1837 Hardlock of Cotton: Historical Review and Perspectives

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Five years after the first report associating hardlock of cotton with *Fusarium* species, and the completion of Koch's postulates with *Fusarium verticillioides* as the hardlock causal agent, the hypothesis proposing that *Fusarium* infects bolls through cotton flowers is difficult to track. In this review, we evaluate our research progress during the last five years and future activities. At this point in time we can state that 1) the disease is associated with lower temperatures between midnight and 6 am, high plant density, insect damage, high nitrogen rates, and seed rot; 2) there is an evident relationship between weather conditions and disease incidence, which is dependent on the temperature fluctuation rather than humidity levels; 3) disease control and yield increase is achieved by fungicide application during bloom; 4) insecticides seem to help to control hardlock when used in combination with fungicides their efficacy (when applied separately) is still not clear; 5) insects, mostly thrips and bumblebees, contribute to inoculum transport and spread; 6) a thiophanate-methyl based fungicide is being used successfully in Florida and is in the process of being registered for the entire United States and; 7) stinkbugs have to be controlled or similar "hardlock" symptoms can be observed from their damage. Despite the difficulties with the etiology, disease management has been shown to be feasible. Future research goals are discussed.

Keywords: *Cotton, hardlock, Fusarium, fiber, lint, flower-infecting-fungi.*

Introduction

The cultivation of cotton in the Southeastern United States, particularly the coastal South and the Florida panhandle, has been compromised by the presence of hardlock/boll rot. Hardlock is a disease that affects the normal expansion of fibers as the boll opens. In other words, the fibers do not fluff out and are not harvestable by spindle pickers (Figure 1). When the spindles hit hardlocked bolls, the locules drop to the ground. The harvest of hardlocked bolls by other methods (strippers) proved equally ineffective due to lower quality lint and subsequent discounts. Hardlocked cotton strings out during mechanical harvesting and will give the appearance of unprofessional harvesting procedures. Gin trash and affected seed from hardlocked bolls are of concern for livestock feeding due to the possible presence of toxins. A University of Florida group pioneered the investigations concerning this cotton disease. Marois and collaborators established the basis for the investigations and initiated the process of determining the disease etiology (Marois et al., 2002). In the first phase of this work, bacteria and fungi were isolated from fibers, seeds, and peduncles of diseased bolls. It was clear from the beginning that the overwhelming organisms found associated with the disease were fungal, particularly from the genera *Fusarium, Cheatomium, Pestalotia, Cunninghamella, Nigrospora, Aspergillus,* and *Phoma*. *Fusarium* was immediately singled out as the most common fungus isolated from surface sterilized peduncles. Most of the isolates were identified as *Fusarium verticillioides*. The fact that *Fusarium verticillioides* cultures produce reddish secondary metabolites in culture corroborated the observation that most of the hardlocked fiber would exhibit a pink coloration when bolls begin to crack at maturity. The University of Florida group also has determined that the disease is associated with a) high nitrogen and plant density; b) high temperature and humidity; c) insect damage; and d) seed rot. As a consequence of this work, the disease was denominated simply as "Fusarium hardlock," possibly a subset of a larger hardlock complex.

The ultimate etiological evidence, which will demonstrate all stages of flower penetration and ovary colonization by *Fusarium,* is yet to be achieved. Studies carried out in South Carolina by a Clemson University group considered that the seed rot and hardlock were related and, as a consequence, have isolated a large number of bacteria after extensive sampling. No specific bacterial or fungal pathogen was identified (Jones, et al., 2000). It is our goal to monitor the infection of *Fusarium* inoculated cotton flowers and show the step-by-step colonization process. We have evidence that the interaction of *Fusarium* and cotton flowers has evolved in an analogous manner as other plant pathogenic fungi. Three major groups of flower-infecting fungi are known: a) group 1, opportunistic, unspecialized pathogens causing necrotic symptoms such as blossom blights; b) group 2, specialist flower pathogens that infect inflorescences either through the gynoecium; or c) group 3, which includes pathogens that systemically invade the apical meristem (Ngugi & Scherm, 2006). Our experience with hardlock and cotton flower development suggests that we are dealing with a plant parasitic fungus that belongs to group 2. Infection by *Fusarium verticillioides* does not seem to affect flowers in a way that complete host sterilization is reached, with the possible exception of flower abortion. Host range, mode of spore transmission, degree of host sterilization as a result of infection, and whether or not the fungus undergoes an obligate sexual cycle are some of the characteristics used to separate flower invaders. Complete host sterilization was not observed in cotton flowers associated with hardlock. Group 2 includes the diseases ergot of rye (caused by *Claviceps purpurea*) and corn smut (caused by *Ustilago maydis*). Fusarium head blight is currently the most known *Fusarium*-related disease (caused by *Fusarium graminearum (Giberella zeae)*), that requires flower infection. Our hypothesis is that *Fusarium* penetrates through the tender tissues of the stigma or style to reach the ovary or alternatively colonizes the germinating pollen and gains entry into the ovary. These possibilities will be discussed in the appropriate section of this review.

