EXPERIMENTAL INSECT VECTORS OF THE CUCURBIT YELLOW VINE PATHOGEN, *SERRATIA MARCESCENS*

BY

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Sultanate of Oman

1990

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
December, 2002

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ACKNOWLEDGMENTS

I would like to thank my major professor, Dr. Jacqueline Fletcher, for her valuable assistance in guiding me during my thesis work, for her contribution and for her thorough review of this manuscript. My sincere gratitude thanks to Dr. Astri Wayadande for her keen supervision, suggestions, practical help and thorough review of this thesis. I appreciate greatly the guidance of my other committee members, Dr. Forrest Mitchell and Dr. Jack Dillwith, for their valuable ideas and expertise.

The invaluable assistance of Dr. Michael Berg, former postdoc, is profoundly appreciated. I would like also to thank my laboratory colleagues; Blake Bextine, former PhD student, Quan Zhang and Kikin Mutaqin, as well as all faculty, staff, students and friends of the Entomology and Plant Pathology Department at Oklahoma State University for their friendship and kindness.

My special thanks to my dear father and to my beloved mother, to my beloved wife, Samira Al-Zadjali, and to the ones who made all of this meaningful and purposeful, my children, Anfal, Saja and Abdullah. My special thanks also to my brothers, sisters and all my family members for their support and supplication.

At the end many thanks to the Ministry of Higher Education and the Ministry of Agriculture and Fisheries, Sultanate of Oman (MOAF), for their financial support. A word of appreciation to Dr. Ahmed Al-Rawahai, the former minister of MOAF, for his nomination of me to do my Master's Degree and to Mr. Khalfan Al-Naibai, the Undersecretary of MOAF, for his great support.

Above all, I thank the almighty ALLAH, Who said in Chapter 14 (Ibrahim) in The Holy Book (Quran), "And when your Lord proclaimed: If ye give thanks, I will give you more."

LIST OF TABLES

Table Page

Chapter 2

Appendix A

Appendix B

Appendix C

Chapter 1

Literature Review

I. Cucurbits

I.1. Cucurbits Taxonomy

The term "cucurbit" was originally coined for only the cultivated species within the family Cucurbitaceae (49), but it denotes all species within the Cucurbitaceae. Most species of this family are frost intolerant, predominantly tendril-bearing vines found throughout the tropics and sub-tropics of Africa, southeastern Asia and the Americas. Most species cannot survive year-round in temperate climates, so they are cultivated as seed-producing annuals (60).

The family Cucurbitaceae is taxonomically isolated from other plant families. It can be divided in two subfamilies: the Cucurbitoideae, with one style in the female flowers, and the Zanonioideae, with three styles (there are no species of Zanonioideae of economic importance). The subfamily Zanonioideae includes cucurbits that are grown throughout Asia for medicinal purposes. There are about 118 genera and over 825 species in the Cucurbitaceae family (35). Among these are four genera of economic importance, watermelon, cucumber, muskmelon and squash (60).

The seedlings of most cucurbits are epigean, germinating with the tips of the cotyledons initially inverted but later erect. Cucurbits generally have a strong taproot that can extend 1-2 m into the soil. Often, many secondary roots occur near the soil surface. The sieve tubes in the secondary phloem are the largest found in angiosperms. The stem of the usual cucurbit is herbaceous, centrally

hollow, sap-filled, and branched. Cucurbit leaves are simple, palmately veined and shallowly to deeply three to seven lobed. There is typically one leaf per stem node. The fruits of cucurbits are extremely diverse in many characteristics including size, shape, color, and ornamentation (60).

I.2. Economic Importance of Cucurbits

Nine genera of cucurbits including thirty species are produced as food crops (49). Food and Agriculture Organization (FAO) statistics (22) show that the most widely cultivated cucurbit in the world is watermelon, with a total production of about 63 million tons, followed by cucumber (29 million tons), muskmelons and other melons (19 million tons) and *Cucurbita* spp. including squash, pumpkins, and gourds (15 million tons). The country with the highest cucurbit production of all four is China, which produces 60% of the world's watermelons, 55% of the cucumbers, 31% of muskmelons and other melons and 20% of the squashes, pumpkins and gourds (22).

The United States Department of Agriculture reported that Oklahoma and Texas yield an annual value of cucurbits greater than $100 million and occupying approximately 40,000 hectares. The watermelon area harvested in 1999 for fresh market in Oklahoma was 3,035.25 hectares, of which 675,000 CWT (hundred weight, which equals 100 pounds or about 45.4 kilograms) were produced at a value of 7.30 dollars per CWT. In Texas, 15,054.84 hectares were harvested, producing 7,440,000 CWT with a value of 3.98 dollars per CWT (1).

I.3. Diseases of Cucurbits

There are more than 200 known cucurbit diseases of diverse etiologies (75). Among the bacterial diseases are those caused by species of *Erwinia,* such as bacterial wilt, caused by *E. tracheiphila*, which affects mainly muskmelon but also cucumber and squash. *E. carnegieana* causes bacterial rind necrosis in muskmelons and watermelons. *E. carotovora* causes soft rot of cucurbit fruits, particularly cucumbers and muskmelons. *E. ananas* causes brown spot in muskmelons. Other bacterial diseases are leaf spot caused by *Xanthomonas campestris* pv. *cucurbitae*, angular leaf spot caused by *Pseudomonas syringae* pv. *lachrymans* and bacterial fruit blotch caused by *Acidovorax avenae* sp. *citrulli* (60).

Fungal diseases include damping off caused by *Pythium* spp., *Phytophthora* spp., *Rhizoctonia solani,* and other fungi. Other fungal diseases include target leaf spot, alternaria leaf blight, anthracnose, Cercospora leaf spot, powdery and downy mildew, charcoal rot, scab, different types of rots, Fusarium and Verticillium wilt (37,75).

Important viral diseases of cucurbits include cucumber mosaic, cucumber green mottle mosaic, squash mosaic, and watermelon mosaic (37,60). Another viral disease, cucurbit latent, is transmitted by the aphid *Myzus persicae* in a non persistent manner. This virus is restricted to California, where it infects muskmelon, watermelon, cucumber, and squash. Cucumber wild mosaic virus is

transmitted by the western striped cucumber beetle (*Acalymma trivittata*) and cucumber vein yellowing virus is transmitted by the sweet potato whitefly, *Bemisia tabaci* (19,20).

I.4. Insect Vectors of Cucurbit Pathogens

The most important insect pests are those that primarily cause feeding damage on the plant but may also cause secondary damage by transmitting pathogens (60). Among those are aphids, the most important insect vectors on cucurbits. The melon aphid, *Aphis gossypii* (Glover), which attacks watermelon, muskmelon, cucumber, squash and other cucurbits, is a serious direct pest of melon and an important vector of non-persistent viruses such as cucumber mosaic virus (CMV), watermelon mosaic virus 2 (WMV-2) and zucchini yellow mosaic virus (ZYMV) (43). The green peach aphid, *M. persicae*, attacks cucurbits also, but prefers other host plants; these aphids migrate from one plant to another seeking a more preferred host, thus spreading viruses such WMV-2 and CMV (9).

Cucumber beetles are another important group of insect vectors on cucurbits. Three species are known to transmit pathogens: the striped cucumber beetle, *Acalymma vittatum* (Fabricius), the banded cucumber beetle, *Diabrotica balteata* (Le Conte) and the spotted cucumber beetle, *D. undecimpunctata howardi* (Barber). *A. vittatum* attacks the young seedlings by consuming the stems and cotyledons. Adults later feed on the leaves, vines and fruits and larvae feed on the roots. *A. vittatum* and *D. undecimpunctata howardi* transmit *E.*

tracheiphila, the causal agent of bacterial wilt (60). Feeding by these beetles makes deep wounds in the plant and bacteria that over-winter in the digestive tract of the beetles emerge within insect feces and enter the wounds. The bacteria multiply within the xylem vessels of the plant until water movement is blocked. Bacteria can survive for one to two months after host plant death but cannot survive the winter in any location other than the cucumber beetle's digestive tract (68). Adults of *D. balteata* and *D. undecimpunctata howardi* feed on leaves and transmit bean mild mosaic carmovirus, bean chlorotic mottle bromovirus, bean pod mottle and cowpea severe mosaic comovirus. *A. trivittata, A. thiemei thiemei* and *D. undecimpunctata undecimpunctata* transmit squash mosaic virus (9).

The squash bug, *Anasa tristis,* is considered a serious insect pest on cucurbits. It feeds by penetrating its stylets intra-cellularly in the vascular tissues (3,50). Squash is the preferred host for squash bugs but in the absence of squash they may feed on cucumber, melon, watermelon, or other cucurbits. Cucurbit seedlings are the targets for *A. tristis* because of the voracious feeding of over-wintered adults and newly hatched nymphs (3). Squash bugs induce Anasa wilt of cucurbits (59). The damage begins on a single leaf and progresses throughout the entire plant. Neal (50) showed that wilting is not due to toxin but is a result of localized feeding of *A. tristis.* Members of the Cucurbitaceae family have bicollateral vascular bundles distributed in a ring around the stem, and because each of the vascular bundles is independent, destruction of all the

bundles is necessary for complete loss of water transport (50). The insect usually has two generations per season but favorable environmental conditions in Oklahoma result in the development of a partial or complete third generation (23).

Thrips, including *Frankliniella occidentalis*, *Heliothrips femoralis,* and other species are destructive to melon and cucumber and are important vectors of tospoviruses (60), such as those causing watermelon silver mottle and zucchini lethal cholorosis (47).

Leafhoppers are important vectors of cucurbit pathogens. These insects, which are capable of long distance movement, may migrate 400 miles or more from the winter breeding areas to cultivated fields (24). The beet leafhopper, *Circulifer tenellus,* may transmit curly top virus when it feeds on cucurbits, although it prefers other host plants (60).

Other major insect vectors on cucurbits are the whiteflies. Three distinct cucurbit viruses have been reported to be transmitted by whiteflies: beet pseudo yellows (BPY), lettuce infectious yellows (LIY), and cucurbit yellow stunting disorder (CYSD). BPY virus causes severe losses in greenhouse-grown cucurbit crops throughout North America, Europe, and Asia. It has been reported from France, the Netherlands, Japan, Italy, Spain, England, Australia, and Bulgaria. Since 1982, the incidence in melon crops under protected environments and outdoors on the Mediterranean coast of Spain has continually increased, inducing considerable economic losses. The virus has a wide host range of important crop, weed, and ornamental hosts. BPYV is transmitted by

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Trialeurodes vaporariorum in a semi-persistent manner. A different whitefly transmitted virus, LIYV, was reported from the desert regions of California and Arizona in 1981. The virus, transmitted specifically by the A biotype of *B. tabaci*, has a wide range of important crop hosts. The virus has been also found in Texas and Mexico. In the early 1980s, a yellowing and stunting disorder of cucurbits was noticed in the Middle East (Jordan, United Arab Emirates and Turkey) (20).

The CYSD has a narrow host range, mainly in the Cucurbitaceae. CYSD is transmitted specifically by the B biotype of *B. tabaci* and is retained by the vector for 10 days (20). *T. vaporariorum* can transmit muskmelon yellows virus to melon and cucumber and *B. tabaci* can transmit squash leaf curl virus and lettuce infectious yellows virus (60).

II. Cucurbit Yellow Vine Disease (CYVD)

A disease of unknown etiology was first observed in 1988 on squash (*Cucurbita pepo* var. melopepo) and pumpkin (*Cucurbita pepo* var. pepo) in Oklahoma (11). The disease, known as cucurbit yellow vine disease (CYVD), has been associated with a rod-shaped plant phloem-resident bacterium.

II.1. Host Range and Geographic Distribution

CYVD was first observed in squash and pumpkin and was observed in other cucurbits only in 1991, when it destroyed early planted watermelon (*Citrullus lanatus* (Thunb.) and muskmelon (*Cucumis melo* L.) in central Texas and Oklahoma (12). The susceptibility level among cucurbits is in the following descending order: squash, pumpkin, watermelon, and muskmelon. Other wild cucurbits and various gourds have not been found affected by CYVD (53,54).

Initially, the geographical range of the disease appeared to be generally confined to the Cross Timbers region of central and northeastern Oklahoma and north central Texas. In 1997-98, CYVD was diagnosed in commercial fields of watermelon and muskmelon from east Texas (Post Oak Savannah) and all cucurbit-growing areas of Oklahoma. In summer of 1998, symptoms of CYVD were recorded in one watermelon (Hardeman County) and three pumpkin fields (Rhea and Morgan Counties) in Tennessee, where the leaves turned yellow and affected plants exhibited phloem discoloration (7). The disease has recently been reported in Arkansas (58), Kansas (unpublished data) and Colorado (unpublished data). In addition, the disease was found in Massachusetts, which extends the known geographical range of CYVD significantly to include the New England area (74).

II.2. Symptoms of CYDV

Symptoms of CYVD generally appear about two weeks before the harvest. General and rapid yellowing of the leaves are the main symptoms, followed by decline, which occurs gradually and ultimately leads to the death of the vines. However, some infected plants showed no chlorosis, but wilted and collapsed within a day. There are no symptoms associated with the fruit or flowers of squash and pumpkin. In the early stages, a golden to honey brown discoloration of the phloem in the primary root and in the crown is usually observed (11). Similar symptoms occur in watermelon; however, the watermelon fruits become distinctly chlorotic and are difficult to sell (12).

II.3. Etiology of the CYVD Bacterium

Since CYVD was first observed in central Texas and Oklahoma in 1988, a concerted effort was made to isolate and identify its causal agent and to determine the means by which it is disseminated to host plants. A number of disease-causing organisms and abiotic conditions were investigated as the possible cause of CYVD. Among these were herbicide damage, nutrient imbalance, *Fusarium* wilt, charcoal rot, *Monosporascus* vine decline, and various common seed-borne pathogens. An exhaustive study by Bruton et al. (12) included attempts to isolate fungal and prokaryotic organisms from surface sterilized sections of infected muskmelon and watermelon and to specifically detect various candidate pathogens using serological and molecular methods. A

large number of fungi, mostly of the genus *Fusarium*, and 22 species of bacteria, most in the genera *Enterobacter* and *Erwinia,* were isolated. However, there was no consistent correlation between the presence of any fungus or bacterium and the occurrence of symptoms in plants. A suggestion that mollicutes might be the causal agent was supported by circumstantial evidence of insect transmission, the necrosis of host plant phloem tissues, and the inability to isolate any organism consistently associated with CYVD symptomology. Moreover, Dienes' staining of cross sections of infected plant tissues was consistent with the presence of spiroplasma or an uncultivable phytoplasma (12).

