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## Differentiating pollen from four species of *Gossypium*

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Cotton (*Gossypium*, Malvaceae) has been spun, woven and dyed since prehistoric times. Four cotton species are economically important; these are *Gossypium arboreum* (tree cotton), *G. barbadense* (American pima cotton), *G. herbaceum* (levant cotton) and *G. hirsutum* (American upland cotton). Previous research has been conducted examining the pollen grains of the Malvaceae and there is a key that differentiates the four economically important species of *Gossypium* by their pollen grains. However, the cotton pollen found in boll weevils, *Anthonomus grandis* Boheman, and other insect pests cannot be keyed to the species using the published key. The objective of this research was to determine if the pollen grains of these four species could be differentiated and develop a key that works for cotton pollen found in insect pests. Flowers of the four taxa were collected from USDA greenhouses and fields and dried. Both unacetolysed and acetolysed pollen grains were examined with light and scanning electron microscopy. The length and width of 300 pollen grains and 100 processes (spines) of each taxon were measured. There were no size differences between the acetolysed and the unacetolysed grains. *Gossypium barbadense* and *G. hirsutum* represent the largest grains (mean = 106.7 and 94.9  $\mu\text{m}$ , respectively) and the longest processes (mean = 17.7 and 15.2  $\mu\text{m}$ , respectively). Differentiation of these taxa can be useful in the determination of the origin of insect pests that attack cotton when different cotton species are grown within a region. However, additional research is needed on the varieties and cultivars of cotton.

**Keywords:** pollen; *Gossypium*; cotton; boll weevils; Texas

### 1. Introduction

Insect pests, such as the boll weevil (*Anthonomus grandis* Boheman), still economically impact the growing of cotton (*Gossypium*) in the southern USA. Previous studies have reported cotton pollen in the gut of trapped boll weevils (Cate and Skinner 1978; Jones et al. 1993; Hardee et al. 1999; Jones and Coppedge 1999). Unfortunately the cotton pollen found in these reports was not identified to the species level. Frequently, it is assumed that any cotton found in the gut of trapped weevils comes from feeding on the cotton nearest the trap where the weevils were captured.

During August 2007, over 150 boll weevils were unexpectedly captured in pheromone traps across an extensive area of the Southern Rolling Plains (SRP) eradication zone of Texas. This eradication zone was considered essentially weevil-free since 2003. In order to mitigate this unexpected re-invasion, expenses of more than \$1M were incurred to increase the number of traps and insecticide applications during the fall of 2007 (Kim et al. 2010). Some of the weevils captured during this event contained cotton pollen. Since two

species of cotton are commercially grown in Texas, the capability of identifying the cotton pollen found in the weevils could help determine the source zone of the weevils in the SRP.

There are about 50 species of cotton (Brubaker et al. 1999). Cotton is a shrub that grows natively in tropical and subtropical areas around the world. Four of these species were domesticated independently as a source of fibres, *Gossypium arboreum* L. (tree cotton), *G. barbadense* L. (American pima cotton), *G. herbaceum* L. (levant cotton) and *G. hirsutum* L. (American upland cotton) (Brubaker 2002). DNA sequencing has suggested that *Gossypium* arose about 10–20 Ma ago (Wendel and Albert 1992; Seelanan et al. 1997).

These four cotton species have two different origins. *Gossypium herbaceum* and *G. arboreum* are Old World cottons that originated from the African-Arabian gene pool. They are both diploid species (i.e. they have two sets of chromosomes). *Gossypium hirsutum* and *G. barbadense* are New World species and are indigenous to Mesoamerica and South America. They are polyploid (i.e. they have more than two sets of chromosomes) (Saad 1960).

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In the USA, cotton is mainly grown in the southern States in what is called the cotton belt. This includes Alabama, Arizona, Arkansas, California, Georgia, Louisiana, Mississippi, North Carolina, Tennessee and Texas. The primary cottons produced within the cotton belt are *G. hirsutum* and *G. barbadense* (the New World species). Although most of the states within the cotton belt grow only *G. hirsutum*, Arizona, California, New Mexico and Texas grow both *G. hirsutum* and *G. barbadense*.