Our primary objective is to compile the literature about hardlock and introduce details about our investigations and/or hypotheses currently under evaluation. Our objectives are a) to verify the progress achieved towards the establishment of the hardlock etiology; b) to establish a foundation to separate "Fusarium hardlock" and "Boll rot" as two distinct disease complexes; c) to ascertain the progress toward disease forecasting; d) to examine the progress in disease control; and e) to determine the impact of hardlock on the farm economy.

Assuming that *Fusarium* is depleting nutrient/energy (Figure 2) from the seed during boll development, we could easily explain why fibers look aged (flattened) or deformed (bent) as shown in recent scanning electron microscopy micrographs (Figure 3). However, the most conspicuous difference observed between healthy fibers and hardlocked fibers was found on the epidermis and palisade layer cells. Signs of disruption and digestion were visible only on hardlocked cottonseed. In contrast, healthy fibers exhibit long and cylindrically shaped fibers. Some researchers tend to categorize hardlock as an abiotic disease caused by nutrient deficiency. The fact that the depletion of nutrients is, in this case, due to the infection by *Fusarium* would place hardlock under biotic diseases; we do not rule out the influence of other abiotic factors that may be favorable to hardlock.

Although chemical control of flower diseases is believed to be difficult due to the fact that some fungicides may damage flower structures such as stigmatic surface or anthers (Wetzstein, 1990; Weiguang et al., 2003; Bristow and Windom, 1987; Church and Williams, 1978), we have been successful in reducing hardlock and increasing yield through fungicide applications during bloom. At first, in 2001, the fungicide benomyl was applied to control hardlock and no significant yield increases were verified (Marois et al., 2002). However, the application of fungicides during distinct growth phases of the cotton crop was then initiated and looked promising. Fungicide applications during bloom and boll opening (Figure 4) showed some significant improvements in controlling hardlock and was clearly better when bloom applications were performed (Wright et al., 2003). In 2006 and 2007 the importance of fungicide application during bloom were confirmed (Leite et al., 2007); however the application of insecticides also showed promising results (Mailhot, 2007). Although fungicides and insecticides continued offering better yields and more hardlock control, the complete elimination of the problem was not attained. In addition, there was a continuous fluctuation of hardlock severity that was apparently due to weather conditions (Marois et al., 2005).

Pollen germination, yield, hardlock

Cotton yield is highly impacted by environmental stress–related conditions at the flower structures level (Figure 5). Apparently, the key to understanding the effect lies on mechanisms of pollen development, pollen germination, pollen tube growth, fertilization, and consequent boll development. The optimum germination range for cotton pollen is between 28 to 31°C and under field conditions, during summer, temperatures may reach as high as 39°C (Burke et al., 2004). This is so critical that the ability of pollen to germinate and to tolerate high temperatures is being suggested as a desirable characteristic for cotton genetic improvement. Higher pollen germination percentages and longer pollen tubes above 32°C indicate a tolerance to high temperatures (Kakani et al., 2005).

Progress in identifying the sensitivities of these developmental stress responses has been slowed down in part by the lack of a reliable method for germinating cotton (*Gossypium* spp.) pollen *in vitro*. A group in Texas is reporting that they are successfully germinating cotton pollen *in vitro* utilizing an agarose/sucrose-based medium amended with gibberellic acid (Burke et al, 2004), among others components. Classically, germination media for pollen would have only sucrose as the main component regulating osmosis. In general, the new medium increased germination and decreased rupturing of pollen. The best pollen germination was observed at a relative humidity of 80%, and ruptured pollen units were registered as the relative humidity increased to 100%. In Florida, minimum humidity usually occurs midday and generally exceeds 50%. On a typical summer day, relative humidity is between 50-70% but often approaches 90% by dawn (Black, 2003). Contrary to what we have predicted, our experience indicates that pollen germination does not require increased humidity. We have also noticed that pollen disruption increases whenever humidity is high. Our results suggest that we should avoid inoculation of flowers with liquid preparations (conidia suspensions). We are currently inoculating by rubbing conidia against the flower stigma with a cotton swab. Alternatively, we are trying inoculations using lyophilized conidia covered with a protective layer provided by skim milk, which maintains viability (Larena et al., 2003). Our goal is to evaluate percent hardlock after spray inoculating dried conidia onto cotton flower structures during bloom. Like Florida, cotton growing areas of China experience (Liu et al., 2006) unexpected periodic episodes of extreme heat stress usually in July and August, the peak time of cotton flowering and boll loading, which results in lower boll set and lint yield.

