# PATHOGENICITY OF FOUR *SERRATIA MARCESCENS* STRAINS TO THE PEA APHID, *ACYRTHOSIPHON PISUM*, AND THE SQUASH BUG, *ANASA TRISTIS*

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# Literature Review

## I. Cucurbitaceae

### Introduction

The family Cucurbitaceae encompasses 118 genera and over 825 species worldwide (Jeffrey 1990). It is divided into two subfamilies, the Zanonioideae and the Cucurbitoideae, of which only members of Cucurbitoideae are of economic importance. Most species originated in warm parts of Africa and southeastern Asia, though the genus *Cucurbita* is of new world origin (Yamaguchi 1983). Of the 825 species, a few are common in agriculture; they include cucumber, cantaloupe, watermelon, squash, pumpkin, and various gourds. Agricultural species are grown most commonly for their fleshy fruits. The leaves, shoots, flowers, seeds, and roots are also used as food in some parts of the world (Yamaguchi 1983). The seeds are of particular interest for their very high oil content, an average of 45% oil in some *Cucurbita* sp. (Pangalo 1930). Cucurbits are even grown in Germany as an oilseed crop (Whitaker et al., 1986).

The genera *Cucumis*, *Citrullus*, and *Cucurbita* within the family Cucurbitaceae are the main focus of this study. The genus *Cucumis* includes cucumber, cantaloupe,

honeydew, and muskmelon, while the genus *Citrullus* is composed of watermelon. Both *Cucumis* and *Citrullus* originated in parts of Africa and Asia, and were distributed throughout Africa, Asia, and southern Europe through trade. Watermelon was introduced from south-central Africa to the Mediterranean region thousands of years ago. It was later taken from India to China and introduced to the Americas (Yamaguchi 1983). Watermelon is the most widely cultivated cucurbit in the world, followed by cucumber, melon, squash and pumpkin (FAO 1999). China produces most of the world's cucurbits.

There are five recognized species of *Cucurbita*: *C. pepo*, *C. ficifolia*, *C. moschata*, *C. maxima*, and *C. mixta* (Whitaker et al., 1950). Within these species are hundreds of varieties, which include different kinds of squash and pumpkin. The genus *Cucurbita* originated in the New World and includes species that are among the oldest cultivated plants in the Americas, dating back to around 2000 B.C. in the Chicama Valley of Peru (Whitaker et al., 1950). They grow wild from South America to as far north as the southern United States, but the largest concentration of species is found between Mexico City and Guatemala (Whitaker et al., 1986). Some believe that the origin of cucurbits in the Americas is located in this region of Mexico due to the large number of wild relatives there (Whitaker et al., 1986). Members of *Cucurbita* were introduced in the early 16$^{th}$ century to Europe, where they are now widely cultivated.

## Biology

Cucurbits are generally tropical to semitropical plants and are unable to withstand freezing temperatures (Whitaker et al., 1986). Most are annuals, but a few are perennials. Many species are climbing or trailing dicotyledonous plants, though summer and winter squash are actually more bush-like and do not produce tendrils for climbing (Whitaker et al., 1986). Seedlings are epigean, germinating with the cotyledons initially inverted in the shape of a hook. They require vast amounts of water, especially while fruits are developing, and have deep taproots that extend 1-2 m into the soil. Cucurbits usually have herbaceous stems that contain the largest sieve tubes found in angiosperms. Leaves are simple and palmately veined and contain an average of three to five lobes that vary in shape, depth, and size (Yamaguchi 1983). Only one leaf is present per node. They produce pepo fruits that vary considerably in shape and color, and are the largest fruits in the plant kingdom (Yamaguchi 1983).

## Economic Importance

Thirty species of cucurbits in nine genera are produced as food crops (Nayar et al., 1998). It is difficult to assess their full economic value, as many are grown on small farms and in backyard gardens and are consumed by the growers or sold at local and roadside markets. However, data are available for cucurbits produced on large farms. In the United States, members of the genus *Cucurbita* are important commercial crops. Among these, watermelon, cantaloupe, and squash are most prevalent, occupying more than 55,000 acres in Texas and Oklahoma (USDA 2003). The annual value of cucurbits produced in these two states alone exceeds $24 million (USDA 2003).

Because of the value of cucurbits, any insect or pathogen that causes damage to these crops is considered a serious threat. Insects that transmit pathogens are of particular concern to farmers.

Many pathogens are transmitted to cucurbits by insects. Examples of viruses include those transmitted by aphids, beetles, leafhoppers, and whiteflies. Some viruses transmitted by aphids are *Watermelon mosaic potyvirus* and *Cucumber mosaic virus*, the latter of which is transmitted by 60 insect species. Viruses transmitted by beetles are *Cucumber green mottle mosaic tobamovirus*, *Wild cucumber mosaic tymovirus*, and *Squash mosaic comovirus*. Whiteflies transmit *Cucumber vein yellowing virus*, *Melon leaf curl bigeminivirus*, *Squash leaf curl bigeminivirus*, *Watermelon chlorotic stunt bigeminivirus*, *Beet pseudo-yellows closterovirus*, and *Watermelon curly mottle bigeminivirus*. Leafhoppers transmit *Beet curly top hybrigeminivirus* and thrips transmit *Watermelon silver mottle virus*.

## II. Insect Vectors of Plant Pathogens

### Introduction

A vector is an organism, such as an insect, nematode, or fungus, which is capable of transmitting a pathogen from one host organism to another. The vector transports the pathogen either internally, in the digestive tract, body cavity and/or internal organs, or mechanically on the mouthparts, legs or other surfaces. Three modes of transmission have been described for plant pathogens transported internally: foregut-borne, circulative, and propagative (Nault 1997). Foregut-borne transmission occurs when a pathogen colonizes or attaches to the foregut of the insect and is transmitted to the host plant from the mouthparts during feeding (Nault 1997). In circulative transmission, the pathogen invades the gut tissue and migrates to the insect's salivary glands, from which it can be transmitted to the host plant (Nault 1997). In the case of propagative transmission, the pathogen can multiply in the vector organism (Nault 1997). Most plant pathogenic prokaryotes do not require a vector to be transmitted from plant to plant. However, some bacteria, such as those that cause Pierce's disease and clover club leaf, require a vector. The vector-pathogen relationship is an important component in the epidemiology of these plant diseases.

Vectors are of great importance to pathogens that are limited to a specific tissue in the plant, such as the phloem or xylem. These pathogens are unable to directly infect other plants. Instead, they utilize insects that feed on the vascular tissue as a mechanism of movement. For example, some phloem-feeding leafhoppers (Homoptera) are efficient vectors of several plant pathogens. They feed frequently, usually on plants from a

specific group or family, by inserting their stylets directly into the phloem tissue. Their ability to probe specific tissues is one aspect that enables them to be good vectors.