The new disease was later consistently associated with the presence of a phloem-resident walled bacterium, originally designated a bacterium-like organism (BLO) because the organism had not yet been cultured (12). Electron microscopy of phloem tissue of CYVD-diseased plants revealed rod-shaped forms of 0.25-0.5 um in width and 1.0-3.0 um in length surrounded by a triple-layered cell envelope (11,12).

The polymerase chain reaction (PCR) was used for DNA amplification from bacterium-like organisms (2). An initial primer pair designed based on sequences of the citrus-greening BLO amplified a 0.15-kilobase (kb) fragment from the DNA of symptomatic plants and not from asymptomatic plants, suggesting that the amplified DNA was of prokaryotic origin. A primer pair designed to amplify nonspecific prokaryotic 16S rDNA amplified a 1.5 kb DNA fragment in both symptomatic and asymptomatic plants. The fragment of 1.5-kb from asymptomatic plants corresponded to chloroplast 16S rDNA, while the

isolates fell within the species *S. marcescens* (58). Later DNA-DNA hybridization tests confirmed it as *S. marcescens* (Fletcher, unpublished).

II.4. *Serratia marcescens*; CYVD Pathogen

S. marcescens is a Gram negative, coliform bacterial species of the family Enterobacteriaceae. A non spore-forming facultative anaerobe with peritrichous flagella, *S. marcescens* is distinguished from other enterobacteria by the production of some interesting compounds such as the surfactant sarawettin, different marcescins, extracellular proteases and chitinases that have been the focus of intensive research (36). *S. marcescens* was long considered to be an innocuous saprophytic microorganism of soil and water habitats, and its plant growth-promoting features made it a focus of research for potential biocontrol applications (67). In addition, however, it is an opportunistic pathogen of humans and has been responsible for many deaths (33). *S. marcescens* also is pathogenic to over 70 insect species, in which it causes reduced longevity, which is of concern for beneficial insects such as honeybees (21) and silkworms, *Bombyx mori* (66). Lethal doses of *S. marcescens* strains are variable; strain RH3 is highly lethal with a LD_{50} of 0.43 cells for the greater wax moth *Galleria mellonella*, whereas strain BR4 had a LD_{50} of 300,000 cells (13).

Although its association with plants has been mostly beneficial, two cases of plant pathogenicity for *S. marcescens* have been described in crown rots of both sainfoin (64) and alfalfa (42). In these instances, *S. marcescens* was reported to be part of a pathogen complex.

II.5. Vascular-Restricted Bacteria similar to CYVD

II.5.1. Phloem-Restricted Bacteria

Phloem-limited bacteria have been identified as the causal organisms of diseases of several crops. Some examples of phloem restricted walled bacteria are those causing citrus greening, clover club leaf, papaya bunchy top and marginal chlorosis of strawberry.

Citrus greening disease (CGD) is a major cause of crop loss in many parts of Asia and Africa. The disease is caused by *Liberobacter asiaticum* and *L. africanum*. Symptoms include yellowing of normal sized leaves along veins, small, lopsided, and bitter fruit, and poorly developed roots. Transmission of CGD from sweet orange to periwinkle was accomplished by dodder and grafting and the inoculated test plants developed yellowing symptoms within 3 months (26). The disease organism was originally thought to be a mycoplasma-like organism (now referred to as phytoplasma) (34), but after further investigation, the causal agent was identified as a bacteria-like organism (BLO) that was restricted to the phloem sieve tubes. Several strains of the BLO have been identified using DNA analysis (73). The CGD causal organisms from Africa and Asia had slight genome differences, but Jagouiex et al. (34), placed the BLOs of both strains in the *Proteobacteria.* The phloem-restricted citrus greening bacterium is transmitted in a persistent manner by the psyllids *Trioza erytreae* (Del Guercio) and *Diaphorina citri* (Kuwayama) (15).

Clover club leaf (CCL) disease affects the leaves of crimson clover, *Trifolium incarnatum* (L.), and other plants such as periwinkle (6). The disease delayed the opening of young leaflets and caused a yellowing of the leaves. Evidence suggested that the pathogen was not a virus, as originally supposed, but rather a rickettsia-like organism (RLO). The CCL bacterium has not been successfully grown on artificial media. The RLO was collected first in *Agalliopsis novella* (Say), captured near woods, and by using *A. novella,* the pathogen was transmitted into healthy plants. The CCL bacterium also was transmitted to 99% of the leafhopper progeny (41), through as many as 21 generations.

Papaya bunchy top (PBT) disease, an important disease in the Antilles and in Trinidad, was originally thought to be caused by phytoplasma (17). This disease organism, which causes diffuse chlorosis in young leaves and reduction of normal leaf blade expansion, was restricted to the periphery of the papaya phloem. The causal organism was later identified as a phloem-limited bacterium, but to date it has not been cultured. Using DNA sequence analysis, a *Proteobacterium* of the genus *Rickettsia* was identified as the causal agent of PBT (18). Recently, electron microscopy of tissues infected with the papaya bunchy top bacterium has shown that it is not the phloem but the lactifers that harbor rickettsial colonies (18). PCR was used successfully to detect the pathogen in symptomatic plants. Two leafhopper species, *Empoasca papayae* (Oman), found in the Antilles, and *E. stevensi* (Young), found in Trinidad, were consistently associated with the disease, and later were identified in transmission tests as vectors of the pathogen (17).

Marginal chlorosis of strawberries was first seen in Spain in 1984 and in France in 1988 (52,76). The disease affected all strawberry cultivars tested. An un-culturable BLO, observed in diseased plants by electron microscopy, was phloem-limited and consistently associated with the disease. Sequencing of PCR-generated rDNA allowed for comparisons with other phloem-limited bacteria. Because of significant differences with other organisms, the bacterium was designated a new organism and assigned the name *Candidatus Phlomobacter fragariae.* The new bacterium was placed phylogenetically in the group 3 of the gamma subclass of *Proteobacteria.* Research at OSU (2) and in France (76) has shown that the bacterium causing marginal chlorosis of strawberry and the bacterium associated with CYVD of cucurbits are different, although they are the only known plant pathogenic gamma-proteobacteria. Different leafhoppers and psyllids collected during several years in and around infected strawberry fields were not found to carry *C. P. fragariae*. However, the *C. P. fragariae spoT* sequence is easily detected in whiteflies proliferating on *P. fragariae* -infected strawberry plants under confined greenhouse conditions but not on control whiteflies, indicating that these insects can become contaminated with the bacterium (76).

II.5.2. Xylem-Restricted Bacteria

Xylem limited bacteria are often referred to as rickettsia - like bacteria. The identity of the pathogen causing Pierce's disease (PD) of grapes as the bacterium *Xylella fastidiosa* was not revealed until the 1970's. The bacterium blocks the xylem vessels of the plant, causing dryness, or scorch to the leaves. *X. fastidiosa* also causes almond leaf scorch, alfalfa dwarf, oleander leaf scorch, and citrus variegated chlorosis. The pathogen is transmitted by 23 species of leafhoppers, but the blue-green sharpshooter, *Graphocephala atropunctata*, and the glassy winged sharpshooter, *Homalodisca coagulata*, are major vectors of the pathogen. The pathogen is transmitted also by three species of xylem feeding spittlebugs (Family Cercopidae) (56,69). *X. fastidiosa* is non-circulative in the insect vectors, but is retained in the foregut region for several weeks to life (8).

Cucurbit lethal yellowing (CLY) disease is one of the major plant diseases of muskmelon, squash, and pumpkin in the mid-west and eastern United States (10). The disease, which is also referred to as bacterial wilt, is characterized by sudden wilt, yellowing or necrotic foliage, curled leaves at the growing tip, and often plant death (10). The causal agent is *E. tracheiphila*, a Gram-negative, rod shaped, and motile bacterium with peritrichous flagella. The striped cucumber beetle, *A. vittatum*, and the spotted cucumber beetle, *D. undecimpunctata howardi*, transmit the bacterium either through their fecal matter or mouthparts when they feed and cause wounds in plants (39). The beetles over-winter as adults and move into cucurbit fields in late April or early May (10). The pathogen

multiplies at the wound site, enters the xylem vessels, and then moves down the petiole becoming systemic (37).

Another xylem-limited pathogen causes phony disease of peach (PP). The bacterium is also transmitted by xylem feeding leafhoppers (70,71).

III. Insect Vector Phytopathogen Relationships

Insect vector-bacterial interactions have been described (section II-5). Of over 200 pathogens characterized as insect-transmitted, about three-fourths are viruses. Mollicutes, bacteria, and fungi make up the other one-fourth. Ten orders of insects are phytophagous, but two (Hemiptera and Coleoptera) are dominant with respect to the number of vector insect species contained within the order and the number of plant viruses transmitted by them (48).

III.1. Hemiptera: The order Hemiptera is divided into two suborders, Homoptera and Heteroptera.

III.1.1. Homoptera:

Homoptera members have piercing-sucking mouthparts and cause limited damage when they feed on plants. Many Homopterans are tissue-specific feeders, favoring the plant phloem as their feeding site (48). The Homoptera include two insect taxa, Auchenorrhyncha and Sternorrhyncha (formerly suborders). The Auchenorrhyncha include the tree hoppers (Membracidae), the plant hoppers (Fulgoroidea), the leafhoppers (Cicadellidae) and the spittlebugs (Cercopidae). The Sternorrhyncha include the psyllids (Psyllidae), the whiteflies (Aleyrodidae), the aphids (Aphididae) and the mealybugs (Pseudococcidae).

III.1.2. Heteroptera:

The suborder Heteroptera includes members who have much larger stylets than those of aphids and leafhoppers, and probably cause more damage to plant tissues during stylet penetration (3). Six Heteropteran families are reported to transmit plant diseases. Members of the Pentatomidae transmit the fungus *Nematospoa coryli* (Pelglion), which causes yeast-spot of soybean (16, 25). Another Pentatomid, *Oebalus pungnax*, transmits *Fusarium oxysporum*, causing kernal discoloration on rice (40). In the family Miridae, *Lygus lineolaris* (*pratensis*) transmits the spinach blight virus (45), the bacteria causing fire blight (*Erwinia amylovora*) (38,65) and celery heart rot (*E. carotovora*) (38), and the seed and pollen associated (62) potato spindle tuber viroid (63). The fifth agent, velvet tobacco mottle sobemovirus, is transmitted by *Cyrtopeltis nicotianae* (28,29,30,57). In the family Piesmatidae, *Piesma quadratum* transmits beet leaf curl rhabdovirus (61) and the rickettsia-like organism that causes sugar beet latent rosette (51,61). Both pathogens are propagative in *Piesma*. A similar disease of sugar beets, called "savoy" in United States, is transmitted by *P. cinerea* (14). In the family Tingidae, *Stephanitis typica* transmits the coconut root phytoplasma (44). In the family Lygaeidae, *Nysius* spp. transmits centrosema mosaic virus (72) and in the family Coreidae, *A.tristis* transmits the CYVD pathogen (4,5).

III.2. Coleoptera: Among mandibulate insects, only beetles are important as vectors because their adults retain the chewing mouthparts of their larval stages and continue to feed on plants. This is important because transmission of plant pathogens is achieved by dispersal of winged adults (48). Some important vector beetle families are Chrysomelidae, Coccinellidae, Cucrculionidae and Meloidae (48), Scolytidae, and Cerambycidae. The ability of beetles to transmit viruses is accomplished by the regurgitation of infective virus from the foregut, as they do not have typical salivary glands. Beetle transmissible viruses are translocated in the xylem of plants and can infect unwounded plant cells (27). One of the virus diseases commonly transmitted by a number of coleopterans, *A. trivittata, A. thiemei thiemei, D. undecimpunctata undecimpunctata; D. bivittula; Epilachna chrysomelina*; and *E. paenulata*; is squash mosaic virus (9). Another example is solanum nodiflorum mottle virus, which is transmitted by the coccinellids: *Epilachna vigintioctopunctata paradalis*, *E. vigintioctopunctata vigintioctopunctata,* and *E. guttatopustulata* (32).

VI. Mechanisms of Insect Transmission of Plant Pathogens

Most of the information available about vector phytopathogen relationships is from vector-virus studies. Mechanisms of insect transmission of plant pathogens are different in Homopterans than in Hemipterans, but generally, five mechanisms are described (48). Non-persistent transmission, which describes the relationship of many aphid transmitted viruses, is also referred to as stylet borne. The pathogen is confined to the inner stylets of the insect mouthparts and

is not transmissible by its vector after approximately 48 hours. In semi persistent transmission, the pathogen is attached in the precibarium or cibarium of the foregut, is transmissible by its vector up to 48 hours, and there is a retention time of up to 48 hours. This mechanism is referred to as foregut borne. In non-circulative transmission, the pathogen colonizes the foregut and is transmissible for the life of the insect. In the circulative mode, the pathogen enters the body through ingestion and migrates from the gut lumen through the hemocoel to the salivary glands. Finally, in propagative transmission, the pathogen multiplies in the body of the insect, which serves as an alternate host for the pathogen (48).

Recently, Gray and Banerjee, (31) proposed new terminology for describing the non-circulative and circulative modes of transmission. In non-circulative, the pathogen does not cross vector cell barriers and is carried externally either on the vector surface or on the cuticle lining of the vector's mouthparts or foreguts (31). Circulative pathogens are transported across the alimentary canal epithelium and are carried internally within the vector hemocoel (31).

Non-circulative viruses may be subdivided into semi-persistent and non-persistent. Semi-persistent viruses are associated with the foregut and are retained for up to 48 hrs (31). Transmission efficiency is directly proportional to the acquisition access period (AAP), which suggests that the virus is bound stably and accumulates until binding sites are saturated (31). The non-persistent viruses, associated with the stylets of the vector, are retained for only a few hours and are easily lost during feeding probes (31). The relationship between

the transmission efficiency and the acquisition feeding period is inversely proportional (31). Examples of non-circulative viruses are potyviruses and caulimoviruses (31). Aphids, whiteflies and leafhoppers, as well as nematode vectors, transmit some plant viruses in a non-circulative manner (31).

