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Effect of the Easiflo Cottonseed Processing Method on Recovery of *Xanthomonas axonopodis* pv. *malvacearum*

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ABSTRACT

Laboratory studies were conducted to compare the effects of acid delinting and Easiflo treated cottonseed on the survival of *Xanthomonas axonopodis* pv. *malvacearum* (*Xam*), causal agent of bacterial blight of cotton. Symptomatic bolls of the susceptible cultivars Paymaster 2326 RR and/or All-Tex Xpress RR were sampled from plots artificially inoculated with *Xam*. In 2007, seed of Deltapine 164B2RF, were obtained from a field naturally infested with *Xam*. Seeds from each cultivar were either mechanically delinted and treated with the Easiflo coating or subjected to acid delinting and placed on potato carrot agar. Resulting yellow, mucoid colonies characteristic of *Xam* were tested for pathogenicity on susceptible cotton seedlings. In 2006, seed from artificially inoculated bolls, receiving the Easiflo treatment had a greater frequency of *Xam* (2.7%), than seed receiving the acid treatment (0%). No differences in *Xam* isolation frequency were observed between mechanically delinted seed treated with Easiflo and acid delinted seed, when bolls were naturally infested with *Xam*. Overall, neither method completely eradicated *Xam* from the seed, which may serve as initial inoculum in the development of bacterial blight in the field.

KEY WORDS: *Xanthomonas axonopodis* pv. *malvacearum*, angular leaf spot, bacterial blight, black arm, seed borne

INTRODUCTION

Bacterial blight, caused by *Xanthomonas axonopodis* pv. *malvacearum* (*Xam*) (synonym = *Xanthomonas campestris* pv. *malvacearum* (Smith) Dye) (Vauterin et al., 2000) is an important disease of cotton (*Gossypium hirsutum* L.) that causes economic damage throughout the world (Hillocks 1992). The bacterium is capable of infecting

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cotton at all stages of growth. Symptoms such as seedling blight, angular leaf spot (Figure 1), black arm (Figure 2), and boll rot (Figure 3) are associated with specific developmental stages (Verma et al., 1986). The disease was first reported in the United States in 1891 (Atkinson 1891). Bacterial blight losses in the United States are typically below 1% (Blasingame 2000). However, yield losses of 10 to 50% have been reported (Thaxton and El-Zik 2001; Verma 1986). Although sporadic, severe Bacterial blight epidemics have been observed on the Southern High Plains of Texas (Boman, personal observation).

Bacterial blight management is achieved through the use of resistant cultivars (Bayles and Verhalen 2007), and cultural methods such as sanitation and acid-delinting of seeds (Bain 1939). The initial development of resistant cultivars was slow due to the complex race structure of *Xam.* At present, 19 races of *Xam* have been identified (Ruano and Mohan 1982), with race 18 being most prevalent (Allen and West 1991; Hussian 1984; Thaxton et al., 2001; Verma and Singh 1975). *Gossypium. hirsustum* lines immune to bacterial blight have been identified (Bird 1960; Bird 1962); however, a large number of commercial cultivars currently being grown are susceptible to infection (Nichols et al., 2007; Sagaram et al., 2003; Thaxton et al., 2001; Wheeler et al., 2007).

The bacterium can survive in the field on debris from previously harvested crops. However, initial inoculum can also be seed borne (Mohan 1983). Viable propagules of *Xam* can be recovered from cottonseed for periods of more than two years when stored at 5^{o}C (Mehta et al., 2005). Studies have reported that seed infection rates as low as 2% can lead to destructive epidemics within a field (Brinkerhoff and Hunter 1963). The use of fungicides such as mancozeb and copper oxychloride were previously evaluated to aid in the elimination *Xam* from cottonseed. However, the results were inconclusive and cost prohibitive (Jeyachandran and Shanmugan 1979). Currently, seed companies in the U. S. acid delint gin run cottonseed to remove remaining lint on the seeds in preparation for planting. The process of acid delinting was first used for disease control in 1911 (Gregory et al., 1999).

Figure 1. Characteristic foliar symptoms of bacterial blight of cotton. Note appearance of angular lesions on the lower leaf surface.

Figure 2. Systemic infection of *Xanthomonas axonopodis* pv. *malvacearum* resulting in black arm symptoms on cotton.

Figure 3. Characteristic boll rot symptoms caused by *Xanthomonas axonopodis* pv. *malvacearum*. Note the water-soaked appearance of the boll.