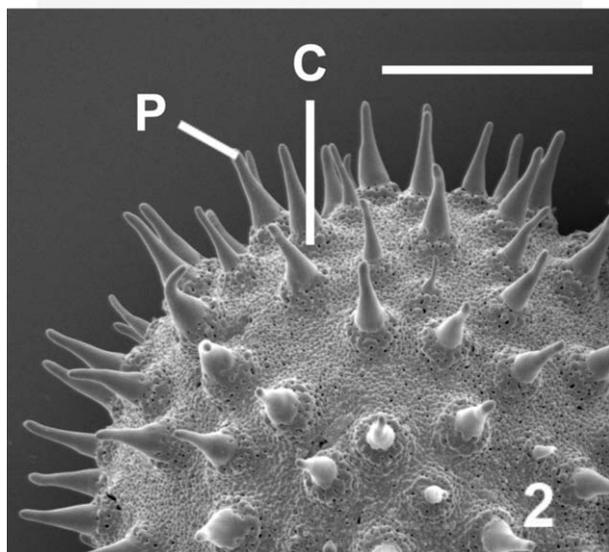
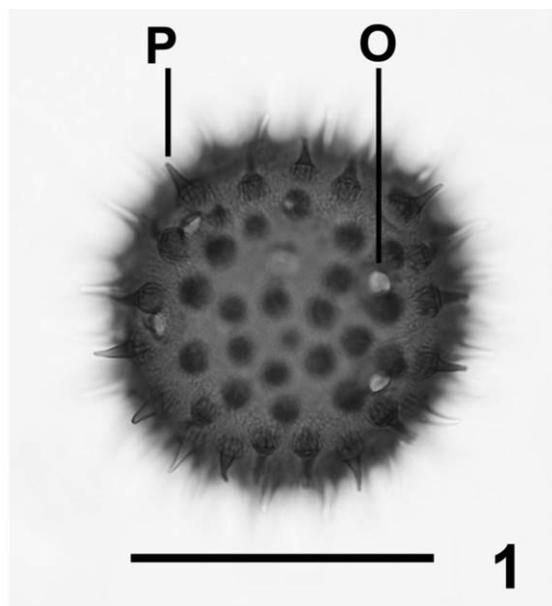


Plate 1. Figure 1. A pollen grain of *Gossypium barbadense* photographed using light microscopy, with a pore (O) and a process or spine (P) indicated. Scale bar = 100  $\mu\text{m}$ . Figure 2. A *Gossypium barbadense* pollen grain photographed with scanning electron microscopy, with a process or spine (P) and a cushion (C) indicated. Initial magnification  $\times 3500$ . Scale bar = 30  $\mu\text{m}$ .

Cotton pollen grains are large, spherical, echinate, and porate having a ring of pores (Plate 1). Each cotton flower produces an average of 30,000 to 40,000 pollen grains, representing a mass of 19 to 26 mg of fresh pollen (Vaissière and Vinson 1994). They are easy to recognise to the genus but not to the species level.

Past morphological research on Malvaceae pollen has been conducted (Saad 1960; Christensen 1986; Naggar 2003). Saad (1960) even includes a key to the four economically important cotton species. However, when examining cotton in weevils and other insect pests, the taxa of cotton could not be keyed out using the existing key. The objectives of this study were to determine if these taxa could be identified by their pollen grains and develop a dichotomous key of the pollen grains of these four taxa.

## 2. Methods and materials

### 2.1. Pollen collection

Flowers of *G. arboreum*, *G. barbadense*, *G. herbaceum* and *G. hirsutum* were collected from USDA-ARS greenhouses and fields in College Station, Texas (Figure 1) on 19 February, and 11, 16 and 25 June 2009. When possible, the staminal column of several flowers of each taxon with the same collection data were removed and placed into a manila packet. The packets were put into a dryer at 32° C and the flowers were dried thoroughly for 3–4 days (Jones et al. 1995).