Circulative viruses can be subdivided into two groups. Propagative viruses replicate in their arthropod vectors, while non-propagative viruses, including luteoviruses and the single membrane enamovirus, do not (31). The circulative pathway involves ingestion into the gut lumen followed by uptake by midgut or hindgut epithelial cells. Virus eventually is released into the hemocoel, and later becomes associated with the salivary glands (31).

Insect transmission of pathogens requires three stages. The acquisition access period (AAP) is the time required for an insect to ingest and begin harboring a pathogen. The latent period (LP) is the period after a pathogen has been acquired, when the pathogen is present within the insect but is not transmissible. The inoculation access period (IAP) is the time required for the inoculative insect to feed on the healthy host plant and to introduce the pathogen.

V. Research Objectives

Several types of circumstantial evidence supported the possibility that the CYVD bacterium is transmitted in nature by an insect vector. Early work on CYVD showed that the pathogen was neither soil borne (11) nor seed borne (54). In the field, a fringe pattern of disease distribution was noted; symptoms appeared first in plants at the edge of the field, and then moved progressively toward the center of the field. In severe epidemics, the entire crop may be lost.

The possible role of insects as vectors was first recognized in research conducted at the Wes Watkins Agricultural Research and Extension Center in Lane, Oklahoma. Soil fumigation with methyl bromide at $67g/m^3$ soil did not lower the incidence of CYVD. A plastic mulch experiment was conducted to determine if squash bug and aphid populations and ground cover, alone or in combination, affected the incidence of CYVD. Regardless of the mulch treatment used, foliar insecticide treatments reduced the incidence of CYVD (11). These data are consistent with the interpretation that the CYVD pathogen is transmitted by an insect vector and is not soil-borne. However, in early transmission tests, a number of different cucurbit insect pests failed to pass on the pathogen (54,12).

A field experiment was designed to test the hypothesis that insects are involved in the epidemiology of the disease (5). The occurrence of the CYVD bacterium in uncovered squash plants was compared to that in plants covered with mesh. Three percent of uncovered plants and 0% of covered plants developed foliar symptoms. Using PCR, 25% of uncovered plants and 0% of

covered plants were positive (5). These data suggest that insects are involved in the disease, and possibly in the transmission of the bacterium.

Recently, transmission of *S. marcescens* was achieved using *A. tristis* as an experimental vector. Transmission rates up to 60% were obtained using single inoculative insects by modifying the artificial acquisition source or feeding bugs on plants naturally infected with *S. marcescens* as acquisition source. Transmission required little or no latent period, and insects were able to transmit within 48 hours of acquisition. Moreover, transmission persisted for at least three weeks (5). The absence of *S. marcescens* in the hemolymph of 20% of the transmitting squash bugs suggested that *S. marcescens* is not circulative. However, its presence in 40% of the insects hemolymph may reflect a circulative route for transmission by the insects, or may instead result from bacterial infection of the insect (5).

Although the squash bug is a confirmed vector of S *marcescens*, other insects such as leafhoppers were considered candidates as vectors of CYVD for several reasons. Several species of leafhoppers, especially *Exitianus exitiosus* (Uhler), were common in fields where CYVD was found. Furthermore, many leafhoppers feed in the phloem, where the CYVD pathogen is located. When the original yellow vine PCR primer pair, YV1 and YV2, was used to screen field collected insects for the presence of the CYVD pathogen a band of the expected size was amplified in numerous samples of *E. exitiosus* collected from grassy plots in Stephenville, Texas. This species was found to be a carrier of a bacterium reacting with the YV1 and YV2 primers (Mitchell, unpublished), with

6% of field collected *E. exitiosus* testing positive by PCR (5). Sequencing of the amplified DNA fragment, however, revealed that it was significantly different from that of the CYVD bacterium and, in fact, was homologous to a region of the 16S rRNA gene of an enteric bacterium (*Bacillus Euscelidius variegatus* (Kirshbaum) (2), which occurs in many leafhoppers (55). Positive PCR results were obtained also with a small number of field-collected painted leafhoppers (*Endria inimica*), and the green sharpshooter, *Draeculacephala minerva* (Lori Green, unpublished). However, bacteria were not identified from these samples, leaving open the possibility that they, too, were insect symbionts.

Other phloem-feeding insects such aphids and other heteropterans were also considered as possible vector candidates.

Leafhoppers and aphids have the potential ability to probe the phloem and have a history of transmitting other vascular limited phytopathogens, particularly phloem-limited pathogens. The way these pathogens are transmitted led us to the hypothesis that leafhoppers and aphids may be involved in CYVD transmission. We included the western tarnished plant bug, *Lygus hesperus* (Knight), in our studies because of the close similarity of its feeding behavior to that of *A. tristis*, lacerating the leaf tissue with sickle-like mandibles to suck the plant sap, and because of the involvement of other Miridae members in transmission of other phytopathogens.

The research objective of this study was to test three groups of hemipterans; aphids, leafhoppers and the western tarnished plant bug; for their ability to transmit *S. marcescens* under artificial conditions using a feeding medium detection assay.

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Chapter 2

Transmission of *Serratia marcescens* by aphids, leafhoppers and the western tarnished plant bug

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Abstract

Cucurbit yellow vine disease (CYVD) causes yellowing, rapid wilting and death of susceptible cucurbit plant hosts. The causal agent of CYVD, *Serratia marcescens,* colonizes the host plant phloem, suggesting possible transmission by phloem-feeding insects. The squash bug, *Anasa tristis*, was previously identified as a field vector of *S. marcescens.* Various hemipteran insects, five aphid species, five leafhopper species and one true bug were tested for their ability to transmit *S. marcescens* using artificial feeding systems. Aphids and leafhoppers were fed on feeding sachets containing *S. marcescens* Z01A at 1.0 x 10^5 CFUs/ml. For the true bug, *Lygus hesperus,* Z01A-amended artificial diet was placed in un-stretched parafilm feeding pouches for acquisition and inoculation. Insects were given a 24-48 hr acquisition access period (AAP) and, immediately following, a 24-48 hr inoculation access period (IAP). Thirty seven percent of the sachets exposed to *S. marcescens*-fed *Circulifer tenellus*, 32.6% of the sachets exposed to bacteria-fed *Exitianus exitiosus* and 66.6% of the

sachets exposed to bacteria-fed *Endria inimica* were positive by PCR. Thirty three percent of the pouches exposed to *S. marcescens*-fed *L. hesperus* were positive by PCR. The aphids *Acyrthosiphon pisum, Myzus persicae, Aphis gossypii, A. nerii* and *Diaurapis noxia* and the leafhoppers, *Dalbulus maidis* and *Macrosteles quadrilineatus* did not transmit *S. marcescens* to artificial sachets under these conditions. These results suggest possible transmission specificity of *S. marcescens* and demonstrate that *E. exitiosus, C. tenellus, E. inimica,* and *L. hesperus* are experimental vectors.

Introduction

Cucurbit yellow vine disease (CYVD), a relatively new disease of squash, melons and pumpkins, has been associated with the presence of phloem–inhabiting bacteria that were recently identified as *Serratia marcescens* (43). Symptoms include yellowing of foliar tissue, especially at the crown, rapid collapse of the plants and discoloration of phloem tissue.

The disease was first observed in 1988 in squash and pumpkin, and was observed in other cucurbits only 1991, when it destroyed early planted watermelon (*Citrullus lanatus* (Thunb.) and muskmelon (*Cucumis melo* (L.)) in central Texas and Oklahoma (9). Susceptibility among cucurbits is highest in squash, followed by pumpkin, watermelon, and muskmelon. Other cucurbits, such as cucumber and various gourds, are not affected by CYVD in nature (36).

The geographical range of the disease includes the Cross Timbers region of central and northeastern Oklahoma and north central Texas, east Texas (Post Oak Savannah), all cucurbit-growing areas of Oklahoma, Tennessee, (5), Arkansas (43), Kansas (unpublished data), Colorado (unpublished data) and Massachusetts (58).

S. marcescens is a cosmoplitan pathogen inhabiting a number of ecological niches, including soil and water (43). It has been documented as a plant endophyte (20), opportunistic human and animal pathogen (12) and insect pathogen (14,49,50)

Although its association with plants has been mostly beneficial, two cases of plant pathogenicity for *S. marcescens* have been described in crown rots of sainfoin (48) and alfalfa (25). In both of these cases *S. marcescens* was reported to be part of a pathogen complex.

Several types of circumstantial evidence support the possibility that the CYVD bacterium is transmitted in nature by an insect vector. Early work on CYVD showed that the pathogen was neither soil borne (8) nor seed borne (36). A fringe pattern of disease distribution was noted; symptoms appear first in plants at the edge of the field, then more progressively toward the center of the field. In severe epiphytotics the entire crop may be lost. The possible role of insects as vectors was first recognized in research conducted at the Wes Watkins Agricultural Research and Extension Center in Lane, Oklahoma. Soil fumigation with methyl bromide at $67g/m^3$ soil did not lower the incidence of CYVD. A plastic mulch experiment was conducted to determine if squash bug (*Anasa tristis*, Heteroptera, Coreidae), aphid populations and ground cover, alone or in combination, affected the incidence of CYVD. Regardless of the mulch treatment used, foliar insecticide treatments reduced the incidence of CYVD (8). These data are consistent with the interpretation that the CYVD pathogen is transmitted by an insect vector and is not soil-borne (8). Results of a recent *A. tristis* exclusion experiment were consistent with the interpretation that the CYVD pathogen is transmitted by an insect (4).

Relatively high populations of squash bugs, *A. tristis*, were observed in areas of high CYVD incidence. Recently, insect transmission of S. *marcescens* was achieved experimentally using *A. tristis* as the vector (3,4,36). Transmission rates up to 60% were achieved using single inoculative insects by modifying the artificial acquisition source or feeding insects on plants naturally infected with *S. marcescens*. Transmission requires little or no latent period, and insects were able to transmit within 48 hrs of acquisition. Moreover, transmission capability persisted for at least three weeks (4).

However, although the squash bug is a confirmed vector of *S. marcescens*, other insects such as leafhoppers were considered candidates as vectors of CYVD because many phloem-associated pathogens are transmitted by leafhoppers (Cicadellidae) and about 130 species of leafhoppers are known to be vectors. Furthermore, leafhoppers of certain families feed in the phloem, where the CYVD pathogen is located. When the original yellow vine PCR primer pair, YV1 and YV2, was used to screen field-collected insects for the presence of the CYVD pathogen a band of the expected size was amplified in numerous samples of the gray lawn leafhopper, *Exitianus exitiosus* (Uhler), collected from grassy plots in Stephenville, Texas. Sequencing of the amplified DNA fragment, however, revealed that it was significantly different from that of the CYVD bacterium and, in fact, was homologous to a region of the 16S rRNA gene of an enteric bacterium (*Bacillus Euscelidius variegatus* (Kirshbaum)) (2), which occurs in many leafhoppers (39). Positive PCR results were obtained also with a small number of field-collected specimens of the painted leafhopper, *Endria inimica*

(Say), and of the green sharpshooter, *Draeculacephala minerva* (Ball) (Lori Green, unpublished data). However, confirmation of bacterial identity was not obtained from these samples, leaving open the possibility that they, too, were enteric bacteria. However, in a different field experiment conducted later to identify leafhoppers naturally carrying *S. marcescens*, leafhoppers collected during 1998 and 1999 from three locations in central Oklahoma were screened by PCR using a re-designed and *S. marcescens* specific primer pair, YV1/YV4 (4). *E. exitiosus* was collected in the highest numbers but less than 1% of this species was carrying *S. marcescens*.

Aphids are also well-documneted plant pathogen vectors. Several species transmit phloem-restricted luteoviruses, though none are known to transmit plant pathogenic bacteria.

As vectors of plant pathogens, heteropterans (true bugs) are not considered to be important. Heteropteran stylets are much larger than those of homopterans (aphids and leafhoppers) and can cause significant damage to the plant tissues (54). Only a few heteropterans have been reported to transmit plant pathogens (13,15,16,17,24,27,28,35,42,45,46,47,52) and the causal agent of sugerbeet latent rosette is the only phloem-restricted bacterium known to be transmitted by a heteropteran (35,45).

Lygus hesperus has a very a wide host range and is an important pest of many economically important crops including members of the Cucurbitaceae (cucumber, squash and watermelon), Solanaceae (potato, tomato, eggplant and pepper), Umbelliferae (carrot, celery, fennel, parsley, coriander (Italian parsley) and parsnip), Asteraceae (endive, artichoke, lettuce, escarole and salisfy), Brassicaceae (cabbage, chinese cabbage, broccoli, mustard, radish, horseradish and turnip), and Amaranthaceae (spinach, sugar beet) (55) and Swiss chard), Fabaceae (pea, faba bean and lima bean), Asparagaceae (asparagus), Alliaceae (onion), Convolvulaceae (sweet potato), Poaceae (corn) and Leguminoceae (cowpea). Field crop hosts include alfalfa, cotton and safflower. Strawberries and other fruit and flower seeds are also attacked by *Lygus* bugs (10).

These phloem-feeding insects, such as aphids, leafhoppers (57) and true bugs, might be natural or experimental vectors of *S. marcescens.* Therefore, the research objective of this study was to test three groups of hemipterans; aphids, leafhoppers and the western tarnished plant bug; for their ability to transmit *S. marcescens* under artificial conditions using a feeding medium detection assay.

By characterizing the *S. marcescens* vector range, we will be able to better understand the relationships of the bacteria with their insect vectors. This work will provide a basis for understanding the specificity between *S. marcescens* and its putative insect vectors, which may in turn contribute to the development of control strategies for the pathogen.

Materials and Methods

Insect sources and maintenance. Five aphid species (family Aphidae, subfamily Aphidinae) were tested for their ability to transmit *S. marcescens*. The melon aphid, *Aphis gossypii* (Glover), and the oleander aphid, *A. nerii* (Bayer de Fonscolombe), are of the tribe Aphidini, whereas the pea aphid, *Acyrthosiphon pisum* (Harris), the Russian wheat aphid, *Diuraphis noxia* (Mordvilko), and the green peach aphid, *Myzus persicae* (Sulzer), are of the tribe Macrosiphini.

