1835 Transmission and Importance of *Pantoea ananatis* During Feeding on Cotton Buds (*Gossypium hirsutum* L.) by Cotton Fleahoppers (*Pseudatomoscelis seriatus* Reuter)

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ABSTRACT

Cotton fleahoppers collected from various hosts in the field or raised on green beans in the laboratory were found to be readily infested with *Pantoea* spp. These isolates caused a severe internal boll rot when introduced into developing fruit via a small puncture wound inflicted using a 28-guage needle. Buds or bolls that abscised following fleehopper feeding consistently showed ovary rot, including the wall, and contained concentrations of 10^8 to 10^{10}cfu/g of *Pantoea*spp., indicating that bacterial rot of the ovary contributed to abscission. A collection of *Pantoea*isolates from both insects and diseased buds were analyzed using API 20E test strips, fatty acid methyl ester profile analysis and base sequences of 16S ribosomal DNA. The polyphasic analysis showed that most isolates were best classified as *Pantoea ananatis*. Fleahoppers fed on beans contaminated with rifampicin-resistant variants of either *P. ananatis* or *P. agglomerans* acquired and transmitted *P. ananatis* but not *P. agglomerans*. These data indicated that *P. ananatis* has developed an endophytic relationship with the cotton fleahopper that resulted in the presence of this bacterium in most of these insects and that transmission of bacteria during feeding on cotton buds was common.

KEYWORDS Bud abscission, cotton, cotton fleahopper, *Gossypium hirsutum*, *Pantoea agglomerans*, *Pantoea ananatis*, bacterial vector, feeding

INTRODUCTION

Cotton fleahoppers (*Pseudatomoscelis seriatus* Reuter) and other bugs that have piercing-sucking mouthparts have been or have become major pests of cotton. In 2004, these pests were ranked among the top five insect causes of cotton yield losses: Lygus bug, #2; stink bugs, #3; and cotton fleahopper, #5 (Williams, 2005). In 1999, the cotton fleahopper was ranked as the top cause of cotton yield losses, primarily because of severe losses in Texas in that year (Williams, 2000).

While cotton fleahoppers occur throughout the Cotton Belt, losses typically occur mostly in Texas followed by Oklahoma, Louisiana, Kansas, and Arizona. This is probably due to its distribution and the fact that the insects generally prefer weed hosts and move to cotton only when favored weed hosts are not available (Beerwinkle and Marshall, 1999). In Central Texas, the insect overwinters as diapausing eggs inserted into stems of croton (*Croton capitatus* Michx.), its preferred fall host. When nymphs hatch in the spring, they move to early season weeds such as horsemint (*Monarda punctata* L.). Later generations move to cotton that is starting to square in late May and early June when the horsemint and other spring weeds begin to senesce. When cotton starts to mature, fleahoppers move back to croton for late summer and fall generations (Beerwinkle and Marshall, 1999).

Several observations indicate that fleahoppers transmit pathogenic microorganisms to flower buds, and that subsequent infections cause ethylene bursts resulting in abscission of buds and young bolls. Multiple reports discuss that cotton fleahoppers are infested with various fungi and with bacteria putatively identified as *Xanthomonas* and *Pseudomonas* spp. (Duffey and Powell, 1979; Grisham et al., 1987; Martin et al., 1987), unfortunately criteria for the identification of the bacteria were not provided. The microorganisms were isolated from salivary glands as well as whole insects. When fleahoppers were fed 5% sucrose containing *Xanthomonas campestris* pv. *malvacearum*, they subsequently transmitted the bacteria into cotton plants causing disease symptoms on leaves and stems (Martin et al., 1988c). Terminal bud explants of cotton planted in agar in 25-ml flasks showed a burst in ethylene production when infested with fleahoppers, or when inoculated with microorganisms associated with the insect (Duffey and Powell, 1979; Grisham et al., 1987; Martin et al., 1988a). Pectinase preparations from salivary glands also caused a burst in ethylene synthesis (Martin et al., 1988b). Ethylene bursts are symptomatic of tissue necrosis incited by microbial infections of plant tissues (Hilsop et al., 1973; Pegg, 1976).