**Process of Transmission**

Transmission of pathogens by a vector is divided into three phases: acquisition, latency, and inoculation. Acquisition occurs the moment an ingested pathogen invades gut epithelial cells. In some cases a pathogen can be acquired congenitally, through the infected ovary of the female. The term acquisition access, used when referring to an insect acquiring a pathogen through feeding, is the period in which an uninfected vector has access to a source of inoculum. During this time the vector is not necessarily able to transmit the pathogen to plants. In the laboratory, pathogens can also be introduced artificially into the vector through injection directly into the hemocoel. The second phase of transmission, the latent or the incubation period, is the time required between vector acquisition and transmission (Purcell 1982). The pathogen colonizes the insect gut and may enter the hemolymph, in which it can circulate throughout the vector's body. Some pathogens multiply in the gut or hemolymph during the latent period. Other pathogens multiply in the vector with no apparent latent period, as is the case with Pierce's disease, in which the causal bacterium, *Xylella fastidiosa*, colonizes the foregut (Purcell 1982) and is transmissible to the plant upon the next feeding. A pathogen that must move into the hemolymph can have a longer latent period than one that does not, due to the time consuming process of crossing the gut barrier and circulating in the hemolymph until it reaches a location from which it can be transmitted. The length of the latent period is influenced by the means of acquisition. For example, the latent period for bacteria

acquired through feeding may be longer than that for a bacterium injected directly into the hemocoel. The third phase is the inoculation phase, during which the insect introduces the pathogen to a new host plant.

There can be differences in efficiency of both inoculation and acquisition due to variations in the amounts of inoculum acquired and injected into the plant by the insect vector (Purcell 1982). Another important factor is the virulence of the inoculum in infecting plants and/or the vector. Some plant hosts are readily inoculated but do not support sufficient inoculum reservoirs to infect feeding insects. These "dead end hosts" are not important in the life cycle of the pathogen. Plant age may also influence susceptibility to infection; in many cases young plants are more susceptible than older plants (Purcell 1982).

**Cucurbit Vectors and Pathogens**

There are many important species of vector insects that affect cucurbits. Among them, the melon aphid, *Aphis gossypii* (Glover), is an important vector of *Cucumber mosaic virus* (CMV), *Watermelon mosaic virus* (WMV-2), and *Zucchini yellow mosaic virus* (ZYMV) (Martin et al., 2000). Pea aphids, *Acyrthosiphon pisum,* are also vectors of ZYMV (Hunter and Ullman 1992). The green peach aphid, *Myzus persicae*, is capable of spreading WMV-2 and CMV (Brunt 1996).

Beetles are another important group of insect vectors. Cucumber beetles of three species transmit pathogens: the striped cucumber beetle, *Acalymma vittatum* (Fabricius), the banded cucumber beetle, *Diabrotica balteata* (Le Conte), and the spotted cucumber beetle, *D. undecimpunctata howardi* (Barber). Two of these species transmit the

bacterium *Erwinia tracheiphila*, which causes bacterial wilt (Robinson et al., 1997). Cucumber beetles also transmit *Bean chlorotic mottle bromovirus*, *Bean pod mottle virus*, *Bean mild mosaic carmovirus*, *Cowpea severe mosaic comovirus*, and *Squash mosaic virus* (Brunt 1996).

Thrips are important vectors of *Watermelon silver mottle virus* and *Zucchini lethal chlorosis virus* (Moyer 1999). The beet leafhopper, *Circulifer tenellus*, is an important vector that transmits *Curly top virus* to cucurbits and other host plants (Robinson et al., 1997). Another major vector group is whiteflies, which transmit three cucurbit viruses: *Lettuce infectious yellows*, *Beet pseudo yellows*, and *Squash leaf curl* (Duffus et al., 1986, Duffus 1965, Cohen et al., 1983).

## III. Cucurbit Yellow Vine Disease

### Discovery and Host Range

In 1988 a new disease of squash and pumpkin was observed in Oklahoma (Cartwright et al., 1993). The disease, which caused yellowing and wilting of plants, was called yellow vine decline (YVD), a name that was later changed to cucurbit yellow vine disease (CYVD). In 1991 a similar, possibly identical, disease was found on watermelon and cantaloupe in Texas and Oklahoma (Bruton et al., 1993). Disease incidence ranged from 5-50% in watermelons grown in central Texas in 1994 (Bruton et al., 1997). The disease was found on watermelons in eastern Texas in 1995 and, during the years of 1995 and 1996, was severe in some areas of Texas and Oklahoma (Bruton et al., 1997). Originally thought to be confined to Oklahoma and Texas, CYVD has since been confirmed in Tennessee, Massachusetts, Arkansas, Colorado, Kansas, Nebraska, Missouri, Connecticut, Michigan, and Kentucky (Bost et al., 1999, Pair et al., 2000, Nesmith 2002, Pair et al., 2003, Boucher 2005, Hausbeck 2004). In 1998 transmission electron microscopy showed the presence of a rod-shaped bacterium in the phloem sieve tube elements of symptomatic plants (Bruton et al., 1998). The causal agent was later identified as *Serratia marcescens* (Rascoe et al., 2003, Zhang et al., 2003).

Of the cucurbits susceptible to CYVD, squash and pumpkin are most susceptible, with watermelon slightly less so and cantaloupe the least susceptible. Disease in cantaloupe usually appears only in crops grown adjacent to infected watermelon fields, and even then, incidence is usually under 10% (Bruton et al., 1997). So far CYVD has never been found affecting cucumber, gourds, or wild cucurbits in nature. CYVD appears to affect early-planted crops more severely than those planted in late June. The

normal range of CYVD incidence in early planted watermelon is 20-60%, but can reach 100% in some fields planted in April and early May (Bruton et al., 1997). Late-planted crops have little to no incidence of disease (Bruton et al., 1997). CYVD may cause severe economic losses in commercially grown cucurbits, ranging between 5 and 100% (Pair et al., 2004). In Oklahoma and Texas, where cucurbits account for more than 40,000 hectares of farm land and more than $100 million dollars in value (Bruton et al., 1998), losses could range between $5 and $100 million dollars annually.

**Symptoms**

The symptoms of CYVD are similar in squash, pumpkin, watermelon, and cantaloupe. As a phloem colonizer, *S. marcescens* interferes with the plant's ability to transfer photoassimilates to the root system, resulting in wilting and eventual plant death (Bruton et al., 1998). Immature plants affected with CYVD may wilt and collapse in a single day without yellowing (Bruton et al., 1995). More mature plants usually develop symptoms about two weeks prior to harvest. First, leaves change color, turning lime green to bright yellow. Then the entire plant turns yellow, taking about two to three days to become fully chlorotic. Fruit on watermelon begins to lose chlorophyll, but fruit and flowers on other hosts do not appear to be affected. Internode lengths are reduced, causing stunting, and leaves fail to expand properly, resulting in inward curling. Most leaves die seven to ten days after the first symptoms appear. The root system is typically symptom free in early stages of infection, but in later stages deteriorates rapidly (Bruton et al., 1995). CYVD resembles other vine decline diseases except that crown lesions do not develop and there is a honey-brown discoloration of the phloem, most intense at the

crown but occasionally found throughout the plant (Bruton et al., 1995). Fusarium wilt also causes vascular discoloration, but it is distinct from that of CYVD in that it is dark reddish-brown and occurs predominately in the xylem. Wilted CYVD-affected plants do not recover turgidity at night, as is seen with Fusarium wilt.