A. pisum, obtained from J. Dillwith (Oklahoma State University), was reared on 7-10 day old faba bean seedlings (*Vicia faba* (L.) Windsor cv.) and *D. noxia*, obtained from C. Baker (USDA-ARS, Stillwater, OK), was reared on 10-15 day-old wheat seedlings, *Triticum durum* (Desf.). Both colonies were maintained in mesh-screened cages (50 x 25 x 45 cm) in a growth room at 27°C, 12L:12D and 50% RH. *M. persicae*, *A. gossypii* and *A. nerii* were collected in the summer of 2001 from natural populations in vegetables, field crops and weeds at the Oklahoma State University farm, Stillwater, OK and used immediately.

Five leafhopper species were selected from the group Cicadomorpha, series Auchenorrhyncha, family Cicadellidae, subfamily Deltocephalinae: the beet leafhopper, *Circulifer tenellus* (Baker), the corn leafhopper, *Dalbulus maidis* (Delong and Wolcott), the aster leafhopper, *Macrosteles quadrilineatus* (Forbes), the gray lawn leafhopper, *Exitianus exitiosus* (Uhler), and the painted leafhopper, *Endria inimica* (Say).

D. maidis and *C. tenellus* were maintained in colonies on 10-15 day-old corn plants, *Zea mays* (L.), and 7-10 day-old sugar beet plants, *Beta vulgaris,* respectively, as described above. Natural populations of *M. quadrilineatus*, *E. exitiosus* and *E. inimica* were collected in spring and summer 2001 using sweep nets (BioQuip, Gardena, California), from Bermuda grass in field borders and on the OSU campus. Insects were maintained in cages on 10-15 day old corn, barley, *Hordeum vulgare* (L.) and wheat in mesh-screened cages as described above.

L. hesperus was provided by E. Backus, University of Missouri, where the insects were reared on a soybean based liquid diet (Bioserv, *Lygus* diet, Philadelphia, PA). At OSU, the insects were maintained at 27°C, constant light and 45-50% RH in round plastic tubs (diameter 20.5 cm and length 13 cm) covered at the top with a muslin cloth and provided with the same soybean based liquid diet. To prepare one liter of liquid diet, 15.63% of the dry diet was homogenized with 520 ml water in a blender for 10 sec. Four chicken eggs were added and blended 35 sec. The liquid diet was made fresh every 7-10 days to maximize insect feeding, and was provided in 10-15 cm rectangular pouches of un-stretched parafilm sealed by an Impulse heat sealer (Research Product International Corp., Mount Prospect, IL). Each pouch contained 40 ml liquid. Filled pouches were stored at 8°C before being warmed to room temperature for use.

Bacterial cultures and maintenance. *S. marcescens* strain Z01-A was isolated originally on purple agar (Difco, Detroit, MI) from a CYVD affected zucchini plant collected in Oklahoma (2). The strain was triply cloned for population homogeneity and frozen in aliquots at –80°F in 30% glycerol. Bacteria were grown to log phase at 28°C in LB broth (Fisher Biotech, Fair Lawn, NJ) and diluted to 10^5 cells / ml for use in all tests.

Optimization of diets. Most leafhoppers were fed D10 feeding solution (100 mM sucrose, 11 mM fructose, 164 mM potassium phosphate (K_2HPO_4) and 0.29 mM magnesium chloride ($MgCl_2$) (1). However, an experiment was done to evaluate survival of one of the leafhoppers tested, *E. exitiosus*, on two concentrations (6 and 12%) of sucrose supplemented with 1% fetal bovine serum (FBS) (Invitrogen Corp.) and at different pH (6.5, 6.87, 7.0, and 7.5). The insects were confined in 29.5 ml clear plastic medicine cups (4.5 cm diameter, 3.5 cm length) (Baxter Heathcare Corp., Deerfield, IL). The cup was corked at one side and the bottom of the cup was removed and covered with mesh fabric for ventilation. A square of parafilm (American National Can, Greenwich, CTI) was stretched four times its original size over the rim of the plastic cup and sterilized by rinsing with 70% ethanol. A volume of 700-900 ul of the artificial diet was introduced and covered with a second layer of stretched parafilm (26,30,37,56).

Optimization of L. hesperus artificial feeding system. Although *L. hesperus* was reared on a soybean based liquid diet as mentioned above, this diet was inconvenient for transmission experiments because its density made DNA extraction difficult. Therefore, *L. hesperus* survival on other diet formulations containing varying sucrose and agarose concentrations was evaluated. Six treatments were used: 5% sucrose + 0.5 % agarose, 5% sucrose + 1.0% agarose, 5% sucrose + 1.5% agarose, 5 % sucrose + 2 % agarose, deionized water alone and no diet. Thirty insects, divided into three replications (ten insects in each replication), were used for each treatment. Insects were maintained at 27°C, constant light and 45-50% RH. The number of surviving insects was recorded after 24, 48, 72, and 96 hrs. The 5% sucrose + 1.5% agarose combination yielded the highest survival, but the agarose density hampered DNA extraction. When sucrose was used alone at concentrations of 5% or 10%, 5% sucrose provided optimal balance of insect survival and ease of DNA extraction and subsequently was used for all subsequent transmission experiments.

Feeding activity. Among the leafhopper species, *E. exitiosus* was selected to test whether the insects were feeding from the medium in the membrane. *E. exitiosus* adults (sex undetermined) were confined in feeding sachets described above, except that the sachet contained no feeding solution. Four sachets, each containing 10 leafhoppers, were maintained at 27°C, constant light and 45-50% RH. The number of living and dead insects was assessed after 18 hrs.

To test whether *L. hesperus* was feeding from the medium in the membrane, five to ten insects were introduced into cylindrical plastic cup cages (diameter 4 cm, length 6 cm), the open ends of which were covered with muslin cloth and sealed with rubber bands over the cup rim. Some insects were provided deionized water-filled pouches and others received no food or water for 24 hrs.

Pathogenicity of S. marcescens on the insects. The effect of *S. marcescens* on longevity of several aphid and leafhopper species and of the western tarnished plant bug was checked by assessing mortality of those insects fed in an artificial membrane system on either 20-30% sucrose (aphids) or 5-10% sucrose (leafhoppers and *L. hesperus*) (controls), or on sucrose amended with 10^5 bacteria / ml of *S. marcescens*. The number of dead insects was recorded after a 24 hr acquisition access period (AAP) and a 24 hr inoculation access period (IAP).

Insect acquisition of S. marcescens. Feeding medium detection assays were used for correlation of the ability of several insect species to transmit *S. marcescens* under controlled conditions (26,37,56). For aphids and leafhoppers, artificial feeding sachets were constructed as described above. For aphids, the sachets were filled with 20-30% sucrose supplemented with 1% FBS and containing *S. marcescens* at a concentration of 10^5 bacteria / ml. Twenty to fifty adult aphids were introduced into each sachet cup and allowed to feed for a 24 hr AAP. Control insects fed upon sucrose solution without bacteria. The insects were maintained at 27°C, constant light and 45-50% RH.

The leafhoppers were fed on D10 medium or on 6% sucrose supplemented with 1% FBS and containing *S. marcescens* 10^5 bacteria/ml. Five to fifteen leafhoppers were introduced into each sachet and allowed to feed for a 24 hr AAP (Fig. 1). Control insects fed upon D10 or sucrose solution without bacteria. The insects were maintained at 27°C, constant light and 45-50% RH.

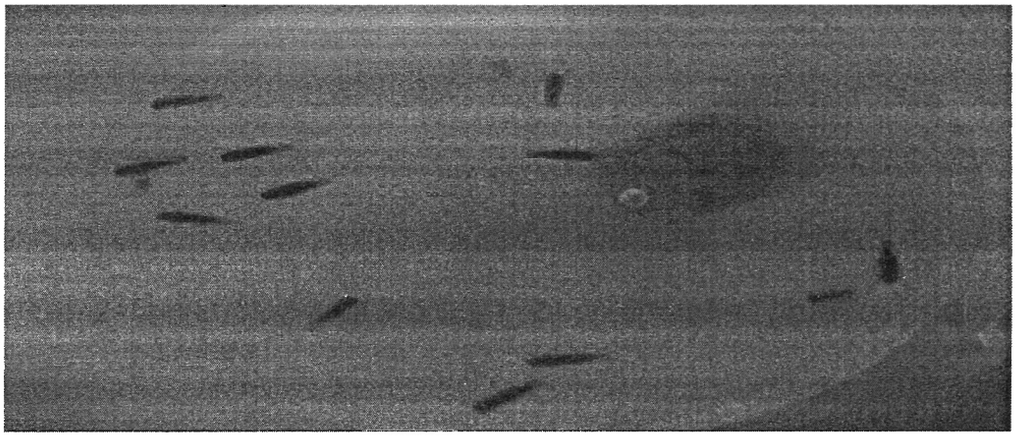

Fig. 1. *Macrosteles quadrilineatus* feeding from artificial membrane feeding sachet.

Un-stretched parafilm feeding pouches used for *L. hesperus* contained 5% sucrose supplemented with 1% fetal bovine serum (FBS) (Invitrogen Corp.) for support of *S. marcescens*. Treatment pouches also contained *S. marcescens* at 10^5 bacteria / ml. Insects were confined in cylindrical plastic cages (diameter 4 cm, length 6 cm), sterilized overnight in 10% Clorox. Pouches (as described above) were filled with two ml (five insects) to three ml (10 insects) of liquid diet, with or without *S. marcescens,* and placed on top of the muslin cloth cage cover. Five to ten insects were introduced into each cage as described above and

allowed to feed through the muslin and parafilm for a 24-48 hr AAP at 27°C, constant light and 45-50% RH (Fig. 2).

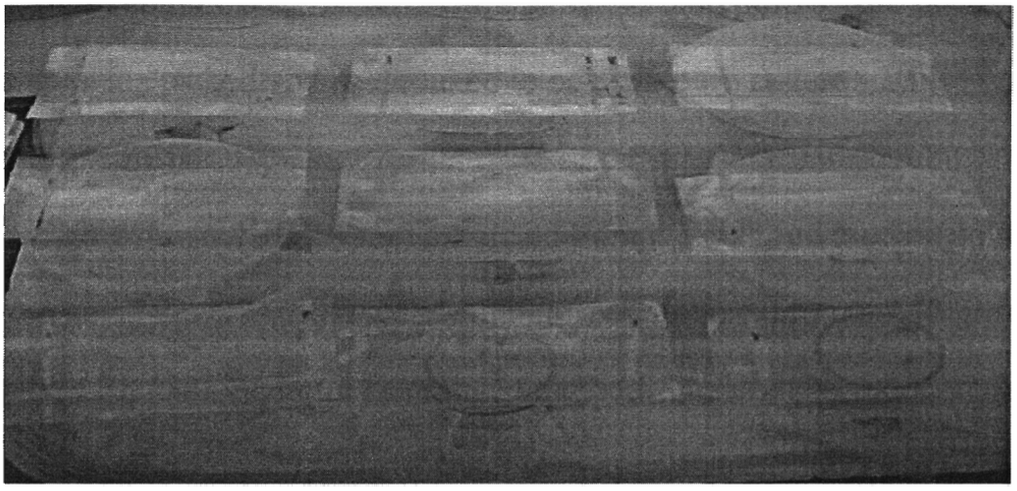

Fig. 2. *Lygus hesperus* feeding from un-stretched parafilm feeding pouches.

Insect inoculation. After the AAP, aphids and leafhoppers were transferred to new sachets containing D10 (1) or 6-10% sucrose supplemented with 1% FBS for a 24 hr inoculation access period (IAP) and *L. hesperus* were transferred to new pouches containing 5% sucrose. Solutions were then collected with a sterile micro pipette, placed into 5 ml LB broth, and incubated, in a shaking incubator (Queue, Lorton, VA) at 28°C + 220 rpm. Bacterial growth was assessed visually by turbidity 2-3 days after inoculation.

The identity of bacteria growing in the sachet-inoculated LB broth was tested by PCR as described below, using the *S. marcescens* specific primer pair, YV1/YV4. In some cases bacteria were streaked onto purple agar plates and single colonies picked and re-streaked to achieve pure cultures, which were subjected to PCR.

DNA extraction. DNA was extracted from the cultured feeding solution samples using the CTAB (hexadecyltrimethylammonium bromide) (Sigma Chemicals Pty Ltd., St. Louis) extraction method. The sample was centrifuged (27,000 x g, 20 min, 4°C) and the pellet re-suspended in 3 ml of 2.5% CTAB (1.4 M NaCl, 1% PVP, 0.02 M EDTA, 0.1 M Tris-HCl, pH=8.0) (Doyle buffer), followed by incubation (55-60°C, 30 min). The suspension was extracted with one volume of chloroform: isoamyl alcohol (24:1, v/v), mixed thoroughly for 2-3 min, and centrifuged (9,500 x g, 10 min, 20°C). The upper phase was placed into a clean 15 ml Corex tube, and isopropanol was added at one-half the original volume to precipitate the DNA. The suspension was incubated at –20°C for 1 hr then centrifuged (31,000 x g, 20 min, 4°C). The supernatant was discarded, 70% ethanol was added to pellet the DNA, and the liquid was centrifuged (8,800 x g, 10 min, 4°C) and the supernatant discarded. The DNA was dried by inverting the tubes for 1 hr, then re-suspended with 100 ul of either Tris EDTA (TE) (10mM Tris-Hcl, pH:8.0, 1mM EDTA) buffer or DNase-RNase free 0.2 uM filtered water.