### 2.2. Pollen processing

*Unacetolysed pollen:* A glycerin-stain solution was made of 20 ml of glycerin and four drops of Safranin O stain. This solution was put into a small vial then stirred. Two drops of the glycerin-stain were placed onto a glass slide. A dehiscent anther (open and releasing pollen) was placed over the slide or a prepared and labelled scanning electron microscope (SEM) stub (Jones et al. 1995) and gently tapped to dislodge the pollen grains onto the slide or stub. A cover slip was placed over the drop on the slide and the perimeter of the cover slip was sealed to the slide with nail polish. Stubs were placed in a dessicator.

*Acetolysed pollen:* Ten to 20 anthers were removed and placed into individual 12 ml uniquely-marked conical centrifuged tubes. To soften the anthers, 4 ml of a 5% KOH (v/v) solution was added to each tube and the contents were stirred. The centrifuge tube was then filled with distilled water. Samples were centrifuged for 3 min at 1,060  $\times g$ , the supernatant decanted, and the pollen residue mixed for 15 s. Distilled water was added to each tube and the contents were poured through a 450  $\mu\text{m}$  stainless steel mesh screen into a 150 ml plastic beaker. The contents in the



Figure 1. A map of the USA showing Texas (in grey) and College Station (C), where the cotton (*Gossypium*) flowers were collected and the pollen processed and measured.

beakers were poured back into its original centrifuge tube. Distilled water was added to each sample. The samples were centrifuged, decanted and mixed (Jones et al. 1995; Jones and Greenberg 2009). This process was repeated three times. Samples were acetolysed (Erdtman 1960, 1963; Jones et al. 1995; Hardee et al. 1999; Jones and Coppedge 1999). After 7 min, 5 ml of glacial acetic acid was added to each sample and the samples were again centrifuged, decanted and vortexed. Next, the samples were rinsed three times with water and once with 5 ml of 100% ETOH. A small amount of pollen residue was dropped onto a marked SEM stub. Two drops of Safranin O stain and an additional 5 ml of 100% ETOH were added to the residues. Finally, the samples were transferred into 5 ml vials and three drops of glycerin were added to each sample. The samples were left overnight on a warm hot block (25°C). The next day, the residue was stirred for 15 s. A single drop was placed onto a glass slide, allowed to spread slightly and a cover slip applied. Pollen grains were measured within two weeks after processing. Scanning electron microscope stubs were dried in a desiccator, coated with 450 Å of gold palladium and examined with either a JOEL T6400 or the Quanta 600F FEI scanning electron microscope.

The length of 200 unacetolysed and 100 acetolysed pollen grains, and the length and width of 100 processes from each taxon were measured using an AX70 Olympus compound light microscope (LM). The mean size of the pollen grains and processes were calculated for each species. ANOVA was used to determine any significant differences.

To measure the pollen grains and processes, the focus was on the outer exine of the grain so that its entire spherical shape could be seen. The center exine was not in focus. The processes and the cushions were not included when measuring the whole pollen grain. A single process at 3 or 9 o'clock that was entirely in focus and perpendicular to the exine edge was measured from the base of the process (not including the cushion) to the tip of the process (Plate 1, figure 2).

### 3. Results

*Gossypium barbadense* and *G. hirsutum* had the largest pollen grains (mean, 106.7 and 94.9  $\mu\text{m}$ , respectively) and *G. arboreum* and *G. herbaceum* had the smallest (mean, 84.8 and 80.4  $\mu\text{m}$ , respectively) (Table 1). Both *G. barbadense* and *G. hirsutum* contained pollen grains over 100  $\mu\text{m}$  in diameter. None of the pollen grains of *G. arboreum* or *G. herbaceum* ever measured over 99  $\mu\text{m}$  (Table 1). There was no significant difference between the sizes of the acetolysed and unacetolysed pollen grain diameters per taxon (ANOVA,  $p > 0.05$ ).