Hardlock and *Fusarium* species

Fusarium hardlock is attributed to *Fusarium verticillioides* (synonym, *F. moniliforme*; teleomorph, *Gibberella moniliformis*) *(*Marois & Wright, 2004). Recently, another species, *F. proliferatum* (Leite et al., 2007a), was identified based on comparing a sequenced internal transcribed spacer region of genomic DNA from *Fusarium* cultures isolated from infected bolls (Gardes and Bruns, 1993). The molecular identification of all isolates will continue and we will also include sequence data of the translation elongation factor-1 alpha gene, which is successfully being used to classify *Fusarium* species (Knutsen et al., 2004). *F. verticillioides* and *F. proliferatum* are known as the most common fungi associated with Fusarium ear rot, a corn disease (Foley, 1962; Munkvold, 2003). Our findings support the contention that Fusarium hardlock of cotton is also a result of the association of these fungi; whether other *Fusarium* species are involved remains to be ascertained.

Temperature, humidity and hardlock severity, developing a predictive system

At this point, we know that hardlock is associated with infection by *Fusarium* sp. Furthermore, hardlock severity is influenced by weather conditions that are optimal for conidia germination. In addition, for the same reasons, optimum temperature and humidity are necessary for pollen germination (Figure 6). There is a third component to this puzzle which consists of insect species that either are present in the flowers or visit the flowers. We can also assume that the life cycle of these insects is affected by weather conditions.

We have been monitoring weather conditions from first bloom to boll opening. Hardlock severity is assessed at harvest and weather information allows the evaluation of conditions that are conducive to the disease. Our direct approach was based on the comparison of temperature and relative humidity. A negative relationship between the average temperature between 0000 and 0600 h and hardlock severity in bolls resulting from flowers produced on that day was noted. The regression equation was performed as a predictive model. Calculating the predicted hardlock for each day from July 1 to September 1 created an estimated hardlock value for the season that was very similar to the observed severity at harvest. Curiously, relative humidity did not appear to be important (D. Mailhot, unpublished). The validity of having a predictive model for hardlock severity is critical as it may offer insights into controlling the disease. The fact that humidity was not shown to be significant may be just an indication that humidity, at least in Florida where the model was developed, is not critical as it may not vary as much during bloom. Humidity might be critical where humidity fluctuates considerably. The predictive model research will continue as we have try to perfect it and monitor hardlock in Florida. We currently designed an experiment that will evaluate the incidence of hardlock at three Florida locations: Jay, Marianna and Quincy. We will be using identical plot designs, varieties, and pesticide treatments. We predict that the differences observed will be attributed mainly to weather conditions and local insect populations.

Control of Hardlock

Path analysis (Marois et al., 2005) was used to help discern the interactions of the yield components. There was a negative correlation across the treatments between the severity of leaf disease and leaf area index ($r = -0.87$ and $p < 0.001$). There was a positive correlation between leaf area index and yield ($r = 0.51$ and $p < 0.006$), as well as a negative correlation between hardlock severity and yield ($r = -0.42$ and $p < 0.03$). Severity of leaf disease was negatively correlated with the number of bolls ($r = -0.49$ and $p < 0.008$)

and a positive correlation existed between leaf area index and the number of bolls ($r = 0.36$ and $p < 0.06$). Finally, there was a negative correlation between severity of leaf disease and yield ($r = -0.51$ and $p < 0.006$).

In the field trials, hardlock severity was never reduced to levels below 15-20%, although the severity fluctuated from year to year (Marois et al., 2006) This could indicate that 1) there is a limit to the efficacy of treatments that we are performing (Figure 7) or 2) there are some plant characteristics that are conducive to the disease, even when highly optimized pesticide applications are performed. If the second possibility is most important, future investigations will demonstrate that trial of different cotton varieties will result in distinct levels of control. For example, commercial varieties of rye less resistant to the flower infecting fungus, *Claviceps purpurea*, exhibit long flowering periods and therefore may be more frequently infected (McMullen and Stoltenow 2002). Cotton varieties with short and heavy flowering periods may decrease the hardlock severity, because the short and heavy flowering periods would reduce the window of opportunity for *Fusarium* conidia to infect. In addition, control measures would target this period only. However, cotton variety evaluations from variety trials have shown no difference in the amount of hardlock.

Perspectives

Fusarium hardlock is a challenging and complex disease. We have certainly made progress in disease management compared to the etiology work. We intend to dedicate considerable time and resources in future endeavors to 1) understand the extent of the *Fusarium* complex associated with Fusarium hardlock; 2) better characterize the weather conditions influencing disease establishment; 3) assess the importance of combined applications of fungicides and insecticides during bloom and the economic benefit of this strategy towards yield increase; 4) complete the etiology investigation by demonstrating the step-by-step colonization of cotton flowers by *Fusarium* isolates; and 5) verify whether we can advance the disease control beyond the base line of 15-20% hardlock severity limit, usually achieved in successful controls. For the first time since the program started, we will be utilizing an exclusion cage in the field. Control plots will be caged with a screen to avoid flower visitors during bloom. Preliminary results obtained in 2006 showed that bumblebees (*Bombus terrestris* L.) transport *Fusarium* conidia to the flowers while visiting (Leite et al, 2007b). We also expect to observe a reduction in the number of other insects, especially thrips, which are also known to transport *Fusarium* conidia (Marois et al., 2004; Mailhot, 2007). Our ultimate goal is to provide the growers with more tools for adequate and economically sound disease control.