DNA amplification. PCR reactions were carried out in 25-ul reaction mixtures containing 10-50 ng of genomic DNA template and 0.2 mM of each deoxynucleoside triphosphate, one Unit of Taq DNA polymerase (Promega, Madison, WI), 0.2 uM forward primer YV1 (5'-GGGAGCTTGCTCCCCGG-3') (2) and 0.2 uM reverse primer YV4 (5'-AACGTCAATTGATGAACGTA TTAAGT-3") (29), in a PCR buffer (10mM Tris-HCl, pH 8.3; 5 nM EDTA; 25 mM KCl; 4mM $MgCl_2$). A PTC-100 thermocycler (MJ Research Inc., Watertown, MA) was programmed for 1 min at 95°C, and 34 cycles of 30 s at 94°C, 1 min at 54°C, and 1 min at 72°C, with a final extension at 72°C for 5 min. A 10-ul aliquot of each PCR sample was separated on a 1.2% horizontal agarose gel and stained with (0.5 ug/ml) ethidium bromide. A 100-bp DNA ladder (Sigma Chemicals Pty Ltd., St. Louis) was used as a size marker. A sample was considered positive for *S. marcescens* if a 308 bp DNA band was visualized with UV light (29).

Detection of S. marcescens in insect bodies. The presence of *S. marcescens* in the insect bodies was assessed after the IAP by grinding 10 aphid bodies (averaging 65 ug/insect), one leafhopper body (averaging 3 mg/insect), or one to three *L. hesperus* (approximately 5 mg per insect) per sample in a 1.5 ml micro-centrifuge tube. The DNA extraction method of Goodwin et al. (18) was used with small modifications. A volume of 125 ul of 2% CTAB (pH 8.0) and 2 ul mercaptoethanol was added for every 1 ml CTAB. The suspension was briefly vortexed and incubated 5 min at 65 ^{0}C, then extracted with one volume of chloroform: isoamyl alcohol (24:1, v/v). The nucleic acids were precipitated with 10 ul 5 M sodium acetate (pH 5.2) and 250 ul of 100% ethanol. DNA

preparations were stored in 50 ul aliquots in TE or DNase-RNase free 0.2 uM filtered water at 4°C.

PCR, using primer pair YV1/YV4, was carried out as described for the bacterial samples. Eight ul aliquots of each PCR sample were separated on 1.2% horizontal agarose gels at 100 volts for one hour and stained with (0.5 ug/ml) ethidium bromide to detect 308 bp bands indicating the presence of *S. marcescens*.

Statistical analysis: The data on transmission of *S. marcescens* by leafhoppers and *L. hesperus* in treatments and controls, as well as the pathogenicity data of *S. marcescens* on aphids, leafhoppers and *L. hesperus,* were analyzed using Chi-square at a confidence level of 0.05 (44).

Results

Optimization of diets. E. exitiosus average mortality was zero after 24 hrs and 20% after 48 hrs when insects were offered 12 % sucrose at a pH of either 6.5 or 7.0 (Table 1). However, average mortality was somewhat higher (30% after 24 hrs and 50% after 48 hrs) when *E. exitiosus* was fed on 12% sucrose at pH 7.5, and highest (40% after 24 hrs and 85% after 48 hrs) on 6% sucrose at pH 6.87. Twelve % sucrose at pH 6.5-7.0 was used as the standard diet for *E. exitiosus*.

Survival rates of *L. hesperus* on liquid diets containing varying concentrations of sucrose and agarose are shown in Table 2. *L. hesperus* that were fed as long as 48 hrs on 5% sucrose + 0.5% agarose or 5% sucrose + 1.5% agarose had mortality rates of zero, but after 72 hrs mortality was 6.6% on 5% sucrose + 0.5% agarose. On 5% sucrose + 2% agarose, the mortality was 10% after 24 hrs and 16.6% after 48 hrs. Mortality was highest on deionized water alone, at 10% after 24 hrs, 20% after 48 hrs and 70% after 72 hrs. On 5% and 10% sucrose, the average mortality rate was 3.3% (Table 3).

Feeding activity. The feeding activity test confirmed that *E. exitiosus* were feeding from the sucrose solutions, whether amended or not amended with bacteria, since all insects denied any feeding diet within 24 hrs (the normal AAP and IAP duration used in the experiments), and 90% of the insects in three of the four sachets died after 18 hrs. The *L. hesperus* feeding activity test showed that insects were feeding from un-stretched parafilm feeding pouches, since 100% of

the insects given no food or water, 20% of the insects fed on water only died within 48 hrs and 70% within 72 hrs (Table 2).

Pathogenicity of S. marcescens on the insects. Mortality of the aphids, leafhoppers and *L. hesperus* was almost always higher when they fed on diets amended with *S. marcescens* than on unamended diets (Fig. 3, 4 and 5). The average mortality for *A. pisum* was not significantly different (X^2=2.5, P=0.05) in bacteria treatments than in controls (84% vs. 79%) (Fig. 4). However, mortality was significantly higher in treatments than in controls for *A. nerii* (X^2=10.3, P=0.05) (31.2% vs. 21.4%), *A. gossypii* (X^2=9.3, P=0.05), (37.5% vs. 23%) and *D. noxia* (X^2=4.9, P=0.05) (41.9% vs. 36%) (Table 4).

The mortality of bacteria-exposed leafhoppers was significantly higher than that of controls (X^2=5.5, P=0.05) with only one leafhopper species, *D. maidis* (20% in treatments vs. 6.3% in the controls) (Fig. 5). Chi-square at P=0.05 was not significant for *C. tenellus* (X^2=1.0, P=0.05) (13.3% vs. 8.5%), *E. extiosus* (X^2=0.06, P=0.05) (29.4% vs. 28.5%), *E. inimica* (X^2=0.03, P=0.05) (27.5% vs. 24.2%), or *M. quadrilineatus* (X^2=0.97, P=0.05) (21.4% vs.14.6%) (Table 5).

The average mortality of bacteria-fed western tarnished plant bug also was not significantly different from that of controls (X^2=0.7, P=0.05) (19.6% vs.17.7%) (Fig. 5; Table 6).

Insect transmission of S. marcescens. Aphids were allowed a 24 hr AAP and 24 hr IAP for 66 sachets, and a 48 hr AAP and 48 hr IAP for 7 sachets (Table 7). Solutions were considered positive if a band of 308 bp was visualized (Fig. 6). All five species tested failed to transmit *S. marcescens* to feeding sachets. However, three of the body samples of *A. gossypii* and two of *A. nerii* tested positive for *S. marcescens* by PCR. All control sachets and insect bodies were PCR-negative. The detailed transmission data of each replication is available in Appendix A (Table 10). Three of the five leafhopper species tested, *C. tenellus*, *E. exitiosus* and *E. inimica*, acquired and transmitted *S. marcescens* from and to artificial sachets.

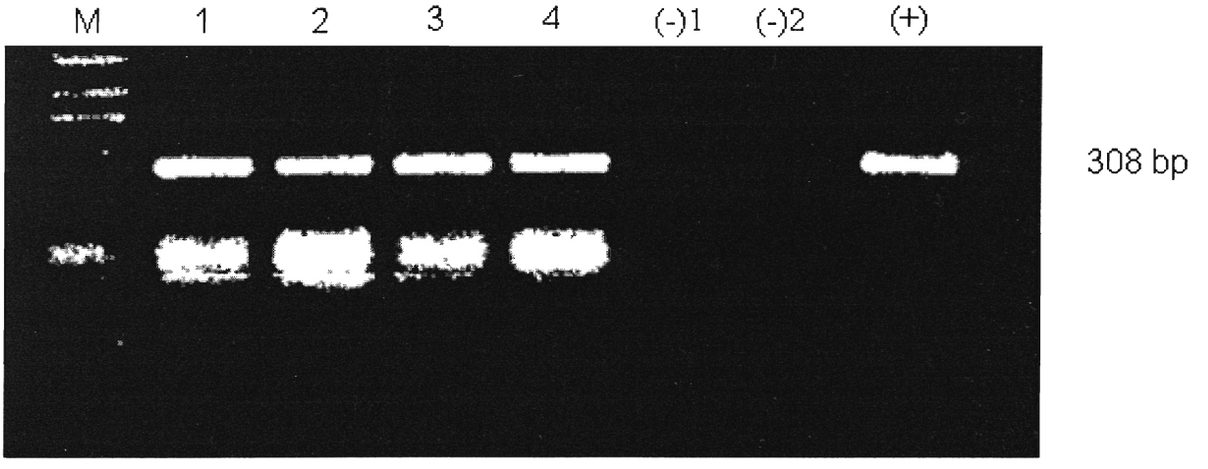

Fig. 6. Agarose gel of Polymerase chain reaction (PCR) products from *Serratia marcescens*-exposed *Exitianus exitiosus* feeding solutions. M=1-kb ladder (Sigma); (1-4) feeding solution treatment; (-)1 = negative control from a healthy source; (-)2 = PCR negative control; (+)= positive control. Expected band size= 308 bp.

Fourteen of 38 (37%) *C. tenellus* sachets, 15 of 46 (32.6%) *E. exitiosus* sachets and four of six (66.6%) *E. inimica* sachets tested were positive by PCR (Table 8). The data were significant when subjected to Chi-square analysis, for *C. tenellus* (X^2=11.8, P=0.05), for *E. exitiosus* (X^2=10.7, P=0.05) and for *E. inimica* (X^2=6, P=0.05). *S. marcescens* was detected also in the bodies of all three species at 77.8% (49/63), 89.1% (41/46) and 100% (4/4), respectively. The other two leafhoppers, *D. maidis* and *M. quadrilineatus,* failed to transmit *S. marcescens* to feeding sachets under these experimental conditions, although 78.3% of the *D. maidis* bodies tested positive for *S. marcescens*. All control sachets and insect bodies were PCR-negative. The detailed transmission data of each replication is available in Appendix A (Table 11).

L. hesperus adults were capable of transmitting *S. marcescens* pouch to pouch. None of the control pouches and the insect bodies tested positive by PCR. Combined data from all experiments show that 33.3% (18 of 54) of the test pouches fed on by *S. marcescens* exposed *L. hesperus* contained the pathogen when tested by PCR (Table 9). The data were significant when subjected to Chi-square at 0.05 (X^2=21.9, P=0.05). The detailed transmission data of each replication is available in Appendix A (Table 12).

The *S. marcescens* transmission rate for *Lygus* was proportional to the length of the IAP, with the percent of positive pouches 10% at 24 hrs IAP, 39.1% at 48 hrs IAP, 66.7% at 72 hrs IAP, and 100% at 96 and 120 hrs IAP (Table 9). Twenty six percent of the *S. marcescens* – fed *Lygus* bodies, but none of the control insects, tested positive by PCR.

Discussion

Prior to this study, many of the insect species used had not previously been assessed for feeding activity on artificial diets. Therefore, it was essential to confirm their feeding by demonstrating mortality in the absence of feeding activity. Thus if insects survived, feeding activity could be inferred. From the feeding test, the death of the insects after 18 hrs without food confirmed that aphids and leafhoppers were feeding from the sachets and *L. hesperus* from the pouches. This result is similar to those observed for the other leafhopper species when given nothing in the sachets, adults died within 24 hours (Wayadande, unpublished). The new diet formulation for *L. hesperus* greatly facilitated DNA extraction without compromising feeding activity.

Although *S. marcescens* is a known insect pathogen (14,23,49,50,51,53), we did not know whether CYVD strains were entomopathogenic. *S. marcescens* had a negative effect on the longevity of 3 species of aphids (*A. nerii*, *A. gossypii* and *D. noxia*) and one leafhopper species (*D. maidis*). The higher mortality rates of insects fed *S. marcescens* may result from a number of bacterial virulence factors among which are enzymes such as protease, gelatinase, lectinase and chitinase, which can adversely affect the insect hosts. These enzymes may inactivate the immune system, allowing the bacteria to reproduce efficiently and leading to septicemia (50,51).

All five aphid species tested failed to transmit the CYVD bacterium sachet-to-sachet, providing no evidence that aphids are a factor in CYVD epidemiology. Previous work (8,9), in which CYVD incidence was unrelated to aphid populations, supports the same conclusion. Aphids are the largest taxon of plant pathogen vectors, transmitting 275 viruses in 19 genera (33). Aphids transmit five virus groups in a non-persistent manner (caulimoviruses, potyviruses, carlaviruses, cucumoviruses and potexviruses), one group (closteroviruses) in a semipersistent manner, two groups (luteoviruses and umbraviruses) in a circulative manner, and one group (rhabdoviruses) in a propagative manner.

All the aphid species selected in this study except for *D. noxia* are insect vectors of important plant pathogens. For example, *A. gossypii* is the vector of many viruses including cucumber mosaic virus (CMV), watermelon mosaic virus 2 (WMV-2), zucchini yellow mosaic virus (ZYMV) and citrus tristeza virus (21). Cucumber mosaic virus, watermelon mosaic virus 2 (WMV-2) and lettuce mosaic virus are transmitted by *M. persicae* (21). *A. pisum* is the vector of pea enation mosaic and yellow bean mosaic viruses (21). *A. nerii* is the vector of araujia mosaic, chili mosaic, papaya ring spot, (types P and W) and sugarcane mosaic viruses (7). However, there are no reports of aphids transmitting bacterial pathogens, and our data are consistent with this conclusion.

In contrast, the CYVD bacterium, *S. marcescens,* was transmitted in the artificial system by three leafhopper species; *E. exitiosus, C. tenellus* and *E. inimica. E. exitiosus* is also an experimental vector of *Spiroplasma kunkelii* and of the maize bushy stunt phytoplasma (31), and is a prevalent leafhopper

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All the aphid species selected in this study except for *D. noxia* are insect vectors of important plant pathogens. For example, *A. gossypii* is the vector of many viruses including cucumber mosaic virus (CMV), watermelon mosaic virus 2 (WMV-2), zucchini yellow mosaic virus (ZYMV) and citrus tristeza virus (21). Cucumber mosaic virus, watermelon mosaic virus 2 (WMV-2) and lettuce mosaic virus are transmitted by *M. persicae* (21). *A. pisum* is the vector of pea enation mosaic and yellow bean mosaic viruses (21). *A. nerii* is the vector of araujia mosaic, chili mosaic, papaya ring spot, (types P and W) and sugarcane mosaic viruses (7). However, there are no reports of aphids transmitting bacterial pathogens, and our data are consistent with this conclusion.

