INSECT TRANSMISSION OF *SERRATIA MARCESCENS*, THE CAUSAL AGENT OF CUCURBIT YELLOW VINE DISEASE

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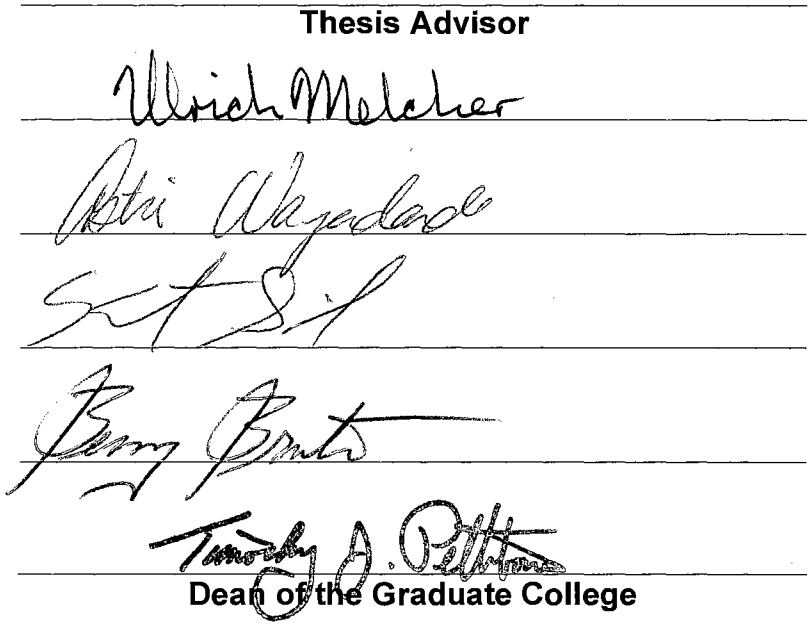

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Literature Review

I. The Crop: Cucurbit

I-1: Cucurbit Biology

The term "cucurbit" was originally coined for cultivated species within the family *Cucurbitaceae* (47), but has evolved to include all species of *Cucurbitaceae*. The members of this family are frost sensitive, predominantly tendril-bearing vines that are found throughout the tropical and subtropical regions of the world. Most species can not survive year-round in temperate climates, so they are grown as seed-producing annuals (64).

The family *Cucurbitaceae* consists of two subfamilies, about 118 genera, and 825 species (39). The subfamily *Zanoniodeae* includes many cucurbits that are grown throughout Asia for medicinal purposes. The subfamily *Cucurbitoideae* includes the four major cucurbit crops: watermelon (*Citrullus lanatus*), cucumber (*Cucumis sativus*), melon (*Cucumis melo*), and squash (*Cucurbita pepo*), along with many other less economically important crops.

Members of the *Cucurbitaceae* family are not closely related to any other family of plants (64). Although members of the family are rather diverse, some generalizations can be made about them. The seedlings of most cucurbits are epigean, germinating with the tips of the cotyledons initially inverted but later

erect. Cucurbits generally have a strong tap root that can extend 1 to 2m into the soil. Often, many secondary roots occur near the soil surface. The sieve tubes in the secondary phloem are the largest found in angiosperm plants. The stem of the typical 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. The fruit, especially those of squash, are quite large. The world's largest pumpkin, grown in 1995, weighed 440 kg.

I-2: Economic Value of Cucurbits

Thirty species in nine cucurbit genera are produced as food crops (47). Many other genera and species are cultivated in the tropics for their medicinal and therapeutic properties; these cucurbits are widely used in tropical medicine. Watermelon leads all the cucurbits in U.S. and worldwide production, both in tonnage and cultivated land area (78). In 1994, 1,824,000 ha of cropland were harvested, worldwide, to produce 29,360,000 tons of watermelon. The U.S. alone harvested 84,000 ha, producing 1,814,000 tons. Next in total world production were cucumber and melon, and then squash and pumpkin. Production of these crops has increased over the past 15 years. Worldwide, squash and pumpkin yields have increased from 5.8 to 8.4 million tons with acreages expanding from 564,000 to 668,000 ha. Melon production worldwide has increased from 8.6 million tons and 594,000 ha to 13.9 million tons and

803,000 ha (64).

China leads the world in production of all four of the main cucurbit crops. In 1990, the Chinese Agricultural Department estimated that 30% of the country's vegetable cropland was devoted to cucurbit production (79). China also produces other cucurbits, such as luo-han-guo, the fruit of which is dried and used for medicinal purposes.

I-3: Cultural Requirements

Seed germination of the cucurbits generally requires warm temperatures (25°C-30°C) for about 3-4 days (42), although emergence can occur more quickly if soaking techniques or various fungicide treatments are used. If conditions are too warm seed proteins can be denatured, resulting in a low percentage of germination (71). Genetically improved varieties of different cucurbit species, more tolerant to extreme climate conditions, have been developed. To prevent soil-borne diseases, such as damping off, fungicide seed treatments are often applied by seed companies (64). Hot water seed treatments (55°C) can also be used to control seed-borne diseases.

Many cultural practices promote the successful production of cucurbits as food crops. Usually, the previous field crop is plowed under after harvest and allowed to decay, although squash and cucumber have been grown successfully using no-till planting, with application of herbicides to control weeds (64). When tilling is practiced, lightweight tillers, such as rototillers, must be used for soil preparation because the fields are generally too wet and/or sandy for large

machinery to be used. Plastic mulch can be used to warm the soil, to conserve moisture, and to reduce the leaching of nutrients from the soil.

Winter cover crops can be grown in the off-season to add organic matter and to prevent erosion of the soil (28). Rows are often prepared by raising beds (18 inches) to improve drainage and to modify temperatures. Before planting, pretreatment herbicides should be used to kill any weeds that would compete with the cucurbit being cultivated. Planting should be done after the danger of late frost has passed, since cucurbits are frost sensitive.

Historically, Native Americans and Africans practiced interplanting, a system in which cucurbits were planted in a field along with other crops, such as corn and wheat (64). In most places today, cucurbits are planted in monocultures, but in many parts of Latin America intercropping is still widely practiced. Legume/cucurbit intercropping and marigold/cucurbit intercropping systems were tested in Honduras to determine the advantages and disadvantages as compared to cucurbit monocultures (58). The cultivated monoculture crop produced a higher yield but the effects of nematode root gall were not different.

Emergence of cucurbit seedlings may be affected by the way the seeds are planted (78). In large production areas where planting machinery is used, one seed is deposited at each site; however, in hand planting, 3 to 7 seeds often are placed in one hole. Later, seedlings are thinned leaving only the most vigorous plant. In general, the rate of seedling emergence is dependent upon the depth at which the seed is planted.

I-4: Fruit and Seed Production

The most common form of sexual expression in cucurbits, as in cucumber, bottle gourd, bitter gourd, watermelon, squashes, and others, is monoecy, in which staminate and pistillate flowers are produced separately on the same plant. Bees and other day-flying insects are needed to pollinate most species of *Cucurbitaceae*. However, the flowers of some species, such as the white flowered bottle gourd, remain open at night and are pollinated by moths.

The maturity of cucurbits is recognized by the appearance of the "slip", an abscission layer that forms, causing the fruit to naturally detach from the plant (64). Cucurbits harvested before maturity will have a jagged scar at the stem end of the fruit. The market value of the fruit will decrease because the low sugar content will give the fruit a poor flavor.

I-5: Diseases of Cucurbits

Cucurbits are susceptible to a variety of diseases. Infections caused by bacteria, viruses, mollicutes, and fungi are common (7,80). The diseases range from damping off at the beginning of germination to postharvest rots. Pathogen control is necessary for the efficient production of cucurbits.

Control of many diseases has come from the development of genetically resistant cultivars of cucurbits. In 1911, the USDA released a watermelon cultivar resistant to *Fusarium* wilt. Since then, cultivars of cucurbits resistant to powdery mildew, scab, cucumber mosaic virus, bacterial wilt, angular leaf spot, anthracnose, downy mildew, and other diseases have been developed (78).

Most of the work with resistant cucurbit cultivars has been done with cucumbers. Development of resistance has been accomplished using both traditional plant breeding and biotechnology methods. When no resistant cultivars are available various preventative control measures must be used. Crop rotation and seed treatments may be more effective, but chemical pesticides are often necessary to reduce crop loss.

I-6. Arthropod Pests of Cucurbits

Species of the phylum Arthropoda belonging to two classes, Acari and Insecta, are considered pests of cucurbits (80). Within class Acari two species of spider mites (Acari: Tetranychidae), the twospotted spider mite, *Tetranychus urticae* Koch, and the carmine mite, *T. cinnabarinus* Boisduval, cause significant damage to cucurbits by puncturing cells of the leaves, interrupting the normal production of photosynthate. The wounds result in chlorosis followed by desiccation and shriveling of leaves.

Several insect species belonging to the orders Hemiptera, Thysanoptera, Coleoptera, Diptera, and Lepidoptera are also cucurbit pests (80). Hemipteran pests are classified in two suborders; Homoptera and Heteroptera. Homopteran pests include aphids (Hemiptera: Aphididae), such as the melon aphid, *Aphis gossypii* Glover, the green peach aphid, *Myzus persicae* (Sulzer), and the cowpea aphid, *Aphis craccivora* Koch. These insects cause damage by contamination with excrement and by serving as vectors of plant pathogens (mainly viruses). Whiteflies (Hemiptera: Aleyrodidae), such as the sweetpotato

whitefly, *Bemisia tabaci* (Gennadius), the silverleaf whitefly, *B. argentifolii* Bellows and Perring, and the greenhouse whitefly, *Trialeurodes vaporariorum* (Westwood), damage tissues by direct feeding, by contamination with excrement, by transmission of plant viruses, and by inducing physiological or phytotoxic disorders in the plant. Only one heteropteran, the squash bug, *Anasa tristis* DeGeer (Hemiptera: Coreidae), is a pest of cucurbits. This insect physically interrupts the plant's vascular system and transmits a bacterial pathogen, which is discussed in this thesis.

Three species of thrips (Thysanoptera: Thripidae); the western flower thrips, *Frankliniella occidentalis* (Pergande), the onion thrips, *Thrips tabaci* Lindeman, and the melon thrips *T. palmi* Karny, damage plant leaves and flowers by feeding and are important vectors of tospoviruses (80). Only one coleopteran insect, the striped cucumber beetle, *Acalymma vittatum* (Fabricius) (Coleoptera: Chysomelidae), is a pest of cucurbits (80). Damage by this insect is due to destruction of leaf tissue through feeding and by transmission of the bacterium *Erwinia tracheiphila*, which causes a bacterial wilt. Leaf-miners (Diptera: Agromyzidae), *Liriomyza* spp., are secondary pests of cucurbits and damage is caused by feeding, which kill localized cells and allows entry by pathogens. The squash vine borer, *Melittia cucurbitae* (Harris) (Lepidoptera: Sesiidae), burrows into vines and stalks, causing plants to wilt.

II. The Disease: Cucurbit Yellow Vine Disease (CYVD)

II-1: Observations of a Disease of Unknown Etiology

In 1988, a new condition, cucurbit yellow vine disease (CYVD), caused vine decline in summer squash (*Cucurbita pepo* var. *melopepo*) and pumpkin (*C. pepo* var. *pepo*) in Oklahoma (14). The same symptoms were found in early season watermelon in both Oklahoma and central Texas in 1991. This disease was distinctly different from other vine declines of summer squash and pumpkin. Symptoms, which generally appeared two weeks prior to harvest, included general and rapid yellowing of the leaves that appeared over a 3-4 day period, followed by gradual decline and death of the vines. Some infected plants exhibited no yellowing and wilted and collapsed in one day. Other than the general yellowing, there were no typical viral symptoms associated with the leaves, fruit or flowers. Root rot was not present in the early stages but there was a golden to honey brown discoloration of the phloem, which occurred in the primary root and in the crown. Symptoms resembled those reported in diseases caused by phloem-associated bacteria in other crops.

II-2: Host Range and Geographic Distribution

CYVD has not been found in all cucurbits. Thus far, field occurrence of the disease has been limited to squash, pumpkin, watermelon, and cantaloupe (55). Field plantings of cucumbers and small gourds have not been found to be affected, even when growing in close proximity to CYVD-affected crops.

Until 1997, CYVD had been diagnosed only within the Cross Timbers region, an area with distinctive vegetation in north central Texas and central Oklahoma (12). The disease had not been found in other important melon growing areas such as the Rio Grande valley or Pecos, Uvalde, and Dilley, Texas. In 1997-98, however, CYVD was confirmed in all of the melon growing areas of Oklahoma, causing an estimated 30-70 % loss in affected fields, and in Anderson Co., in east Texas, areas outside the Cross Timbers region. Disease symptoms resembling those of CYVD were later confirmed in Arkansas (J.C. Correll personal communication), Tennessee (11), Massachusetts (77), and Kansas (Tisserat, personal communication) by specific polymerase chain reaction (PCR).

II-3: Diseases Similar to CYVD

Phloem-associated bacteria have been identified as the causal organisms of diseases in other crops that have symptoms similar to those of CYVD. Citrus greening, clover club leaf, papaya bunchy top, and strawberry marginal chlorosis express the greatest similarities because they are all caused by phloem-associated bacteria.

Citrus greening disease (CGD) is a major cause of crop loss in many parts of Asia and Africa. Symptoms of the disease include veinal-pattern yellowing of normal sized leaves, small, lopsided, and bitter fruit, and poorly developed roots. Transmission of the CGD causal organism from sweet orange to periwinkle was accomplished by dodder and grafting (29), and the inoculated test plants

developed yellowing symptoms within 3 months. The disease organism was originally thought to be a mycoplasma-like organism (MLO, now called phytoplasma) (38), but after further investigation, it 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 (75). Strains of the GCD causal organism from Africa and Asia had slight genome differences, but (38) placed the BLOs of both strains in the phylum *Proteobacteria*. The psyllids *Trizoa erytreae* and *Diaphorina citri* were identified as vectors of the CG bacterium (33).

Clover club leaf disease (CCLD) affects the leaves of crimson clover and other plants (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. Using the leafhopper *Agalliopsis novella* the pathogen was transmitted into healthy plants. The CCL bacterium also was transmitted to 99% of the leafhopper progeny (43), through as many as 21 generations.

Papaya bunchy top disease (PBTD), an important disease in the Antilles and in Trinidad, causes diffuse chlorosis in young leaves and reduction of normal leaf blade expansion. This pathogen, which was restricted to the periphery of the papaya phloem, was originally thought to be a phytoplasma (21) but was later identified as a phloem-limited bacterium. Using DNA sequence analysis, a *Proteobacterium* of the genus *Rickettsia* was identified as the causal agent of

PBTD (22). To date it has not been cultured, but PCR was used successfully to detect the pathogen in symptomatic plants. Two leafhopper species, *Empoasca papayae* Omen, 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 (22).

Marginal chlorosis of strawberries was first seen in Spain in 1984 and in France in 1988 (50,81). The disease affected all strawberry cultivars tested. An unculturable phloem-limited bacterium-like organism (BLO), observed in the phloem of diseased plants by electron microscopy, was 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 *Fragaria* x *ananassa*. The new bacterium was placed phylogenetically in group 3 of the gamma subclass of *Proteobacteria*. Research at OSU (2) and in France (81) has shown that the bacterium causing marginal chlorosis of strawberry and the bacterium causing yellow vine of cucurbits are different, although they are the only known plant pathogenic gamma-proteobacteria.

Ratoon stunting disease, caused by *Clavibacter xyli* ssp. *xyli*, is one of the most important diseases of sugarcane (20). The pathogen is probably present wherever sugarcane is produced (65). However, poor expression of symptoms, which include stunted growth and thin stalks, in infected plants makes disease diagnosis difficult. Additionally, the difficulty in symptom recognition increases the risk of pathogen dissemination through germplasm exchange. Accurate

detection methods are an important component of containing spread of the pathogen. Of the several methods of detection that have been developed, tissue blot enzyme immunoassay (37) and PCR (25,56) were the most accurate. Development of resistant cultivars has decreased the spread of *C. xyli* (15,35,37). Although the causal bacterium is apparently xylem-limited, an insect vector is not known to be involved in its transmission. Within sugarcane fields, *C. xyli* can be transmitted mechanically from one plant to another (15,37).

Xylella fastidiosa is the bacterium that causes a lethal condition of grape vines called Pierce's disease (60). The bacterium blocks the xylem vessels of the plant causing leaves to dry or scorch. Infected vines can die in one to two years. *X. fastidiosa* also causes almond leaf scorch, phony peach disease, alfalfa dwarf, oleander leaf scorch, and citrus variegated chlorosis. The principal vector of *X. fastidiosa* in California, for many years, was the blue-green sharpshooter (*Graphocephala atropunctata*). Because this insect is a poor flyer the pathogen was disseminated only short distances. However, in 1990, the glassy-winged sharpshooter (*Homalodisca coagulata*), a native of the southeastern United States, was found in California (61). *H. coagulata* is capable of transmitting *X. fastidiosa*, is a good flyer, frequently appears in high numbers, and is able to survive cold temperatures. This combination of factors has allowed *H. coagulata* to become the most important *X. fastidiosa* vector. *X. fastidiosa* is non-circulatively transmitted by both sharpshooters, attaching in the foregut and remaining transmissible for the life of the insect vector.

II-4: Identification of the CYVD Pathogen

A number of pathogens or abiotic conditions had been proposed as possible causes of CYVD, as reported by Bruton et al. (12) and Pair et al. (55). Among these were herbicide damage, nutrient imbalance, *Fusarium*, *Monosporascus*, and various common seed-borne pathogens. In the early stages of investigation, however, no fungus, prokaryote, or virus was consistently associated with the disease. Research at the Wes Watkins Agricultural Research and Extension Center in Lane, Oklahoma showed that insecticide treatments, such as soil fumigation with methyl bromide at 67 g/m^3 soil, did not lower the incidence of CYVD (15). A mulch experiment was conducted to determine if insect populations and ground cover, alone or in combination, affected the occurrence of CYVD. Irrespective of the mulch used, foliar insecticide treatments reduced the incidence of CYVD. These data are consistent with the interpretations that an insect was involved in the disease and that the disease was not caused by a soil-borne pathogen. In early tests, a number of different cucurbit insect pests failed to transmit a CYVD causing pathogen (12,53).