*Gossypium barbadense* (Plate 1, figure 2) and *G. hirsutum* had the longest and widest processes (spines) (means = 17.7  $\mu\text{m}$  long, 10.7  $\mu\text{m}$  wide and 15.2  $\mu\text{m}$  long and 12.1  $\mu\text{m}$  wide, respectively) (Table 2). *Gossypium herbaceum* had the shortest processes (9.4  $\mu\text{m}$ ) (Table 2).

The shape of the processes of *G. arboreum*, *G. herbaceum* and *G. hirsutum* were conical. However, the cushions (the base of the process) were different. *Gossypium arboreum* had small, smooth cushions,

Table 1. The mean, maximum and minimum length in  $\mu\text{m}$  of the pollen grains from the four economically important cotton (*Gossypium*) species, *G. arboreum*, *G. barbadense*, *G. herbaceum* and *G. hirsutum*.

Species	Mean	Maximum	Minimum
<i>Gossypium arboreum</i>	84.8	96	64
<i>Gossypium barbadense</i>	106.7	125	77
<i>Gossypium herbaceum</i>	80.4	99	64
<i>Gossypium hirsutum</i>	94.9	109	77

Table 2. The mean, maximum (Max.), and minimum (Min.) of the length and width in  $\mu\text{m}$  of the processes of the pollen grains from the four economically important cotton (*Gossypium*) species, *G. arboreum*, *G. barbadense*, *G. herbaceum* and *G. hirsutum*.

Species	LENGTH			WIDTH		
	Mean	Max.	Min.	Mean	Max.	Min.
<i>Gossypium arboreum</i>	10.6	14	9	9.2	12	7
<i>Gossypium barbadense</i>	17.7	22	14	10.7	14	9
<i>Gossypium herbaceum</i>	9.4	12	7	9.3	12	7
<i>Gossypium hirsutum</i>	15.2	19	12	12.1	19	9

*G. herbaceum* had textured cushions, and *G. hirsutum* had large and slightly textured cushions. The processes and cushions of *G. barbadense* were distinctive from the other three (Plate 1, figure 2). The processes were bottle-shaped, and the cushions were large and smooth.

#### 4. Discussion

The difference in the size of the pollen grains among the different species of *Gossypium* can be due to the differences in chromosome number. Both *G. barbadense* and *G. hirsutum* are polyploid with 52 chromosomes, while *G. arboreum* and *G. herbaceum* are diploid with 26 chromosomes (Saad 1960). The fact that pollen grains increase in size as the chromosome numbers increase is well known and documented in the literature (Christensen 1946; Gould 1957; Celt 1960; Nagl 1978; Recupero and Russo 1980; Small 1983; Jacob and Pierret 2000).

There was no significant difference in the acetolysed and unacetolysed pollen grain diameters per taxon (ANOVA,  $p > 0.05$ ). It is known that pollen grains mounted in glycerin jelly swell by 1.25–1.5 times their size (Faegri and Deuse 1960; Moore et al. 1991). Although the grains were measured within two weeks after they were put into glycerin, it is possible that since both the acetolysed and unacetolysed grains were

in glycerin any enlargement due to the glycerin was uniform for both processes.

Although the mean measurements for the four cotton taxa fall within the range of those measured by Saad (1960), the size range of the four taxa are much larger and the mean is smaller than those measured by the latter author. The measurements are also larger than those taxa measured by Christensen (1986) and Naggar (2003), and vary greatly from the measurements made by Chaudhuri (1965). However, our study agrees with Saad (1960) in that *G. barbadense* and *G. hirsutum* had the largest pollen grains and *G. herbaceum* and *G. arboreum* had the smallest.

These differences can be accounted for by several factors. First, the number of pollen grains measured influences the mean and the range of the pollen grains measured for each taxon. We measured 300 grains, 200 acetolysed and 100 unacetolysed for each taxon. Christensen (1986) measured 15 grains, Saad (1960) measured 50–100, Naggar (2003) measured 22–45 and Chaudhuri (1965) measured 10 pollen grains per taxon.

