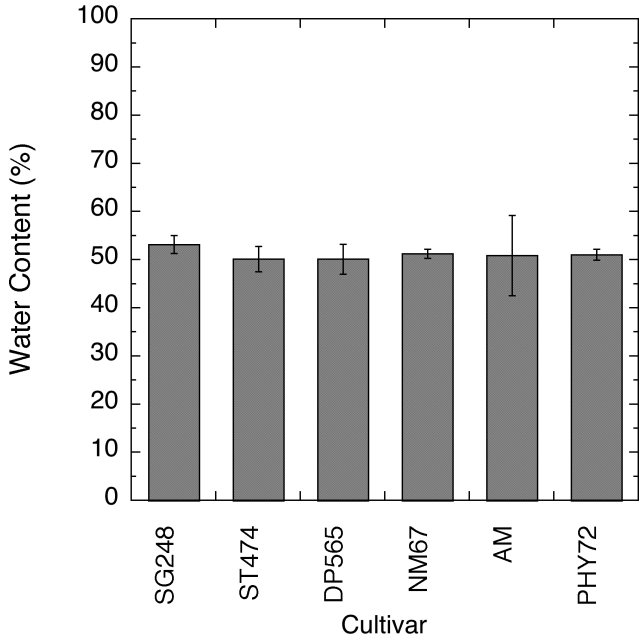

Figure 3. The water content of cotton pollen at dehiscence from Suregrow 248, Stoneville 474, Deltapine 565, NM67, Acala Maxxa and Phytogen 72 cotton cultivars. Error bars represent the standard error of five replications.

Having shown that the pollen started with equal internal water contents, experiments were performed to determine if pollen tube length development was impacted by the humidity surrounding the pollen during germination.

HUMIDITY EFFECTS ON POLLEN TUBE LENGTH DEVELOPMENT

Pollen germination was evaluated in 25 and 80% RH environments. The 80% RH level was obtained by using a 1.7 liter storage container (Rubbermaid Save and Serve) containing a saturated solution of NH_4SO_4 as described by Gawel and Robacker (1986). The 25% RH was the humidity level of the laboratory in which the experiments were performed. Flowers were harvested between 0930 and 1030 h from the greenhouse plants and placed in Ziploc plastic bags for transport into the laboratory. Upon returning to the laboratory, the pollen was collected by gently tapping the inverted flower. The pollen was germinated in vitro at 28°C according to the procedure of Burke *et al*. (2004) with half of the pollen placed in a 25% RH environment and the other half placed in an 80% RH environment. The pollen was incubated on the media for 1 h prior to analysis. Pollen germination was determined microscopically using a Leica MZ6 modular stereomicroscope. The percent germination was determined for samples from

3 replicate experiments. The SG248, STV474, DP565, and NM67 showed 35-40% reductions in pollen tube length when germinated in a 25% RH environment compared with the 80% RH environment (Fig. 4). The low humidity was more deleterious to the Acala Maxxa and PHY72 pollen as shown by the 60-65% reductions in pollen tube lengths in the 25% RH environment. These results support the hypothesis that the Acala Maxxa and PHY72 may lose internal water more rapidly than the SG248, STV474, DP565, and NM67. This water loss appears to reduce germination and pollen tube development in vitro.