PCR primers specific for CYVD affected plants were developed (2). Initially, a primer pair based on sequences of the citrus greening BLO amplified a specific product. Sequencing that fragment showed clearly that the amplicon was a bacterial gene and suggested that the disease was related to the presence of a phloem-resident bacterium. Primers more specific for CYVD (YV1, YV2, and YV3) were designed from the amplicon sequence and used to detect the

pathogen in plant and insect samples. This was an important step in monitoring for the causal agent because the phloem-limited bacterium had not yet been cultured.

When the YV1 and YV2 primers were used to screen field-collected insects for the presence of the CYVD bacterium a band of the expected size was amplified in numerous samples of the leafhopper *Exitianus exitosus* collected from grassy plots near Stephenville, Texas. Sequencing of the 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 a known insect parasite. This bacterium, BEV, occurred in many leafhoppers (59). This finding generated the need to develop primers that would discriminate between BEV and the CYVD bacterium. Primers YV1 and YV4 now amplify the CYVD bacterium but not BEV, while primers BEV1 and BEV2 amplify BEV but not the CYVD bacterium (45).

For some time the CYVD pathogen was referred to as a BLO because early attempts to culture it were unsuccessful, making further characterization difficult. However, by PCR the bacterium was consistently associated with disease symptoms. Later it was cultured from a symptomatic zucchini plant, and has since been cultured from watermelon, zucchini, pumpkin, and cantaloupe (Mitchell unpublished; Fletcher unpublished). Koch's postulates were recently completed with the CYVD bacterium (Bruton et al. manuscript in preparation). When a pure culture of the CYVD bacterium was introduced into healthy plants through needle inoculation or the use of an insect vector, *A. tristis*, disease

symptoms were observed. The CYVD bacterium was recovered from the symptomatic, inoculated plants and identified by PCR and DNA sequencing as the same bacterium that was introduced.

Nucleotide sequence analysis based on 16s rRNA sequence suggested that the causal agent of CYVD was a gamma-3-proteobacterium, with *Serratia marcescens* being the closest identified relative (2). Biological and nucleic acid sequence analysis of two CYVD bacteria strains, one isolated from watermelon (W01) and the other from zucchini (Z01), and two reference strains allowed for further characterization of the CYVD bacterium (63). The highly conserved *gro*E and 16S rDNA genomic regions of the bacteria were amplified by PCR and directly sequenced (Rascoe, manuscript in preparation). The CYVD isolates were compared by sequence analysis to eight other bacterial isolates from diverse ecological niches, preliminarily identified by others as *Serratia* sp. Both CYVD isolates and six of the eight reference isolates were decisively identified as *S. marcescens* species.

Despite near-identity of the two nucleic acid sequences (*gro*E and 16S rDNA) among the *S. marcescens* strains from cucurbits and those from other niches, the CYVD strains differed significantly from the others in their biological attributes. In fatty acid profiling (FAME) the CYVD isolates clustered together, well isolated from the reference strains and database *Serratia* strains. In addition, CYVD isolates lacked a number of metabolic features characteristic of *S. marcescens*, as shown by substrate utilization assays (BIOLOG, Vitek, and API). Zhang et al. (personal communication) recently examined *S. marcescens* strain

variations by rep-PCR, in which repetitive sequences found commonly in bacteria and distributed apparently randomly within the genome are amplified to reveal profile differences among bacterial strains. The rep-PCR patterns revealed that the cucurbit strains of *S. marcescens* were very similar to one another, but differed significantly from strains from other niches, which allow strain difference to be detected through rep-PCR pattern grouping.

II-5: Control of CYVD

Currently there are no effective control tactics for CYVD. No resistant or tolerant varieties have been developed, although some varieties seem to be more or less susceptible to CYVD. In 1997-98, incidence was significantly influenced by both cultivar and ploidy level when 21 watermelon cultivars were field screened. Triploids (3n) and tetraploid (4n) lines had much lower incidences of CYVD than the open-pollinated (2n) and hybrid (2n) lines (19).

Cultural and chemical control of insect vector(s) of plant pathogens may reduce incidence of corresponding disease. Since our work has shown that *A. tristis* is a field vector of *S. marcescens* (Pair et al., unpublished and Wayadande et al., unpublished), measures that reduce squash bug populations or plant access could be effective as disease reduction strategies. While the use of fabric row covers excluded insect vectors, it also excluded insects that are necessary for pollination, thus reducing fruit production (5). The use of trap crops to attract pest insects away from a primary target crop is another management strategy (36). The squash variety 'Lemon Drop' was used effectively to attract cucurbit

pests away from watermelon and cantaloupe (52). Different colored plastic mulches have also shown promise in reducing squash bug populations in watermelon crops (54).

III. The Pathogen: *Serratia marcescens*

The identification of the CYVD pathogen, a phloem-associated bacterium, as *S. marcescens* identified a new niche for this cosmopolitan microbe. *S. marcescens* belongs to the family Enterobacteriaceae, gram negative microbes that have peritrichous flagellae and a G+C content of 53-59 mol% (72). Many strains of *S. marcescens* produce a protein pigment, prodigiosin, which gives the cell cultures a striking red hue and may play roles in respiration and antibiotic activity. However, not all strains of *S. marcescens* produce prodigiosin and within strains prodigiosin production can vary depending on the nutritional environment. The CYVD strains of *S. marcescens* do not produce prodigiosin under the conditions tested to date (63,Bruton et al., in preparation).

S. marcescens has been isolated from plants, or from soil in association with plants, where they may play beneficial roles. For example, *S. marcescens* strains that inhabit the rhizosphere may act as plant growth promoting rhizobacteria (PGPR), enhancing plant growth and productivity and even protecting plants from soil-borne pathogens (23,76). When *S. marcescens* 9M5 was inoculated into the rhizosphere of Kentucky bluegrass at a rate of 10^9 cfu/ml, 50% suppression of summer patch disease, which is caused by *Magnaporthe poae*, was observed (41). Second, endophytic strains of *S. marcescens* with potentially beneficial effects on the plant have been isolated from roots and stems of cotton plants (76) and from xylem vessels of citrus (32) and rice (34). Two reports of *S. marcescens* as a plant pathogen have also been made after crown rots of alfalfa (44,73) and sainfoin (68) were attributed to a disease

complex which included *S. marcescens*. However, the identification of the pathogen in these cases was done without the benefit of current molecular tools.

S. marcescens has also been isolated from soil, water, food products, and infections of humans and animals, including insects. It has been identified as an entomopathogen in over 70 insect species and has been isolated from over 30 insect species.

S. marcescens pathogenicity in insects may result from a number of virulence factors. The bacteria produce several enzymes (protease, gelatinase, lecithinase, and chitinase) that adversely affect the insect hosts. These enzymes may inactivate the host immune system, allowing bacteria to reproduce efficiently and leading to septicemia. When small amounts of the bacterium are ingested by an insect host, pathogenicity does not occur because the alimentary canal is not a suitable environment for the bacteria (69,70). However, when large doses of the bacterium are ingested under laboratory conditions, many insect species are susceptible to infection. A red strain of *S. marcescens* was isolated from healthy, diseased, and dead individuals of *Heliothis virescens* (Lepodoptera: Noctuidae) laboratory colonies (69). This strain was transmitted vertically (from parent to offspring, in or on the egg) and horizontally (from individual to individual at any age after birth). The feeding of *S. marcescens* to adult moths did not cause significant mortality (70). However, the number of eggs laid and the rate of hatch was reduced when *S. marcescens* was present.

The use of *Serratia* species for biological control of invertebrates has been explored. *S. entomophila* is marketed commercially as a microbial control agent

for the grass grub (*Costelytra zealandica*). Pathogenic strains of this *Serratia* species adhere in the foregut of the grass grub larvae and cause symptoms associated with amber disease. *S. marcescens* has been tested as a biological control agent for several insects in the order Diptera, including the tse tse fly, *Glossina morsitans* (40,57); the sheep blowfly, *Lucilia sericata* (51); and the house fly, *Musca domestica* (4).

IV. Potential Insect Vectors of *S. marcescens*

IV-1. Homopterans

The majority of phloem-associated bacteria are transmitted by insects, most are transmitted by homopterans, mainly leafhoppers and psyllids (46). Walled bacteria that cause diseases similar to CYVD, such as the agents of citrus greening, clover club leaf, papaya bunchy top, and strawberry marginal chlorosis, are transmitted by either leafhoppers or psyllids. Mollicutes, spiroplasmas and phytoplasmas, are transmitted by leafhoppers, planthoppers, and psyllids.

Leafhoppers are important to consider as potential vectors of *S. marcescens* because the CYVD bacterium is phloem-associated. *Exitianus exitiosis* is a prevalent leafhopper throughout the central United States throughout the summer months and was found to be a carrier of *S. marcescens* (Mitchell, unpublished). However, *E. exitiosis* failed to transmit the pathogen in experimental trials. Other leafhopper taxa, most notably *Macrosteles* sp. and *Endria* sp., also were considered as potential vectors because they occur somewhat frequently throughout the growing season, but *S. marcescens* has not been detected in these or any other species (Wayadande, personal communication).

IV-2. Heteropterans

Only seven plant pathogens are reported to be transmitted by

heteropterans. The fungus *Nematospoa coryli* Peglion, which causes the seed disease yeast-spot of soybeans, is transmitted by several members of the family Pentatomidae (18,26). The mode of transmission was not reported; however, the pathogen was found in several organs of the insects. *Dysdercus* spp. (Hemiptera: Pyrrhocoridae) also reported to transmit *N. coryli,* but to cotton, in which it causes internal boll disease (27), but this report was not confirmed.

Plant pathogens are transmitted by three members of the family Lygaeidae. The Centrosema mosaic virus, thought to be a potexvirus, is proposed to be transmitted mechanically by *Nysius* spp. (74). The phloem-associated potato spindle tuber viroid is transmitted by *Lygus lineolaris* (67), although this insect is not thought to be an important vector species. The beet leaf curl rhabdovirus (66) and the sugarbeet latent rosette rickettsia-like organism (49) are transmitted by the beet leaf bug, *Piesma quadratum*, and are propagative within the insect. Transmission of one other virus, the velvet tobacco mottle sobemovirus, by *Cyrtopeltis nicotianae* of the family Miridae, is well documented and the mode of transmission is thought to be circulative (30,31,62).

V. An Insect Vector of the CYVD Pathogen: *Anasa tristis*

V-1: Classification

The squash bug, *Anasa tristis* DeGeer (Hemiptera: Coreidae), is a true bug. Hemipterans are now separated into two suborders; the heteroptera (true bugs) and the homoptera (leafhoppers and aphids). All heteropterans have piercing-sucking mouthparts and feed on the fluid of plants or animals. Heteroptera have two pairs of wings, and the forewings differ in structure from the hindwings. The forewings of heteroptera are partly leathery and partly membranous, a characteristic that easily distinguishes them from other insect groups. All heteropteran insects develop through incomplete metamorphosis.

V-2: *Anasa tristis* Biology

A. tristis occurs throughout North America and is considered a major pest of cucurbits. Adults overwinter in sheltered areas, such as under wooden planks, buildings, hay bales, and bark (3), emerging in the spring to find susceptible host plants. In the northern U.S., *A. tristis* is univoltine, having only one generation per year. However, in the southern U.S. it is multivoltine, making it difficult to control because of the varied age structure (24). Eggs are generally laid on the underside of leaves and along stems of the host plant. When eggs hatch, first instars generally remain aggregated and do not require food for development (16). Second stage nymphs require adequate food for development, as do 3rd, 4th, and 5th stage nymphs. Throughout their development *A. tristis* immatures

and adults tend to aggregate and their intense feeding activity causes a condition called Anasa wilt, in which water stress results from the interruption of xylem transport from *A. tristis* salivary sheath blockage and stylet severing of the vessels (48).

A. tristis feeding involves piercing of the plant's epidermis by the stylets and intracellular penetration to the mesophyll or vascular tissues (3). Electronic monitoring of feeding behavior revealed that 1st instar squash bugs are capable of sustained phloem ingestion (9). *A. tristis* prefer to feed on pumpkin and squash (8,17). Survival is possible on less-suitable hosts such as watermelon, but the insects are unable to survive and reproduce on plants other than cucurbits.

V-3: *A. tristis* as a vector

The squash bug is an unusual vector and is only the second reported vector of a phloem-associated bacterium to be identified. The majority of phloem-associated insect-transmitted plant pathogens are disseminated by insects belonging to the suborder Homoptera, mainly leafhoppers and aphids. These insects are relatively small and have relatively small stylets that allow penetration of host sieve elements causing minimal physical damage or disruption of plant cells. Heteropteran stylets are large, relative to those of leafhoppers or aphids, and probably cause significant physical damage to host tissue during stylet penetration (3).

VI. Insect Transmission of Plant Pathogens

Insects play an important role in the transmission of plant pathogens (10). Over 200 pathogens have been characterized as insect transmitted, and of these about three-fourths are viruses. Mollicutes, walled bacteria, and fungi make up the other one-fourth.

Five mechanisms of insect transmission of plant pathogens have been described (1,10,46). In non-persistent transmission, the pathogen does not enter the body of the insect but rather is confined to the inner stylet surface of the insect foregut and the pathogen is transmissible for only a short period (<48 hr). This type of transmission is characteristic of most aphid-transmitted pathogens. Cucumber mosaic virus and alfalfa mosaic virus, which are transmitted by the aphid *Myzus persicae*, are examples of non-persistently transmitted pathogens. In the second type, semi-persistent transmission, the pathogen is confined to the foregut of the insect similar to non-persistent transmission, however, pathogens attach in the pre-cibarium or cibarium of the foregut. These pathogens are transmissible for only a short period (<48 hr). An example of semi-persistent transmission is maize chlorotic dwaft virus transmission by *Graminella nigrifrons*. In the third type, non-circulative transmission, the pathogen colonizes the foregut of the insect and is usually transmissible for the life of the insect. This method is exemplified by sharpshooter transmission of *Xylella fastidiosis*, previously described in section II-3. In the fourth type, circulative transmission, the pathogen enters the body of the insect via ingestion and travels without multiplying from the gut lumen through the hemocoel to the salivary glands,

where it can be introduced into the plant via the saliva of the insect. In the fifth type, propagative transmission, the pathogen reproduces in the insect, which is considered an alternate of the pathogen. The aster yellows phytoplasma, which affects a broad plant host range including aster, celery, squash, cucumber, wheat, and barley, is transmitted in this way via leafhoppers. Leafhopper and psyllid vectors propagatively transmit the walled bacteria causing citrus greening, clover club leaf, and papaya bunchy top.

There are three stages of insect transmission of pathogens: the acquisition access period (AAP), the latent period (LP), and the inoculation access period (IAP) (46). The AAP is the time required for an insect to ingest and begin harboring a pathogen. The LP is the period of time after a pathogen has been acquired, when the pathogen is present within the insect but is not transmissible. Propagative pathogens are multiplying within the body of the insect during this period. The last period, IAP, is the access time for the inoculative insect to feed on the healthy host plant and actually introduce the pathogen into it. The AAP, LP, and IAP differ in duration depending on the pathogen, the insect and the host plant.

VII. Research Objectives

At the onset of this research project, little was known about the causal agent or epidemiology of cucurbit yellow vine disease (CYVD). Two previous studies, in which the effects of soil fumigation and foliar insecticides applications on disease incidence were tested, indicated that the CYVD pathogen might have a relationship with insects (13). In this project, the importance of insects in the newly discovered disease was investigated. The primary objective of the work was to learn more about CYVD and its epidemiology, as a basis upon which to develop effective management recommendations.

The first experiments reported in this thesis, described in Chapter 2, were designed to investigate the hypothesis that an insect was involved in the epidemiology of the disease. To test this hypothesis, above-ground insects were excluded from test plants to determine if incidence of the disease and occurrence of the causal agent was affected.

The facts that (1) the causal agent of CYVD was associated with plant phloem and (2) homopterans, mainly leafhoppers, transmit the majority of phloem-associated pathogens, led to the hypothesis that a leafhopper vector was involved in the transmission of the CYVD pathogen. To investigate the potential role of leafhoppers as vectors of the CYVD pathogen, natural populations of leafhoppers collected near CYVD-affected cucurbit fields were screened for the pathogen. The results of these assays are reported in Appendix A.

Also of potential importance in CYVD epidemiology was the role of

alternate plant hosts that could serve as pathogen sources and/or as over-wintering hosts for the CYVD pathogen. The existence of such plant hosts was investigated by screening selected plants growing near cucurbit fields for the pathogen. These studies are presented in Appendix B.

After transmission studies conducted by other researchers demonstrated the capability of *Anasa tristis*, the squash bug, to transmit the bacterium *Serratia marcescens*, causal agent of CYVD, further investigation into the relationship between the vector and the pathogen was warranted. Such studies were hampered by the lack of a convenient system by which insect/pathogen interactions could be examined. No artificial feeding system (AFS) was available for this insect and attempts to use existing AFSs developed for closely related insects, those within the same order, failed. In this work, observations of *A. tristis* feeding behavior and adaptation of existing AFSs led to the development and application of a novel AFS for this insect, which is described in Chapter 3.

We hypothesized that *S. marcescens* is transmitted in a non-circulative manner by *A. tristis*. In the study reported in Chapter 4, we characterized the mode and parameters of transmission of the pathogen by *A. tristis*.

Experiments on the mode of pathogen transmission included investigations into the time period necessary for pathogen acquisition by the vector (AAP), the time period in which the vector is capable of transmitting the pathogen (IAP), and the latent period required between AAP and IAP (LP). *A. tristis* was allowed to feed on an artificial acquisition source, followed by feeding on potential inoculation targets, both artificial and plant, for consecutive defined

periods. The percentage of insects that transmitted *S. marcescens* in the artificial system was determined.

The persistence of the pathogen in the insect is an important component of the relationship between a pathogen and its insect vector. The length of time in which the CYVD pathogen was detectable by the polymerase chain reaction in the bodies of insects that did or did not transmit, was determined.

Determining the route of pathogen movement in the body of the insect vector is useful in understanding how the pathogen is transmitted. Transmission of circulative pathogens requires the pathogen to enter the midgut of the alimentary canal, enter the hemocoel of the insect by traversal of the gut wall, migrate to and enter the salivary glands, and inoculate a new host after insect salivation (46). In contrast, non-circulative pathogens attach to the foregut wall and remain there. The foregut of an insect, formed by invagination of exo-cuticle, is shed when the insect molts. Non-circulative pathogens are lost along with the exo-cuticle during molting, resulting in the loss of transmissibility.