In contrast, the CYVD bacterium, *S. marcescens,* was transmitted in the artificial system by three leafhopper species; *E. exitiosus, C. tenellus* and *E. inimica. E. exitiosus* is also an experimental vector of *Spiroplasma kunkelii* and of the maize bushy stunt phytoplasma (31), and is a prevalent leafhopper

throughout the central United States during the summer months. It was found to be a carrier of *S. marcescens* (Mitchell, unpublished), with 6% of *E. exitiosus* collected from the field-testing positive for *S. marcescens* by PCR (4). *C. tenellus* is a vector of *Spiroplasma citri* and beet curly top virus, both of which are phloem limited in cucurbits. *E. inimica,* a vector of wheat striate mosaic virus and an experimental vector of the celery-infecting strain of aster yellows (11), is found somewhat frequently throughout the growing season in Oklahoma.

The ability of these three leafhoppers to transmit *S. marcescens* may be explained by the fact that they have highly modified mouthparts suitable for piercing and sucking sap from their host plants, and that they insert their stylets intracellulary into the vascular tissues. However, neither *D. maidis*, the vector of *S. kunkelii*, the maize bushy stunt phytoplasma (MBSP) and the maize rayado fino marafivirus (MRFV) (32), nor *M. quadrilineatus,* the vector of aster yellows phytoplasma, were capable of transmitting the bacterium in this artificial system. These results suggest that there is a degree of specificity between *S. marcescens* and its insect vectors. Although *C. tenellus*, *E. exitiosus* and *E. inimica* all feed on cucurbits, and this study demonstrates they are all experimental vectors of *S. marcescens* under the conditions tested, their role in transmitting *S. marcescens* in the field or in natural spread of CYVD has yet to be assessed. The presence of *S. marcescens* in the test feeding solution is presumably due to the extravasation of the pathogen from the food canal during feeding. The PCR screening of the artificial diets provides an easy and reliable method to test insect inoculativity and enable large-scale screening.

It was demonstrated that *L. hesperus* is an experimental vector of *S. marcescens* under the conditions of these experiments. Detection of *S. marcescens* in liquid diet fed on by bacteria-exposed insects indicates that the pathogen was extravasated from the food canal into the solution during feeding. The transmitted bacteria must have been acquired during the AAP. The percentage of test pouches positive for *S. marcescens* ranged from 10-100%, depending on the length of the IAP. Because each test pouch was exposed to multiple (5 or 10) insects, the rate of transmission of individual insects given a 96 or 120 hr IAP could have been as low as 10% or as high as 100%.

To our knowledge, this is only the sixth report of phytopathogen transmission by an insect in the family Miridae, possibly because the Miridae are not significant vectors or because few studies have been done (15,16,17,42). The spinach blight virus (tomato fern leaf virus) is transmitted mechanically by the tarnished plant bug, *Lygus lineolaris (pratensis)* (28). The fire blight bacterium, *Erwinia amylovora,* is transmitted to pear fruits by *Lygus* spp. (24,52). *E. carotovora,* the causal agent of celery heart rot, is transmitted by *L. lineolaris*, which carries the bacteria externally on its legs (24). The seed and pollen borne potato spindle tuber viroid is also transmitted by *L. lineolaris* (45,46), and the velvet tobacco mottle sobemovirus is transmitted by *Cyrtopeltis nicotianae* in Australia. The latter report is the only one of natural transmission, and in this case the pathogen is transmitted circulatively (15,16,17,42). *C. nicotiana* was found to also transmit solanum nodiflorum mottle virus (19).

In this work, *S. marcescens* was detected in some but not all, of the bodies of insects that fed on bacteria. Detection of *S. marcescens* in insect bodies is not a reliable indicator of the ability of the insects to transmit the CYVD pathogen. The presence of the bacterium in the whole body samples of *D. maidis*, despite this species' failure to transmit, indicates that this species did acquire the pathogen but was not able to extravasate (expel) the bacteria from food canal. However, this is not surprising since ingestion of the pathogen very likely occurs during feeding. Testing the insect's bodies by PCR showed that 10 - 44.4% carried *S. marcescens.* These percentages are conservative, and optimization of the extraction procedure could reveal higher inoculation rates.

The fact that three leafhoppers (*C. tenellus*, *E. exitiosus* and *E. inimica*) and *L. hesperus* transmit *S. marcescens,* while two leafhoppers (*D. maidis* and *M. quadrilineatus*) and five species of aphids do not may be due to one or more of several factors. One parameter may be the attachment and detachment of *S. marcescens* to and from the foregut lining of the insects during the feeding. Another insect-transmitted vascular pathogen, *X. fastidiosa,* attaches to the surface of the cibarial pump, in the food meatus and in the groove of the cibarium floor connecting the food meatus with the esophagus (6,22,38,40). The bacterium was also seen attached to the pump diaphragm.

There are other interesting similarities between *S. marcescens* and *X. fastidiosa* and their respective insect vectors, *A. tristis* and several sharpshooters. Both lack a latent period required for transmission; *S. marcescens* was transmitted within 24 hrs and *X. fastidiosa* in as little as 2 hrs by *Graphocephala atropunctata* (Say) (37), indicating that even small numbers of bacteria that are attached in the food canal distal to the cibarial valve are sufficient for transmission. The mode of transmission is non circulative for *X. fastidiosa* and may be the same for *S. marcescens*. Sharpshooter vectors of *X. fastidiosa* transmit for life, while squash bugs transmit *S. marcescens* for at least 21 days and *L. hesperus* transmit *S. marcescens* for at least 5 days. This is consistent with pathogen retention in the vector, suggesting a non-circulative pathway. *X. fastidiosa* cells secrete a polysaccharide matrix that helps in extraction of nutrients from the xylem sap and protect on of the bacteria from being flushed out by the rapidly moving solute stream during feeding (6,22,38,40,41). A similar matrix may be produced by *S. marcescens.*

The fact that *L. hesperus* transmits in a manner similar to that used by the squash bug, *A. tristis*, at least in an artificial system, opens the possibility that the *Lygus*-*S. marcescens* system could be used as a model for *A. tristis* and *S. marcescens.* Working with *L. hesperus* is more convenient than working with *A. tristis* because an artificial liquid diet has been developed for the former. At present, the only artificial feeding system known for *A. tristis* is vacuum infiltrated cubes of excised squash fruits (3). DNA extraction is also easier from a liquid diet

than from squash cubes. However, the transmission of *L. hesperus* to plants should be assessed.

Acknowledgments

I would like to thank Dr. J. Dillwith, Department of Entomology and Plant Pathology, OSU, for providing colony reared *A. pisum*, Cheryl Baker, USDA-ARS, Stillwater, for providing *D. noxia* and Elaine Backus, University of Missouri, for providing *L. hesperus.* I would like also to acknowledge funding from the USDA Southern Regional Integrated Pest Management Program and the Oklahoma Agricultural Experiment Station.

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Table 1: Mortality of *Exitianus exitiosus* on sucrose solutions at different concentrations and pH.

% Sucrose pH	Number of leafhoppers per sachet	Cumulative % Mortality			
		24-48 hr	48-96 hr	96-144 hr	After 144 hr
6 % pH 6.87	10	40	70	80	100
	10	40	100	-	-
Average	10	40	85	90	100
12% pH 6.5	10	0	20	90	100
	10	0	20	90	100
Average	10	0	20	90	100
12% pH 7.0	10	0	20	70	100
	10	0	20	90	100
Average	10	0	20	80	100
12% pH 7.5	10	30	70	100	-
	10	30	30	80	100
Average	10	30	50	90	100

Table 2: Mortality of *Lygus hesperus* on liquid diets with different sucrose and agarose concentrations.

Diet contents	No. of insects tested*	Cumulative % Mortality				Average % mortality for 24 hr
		24 hrs	24-48 hrs	48-72 hrs	72-96 hrs	
5 % sucrose + 0.5% agarose	30	0	0	6.6	6.6	1.7
5 % sucrose + 1.0 % agarose	30	0	6.6	6.6	9.9	2.5
5 % sucrose + 1.5 % agarose	30	0	0	0	3.3	0.8
5 % sucrose + 2 % agarose	30	10	16.6	16.6	16.6	4.2
Water	30	10	20	70	85	21.3
No pouch	10	40	100			25

* Data from three replications (ten insects each) were pooled.

Table 3: Mortality of *Lygus hesperus* on two sucrose concentrations.

Diet contents	No. of insects tested*	Cumulative % Mortality				Average % mortality for 24 hr
		24 hrs	24-48 hrs	48-72 hrs	72-96 hrs	
5 % sucrose	30*	0	0	6.6	13.2	3.3
10 % sucrose	30	3.3	9.9	13.2	13.2	3.3

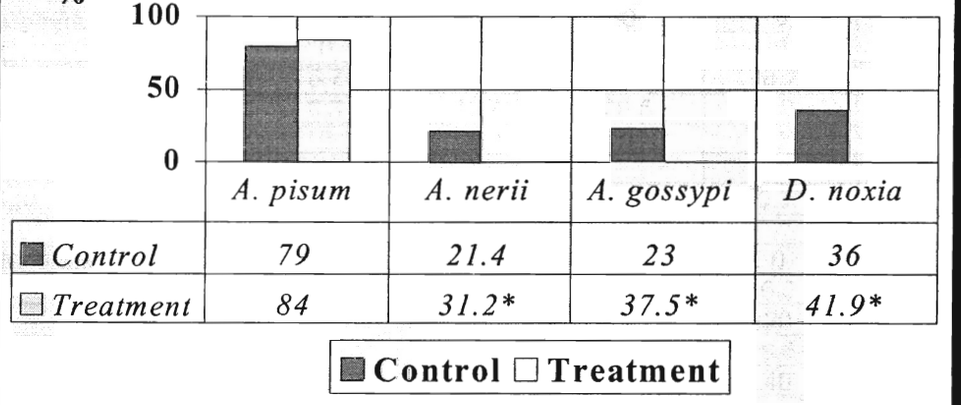
* Data from three replications (ten insects each) were pooled.

Table 4: Mortality of aphids after feeding on *Serratia marcescens*.

Species	Rep.	Control[a]			Treatment[b]		
		M[c] % after AAP	M[c] % after IAP	Average M %	M[c] % after AAP	M[c] % after IAP	Average M %
Acyrthosiphon pisum	1	ND[d]	79		ND	84	
Average		ND	**79**	**79**	ND	**84**	**84**
Aphis nerii	1	ND	59	59	ND	79	79
	2	3.3	19.8	11.5	10	25.9	18
Average		**3.3**	**39.4**	**21.4**	**10***	**52.5***	**31.2***
Aphis gossypii	1	32.7	27.3	30	46	34	40
	2	22	10	16	40	30	35
Average		**27.4**	**18.7**	**23**	**43***	**32***	**37.5***
Diuraphis noxia	1	27.2	65.5	46.4	41.8	40	40.9
	2	28	23	25.5	73.8	12	42.9
Average		**27.6**	**44.3**	**36**	**57.8***	**26***	**41.9***

[a] AAP was on sachets containing sucrose.
[b] AAP was on sachets containing sucrose supplemented with *S. marcescens* Z01-A at 10^5 bacteria/ml. IAP sachet solutions were added to culture medium.
[c] Mortality.
[d] Not done.
* Significantly different (P=0.05)

Fig. 3. Mortality of aphids after feeding on *S. marcescens* Z01-A



	A. pisum	*A. nerii*	*A. gossypi*	*D. noxia*
Control	*79*	*21.4*	*23*	*36*
Treatment	*84*	*31.2**	*37.5**	*41.9**

Control Treatment

* Significant at P=0.05 (Chi-Square).

Table 5: Mortality of leafhoppers after feeding on *Serratia marcescens*.

Species	Rep.	Control[a]			Treatment[b]		
		M[c] % after AAP	M[c] % after IAP	Average M %	M[c] % after AAP	M[c] % after IAP	Average M %
Dalbulus maidis	1	0	0	0	0	33.3	16.6
	2	0	0	0	0	33.3	16.6
	3	6.7	3.3	5	6.7	33.3	20
	4	6.3	46.7	26.5	10	83.3	46.6
	5	0	0	0	0	0	0
Average		**2.6**	**10**	**6.3**	**3.3***	**36.6***	**20***
Circulifer tenellus	1	13.3	11.5	12.4	0	10	5
	2	0	20	10	6.6	26.6	16.6
	3	13.3	0	6.7	20	8.3	14.2
	4	6.6	0	3.3	6.6	14.3	10.5
	5	20	0	10	40	0	20
Average		**10.6**	**6.3**	**8.5**	**14.6**	**11.8**	**13.3**
Exitianus exitiosus	1	10	51	30.5	12.5	37.5	25
	2	1.1	20	10.6	10	20	15
	3	25	20	22.5	37.5	20	28.8
	4	60	30	45	18.8	76	47.4
	5	40	28	34	42.5	18.8	30.7
Average		**27.2**	**29.8**	**28.5**	**24.3**	**34.5**	**29.4**
Endria inimica	1	50	30	40	60	25	42.5
	2	16.7	0	8.4	0	25	12.5
Average		**33.4**	**15**	**24.2**	**30**	**25**	**27.5**
Macrosteles quadrilineatus	1	8	7.5	7.8	10	17.5	13.8
	2	19.4	23.4	21.4	57.7	0	28.9
Average		**13.7**	**15.5**	**14.6**	**33.9**	**8.8**	**21.4**

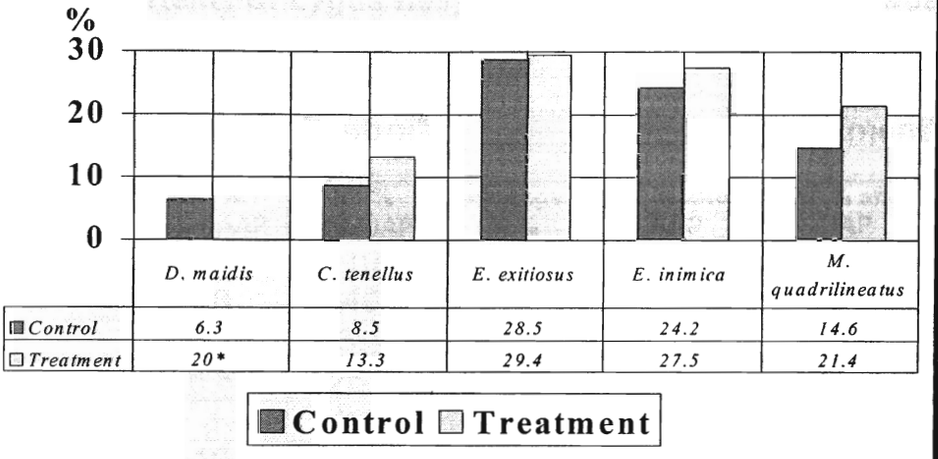
[a] AAP was on sachets containing D10 or sucrose.