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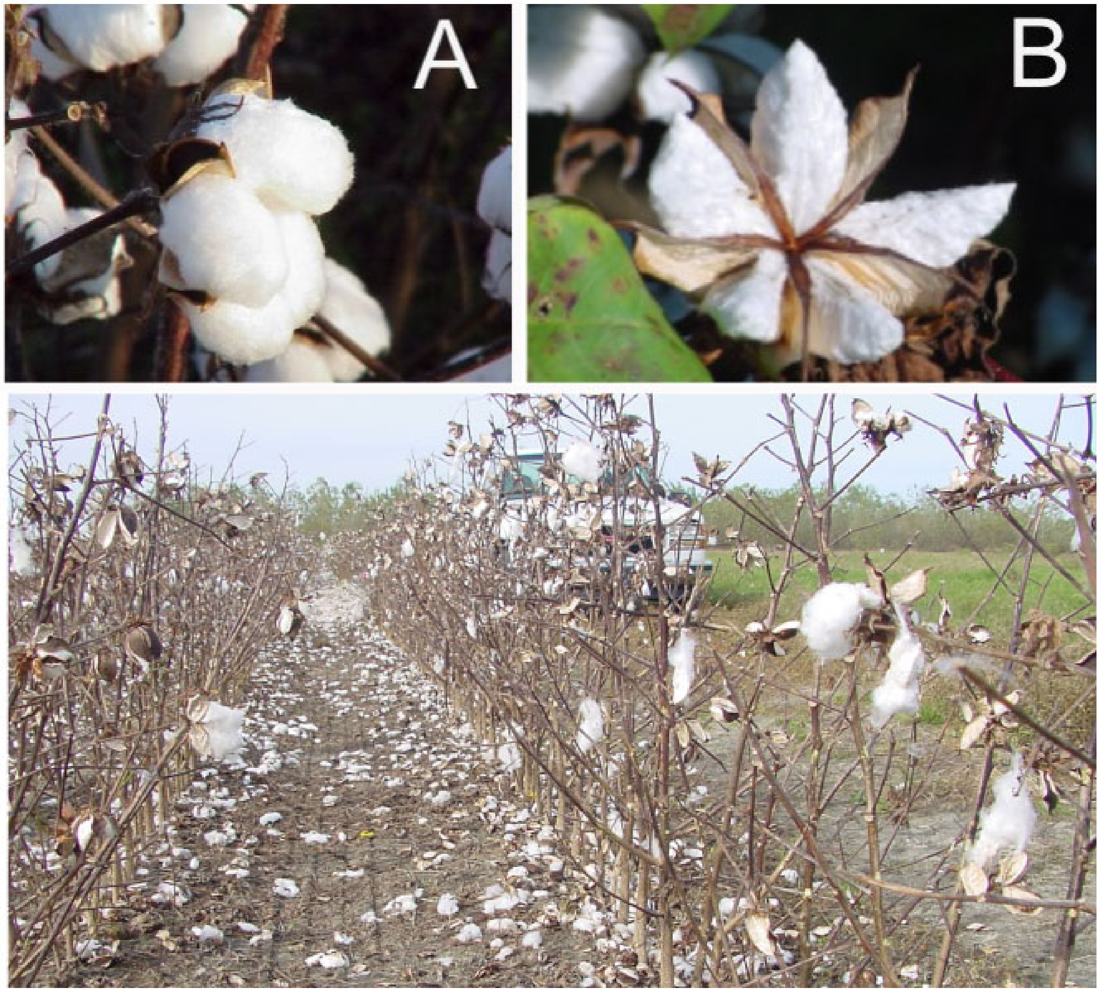

Figure 1 – Typical healthy looking harvestable cotton boll (A), and a hardlocked, mechanically not harvestable, cotton boll (B). Notice that a characteristic fusarium hardlock will exhibit failure to expand fibers from the very first day of boll opening. Frequently, bolls do not present any sort of discoloration at this point. Hardlock causes poor harvest practices because the cotton picker is not effective in collecting hardlocked bolls.