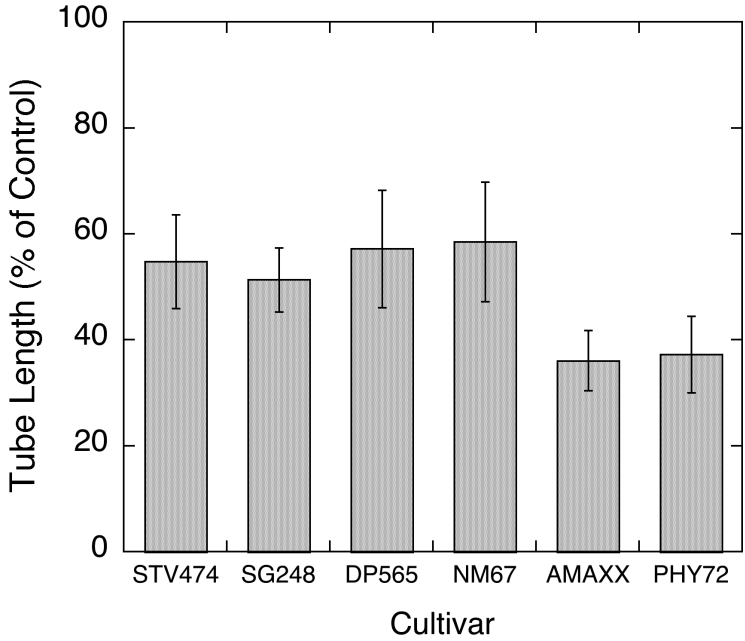

Figure 4. The effect of humidity on in vitro pollen tube length development of cotton pollen from Suregrow 248, Stoneville 474, Deltapine 565, NM67, Acala Maxxa and Phytogen 72 cotton cultivars. Germination at 25% relative humidity was compared with germination at 80% relative humidity. Error bars represent the standard error of three replications.

POLLEN WATER UPTAKE

The rate of water movement into pollen was evaluated by monitoring the time required for the pollen to rupture in an aqueous medium. Flowers were harvested between 0930 and 1030 h from the greenhouse plants and placed in Ziploc plastic bags for transport into the laboratory. Upon returning to the laboratory, the flower petals were folded back and the anthers dipped into 3-4 drops of a 0.8 M sucrose solution on a glass microscope slide. A cover slip was immediately placed on the slide and the time required for the pollen grains (a minimum of 100 grains per field of view) to rupture was determined microscopically using a Leica MZ6 modular stereomicro-

scope. The time to the first pollen grain rupture was determined for 41 flowers for each cultivar. The pollen from field-grown cotton was evaluated using flowers harvest at 0930 and 1330 h to determine if the time to first rupture changed over time.

If the assumption that water is lost more readily from the Acala Maxxa and PHY72 pollen than the SG248, STV474, DP565, and NM67 pollen is correct, then it is reasonable to hypothesize that water movement into the Acala Maxxa and PHY72 pollen may occur more rapidly than the SG248, STV474, DP565, and NM67 pollen. We chose to test this hypothesis by evaluating the rate of water uptake into the pollen. This was accomplished by monitoring the swelling and rupturing of the pollen grains in aqueous media. Burke (2002) reported the hypersensitivity of cotton pollen to water and that pollen grains placed in water would swell and rupture in seconds to minutes. In order to optimize the detection of genetic differences in pollen water uptake between cotton lines, we evaluated a range of osmotic media to slow the rate of pollen popping and maximize the difference should they exist. We observed optimum differences in the rate of pollen popping using a 0.8 M sucrose solution. A 2 to 4-fold difference in the time required to rupture the pollen was observed among these six cotton lines (Fig. 5). Although the absolute time required to rupturing of the pollen grain varied between the greenhouse and field-grown cotton, the ranking of the lines was identical. These findings further support the hypothesis that there exists genetic difference in the ability of pollen to retain internal water levels and maintain pollen viability.

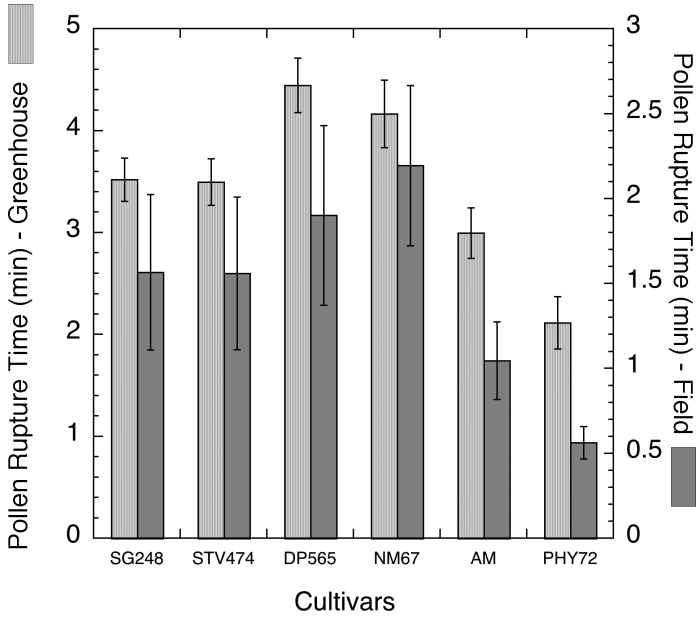

Figure 5. The time to first pollen grain rupture when cotton pollen from greenhouse-grown (light grey) and field-grown (dark grey) Suregrow 248, Stoneville 474, Deltapine 565, NM67, Acala Maxxa, and Phytogen 72 cotton cultivars were placed in 0.8 M sucrose. Error bars represent the standard error.