Acid delinting is the process of exposing gin run cottonseed to a wet sulfuric acid (H_2SO_4), or a gaseous hydrochloric acid (HCl) solution (Cherry and Leffler 1984; Delouche 1986). This process facilitates the removal of low quality and less dense seed, and allows the seeds to be more uniformly coated with seed treatments. In addition, this process aids in the flowability of the seeds through air and vacuum planters, and is believed to kill most microorganisms found on the seed surface (Bain 1939). However, acid delinting uses large quantities of concentrated acid (Fink 1964) and is considered a source of environmental concern (Gregory et al., 1999). Concerns due to worker protection standards and hazardous waste disposal have resulted in an increased interest

in safer processing methods. One such system is the Easiflo cottonseed processing system.

Easiflo coated cottonseed was first designed as an alternative to fuzzy cottonseed feed for better handling, storage, and shipping characteristics (Laird et al., 1997). The Easiflo seed coating process is a combination of mechanical delinting and the addition of a polymer-based coating (Olivier 2005). Studies have reported that varying rates of corn starch have little to no effect on germination (Williams et al., 1999). Studies evaluating the field performance of Easiflo treated cottonseed have been conducted. Olivier (2005) determined that seedling emergence was reduced with the Easiflo system than with acid delinted seeds. However, final stands for the two treatments were similar (McMichael et al., 2004). Since the Easiflo seed processing system circumvents the use of acid, there is an increased potential for a resurgence of seed borne pathogens, such as *Xam*.

The objective of this research was to determine the survival of *Xam* on cottonseed treated with the Easiflo seed processing system compared to the more standard acid delinting procedure.

MATERIALS AND METHODS

Field Parameter. The glyphosate-tolerant cotton cultivars 'All-Tex Xpress RR' and 'Paymaster 2326 RR' were chosen because of their varying levels of genetic resistance to bacterial blight. All-Tex Xpress RR is considered a moderately susceptible cultivar; whereas, Paymaster 2336RR is highly susceptible (Sagaram et al., 2003). Field trials were conducted at the Texas AgriLife Research Station at Halfway, TX, in 2006 and 2007. The field had no history of bacterial blight in the previous three years. Plots were four rows wide by the length of the field (~0.2 ha per cultivar). Plots were sprayed with a pathogenic *Xam*, race 18 isolate at a rate of 1×10^6 colony forming units/ml (CFU). The organosilicone surfactant Silwet L77 (0.2% v:v, Helena Chemical Co., Collierville, TN) was added to the solution and 470 L of solution applied/ha (Wheeler et al., 2007). This procedure was developed for leaf symptom development, and was not optimized for boll infection. Applications of *Xam* were made on 10-July, 19-July, 4-Aug., and 18-Aug. in 2006. In 2007, applications were made on 20-July and 10-Aug. However, there was a problem with tank contamination on the first application resulting in lack of symptom development. To obtain infected seeds in 2007, bolls were injected with *Xam* at a concentration of 1×10^6, 1×10^7, and 1×10^8 CFU/mL. A syringe (Becton, Dickinson and Co. 1 mL Tuberculin Slip Tip, Franklin Lakes, NJ) was used to inject the bolls with 0.2 mL of each *Xam* concentration. Six hundred bolls were injected with the 1×10^7 concentration on 30-Aug., while 200 bolls were injected with the 1×10^6 and 1×10^8 on 5-Sept. Following the applications of *Xam* inoculum, bolls were tagged and subsequent selections were made on the basis of boll infection. In 2007, cotton lint and seeds were bulk harvested from a production field near Midkiff, TX, in which a natural bacterial blight epidemic was observed. The susceptible cultivar 'Deltapine 164 B2RF' was planted at this location. With 2007 being the only growing season that this cultivar was utilized, two separate seed lots (n = 1,000) were tested to determine the reliability of the results. Tagged bolls were hand harvested on November 18 and November 7 in 2006 and 2007, respectively. Boll samples were stored at ambient temperature in paper bags in the laboratory prior to processing on a 10-saw laboratory gin to obtain the fuzzy seeds. Seeds from the multiple sampling dates were combined in order to have enough seed to

administer each treatment. Fuzzy seeds for each cultivar were divided into two separate portions (approximately 454 g), and were acid delinted, or mechanically delinted followed by the application of Easiflo.