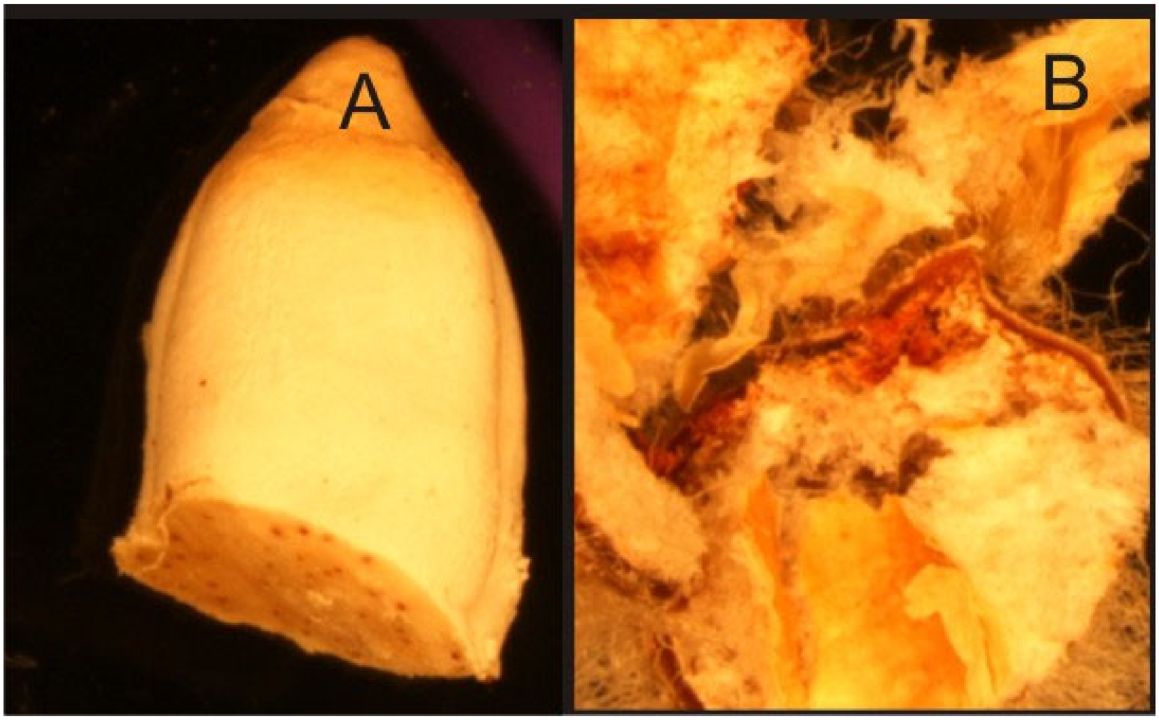

Figure 2 – Healthy (A) and hardlocked (B) cottonseed. Hardlocked cotton bolls frequently exhibit nearly empty cottonseeds, often called "hollow seeds," which are partially digested and have lost their anatomical integrity. The overall appearance suggests that the cottonseed was invaded by an active organism able to utilize seed reserves in order to survive and grow. In addition, tissues are highly compromised and the separation of cottonseed material from the cotton fibers is not possible.

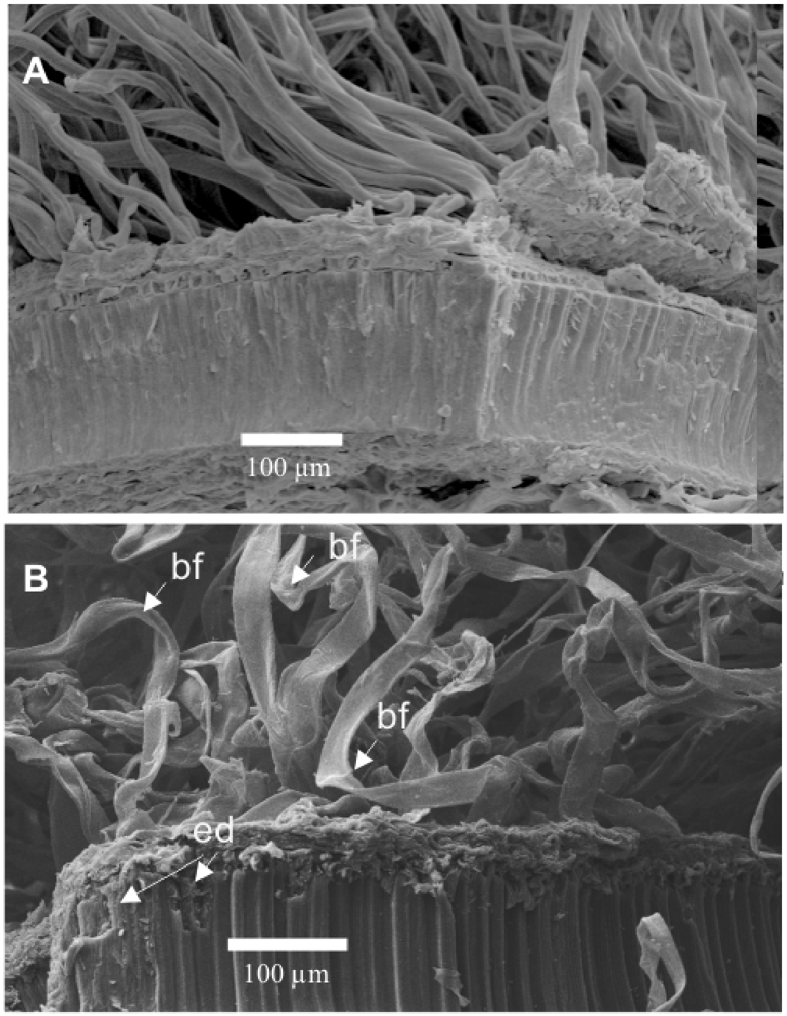

Figure 3 – Scanning electron microscopy of healthy (A) and hardlocked (B) cotton bolls. Healthy fibers are round in shape and the epidermis and integument (seed coat) are intact. In contrast, hardlocked fibers are bent (bf) and the epidermal and integument layers are eroded and digested (ed). Pictures credit: Eduardo Alves – University of Lavras, Brazil.

