TRANSMISSION OF BACTERIAL BLIGHT OF COTTON, *XANTHOMONAS CAMPESTRIS* PV. *MALVACEARUM*, BY FEEDING OF THE COTTON FLEAHOPPER: IMPLICATIONS FOR STRESS ETHYLENE-INDUCED SQUARE LOSS IN COTTON

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ABSTRACT

Using a model system of cotton and the causative agent of bacterial blight, *Xanthomonas campestris* pv. *malvacearum* (Smith) Dye (XCM), microbial transmission by feeding of the cotton fleahopper, *Pseudatomoscelis seriatus* (Reuter) (CFH), was studied. In controlled feeding tests, artificially infested CFH infected 51% of the plants they attacked. Of these infected plants, 81% exhibited symptoms of the disease, while 19% did not exhibit symptoms, but contained the bacterium. After feeding to acquire XCM, salivary glands and alimentary tracts were dissected and assayed for the presence of XCM. The bacterium was reisolated from 60% of the salivary gland pairs examined, and 95% of the alimentary tracts that were analyzed. In addition, viable bacteria were isolated from 76% of CFH fecal spots which were collected within a 24 h period after insects fed on the bacterium. XCM was present in 73% of the aliquots taken from a solution which was used to surface sterilize CFH after feeding on the bacterium. The implications of CFH as vectors of microorganisms to cotton and the resulting increase in stress ethylene production by the plant are discussed.

Key Words: Cotton fleahopper, cotton, bacterial blight, transmission, abscission, ethylene.

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INTRODUCTION

An important early-season pest of cotton, *Gossypium hirsutum* L., in certain growing areas is the cotton fleahopper (CFH), *Pseudatomoscelis seriatus* (Reuter). This insect has piercing-sucking mouthparts which probe and penetrate the plant during the course of the insect's feeding (Reinhard 1926; Mauney and Henneberry 1979). The CFH attacks the terminal portions of the plant, and can cause stem lesions, abnormal branching, stunting, malformed leaves, and excessive shedding of bolls and small squares (Ewing 1929; Painter 1930).

The plant hormone ethylene has been shown to play an important role in the natural abscission of fruit in many plants, including cotton (Lipe and Morgan 1973). When plants are subjected to environmental stresses, such as insect feeding, ethylene is produced at higher than normal rates. This 'stress ethylene' can cause flower bud abscission and inhibit internodal growth (Abeles 1973).

Several reports have shown that CFH promote ethylene production while feeding on detached apical buds of cotton (Duffey and Powell 1979; Grisham et al. 1987).

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In addition, Duffey and Powell (1979) found that many CFH infested buds were contaminated with microorganisms that were not present in control buds, suggesting that CFH may act as a vector of these microbes. Martin et al. (1987) confirmed that CFH salivary glands and alimentary tracts can contain a variety of microorganisms. Thus, CFH may vector both pathogenic and nonpathogenic microorganisms to cotton. Because increased ethylene production by diseased plants is a well known phenomenon (Williamson 1950; Abeles 1973; Pegg 1976), microorganisms carried by CFH may enhance the induction of stress ethylene.

Bacterial blight of cotton, caused by the bacterium *Xanthomonas campestris* pv. *malvacearum* (Smith) Dye (XCM), can be a serious problem in many areas of the world where cotton is produced. The pathogen is easily spread, and can infect all aerial portions of the plant (Innes 1983). Although wind, splashing rain and irrigation water are the main means of dissemination, transmission by insects can also occur (Logan and Coaker 1960; Borkar et al. 1980). Insects may vector inoculum or create feeding and oviposition wounds through which the pathogen may enter.

Using XCM and cotton as a model system, the objective of this study was to determine if CFH are capable of transmitting microorganisms via their feeding habits. There is no evidence that CFH are important vectors of XCM, and we do not suggest that they are. In fact, bacterial blight of cotton has been virtually eliminated in the U.S. through the development of resistant varieties and cultural practices such as sanitation (Brinkerhoff and Fink 1964), seed treatment (Hunter and Brinkerhoff 1964), and quarantine (Schnathorst 1966). However, due to the virulence of XCM, and the easily discernible symptoms it causes in cotton, it is a useful organism for transmission studies.

MATERIALS AND METHODS

Plants

Cotton plants, cv. MacNair 220, were grown in a growth chamber in 1.5 liter plastic pots (3 plants per pot) containing Schertz potting mix (K. F. Schertz, pers. comm.). This variety of cotton is highly susceptible to attack by XCM (L. S. Bird, pers. comm.). The growth chamber was generally maintained at 30°C, ambient relative humidity, and a L:D 14:10 photoperiod. The conditions of the chamber were slightly altered after the plants were inoculated with the bacterium (see below). Susceptibility of the plants under these environmental conditions were confirmed prior to the beginning of this study.