CYVD symptoms were originally attributed to factors such as herbicide damage, nutrient imbalance, virus infection, Fusarium wilt, charcoal rot, Monosporascus vine decline, and seed-borne pathogens (Pair et al., 1998). Fusarium wilt is the most prominent soil-borne disease of watermelon in Oklahoma and Texas (Bruton et al., 1995). *Fusarium oxysporum* is capable of infecting cucumber, cantaloupe, and watermelon (McMillan 1986), and also infects squash and pumpkin. *Fusarium* was isolated from CYVD-affected plants along with several other fungi, but none were consistently associated with CYVD. In 1991 *Lettuce infectious yellows virus* (LIYV) was reported infecting cantaloupe and honeydew melons in north central Texas, watermelon in central Texas, and squash and cushaw along the gulf coast of Texas (Halliwell et al., 1992). However, the whitefly (*Bemisia tabaci*) vector of LIYV was not found in infected melon fields and the cause of CYVD was determined to not be LIYV (Pair, unpublished data). DNA hybridizations of CYVD-affected samples were negative for LIYV, beet curly top virus, whitefly-transmitted geminiviruses, and *Spiroplasma citri* (Bruton et al., 1995). Vine decline caused by *Macrophomina phaseolina* was the predominant disease of cantaloupe in Texas in 1980 (Bruton et al., 1987) and root rot/vine decline caused by *Monosporascus cannonballus* was a major pathogen in cantaloupe in 1985 (Bruton et al., 1995). However, *M. phaseolina* was not consistently associated with CYVD and inoculations with the pathogen did not result in yellow vine

symptoms (Bruton et al., 1995). *M. cannonballus* was not isolated from affected cucurbits in Oklahoma and Texas, so it was also eliminated as the cause of CYVD (Bruton et al., 1995). Another potential cause of the symptoms seen in cucurbit crops was Anasa wilt, a condition caused by the feeding of numerous squash bugs on a plant and characterized by wilting, delayed maturity, reduced yield, and death due to vascular blockage (Neal 1993). Squash bug feeding may contribute to a set of CYVD symptoms that include wilting and collapse of the plant, but is not the sole factor in CYVD symptoms (Bruton et al., 2003). Eventually, a phloem-colonizing bacterium, *S. marcescens*, was isolated from diseased plants, and completion of Koch's postulates proved it to be the pathogen responsible for CYVD (Bruton et al., 2003). *S. marcescens* causes phloem discoloration and reduction in internode length, features not associated with Anasa wilt. It is possible that a combination of both Anasa feeding damage and *S. marcescens* colonization is necessary to cause the complete symptoms of CYVD, though further investigation is needed to confirm that hypothesis (Wayadande personal communication, 2006).

## IV. *Serratia marcescens*

### Introduction

The genus *Serratia* belongs to the family Enterobacteriaceae, and is composed of many species. A few species, including *S. marcescens*, produce a water-insoluble, nondiffusible red pigment called prodigiosin. Historically, all red bacteria were thought to be *S. marcescens*, but red pigmentation is not unique to *Serratia*; it is also found in several other genera including *Vibrio*, *Pseudomonas*, *Alteromonas*, *Streptomyces*, and *Nocardia* (D'Aoust et al., 1974, Gandhi et al., 1973, Gerber et al., 1976).

Accounts of red spots on food go as far back as Diodorus of Sicily in 60 B.C. and Quintus Curtius Rufus in 332 B. C. (Grimont and Grimont, 1978). Superstitions about the 'blood' on bread and consecrated wafers haunted people in antiquity and continued to cause anxiety into the early 19$^{th}$ century. In 1819 a case of red spots on corn polenta terrorized the people of Padua, Italy. Less than five years later, Bizio and Sette independently demonstrated that the 'blood' was caused by a microorganism that Bizio named *Serratia marcescens* and Sette named *Zaogalactina imetrofa* (Bizio 1823 as cited in Grimont and Grimont, 1978, Sette 1824 as cited in Grimont and Grimont, 1978). In 1848 Ehrenberg described a motile organism causing red spots on food in Germany, which he named *Monas prodigiosa* (Ehrenberg 1849 as cited in Grimont and Grimont, 1978). Koch isolated a red pigmented bacterium from a monkey in 1884, naming it *Bacillus indicus* (Eisenberg 1886 as cited in Grimont and Grimont, 1978). By 1903, more than 76 bacterial species, including *Serratia* species, were known to produce a red or pink pigment (Grimont and Grimont, 1978). In 1918, Buchanan revived the generic name *Serratia* (Buchanan 1974), which is now universally accepted. Because the

original organisms studied by Bizio, Sette, and Ehrenberg were lost it is not certain whether any of these bacteria were actually *Serratia*.

**Biology**

*S. marcescens* is a gram-negative, spore-forming bacterium that grows at temperatures between 4°C and 40°C (Yannelli et al., 1987). *S. marcescens* can be flagellate or non-flagellate, depending on the medium in which it is grown. When flagella are present, they are peritrichous and their number can vary. In addition to flagella the bacterium also possesses pili, which are essential in adherence to host cells (Hejazi et al., 1997). The organism is Voges-Proskauer positive, indicating that it is able to ferment glucose, and can reduce nitrate and utilize citrate as the sole carbon source for metabolism. *S. marcescens* tests negative for chytochrome oxidase and indole production (Yannelli et al., 1987). It is capable of utilizing a wide variety of nutrients and can grow in diverse, sometimes extreme, environments. *S. marcescens* can grow in certain disinfectants, antiseptics, and double-distilled water (Marrie et al., 1981, Parment et al., 1986, Nakashima et al., 1987), as well as in anaerobic, semi-anaerobic, and aerobic conditions (Hejazi et al., 1997). *S. marcescens* thrives in many diverse environments partly due to its production of extracellular products, such as chitinase, DNAse, gelatinase, esterase, caseinase, lecithinase, several proteases, hemolysin, nuclease, lipase, metalloprotease, and serrawettin (Hines et al., 1988, Matsuyama et al., 1985, Matsuyama et al., 1992, Matsuyama et al., 1986, Yannelli et al., 1987, Marty et al., 2002, Hertle et al., 1999, Eaves et al., 1963). Working in concert, these enzymes and other compounds allow the bacterium to convert organic matter into metabolites. Chitinase is of particular

importance because chitin is the second most abundant naturally occurring polymer, after cellulose. Chitin is the primary component of arthropod exoskeletons and also occurs in cell walls of fungi. Serrawettin is important because it acts as a surfactant, facilitating colonization of surfaces (Matsuyama et al., 1985, Matsuyama et al., 1992, Matsuyama et al., 1986).

**Habitats and Host Range**

*S. marcescens* is an extremely versatile microbe that is commonly found in food, water, soil, animals, and plants (Grimont and Grimont, 1978). The bacterium is found in milk and other dairy products (Roussel et al., 1969 as cited in Grimont and Grimont, 1978, Wilson 1963 as cited in Grimont and Grimont, 1978, Grimes et al., 1931), and is a common contaminant of seafood, such as oysters and fresh fish (Jay 1970).