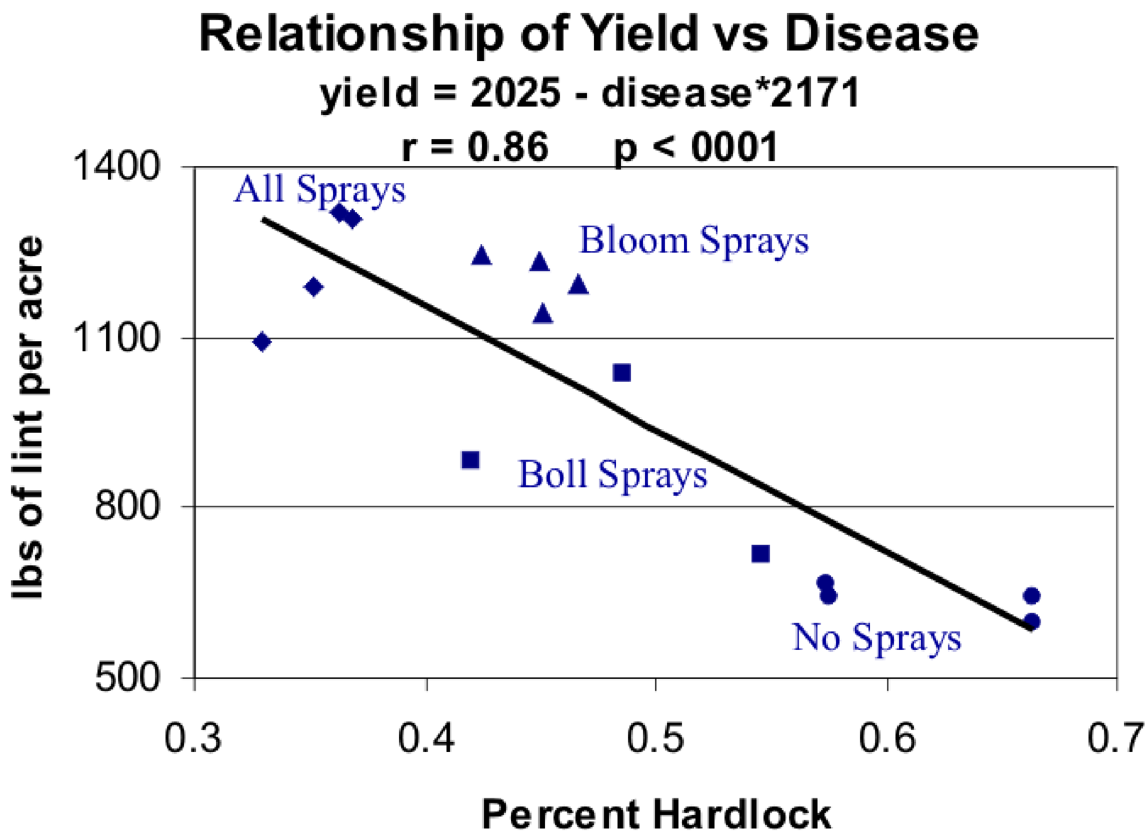

Figure 4 – Lint yield and incidence of hardlock. Since 2003, field experimentation showed that strategic fungicide applications during bloom presented the highest economical return (Marois and Wright, 2003). These results have been confirmed in subsequent years.