[b] AAP was on sachets containing sucrose supplemented with *S. marcescens* Z01-A at 10^5 bacteria/ml. IAP sachet solutions were added to culture medium.

[c] Mortality.

* Significantly different (P=0.05)

Fig. 4. Mortality of leafhoppers after feeding on *Serratia marcescens* Z01-A



	D. maidis	*C. tenellus*	*E. exitiosus*	*E. inimica*	*M. quadrilineatus*
Control	6.3	8.5	28.5	24.2	14.6
Treatment	20*	13.3	29.4	27.5	21.4

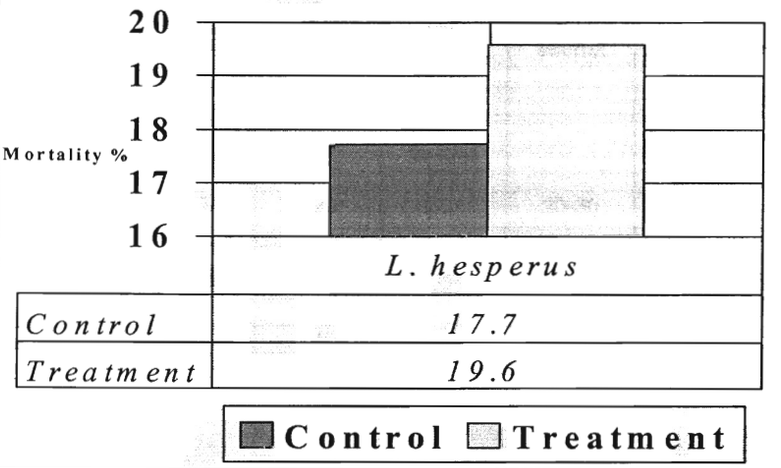
* Significant at P=0.05 (Chi-Square).

Table 6: Mortality of *Lygus hesperus* after feeding on *Serratia marcescens*.

Rep.	Control[a]			Treatment[b]		
	M[c] % after AAP	M[c] % after IAP	Average M %	M[c] % after AAP	M[c] % after IAP	Average M %
1	4	10	7	2	12	7
2	50	40	45	30	50	40
3	6.6	3.3	5	10	6.6	8.3
4	6.6	26.7	16.7	6.6	16.7	11.7
5	3	46.7	24.9	30	30	30
6	10	30	20	6.6	23.3	15
7	6.6	56.7	31.7	16.7	53.3	35
8	ND[d]	5.3	5.3	ND	25.3	25.3
9	6.7	.66	3.7	6.6	1.3	4
Total	11.7	24.4	**17.7**	13.6	24.3	**19.6**

[a] AAP was on un-stretched parafilm feeding pouches containing sucrose.
[b] AAP was on un-stretched parafilm feeding pouches containing sucrose supplemented with *S. marcescens* Z01-A at 10^5 bacteria/ml. IAP pouch solutions were added to culture medium.
[c] Mortality.
[d] Not done.

Fig. 5. Mortality of *Lygus hesperus* after feeding on *Serratia marcescens* Z01-A



* Significant at P=0.05 (Chi-Square).

Table 7: Aphid acquisition and inoculation of *Serratia marcescens* using artificial feeding sachets.

Species	Rep	Length of AAP-IAP (hr)	Control[a]			Treatment[b]			Treatment [c] No. of PCR (+) /No. of insects
			No. of insects/ sachet	Total no. of insects	No. of IAP sachets (+) by PCR /No. tested (%)	No. of insects/ sachet	Total no. of insects	No. of IAP sachets (+) by PCR /No. tested (%)	
Acyrthosiphon pisum	3	24-24	20-30	520	0/21	20-30	520	0/21	
Total					**0/21**			**0/21 (0)**	
Myzus persicae	1	48-48	40	240	0/6	40	240	0/6	
Total					**0/6**			**0/6 (0)**	
Aphis nerii	4	24-24	30-55	830	0/17	30-55	830	0/17	2[d]
Total					**0/17**			**0/17 (0)**	
Aphis gossypii	1	24-24	50	150	0/3	50	150	0/3	2[d]
	1	48-48	50	50	0/1	50	50	0/1	1[d]
Total				**200**	**0/4**		**200**	**0/4 (0)**	
Diuraphis noxia	2	24-24	25-50	600	0/17	25-50	815	0/25	
Total					**0/17**			**0/25 (0)**	

[a] AAP was on sachets containing sucrose.

[b] AAP was on sachets containing sucrose supplemented with *S. marcescens* Z01-A at 10^5 bacteria/ml. IAP sachet solutions were added to culture medium and bacteria were identified by PCR. Solutions in which bacteria did not grow were not tested by PCR.

[c] Insect bodies tested.

[d] Each sample contained 10 individuals.

Table 8. Leafhopper acquisition and inoculation of *Serratia marcescens* using artificial feeding sachets.

Species	Rep	Length of AAP-IAP (hr)	Control[a]			Treatment[b]			Treatment[c] No. of PCR (+) /No. of insects (%)
			No. of insects/ sachet	Total no. of insects	No. of IAP sachets (+) by PCR /No. tested (%)	No. of insects/ sachet	Total no. of insects	No. of IAP sachets (+) by PCR /No. tested (%)	
Dalbulus maidis	9	24-24	2-11	200	0/28	2-11	226	0/30 (0)	13/18 (72.2)
	1	48-72	8	8	0/1	8	8	0/1 (0)	5/5 (100)
Total				**208**	**0/29**		**234**	**0/31 (0)**	**18/23 (78.3)**
Circulifer tenellus	7	24-24	5-7	123	0/21	5-7	214	12/34 (35.3)	39/53 (73.6)
	1	48-48	5	15	0/3	5	15	1/3 (33.3)	6/6 (100)
	1	72-72	5	5	0/1	5	5	1/1 (100)	4/4 (100)
Total				**143**	**0/25**		**234**	**14/38 (37)**	49/63 (77.8)
Exitianus exitiosus	4	24-24	5-8	52	0/9	5-8	151	13/29 (44.8)	26/29 (90)
	2	48-48	10	170	0/17	10	170	2/17 (12)	15/17 (88.2)
Total				**222**	**0/26**		**321**	**15/46 (32.6)**	41/46 (89.1)
Endria inimica	2	24-24	2-5	25	0/4	2-5	27	4/6 (66.6)	4/4 (100)
Total				**25**	**0/4**		**27**	**4/6 (66.6)**	**4/4 (100)**
Macrosteles quadrilineatus	1	48-48	5	40	0/8	5	40	0/8 (0)	0/8
	2	24-24	2-8	16	0/3	3-5	11	0/3	
Total				**56**	**0/11**		**51**	**0/11 (0)**	**0/8**

[a] AAP was on sachets containing D10 or sucrose.

[b] AAP was on sachets containing sucrose supplemented with *S. marcescens* Z01-A at 10^5 bacteria/ml. IAP sachet solutions were added to culture medium and bacteria were identified by PCR. Solutions in which bacteria did not grow were not tested by PCR.

[c] Insect bodies tested.

Table 9. *Lygus hesperus* acquisition and inoculation of *Serratia marcescens* using un-stretched parafilm feeding pouches.

No. of Reps.	Length of AAP-IAP (hr)	Control[a]			Treatment[b]			Treatment[c]
		No. of insects/ pouch	Total no. of insects	No. of IAP pouches (+) by PCR /No. tested (%)	No. of insects/ pouch	Total no. of insects	No. of IAP pouches (+) by PCR /No. tested (%)	No. of PCR (+) /No. of insects (%)
2	24-24	10-30	300	0/20	10-30	300	2/20 (10)	ND[d]
1	48-24	10	30	0/3	10	30	0/3 (0)	3/12 (25)
3	48-48	10	230	0/23	10	230	9/23 (39.1)	12/27 (44.4)
1	48-72	10	30	0/3	10	30	2/3 (66.7)	2/12 (16.7)
1	48-96	10	30	0/3	10	30	3/3 (100)	3/18 (16.7)
1	48-120	10	30	0/3	10	30	2/2 (100)	1/10 (10)
Total			650	0/55		650	18/54 (33.3)	21/79 (26.6)

[a] AAP was on un-stretched parafilm feeding pouches containing sucrose.

[b] AAP was on un-stretched parafilm feeding pouches containing sucrose supplemented with *S. marcescens* Z01-A at 10^5 bacteria/ml. IAP pouch solutions were added to culture medium and bacteria were identified by PCR. Solutions in which bacteria did not grow were not tested by PCR.

[c] Insect bodies tested.

[d] Not done.

Summary

In 1988, the cucurbit yellow vine disease (CYVD) devastated squash and pumpkins in Texas and Oklahoma. In 1991 it was found also infecting melons (Bruton et al. 1995, 1998). The disease has been associated with the presence of phloem–inhabiting bacteria that were identified as *Serratia marcescens* (Rascoe et al., 2000). The natural vector was determined to be the squash bug, *Anasa tristis* (Bextine et. Al. 2000, Bextine 2001).

Since CYVD is a phloem-associated bacterium (Rascoe et al., 2000), it was logical to test other hempiterans, particularly homopterans, for their ability to transmit *S. marcescens*. Homopterans, which have a long history in transmitting phloem limited pathogens, have small stylets that do not disturb the phloem during ingestion of fluids (57), hence are capable of transmitting pathogens.

This research project was conducted to test several homopteran insects, and one heteropteran, for transmission under laboratory conditions. Ten homopterans were selected. The five aphid species were *Aphis gossypii, A. neriii*, *Acyrthosiphon pisum, Myzus persicae* and *Diuraphis noxia*, and the five leafhopper species were *Circulifer tenellus*, *Exitianus. exitiosus*, *Endria inimica, Dalbulus maidis* and *Macrosteles quadrilineatus*. In addition, I chose to test a heteropteran insect, *Lygus hesperus*, whose feeding mode is similar to that of *A. tristis*. An artificial feeding system assay was selected because it is easy to use and most of the insects used in this study do not feed on cucurbits.

None of the five species of aphids tested were able to transmit *S. marcescens* from the feeding sources under the conditions tested. In contrast, three of the five leafhopper species tested (*C. tenellus*, *E. exitiosus* and *E. inimica*) transmitted *S. marcescens* from the feeding sachets. Two leafhopper species (*D. maidis* and *M. quadrilineatus*) did not transmit the bacterium under these conditions. *L. hesperus* adults were capable of transmitting *S. marcescens* pouch to pouch. The transmission rate of *L. hesperus* was proportional to the length of the IAP: the greater the length of time post acquisition, the higher the percent of PCR positive pouches. These data suggest some degree of transmission specificity as all of the five aphid species and two out of five leafhopper species failed to transmit.

Currently, *A. tristis* is the only identified competent vector of *S. marcescens.* The fact that none of the five aphid species tested were able to transmit *S. marcescens* from sachet to sachet suggests that aphids do not have a role in the spread of the pathogen. There is a possibility that the three leafhoppers shown capable of transmission, or *L. hesperus*, play a role in the field in spreading the disease, particularly since they feed on cucurbits. However, this must be assessed in future research.

The fact that *L. hesperus* transmits in a manner similar to that used by the squash bug, *A. tristis*, at least in an artificial system, opens the possibility that *Lygus-S. marcescens* system could be used as a model for *A. tristis* and *S. marcescens.* Working with *L. hesperus* is more convenient than working with *A. tristis* because an artificial liquid diet has been developed for the former. At

present, the only artificial feeding system known for *A. tristis* is vacuum infiltrated cubes of excised squash fruits (Bextine 2001). DNA extraction is also easier from a liquid diet than from squash cubes.

Five aphid species and the leafhoppers *D. maidis* and *M. quadrilineatus* were unable to inoculate *S. marcescens* to artificial sachets. It is useful to consider possible explanations for the pathogen-vector specificity observed in these experiments.

The Pierce's disease bacterium, *Xylella fastidiosa,* has been reported to attach to the lining of the cibarial pump, in the food meatus and in the groove of the cibarium floor connecting the food meatus with the esophagus of its vector, the sharpshooter *Homalodisca coagulata* (Brlansky et al. 1983, Hill and Purcell, 1995, Purcell et al. 1979). It is possible that *S. marcescens* also attaches to the foregut lining of vector species, but not of aphids or other insects. This hypothesis must be further tested.

There are interesting similarities between *S. marcescens* and *X. fastidiosa* and their respective insect vectors, *A. tristis* and several sharpshooters. Both lack a long latent period during transmission; *S. marcescens* was transmitted within 24 hrs and *X. fastidiosa* in as little as three hrs, indicating that even small numbers of bacteria that are attached in the food canal distal to the cibarial valve are sufficient for transmission. The mode of transmission is non circulative in both cases. Sharpshooter vectors of *X. fastidiosa* transmit for life. The data from this study indicate that squash bugs transmit *S. marcescens* for at least 21 days and *L. hesperus* transmits for at least 5 days; in both these cases it is possible

that transmission capability persists for life, since these experiments were not designed to reveal this information.

X. fastidiosa cells secrete a polysaccharide matrix that helps in extraction of nutrients from the xylem sap and protecting the bacteria from being flushed out by the rapidly moving solute stream during feeding (Brlansky et al. 1983, Hill and Purcell, 1995, Purcell et al. 1979). A similar matrix may be produced by *S. marcescens.*

S. marcescens is known to be an insect pathogen. For example, in *Heliothis virescens* adults, bacterial infection caused 90.1% mortality (Sikorowski and Lawrence 1998). However, it was not known whether CYVD strains had this property. Pathogenicity tests of *S. marcescens* on the three different insect groups revealed that *S. marcescens* does have a negative impact on the longevity of aphids, leafhoppers and *L. hesperus.*

The higher mortality rates of insects fed *S. marcescens* may be due to one or more of a number of bacterial virulence factors, among which are enzymes such as protease, gelatinase, lectinase and chitinase, which can adversely affect the insect hosts. These enzymes may inactivate the immune system allowing the bacteria to reproduce efficiently and leading to septicemia (Sikorowski et al, 1998 and 2001).