Seed Preparation. The acid delinted seeds were processed at the Bayer CropScience laboratory in Idalou, TX. The process involved placing the fuzzy, gin-run cottonseed in acid-resistant mesh bags, and then coating the seeds with a dilute 19% H_2SO_4 solution. The wetted seeds were then placed in a tumbling dryer for approximately 40 min at a temperature of 60°C. The delinted seeds were exposed to sodium carbonate (Na_2CO_3) to neutralize the remaining H_2SO_4 on the seed coat. Seeds were placed back into the tumbling dryer and dried at 49°C until the seeds obtained a suitable moisture level (5-10%).

The Easiflo treatment on the cottonseed was applied at the Seed Physiology Laboratory at Texas Tech University in Lubbock, TX. The fuzzy, gin-run cottonseed were placed in a proprietary mechanical delinter (Cotton Incorporated, Cary, NC) until >90% of the fuzz was removed from the body of the seeds. For the Easiflo process to be efficient, it is desirable to have the only remaining fuzz on the micropilar and chalazal caps of the cottonseed. After the seeds were mechanically delinted, the seeds were weighed and a talc, corn starch, and water mixture (2.0%, 0.5%, and 5.0% by weight, respectively) was applied to the seed using a modified Hege seed treater. Seeds were dried for approximately five minutes using a forced air blower maintained at 40°C.

Bacterial Isolation. To test individual seeds for *Xam* infection, each seed was placed on potato carrot dextrose agar with peptone and yeast extract (PCA) composed of $MgSO_4$ (0.3 g), $CaCO_3$ (0.2 g), technical grade agar (10.0 g), commercial grade potato dextrose agar (40.0 g), peptone (2.5 g), commercially canned carrot juice (15 mL), and yeast extract (0.5 g) per liter of distilled water. Bacterial colonies from individual seeds (900 seeds/treatment in 2006 and 1,000 seeds/treatment in 2007) were streak plated onto a fresh PCA plate. Bacterial colonies from the streaked plates were serial diluted by placing a loop of bacteria into a vial filled with 10 mL sterile distilled water. The serial dilution process resulted in a 1×10^{-7} stock solution. A 0.1 mL aliquot of the stock solution was pipetted onto a PCA plate, spread over the plate using a sterilized glass rod and incubated at room temperature on a laboratory bench for 48 to 72 hours. Transfers from pure colonies were placed in a vial of permanent, freezable media consisting of tryptone (10 g), yeast extract (5 g), NaCl (0.5 g), K_2HPO_4 (6.3 g), KH_2PO_4 (1.8 g), sodium citrate (0.45 g), $MgSO_4\cdot7H_2O$ (0.09 g), $(NH_4)_2SO_4$ (0.09 g), glycerol (50.6 g) per liter of distilled water. Each vial was placed on a wrist action shaker (Burrell Scientific, Pittsburg, PA). Sub-samples of the bacterial isolates were streaked onto additional PCA plates for confirmation, and vials were frozen for storage at -18°C.

Pathogenicity Tests. Pathogenicity tests were conducted using the yellow, mucoid bacterial colonies characteristic of *Xam* (Bradbury 1986) recovered from field infested or inoculated seed. Cotton seedlings (Paymaster 2326RR) grown in flats and placed in a growth chamber (Percival Scientific Inc. Model AR-75L, Perry, IA) maintained at 27 ± 2°C. Following cotyledon emergence, the plants were removed from the growth chamber and divided into three replicates for each sample. This process was repeated for each location from both growing seasons. An autoclaved toothpick was used to scratch each cotyledon with a sample of each bacterial isolate. Following inoculation, the plants were

placed in a dew chamber (Percival Scientific Inc. Model I-35D2L, Perry, IA) maintained at 24°C and >90% RH for 24 hours and then transferred back into the growth chamber. After a two-week incubation period, plants were rated on the basis of symptom development using a qualitative scale, where a positive reaction was determined if angular, water-soaked lesions had developed away from the inoculation point; whereas, a negative reaction was characterized by discoloration of the inoculation point. All plant material was discarded after being rated.

Data Analysis. The experimental design was a binomial test consisting of an infected or non-infected proportion. Data were analyzed using a chi-squared analysis in the Mixed Procedure of SAS (v.9.1, SAS Institute, Inc., Cary, NC). A test of proportions ($\alpha = 0.05$) was utilized to test the infected proportion versus the non-infected proportion of a cultivar. Specifically, a proportions test performs two hypothesis tests of the difference between two binomial proportions. The output includes two types of hypothesis tests, one based on a normal approximation; whereas, the other is Fisher's exact test.