Development of Bacterial Marker

Isolates of XCM (races 1 and 2) were obtained from L. S. Bird, Texas A&M University, and maintained on potato-carrot-dextrose agar (PCDA) (Bird 1966). The races were combined and, for several generations, subjected to increasing levels of streptomycin sulfate incorporated into PCDA. A strain resistant to this antibiotic at a concentration of 2 mg/ml was developed and used in the present study (abbreviated as XCMr). All subsequent isolations of XCMr were carried out on King's B agar medium (King et al. 1954) containing 2 mg/ml streptomycin sulfate (KBAstr).

Rearing and Treatment of Insects

Fleahoppers were hatched and reared in the laboratory from overwintering eggs in stems of the preferred host plant, *Croton capitatus* (Michaux) (Gaylor and

Sterling 1975). After hatching, the insects were maintained in 1.89-liter paper cans covered with nylon organza. The fleahoppers were fed fresh green bean pods (*Phaseolus* sp.) and slices of potato tubers (*Solanum tuberosum* L.) every 2 to 3 days.

Because *Xanthomonas* spp. has been shown to be associated with CFH (Martin et al. 1987), we conducted an experiment to ensure that there were no streptomycin-resistant *Xanthomonas* spp. indigenous to the laboratory-reared CFH used in this study. Five 3rd and 4th instar nymphs were ground by hand in a Potter-Elvehjem tissue grinder containing ca. 2 ml sterile, distilled water. Triplicate 0.1 ml aliquots of this solution were removed and spotted onto KBAstr. This procedure was repeated three times (a total of four replications).

To acquire the bacterium, CFH nymphs were allowed to feed on a suspension of XCMr in a 5% sucrose solution. Turbid suspensions were made using 2- to 4-day-old bacterial cultures. This solution was spotted onto strips of sterile paper toweling (ca. 6.0 × 1.5 cm) that were placed in petri dishes (2 strips per dish). Third and fourth instar CFH nymphs were collected via aspirator and held without food or water for ca. 3 h in petri dishes. The insects were then transferred to the petri dishes containing the paper strips inoculated with XCMr (10 to 15 CFH per dish), and allowed to feed for 24 h. The dishes were checked periodically, and the paper strips were re-spotted with the XCMr/sucrose solution if they were dry or close to dryness. After feeding and acquiring XCMr in this manner, the CFH were termed 'infested' (abbreviated CFHi). To confirm that the drying of the paper was primarily due to CFH feeding and not to evaporation, paper strips moistened with sucrose solution were placed into petri dishes without insects.

The efficacy of the above procedure was confirmed by assaying the salivary glands and alimentary tracts of 20 CFHi for the presence of XCMr after the feeding period. Insects were first surface sterilized for 30 s in a 0.525% sodium hypochlorite/0.1% Triton X-100 solution, followed by a 30 s rinse in sterile distilled water. Using microsurgical techniques as described in Martin et al. (1987), the alimentary tract and salivary glands were removed, externally rinsed as described above, and mascerated onto KBAstr. In addition, sixty 0.1 ml aliquots from at least 5 different rinse solutions were assayed for the presence of XCMr.

Another check included an examination of CFHi fecal material for the presence of XCMr. After the above feeding procedure, 20 insects were externally rinsed as described, and placed individually in sterile petri dishes lined with a circular piece of sterile filter paper. The insects were provided with a small moistened sterile cotton ball as a source of water. To prevent wetting from the cotton ball, a small section of the filter paper was removed from its perimeter. The cotton was positioned so that it did not contact the lining. After ca. 24 h a total of 25 fecal spots (at least one from each insect) were cut from the filter paper and placed on KBAstr.

Several methods were employed to eliminate external and fecal contamination as possible modes of transmission. After acquiring XCMr, the fleahoppers were surface sterilized as described, and a small drop of Elmer's® carpenter's wood glue was placed over the anus with a small insect needle (this type of glue was used because of its fast-drying nature). When necessary, the insects were lightly anesthetized with nitrogen until the glue was completely dry.

Inoculation of Plants

Using a small camel's-hair brush, 2 to 3 CFHi, prepared as described above, were placed onto the terminals of each of 41 cotton plants at the 4 to 5 true leaf

stage. The plant terminals were then enclosed in nylon organza bags which were sealed at the bottom with twist ties. Prior to returning the plants to the growth chamber, the temperature of the chamber was changed to a 34°:22°C L:D regime. This temperature regime favors the development of XCM in cotton (Stoughton 1928). Relative humidity was increased to ca. 40%, while photoperiod remained unchanged. After allowing CFHi to feed on the plants for 72 h, the bags and insects were removed. The plants were then held for 21 days and monitored daily for disease symptoms (e.g. water-soaked spots on the leaves or blackish lesions on stems or small bolls). Three groups of controls were also monitored: 10 plants which were toothpick-inoculated with the XCMr/sucrose solution (Bird 1982); 10 plants which were infested with CFH which were fed 5% sucrose solution without XCMr; and 10 plants which received no treatment.