In a study by Grimont, 50% of all *Serratia* strains found in both freshwater and saltwater were in the species *S. marcescens* (Grimont 1977). Of the *S. marcescens* strains inhabiting water, both pigmented and non-pigmented forms were identified (Grimont 1977). *S. marcescens* is frequently found in the rhizosphere, where it is associated with suppression of soil-borne fungal pathogens. In one example, chitinolytic activity of *S. marcescens* reduced the incidence of summer patch disease in Kentucky bluegrass, caused by the fungus *Magnaporthe poae,* by 50% or more (Kobayashi 1995). *S. marcescens* was identified as a potential biocontrol agent of *Sclerotium rolfsii,* causing up to 75% disease reduction in beans under greenhouse conditions, and reduced incidence of damping-off caused by *Rhizoctonia solani*, by 50% (Ordentlich 1987). In the case of *S.*

*rolfsii,* the *S. marcescens* cells colonized root surfaces and competed with the fungal pathogen by inhibiting its germination up to 50% (Ordentlich 1987).

Many cold-blooded vertebrates are susceptible to *Serratia* infection. A contagious, subacute, nodular infection is caused by the bacterium in a reptile, *Anolis equestris* (Duran-Reynals et al., 1937 as cited in Grimont and Grimont, 1978). *Serratia* also causes subcutaneous abscesses in iguanas, arthritis in geckos, and septicemic and subcutaneous ulcerative disease in the painted turtle, *Chrysemys picta* (Boam et al., 1970, Capponi et al., 1956 as cited in Grimont and Grimont, 1978, Jackson et al., 1976 as cited in Grimont and Grimont, 1978).

*S. marcescens* has been isolated from the digestive tract of hens and contaminated chicken carcasses (Izawa et al., 1971 as cited in Grimont and Grimont, 1978). The bacterium also causes septicemia in other warm-blooded animals, such as horses, pigs and goats (Deom et al., 1953 as cited in Grimont and Grimont, 1978, Wijewanta et al., 1970, Brisou et al., 1959 as cited in Grimont and Grimont, 1978), and abortion and chronic mastitis in cattle (Smith et al., 1970, Barnum et al., 1958).

Organisms thought to be *S. marcescens*, isolated occasionally from human lesions, were thought to be a curiosity until scientists discovered that nonpigmented *Serratia* strains were capable of causing human infection. *S. marcescens* has since been recognized as a nosocomial human pathogen of emerging importance during the last three decades. The greatest occurrence of human infection is in hospitals, where the organism has developed resistance to many antibiotics and is capable of surviving in many habitats, including in common disinfectants. *S. marcescens*-induced diseases in humans were once thought limited to patients with compromised immune systems, but the bacterium is

now recognized as an etiological agent in many kinds of infections (Hejazi et al., 1997) including respiratory tract and urinary tract infections, septicemia, meningitis, wound infections, infective endocarditis, pneumonia, lung abscesses, emphysema, septic arthritis, osteomyelitis, peritonitis, and sinusitis (Wilfert et al., 1968, Davis et al., 1970, Cohen et al., 1980). Endocarditis may occur following cardiac surgery (Cohen et al., 1980), while urinary and respiratory tract infections can be associated with contaminated tracheotomy tubes and indwelling catheters (Wilkowske et al., 1970). In rare cases patients can acquire *S. marcescens* through infected donor blood during a transfusion (Heltberg et al., 1993, Hogman et al., 1993). Transfusion-associated *S. marcescens* infections result in severe and sometimes fatal septic shock (Jeppsson et al., 1984).

*S. marcescens* also has been isolated from plants. Grimont and Grimont (1978) isolated *Serratia* species, predominantly *S. marcescens* and *S. liquefaciens*, from eucalyptus, pistachio, bitter cherry, acacia, coconuts, sorghum, grass, mushrooms, tomatoes, leeks, green onions, Brussels sprouts, lettuce, broccoli, artichokes, radishes, spinach, carrots, and figs. They theorized that bacteria living in the soil somehow colonized plants that grew in the same sites. They believed that the bacterium was an epiphyte capable only of opportunistic infection. Recently, *S. marcescens* was identified as a beneficial endophyte when strains of the species isolated from several rice varieties were found capable of fixing nitrogen and developing an intimate relationship with their host plant (Gyaneshwar et al., 2001).

*S. marcescens* is also an insect pathogen and has been recovered frequently from insects. More than 70 species of insects were susceptible to infection, though most of these were experimental (Table 1). Six orders containing insects that frequently harbor

*Serratia* are Orthoptera, Isoptera, Coleoptera, Hymenoptera, Diptera, and Lepidoptera. *S. marcescens* also occurs in other insect orders, but not as often.

Much of the damage caused by *Serratia* in insect hosts stems from the production of exoenzymes such as proteinase and chitinase, which are toxic to insects (Kaska 1976, Lysenko 1976). Sometimes the bacterium can find natural entry into the hemocoel through wounds in the gut lining. Spontaneous gut rupture, found to occur in 10% of grasshoppers, allowed the bacterium to enter the hemocoel and cause septicemia by overwhelming the insect's body with high numbers of cells (Bucher 1959). Another mode of entry is mechanical inoculation by parasitic organisms: for example, the hymenopterous parasite *Itoplectis conquisitor* introduces the bacteria by means of the ovipositor into several lepidopteran host species (Bucher 1963).

The role of insects in transmitting plant pathogens is important in plant disease epidemiology. The transmission of *S. marcescens* to plants by insects was first described for the fig wasp, *Blastophaga psenes*, which transported the microbe between fig plants mechanically during pollination (Caldis 1927, Phaff and Miller, 1961, Smith et al., 1931, Grimont and Grimont, 1978). The host plant in this case was a variety of fig from which the bacterium has been repeatedly isolated (from the pollinated edible fruit) since 1927 (Caldis 1927).

## V. *Anasa tristis*

### Introduction

The common squash bug is a member of the order Hemiptera and the family Coreidae. In some parts of North America it is mistakenly called a stink bug. *A. tristis* was first described by De Geer in 1773 as *Cimex tristis*. In 1788 Gmelin re-named the species as *Cimex moestus*. Other synonyms in later years were *Coreus rugator* (Fabricius 1803), *Coreus ordinatus* (Say 1825), and *Oriterus destructor* (Hahn 1831). In 1862 the species *tristis* was placed in the genus *Anasa* by Stål (Beard 1940).

### Biology

The squash bug is found throughout the Western Hemisphere, extending from Canada to South America (Beard 1940). It feeds on a variety of plants within the family Cucurbitaceae, including squash, pumpkin, watermelon, cantaloupe, and gourds. Its preferred hosts are summer squash and pumpkin (Beard 1940).

The life cycle of *A. tristis* is simple. An individual develops from egg, to nymph, to adult in a single season. The nymphal stage includes five instars (Beard 1940). The first molt occurs 3 days after hatching, and the entire process from first instar to adult takes about 36 days, depending on weather (Weed et al., 1902). There is usually only one generation per year in the northern U. S. and two to three in the southern states (Fargo et al., 1988). Because squash bugs mate and lay eggs throughout the summer months, several generations overlap and all stages of bugs are present at the same time in the field. Females cease laying eggs in August and find suitable locations to overwinter (Weed et al., 1902).