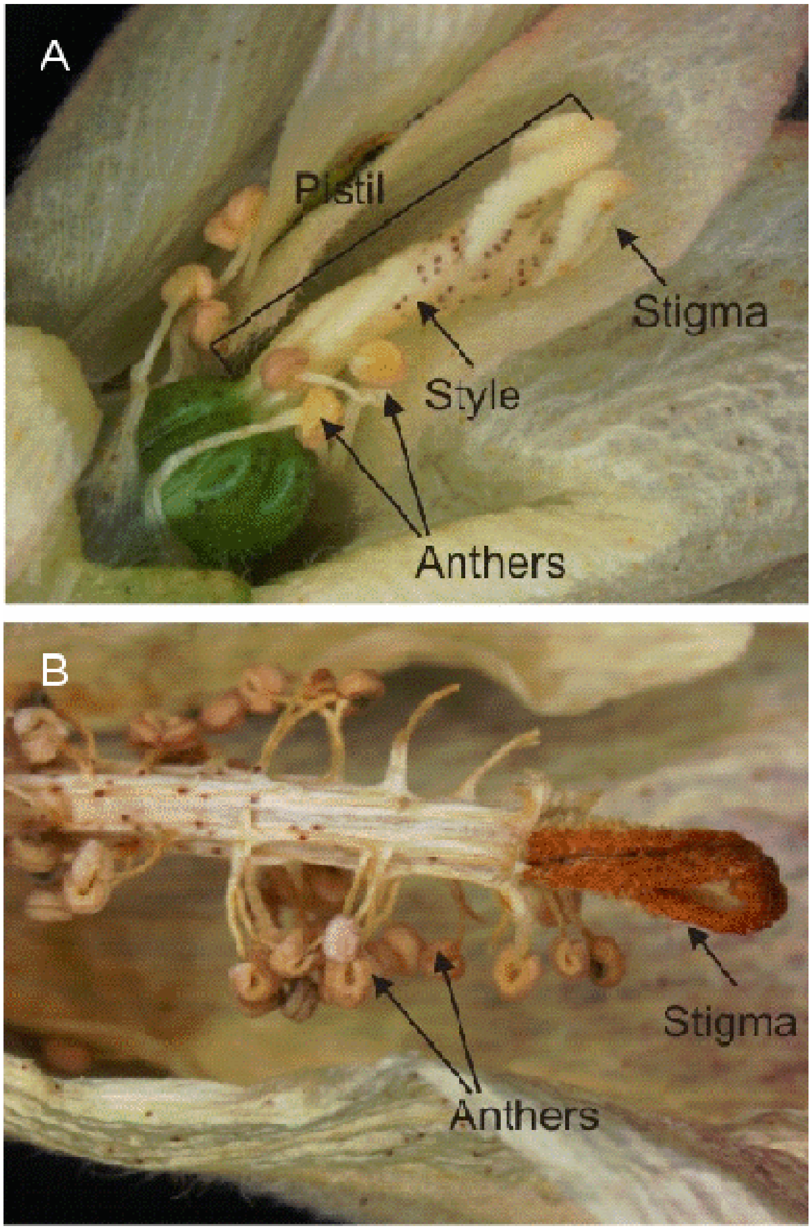

Figure 5 – Cotton flower anatomy prior to opening (A) and a three-day-old cotton flower (B) sexual structures. Notice that in early stages the style is not surrounded by anthers. That complete maturation occurs within a few hours and late afternoon of the first day the fertilization usually has occurred. Pollen germination is believed to be the pathway which allows the colonization of the ovary. On the left side (B), we can visualize the increase in pinkish pigmentation that takes place after fertilization.