At the end of the 21 day incubation period, all plants were assayed for the presence of XCMr. At least two sections of leaf (ca. 2 cm^2) and stem tissues (ca. 2 cm long) were removed from each plant with a sterile razor blade, and carefully minced individually on a glass slide in 2 to 3 drops of distilled water. Three aliquots of an unspecified volume were removed from each solution with an inoculating loop and transferred to KBAstr. Agar plates were incubated at room temperature and examined for the presence of XCMr daily for up to 10 days. The bacterium was recorded as not present if no growth occurred within this 10 day incubation period.

All of the procedures described above were performed using sterile technique. When possible, handling of the bacterium, insects, and plant tissues was carried out under a laminar flow hood.

RESULTS AND DISCUSSION

Laboratory-reared 3rd and 4th instar CFH used in this study did not contain an indigenous streptomycin-resistant *Xanthomonas* spp. In fact, very little growth was noted on KBAstr plates that were inoculated with CFH homogenate. The growth that occurred on these plates was usually confined to 1 to 2 species of fungi. An incubation period of 5 to 7 days was generally required for the appearance of these contaminants, and their growth was very slow and somewhat limited.

CFH fed readily on the 5% sucrose/XCMr solution. During the 24 h exposure period, the paper strips had to be re-moistened with this solution 2-3 times. Control plates containing only moist paper strips remained wet for the duration of the exposure period. Thus, the drying of the paper was primarily due to consumption of the liquid by CFH. This feeding method has also been used successfully to load CFH with ^{32}P for predation studies (R. G. Breene and W. L. Sterling, pers. comm.).

Isolation of XCMr from the Insect

After feeding on XCMr in sucrose, the CFH were highly contaminated, both internally and externally (Table 1). The alimentary tract was the most highly infested tissue examined (95%), while the salivary glands were contaminated with XCMr to a lesser extent (60%). The bacterium also remained viable after passing through the alimentary canal of the insect. Seventy-six percent of CFH fecal spots collected within 24 h of acquiring the bacterium contained XCMr. XCM, as well as three other pathogenic species of *Xanthomonas*, have also been shown to remain

viable after consumption and excretion by several other species of insects (Borkar et al. 1980; Kaiser and Vakili 1978). Borkar et al. (1980) also demonstrated that *Xanthomonas* spp. can retain their pathogenicity for up to 8 days after excretion by insects. In addition, other plant pathogenic bacteria have been shown to be able to survive within insects. For example, *Erwinia amylovora* (Burill) Wilson et al., was found to survive in the intestinal tract of several insects for many days, and was also transferred from contaminated females of the fly, *Musca domestica* L., to the exterior of eggs (Ark and Thomas 1936).

Table 1. Number of salivary gland pairs, alimentary tracts, fecal spots, and L., to external rinse aliquots from the cotton fleahopper, *Pseudatomoscelis seriatus*, containing streptomycin-resistant *Xanthomonas campestris* pv. *malvacearum* (XCMr). The fleahopper was artificially infested with XCMr by allowing it to feed for 24 h on a suspension of the bacterium in 5% sucrose. Bacterial isolations were made on King's B medium containing 2 mg/ml streptomycin sulfate.

	Number Contaminated With XCMr	% of Total
Salivary Gland Pairs	12a*	60
Alimentary Tracts	19a	95
Fecal Spots†	19a	76
External Rinse Aliquots	44b	73
Control‡	0	—

* Numbers followed by the same letter are not significantly different ($\alpha < 0.01$; X^2 pairwise comparison).

† After feeding to acquire XCMr, fleahoppers were externally rinsed and held in individual petri dishes lined with filter paper. After 24 h, fecal spots were cut from the filter paper and placed onto the agar medium.

‡ Control insects were allowed to feed on 5% sucrose solution without XCMr.

External body parts of many of the CFH were also infested with XCMr after the feeding period (Table 1). Kaiser and Vakili (1978) found that, depending on the particular isolate, pathogenic *Xanthomonas* spp. could remain viable and pathogenic for up to 19 days on the surface of two species of living beetles. In addition, they found that the number of pathogenic bacteria in surface washes of these insects remained high (10^5 to 10^6) for 14 days, when the tests were discontinued. In the present study, aliquots were removed from rinse solutions in which several insects were washed. Thus, we cannot make inferences about the level of external contamination of individual insects. However, data presented in Table 1 confirm that most of the CFH came into contact with and consumed the bacterium.