Adults of both sexes overwinter in field debris, wood piles, and other protected spaces before emerging in the spring (Weed et al., 1902). In June, they begin to congregate in squash fields, where they mate. Females deposit eggs shortly afterward in irregularly shaped patches on the underside of host plant leaves. A single female can lay up to 300 eggs in a lifetime (Beard 1940, Surface 1902), usually laying several batches of around 15-35 eggs at a time. The eggs hatch in about 10-16 days (Surface 1902).

Squash bugs have piercing-sucking mouthparts and feed by inserting stylets intra-cellularly into the host tissue (Beard 1940, Neal 1993). They use a combination of olfactory, contact chemical and gustatory receptors to assess host plant suitability before feeding (Cook et al., 1999), and must be in direct contact with the plant during this process. Host preferences are reflected in reproduction and longevity (Bonjour et al., 1993). Females confined on pumpkin or squash spent most of their adult life in the ovipositional phase and exhibited very short postoviposition periods resulting in large quantities of eggs being deposited, while females fed on watermelon had longer postoviposition periods and slightly lower egg production (Bonjour et al., 1993). Females fed on muskmelon spent a large amount of time in the premating phase which detracted from egg deposition and those fed on cucumber plants generally failed to deposit eggs (Bonjour et al., 1993). These data suggest that squash bug populations would increase faster on pumpkin and squash based on the fecundity values on these two plants.

When in contact with a potential host, the insect probes the plant surface. If suitable chemical cues are detected, surface probing is followed by insertion of the stylets into the plant tissue to further test for suitability by means of gustatory cues. Once a

suitable food source is identified, the insects begin to feed, probing the plant tissue until they penetrate a vascular bundle, where they use their stylets to ingest fluid from the xylem, phloem, or occasionally other tissues (Bonjour et al., 1991).

**Pest Status**

*Anasa tristis* is the most destructive pest of cucurbit crops in mid to late summer throughout North America (Weed et al., 1902, Beard 1940). The direct damage to the vascular bundles, particularly to the xylem, which interrupts water transport, leads to a condition known as Anasa wilt (Robinson et al., 1931). Wilting begins in the leaves and extends into the stem. Summer squash and pumpkin are most at risk because they are preferred hosts (Bonjour et al., 1993), but the insects can also attack watermelon and cantaloupe. Nymphs aggregate to feed on leaves, causing a mottled appearance (Beard 1940). Other symptoms of squash bug damage are brown spots on leaves, wilting, delayed fruit maturity, reduced yield, necrosis of entire leaves, and gradual plant death (Beard 1940, Pair et al., 2004). Damage to plants was once thought to be a result of toxic saliva injected into the host (Surface 1902), but it is now attributed to vascular blockage by damaged cells and debris (Neal 1993).

The squash bug's pest status was recently amplified by the discovery of its ability to transmit the CYVD pathogen to cucurbits (Bruton et al., 2003). In early studies on the nature of CYVD, insecticide treatments lowered incidence of CYVD while soil fumigation failed to have any effect, suggesting that insects had a direct association with CYVD, either as vectors or as stress factors (Bruton et al., 1995). The hypothesis that insects were somehow involved was strengthened by evidence that insect exclusion

prevented disease in mesh-covered field squash plants, while 20 to 25% of uncovered plants (exposed to insects) were PCR positive for presence of the bacterium (Bextine et al., 2001). Transmission tests with several cucurbit pests, specifically cucumber beetles, aphids, and four families of leafhoppers, were negative (Bruton et al., 1998, Pair et al., 1993), but ultimately the squash bug was implicated in transmission of *S. marcescens* to several cucurbit species and completion of Koch's postulates demonstrated the insect's role as a vector of *S. marcescens* (Bruton et al., 2003).

Inoculation of greenhouse plants by infected squash bugs led to rapid wilting and premature death of plants prior to harvest (Pair et al., 2004). Squash bugs harbor the bacterium through the winter and become a source of re-infection to new crops in spring (Pair et al., 2004). Recently, the CYVD pathogen was discovered to be retained by molting squash bug nymphs into the adult stage, suggesting presence in the hemocoel and the possibility of a circulative relationship with the insect (Wayadande et al., 2005). Retention of the pathogen through molts would greatly increase the time frame in which the insect is able to transmit the bacterium. Though the exact mechanisms of transmission are not completely understood, it is clear that the vector and the pathogen pose a serious threat to cucurbits.

Recent research has revealed an intimate association of *S. marcescens* with the squash bug, *Anasa tristis*. Transmission of *S. marcescens* is the first known case in which *A. tristis* has been identified as a vector of a plant pathogen. No other coreids have been associated with bacterial plant diseases. This interaction is the focus of the present study.

# VI. Trypanosomatidae

## Introduction

The family Trypanosomatidae belongs to the order Kinetoplastida in the phylum Protozoa and includes the genera *Endotrypanum, Trypanosoma, Leishmania, Leptomonas, Herpetomonas, Crithidia, Blastocrithidia*, *Rhyncoidomonas, Wallaceina,* and *Phytomonas* (McGhee and Cosgrove 1980, Molyneux and Ashford 1983). The genera are divided into three groups: insect-infecting, vertebrate-infecting, and plant-infecting trypanosomatids. In the two groups that infect vertebrates (*Trypanosoma* and *Leishmania*) and plants (*Phytomonas*), the vectors are commonly insects, often in the order Hemiptera.

Trypanosomatids are found worldwide and infect both animal and plant hosts, in which they may or may not cause disease (McGhee and Cosgrove 1980). Most are found within animal and plant hosts, but in some cases can exist as free-living organisms for a short period of time (Corwin 1962, Riley 1978, Clark 1959). Some of the pathogenic trypanosomatids are transmitted by insects of the Hemipteran families Coreidae, Lygaeidae, Pentatomidae, and Reduviidae (Dollet et al., 1982, Ayala et al., 1975, McGhee and Hanson, 1971, McGhee and Postell, 1982, McGhee and McGhee, 1971, Kastelein 1985, Louise et al., 1986). The best known trypanosomatids that cause human disease are *Trypanosoma cruzi*, causal agent of Chagas' disease, and *Trypanosoma brucei*, causal agent of African sleeping sickness (Chagas 1909, Bruce 1903), which are transmitted by a reduviid, *Rhodnius prolixus*, and the tsetse fly, *Glossina* sp., respectively. Other trypanosomatids in the genera *Blastocrithidia, Leptomonas, Herpetomonas, Crithidia* and *Rhyncoidomonas* infect primarily insects.