Appendix A

Aphids, leafhoppers and *Lygus hesperus* acquisition and inoculation of *Serratia marcescens*

The following tables are expanded data of aphid, leafhopper species and *L. hesperus* corresponding to Tables 7, 8 and 9 in Chapter 2.

Table 10. Aphid acquisition and inoculation of *S. marcescens* using artificial feeding sachets.

Species	Rep.	Length of AAP-IAP (hr)	Control[a]			Treatment[b]			Treatment[c] No. of PCR (+) /No. of insects
			No. of insects/ sachet	Total no. of insects	No. of IAP sachets (+) by PCR /No. tested (%)	No. of insects/ sachet	Total no. of insects	No. of IAP sachets (+) by PCR /No. tested (%)	
Acyrthosiphon pisum	1	24-24	20	100	0/5	20	100	0/5 (0)	
	2	24-24	20	120	0/6	20	120	0/6 (0)	
	3	24-24	30	300	0/10	30	300	0/10 (0)	
Total				**520**	**0/21**		**520**	**0/21 (0)**	
Myzus persicae	1	48-48	40	240	0/6	40	240	0/6 (0)	
Total				**240**	**0/6**		**240**	**0/6 (0)**	
Aphis nerii	1	24-24	55	330	0/6	55	330	0/6 (0)	
	2	24-24	55	330	0/6	55	330	0/6 (0)	
	3	24-24	50	50	0/1	50	50	0/1 (0)	
	4	24-24	30	120	0/4	30	120	0/4 (0)	2[d]
Total				**830**	**0/17**		**830**	**0/17 (0)**	
Aphis gossypii	1	24-24	50	150	0/3	50	150	0/3 (0)	2[d]
	2	48-48	50	50	0/1	50	50	0/1 (0)	1[d]
Total				**200**	**0/4**		**200**	**0/4 (0)**	
Diuraphis noxia	1	24-24	25-50	375	0/8	25-50	465	0/16 (0)	
	2	24-24	25	225	0/9	25-50	350	0/9 (0)	
Total				**600**	**0/17**		**815**	**0 /25 (0)**	

[a] AAP was on sachets containing sucrose.

[b] AAP was on sachets containing sucrose supplemented with *S. marcescens* Z01-A at 10^5 bacteria/ml. IAP sachet solutions were added to culture medium and bacteria were identified by PCR. Solutions in which bacteria did not grow were not tested by PCR.

[c] Insect bodies tested.

[d] Each sample contained 10 individuals.

Table 11. Leafhopper acquisition and inoculation of *S. marcescens* using artificial feeding sachets.

Species	Rep.	Length of AAP-IAP (hr)	Control[a]			Treatment[b]			Treatment[c] No. of PCR (+) /No. of insects (%)
			No. of insects/ sachet	Total no. of insects	No. of IAP sachets (+) by PCR /No. tested (%))	No. of insects/ sachet	Total no. of insects	No. of IAP sachets (+) by PCR /No. tested (%)	
Dalbulus maidis	1	24-24	7	14	0/2	7	28	0/4 (0)	ND[d]
	2	24-24	7	42	0/6	7	42	0/6 (0)	ND
	3	24-24	11	66	0/6	11	66	0/6 (0)	ND
	4	24-24	3	3	0/1	3	3	0/1 (0)	ND
	5	24-24	3	3	0/1	3	3	0/1 (0)	3/3 (100)
	6	24-24	5	30	0/6	5	30	0/6 (0)	2/4 (50)
	7	24-24	8	24	0/3	8	24	0/3 (0)	4/7 (57.1)
	8	48-72	8	8	0/1	8	8	0/1 (0)	3/5 (75)
	9	24-24	8	16	0/2	10	20	0/2 (0)	1/3 (33.3)
	10	24-24	2	2	0/1	2	2	0/1 (0)	1/1 (100)
Total				**208**	**0/29**		**226**	**0/31 (0)**	**14/23 (61)**
Circulifer tenellus	1	24-24	7	14	0/2	7	28	0/4 (0)	ND
	2	24-24	7	14	0/2	7	28	4/4 (100)	ND
	3	24-24	7	14	0/2	7	28	6/10 (60)	ND
	4	24-24	7	21	0/3	7	70	0/6 (0)	ND
	5	24-24	5	30	0/6	5	30	0/3 (0)	18/30 (60)
	6	24-24	5	15	0/3	5	15	1/4 (25)	10/10 (100)
	7	24-24	5	15	0/3	5	15	1/3 (33.3)	11/13 (84.6)
	8	48-48	5	15	0/3	5	15	1/3 (33.3)	6/6 (100)
	9	72-72	5	5	0/1	5	5	1/1 (100)	4/4 (100)
Total				**143**	**0/25**		**234**	**14/38 (37)**	**49/63 (77.8)**
Exitianus exitiosus	1	48-48	10	80	0/8	10	80	0/8 (0)	7/8 (87.5)
	2	48-48	10	90	0/9	10	90	2/9 (22.2)	8/9 (88.9)
	3	24-24	8	8	0/1	8	8	1/1 (100)	1/1 (100)
	4	24-24	5	10	0/2	5	10	2/2 (100)	4/4 (100)
	5	24-24	5	20	0/4	5	120	10/24 (41.7)	21/24 (87.5)
	6	24-24	7	14	0/2	5-8	13	0/2 (0)	ND
Total				**222**	**0/26**		**321**	**15/46 (32.6)**	**41/46 (89.1)**
Endria inimica	1	24-24	5	20	0/2	5	20	¾ (75)	4/4 (100)
	2	24-24	2-3	5	0/2	**2-5**	**7**	**½ (50)**	ND
Total				**25**	**0/4**		**27**	**4/6 (66.6)**	**4/4 (100)**
Macrosteles quadrilineatus	1	48-48	5	40	0/8	5	40	0/8 (0)	0/4
	1	24-24	2-8	16	0/3	3-5	11	0/3 (0)	ND
Total				**56**	**0/11**		**51**	**0/11 (0)**	**0/8 (0)**

[a] AAP was on sachets containing D10 or sucrose.

[b] AAP was on sachets containing sucrose supplemented with *S. marcescens* Z01-A at 10^5 bacteria/ml. IAP sachet solutions were added to culture medium and bacteria were identified by PCR. Solutions in which bacteria did not grow were not tested by PCR.

[c] Insect bodies tested.

[d] Not done

Table 12. *Lygus hesperus* acquisition and inoculation of *Serratia marcescens* using un-stretched parafilm feeding pouches.

Rep.	Length of AAP-IAP (hr)	Control[a]			Treatment[b]			Treatment [c] No. of PCR (+) /No. of insects (%)
		No. of insects/ sachet	Total no. of insects	No. of IAP sachets (+) by PCR /No. tested (%)	No. of insects/ sachet	Total no. of insects	No. of IAP sachets (+) by PCR /No. tested (%)	
1	48-48	10	50	0/5	10	50	1/5 (20)	ND
2	24-24	10	150	0/15	10	150	1/15 (6.7)	ND[d]
3	48-24	10	30	0/3	10	30	0/3 (0)	3/12 (25)
4	48-48	10	30	0/3	10	30	2/3 (66.7)	1/9 (11.1)
5	48-72	10	30	0/3	10	30	2/3 (66.7)	2/12 (16.7)
6	48-96	10	30	0/3	10	30	3/3 (100)	3/18 (16.7)
7	48-120	10	30	0/3	10	30	2/2 (100)	1/10 (10)
8	48-48	10	150	0/15	10	150	6/15 (40)	11/18 (61.1)
9	24-24	30	150	0/5	30	150	1/5 (20)	ND
Total			**650**	0/55		**650**	**18/54 (33.3)**	**21/79 (26.6)**

[a] AAP was on un-stretched parafilm feeding pouches containing sucrose.

[b] AAP was on un-stretched parafilm feeding pouches containing sucrose supplemented with *S. marcescens* Z01-A at 10^5 bacteria/ml. IAP pouch solutions were added to culture medium and bacteria were identified by PCR. Solutions in which bacteria did not grow were not tested by PCR.

[c] Insect bodies tested.

[d] Not done

Appendix B

Transmission of other strains of *Serratia marcescens* by *Circulifer tenellus*

The fact that no differences were detected among cucurbit strains of *Serratia marcescens* means they probably evolved from one ancestor. However, other *S. marcescens* strains do differ from cucurbit strains in a number of important ways. For example, strain H01-A, isolated from a human subject, possesses several metabolic enzymes lacking in cucurbit strains, and has a fatty acid profile quite different from that of CYVD strains as well (Rascoae et al, 2001). Therefore, in addition to testing strain Z01-A of *S. marcescens*, strain H01-A was also tested for transmission by *Circulifer tenellus* (Baker). Another issue of concern in our research program was whether experimental manipulation of cucurbit strains to facilitate tracking would affect their ability to be transmitted. To address this issue, we also tested a strain that incorporated the gene for green fluorescent protein (GFP)(Z01-A-GFP), and a rifampicin-resistant strain (Z01-A-rif.).

C. tenellus adults were reared and maintained as described in Chapter 2. *S. marcescens* modified with GFP was produced by Michael Berg. Cultures were frozen at –80°F in LB broth until use. Rifampicin resistant cultures, selected on rif-amended agar medium by John Rascoe, were maintained on LB plates containing 25 ug/ml rifampicin and frozen until use. The human strain, H01-A, was obtained from D. Adamson, Medical Arts Laboratory, Oklahoma City, OK,

cultured on LB agar, and stored at –80°F until use. Acquisition and inoculation access periods and detection were as described in Chapter 2. GFP was also detected by fluorescence microscopy by placing samples on a glass slide and examining with a Nikon camera with a fluorescence attachment at a wavelength of 488. All three strains were identified as *S. marcescens* by PCR as described in Chapter 2.

Table 13. *Circulifer tenellus* acquisition and inoculation using other strains of *Serratia marcescens*.

Species	Bacterial Strain	Length of AAP-IAP (hr)	Control[a]			Treatment[b]			Treatment[c] No. of PCR (+) /No. of insects (%)
			No. of insects/ sachet	Total no. of insects	No. of IAP sachets (+) by PCR /No. tested (%)	No. of insects/ sachet	Total no. of insects	No. of IAP sachets (+) by PCR /No. tested (%)	
Circulifer tenellus	ZO1-A-GFP	24-24	5	3	0/1	5	15	1/3 (33.3)	9/9 (100)
	ZO1-A-Rif^R	24-24	5	3	0/1	5	15	1/3 (33.3)	10/10 (100)
	H01A	24-24	5	3	0/1	5	15	1/3 (33.3)	10/10 (100)

[a] AAP was on sachets containing D10 or sucrose.
[b] AAP was on sachets containing sucrose supplemented with *S. marcescens* at 10^5 bacteria/ml. IAP sachet solutions were added to culture medium and bacteria were identified by PCR. Solutions in which bacteria did not grow were not tested by PCR.
[c] Insect bodies tested.

Table 14: Mortality of *Circulifer tenellus* after feeding on three strains of *Serratia marcescens*.

Species	Bacterial Strain	Control[a]			Treatment[b]		
		M[c] % after AAP	M. % after IAP	Average M. %	M. % after AAP	M. % after IAP	Average M. %
Circulifer tenellus							
	Z01-A- GFP	20	0	**10**	6.6	13.3	**10**
	Z01-A-Rif^R	0	0	**0**	13.3	20	**16.6**
	H01A	20	0	**10**	6.6	21.4	**14**

[a] AAP was on sachets containing sucrose.
[b] AAP was on sachets containing sucrose supplemented with *S. marcescens* at 10^5 bacteria/ml. IAP sachet solutions were added to culture medium.

The culture of Z01A-GFP glowed under fluorescence microscopy, whereas untransformed Z01A did not fluoresce. *C. tenellus* was able to transmit all three different strains after 24 hrs from sachet to sachet under the controlled conditions. All strains were detected in the *C. tenellus* bodies when tested by PCR, indicating that the insect fed on the inoculum and acquired the bacterium. These results suggest that the mechanism of *S. marcescens* transmission is the same for these three strains of the bacterium. This finding is important for two reasons: differential transmission among these three strains might reflect *S. marcescens* adaptation to the cucurbit ecosystem, thus providing clues as to the origin of the cucurbit strains. Second, the knowledge that GFP and rif- marked strains retain transmissibility will allow their use in experiments designed to identify factors such as adhesins and the genes involved in transmission of cucurbit strains.

Appendix C

Transmission of *Serratia marcescens by Circulifer tenellus* nymphs

Pathogens acquired by immature stages of many vector insect species are transmitted by adults following a molt. *Circulifer tenellus* nymphs were tested for their ability to transmit *Serratia marcescens*. Four sachets were prepared, with 5 nymphs of the 4th or 5th instar stage in each sachet. Two sachets contained *S. marcescens* – amended feeding solution, and the other two contained only feeding solution, as controls. The insects were allowed 24-24 hrs each for AAP and IAP.

Table 15. *Circulifer tenellus* nymph acquisition and inoculation of *Serratia marcescens* using artificial feeding sachets.

Species	Rep	Length of AAP-IAP (hr)	Control[a]			Treatment[b]			Treatment [c] No. of PCR (+) /No. of insects (%)
			No. of insects/ sachet	Total no. of insects	No. of IAP sachets (+) by PCR /No. tested (%)	No. of insects/ sachet	Total no. of insects	No. of IAP sachets (+) by PCR /No. tested (%)	
Circulifer tenellus	1	24-24	5	10	0/2	5	10	½ (50)	8/8 (100)

[a] AAP was on sachets containing sucrose.
[b] AAP was on sachets containing sucrose supplemented with *S. marcescens* Z01-A at 10^5 bacteria/ml. IAP sachet solutions were added to culture medium and bacteria were identified by PCR. Solutions in which bacteria did not grow were not tested by PCR.
[c] Insect bodies tested.

The nymphs of *C. tenellus* were able to transmit *S. marcescens* from sachet to sachet after 24 hrs IAP. One of two sachets was positive by PCR. *S. marcescens* was detected by PCR in the bodies of all the insects tested.

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