Second, treated pollen grains generally grow in size. Naggar (2003) measured untreated dry pollen. Saad (1960), Chaudhuri (1965), and Christensen (1986) all measured acetolysed grains. Christensen (1986) used silicon oil while Saad (1960), Chaudhuri (1965) and Naggar (2003) used glycerin jelly. The measurements in this study were from both dry (unacetolysed) and acetolysed grains in glycerin, and there were no significant differences between the unacetolysed and acetolysed measurements.

Third, the size of pollen grains is affected by chemical treatment, mounting media and whether or not the samples were from herbarium specimens or fresh flowers (Christensen 1946; Andersen 1960; Punt 1962; Reitsma 1969). Chaudhuri (1965), Christensen (1986) and Naggar (2003) all used fresh and herbarium specimens in their studies on Malvaceae but did not indicate which was used for *Gossypium*. Saad (1960) measured fresh *G. barbadense* but used herbarium specimens for the others. Flowers for this study were fresh from the field or greenhouses and were not from herbarium specimens but were dried for three days prior to measuring and processing.

Finally, the variation in pollen size can be due to a number of mechanical variables. These include instrumental error, optical interference, natural variations, distance measures that are not parallel to the plane of the eyepiece micrometer and small deviations in depth of field resulting in large deviations in measurements (McAndrews and Swanson 1967; Christensen 1986). In most cases these variations are almost impossible to prevent.

Although the process lengths in this paper are larger than Saad's (1960) measurements, they are in

agreement in that *G. barbadense* had the longest processes while *G. herbaceum* had the smallest. Chaudhuri (1965) found the processes of *G. barbadense* were smaller than the processes of *G. herbaceum* and larger than those of *G. hirsutum*. The process length depends entirely on where the process is measured and how easily the measurement can be made. The processes measured in this study were from the base of the process, not including the cushion, to the tip.

Since there was some overlap in the maximum and minimum sizes of the pollen grains among the different taxa, the processes and cushions may need to be examined. Although, the cushions can be seen with careful examination when using LM, they are more easily seen with scanning electron microscopy. Because of the difficulty in seeing the cushions with LM, they are not used in the key.

#### 4.1. Key to differentiate the four species of *Gossypium* using acetolysed pollen grains

1. Pollen grain diameter of 97  $\mu\text{m}$  or larger (excluding processes and cushions)

- (1) Processes (spines) longer than 19  $\mu\text{m}$   
*G. barbadense*.
- (2) Processes (spines) shorter than 18  $\mu\text{m}$   
*G. hirsutum*.

1. Pollen grain diameter smaller than 97  $\mu\text{m}$  (excluding processes and cushions)

- (1) Processes (spines) longer than 12  $\mu\text{m}$   
*G. arboreum*.
- (2) Processes (spines) 12  $\mu\text{m}$  or shorter  
*G. herbaceum*.

#### 5. Conclusions

Each of the four species of *Gossypium* was differentiated based on the pollen grain morphology. The ability to differentiate among the different species of cotton based on pollen morphology (size, processes, cushions, etc.) is important not only for the taxonomy of *Gossypium* but also so that cotton pollen found on insect pests can be identified to species level. Further research is needed to examine all species of *Gossypium* and Bt (*Bacillus thuringiensis*) cottons, to determine how well pollen morphology (size, process length and shape, cushions, etc.) can be used to differentiate the species within the entire genus. Proper identification of the cotton species can help in determining the origin of boll weevil re-infestation when different species of cotton are grown within a state or eradication zone.

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#### Author biographies



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HALI McCURRY graduated in 2006 from Memorial High School, Houston, TX. She received her BS in biology in 2010 from Texas A&M University, College Station, TX. Hali did an internship under Dr Gretchen D. Jones (USDA-ARS, APMRU) during the summer of 2009. She researched the differences in the morphology of cotton, *Gossypium* spp. pollen. Hali is currently a Department Supervisor at JC Penney, but maintains a strong interest in Biology. She also enjoys racing thoroughbred horses with her mother. She is pictured with her "friend" Notorious Delight that she raised from birth.

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