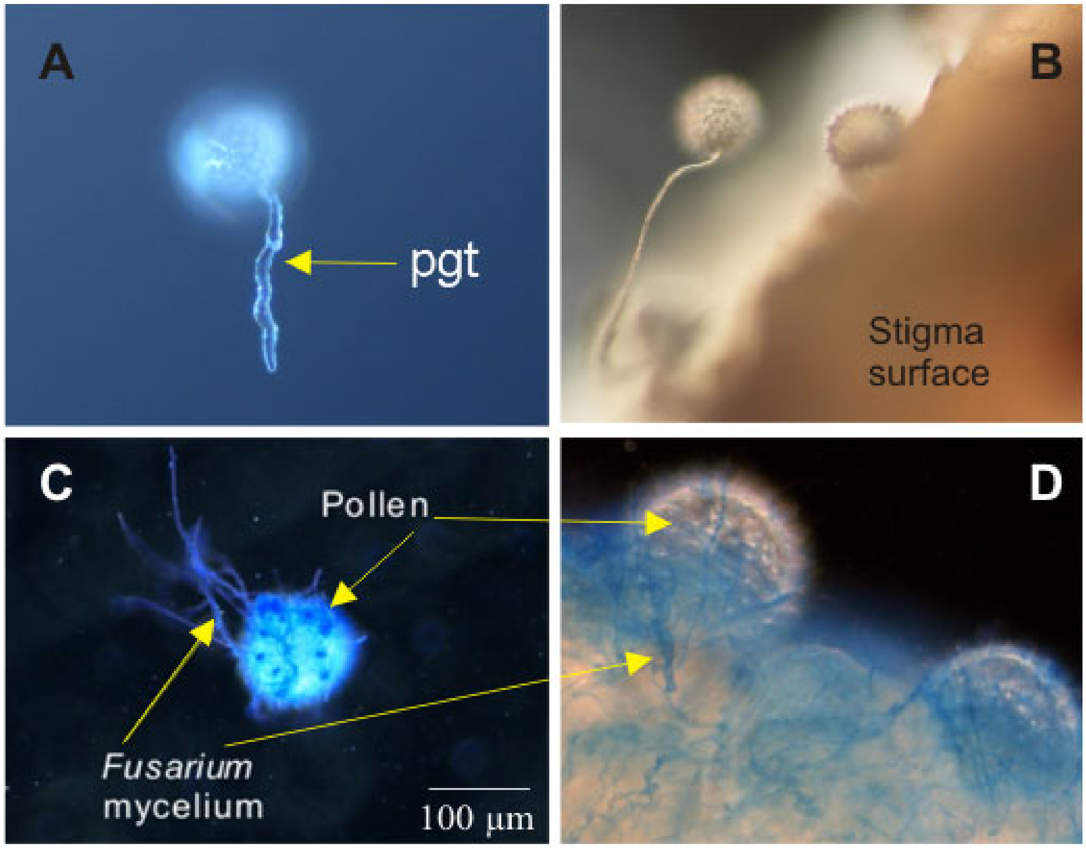

Figure 6 – Pollen germination on the surface of an artificial sucrose-based medium (A) and pollen germinating on the surface of a cotton flower stigma (B). The bottom pictures are showing pollen colonized by *Fusarium verticillioides* from inoculated flowers (C and D). At this point, we are not sure whether internal colonization of the pollen is also taking place.

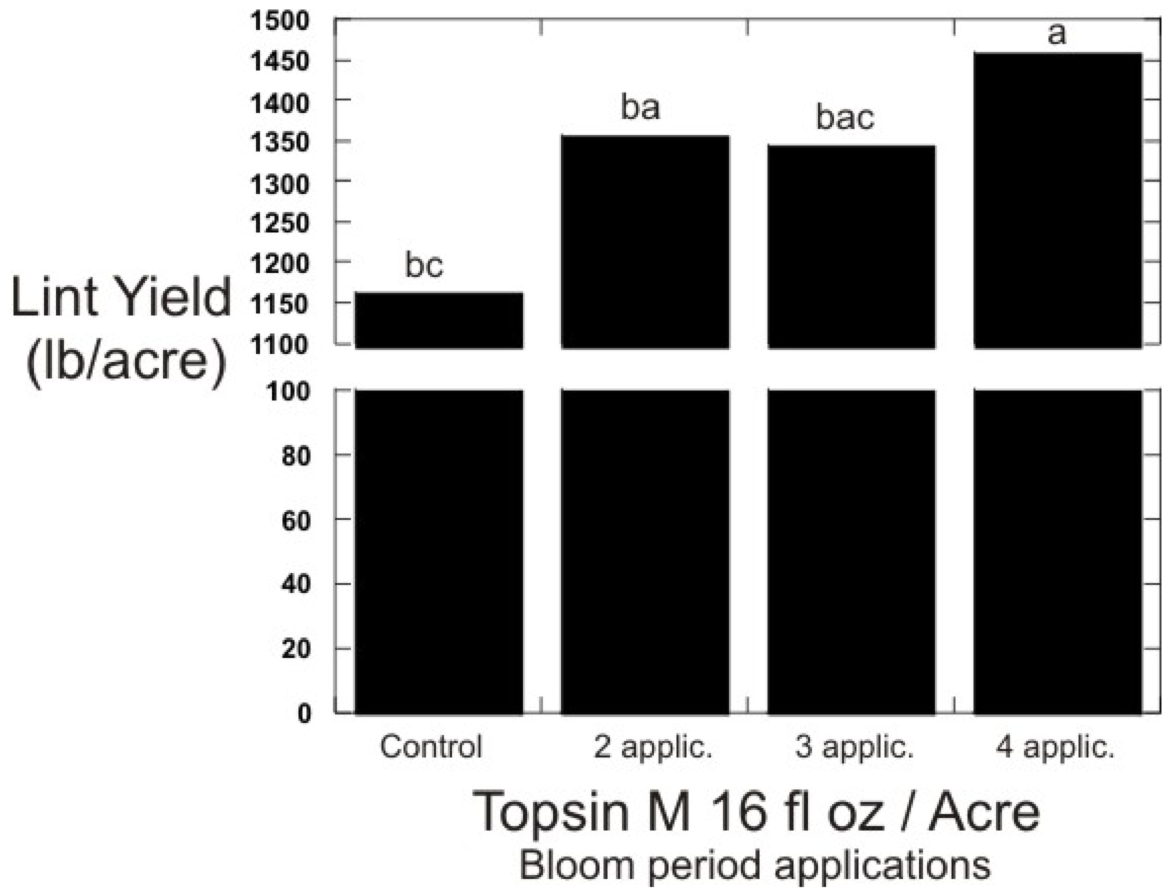

Figure 7 – Lint yield of cotton DP 555 treated with the fungicide Topsin M (Thiophanate-methyl) during the bloom period. Four weekly applications resulted in significant yield increase. The experiment was carried out at NFREC-IFAS Univ. of Florida, Quincy, FL. Four consecutive applications resulted in 25% yield increase as compared to the untreated control.