Our hypothesis that *S. marcescens*, the CYVD pathogen, was transmitted in a non-circulative manner was investigated by testing for the pathogen in the hemolymph of *A. tristis* that transmitted it and by testing the capability of *A. tristis* to transmit the bacterium after molting.

The research presented in this thesis provides important information about the nature and transmission of CYVD and its relationship with recently confirmed insect vector, *A. tristis*.

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Chapter II

Effect of Insect Exclusion on the Incidence of Cucurbit Yellow Vine Disease and of the Associated Bacterium in Squash

Abstract

Cucurbit yellow vine disease (CYVD), associated with a phloem-limited bacterium, causes rapid wilting and death in affected plants. In a previous study, experimental insecticide-treated plots had lower incidence of CYVD than untreated plots, suggesting that insects were involved in the transmission of the bacterium. In the study reported here, we compared incidence of CYVD and PCR detection of the CYVD bacterium in not-covered squash plants (*Cucurbita pepo* var. melopepo) with plants covered with fine mesh fabric secured such that insects were excluded. Rows of squash were covered with row mesh cover that was stretched over hoops and anchored in the soil. The row cover was removed after either 40 or 50 days, at which time all plants were destructively sampled by harvesting the crown and root. In the first experiment, 3% of the not-covered plants had foliar symptoms, 7% were positive using Dienes' stain, and 25% were positive when analyzed by PCR using specific primers. No covered plants were positive by any detection method. No plants in the second experiment had foliar symptoms or tested positive using Dienes' stain. However, 20% of not-covered

and 0% of covered plants were PCR positive. These data support the hypothesis that insects were involved in the transmission of the bacterium.

Introduction

Cucurbit production in Texas and Oklahoma accounts for over one hundred million dollars in annual grower income (3). In 1988, a newly discovered vine decline disease in squash and pumpkin was observed in Oklahoma and north central Texas (5). Similar disease symptoms and serious crop losses were observed in watermelon in Oklahoma and north Texas in 1991. The disease, called cucurbit yellow vine disease (CYVD) (3), is distinctly different from other vine declines of squash (*Cucurbita pepo* var. *melopepo*) and pumpkin (*Cucurbita pepo* var. *pepo*) (13). Two distinct symptoms are exhibited by diseased plants (3). In early season, following fruit-set and beginning fruit enlargement, there is often a sudden wilting and death of the plant in a single day without leaf yellowing. The more prominent symptoms of leaf yellowing and gradual decline generally occur about 7-14 days prior to harvest. A honey brown discoloration of the phloem is prominent in cross-section of the primary root and crown; this symptom was often, but not always, observed throughout the terminal portion of the vine section as well. Root rot is not a symptom of CYVD, but secondary root invaders may cause tissue breakdown as plants decline (3). CYVD has not been observed in cucumbers, but has occurred frequently in summer and winter squash, pumpkin, watermelon, and cantaloupe (11).

Early hypotheses of the cause of CYVD included numerous biotic and abiotic causes, as noted in previous publications (3,4,5). Among these were herbicide damage, nutrient imbalance, fusarium wilt, charcoal rot, monosporascus vine decline, and lettuce infectious yellows virus. However, none

of these were consistently associated with the incidence of CYVD (3). Dienes' stain, previously reported to be specific for mollicutes (6), consistently stained the phloem of symptomatic plants, but no other evidence of spiroplasma or phytoplasma involvement was found (3). Using electron microscopy, CYVD symptoms were consistently associated with the presence of a phloem-inhabiting bacterium, and polymerase chain reaction (PCR) primers were designed to detect this organism (2,8). Using these primers, the bacterium was consistently detected in symptomatic plants and not in asymptomatic plants. The use of PCR facilitated the detection of the CYVD bacterium in cucurbit crops (3) and insects (unpublished data).

In related studies, soil fumigation did not affect the incidence of CYVD and foliar insecticide treatments lowered the disease incidence (4), leading to the hypothesis that the CYVD bacterium is transmitted by an insect vector. However, preliminary transmission tests performed with a number of different cucurbit insect pests failed to move the bacterium from infected to healthy plants (4,10).

Row covers are used in horticultural crops as barriers against insects and associated diseases. Research in melon crops has demonstrated that disease transmission by insects is reduced by use of floating row covers made from Reemay, a spun, bonded polyester material, thin enough to allow sunlight and air flow, but fine enough to exclude insects (9,12). In this study, to further evaluate the hypothesis of insect transmission of the CYVD bacterium, an insect exclusion experiment was used to test whether physical exclusion of above-ground insects

from cucurbit plants affected the incidence of disease and/or the presence of the CYYD bacterium in yellow squash.

Materials and Methods

Experiment one. Insect exclusion tests were performed at the Oklahoma State University Plant Pathology Farm in Stillwater, Oklahoma. A randomized complete block design, with four replications of two treatments (covered rows and not-covered rows), was used. Yellow squash (*Cucurbita pepo* var. *melopepo* cv. 'Lemon Drop') was chosen as a test species because of its known susceptibility and because vines of this species are more restricted in growth habit than those of watermelon and pumpkin, facilitating complete physical exclusion of above-ground insects. Prior to the first experiment, plots were disked and the pre-plant herbicides, Alanap and Prefar, were applied by a CO_2 wheelbarrow plot sprayer (2.18 l/hectare). Seeds were planted on May 24, 1999, at a depth of approximately 3-cm, two seeds per hill, at 0.45-meter intervals in eight 25-m rows. On the same date, two rows of two-week-old, greenhouse-grown seedlings of the same cultivar were transplanted, one per hill, at 0.45-meter intervals around the perimeter of the test plot. These borders were planted to reduce the possibility of an edge effect; that is, a higher incidence of disease in the outer rows due to greater accessibility by insects. Within 24 hr of seed sowing, four of the eight rows were covered with Reemay (Reemay Inc., Old Hickory, Tennessee) (1.70 m x 27 m, 15.6 g/m^2), stretched over flexible PVC arches placed at 4 m intervals, and sealed and anchored by covering the fabric edges with soil. The Reemay was checked twice a week to ensure that insects could not gain access. Two 7.62 cm x 12.70 cm Sensor^{TM} yellow sticky traps (Whitmire Micro-Gen, St. Louis, Missouri) were placed in each row, covered and

not-covered. These traps were suspended 3 m apart, 20 cm above the soil surface by a 30 cm piece of 14 gauge galvanized steel wire (Anchor Wire Corp., Goodlettsville, Tennessee) that was anchored in the soil under the row cover. The traps were checked each week without disturbing the Reemay over the covered rows to monitor insect presence. Soaker hose (Colorite Plastic Co., Ridgefield, NJ) was deployed along the length of each treatment and control row prior to seeding and row covering, and plants were watered as needed. Border plants were watered directly by hose.

Sample collection. Row covers were removed when first symptoms of CYVD occurred (50 days post-plant) and foliar symptoms were noted. The crown and root of every plant, in both experiment and border rows, were collected and stored in plastic bags at 4°C for up to five days. Samples were screened for the presence of the CYVD bacterium by Dienes' stain of hand-cut crown sections, following the protocol of Deeley (3,6), and by PCR using a primer set (YV 1-4) specific for the CYVD bacterium (2,8).

Experiment two. In the second experiment squash seeds were planted on July 15, 1999. As in the first experiment, a randomized complete block design with four replications of two treatments (covered rows and not-covered rows) was used. However, slight modifications from the design of the first experiment were made. To maximize the likelihood of infection, rows were arranged as the edges of a rectangle with nothing planted in the center, such that all were at the borders of the field. Samples in the second experiment were collected after 40 days instead of 50 days, even though symptoms had not appeared in any of the

plants, because high summer temperatures and lack of precipitation were beginning to negatively impact all plants. The samples were stored and tested in the same manner as for the first experiment.

DNA extraction and PCR. DNA was extracted from the plant samples with a GeneClean II extraction kit (Bio 101, Vista, CA), but using a protocol modified from that of the manufacturer. Approximately 0.2 g of crown tissue was ground with a plastic pestle in 160 μl of Lytic Buffer in a 1.5 ml microfuge tube. Forty μl of 5% sodium sarkosyl was added and the tube incubated at 55°C, 6 min, and centrifuged (13,200 x g, 6 min) to pellet cellular debris. Meanwhile, 400 ul of GC Spin Glassmilk was added to a spin column, a specialized microfuge catch tube with a removable size 300 filter (0.45 microns). Supernatant was transferred to the spin column, incubated 5 min (inverted once per minute), and centrifuged (10,000 x g, 30 sec, RT). The filter, containing the silica beads and sample DNA, was replaced in the catch tube and washed twice with 400 μl GC Spin New Wash. After the second wash, the tube was centrifuged 1 min, 13,200 x g, to remove any remaining ethanol. In a fresh catch tube, 100 μl of GC spin elution solution was added to the filter and agitated to resuspend the beads before centrifuging 13,200 x g, 1 min. The eluent, containing extracted DNA, was saved and kept refrigerated until PCR was run.

PCR reactions were carried out in 25-μl reaction mixtures containing 10 to 50 ng of genomic DNA template, 0.2mM of each dNTP, 0.625 U T*aq* DNA polymerase (Promega Corp., Madison, WI), and 0.2 μM of the forward primer YV1 (5' GGGAGCTTGCTCCCCGG 3') (2), and the reverse primer YV4 (5'

AACGTCAATTGATGAACGTATTAAGT3') (8), in a PCR buffer (10mM Tris-HCl, pH 8.3, 5nM EDTA, 25mM KCl, 4mM $MgCl_2$). A PTC-100 thermocycler (MJ Research Inc., Watertown, MA) was programmed for 3 min. at 95°C, followed by 34 cycles of 30 sec. at 95°C, 30 sec. at 55°C, and 2 min at 72°C, with a final extension at 72°C for 10 min. A 10-μl aliquot of each PCR sample was separated on a 1.2% horizontal agarose gel at 10 V/cm for 120 min in TBE buffer (0.1 M Tris-HCl, pH 8.0, 0.1 M boric acid, 2 mM EDTA). A 100 bp-DNA ladder was used as a size marker. The gel was stained with ethidium bromide (0.5 μg/ml). A sample was considered positive for the presence of the CYVD bacterium if a 308 bp DNA band was visualized using UV light.

Analysis of field data. Data on the incidence of the CYVD bacterium in both experiments were analyzed using two statistical tests. The exact binomial test (1) is based on the normal approximation of populations to the binomial distribution. That is, if row cover has no effect on incidence of the bacterium, we would expect percent incidence between the two test plot populations (covered and not-covered) to be the same. The exact binomial test determines the probability that differences in means would occur to varying degrees. Fisher's exact test determines whether there is any relationship between two categorical variables and the right tailed version of the test is used when the alternative to independence is that there is positive association between the variables. In this case the variables are covered/not-covered and incidence/no incidence.

Results

Experiment one. Of the 70 plants grown under row cover, none appeared symptomatic or tested positive by Dienes' stain or by CYVD-specific PCR (Table 1). Of the 59 plants collected from the not-covered rows, two showed strong CYVD foliar symptoms including yellowing, wilting, and general and rapid decline of the vine. Four of the 59 plants (7%), including the two with foliar symptoms, had phloem discoloration and tested positive by Dienes' stain, and 15 plants (25.42%), including the seven that tested positive by Dienes' stain, were PCR-positive for the presence of the CYVD bacterium. PCR-positive samples occurred in three of the four not-covered test rows. Additionally, 11 of the 170 plants (6.5%) in the border rows were symptomatic and 35 of the 170 plants (20.6%) tested positive for the CYVD bacterium by PCR.

Although the CYVD bacterium was not detected in any of the covered rows, plants in these treatments were smaller than their not-covered counterparts and slightly yellowed. However, the slight yellow appearance was distinguishable from CYVD symptoms. The fruit in these rows were smaller and misshapen, probably because wind pollination of cucurbits is inefficient (13) and insect pollination was prevented by the row cover. No insects that would commonly feed on cucurbits, spotted cucumber beetles (*Diabrotica undecimpunctata howardi*), striped cucumber beetles (*Acalymma vittatum*), squash bugs (*Anasa tristis*), or leafhoppers were found on yellow sticky traps under the row cover, in fact, only small flies (Diptera) were found. The fact that no potential insect vectors were found on the yellow sticky traps confirmed the effectiveness of the

row cover. In not-covered rows, in addition to the small flies, the leafhoppers *Exitianus exitiosis* and *Macrosteles* sp. were collected in moderate numbers at all collection times throughout the season. Several other cucurbit pests including squash bugs, spotted cucumber beetles, striped cucumber beetles, and several other unidentified leafhopper species were inconsistently present in low densities. During the 50 days after planting, weeds were not controlled in either treatment and became quite numerous.

The exact binomial test considered individual plants as the experimental units, and was based on the probability that 0% incidence would occur in the covered plants if the row cover had no effect on the outcome. Using this test, the difference in incidence of the CYVD bacterium between the covered (25.42%) and not-covered (0%) plant populations was highly significant ($p<0.001$).

The right-tailed version of Fisher's exact test was used to determine if there was a relationship between the two categorical variables, covered/not-covered and incidence/no incidence. In this test, each of the four rows, rather than each individual plant, was considered as an experimental unit because rows, not individual plants, were covered. Using this test, the difference in incidence of the CYVD bacterium between the treatments was significant at the 93% level ($p=0.071$), a confidence level considered acceptable because of the small sample size (4 rows) and the nature of this statistical test.

Experiment two. Stand establishment was poor and plant growth less vigorous in the second experiment than in the first, possibly because the average temperature over the course of the experiment was relatively cool (25.29°C)

during the first experiment and much warmer (30.48°C) in the second. In addition, total precipitation was 27.86 cm during the first experiment, but only 3.15 cm during the second. Yellow sticky traps again indicated that the row cover was effective in excluding insects. Only small flies were found on the traps in covered rows. Several cucurbit insect pests, including squash bugs, spotted cucumber beetles, striped cucumber beetles, and several unidentified leafhopper species were inconsistently observed in not-covered rows in low densities. The leafhopper species *E. exitiosis* and *Macrosteles* sp were collected in moderate numbers throughout the growing season.

No plants had CYVD symptoms or tested positive with Dienes' stain in either covered or not-covered rows. When screened by PCR, none of the 22 covered plants (0%) tested positive, while three of the 15 not-covered plants (20%) tested positive (Table 2). The difference between the expected and observed proportion was significant (exact binomial test, $p<0.007$; Fisher's exact test, $p=0.214$).

Discussion

During the course of this study, the CYVD bacterium was not detected by PCR in yellow squash plants protected by row cover, while the bacterium was detected in 20-25% of not-covered plants. It must be noted that the row cover restricted access to plants not only by insects, but also by birds, rodents and other animals, thereby altering environmental conditions and causing higher soil and air temperatures and higher humidity (7). However, when the row cover experiments results are considered in conjunction with those of previous work showing disease suppression by insecticide application (4), a clear association between the restriction of insect access to plants and lack of the putative pathogen emerges. Thus, the data presented here support the hypothesis that the CYVD bacterium is insect-transmitted. Furthermore, low incidence of the CYVD bacterium in commercial fields is not unusual. Disease incidence can range from less than 5% to 100% (5). This fluctuation in percent incidence along with fluctuation in insect population and natural distribution probably accounted for the lack of CYVD in some not-covered rows. Randomized complete block design was used to control for these natural inconsistencies.

Dienes' stain, initially applied to screen for mollicutes (spiroplasmas and phytoplasmas) in plant tissues (6), has consistently given positive results with CYVD-affected plants exhibiting phloem discoloration (3). All plants that tested positive by Dienes' stain also tested positive by PCR. In addition, several Dienes' stain negative plants tested positive by PCR. Among available assays, PCR is the most reliable and most sensitive test for the presence of CYVD bacterium.

The small percentage of PCR-positive, but asymptomatic field plants may have reflected early stages of infection by the CYVD bacterium.

The experiments reported here were designed to determine if exclusion of above-ground insects could affect CYVD incidence and/or the occurrence of the CYVD bacterium in exposed plants. The absence of CYVD in covered plots suggests that these exclusion methods might be adapted for use by growers. However, several complications would have to be overcome. Both covered and not-covered plants flowered and bore fruit, but covered plants produced fewer fruits. Squash that were produced were small and misshapen because of the lack of adequate insect pollination (13). Using row cover to protect plants from insect feeding prior to flowering, then removing it to allow insect pollination to occur, may protect the plants from CYVD early in the season but allow for normal fruit production later. Insect pollinators could also be added under the row cover during flowering; however, harvesting from under the row cover would be difficult. Despite pre-plant herbicide applications, weeds were a considerable problem and would be difficult to control without disrupting the row cover. Maintenance of the row cover during the experiment was time consuming; constant attention was necessary to quickly repair small tears in the fabric, which otherwise could have allowed for insect entrance.

The original intent of our studies was to determine if excluding above ground insects from yellow squash plants would reduce incidence of the disease and/or occurrence of the CYVD bacterium. Our results showed a complete absence of both the disease and the associated bacterium in covered rows,

providing strong circumstantial evidence for insect involvement in the transmission of the bacterium associated with CYVD.

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Table 1. Influence of row covering on incidence of CYVD and detection of the associated bacterium in yellow squash (*Cucurbita pepo* var. *melopepo* cv. 'Lemon Drop') for the first exclusion experiment[a].

Treatment (row)	Plants (no.)	Symptomatic Plants (no.)	Dienes' Stain Positive (no.)	[b]PCR Positive (no.)[c]	Incidence by PCR (%)[d]
Covered Rows					
1	26	0	0	0	0.0
2	11	0	0	0	0.0
3	19	0	0	0	0.0
4	15	0	0	0	0.0
Total Covered	70	0	0	0	0.0
Not-Covered Rows					
1	15	2	3	7	46.7
2	19	0	1	4	21.1
3	15	0	0	0	0.0
4	10	0	0	4	40.1
Total Not-Covered	59	2	4	15	25.4

[a] Planted May 24, 1999.
[b] Polymerase chain reaction.
[c] Categorical variables are different by Fisher's exact test ($p=0.071$).
[d] Means are significantly different by exact binomial test ($p<0.001$).

Table 2. Influence of row covering on incidence of CYVD and detection of the associated bacterium in yellow squash (*Cucurbita pepo* var. *melopepo* cv. 'Lemon Drop') for the second exclusion experiment[a].