Bell et al. (2006) further characterized the microorganisms associated with cotton fleahoppers and provided evidence for their involvement in leaf and flower bud abscission. The insects were collected from two weed hosts, horsemint (*Monarda punctata* L.) and croton (*Croton capitatus* Michx.), and cotton (*Gossypium hirsutum* L.) at seven intervals during the 2005 cotton growing season in Texas. Most fleahoppers yielded an infective dose of bacteria opportunists, when washed in sterile water, to cause severe seed rot and internal boll rot of 13- to 15-day-old cotton bolls puncture inoculated with the wash water. Most bacterial isolates, especially those from fleahopppers collected from croton the host plant on which they overwinter, were *Pantoea spp.* Cotton fleahoppers raised on green beans in the laboratory, starting with eggs embedded in croton stems, also were frequently infested with *Pantoea* species. The insects transmitted these *Pantoea* species to leaf and flower buds and young bolls when the insects were caged over fruiting branches. Buds or bolls that abscised consistently showed rotting of the ovary, including the wall, whereas buds or bolls that were retained on the plant had healthy ovaries, indicating that bacterial rot of the ovary contributes to abscission. Initial efforts to identify the *Pantoea* isolates to species were inconclusive.

Pantoea species have been distinguished by numerical taxonomy using API systems (Mergaert et al., 1984), fatty acid profiles (Mergaert et al., 1993; Kado, 2006) and 16S rDNA analyses (Kwon et al.,1997; Mergaert et al., 1999). In this study we used all of these techniques to characterize *Pantoea* isolates from laboratory reared insects and diseased buds resulting from feeding of these insects. In addition, we determined whether rifampicin resistant derivative strains of *P*. *ananatis* and *P. agglomerans* could be taken up by fleahoppers during feeding on beans contaminated with the bacteria and later be transmitted to cotton buds over which the insect was caged. Finally, we discuss the probability that a strain of *P. ananatis* is a natural endophyte of the cotton fleahopper.

MATERIALS AND METHODS

Bacterial Transmission and Damage from Laboratory-Reared Insects

Fleahoppers were reared in the laboratory using methods similar to those described by Beerwinkle and Marshall (1999). Newly emerged adults were caged over fruiting branches using a Styrofoam cylinder enclosed with a section of nylon mesh hose fitted over the cylinder and fruiting branch stem and tied at both ends after 3 unsexed fleahoppers were placed in the cage. After 7-9 days, the insects were removed from the cages. Buds were

sectioned longitudinally with a razor blade and examined for tissue necrosis in the anthers, stigma, and ovary (including the wall). Both insects and sectioned tissues from individual cages were tested separately for the presence of seed-rotting bacteria.

Infestation with Marked *Pantoea* spp.

Newly emerged adults reared in the laboratory were fed fresh green beans (*Phaseolus vulgaris* L.) that had been washed in a sodium bicarbonate solution and then dipped either in suspensions (ca. 2 x 10^7 cfu/ml) of a rifampicin resistant strain of *P. agglomerans* (Sc 1-R), or were fed fresh or sterilized (121ºC, 15 min, 1 kg/cm^2) green beans dipped in a suspension of *P. ananatis* (7-1R; a natural variant from a cotton fleahopper) in 50, 100 or 200 ppm rifampicin in water and placed in Petri dishes. After 4 days and 48 hrs, respectively, the insects were transferred to fresh beans without pathogenic bacterial contamination and after an additional 24 hrs they were caged over cotton squares as described previously.

Isolation of Bacteria from Insects and Buds or Bolls

Each insect was placed in 2 ml sterile distilled water and agitated periodically with a vortex mixer. After 1, 24, and 48 hr, a 10 µl loop full of wash water was streaked on Trypticase Soy Agar (TSA) amended with 100 ppm cycloheximide to suppress fungal growth. The media was amended with 200 ppm rifampicin to detect the rifampicin resistant strains. Two or three seeds from diseased locks or longitudinally sectioned buds and small bolls were soaked in sterile distilled water for 1-2 hr with intermittent agitation. A 10 µl sample was streaked on the media. Isolated bacterial colonies of different types and from separate insects or bolls/buds were both used for identification and to test for pathogenicity and virulence. An emphasis was given to the most prevalent bacteria from a sample.