RESULTS AND DISCUSSION

Significant differences ($P \leq 0.05$) were observed between the acid and Easiflo treatments for Paymaster 2326RR in 2006 for all bacterial isolates obtained from yellow, mucoid colonies that were associated with the seeds, and for pathogenic *Xam* isolates (Table 1). Acid delinting eliminated all yellow, mucoid colonies from the seeds tested; whereas, colonies were recovered from 3.0% of the seed treated with Easiflo. The isolation frequency of *Xam* recovered from seed, as exhibited by a positive reaction in the pathogenicity test, was 2.7% (Table 1). In 2007, the isolation frequency of bacteria for the two treatments was similar for Paymaster 2326RR. However, a greater number (2.4%) of yellow, mucoid colonies were recovered from acid delinted seed when compared to Easiflo for All-Tex Xpress RR (Table 1). Likewise, there was a 2.0% increase in the number of *Xam* isolates identified from the pathogenicity test for acid delinted seed than Easiflo treated seed (Table 1). The bacteria were injected directly into the bolls for this set of seed, which may have bypassed the partial resistance exhibited by All-Tex Xpress RR to the disease (Sagaram et al., 2003). It is possible that the distribution of the bacteria was different within the seed for the two inoculation techniques. The leaf spray may have resulted in more of an external infestation of seed and the syringe inoculation may have caused more internal infection of seed. No differences in isolation frequency were observed between treatments for either of the Deltapine 164 B2RF seed lots obtained from the natural epidemic from 2007 (Table 1). The overall seed infection was low for the natural epidemic, where bolls were collected without regards to Bacterial blight symptoms.

Although sporadic in nature, severe bacterial blight epidemics can occur. In 2011, widespread occurrences of the disease were reported in Arkansas (Rothrock et al., 2012) and Mississippi (Allen 2012). Spray inoculations of *Xam* inoculum are effective at inciting temporary leaf symptoms and low percentages of boll rot (Wheeler et al., 2007). Adequate disease development was observed following spray inoculations in 2006. However, spray applications were unsuccessful in initiating disease in 2007 due to a chemical contamination in the spray tank. Therefore, bolls were artificially infected by injecting *Xam* concentrations with a hypodermic syringe. Overall, injecting bolls with *Xam* was detrimental to boll retention with 58.3 to 72.0% of the bolls abscising (data not

presented). As a result, inoculation rates were combined to provide a sufficient amount of seeds with which to administer treatments. Populations of *Xam* were generally greater when bolls were injected compared to spray inoculations. There is evidence that *Xam* can reside on the seed surface, or below the seed coat (Bain 1939; Brinkerhoff and Hunter 1963). Furthermore, acid delinting is capable of decreasing *Xam* on the seed surface; however, the overall effect on internal infections is unclear. It appears that internal seed infections resulted from the boll injections, as neither treatment with acid nor Easiflo affected the isolation frequency. In contrast, acid delinting had a significant effect on *Xam* isolation frequency from bolls collected from plots receiving spray inoculation. Although no differences between the treatments were observed in either of the seed lots from the natural epidemic, *Xam* populations were consistently greater for the Easiflo treated seed. With such low infection frequencies, it may have been necessary to isolate from more seed to obtain significant differences.

Based on this study, Easiflo treated seed does not appear to differ from acid delinting in negating the transmission of *Xam* in infested planting seed. The data from 2006 show that in a year with relatively unfavorable environmental conditions for disease development, acid delinting can reduce the likelihood of disease development (Table 1). This is due to the fact that the poor disease development conditions did not lead to a systemic infection that would infect the seed internally. Therefore, the pathogen affected the seed externally, and was removable with the use of acid delinting, but not the Easiflo process.

The data collected in 2007 shows drastic differences from those seen in 2006 (Table 1). This may be attributed to above average precipitation during the growing season in 2007. Observations from the West Texas Mesonet Plainview Station (www.mesonet.ttu.edu/mesonet-precipitation.htm) show that rainfall received during 2006 was similar to the 10-year average; whereas, rainfall amounts in 2007 were 48% above the long-term average. The acid delinted portion of all three of the cultivars tested resulted in greater infection rates than those experienced in 2006, and in one case had a greater infection rate than the Easiflo treated seed of the same cultivar (All-Tex Xpress RR). With that said, the same conclusion concerning the Easiflo system can be made in that it does not eliminate transmission of *Xam* on infested cottonseed. While both treatments experienced greater infection rates in 2007, the results exhibited by the seed treatments in 2006 are evidence of the ability of the acid delinting process to remove external infestations while the Easiflo system was not able to remove external *Xam* either year, or probable internal infection that was encountered in 2007. Also, acid delinting did not appear to impact internal *Xam* infections, as the bacterium was likely protected within the seed.