COMPETITIVE POLLINATION

The relative time required for the pollen to germinate and pollen tubes to reach the ovules was evaluated by competitive pollination. Competitive pollination was evaluated in a greenhouse with well-watered plants. Pollen from Suregrow 248, Stoneville 474, Deltapine 565, NM67, Acala Maxxa, and Phytogen 72 were co-pollinated with pollen from the glandless cotton Gregg 65. Gregg 65 flowers were sterilized according to the procedure of Burke (2002). Anthers on a flower from Gregg 65 and a test line were simultaneously rubbed on the recipient stigma. The resulting boll was allowed to mature and seed were harvested for analysis. Seeds were planted in soil flats, placed in a growth chamber set to 30°C, and hypocotyls were evaluated for gossypol glands two weeks after planting. Only bolls with 20 or more seeds were evaluated.

Figure 6 shows the percentage of glandless offspring. The results showed that pollen from PHY72, Acala Maxxa, and SG248 germinated more rapidly and/or pollen tubes grew more rapidly than STV474, DP565, and NM67 allowing approximately 70% of the resulting seeds to be glanded. The STV474, DP565, and NM67 pollen had similar germination and growth rates to those of the Gregg 65. This is shown by the 50:50 split in glanded and glandless offspring. The results suggest that pollen that is sensitive to relative humidity levels (Fig. 4) not only will lose water more rapidly in dry environments but will hydrate and germinate more rapidly in moist environments (Fig. 6).

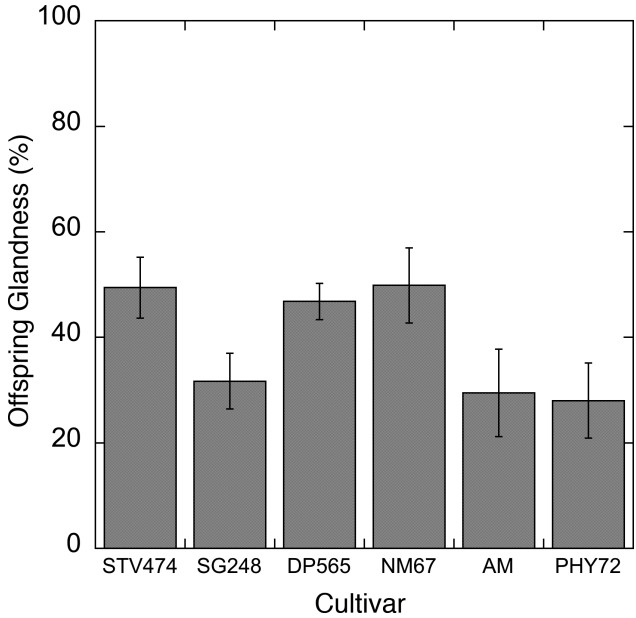

Figure 6. The percent glandless cotton plants obtained from Gregg 65 glandless cotton that was co-pollinated with pollen from Gregg 65 and pollen from either Suregrow 248, Stoneville 474, Deltapine 565, NM67, Acala Maxxa or Phytogen 72 cotton cultivars. Error bars represent the standard error.

SUMMARY

Genetic diversity in reproductive abiotic stress tolerance has been reported for cotton based upon the percentage of anther dehiscence of mature pollen in adverse environments. This study investigated the abiotic stress tolerance of mature pollen and identified genetic variability among six cotton lines. Similar high temperature sensitivities were observed for the SG248, STV474, DP565, NM67, Acala Maxxa, and Phy72 pollen. Genetic diversity in pollen viability was observed following a 6.5 h exposure to 25% RH. NM67, DP565, and SG246 exhibited less inhibition of pollen germination than STV474, Acala Maxxa and PHY72. Similar pollen water contents were observed for all lines. Genetic diversity in pollen tube length development at 25% RH compared with 80% RH was observed. Acala Maxxa and Phy72 pollen produced tube lengths of 35-40% of controls at 80% RH, while STV474, SG248, DP565, and NM67 exhibited tube lengths 50-60% of controls. Pollen water uptake studies showed faster uptake in PHY72 and Acala Maxxa than the other lines. Competitive pollinations showed faster germination of PHY72, Acala Maxxa and SG248 pollen compared to STV474, DP565 and NM67. These findings show genetic differences in cotton pollen sensitivities to water uptake and water loss. Our findings provide breeders with a previously unexplored reservoir of genetic diversity associated with reproductive abiotic stress tolerance.

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