Inoculation of Bolls with Wash Water or Bacteria

Wash water from each insect was used to test for the presence of pathogens. Fibermax 958 and Deltapine 493 plants were grown in the greenhouse under a rigid insect pest control regime. Opened flowers were tagged daily so that bolls of a specific age could be inoculated. A 40 µl drop of wash water from an insect was placed over the suture of a 13- to 15- day-old boll midway between the apex and base. A 28-gauge needle was placed through the drop and then 2-3 mm into the boll passing through the suture. Bolls were sectioned longitudinally through the suture at 7 or more days after inoculation to observe disease progress, or bolls were allowed to mature and open, and then final symptoms were observed. Suspensions of bacteria in sterile water were also inoculated into bolls to confirm pathogenicity.

Characterization of Bacteria

Remote colonies of bacteria that were purified, inoculated, and then recovered from diseased bolls were used for characterization and/or identification. Colony morphology was observed on TSA, King's B-pectin agar (KBP), and potato dextrose agar containing 0.8 g/L of fine $CaCO_3$ (PDAC). Anaerobic growth was determined on a medium that consisted of peptone, 2.0 g; NaCl, 5.0 g; KH_2PO_4, 0.3 g; agar, 3.0 g; bromothymol blue (1% aqueous solution), 3.0 ml; glucose, 1.0 g; per liter of water. Ingredients were dissolved with minimal heat, and 5 ml of the solution was dispensed into 13 ml tubes before sterilizing at 121°C for 15 min. The tubes were stabbed with a bacterial suspension using a plastic probe, and the medium was covered immediately with sterile mineral oil. Anaerobes acidified the medium

turning it yellow within 4 to 8 hr at 30°C; tubes were scored for anaerobic growth after 24 hr. Other phenotypic tests were performed using protocols described by Schaad et al. (2001). Representative isolates of different groups of bacteria determined from the above criteria were submitted to the Texas Plant Disease Diagnostic Laboratory (http://plantpathology.tamu.edu/extension/tpddl/tpddl.asp) for fatty acid methyl ester (FAME) profile analysis. Possible species identification was determined by best fit (similarity index) to the database for bacteria, Sherlock Version 4.5 (0209B); TSBA 40 4.10. Gram negative isolates that grew anaerobically were tested with the API20E strip (Biomérieux, Hazlewood, MO) to determine possible species identification. The type strains of *P. agglomerans* (ATCC 27155) and *P. ananatis* (ATCC 33244) were used as controls.

16S Ribosomal DNA Sequencing

A universal degenerate primer set with added endonuclease digestion sites (underlined) (16SFXbaI – 5' GGTCTAGAAGAGTTTGATCMTGGCTCAG 3'; 16SRNotI – 5' CGGCGGCCGCACGGGCGGTGTGTACA 3') was used to amplify a 16S ribosomal DNA (rDNA) Polymerase Chain Reaction (PCR) product (Medrano and Bell 2007). A 1.5 K base pair product that was predicted based on *E. coli* positioning was ligated into the XbaI/NotI sites of the pDrive cloning vector (New England Biolabs Inc., Beverly, MA) and then transformed into *E. coli* strain ER2267 (New England Biolabs Inc., Beverly, MA) by $CaCl_2$ transformation (Sambrook et al., 1989). A Qiagen kit (Valencia, CA) was used for all PCR experiments with an amplification protocol that consisted of an initial denaturation step at 96°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min, and then a final extension at 72°C for 5 min using a PTC-200 DNA Engine Cycler (MJ Research Inc., Waltham, MA). Sequencing was performed by the Institute of Developmental and Molecular Biology, Gene Technologies Laboratory at Texas A&M University, College Station, TX. The derived 16S rDNA gene sequence data from both strands was edited and assembled using Sequencher 4.0.5. A phylogenetic analysis was performed using MEGA3 (Kumar et al., 2004).