Table 1. Effect of two cottonseed processing methods on isolation frequency of *Xanthomonas axonopodis* pv. *malvacearum* (*Xam*) from seed artificially or naturally infested in 2006 and 2007.

Year, cultivar	Yellow, mucoid colonies			Positive pathogenicity test (*Xam*)		
	Acid delinted	Easiflo treated	*P*-value	Acid delinted	Easiflo treated	*P*-value
	- - - - - - (%) - - - - - -			- - - - - - (%) - - - - - -		
2006[a] Paymaster 2326 RR	0.0	3.0	<0.0001	0.0	2.7	<0.0001
2007[b] Paymaster 2326 RR	2.2	1.8	NS	1.8	1.1	NS
2007 All-Tex Xpress RR	3.8	1.4	0.001	3.1	1.1	0.003
2007 Deltapine 164 B2RF (Lot 1)[c]	0.7	1.4	NS	0.6	0.9	NS
2007 Deltapine 164 B2RF (Lot 2) [c]	0.5	1.2	NS	0.4	1.0	NS

[a] A total of 900 seeds were tested for each treatment. Seeds were obtained from artificially infecting plants via foliar applications of *Xam* inoculum as described by Wheeler et al. (2007).
[b] A total of 1,000 seeds were tested for each treatment. Seeds were obtained from bolls injected with *Xam* concentrations.
[c] Two separate seed lots of Deltapine were tested from a naturally infested field to determine reproducibility.

CONCLUSIONS

Evaluations from these studies indicate that bacterial blight development in bolls differs greatly by the type of inoculation method used. While severe bacterial blight symptoms can be observed following natural infections, the sporadic nature of the disease often requires artificial inoculation to ensure disease development. Spray inoculation with *Xam* suspensions were effective at inciting bacterial blight on leaves, but have poor penetration within bolls. Furthermore, the sensitivity of *Xam* to tank contaminants experienced in this study necessitated the need for an additional inoculation method. Injections of *Xam* should result in more severe boll rot, as the bacterial inoculum is introduced directly to the developing boll. In these studies, injections with concentrations of *Xam* did result in adequate levels of boll rot development; however, the injections themselves reduced boll retention. Additional research in the different inoculation methods is warranted, to better understand the mechanism of boll infection by *Xam.*

Overall, results from the comparisons made between acid delinting and the Easiflo seed coating system were inconclusive. The use of Easiflo did not appear to differ from the acid delinting process as it relates to reducing *Xam* populations on infested seed. The initial low populations of the bacterium on seed from 2006 were completely removed via acid delinting. The use of the Easiflo system did not reduce *Xam* populations below levels that could result in field epidemics. When internal seed infection occurred under favorable environmental conditions, and/or boll injections with *Xam*, as was the case in 2007, neither acid delinting, nor treatment with Easiflo had any effect on isolation frequency of *Xam* from Paymaster 2326RR. Contradictory to what was observed in 2006 for Paymaster 2326RR, the isolation frequency of *Xam* from All-Tex Xpress RR was greater for acid delinted seed than for Easiflo-treated seed. Differences in reaction to the treatments between Paymaster 2326RR and All-Tex Xpress RR cultivars during the 2007 trial could be attributed to varying response between the cultivars to injections. This could potentially be explained by differences in boll size, carpel wall thickness, or sensitivity to breach in the carpel wall of the two cultivars. These factors could influence *Xam* development independently, thus differences in boll infection may have been observed. Although no observable differences between treatments for the two naturally infected Deltapine 164 B2RF seed lots were observed, isolation frequencies of *Xam* were numerically lower from seed treated with acid. Additional studies utilizing naturally infected seed with a larger number of observations need to be conducted.

Further research is warranted to better understand the effect of Easiflo on cottonseed infected with *Xam*. Another area of interest that should be examined is the possible contamination of Easiflo seed coating equipment by *Xam* infected seed during the Easiflo coating process. Additional studies are also necessary to examine the impact of Easiflo on other seed transmitted pathogens.

The current logic that acid delinting completely removes *Xam* from infected cottonseed appears to only apply to infestations of the seed coat by the bacterium. The development of an assay to test internal portions of the seed, such as the embryo, is needed to determine the extent of *Xam* infections. Strategies to minimize the spread of *Xam* must include sanitation and the use of high quality pathogen-free seed. Fields known to be infested with *Xam*, or exhibiting symptoms of bacterial blight should not be harvested for seed, nor should gin trash from such fields be land applied for disposal.

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