Treatment (row)	Plants (no.)	Symptomatic Plants (no.)	Dienes' Stain Positive (no.)	[b]PCR Positive (no.)[c]	Incidence by PCR (%)[d]
Covered Rows					
1	7	0	0	0	0.0
2	10	0	0	0	0.0
3	3	0	0	0	0.0
4	2	0	0	0	0.0
Total Covered	22	0	0	0	0.0
Not-Covered Rows					
1	8	0	0	2	25.0
2	3	0	0	0	0.0
3	3	0	0	1	33.3
4	1	0	0	0	0.0
Total Not-Covered	15	0	0	3	20.0

[a] Planted July 15, 1999.
[b] Polymerase chain reaction.
[c] Categorical variables are different by Fisher's exact test ($p=0.214$).
[d] Means are significantly different by exact binomial test ($p<0.007$).

Chapter III

Artificial Feeding System for the Squash Bug,

Anasa tristis (Heteroptera: Coreidae)

Abstract

The squash bug, *Anasa tristis* DeGeer (Heteroptera: Coreidae), occurs throughout North America and is considered a major pest of cucurbits. It has been confirmed as a vector of *Serratia marcescens*, the causal agent of cucurbit yellow vine disease (CYVD). Investigation of pathogen-vector interactions was hampered by the lack of a convenient artificial feeding system (AFS). Squash bugs did not feed on traditional feeding sachets filled with a liquid diet used for feeding homopertans, or in an AFS containing a diet developed for rearing tarnished plant bugs, *Lygus hesperus*, heteropterans closely related to *A. tristis*. However, cubes of excised squash fruit, vacuum infiltrated with a suspension of the desired diet and offered above a screen barrier, were accepted. During a 48 hr testing period, all cube-fed adult insects suvived. However, during the same period, 35% of those fed on the Meridic diet offered in parafilm bags (a system used for rearing *L. hesperus*), and 40% of those fed on 5% sucrose, offered in sachets, died. When blue dye was added to the diets as a marker, 75% of insects fed on infiltrated cubes defecated blue fluid, an indication of feeding. No blue defecation occurred when dye-amended diets in the other two systems were offered. The *A. tristis* AFS developed in this study provides a convenient system for studying the feeding behavior of squash bugs and may provide an alternative

to cucurbit plants for studying the etiology of CYVD disease.

Introduction

The squash bug, *Anasa tristis* DeGeer (Heteroptera: Coreidae), occurs throughout North America and is considered a major pest of cucurbits. *A. tristis* feeding involves piercing of the plant's epidermis by the stylets and intracellular penetration to the mesophyll or vascular tissues (2). Immature and adult squash bugs tend to aggregate, causing a condition called Anasa wilt, in which water stress is caused by the interruption of xylem transport from *A. tristis* salivary sheath blockage and stylet severing of the vessels (9).

A. tristis was reported (10) and confirmed (Bextine et al., manuscript in preparation) as a vector of *Serratia marcescens*, the causal agent of cucurbit yellow vine disease (CYVD). CYVD is characterized by rapid and general yellowing of leaves appearing over a 3-4 day period, followed by gradual or rapid decline and death of the vine, in several cucurbit crops (3). This bacterium has been cultured from watermelon, zucchini, pumpkin, and cantaloupe (Mitchell and Fletcher, unpublished), all suitable hosts for *A. tristis*.

Although artificial feeding systems (AFS) are available for many homopteran insects such as leafhoppers, aphids, and whiteflies, few have been developed for heteropterans because the nutritional requirements of the latter are more complex (5). AFSs, along with artificial diets, have been developed for the tarnished plant bug, *Lygus hesperus* (4,6,7), the reduviid bug, *Triatoma infestans* (12), and the southern green stink bug, *Nezara viridula* (11). In the latter case, the AFS was instrumental in associating insect feeding with microorganism transmission.

The relationship between the vector, *A. tristis*, and the pathogen, *S. marcescens*, is an important part of the etiology of CYVD. Investigation of pathogen-vector interactions of *S. marcescens* and *A. tristis* was hampered by the lack of a convenient AFS by which pathogen acquisition could be assured. Preliminary attempts in our laboratory to feed *A. tristis* on known hemipteran artificial diets were unsuccessful. The goal of this study was to develop an AFS to maintain squash bugs during research studies and to provide a convenient microorganism acquisition source. Specifically, we compared feeding acceptance and survivorship of *A. tristis* on a new AFS, diet-infiltrated squash cubes, and on traditional AFSs.

Materials and Methods

Insect colony maintenance. Colonies of *A. tristis*, initiated with adults collected near Lane, OK, were maintained in screened cages (50 X 25 X 45 cm) in a growth room at 27°C, 12L:12D, and 45-50% RH. Colony insects were reared on pumpkin plants (*Cucurbita pepo* L. var. *pepo* "Connecticut Field") replaced at weekly intervals and supplimented with washed squash fruit (*C. pepo* L. var. *melopepo*).

Preliminary tests of existing hemipteran AFSs and artificial diets. Several AFSs and artificial diets used for sustaining other hemipteran insects were offered to *A. tristis* and observations of feeding were made. Feeding sachets, or stretched-parafilm membrane feeding systems, commonly used for studying leafhopper, aphid, and whitefly feeding (8), consisted of five insects individually placed into five 29.5 ml flexi-cup medicine cups (Baxter Heathcare Corp., Deerfield, IL) covered by one layer of parafilm (American National Can, Greenwich, CT) stretched to 4X its original size over the medicine cup. A volume of 0.5 ml of 5% sucrose (pH 7.0) was placed on the outer surface of the stretched parafilm and covered with another layer of stretched parafilm to enclose the solution (8). These apparatuses were maintained at 27°C, 12L:12D, and 45-50% RH. Another five insects were offered a modification of this membrane feeding system in which squash slurry, made by liquefying whole squash fruit with sterile tap water (approximately 10 g squash fruit/20 ml water) in a blender, was brushed onto the insect-facing surface of the membrane (1 ml squash slurry/16 cm^2 membrane surface) as a possible gustatory cue.

Non-membrane systems were also tested by offering *A. tristis* semi-solid preparations of agarose or gelatin dissolved by heating to a boil either agarose (5%) or gelatin (3%) in sterile tap water. Ten ml of solution was poured into the bottom of 60 X 15 mm petri dishes (11). A 1 mm fiberglass screen barrier was placed over the petri dish bottom, and five insects/dish were confined on top of the screen under the dish lid for 48 hr at 27°C, constant light, and 45-50% RH. In one experiment the agarose and gelatin preparations were amended with squash fruit slurry, prepared as previously described, to 10% of the final preparation weight. They were offered to five insects each in separate apparatuses for 48 hr.

Development of the A. tristis artificial feeding system. Cubes of squash fruit (approximately 6 mm^3) were used as the feeding source. After washing the intact fruit with hand soap and reverse osmosis (RO) water, the fruit was cut into 6 mm slices with a sterile razor blade. The epidermis was removed and cubes were excised from the cortex. For infiltration, squash cubes were submerged in RO water containing blue food coloring (McCormick and Co., Inc, Hunt Valley, MD) (0.1ml/10ml H_2O) at a rate of 1 cube per ml in a 500ml Erlenmeyer vacuum flask. A vacuum force was applied for 5 sec., during which negative pressure forced air from the intercellular spaces of the tissue, and as the vacuum was released the blue colored water entered the intercellular spaces.

Adult squash bugs were confined individually with a 6 mm^3 squash cube using an apparatus similar to one described by Ragsdale et al. (11). The insect was placed in the bottom half of a 60 X 15 mm petri dish. A 70 X 70 mm square of 1mm fiberglass screen was placed over the dish, the squash cube was

positioned at the center of the screen, and the petri dish lid was positioned on top. The squash cube was offered above the insect, rather than below, to avoid fecal contamination and because *A. tristis* is an abaxial feeder. The feeding apparatuses were incubated in a humidity chamber (placed on a stand in a covered Rubbermaid shoebox containing water at a depth of approximately 1 cm) to minimize cube desiccation with constant light and at 27°C.

AFS acceptance trial. The three AFSs (the homopteran membrane feeding system, the tarnished plant bug feeding system, and the newly developed *A. tristis* AFS described above) amended with identical ratios of blue food coloring were tested for *A. tristis* acceptance in one replication over a 48 hr period at 27°C, constant light, and 45-50% RH. In the homopteran membrane feeding system, 20 adult *A. tristis* were placed individually into the apparatuses and, 0.5 ml of 5% sucrose (pH 7.0) containing blue food coloring (0.1 ml/10 ml sucrose) was placed on the outer surface of the stretched parafilm and covered with another layer of stretched parafilm (8). The tarnished plant bug feeding system (4) consisted of Meridic diet, a lima bean meal and wheat germ based formulation containing additional nutrient supplements, and containing blue food coloring (0.1 ml/10 ml diet) enclosed in unstretched parafilm bags as described by Debolt and Patana (7). This diet was offered individually to twenty single insects, in parafilm bags on mesh screening in six-well tissue culture plates with 35-mm wells. The *A. tristis* feeding system developed as part of this study, which is described above, was also offered individually to 20 *A. tristis*. For all three feeding systems filter paper (Whatman no. 4), cut to fit the bottom of the

apparatus, allowed collection of the droplets of squash bug excretory fluid. If this excretory fluid was blue, squash bugs were assumed to have fed on the offered diet. Insect mortality over the 48 hr period was recorded.

Data analysis. For both detection of blue excretory fluid and insect mortality, analysis of variance (ANOVA) was used to determine differences (critical *P*-value=0.05) in means between treatments. The Least Significant Differences (LSD) method was used to separate differences because more than two treatments were tested.

Results

In our preliminary experiments, when squash bugs were placed in several AFSs and offered artificial diets known to be acceptable to other hemipteran insects, neither stylet insertion nor test probing was observed. These insects did not feed through any membrane based feeding system, nor would they feed on the semi-solid agarose or gelatin formulations. Squash bugs began to feed on squash cubes in the newly developed AFS within one hr of introduction.

Squash bugs fed readily on blue water-infiltrated squash cubes, with 15 of 20 (75%) test insects defecating blue fluid on filter paper after 48 hr (Table 1), whereas none of the insects offered the other two systems/diets defecated blue fluid ($P<0.001$). None of the twenty insects offered blue water-infiltrated cubes in our AFS died during the 48 hr period, compared to 7/20 (35%) of those offered the Meridic diet in unstretched parafilm bags and 8/20 (40%) of those offered 5% sucrose in feeding sachets. Mean mortality of squash bugs fed in the *A. tristis* AFS was different than the other two AFSs ($P<0.001$)

Discussion

In our preliminary studies, squash bugs did not feed in the known homopteran or heteropteran artificial feeding systems tested. It is possible that physical, tactile, or gustatory cues, present on or in plants, are not present on these membranes, but neither supplementation of the diet with macerated squash fruit, nor coating the stretched membrane with squash fruit slurry, induced squash bug feeding behavior. Our *A. tristis* AFS was developed in response to observation of the insects feeding readily on squash fruit in colonies.

The ability of squash bugs to feed readily from infiltrated cubes was demonstrated by the fact that 75% of the insects tested were determined to have fed within a 48 hr period. These data indicated that this system might be suitable for the oral delivery of *S. marcescens* to *A. tristis* in acquisition experiments. *A. tristis* acquisition of *S. marcescens* in this artificial system has since been demonstrated by transmission experiments in which squash cubes were infiltrated with a bacterial suspension (Bextine, manuscript in preparation). The use of squash cubes as inoculation targets also facilitated the detection of the pathogen by polymerase chain reaction, as the cube size was ideal for DNA extraction.

The *A. tristis* AFS developed in this work is only partially artificial because the primary nutrient source is the squash fruit, a natural squash bug food that is chemically undefined. Development of an artificial diet to be used in the AFS would offer better control of the feeding medium; however, the cube system is

well-suited for the purpose of acquisition and inoculation of *S. marcescens* in pathogen transmission studies.

The demonstration of *A. tristis* as a vector of *S. marcescens*, the causal agent of CYVD, necessitated better understand the relationship between the insect and the pathogen. The cultivation of the bacterium from infected plants ensured a reliable source of the pathogen, but a system of microorganism delivery to the insect was needed. The *A. tristis* feeding system described here serves as a convenient method to study pathogen transmission by the squash bug. Using fruit cubes rather than infected plants as inoculum sources for *A. tristis* acquisition has several advantages; the fruit is readily available from stores, eliminating the need for plant care, and the amount and titer of the bacterial inoculum can be controlled and standardized in the infiltration step. Environmental conditions are also easier to control in the AFS than in a plant system. The cube system allows the interaction between the pathogen and the insect to be studied without the added dimension of the plant. However, eliminating the plant from the system does remove an important aspect of pathogen transmission. The pathogen titer in the cubes is probably higher than would be expected in the plant. Because the CYVD pathogen is phloem-associated, testing *S. marcescens* transmission by *A. tristis* in the infiltrated squash cube AFS would not reflect acquisition or inoculation of the pathogen in a plant system. The vacuum infiltration of a squash cube saturates all intercellular spaces, which would not be true in plant infection, and does not assure bacterial

entry into phloem sieve tubes However, the feeding system developed for *A. tristis* in this work provides a tool that will facilitate the study of CYVD.

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Table 1. Preference and suitability of three different artificial feeding systems by the squash bug, *Anasa tristis*.

Treatment[c]	Blue Defecation[a]		Mortality[b]	
	Total	Mean[d]	Total	Mean[d]
A. tristis feeding system with squash cube diet[e]	15/20	75a	0/20	0a
Lygus system with Meridic artificial diet[f]	0/20	0b	8/20	40b
Feeding sachets with 5% sucrose diet[g]	0/20	0b	7/20	35b

[a]Defecation: excretion of blue fluid was taken as a sign that *A. tristis* had fed on blue dye-amended diet in the artificial system tested.
[b]Mortality: proportion of insects that died within the 48 hr experimental period.
[c]Offered diet for 48 hr at 27°C, constant light, and 45-50% RH.
[d]Means followed by the same letter within a column are not significantly different (ANOVA, LSD, $p \leq 0.05$).
[e]*A. tristis* were offered 50 mm^3 squash cubes as a feeding source.
[f]*A. tristis* were offered unstretched parafilm bags containing the Meridic diet.
[g]*A. tristis* were offered parafilm membrane feeding sachets with 5% sucrose.

Chapter IV

Parameters of *Serratia marcescens* Transmission by *Anasa tristis* (Heteroptera: Coreidae)

Abstract

Symptoms of cucurbit yellow vine disease (CYVD) are phloem discoloration, chlorosis, rapid wilting and death of infected squash, pumpkin, watermelon, and cantaloupe. The causal agent of CYVD, *Serratia marcescens,* can be transmitted by the squash bug, *Anasa tristis.* In this study, we characterized basic parameters of *S. marcescens* transmission by *A. tristis.* Adult *A. tristis* of both sexes acquired *S. marcescens* by feeding on bacteria-infiltrated cubes of squash fruit and transmitted the pathogen after 24 hr. They retained *S. marcescens* and were able to transmit the bacterium up to 21 da post acquisition (DPA), the duration of the experiments. Most transmissions occurred between 2 and 8 DPA when a cube of squash fruit was the target and between 11 and 21 DPA when a pumpkin plant was the target. *S. marcescens* was detected inconsistently in the hemolymph of bacteria-fed insects; its presence there did not correspond with ability to transmit. Eggs laid by female *A. tristis* that had transmitted *S. marcescens* tested negative for the bacterium by specific PCR. Our data on the transmission of *S. marcescens* by *A. tristis* is consistent with, but not proof of, a hypothesis of non-circulative pathogen transmission.

Introduction

Cucurbit yellow vine disease (CYVD) is characterized by rapid and general yellowing of leaves appearing over a 3-4 day period, followed by gradual or rapid decline and death of the vine, in several cucurbit crops (6). In some cases, however, infected plants exhibit no yellowing or wilt but collapse in one day. A golden to honey brown discoloration of the phloem is observed in cross-sections of infected plant crowns. Symptoms resemble those reported in diseases caused by phloem-associated organisms in other crops, such as those causing citrus greening disease and clover club leaf disease. The bacterial pathogens of both of these diseases are transmitted by homopterans (6).

The presence of a walled bacterium, later identified as *Serratia marcescens* (21), was consistently associated with CYVD symptoms (1). *S. marcescens* isolates have since been cultured from CYVD-symptomatic watermelon, squash, pumpkin, and cantaloupe (Mitchell, Fletcher, unpublished). Koch's postulates were completed using *S. marcescens* strain Z01, from zucchini, confirming that *S. marcescens* is the causal agent of CYVD (Bruton et al. manuscript in preparation).

Preliminary evidence from experiments in which insecticides were used to suppress insect populations (7), and from analysis of in-field disease distribution of affected plants (6), led to the hypothesis that *S. marcescens* is insect transmitted. In field studies, when row cover fabric was used to exclude insect access to yellow squash plants (*Cucurbita pepo* var. *melopepo*) (3), none of the covered plants had yellow vine symptoms or evidence of bacterial infection,

whereas up to 46% of the uncovered plants tested positive for *S. marcescens* by PCR. Transmission of *S. marcescens* by insects was eventually demonstrated first by Pair et. al. (17), who caged squash bugs (*Anasa tristis*) on symptomatic pumpkin plants, later transferring the insects to healthy test plants for an inoculation access period (IAP).

Squash bugs occur in high numbers in cucurbit fields throughout the growing season (18). The species occurs throughout North America and is a major pest of most cucurbits. In the northern U.S., *A. tristis* is univoltine, having only one generation per year. However, in the southern U.S. it is multivoltine, making it difficult to control because of the varied age structure within the natural population (11). When feeding, *A. tristis* pierce the plant's epidermis with their stylets and penetrate intracellularly to the mesophyll or vascular tissues (2). Electronic monitoring of feeding behavior of first instars revealed that first instar *A. tristis* is capable of sustained phloem ingestion (5).

Insects belonging to the suborder homoptera, mainly leafhoppers and aphids, disseminate the majority of insect-transmitted plant pathogens (14). Relative to *A. tristis* these insects are small and their stylets penetrate host sieve elements causing minimal physical damage or disruption of plant cells. Pathogen transmission by a heteropteran is very unusual. Heteropteran stylets are large, relative to those of leafhoppers or aphids, and probably cause significant physical damage to host tissue during stylet penetration (2). Only one phloem-associated bacterium, the sugarbeet latent rosette pathogen, (16), has

been reported to be transmitted by a heteropteran insect, the beet leaf bug, *Piesma quadratum*. This bacterium is propagative within the insect.