RESULTS AND DISCUSSION

Three groups of cotton fleahoppers were grown in the laboratory and tested for the presence of and ability to transmit cotton seed and boll rot pathogens. The first group, reared in the fall of 2005, was not exposed to any microbes other than those originating from the hatched eggs or the fresh bean diet. Insects were taken directly from the rearing cage and caged over cotton fruiting branches. The second group, reared in the spring of 2006, was fed fresh beans contaminated with a rifampicin resistant strain of *P. agglomerans,* Sc 1-R, which was used previously to show transmission of this *Pantoea* species by southern green stink bugs (Medrano et al., 2007). The contaminated beans were placed in the feeding compartment of the rearing cage in place of the normal washed beans for 4 days when adult fleahoppers first began molting from nymphs. The contaminated beans were replaced with new washed beans for 24 hr before insects were placed in cages over cotton flowering branches. The third group, reared in the fall of 2006, was removed from the rearing cages when adults first appeared and were placed in Petri dishes containing fresh or sterilized (121ºC, 15 min, 1 kg/cm^2) beans dipped in a rifampicin resistant isolate of *P. ananatis,* 7-1R, suspended in solutions of 50-200 ppm rifampicin. After 48 hr, the insects were moved to dishes containing beans free of the bacteria for 24 hr, and then they were caged over cotton fruiting branches. Most of the results from the first group were presented previously (Bell et al., 2006), but are reviewed here for purpose of comparisons.

When newly emerged adults from group 1 were caged over fruiting branches (three insects in each of 34 cages), they caused severe damage to cotton fruiting forms. All 48 pinhead squares were blighted and killed and most abscised. Twenty-four of 29 large flower buds (3-7 mm diameter) died and abscised. Eight of 14 bolls (7-9 mm diameter) became blackened and abscised. Also, protuberances from insect feeding or ovipositing appeared on fruiting branches, leaves and flower petals.

Abscission was consistently associated with necrosis and damage of the ovary wall (Figure 1; Table 1). This symptom in abscised squares also was reported by Mauney and Henneberry (1979, 1984) but was found only occasionally. Both abscised and retained buds showed necrotic spots among the anthers or on the stigma and style. This symptom is considered diagnostic for square abscissions caused by fleahoppers (Mauney and Henneberry, 1979; Williams et al., 1987). Damage to the ovary, however, appeared to be most critical for inciting abscission.

The first group of laboratory reared fleahoppers was frequently infested with seed-rotting bacteria, especially *Pantoea* and *Serratia* spp. (Table 2). In 16 of 34 cages, the major bacterium obtained from insects, and damaged buds and bolls within a cage were the same. The array of pathogenic bacteria transmitted by the laboratory insects (Table 2) was very similar to bacteria found infesting the insects from the field (Bell et al., 2006). The higher frequency of *Serratia* spp. might be due to the rearing conditions or bean diet. Most *Serratia* spp. isolates from fleahoppers showed API 20E test reactions typical of the type species of *Serratia marcescens*. Thus, isolates from fleahoppers were different from the *Serratia marcescens* strain that causes cucurbit yellow vine disease (Rascoe et al., 2003). The *Pantoea* isolates from fleahoppers showed the same range of variation as those previously isolated from stink bugs (Bell et al., 2005; Medrano and Bell, 2006). However, there was a much greater preponderance of isolates categorized as *Pantoea* species 2 by the API 20E System from the fleahoppers. Almost 75% of the isolates from the field or group 1 of the laboratory reared insects were of this type, indicating a possible specialized association of a specific *Pantoea* species with cotton fleahoppers.