The genus *Phytomonas* was discovered in 1909 from the latex of a Euphorb (Lafont 1909). Early researchers theorized that plants were reservoirs for leishmania and trypanosomiasis. During the next two decades numerous reports of trypanosomatids infecting plants appeared in medical and veterinary journals (Dollet 1984). Interest waned in 1925 when a paper was published announcing the non-pathogenicity of a milkweed *Phytomonas* in Maryland (Holmes 1925). It was not until 1930 that Stahel discovered the first economically important disease caused by a member of the genus *Phytomonas* (Stahel 1931). This organism parasitized coffee plants in Surinam and was later proven to be the causal agent of coffee phloem necrosis, a disease that results in plant death (Stahel 1933, Vermeulen 1968). Other economically important *Phytomonas*-induced diseases have since been identified: oil palm marchitez, hartrot of coconut, and empty roots of cassava (Parthasarathy et al., 1976, Dollet et al., 1977, Dollet 1984).

**Biology**

Trypanosomatids vary in shape, from spherical to pyriform, and in size, ranging from 4 to 385µm in length (McGhee and Cosgrove 1980). Despite these differences, all members of the family Trypanosomatidae share a common structure, the kinetoplast, which is a specialized mitochondrion region rich in kinetoplast DNA (kDNA) that can account for up to 20% of the total cellular DNA (McGhee and Cosgrove 1980). In addition to the kinetoplast, most trypanosomatids have a single flagellum. There is one exception; a biflagellate form, described in *Herpetomonas muscarum ingenoplastis*, was found both in culture and inside the insect host (Rogers and Wallace 1971). The relative locations of the flagellar pocket and the kinetoplast, as well as the proportions of total

flagellar length represented by free, attached, and flagellar pocket portions of the flagellum, are important diagnostic features (Clark 1959). However, species designation is difficult due to the lack of sufficient structural differences among genera.

All trypanosomatids have an array of microtubules underlying the plasma membrane that function as a sort of cytoskeleton that helps maintain the shape of the trypanosomatid while allowing for flexibility (McGhee and Cosgrove 1980). The cytoplasm contains numerous ribosomes, some of which are attached to the endoplasmic reticulum. The Golgi apparatus is located near the base of the flagellar pocket and is surrounded by numerous smooth and coated vesicles. Contractile vacuoles are common, but not universal, in trypanosomatids. When they are present, there is only one per cell. The nucleus is typical of that of a eukaryote and is enclosed by a nuclear envelope composed of two unit membranes. In addition to these cellular structures, a few species of trypanosomatids contain intracellular endosymbionts that retain their own DNA similar to that of bacteria (Tuan and Chang 1975). These endosymbionts supply their hosts with essential nutrients, thus enabling the trypanosomatids to inhabit nutritionally poor media (McGhee and Cosgrove 1980).

These organisms have a complex life cycle, existing in different morphological forms (amastigote, promastigote, trypanomastigote, epimastigote, and ophistomastigote) that may be associated with different hosts or environments (McGhee et al., 1971). The promastigote, ophistomastigote, epimastigote and trypomastigote forms consist of an elongate body with a single long flagellum. The spherical amastigote form may or may not have flagella. Transformation from one form to another is generally triggered by environmental cues; for example, *Trypanosoma cruzi* exists as an epimastigote in the gut

of its insect vector and as an amastigote in the blood of its vertebrate host (Chagas 1909). Different forms may be present at the same time in a single host. An example of this is in *Wallaceina brevicula*, from which two morphologically distinct subpopulations were identified in the insect intestine (Frolov and Malysheva 1989, Malysheva and Frolov 1995). These two morphological forms were different life cycle stages of the same species (Podlipaev et al., 1990).

Most transmission of animal and plant-infecting trypanosomatids occurs via an insect vector, in which the trypanosomatids multiply within the insect and are deposited into the plant or animal host through saliva. The infective power of the insect persists even after hibernation and until death. In the case of *Phytomonas*, transmission through grafting has been demonstrated by Stahel, but transmission of a trypanosomatid species through seeds has not been documented. *Phytomonas* can be found within the insect vector hemolymph, salivary glands, and digestive tract (Dollet 1984), while exclusively entomophilic trypanosomatids are found predominately in the alimentary canal (Wallace 1966). In *Trypanosoma*, the trypanosomatids are found within the insect alimentary canal and are transmitted to the animal host through infected feces (Chagas 1909). Transmission is propagative (Dollet 1984).

**Habitats and Host Range**

Insect trypanosomes have wide host specificity in which members of a single genus can be found in several orders of insects and numerous genera of trypanosomatids can be found in a single insect species or genus (Podlipaev 2000). Distribution of trypanosomatids in insect orders is irregular; for example, in hemiptera and diptera there

are 300 described species and undetermined trypanosomatids reported (Podlipaev 2000). About 20 other reports have been made for insects in 7 other orders, but some of them are unreliable (Podlipaev 1990). Water striders in the family Gerridae may be the most commonly infected of all the taxa and harbor an abundance of trypanosomatids (Podlipaev 1985, Podlipaev 1999). Many insects may harbor mixed infections due to the trypanosomatids' low level of specificity and non-specific infection. *Phytomonas* may have diverged from the insect trypanosomatids due to the relationship of commonly infected insects (Hemiptera) with plant hosts (Podlipaev 2000). Hemiptera feed on plant fluids and nonspecific transfer of flagellates may have occurred regularly; in fact, fruit serves as a rich medium for the multiplication of many insect trypanosomatids (Conchon et al., 1989). Over time the insect trypanosomatids may have adapted to take advantage of the plant resources. Leishmania may also have diverged from insect-infecting trypanosomatids in a manner similar to that of *Phytomonas,* but with the trypanosomatids adapting to an animal environment rather than a plant environment (Podlipaev 2000).

The genus *Phytomonas* is divided into two distinct groups: those that inhabit the phloem and those that inhabit the latex cells of lactiferous plants (Dollet 1984, Mitchell 2004). In both groups, infection is usually localized, but can become systemic. Fruits, seeds, and flowers are sometimes infected as is the case in corn, bergamot, mango, and tomato (Serrano et al., 1999). Most *Phytomonas* are found in the families Euphorbiaceae and Asclepiadiaceae, but other families including Apocynaceae, Moraceae, Urticaceae, Sapotaceae, Compositae, and Solanaceae are also infected (Harvey and Lee 1943, Holmes 1931, McGhee and Hanson 1971, McGhee and McGhee 1971). Not all *Phytomonas* are pathogenic to their plant host; many parasitize Asclepiads and Euphorbs

without producing symptoms of disease. Nearly 100 insect species are known to harbor unidentified trypanosomatid flagellates thought to be *Phytomonas*: Miridae, Pentatomidae, Corimelanidae, Lygaeidae, Pyrrhocoridae, Largidae, Stenocephalidae, and Coreidae. Species of Coreidae commonly harbor *Phytomonas* (Sbravate et al., 1989, Godoi et al., 2002), for example, *Phytomonas serpens* is a fruit-infecting trypanosomatid transmitted predominately by coreids (Mitchell 2004). Fruit and seed feeding bugs predominate in field surveys of hosts of *Phytomonas* (Mitchell 2004).

Squash bugs, the subject of this study, are herbivorous insects that often feed on fruits of cucurbits and belong to a family of insects (Coreidae) that are well known hosts of trypanosomatids. During experimental microscopic examination for the presence of *S. marcescens* in abdomen samples of colony-reared squash bugs, we discovered organisms having morphologic and genetic similarity to that of trypanosomatids. These unknown organisms could belong to either the group of entomophilic trypanosomatids or to the genus *Phytomonas*.