The primary goals of this study were to characterize the relationship between *S. marcescens, A. tristis*, and cucurbit plants and to describe parameters of *S. marcescens* transmission. We hypothesized that *S. marcescens* is non-circulatively transmitted by *A. tristis*. To test this hypothesis, we investigated the time period necessary for pathogen acquisition by the vector (acquisition access period or AAP), the time period in which the vector is capable of transmitting the pathogen (IAP), and the lag period required between AAP and IAP (latent period or LP). We also investigated the percentage of *A. tristis* that transmit *S. marcescens* in an artificial system and the length of time in which the CYVD pathogen was detectable by PCR in the bodies of insects that did or did not transmit. We determined by PCR whether *S. marcescens* ingested by squash bugs invades the hemolymph. We also tested the hypothesis that immature *A. tristis* could transmit *S. marcescens* and that the bacteria could be transmitted vertically to offspring through eggs.

Materials and Methods

Insect colony maintenance. Colonies of *A. tristis*, initiated with adults collected near Lane, OK, were maintained in screened cages (50 X 25 X 45 cm) in a growth room at 12L:12D, 27°C, and 45-50% RH. Colony insects were reared on pumpkin plants (*Cucurbita pepo* L. var. *pepo* "Connecticut Field"), changed at weekly intervals, and were supplied with washed squash fruit (*C. pepo* L. var. *melopepo*) at the same time.

Bacterial culture. S. marcescens strain Z01-A was originally isolated (Bruton et al., in preparation) in LD8 broth (8) from the crown phloem tissue of a CYVD-symptomatic zucchini plant that had tested positive with CYVD-specific PCR primers (1). The strain was triply cloned for population homogeneity (Fletcher, unpublished data) and aliquots (500µl) were frozen in LB broth containing 30% glycerol at -80°C. For each experiment, an aliquot was thawed, added to 5 ml LD8 broth, and incubated for 8-12 hr with shaking at 28°C.

Plant cultivation. Pumpkin plants were grown from seed (two per 12 cm diameter pot in Metro-Mix 702 growing medium) under natural light conditions in the greenhouse, where temperatures ranged from 15-32°C.

Feeding assay. An artificial feeding system (AFS) (Bextine, thesis chapter 3) was used for acquisition of *S. marcescens* by *A. tristis* in all experiments and for inoculation in all cube-to-cube transmission experiments. For acquisition access, cubes of squash fruit (approximately 6 mm^3), excised from the interior of surface-sterilized fruit, were inoculated by vacuum infiltration with *S. marcescens* strain Z01-A (1 x 10^5 cfu/ml). An insect was placed in the bottom half a petri dish

with a square of fiberglass screen placed over the dish. The squash cube was positioned at the center of the screen, and the petri dish lid was positioned on top.

Transmission experiments. Experiments were designed to determine if *A. tristis* could transmit *S. marcescens* and to characterize several parameters of pathogen transmission. In all experiments, squash bugs (adult or immature) were placed in feeding apparatuses (Bextine, thesis chapter 3) where they had access to *S. marcescens*-infiltrated squash cubes for a 24 or 48-hr acquisition access period (AAP). Control insects were offered squash cubes that were not infiltrated (cube-to-cube transmission experiment, replication 1) or cubes that were infiltrated with diluted sterile LD8 broth (all other trials).

Polymerase chain reaction. DNA was extracted from squash cubes and insect bodies or parts with a GeneClean II extraction kit (Bio 101, Vista, CA), using a protocol modified from that of the manufacturer (3). These DNA samples were screened for *S. marcescens* by PCR using the *S. marcescens*-specific YV1-4 primer set (13). The detection limit of this DNA extraction method and PCR protocol was determined by testing 500µl samples serially diluted 10-fold to concentrations ranging from 1.6×10^{8} to 1.6×10^{-1} cfu/ml. The presence of *S. marcescens* in the hemolymph was tested by PCR assay of a single leg. Because legs lack any connection to the alimentary canal, bacteria detected in leg samples were assumed to have been in the hemocoel. Molted exuvia were also tested by the method described above. In all experiments involving *A. tristis* adults, gender was recorded.

Cube-to-cube transmission by adult A. tristis. An experiment was designed to test the ability of *A. tristis* adults to transmit *S. marcescens* from one AFS to another over an extended period of time and to determine the location of *S. marcescens* in the insects. Each of three experimental replications consisted of 10 treatment and 10 control insects at 27°C, constant light, and 45-50% RH. After a 24-hr AAP on bacteria-infiltrated squash cubes (treatment) or squash cubes infiltrated with LD8 broth (control), each insect was moved to a clean feeding apparatus containing a fresh, non-infiltrated squash cube. Each day for a total of 21 days, provided the insect lived that long, the insect-exposed squash cube was removed for analysis and replaced with a fresh squash cube for a succession of 24-hr IAPs. The whole insect bodies were individually tested in all three replications, but hemolymph samples were tested only in the second and third replications.

Cube-to-plant transmission by individual and grouped adult A. tristis. Once the capability of adult squash bugs to transmit *S. marcescens* in an entirely artificial system was established, two experiments were designed to test their ability to transmit *S. marcescens* from an artificial acquisition source to a healthy pumpkin plant. The individual cube-to-plant transmission experiment consisted of three replications, each with 10 treatment and 10 control insects. After a 48-hr AAP on bacteria-infiltrated squash cubes (treatment) or cubes infiltrated with LD8 broth (control), insects were confined individually on a single, second true-leaf stage pumpkin plant for 11, 48-hr IAPs. The cages, 25 cm tall, 7.5 cm diameter cylinders, were pressed into pot soil at the bottom and covered with mesh fabric

at the top. In the grouped cube-to-cube transmission experiment, groups of 16 insects were confined in screened cages (50 X 25 X 45 cm) on a succession of 16 pumpkin plants for 3, 10-day IAPs. Insects in both experiments were maintained in a growth room at 27°C, 12L:12D, and 45-50% RH. After exposure to insects, plants were treated with Resmethrin (Whitmire Research Laboratories Inc., St. Louis, MO) to kill surviving insects and maintained un-caged in a greenhouse (15-32°C, natural light, and 45-50% RH) for eight weeks. Plant crown samples were harvested, rinsed and stored at 4°C. Thin sections of crown tissue were evaluated with Dienes' stain (9,3), which highlights callose deposits in the phloem of plants with advanced infection, and by PCR as described above. Whole bodies were tested by PCR in all three replications, but hemolymph samples were tested only in the second and third replications. In all three replications, eggs laid by *A. tristis* females that transmitted *S. marcescens* to plants were collected from the under sides of leaves or the wall of the cage. The eggs were pooled (approximately 25 eggs per replication) and DNA was extracted and tested by PCR as described above. Eggs from control insects were also collected and tested.

Percentage of S. marcescens cube-to-cube transmission by adult A. tristis. This experiment was designed to determine the proportion of adult squash bugs transmitting *S. marcescens* using the artificial system and to determine whether transmission ability was correlated with the presence of *S. marcescens* in insect hemolymph. For this experiment, which consisted of four replications, each with 12 treatment and 12 control insects, six-well tissue culture

plates with 35-mm wells were used instead of petri dishes in the feeding apparatuses. Experiments were conducted at 27°C, constant light, and 45-50% RH. After a 48-hr AAP on *S. marcescens*-infilitrated squash cubes (treatment) or LD8 broth-infiltrated squash cubes (control), the screen separating the insects from the squash cube was replaced and a fresh squash cube was offered. Transfers of fresh squash cubes continued for a succession of 24-hr IAPs, totaling 6 days, provided the insect survived that long. Because previous studies had shown transmission of bacteria to cubes to be greatest between 2 and 8 days post-acquisition (DPA), cubes from 4, 5, and 6 DPA were pooled. DNA was extracted and tested by PCR as described above. For those treatments in which *S. marcescens* was not detected from 4,5, and 6 DPA, the possibility of earlier transmission was tested by pooling the cubes from 1, 2, and 3 DPA. Sex of the test insects was recorded.

Effect of A. tristis life stage on cube-to-cube transmission of S. marcescens. In this experiment, the goal was to test the ability of immature (second instar) squash bugs to transmit *S. marcescens* in the artificial system. Feeding apparatuses were constructed from twenty-well tissue culture plates with 20-mm wells. Two replications were done, the first with 20 treatment and 20 control insects and the second with 24 treatment and 24 control insects. Both replications were at 27°C with constant light and 45-50% RH. Second instar *A. tristis* were given a 48-hr AAP on *S. marcescens*-infilitrated squash cubes (treatment) or LD8 broth-infiltrated squash cubes (control), after which the screen separating the insect from the squash cube was replaced and a fresh, un-

infiltrated squash cube was offered. Transfers of fresh squash cubes continued for a succession of 24-hr IAPs, totaling 6 days, provided the insect survived that long. Each day, observations of the insects' condition and activity were made and any significant event, such as molting, was noted. As in the percentage cube-to-cube transmission experiment, cubes from 4, 5, and 6 DPA were pooled, and DNA was extracted and tested as above. If *S. marcescens* was not detected, cubes from 1, 2, and 3 DPA were pooled and tested.

Effect of molting on cube-to-cube transmission of S. marcescens by A. tristis. This experiment was designed to test the ability of *A. tristis* to transmit *S. marcescens* after molting, a process in which the cuticle of the insect's foregut is shed and replaced. The single replication included 10 treatment and 10 control insects at 27°C, constant light, and 45-50% RH. After a 48 hr AAP on *S. marcescens*-infilitrated squash cubes (treatment) or LD8 broth-infiltrated squash cubes (control), fifth instar insects were caged individually in the AFS with test squash cubes for a succession of 21, 24 hr IAPs. At the completion of the experiment, all squash bugs had molted to adults. Three squash cubes collected from the three IAPs immediately preceding the molt were pooled and tested by PCR and three squash cubes collected from the three IAPs immediately following the molt were pooled and tested. Insect bodies, hemolymph, and exuvia of all the insects were also tested.

Statistical analysis. Ratios of transmission by *S. marcescens*-fed *A. tristis* and control *A. tristis* were compared between treatments using chi-square analysis. Because in all control treatments, *S. marcescens* was not detected in

inoculation targets (squash cubes or plants), *A. tristis* whole bodies, hemolymph samples, nor exuvia, further comparisons of data were made within treatment data independent of the control set.

Results

Using the DNA extraction method and PCR protocol described, *S. marcescens* was detected at a concentration as low as 1.6×10^4 cfu/ml, approximately 8000 cells in a 500µl sample, but not at lower concentrations. The detection of *S. marcescens* in squash cubes and plants was used as the criterion for concluding that insect transmission occurred.

In the cube-to-cube experiment, none (0 of 30) of the control insects transmitted the bacterium, but 63% (19 of 30) of the squash bugs fed on bacteria-infiltrated cubes did (χ^2= 27.8, df= 1, *P*= 0.001) (Table 1). Many of the insects transmitted *S. marcescens* on multiple days, although 97% (18 of 19) of the transmissions occurred between 2 and 8 DPA, with only one transmission occurring outside of this range, at 14 DPA. In all three replications, the bacterium was not detected in any of the bodies or hemolymph from control insects. The bodies of most (14 of 19) insects that transmitted *S. marcescens* tested positive for *S. marcescens* by PCR and the bodies of the majority (6 of 8) of bacteria-fed *A. tristis* that survived 21 days tested positive. Of the *S. marcescens*-fed insects that did not transmit, 10 of 11 bodies harbored the pathogen. Only three out of13 of the hemolymph samples from *A. tristis* that transmitted the bacterium, and two of seven hemolymph samples of non-transmitters, tested PCR-positive for *S. marcescens* (assayed only in the second and third replications).

In the individual cube-to-plant experiment, plants exposed to control insects were consistently negative by all the detection methods used and all of the bodies or hemolymph samples from these insects tested negative. None of

the pumpkin plants exposed to *S. marcescens*-fed squash bugs showed typical foliar CYVD symptoms. However, transmission of *S. marcescens* to test plants by 10% (3 of 30) of *A. tristis* was indicated by phloem discoloration, Dienes' stain, and PCR (Table 2). An additional three transmissions were detected by PCR alone, for a total of six plants (6 of 30), compared to none (0 of 30) in the group exposed to control insects (χ^2= 6.7, df= 1, *P*= 0.01). In most cases, transmissions by bacteria-fed insects to plants occurred later (after 7 or 9 DPA) than those to squash cubes. The bodies of all (6 of 6) insects that transmitted *S. marcescens* to plants tested positive for the pathogen by PCR. Of the bacteria-fed insects that did not transmit, 8 of 14 bodies harbored the pathogen. Some, but not all, hemolymph samples (3 of 4 that were tested) from *A. tristis* that transmitted *S. marcescens* tested positive (assayed only in the second and third replications).

In the grouped cube-to-plant transmission experiment, none of the plants exposed to control insects tested positive, but three of eight treatment plants from the first 10-day period were Dienes' stain and PCR positive (Figure 1). All plants from the second or third 10-day period tested negative.

All three pooled groups of eggs collected from squash bug females that transmitted *S. marcescens* (individual cube-to-plant transmission experiment, replications one and two) tested negative for the bacterium by PCR.

In the transmission percentage experiment, 31.3% (15 of 48) of *S. marcescens*-fed adult *A. tristis* transmitted the bacterium, but the percentage of transmissions varied greatly among the four replications (Table 3). None of the

control insects transmitted *S. marcescens* (χ^2= 17.8, df= 1, *P*= 0.001), nor was the bacterium detected in their bodies or hemolymph. In the first replication, two *S. marcescens*-fed and one control insect died prior to 4 DPA, so in those cases squash cubes pooled from 4, 5, and 6 DPA were not tested. *S. marcescens* was detected in 56.3% (27 of 48) of all treatment insects, including 60% (9 of 15) insects that transmitted the bacterium and 54.5% (18 of 33) of the insects that did not. The bacterium was never (0 of 15) detected in the hemolymph (leg samples) of the insects that transmitted, but occurred in 12.1% (4 of 33) of those that did not transmit. One hemolymph sample from an insect in replication four tested positive even though its whole body sample was negative.

In the immature *A. tristis* transmission experiment, *S. marcescens* was not detected in squash cubes, whole bodies, or hemolymph samples of insects in the control treatment. The bacterium was not detected in any of the 44 tested squash cubes exposed to *S. marcescens*-fed insects (Table 4). However, it occurred in the bodies of 68.2% (30 of 44) of the bacteria-fed insects. Of the 30 insects in which the bacterium was detected, 13 molted and 17 did not. The bacterium was detected in the hemolymph of 27.3% (12 of 44) of the bacteria-fed insects, although the percentage varied greatly between the two replications (5%, or one of 20, in the first replication and 46%, or 11 of 24, in the second replication). Of the 12 insects in which the bacterium was detected in the hemolymph, four molted and eight did not.

In the molting study, *S. marcescens* was not detected in squash cubes, whole bodies, hemolymph samples, or exuvia of insects in the control treatment.

The bacterium was not detected in any of the squash cubes exposed to *S. marcescens*-fed *A. tristis* either before or after molt (Table 5). The bacterium was present in 20% (2 of 10) of the *A. tristis* bodies, and in one insect exuvium but not in any of the insects' hemolymph.

Discussion

The information reported in this paper confirms that *A. tristis*, an unusual vector species, transmits *S. marcescens*, the causal agent of CYVD, in an AFS. The use of an artificial system provided the opportunity to control the experimental conditions such that important basic information relevant to transmission could be collected. Considering the five major insect transmission modes described for other pathogen-vector systems, our data are most consistent with the non-circulative mode of transmission. Our characterization of several parameters of pathogen transmission will aid in the development of management strategies for CYVD.

A. tristis has been reported as a field vector of *S. marcescens* (17, Wayadande et al., personal communication). Although the data reported here do not confirm this insect's natural vector status they establish the biological capability of *A. tristis* to transmit the pathogen. Squash bugs are common pests of many cucurbit species, especially those in which CYVD has been reported. They are found in all areas of the U.S. where CYVD has been confirmed, and are prevalent in the large cucurbit-growing areas of Texas and Oklahoma (18), where CYVD was first described and causes considerable damage to cucurbit crops. The temperate environment in this region supports large populations of the species, and staggered age development contributes to the insects' persistence because certain life stages are less susceptible to chemical control than others (11).

CYVD symptoms differ from those of Anasa wilt, a condition caused by aggregated feeding of *A. tristis* individuals on leaf surfaces (19). In the latter condition interruption of xylem transport results after vessels are blocked by *A. tristis* salivary sheaths and/or severed by stylet puncture, causing water stress (15). Anasa wilt has a slow progression of symptoms, usually occurring over several weeks, in contrast to CYVD, where affected plants can collapse within one to two days after the onset of symptoms. In addition, the phloem discoloration associated with CYVD does not occur with Anasa wilt.

Our data are consistent with the hypothesis that *S. marcescens* is the causal agent of CYVD. Following the steps of Koch's postulates, Bruton et al. (manuscript in preparation) determined that *S. marcescens* was the phloem-associated bacterium associated with CYVD. Our observation of phloem discoloration and detection of *S. marcescens* by PCR in plants in the cube-to-plant experiments provided further evidence that this bacterium is the causal agent of CYVD and that *A. tristis* a vector. However it is possible that the role of *A. tristis* could extend beyond that of a vector because heavy stress caused by squash bug feeding damage, as in Anasa wilt, could contribute to the progression and severity of CYVD symptoms in infected plants.

Our findings are consistent with the hypothesis that the mode of transmission of *S. marcescens* by *A. tristis* is non-circulative transmission, similar to that which occurs in the transmission of *Xylella fastidiosa* by its homopteran vectors (20). *X. fastidiosa* does not move through the insect's body; rather, the bacterium colonizes the foregut of the vector and, if acquired by an adult, is

transmissible for the life of the insect. In our experiments, transmission of *S. marcescens* by *A. tristis* most commonly occurred after 24 hr, although in replication three of the cube-to-plant transmission experiment the first IAP was 48 hrs, so transmission occurring late in this first IAP would be consistent with transmission after 24 hr. In the first cube-to-cube transmission experiment transmissions occurred mainly between 2 and 8 DPA and later transmissions were infrequent. However, in the cube-to-plant transmission experiment, transmissions occurred mainly after incubation periods of one to two weeks. The exception occurred in replication three, in which one insect transmitted in six of ten IAPs, as early as 1-2 DPA and as late as 19-20 DPA. These data are inconsistent with non-persistent and semi-persistent transmission modes or with pathogen transfer via stylet contamination, in which pathogens are transmissible for only a short period (<48 hr). Differences in the incubation period, depending on whether the target was a cube or a plant, could be due to different *A. tristis* feeding behaviors when they feed from different tissues.