Transmission of XCMr to Cotton

Of the 51 plants infested with CFH (41 with CFHi and 10 with untreated CFH), 47 showed typical signs of CFH feeding - plants somewhat spindly and stunted, and newly formed leaves containing holes and tears (Ewing 1929). Thus, despite glue treatment, external washes, and handling, most of the CFH survived and fed on the cotton plants. However, it seems likely that preparation of the insects

probably impaired some of them, and that this may have reduced the number of successful inoculations. On the four plants which did not show evidence of CFH feeding, CFH were either not located or appeared unhealthy and lethargic.

Only 17 of the 41 plants (41%) infested with CFHi exhibited the typical symptoms of bacterial leaf blight after the 21 day incubation period (Table 2). The symptoms were manifested as water-soaked lesions on the foliage, and were associated with the feeding tears caused by the CFH. There were a few plants which exhibited water-soaked lesions on the stems and terminal buds. The bacterium was re-isolated from all of these plants, and from 4 additional plants that did not exhibit disease symptoms. It is likely that the bacterial populations of these 4 plants had not reached levels high enough in order for symptoms to become apparent. Nonetheless, these plants were infected.

Table 2. Number of cotton plants with symptoms of bacterial blight disease and/or the presence of streptomycin-resistant *Xanthomonas campestris* pv. *malvacearum* (XCMr) due to various treatments. Bacterial isolations were made on King's B medium containing 2 mg/ml streptomycin sulfate.

Treatment (N)*	Disease Symptoms Present	XCMr Present
1. Mechanically Inoculated (10)	10a†	10a
2. Treated Insects (41)	17a	21b
3. Untreated Insects (10)	0b	0c
4. Control Plants (10)	0b	0c

* Description of treatments 1-4:
1. Leaves and stems of plants inoculated with XCMr in 5% sucrose solution via sterile toothpick.
2. Plants were infested with cotton fleahoppers which had fed on XCMr in 5% glucose for 24 h.
3. Plants were infested with cotton fleahoppers fed for 24 h on 5% glucose only.
4. Plants not treated with insects or bacterium.

N = Number of plants receiving each treatment.

† Numbers followed by the same letter within a column are not significantly different ($\alpha < 0.01$; X^2 pairwise comparison).

Untreated plants and plants which were infested with control CFH remained free from symptoms of bacterial blight (Table 2). In addition, XCMr was not isolated from any tissues tested from these plants. All of the plants that were mechanically inoculated with XCMr in glucose exhibited disease symptoms. Symptoms on these plants were the first to appear, and were also the most severe. This was probably due to the relatively high number of bacterial cells placed directly on and in the plant with the toothpick, compared to those plants infested with CFHi. XCMr was re-isolated from all of the diseased tissues that were sampled from these plants.

The data indicate that CFH are capable of transmitting XCM by feeding. An insect with similar feeding behaviors, *Lygus vosseleri* Poppius, also was found to have the capacity to disseminate XCM inoculum and incite the disease in cotton (Logan and Coaker 1960). Another plant bug, *L. pratensis* L. was found to transmit the bacteria, *Erwinia carotovora* (Jones) Holland, the cause of celery heart rot, and *E. amylovora*, the causative agent of fire blight of pear trees (Leach 1940). Xanthomonads pathogenic to bean (*Phaseolus vulgaris* L.) and cowpea (*Vigna*

unguiculata [L.] Walp.) have been shown to be associated with feeding damage caused by several foliage-feeding insects, including those with chewing and those with sucking mouthparts (Kaiser and Vakili 1978). XCM was transmitted to cotton by larvae of the spotted bollworm, *Easias* spp., but only after feeding on a pure culture of the bacterium (Borkar et al. 1980).

While this study has shown that CFH can transmit XCM to healthy plants by feeding, the extent to which this insect can transmit this and other microorganisms in nature remains unknown. In the present studies, experimental conditions were made favorable for transmission by using highly contaminated insects, and furnishing them with young, highly susceptible plants to feed on. Even under these conditions only 51% of the plants attacked by CFHi either showed disease symptoms or had tissues with the bacterium present. However, the main objective of this study was to determine whether or not transmission of a microorganism by feeding of the CFH was possible, and in this the study has succeeded. In addition, we found that CFH can become heavily contaminated externally, and that its feces can contain viable XCM.

The results obtained in this study, in addition to several other studies (Duffey and Powell 1979; Grisham et al. 1987; Martin 1987; Martin et al. 1987), provide evidence that CFH can acquire and transmit microorganisms to the cotton plant, and that these microorganisms may contribute to square loss due to increased ethylene production. In addition, there is evidence that the microorganisms need not be viable to induce ethylene synthesis (Martin 1987). Thus, dead bacterial cells or fungal spores carried by CFH, but which cannot be detected, may also contribute to this increased ethylene production. While CFH have not been considered to be important vectors of disease organisms, their microflora likely contribute to ethylene-induced square loss.

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