## VII. Project Proposal

### Introduction

Little is known about the interaction of *S. marcescens* with its insect vector, *A. tristis*. Due to the bacterium's reputation as an insect pathogen, it has been suggested that CYVD strains of *S. marcescens* may have detrimental effects on squash bugs and on other insect species (Bextine 2001), but whether those effects differ in nature or severity from those caused by known insect-pathogenic *S. marcescens* strains, or by strains from other ecological niches, is not known. The question of how the degree of pathogen virulence on a vector species such as *A. tristis* would affect the insects' ability to transmit that pathogen is also unresolved. Information about differences in *S. marcescens* strain pathogenicity could help us determine why the CYVD strain evolved into an insect-transmitted plant pathogen and the other strains did not. In this project, I assessed the pathogenicity of several strains of *S. marcescens* on pea aphids, *Acyrthosiphon pisum*. Although there is no evidence that aphids are vectors of *S. marcescens* (Al-Zadjali, 2000), they are well-characterized insects that can serve as models to provide insight into the bacterium's association with non-vector species. Aphids were used in this study to determine if the CYVD strain of *S. marcescens* is pathogenic to a non-vector species and to answer the question of whether differences in strain pathogenicity exist. This data provided a basis of comparison for the results of the second part of the project in which the same strains were tested on a known vector species, the squash bug. In the second stage of the project, pathogenicity of the *S. marcescens* strains was assessed on squash bugs to identify any differences among the strains in the manner of interaction with the squash bug. A third objective focused on discovering which, if any, organs and tissues

are invaded by *S. marcescens* ZO1. This information will help answer the question of how the ZO1 strain is capable of being taken up by the insect during feeding and later transmitted to the host plant. These data could help determine the route of transmission, and whether the bacterium is foregut-borne or circulative in the insect body. Data collected in these experiments will contribute to the understanding of why the CYVD strain is insect transmitted.

While dissecting squash bugs and searching for *S. marcescens* cells inside the insect bodies, flagellate protozoa were observed swimming in several gut samples. These flagellates resembled members of the family Trypanosomatidae. Since insect-inhabiting microbes could have a significant impact on vector capacity or disease epidemiology, it was important to identify these flagellates. This led me to a fourth and final objective, to identify the squash bug-resident flagellate.

My objectives were:

1. Assess the pathogenicity of four *S. marcescens* strains on the pea aphid, *Acyrthosiphon pisum*
2. Assess the pathogenicity of four *S. marcescens* strains on the squash bug, *A. tristis*
3. Determine which squash bug internal organs are invaded by *S. marcescens*
4. Identify a squash bug-resident flagellate

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elements-based polymerase chain reaction and DNA-DNA hybridization. Phytopathology 93:1240-1246.

Table 1: Insect hosts of *S. marcescens*

| Order | Genus and species | Common name | Type of infection | Disease and symptoms | Authority |
|---|---|---|---|---|---|
| Orthoptera | *Periplaneta Americana* | American cockroach | Natural | No disease | Pai et al., 2005 |
| Orthoptera | *Blattella germanica* | German cockroach | Natural | No disease | Pai et al., 2005 |
| Orthoptera | *Schistocera gregaria* | Desert locust | Natural | Morbidity and death | Lepesme 1937 |
| Mallophaga | *Pediculus humanus* | Human body louse | Natural | No disease | La Scola et al., 2001 |
| Lepidoptera | *Heliothis virescens* | Armyworm | Natural | Death | Matsumoto et al., 1998 |
| Lepidoptera | *Pseudaletia separate* | Tobacco budworm | Natural | High larval mortality | Sikorowski et al., 2001 |
| Hymenoptera | *Apis mellifera* | Honeybee | Natural | High larval mortality | El Sanousi et al., 1987 |
| Diptera | *Drosophila melanogaster* | Fruit fly | Natural and experimental | Morbidity and death | Flyg et al., 1980 |
| Diptera | *Lucilia sericata* | Blowfly | Experimental | Morbidity and death | O'Callaghan et al., 1996 |
| Diptera | *Rhagoletis pomonella* | Apple maggot fly | Natural and experimental | Morbidity and death | Lauzon et al., 2003 |
| Coleoptera | *Listronotus bonariensis* | Argentine stem weevil | Experimental | Death | Jackson et al., 1998 |
| Hemiptera | *Rhodnius prolixus* | Triatomine bug | Experimental | Stops trypanosome development | Azambuja et al., 2004 |

CHAPTER II

# Pathogenicity of *Serratia marcescens* to the Pea Aphid, *Acyrthosiphon pisum*


## Abstract

*Serratia marcescens*, the causal agent of cucurbit yellow vine disease (CYVD), is transmitted in nature by the squash bug *Anasa tristis*. Aphids were used as model organisms in this experiment to test for pathogenic effects of four *S. marcescens* strains. Aphids were fed concentrations of $10^8$ or $10^2$ cells/ml each of either an insect isolate (73-1-1), a human isolate (HO2-A), a rice endophyte (RO2-A), or a CYVD strain (ZO1-A) of *S. marcescens* suspended in 15% sucrose. After a 24 hour acquisition access period, aphids were placed individually on excised fava bean leaves and mortality was assessed every 24 hours for ten days.

Aphids fed sucrose only had a lower mortality rate than that of aphids fed high ($10^8$ cells/ml) concentrations of any of the four *S. marcescens* strains for up to four days post-acquisition. Mortality of aphids exposed to the ZO1-A ($10^2$ cells/ml) treatment did not differ significantly from that of the sucrose-only control at any time during the experiment. These data suggest that the CYVD strain of *S. marcescens* is not significantly pathogenic to the pea aphid in low concentrations, but that high

concentrations of all strains (RO2-A, 73-1-1, HO2-A, and ZO1-A) have a significant effect on the mortality rate of pea aphids, at some time point(s) following inoculation. However, the fact that the CYVD strain ZO1-A displays only mild pathogenicity to aphids may reflect adaptation of the ZO1-A strain to the insect environment.

## Introduction

Cucurbit yellow vine disease (CYVD), which causes yellowing and rapid wilting of cucurbit species (Cartwright 1993) (Figure 1), is caused by a phloem-resident bacterium, *Serratia marcescens* (Bruton et al., 2003). The squash bug transmits the pathogen, which occurs in north and central Texas, central and eastern Oklahoma, and several other states (Bruton et al., 2003, Bruton et al., 1997, Bost et al., 1999, Pair et al., 2000, Nesmith 2002, Pair et al., 2003, Boucher 2005, Hausbeck 2004).