The fact that transmission of *S. marcescens* by *A. tristis* occurred most commonly after 24 hr and as long as days to weeks after acquisition is consistent with a latent period during which the bacteria increase in titer or are processed within the insect before transmission can occur. In the case of *X. fastidiosa*, which is non-circulatively transmitted, the bacteria must accumulate in the foregut before transmission. It is possible that *S. marcescens* accumulation is necessary for transmission by *A. tristis*.

Detection of *S. marcescens* in the hemolymph was not consistently associated with transmission in three separate experiments, further supporting the hypothesis that the pathogen is non-circulatively transmitted. That is, it was not necessary for the bacterium to traverse the insect alimentary canal wall and migrate through the hemocoel to the salivary glands before being transmitted to a new host. *S. marcescens* is known to be an insect pathogen, thus, detection of this bacterium in the hemolymph of some, but not all, of these insects could reflect bacterial invasion of the insect's body irrelevant to pathogen transmission. However, we cannot eliminate the possibility that the bacterium was present in the hemolymph of all the transmitting insects, since it may have occurred in some of them at a level too low to be detected by our PCR (detection limit 1.6×10^4 cfu/ml).

Evidence that *S. marcescens* persists for up to 21 DPA in *A. tristis*, without being detected in the hemolymph, also supports the hypothesis that the bacterium is non-circulatively transmitted. *S. marcescens* was detected in the bodies of some squash bugs that transmitted bacteria as late as 21 DPA. However, there were several cases in which the bacterium was not detected in the bodies of transmitting insects. This could reflect a gradual reduction in the titer of the bacterium to a level undetectable by PCR.

We found no evidence for the presence of *S. marcescens* in eggs of *A. tristis* females that transmitted the bacterium. Although our sample size was insufficient to allow a definitive conclusion, this preliminary evidence suggests that vertical or transovarial transmission of *S. marcescens* does not occur in

squash bugs. Absence of vertical pathogen passage also is consistent with the hypothesis that the mode of bacterial transmission is not circulative.

The percent of *A. tristis* individuals transmitting *S. marcescens* in an artificial system varied greatly. This could be due to the highly plastic feeding habits of individual squash bugs. That is, the variable feeding behavior from one insect to another. The transmission percentage of 31.3% in the percentage cube-to-cube transmission experiment was lower than that of 63.3% in the first cube-to-cube transmission experiment, probably due to the shorter time period allowed for the IAP in the former.

Although the bacterium was detected in bodies of *S. marcescens*-fed second and fifth instar squash bugs, these immature insects did not transmit the pathogen in our experiments. There is precedence for the failure of immature insects to transmit a pathogen that adults readily transmit; two examples in which insects can acquire but not transmit are those of tomato spotted wilt virus (TSWV) transmission by thrips (23) and clover club leaf bacterium transmission by leafhoppers (4). It is possible that the immature forms require different conditions for transmission (AAP, LP, IAP, or environmental conditions) or that transmission may be insect stage-specific (i.e. immature squash bugs may be incapable of transmitting *S. marcescens*). Transmission may require a specific physiological or biochemical attachment site that is present in the adults but absent in immature insects, as seen in the case of thrips thransmission of TSWV, in which enzyme processing of a glycoprotein is a required for transmission (12,22). If attachment in the foregut were necessary for transmission, as in non-

circulative transmission, the pathogen would be transmissible before molt but non-transmissible after molt because an attached pathogen would be removed along with the cuticular lining of the foregut. *S. marcescens* was detected by PCR in the exuvium of one bacteria-fed insect, which is consistent with bacterial attachment in the foregut. Unfortunately, none of the squash bugs in this experiment transmitted the pathogen, so transmissibility and potential retention of the bacterium in the foregut, as detected by PCR in the exuvia, could not be correlated.

The introduction of the bacterium to adult and immature *A. tristis* through *S. marcescens*-infiltrated squash cubes is an artificial system. The concentration of bacteria in the infiltrated squash cubes was greater, and the bacteria were offered from a different tissue, than would be the case if a squash bug were feeding from an infected plant. Thus, interpretations of these data must be made with caution; although they offer insight toward understanding the basic biological parameters involved in the interactions between *S. marcescens* and *A. tristis* that result in pathogen uptake, they do not necessarily reflect dissemination events that occur in the field.

Characterizations of the AAP, LP, IAP, retention time, rate of transmission by adult squash bugs, lack of vertical transmission, and inability of immature insects to transmit are important in understanding the interactions among the pathogen, vector, and plant and are critical to the development of effective management strategies for CYVD. Limiting the AAP, LP, and IAP could provided an exploitable means by which transmission of the bacterium could be disrupted.

The retention time, rate of transmission by adult squash bugs, and lack of vertical transmission are all important factors in the potential spread of the pathogen within and between plants of cucurbit fields, and could be manipulated to impede the dispersal of the CYVD bacterium. Breaking the diapause of adult squash bugs, could be a method to limit primary inoculum, because the CYVD bacterium overwinters in this insect. Our data indicate that the most important squash bug life stage, in relation to transmission of *S. marcescens*, is the adult therefore, stage specific control of *A. tristis* through chemical, mechanical, and biological means could be an efficient way to suppress CYVD incidence.

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Table 1. Cube-to-cube transmission experiment: Detection of *Serratia marcescens* in squash cubes that were exposed to bacteria-fed *Anasa tristis*.

		Day post-AAP[a]																						
SB[b]	A[c]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	Body[d]	Hemo[e]	Sex[f]
1-1	+	-	-	+	+	-	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	B-		
1-2	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	B+		
1-3	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	B+		
1-4	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-							B+		
1-5	+	-	-																				B+		
1-6	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	B+		
1-7	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-							B+		
1-8	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	B-		
1-9	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	B+		
1-10	+	-	-	-	-	-																	B+		
2-1	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-							B+	H-	f
2-2	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	B+	H-	f
2-3	+	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	B+	H+	f
2-4	+	-	-	-	-	-	-	-	-	-	-	-	-										B+	H-	f
2-5	+	-	-	-	-	-	-	-	-	-	-	-	-	-									B+	H-	m
2-6	+	-	-	-	-	-	-	-	-	-	-	-	-	-									B+	H+	m
2-7	+	-	-	+	-	-	+	-	-	-													B+	H-	m
2-8	+	-	-	-	+	-	-	-															B+	H+	f
2-9	+	-	-	-	-	-	-	-	+	-													B+	H+	m
2-10	+	-	-	-	-	-	-	-	-	-													B-	H-	f
3-1	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-							B+	H-	m
3-2	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-							B+	H+	m
3-3	+	-	-	+	-	-	+	-	-														B-	H-	f
3-4	+	-	-	-	-	-	+	+															B+	H-	m
3-5	+	-	-	+	-	-	+	-	-	-	-	-	-	-	-	-							B+	H-	f
3-6	+	-	-	-	-	-	+	+	+	-	-	-	-	-	-	-							B+	H-	f
3-7	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-							B-	H-	m
3-8	+	-	-	-	-	-	-	-	+														B+	H-	m
3-9	+	-	-	-	-	+	-	-	+														B-	H-	f
3-10	+	-	-	-	-	+	-	-	-	-													B-	H-	f

[a]Numbered columns represent days post AAP, + and shaded boxes indicate *S. marcescens* PCR positive squash cubes. If no +/- appear, insect died, so squash cubes were not tested.

[b]Each row represents an individual insect (replication number – insect number). An equal number of control insects were tested but all test squash cubes in those control sets were PCR-negative.

[c]PCR results from the squash cube infiltrated with *S. marcescens* and offered to insects for pathogen acquisition (AAP).

[d]B(+/-) indicates insect body was tested post-mortem.

[e]H(+/-) indicates that insect hemolymph was tested.

[f]Sex of the insect.

Table 2. Cube-to-plant transmission experiment: Detection of *Serratia marcescens* in 8-week-old pumpkin plants exposed to bacteria-fed *Anasa tristis*.

		Days post-AAP[a]													
SB[b]	AAP[c]	1-2	3-4	5-6	7-8	9-10	11-12	13-14	15-16	17-18	19-20	21-22	Body[d]	Hemo[e]	Sex[f]
1-1	+	-	-	-	+	-	-	+	+	+	+		+		f
1-2	+	-	-	-	-	-	-	-	-	-	-		+		f
1-3	+	-	-	-	-	-	-	-	-	-	-		+		m
1-4	+	-	-	-	-	-	-	-	-	-	-		-		f
1-5	+	-	-	-	-	-	-	-	-	-	-		+		f
1-6	+	-	-	-	-	-	-	-	-	-	-		+		m
1-7	+	-	-	-	-	-	-	-	-	-	-		-		m
1-8	+	-	-	-	-	-	-	-	-	-	-		-		m
1-9	+	-	-	-	-	-	-	-	+	-	-		+		m
1-10	+	-	-	-	-	-	-	-	-	-	-		-		f
2-1	+	-	-	-	-	-	-	-	-	-	-	-	-	-	f
2-2	+	-	-	-	-	-	-	-	-	-	-	-	-	-	m
2-3	+	-	-	-	-	+	+	+	+	+	-	-	+	+	f
2-4	+	-	-	-	-	-	-	-	-	-	-	-	-	-	f
2-5	+	-	-	-	-	-	-	-	-	-	-	-	-	-	m
2-6	+	-	-	-	-	-	-	-	-	-	-	-	-	-	m
2-7	+	-	-	-	-	-	-	-	-	-	-	-	-	-	f
2-8	+	-	-	-	-	-	-	-	-	-	-	-	-	-	f
2-9	+	-	-	-	-	-	+	-	-	-	-	-	+	+	f
2-10	+	-	-	-	-	-	-	-	-	-	-	-	+	-	f
3-1	+	-	-	-	-	-	-	-	-	-	-	-	-	-	m
3-2	+	-	-	-	-	-	-	-	-	-	-	-	-	-	f
3-3	+	-	-	-	-	-	-	-	-	-	-	-	-	-	f
3-4	+	-	-	-	-	-	-	-	-	-	-	-	+	+	f
3-5	+	-	-	-	-	-	-	-	-	-	-	-	-	+	m
3-6	+	+	-	+	+	+	-	-	+	+	+	-	+	+	f
3-7	+	-	-	-	-	-	+	-	-	-	-	-	+	-	f
3-8	+	-	-	-	-	-	-	-	-	-	-	-	-	-	m
3-9	+	-	-	-	-	-	-	-	-	-	-	-	+	-	f
3-10	+	-	-	-	-	-	-	-	-	-	-	-	-	-	f

[a]Numbered columns represent days post AAP, + and shaded boxes indicate *S. marcescens* PCR positive plant, + and striped box indicate phloem discoloration and PCR positive plant.

[b]Each row represents an individual insect (replication number – insect number). An equal number of control insects were tested but all test squash cubes in those control sets were PCR-negative.

[c]PCR results from the squash cube infiltrated with *S. marcescens* and offered to insects for pathogen acquisition (AAP).

[d]B(+/-) indicates insect body was tested post-mortem.

[e]H(+/-) indicates that insect hemolymph was tested.

[f]Sex of the insect.

Table 3. Percentage cube-to-cube transmission experiment: Detection of *Serratia marcescens* in squash cubes that were exposed to bacteria-fed *Anasa tristis* 4, 5, and 6 days post-acquisition (DPA), and in *A. tristis* bodies and hemolymph.

	Detection of *S. marcescens*[a]					
	Replication					
	1	2	3	4	Total	Mean
Inoculation target (squash cubes)[b]						
Serratia marcsesens detected in cubes from 4, 5, and 6 DPA, exposed to both male and female insects.	6/12	1/12	5/12	3/12	15/48	31.3
Cubes exposed to males only	2/4	0/4	2/6	¼	5/18	27.8
Cubes exposed to female only	4/8	1/8	3/6	2/8	10/30	33.3
All *Anasa tristis*[c]						
Whole body extract[d]	5/12	2/12	11/12	9/12	27/48	56.3
Hemolymph extract[e]	1/12	0/12	0/12	4/12	5/48	10.4
***A. tristis* that transmitted**[f]						
Whole body extract	2/6	1/1	4/5	2/3	9/15	60
hemolymph extract	0/6	0/1	0/5	0/3	0/15	0
***A. tristis* that did not transmit**[g]						
Whole body extract	4/6	1/11	7/7	8/9	20/33	60.6
Hemolymph extract	1/6	0/11	0/7	3/9	4/33	12.1

[a]Detection of *S. marcescens* by PCR. Data are shown as number positive/number tested. An equal number of control cubes and insects were tested but are not shown because they tested PCR-negative.

[b]Detection of *S. marcescens* by PCR in cubes pooled from 4,5, and 6 DPA.

[c]Detection of *S. marcescens* by PCR in all individual treatment insects.

[d]Whole insect body was tested by PCR.

[e]Insect hemolymph was tested by PCR.

[f]Detection of *S. marcescens* by PCR in insects that transmitted the pathogen.

[g]Detection of *S. marcescens* by PCR in insects that did not transmit the pathogen.

Table 4. Immature cube-to-cube transmission: Detection of *Serratia. marcescens* in squash cubes that were exposed to bacteria-fed *Anasa tristis* 4, 5, and 6 days post-acquisition (DPA), and in *A. tristis* bodies and hemolymph.

	Detection of *S. marcescens*[a]			
	Replication			
	1	2	Total	Mean
Inoculation target (squash cubes)[b]				
Serratia marcsesens detected in cubes from 4, 5, and 6 DPA	0/20	0/24	0/44	0.0
Anasa tristis[c]				
Whole body extract[d]	12/20	18/24	30/44	68.2
molted[e]	4/6	4/13	8/19	42.0
non molted[f]	8/14	8/11	16/25	64.0
Hemolymph extract[g]	1/20	11/24	12/44	27.3
molted	0/6	4/13	4/19	21.1
non molted	1/14	7/11	8/25	32.0

[a]Detection of *S. marcescens* by PCR. Data are shown as number positive/number tested. An equal number of control cubes and insects were tested but are not shown because all tested PCR-negative.
[b]Detection of *S. marcescens* by PCR in cubes pooled from 4,5, and 6 DPA.
[c]Detection of *S. marcescens* by PCR in all individual treatment insects.
[d]Whole insect body was tested by PCR.
[e]Indicates that the insect molted during the study.
[f]Indicates that the insect did not molt during the study.
[g]Insect hemolymph was tested by PCR.

Table 5. Effect of molting on cube-to-cube transmission. Detection of *Serratia marcescens* in squash cubes that were exposed to 5th instar *Anasa tristis* pre- and post-molt, and in *A. tristis* bodies, hemolymph and exuvia.

	Detection of *S. marcescens*[a]	Mean
Inoculation target (squash cubes)[b]		
Serratia marcescens detected in cubes pre-molt[c]	0/10	0%
S. marcsesens detected in cubes post-molt[d]	0/10	0%
Anasa tristis[e]		
Whole body extract[f]	2/10	20.0%
Hemolymph extract[g]	0/10	0.0%
Exuvia extract[h]	1/10	10.0%

[a]Detection of *S. marcescens* by PCR. Data are shown as number positive/number tested. An equal number of control cubes and insects were tested but are not shown because all tested PCR-negative.
[b]Detection of *S. marcescens* by PCR in pooled cubes.
[c]Pooled squash cubes from three days pre-molt.
[d]Pooled squash cubes from three days post-molt.
[e]Detection of *S. marcescens* by PCR in all individual treatment insects.
[f]Whole insect body was tested by PCR.
[g]Insect hemolymph was tested by PCR.
[h]Molted exoskeleton of individual insects were tested b y PCR.

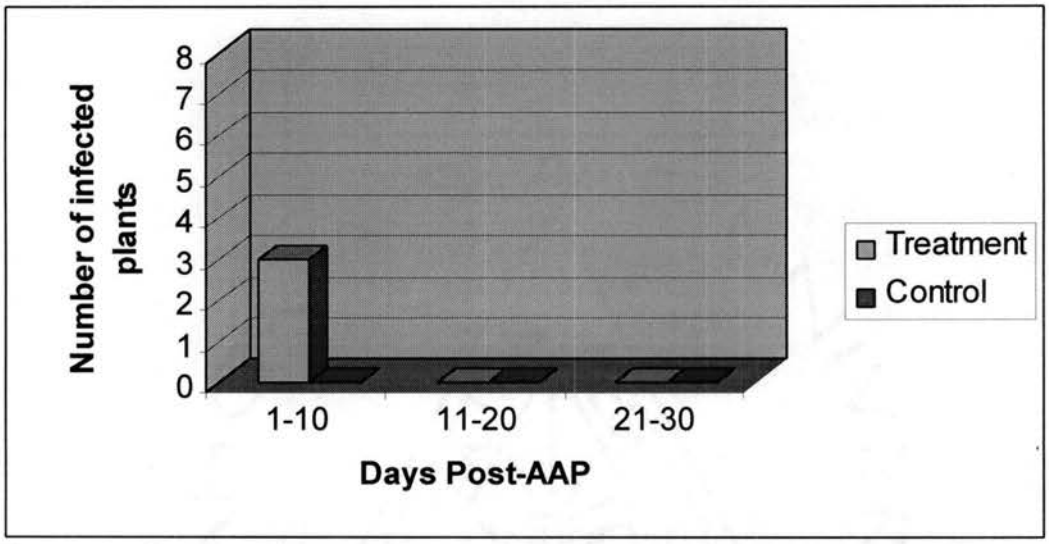

Figure 1. Grouped insect cube-to-plant transmission. Detection of *Serratia marcescens* in groups of pumpkin plants that were exposed to groups of bacteria-fed squash bugs or control squash bugs.

Chapter V

Summary

At the beginning of this research project little was known about the biology of cucurbit yellow vine disease (CYVD), a disease unknown until 1988. In the following decade the causal agent of the disease was identified as a phloem-associated bacterium, which was later identified as *Serratia marcescens*. No control measures were defined for CYVD and this disease was beginning to devastate cucurbit plantings in Oklahoma and Texas. Better understanding of CYVD biology was needed to develop control strategies, which could aid producers whose cucurbit yields were suffering.

Phloem-associated bacteria are often associated with insect vectors and two experiments conducted by Bruton et al. (1998) indicated that an insect was involved in some manner with CYVD occurrence. Soil fumigation did not affect the incidence of CYVD, but foliar application of insecticides lowered the disease incidence. Application of foliar insecticides does not target a specific pest and lack of specificity often leads to killing non-target, beneficial insects, such as natural enemies and pollinators. Therefore, general application of foliar insecticides is not the best management strategy in most disease situations. Identification of an exploitable aspect of CYVD biology for disease control would be an important step in developing a management plan.