The occurrence of seed-rotting bacteria in group 2, which was exposed to the marked strain of *P. agglomerans*, is shown in Table 3. Although the insects had been provided beans contaminated with *P. agglomerans* for 4 days, this strain was not found either from the insects or the abscised buds. One insect removed from a cage over a cotton fruiting branch yielded a rifampicin resistant *Pantoea* isolate, but this isolate was not identified as the Sc 1-R strain of *P. agglomerans.*

Seventeen isolates from fleahoppers, including 13 *Pantoea* isolates, and 16 isolates from diseased buds, including 15 *Pantoea* isolates, were characterized by microbiological tests including API 20E tests strips. Results that are typical of the *Pantoea* isolates are shown for nine isolates which are compared with the type strains of *P. agglomerans* and *P. ananatis* in Table 4. All of the 28 *Pantoea* isolates, except Bud 10 (not shown), were more similar to *P. ananatis* than to *P. agglomerans*. Specifically, all produced indole, and all produced acid from sorbitol and melibiose like the type strain of *P. ananatis*. The isolates varied in ability to produce acetoin (20/27), produce acid from inositol (7/27) and utilize citrate (8/27), which are three other distinguishing characters of the type strain of *P. ananatis*. Four isolates, Bud 7 and FH 8-1 (Table 4) and Bud 19 and Bud 13 (not shown) gave test results identical to those of the type strain of *P. ananatis*. The type strain of *P. ananatis* had a %ID value of only 65.2 with the API 20E System designated *Pantoea* species 2. A similar fit has been observed with most *Pantoea* isolates from insects collected in the field (Bell et al., 2006) as well as those reared in the laboratory.

The isolates shown in Table 4 were also subjected to fatty acid profile analysis (Table 5) and 16S rDNA phylogenetic analysis (Table 6; Figure 2). Although the fatty acid methyl ester profiles of the isolates from fleahoppers were usually most similar to those of *P. ananatis*, the similarity indices generally were very low and not greatly different than those of other species of the Enterobacteriaceae, such as *Cedecia* spp. This is partially due to the type strain of *P. ananatis* having a similarity index of only 0.65 with the database for *P. ananatis.* However, fatty acid profiles of various genera in the Enterobacteriaceae also are very similar and of limited value in identifying species. The 16S rDNA phylogenetic tree (Figure 2) grouped the *Pantoea* isolates from fleahoppers and buds abscised after their feeding with the *P. ananatis* type strain.

The third group of reared fleahoppers was placed in Petri dishes containing either fresh or sterilized green beans that had been dipped in suspensions of the rifampicin-resistant 7-1R isolate of *P. ananatis* isolated from a fleahopper in group 2. The bacteria were suspended in 50, 100, or 200 ppm rifampicin in water for treatment of the beans to favor the establishment of the marked isolate. Six insects (three per cage) from each treatment were placed over cotton fruiting branches. The marked 7-1R isolate was obtained from the insects in two of the twelve cages at nine days after insects were placed in cages over fruiting branches. Diseased buds from three cages also yielded the marked isolate. Most insects and buds contained unmarked isolates of *P. ananatis*. There were no apparent significant differences or trends among treatments. These results confirmed that cotton fleahoppers were capable of taking up and sustaining *P. ananatis*, and also transmitting the bacterium during feeding.

In conclusion, cotton fleahoppers were frequently infested with *Pantoea ananatis* strains that cause severe necrosis and rot of flowering buds, young bolls and seed within older bolls. Other pathogenic bacteria occasionally were carried by fleahoppers and also can contribute to damage caused by the insect. Cotton fleahoppers introduced these bacteria into ovaries of buds and into young bolls where they caused rot and subsequent abscission. The small size of the insect (2.5-3 mm long) might seem to preclude this possibility. However, the proboscis and stylet of the insect is typically about 1.5 mm long, whereas the distance to the ovary from the outside of even a large bud is usually no more than 1mm. The bacterial infections, especially of the ovary, are probably the primary and direct cause of ethylene bursts that occur in squares injured by fleahoppers and contribute to abscission. The pathogenicity of *P. ananatis* strains from fleahoppers to other known hosts of this bacterium, such as pineapple, melons, and onions (Bruton et al., 1991; Gitaitis and Gay, 1997; Lim, 1986) has yet to be determined.

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Table 1. Association of infection and rot of ovary walls with bud and boll abscission

	Frequency of Rot of Ovary Walls in	
Bud/Boll Fate	**Flower Buds**	**Young Bolls**
Abscised	**21/24**	**7/8**
Retained	**0/5[z]**	**0/6**

[z]Necrosis of anthers and/or stigma, but not the ovary, occurred in these buds. (from Bell et al., 2006)