The first objective in the research project reported in this thesis was to test the hypothesis that insects are involved in CYVD. To test this hypothesis, above-ground insects were completely excluded from cucurbit plants in the field. Plants from which insects were excluded had 0% disease incidence, compared to 20-25% incidence of CYVD in plants from which insects were not excluded. This experiment did not prove the role of insects, but did indicate that insect presence was involved in some aspect of the disease.

Because leafhoppers serve as vectors of most phloem-associated bacteria that are insect-transmitted, the knowledge that an insect was involved in the development of CYVD led to the hypothesis that *S. marcescens*, the causal agent of CYVD, was transmitted by an insect vector. Natural populations of leafhopper species were screened by PCR for *S. marcescens* to identify species that carried the bacterium and could therefore be potential vectors. Of 14 leafhopper species tested, only *Exitianus exitiosis* was identified as a carrier of the pathogen. Further investigation is needed to determine if it is a vector of the CYVD bacterium.

We also hypothesized that an alternate plant host was involved in the epidemiology of CYVD. Because alternate hosts often play important roles in the life cycles of pathogens, serving as inoculum sources and/or over-wintering hosts, disrupting the disease cycle by controlling the pathogen's alternate host(s) could constitute an effective management strategy. Possible plant over-wintering sites of the CYVD bacterium include cucurbit field debris and perennial or annual plants. Because domesticated cucurbit plants are annuals, they do not survive

from one season to the next and would not provide an alternate host for bacterial overwintering. Of 29 plant species growing near cucurbit fields and screened by PCR, plants of only two were positive and in each of these cases the presence of *S. marcescens* could not be confirmed. Although no plant species were definitively identified as potential alternate hosts for the CYVD bacterium, our study was not extensive enough to rule out the importance of alternate hosts, a question that will require further investigation.

Pair et al. (personal communication) and Wayadande et al. (personal communication) independently identified the squash bug, *Anasa tristis* (Heteroptera: Coreidae), as a vector of the CYVD bacterium by exposing field collected *A. tristis* to CYVD foliar symptomatic cucurbits and then caging these insects on healthy plants. In each case a proportion of the test plants developed CYVD symptoms. Because *A. tristis* is a major pest of cucurbits and occurs in great numbers in cucurbits throughout the growing season, it was hypothesized to be a significant natural vector of the CYVD bacterium. The concept of a heteropteran such as *A. tristis* serving as a vector, however, is very unusual because most phloem-associated bacteria are transmitted by homopterans. Only one other heteropteran has been was reported to transmit a phloem-associated bacterium. Better knowledge of this unusual pathogen/insect/plant interaction was needed to understand the biology of CYVD so that targeted control tactics could be designed.

Studies of *S. marcescens* transmission by *A. tristis* were hampered because CYVD symptomatic plants were present only during the few months of

the growing season and investigations into the disease using field plant systems were inconvenient and difficult to control. The 1999 cultivation of the bacterium from infected plants ensured a reliable source of the pathogen, but a system of microorganism delivery to the insect was needed. An artificial feeding system (AFS) was developed to serve as a convenient system for the study of pathogen transmission by the squash bug without the added dimension of the plant.

Characterization of the parameters of pathogen transmission by insects is essential for formulating management strategies for insect-borne pathogens. In this work we investigated the time period necessary for pathogen acquisition by the vector (AAP), the time period in which the vector is capable of transmitting the pathogen (IAP), and the lag period required between AAP and IAP (LP). We determined the persistence of the pathogen in adult *A. tristis* for up to 21 da and the correlation between pathogen transmission and bacterial presence in insect hemolymph. We investigated difference in transmission capability between male and female *A. tristis,* as well as, the ability of female *A. tristis* to pass the bacterium to offspring through egg lay. We also examined the vector capability of immature *A. tristis* and the ability of the insect to transmit *S. marcescens* after molting.

Determination of the length of the incubation period is important because it may suggest the type of relationship between the pathogen and the insect. Very long incubation periods (1 to 2 wks) suggests that the pathogen must traverse cellular barriers in the vector, circulating in the hemolymph, whereas very short incubation periods suggest that traversal of these barriers does not occur. In this

investigation, transmission of *S. marcescens* by *A. tristis* most commonly occurred after 24 hr but in a few cases it took as long as days to weeks after acquisition. These data are inconsistent with either non-persistent or semi-persistent transmission, or with pathogen transfer via stylet contamination.

Detection of a pathogen in the hemolymph of transmitting insects shows that the microbe can traverse cellular barriers within the body of the insect vector. *S. marcescens* was detected inconsistently by PCR in the hemolymph of insects that transmitted the bacterium, evidence that it was transmitted by *A. tristis* in a manner other than circulative. Transmission of circulative pathogens require the pathogen to enter the midgut of the alimentary canal, enter the hemoceol of the insect by traversal of the gut wall, migrate to and enter the salivary glands, and then inoculate a new host after insect salivation. *S. marcescens* was detected in a proportion of bacteria-fed insects, whether they transmitted or not. The bacterium is known to be an insect pathogen, thus, its detection in the hemolymph of these insects could reflect bacterial invasion of the insect's body irrelevant to pathogen transmission.

In non-circulative transmission, the pathogen attaches to the foregut wall and remains there. The inability of *A. tristis* to transmit *S. marcescens* after molting suggests a non-circulative mode of pathogen transmission. The foregut of an insect, formed by invagination of exo-cuticle, is shed when the insect molts. Non-circulative pathogens are lost along with the exo-cuticle during molting, resulting in the loss of transmissibility. *A. tristis* did not transmit *S. marcescens*

before or after molt, thus, this study was inconclusive and requires further investigation.

Determining the persistence of the pathogen in the insect is useful in understanding the relationship between a pathogen and its insect vector. *S. marcescens* was detected in the bodies of *A. tristis* for at least 21 da after acquisition, the length of the study. The bodies of some *A. tristis* that transmitted the pathogen (5 of 19) tested PCR negative for the bacterium. This could reflect a gradual reduction in the titer of the bacterium to the point that it was not detectible by PCR. The retention of the CYVD bacterium for as long as 21 da also is consistent with the non-circulative mode of pathogen transmission.

Several other parameters of pathogen transmission were investigated. We hypothesized that immature *A. tristis* could transmit *S. marcescens* because immature stages of many pathogenic insect species transmit the same pathogens as the adults. However, in our research studies, immature (second and fifth instar) *A. tristis* failed to transmit the CYVD bacterium. We also hypothesized that *S. marcescens* could be transmitted vertically through *A. tristis* eggs. However, the pathogen was not detected in eggs layed by *A. tristis* females that transmitted the pathogen.

In some cases of pathogen transmission, there is a difference in the transmission capability of males and females of the vector species. However, no difference in ability of male and female *A. tristis* to transmit *S. marcescens* was observed.

Understanding of these parameters provides important information that is useful for constructing an effective management strategy based on controlling adult *A. tristis*, the life stage that seems to be of greatest importance in *S. marcescens* transmission.

The data collected in this study are consistent with a non-circulative mode of pathogen transmission, similar to that described for the transmission of the bacterium *Xyllela fastidiosis* by its homopteran vectors. *X. fastidiosis* does not move through the insect's body; rather, the bacterium colonizes the foregut of the vector and, if acquired by an adult, is transmissible for the life of the insect.

The data reported in this thesis provide evidence of parameters of CYVD transmission. Further investigation is needed to definitively identify the mode of CYVD pathogen transmission by *A. tristis,* but this species was confirmed as a vector of the CYVD bacterium. In our experiments, an AFS was used for pathogen acquisition, which offer high bacterial titer in non-specific tissue and would not be consistent with acquisition from plants in a field situation. Furthermore, percentage of pathogen transmission to squash cubes was greater than transmission to plants, indicating that acquisition from and inoculation to a plant may influence transmission percentage by adding another dimension to the system. Therefore, the percentage of *A. tristis* able to transmit the CYVD bacterium plant-to-plant must be determined to understand the vector's significance in a field situation. Although this study was not designed to determine field vector status our data suggest that *A. tristis* is probably the most important vector in the field because it is capable of transmission and occurs in

high numbers in cucurbit fields throughout the growing season. To identify other insects that are potential vectors of the CYVD pathogen, insect transmission specificity also needs to be investigated. Finally, assessment of bacterial strain specificity will be necessary to determine if non-CYVD strains of the *S. marcescens* can cause CYVD or if the CYVD *S. marcescens* strain can inhabit other niches. Evaluating both insect and pathogen specificity in relation to CYVD will provide a better understanding of the potential impact of the disease and may uncover other exploitable aspects for disease management.

The data reported here provide important information that enhance our ability to develop effective control strategies for CYVD. The parameters of pathogen transmission described in this research are important in evaluating the impact of *A. tristis* as a vector. Controlling this insect through chemical, mechanical, and biological means could be an efficient way to suppress CYVD incidence.

Appendix A

PCR Screening for *Serratia marcescens*, the Causal Agent of Cucurbit Yellow Vine Disease, to Identify Potential Leafhopper Vectors

Abstract

Cucurbit yellow vine disease (CYVD) is characterized by chlorosis, rapid wilting and death of infected cucurbits and the causal agent, *Serratia marcescens*, is phloem-associated and insect-transmitted. Most insect-transmitted, phloem-associated bacterial pathogens are transmitted by leafhoppers (Hemiptera: Cicadellidae). Leafhopper populations at three locations in central Oklahoma were sampled and leafhopper carriers of *S. marcescens* were identified as a preliminary step in vector identification. During the 1998 and 1999 cucurbit growing seasons, over 2,000 leafhoppers were collected at three sites in Oklahoma and screened for the pathogen by *S. marcescens*-specific PCR. *Exitianus exitiosis* was the species collected in the greatest numbers; however, less than 1% of the *E. exitiosis* carried the pathogen. Individual insects of two other unidentified leafhopper species tested positive in 1998, but these species were collected in low abundance in 1999 and the individuals screened in that year were PCR-negative. In separate studies susceptible pumpkin plants confined with mixed-species groups of field-collected leafhoppers developed no disease symptoms and remained free of *S. marcescens*. Plants exposed to *S.*

marcescens-fed *E. exitiosis* also remained free of the pathogen, but since transmission parameters used in these experiments were not optimized for *E. exitiosis*, the negative results are not conclusive. Further experimentation will be required to confirm or refute a role for *E. exitiosis* in transmission of *S. marcescens*.

Introduction

A phloem-associated pathogen, later identified as the bacterium *Serratia marcescens* (9), was proposed as the causal agent of cucurbit yellow vine disease (CYVD) (1). *S. marcescens* causes rapid and general yellowing of leaves appearing over a 3-4 day period, followed by gradual or rapid decline and death of the vine and a golden to honey brown discoloration of the phloem (4). Some infected plants exhibit no yellowing and wilt and collapse in one day.

Observations of in-field disease distribution of affected cucurbit plants indicated that an insect might transmit the CYVD pathogen (5). Two subsequent experiments were designed to test this hypothesis. In one, the aerial application of insecticides reduced the incidence of CYVD in test fields (5). In the second, row cover material was used to exclude insect access to field plots of yellow squash plants (*Cucurbita pepo* var. *melopepo*) (2). In the second study, covered plants had 0% incidence of yellow vine, whereas up to 46% of the uncovered plants tested positive for the CYVD pathogen, as shown by CYVD-specific PCR.

CYVD symptoms, which are consistent with disruption of nutrient translocation, are similar to those caused by several other phloem-associated phytopathogens. Plant pathogenic mollicutes, spiroplasmas and phytoplasmas, are all phloem-associated and most are transmitted by leafhoppers. About 130 species of leafhoppers are known to transmit plant pathogens, including about 100 plant pathogenic mollicutes. Other similar diseases caused by phloem-associated bacteria are clover club leaf disease (CCLD), transmitted by the leafhopper *Agalliopsis novella* (3), and papaya bunchy top disease (PBTD),

transmitted by the leafhoppers *Empoasca papayae* Oman and *E. stevensi* Young (6).

Molecular techniques, such as the polymerase chain reaction (PCR), have facilitated searches for phytopathogen vectors. For diagnostic purposes, PCR was used to monitor populations of the aster leafhopper *Macrosteles quadrilineatus* for the presence of the aster yellows phytoplasma (7). Also, detection of phytoplasmas, such as that causing ash yellows, in leafhopper individuals allowed investigators to narrow the search for species of leafhoppers that might be vectors (8). Detection of phytoplasmas in the leafhopper species individuals did not prove vector competence of that species because detection could have resulted from the phytoplasma being in the gut of the insect as a result of feeding from infected tissue. However, detection of the pathogen in some species but not others did narrow the range of species that were more likely candidates as vectors.

The objective of this study was to identify leafhoppers naturally carrying *S. marcescens*, the causal agent of CYVD by screening natural leafhopper populations collected near CYVD-affected cucurbit fields for *S. marcescens*. Such species will be considered potential vectors and tested for their ability to transmit the CYVD pathogen.

Materials and Methods

Insect collection, 1998. Insects were collected from two locations (a commercial pumpkin planting, Coyle, OK and a commercial squash planting, Coyle, OK) once each week beginning in June and ending in July. For each collection, three sweep net samples were taken from the grassy areas adjacent to cucurbit fields in ten-meter lines, sweeping the net 25 to 30 times, about 30 cm off the ground. The insects collected from each sweep were placed, live and without sorting, in a capped tube (50 ml) for transport to the laboratory, where they were stored at -20°C.

Insect collection, 1999. Insects were collected from three locations (Oklahoma State University's Entomology and Plant Pathology Research Farm, Stillwater, OK, a commercial pumpkin planting, Coyle, OK, and a commercial squash planting, Coyle, OK) once each week beginning in February and ending in September. For each collection, three sweep net samples were taken as described above. The insects collected from each sweep were collected as described above.

Polymerase chain reaction. For PCR analysis the insects were thawed and sorted by species. Leafhoppers were pooled in groups of five and DNA was extracted and tested by PCR with *S. marcescens*-specific primers YV1 and YV4 (2).

Plant cultivation. Pumpkin plants were sown two seeds per 11.43 cm diameter Geranium pot (Kord Corp., Lugoff, SC) in Metro-mix 702 growing

medium (Scotts-Sierra Horticultural Products Co., Marysville, OH) under natural light conditions in a greenhouse, where temperatures ranged from 15°-32°C.

Pooled-insect transmission experiment. During the 1999 growing season, leafhoppers were collected once a week from the Coyle pumpkin plot by the sweep net technique described above and placed, live and without sorting, in a capped tube (50 ml) for transport to the laboratory. Approximately 50 leafhoppers of mixed species were confined in screened cages (50 X 25 X 45 cm) in a growth room at 27°C, 12L:12D, and 45-50% RH with four healthy 2 wk-old pumpkin seedlings (*Cucurbita pepo* L. var. *pepo* "Connecticut Field") for two weeks. The plants were then removed from the cage, sprayed with Resmethrin (Whitmire Research Laboratories Inc., St. Louis, MO) to eliminate any live insects, and placed in the greenhouse. Plants were observed for CYVD symptoms twice a week. After six weeks, pumpkin plant crowns were harvested by uprooting the plant. Any phloem discoloration was recorded and crown tissue of plants was subjected to DNA extraction and tested by PCR with CYVD-specific primers (1,2).

Sachet-to-plant transmission tests. The ability of *Exitianus exitiosis* adults to transmit *S. marcescens* in an artificial feeding system was tested because the bacterium was detected in field-caught *E. exitiosis*. During a 48 hr acquisition access period (AAP) groups of ten field-collected *E. exitiosis* adults were fed on sachets (Bextine, manuscript in preparation) containing 10% sucrose amended with *S. marcescens* Z01 (1.5×10^6 bacteria per ml), a CYVD strain cultured from zucchini. After the AAP, groups of 100 leafhoppers were successively

transferred to cages containing eight pumpkin plants for ten consecutive 48 hr inoculation access periods (IAPs). Plants were removed from cages after each 48 hr period after lightly shaking them to remove insects. All such plants were treated with Resmethrin and placed in the greenhouse for six weeks. Plants were checked for phloem discoloration and tested by PCR as described above. After 20 da, insects were recaptured using an aspirator, sorted into groups of five to reduce the number of samples, and tested by PCR for the CYVD pathogen.

Results

In the 1998 leafhopper collection 382 individuals were collected from the two locations and tested for the presence of *S. marcescens* (Table 1). Of these insects, eight tested positive for *S. marcescens* and these were confirmed in a second PCR test. One of the eight PCR-positive leafhoppers was an unidentified small gray leafhopper collected on 6/9/98. The other seven PCR-positive leafhoppers all were identified as *E. exitiosis* and all were collected on 6/16/98. Six unidentified rainbow-colored leafhoppers collected on 6/9/98 tested positive in an initial PCR but in second assay on the same extracted DNAs all six were negative, making the previous assay results unreliable. The majority of all insects collected (175 of 382, or 45.8%) were *E. exitiosis*. The percentage of *E. exitiosis* testing positive for *S. marcescens* was 6%. Other leafhoppers, collected infrequently, were; *Stirellus* sp., *Dalbulus* sp., *Macrosteles* spp., *Endria* sp., and several unidentified leafhoppers (Wayadande, personal communication). All of these leafhoppers tested negative. The first recorded symptomatic and PCR positive plants were a squash plant at the Coyle squash plot and a watermelon plant at the Coyle watermelon plot, both collected on 6/16/98.

In the 1999 leafhopper collection, 1399 individuals were collected, of which 793 (56.7%) were *E. exitiosis* (Table 2). Five *E. exitiosis,* collected on 4/6/99 from the Plant Pathology Farm, were the only insects to test positive for the presence of *S. marcescens*. No unidentified small gray leafhoppers and only 3 unidentified rainbow-colored leafhoppers (all PCR negative) were collected in 1999. Other leafhoppers collected infrequently were; *Stirellus* sp., *Empoasca*

sp., *Agallia* sp., *Dalbulus* sp., *Macrosteles* spp., *Erythroneura* sp., *Endria* sp., and several unidentified leafhoppers (Wayadande, personal communication), all of which tested negative for the CYVD bacterium. The first plants to test positive in 1999 were a squash plant from the Coyle squash plot and a watermelon plant at the Coyle watermelon plot, both on 6/11/99, and a squash plant at the Plant Pathology Farm on 6/18/99.

Pumpkin seedlings exposed to field-collected leafhoppers did not develop symptoms of CYVD, nor did they test positive by PCR. Of the 179 field-collected leafhoppers recovered at the end of the experiment, 118 of which were *E. exitiosis,* none tested positive for the CYVD bacterium.

Plants exposed to *E. exitiosis* that fed on sachets containing *S. marcescens* did not show symptoms of CYVD nor did they test positive by PCR. None of the insects collected after the experiment tested positive for the bacterium.

Discussion

The goal of this study was to identify leafhopper species individuals, occurring near CYVD-affected cucurbit plots, that naturally carry *S. marcescens*. These species would be considered likely candidates as vectors. *S. marcescens* was detected in three species of leafhoppers collected from fields adjacent to cucurbit fields affected by CYVD. *E. exitiosis* was the leafhopper species collected in the greatest numbers in both years and was the species in which *S. marcescens* was most often detected. *S. marcescens* was detected in one unidentified small gray leafhopper; however, this species was collected in low numbers in 1998 and not at all in 1999, making it unlikely that it is an important vector species. The positive PCR results obtained for unidentified rainbow-colored leafhoppers were not confirmed in a repeated PCR, making these positives unreliable. Other leafhopper taxa, most notably *Macrosteles* sp. and *Endria* sp., also were collected somewhat frequently throughout the growing season, but *S. marcescens* was not detected in these or any other species.

In our study, the screening for *S. marcescens* in insects was not meant to verify vector status of any insects. Insects carrying the bacterium could be vectors, dead-end hosts, or non-hosts. If the insect was feeding in the phloem of an infected plant at the time of collection, it would probably carry the pathogen in the gut lumen. Such bacteria would not necessarily be transmissible by that species. Detection of the bacterium in insects collected in 1998 occurred around the time disease symptoms and pathogen detection in cucurbit plants was noted. This would be consistent with pathogen presence in the gut lumen as a result of

feeding and would not necessarily indicate that the insects were inoculative. Inoculative insects would be expected to test positive several weeks before plant infections are detected. However, detection of the pathogen in a certain leafhopper species justifies further investigation into that insect's potential role as a vector.

Because the *S. marcescens*-specific primer set YV1 and 4 amplifies DNA from other strains of *S. marcescens,* and would not distinguish CYVD-causing *S. marcescens,* it is also possible that the bacteria we detected in the insects were an entomopathogenic strain and not the CYVD pathogen. *S. marcescens* is a cosmopolitan bacterium with strains that colonize insects (10). It has been identified as an entomopathogen in over 70 insect species and has been isolated from over 30 insect species. When an insect host ingests only small amounts of the bacterium, disease generally does not occur because the alimentary canal is not a suitable environment for the bacteria (10,11). However, when large doses of the bacterium are ingested, which may occur under laboratory conditions, many insect species are susceptible to infection and colonies can be devastated (10). A red-pigmented strain of *S. marcescens* was isolated from diseased and dead individuals of *Heliothis virescens* (Lepodoptera: Noctuidae) laboratory colonies.

Even if *E. exitiosis* and/or other leafhoppers in the collections were vectors of *S. marcescens*, the lack of transmissions in our experiments is not surprising, since parameters of transmission (optimal AAP, IAP and latent period) were unknown. It also was not known when or if the field-collected insects had fed on

infected plants. Optimal temperature, humidity, air flow, and other physical conditions, which are known to affect homopteran feeding and the effectiveness of pathogen transmission, also were not known.

Our experiments indicate that at least three species of leafhoppers collected in the vicinity of cucurbit fields carried *S. marcescens* in their bodies. Of these, *E. exitiosis* was the species most frequently collected, collected in the greatest numbers, and most often carrying *S. marcescens*. Further investigation of the potential of this insect to serve as a vector of the CYVD bacterium is warranted.

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Table 1. Use of PCR detection of *Serratia marcescens*, the causal agent of cucurbit yellow vine disease, to identify potential leafhopper vectors, 1998.

Collection date	Coyle squash plot		Coyle watermelon plot	
	Exitianus exitiosis[a]	Other species[a]	*E. exitiosis*[a]	Other species[a]
6/2/98	0/41	0/35	0/2	0/2
6/9/98	0/15	1/71[b]	0/55	6/58[c]
6/16/98[d]	7/46	0/19	0/10	0/0
6/30/98	0/1	0/22		
7/7/98	0/5	0/0		
Total	7/108	1/147	0/67	6/60
All species	14/382			

[a]Data represents number positive by PCR/number tested.
[b]PCR positive was from an unidentified small gray leafhopper.
[c]PCR positives were all from an unidentified rainbow-colored leafhopper (not confirmed when extracted DNAs were retested by PCR).
[d]First symptomatic plant collected.

Table 2. Use of PCR detection of *Serratia marcescens*, the causal agent of cucurbit yellow vine disease, to identify potential leafhopper vectors, 1999.

Collection date	Plant Pathology Farm		Coyle squash plot		Coyle watermelon plot	
	Exitianus exitiosis[a]	Other Species[a]	*E. exitiosis*[a]	Other species[a]	*E. exitiosis*[a]	Other species[a]
2/8/99	0/12	0/0				
3/1/99	0/1	0/2				
3/29/99	0/80	0/2				
4/6/99	5/77	0/0				
4/12/99			0/5	0/5	0/10	0/109
4/19/99	0/25	0/26				
4/28/99	0/20	0/30				
5/4/99	0/15	0/60				
5/12/99	0/25	0/10	0/45	0/11	0/28	0/3
5/14/99	0/35	0/1				
5/27/99			0/25	0/8	0/25	0/7
6/3/99			0/55	0/4	0/5	0/11
6/11/99[b]			0/46	0/27	0/10	0/3
6/17/99			0/27	0/13	0/1	0/7
6/25/99			0/13	0/15	0/14	0/25
7/2/99			0/26	0/32	0/7	0/27
7/10/99			0/41	0/22	0/8	0/16
7/16/99			0/24	0/21	0/10	0/42
7/23/99			0/5	0/3	0/6	0/2
8/6/99			0/18	0/7	0/28	0/16
8/13/99			0/8	0/2	0/10	0/24
9/3/99			0/1	0/6	0/2	0/7
Total	5/290	0/131	0/339	0/176	0/164	0/299
All species	0/1399					

[a]Data represents number positive by PCR/number tested.
[b]First symptomatic plant collected.

Appendix B

PCR screening for *Serratia marcescens*, the Causal Agent of Cucurbit Yellow Vine Disease, to Identify Potential Alternate Weed Hosts

Abstract

Cucurbit yellow vine disease, CYVD, occurs in several cucurbit crops and is characterized by rapid and general yellowing of leaves appearing over a 3-4 day period, followed by gradual or rapid decline and death of the vine and a golden to honey brown discoloration of the phloem. Transmission of *S. marcescens*, the causal agent of CYVD, by the squash bug, *Anasa tristis*, and the ability of the bacterium to over-winter in this insect have been demonstrated. However, alternate plant hosts, such as perennial weed species, may exist. Over three growing seasons, 1998, 1999, and 2000, weeds growing in field borders were monitored for CYVD-like symptoms. Crown samples of numerous weed species were collected near cucurbit fields and tested by *S. marcescens*-specific PCR. None of the weeds surveyed displayed CYVD-like symptoms. A single plant each of pigweed (*Cyclolorna atriplicifolium*) and lamb's quarters (*Chenopodium album* L.) tested positive in 1998, but these results were not confirmed in subsequent sampling. No other weeds tested were PCR positive in any year. The existence of weed alternate hosts remains a possibility, but further sampling will be necessary for conclusive results.

Introduction

With the advent of molecular technologies, such as the polymerase chain reaction (PCR), it is now easier than in the past to screen large populations of plant and insects for the presence of pathogens. The use of such tools has revolutionized fields such as plant breeding by allowing researchers to screen large populations for specific genetic sequences (3). The same technology is available to other scientific fields, such as entomology and plant pathology. For example, PCR was used to detect *Polymyxa betae* Keskin in sugar beet roots (8) and for monitoring populations of known vectors of the aster yellows phytoplasma (4). PCR screening also was used to detect ash yellows phytoplasmas in populations of leafhoppers for identification of candidate vectors (6).

PCR was used to detect the walled bacterium, *Serratia marcescens,* the causal agent of cucurbit yellow vine disease (CYVD) (1,10), in cucurbit crowns (2). This pathogen is transmitted by and overwinters in the squash bug, *Anasa tristis* (DeGeer) (9, Bextine, manuscript in preparation). Other over-wintering plants of CYVD strains of *S. marcescens* may exist. Because over-wintering hosts often play important roles in the life cycles of pathogens, disrupting the disease cycle by controlling the pathogen's over-wintering host(s) could constitute an effective management strategy. Possible over-wintering sites include cucurbit field debris, insects that either overwinter locally or migrate into and out of the region, and perennial or annual plants that serve as alternate

hosts. Because domesticated cucurbit plants are annuals, they do not survive from one season to the next and would not provide an over-wintering site.

The possibility that weeds could serve as alternate hosts of the CYVD pathogen was evaluated in this project by CYVD-specific PCR screening of weeds growing in close proximity to cucurbit fields. Small-scale weed collections were conducted over the 1998 and 1999 growing seasons. More systematic weed collections in 2000 were designed to enhance the likelihood of pathogen detection in weed species, thereby facilitating the identification of other candidate over-wintering hosts for *S. marcescens*.

Because sampling all natural weed flora in Oklahoma would have been unrealistic, we concentrated on weeds that are the most probable over-wintering hosts based on known interactions with *S. marcescens*. Annual weed species from the families Amaranthaceae and Chenopodiaceae were considered candidate *S. marcescens* alternate hosts because one plant each of pigweed (*Cyclolorna atriplicifolium*) and lamb's quarters (*Chenopodium album* L.) tested positive in 1998. In addition, perennial weed species from these families, which also occur in Oklahoma, were assayed as potential over-wintering hosts.

S. marcescens endophytically colonizes cotton, which is a member of the mallow family (12). Several mallow species occur as weeds in Oklahoma, the most prominent of which are rose mallows (*Hibiscus* sp.) and Cowboy's delight (*Sphaeralcea coccinea*). *S. marcescens* has also been associated with rice (*Oryza sativa* L.) as a beneficial endophyte able to fix nitrogen (5). Weed species related to rice, such as wild grasses belonging to the family Gramineae,

occur in Oklahoma. Alfalfa (*Medicago sativa*) was reported to be infected with *S. marcescens* as part of a pathogen complex causing crown rot (7,11); therefore alfalfa and other members of the family Fabaceae were also assayed for *S. marcescens* by PCR. Several cucurbit weeds that occur in Oklahoma, including pie melon (*Citrullus lanatus*), a perennial, are also potential alternate hosts for *S. marcescens*.

The goal of this study was to identify weed species that could serve as potential alternate hosts for *S. marcescens*. The identification of such weeds could be useful in understanding disease etiology and developing control strategies to suppress CYVD incidence in cucurbits.

Materials and Methods

Weed collection and PCR screening, 1998 and 1999. On September 11, 1998, 32 weed samples were collected from field borders adjacent to a watermelon field in Coyle, Oklahoma in which CYVD incidence was high. Weeds were uprooted and cleaned, leaves and flowers were labeled and pressed for later identification and crown samples were placed in zip-lock freezer bags and stored at -20°C. Samples were thawed and crown tissue was subjected to DNA extraction and tested by PCR with CYVD-specific primers (1,2). If weeds tested positive, they were identified.

On September 4, 1999, 24 weed samples were collected from the same sites as in 1998, and were collected and tested for *S. marcescens* as described above.

Weed collection and PCR screening, 2000. The Plant Pathology Farm in Stillwater, Oklahoma was chosen as the collection site in 2000 because CYVD incidence was as high as 46% in yellow squash plots (*Cucurbita pepo*) at this site over the 1998 and 1999 seasons. Weeds belonging to the candidate groups described above, growing in close proximity to the cucurbit plot, and weeds not identified to have known relationships with *S. marcescens* but growing in great abundance at the site, were collected once a week from May 18, 2000 through August 31, 2000. Each week, between five and ten plants were evaluated for CYVD-like symptoms, uprooted, placed in zip-lock freezer bags, and stored at 4°C. The plants were identified and three aliquots of each crown stored at -20°C. One aliquot was analyzed by PCR as described above. Another aliquot was kept

for bacterial isolation from plants that tested positive. As a positive control, 1 μl of cultured *S. marcescens* was added to the third aliquot and analyzed by PCR. As a negative control, aliquots of known negative plant samples were analyzed.

Results

Of the plants screened for *S. marcescens* in 1998 and 1999, only two tested positive by PCR. One sample each of pigweed and lamb's quarters, collected at the edge of a watermelon field in Coyle, Oklahoma, were identified as carrying *S. marcescens*.

Plants collected in 2000 are shown in Table 1. Of the 104 weeds collected and tested, few were from the chosen candidate alternative plant host groups because few of these were found at the collection site. None of the weeds at the site showed CYVD-like symptoms. Perennial species from the families Amaranthaceae and Chenopodiaceae were not found, but pigweed and lamb's quarters were collected on several occasions. Two mallows, *Hibiscus* sp. and *Sphaeralcea coccinea,* and several members of the Gramineae family, including Bermudagrass (*Cynodon dactylon* (L.) Pers.) and cheat (*Bromus secalinus* L.), were collected on several occasions. Alfalfa and other members of the Fabaceae family, such as Illinois bundleflower (*Desmanthus illinoensis*) and two species of sweetclover (*Melilotus* sp.) were collected regularly. Species belonging to nine other plant families were also collected. No cucurbit weeds were located at or near the Plant Pathology Farm.

None of the plants collected were found to harbor *S. marcescens* when tested by PCR. When DNA was extracted and tested by PCR from plant samples to which a small amount of *S. marcescens* had been added, positive reactions were always obtained. None of the negative control plants tested positive.

Discussion

Weeds belonging to most taxa identified as important possible alternate hosts of the CYVD pathogen, *S. marcescens,* were collected, but except for a single pigweed and a single lamb's quarter sample in 1998, all were negative when tested by CYVD-specific PCR. Many weed species were collected, but sample sizes were low, minimizing the odds of detecting the pathogen in an over-wintering host.

Because *S. marcescens* was detected in pigweed and lamb's quarters in 1998, these species were considered potential alternate hosts for the CYVD pathogen. However, both of these species are annuals and thus would not be probable over-wintering hosts for the bacterium. Our inability to confirm either species as a carrier of *S. marcescens* in subsequent testing forces us to consider the possibility that the 1998 results were erroneous. The PCR reactions could have been false positives, resulting from either contamination or human error, or may have been due to the presence in the plants of a different strain of *S. marcescens,* as this bacterium is known to inhabit several plant species as an endophyte. PCR using the YV1 and 4 primers is specific for *S. marcescens* but does not differentiate between strains (10). Strain identification of bacteria collected from PCR positive weed samples was planned in the 2000 weed-screening project, but since no weeds tested positive in that year, this protocol was not used.

None of the weeds in the test sites, whether collected or not, exhibited CYVD-like symptoms. An alternate host would not necessarily exhibit the same

symptoms that are documented in susceptible cucurbit varieties, and some plant hosts might actually be symptomless carriers.

Weeds are not the only possible sites in which the pathogen could overwinter. Other possible over-wintering niches include crop field debris or insects that either overwinter locally or migrate into and out of the region. Pair et al. (9) reported that *S. marcescens* survives in the squash bug, *A. tristis*, and is transmissible from one season to the next.

Although *S. marcescens* was detected in two weed samples, we cannot conclude that either species is an alternate weed host because these positives were not confirmed. However, we also cannot conclude that an alternate weed host does not exist, because sufficient data to support this were not collected.

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Table 1. PCR assay of selected weed flora collected at the Plant Pathology Farm in Stillwater, Oklahoma in 2000 for the presence of *Serratia marcescens*, the causal agent of CYVD.

Family	*Genus species*	Common Name	Date of Weed Collection[a]															
			5/18	5/25	6/1	6/8	6/15	6/22	6/29	7/6	7/13	7/20	7/27	8/3	8/10	8/17	8/24	8/31
Amaranthaceae	*Cyclolorna atriplicifolium* (Spreng.)	Pig weed	-		-					-	-	3-			-	2-		-
Apocynaceae	*Apocynum cannabinum*	Dogbane					-											
Asclepiadaceae	*Asclepias syriaca* L.	Milkweed			-													
Chenopodiaceae	*Chenopodium album* L.	Lambsquarters	-		-	-					-	-						
Compositae	*Ambrosia artemisiifolia* L.	Ragweed			3-													
	Coreopsis tinctoria Nutt.	Plains Coreopsis									-	-						
	Erigeron strigosus Muhl	Fleabane					-		-	-								
	Helianthus annuus	Sunflower			-		-	-										
	Vernonia baldwini	Ironweed		-	-													
Convolvulaceae	*Convolvulus arvensis* L.	Field Bindweed			-								-					
Cruciferae	*Brassica sp.*	Mustard			-													
Fabaceae	*Cassia fasciculate*	Locust Weed												-				
	Baptisia sp.	Rattleweed						2-		-								
	Medicago lupulina L.	Black medic	-							-			-					
	Desmanthus illinoensis	Illinois bundleflower					-		-	-	-							
	Medicago sativa	Alfalfa	-	-		-	-	-	-	-	-		-		-	-	-	-
	Melilotus alba	White Sweetclover			-	-	-							2-	-			
	Melilotus officinalis	Yellow Sweetclover	-	-					-	-		-		2-	-			
	Schrankia Nuttallii	Mimosa												-				
	Tripholium campestre	Clover												3-			-	-
Gramineae	*Bromus secalinus* L.	Cheat	-	-	-						-							
	Cynodon dactylon (L.) Pers.	Bermudagrass	-							-	-		-	-	-		-	-
	Eleusine indica	Goosegrass		-														
Malvaceae	*Hibiscus sp.*	Unknown Mallow				-	-	-					-					
	Sphaeralcea coccinea	Cowboy's delight	-															
Onagraceae	*Oenothera laciniata L.*	Cutleaf Primrose										-						
Polygonaceae	*Rumex sp.*	Curly Dock	-															
Solanaceae	*Solanum carolinense* L.	Horsenettle				-	-			-	-		-					
Umbelliferae	*Torilis sp.*	Parsley								-								

[a]Shaded boxes indicate that a weed sample was collected and tested, -/+ represents result of PCR. -/+ followed by a number indicates that more than one sample was collected and tested.

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