*S. marcescens*, a Gram-negative, motile bacterium in the family Enterobacteriaceae, occupies many diverse niches, including those of plant endophyte, plant pathogen, human pathogen, animal pathogen, soil and water inhabitant and food contaminant (Grimont 1977, Grimont and Grimont, 1978, Hejazi et al., 1997, Gyaneshwar et al., 2001, Bruton et al., 2003). Certain strains of *S. marcescens* are also pathogenic to more than 70 species of insects (El Sanousi et al., 1987, Matsumoto et al., 1998, Sikorowski et al., 2001, Lauzon et al. 2003). Preliminary evidence from experimental transmission studies suggested that CYVD strains of *S. marcescens* have detrimental effects on squash bugs and possibly also on other insect species, but whether those effects differ in nature or severity from those caused by known insect-pathogenic *S. marcescens* strains, or by strains from other ecological niches, is not known. Information

about differences in *S. marcescens* strain pathogenicity to both plant and insect hosts could help to explain the process by which the CYVD strains evolved into insect-transmitted plant pathogens. Luo et al. (2006) examined pathogenicity of several *S. marcescens* strains on different plant species. Only CYVD-causing strains resulted in stunting of squash plants. All *S. marcescens* strains tested caused necrosis on tobacco, wilt in carrot, and water soaking in onions. Luo et al. 2006 concluded that *S. marcescens* from non-plant niches could cause symptoms in plants, but that their interaction with the plant host was different than that of the CYVD strains. A similar experiment was needed to explore the effects of *S. marcescens* strains from different ecological niches on CYVD vector and non-vector insect species. In this study, we assessed the pathogenicity of several strains of *S. marcescens* on pea aphids, *Acyrthosiphon pisum,* by comparing mortality rates among four strains from different ecological niches. Although aphids are not vectors of *S. marcescens* (Al-Zadjali, 2000), they are well-characterized model insects and could provide insight into the interaction of *S. marcescens* strains with the insect environment.

**Materials and Methods**

*S. marcescens* strains used in this experiment were ZO1-A, HO2-A, RO2-A, and 73-1-1 (Table 1). ZO1-A was isolated from CYVD-diseased zucchini collected in Coyle, OK (Rascoe et al., 2003). HO2-A was isolated from a clinic patient by D. Adamson, Medical Arts Laboratory, Oklahoma City, OK. RO2-A, a non-pathogenic rice endophyte, was isolated from rice by G. Prasad, International Rice Research Institute, the Philippines (Gyaneshwar et al., 2001). The red-pigmented 73-1-1 was obtained from an

insect by Alexander Purcell, University of California, Berkeley. After isolation and culturing in Luria broth (LB), all bacterial samples were suspended in 30% glycerol and stored in 1.5 ml microfuge tubes at -80ºC (Sambrook et al., 1989). For experiments, a single loopful was streaked on LB-agar plates (Sambrook et al., 1989) and incubated in the dark at room temperature for 24 hours.

Aphids (*A. pisum*) were acquired from Dr. Jack Dillwith, Department of Entomology and Plant Pathology, Oklahoma State University. They were reared on fava bean, *Vicia faba*, at 25ºC, 30% humidity, with 14 hours of light and 10 hours of darkness. Fava bean plants were grown from seed and were used prior to flowering as a food source for aphids.

Aphids used in this experiment were adult females of unknown age. To validate the use of unknown age adult aphids, an additional experiment was conducted to determine whether a test group of potentially mixed age aphids was comparable to a group of known-age aphids. In this experiment, the mortality rates of two groups of adult aphids, one adults of unknown age and the other of 10 day old adults, were assessed over a period of 6 days. Aphids of both groups were fed individually on sterile 15% sucrose sachets for 24 hours, after which they were removed and maintained separately on excised fava bean leaves. Data were analyzed using the GLM procedure of SAS ($p=0.05$) and revealed no significant differences in the mortality rate of unknown age adult aphids and 10 day old adult aphids, thus validating the use of unknown age aphids.

Optimization of experimental conditions

Preliminary experiments were conducted to determine sucrose concentrations suitable for bacterial survival. In separate experiments, isolated ZO1-A and HO2-A colonies were picked from the LB agar surface and suspended in 5 ml 15% or 20% sucrose. Twenty percent sucrose was inoculated with ZO1-A only, while 15% sucrose was inoculated separately with ZO1-A and HO2-A. Control colonies were suspended in LB medium. After incubation at 25ºC for 24 hours, they were assessed by darkfield microscopy using an Olympus BH-2 microscope (Tokyo, Japan) at 120x and 480x magnification for motility, morphology, and aggregation. Following observations, 450µl of each treatment was inoculated to 5ml fresh LB medium and incubated for 24 hours at 25ºC. The sub-cultures were observed by microscopy as above and assessed for motility, morphology, and aggregation. Twenty percent sucrose is considered to be more suitable for aphids than 10% sucrose (A. Wayadande, personal communication), however, suitability of sucrose concentrations was not tested on aphids.

Experimental methods

Bacterial colonies (Table 1) collected from LB agar were suspended in 15% sucrose adjusted to pH 7.0. Serial dilutions were performed at a ratio of 1:10 and cell counts were taken to determine the titer of the original bacterial solution, which was then diluted to either $10^2$ or $10^8$ cells/ml. Groups of 30 adult female aphids were placed in feeding sachets, each constructed of a plastic 30 ml (1 fluid oz.) medicine dose cup covered with two layers of stretched Parafilm® membrane between which was inserted 500 µl of the sucrose-plus-bacterium suspension. Control insects were offered 15%

sucrose without bacteria. The aphids were allowed to feed for 24 hours, then removed from the sachets and placed singly in individual square, plastic snap-cages (3”x2”x0.65”) or sachet cups with cardboard lids, both of which were approximately 35ml in volume. Each cage contained a fava bean leaf, excised from an immature plant ranging in age from the 8-leaf to the 12-leaf stage, that served as a food source. Cages were maintained at room temperature (24-26ºC) with approximately 10 hours of daylight and 14 hours of darkness.

Experimental treatments included one 15% sucrose control and eight bacterial treatments consisting of high ($10^8$ cells/ml) and low ($10^2$ cells/ml) titers of four *S. marcescens* strains (Table 1). Because it was not possible to run all treatments at the same time, the experiment was analyzed in a completely randomized design in which one control treatment and three to four experimental treatments, selected randomly, were evaluated on sequential weeks. Controls were used in each incomplete block to assure the integrity of the experiment over time. There were three replications for each treatment. Each treatment consisted of 30 aphids, and three replications comprised a total of 90 aphids per treatment. Additional treatments were selected randomly each week until all had been evaluated. The proportions of insects surviving each day after exposure to the treatments were recorded at 24 hour intervals.

Aphid mortality and body color were assessed at 24 hour intervals for 6 days after acquisition. Mortality was expressed as percent of aphids dead and fecundity was recorded as the number of live, parthenogenetically produced offspring per day. Body color was recorded because, in aphids exposed to the pigmented insect isolate (*S. marcescens* 73-1-1), red color was likely an indicator of bacterial presence in the body.

Data were analyzed using the GLM procedure of SAS followed by Duncan's multiple range test (p=0.05) to compare treatments by day of the experiment.

## Results

### Optimization of feeding solution

After 24 hour incubation in 20% sucrose, 100% of the *S. marcescens* ZO1 cells were plasmolyzed, the cell membrane was constricted and pulled away from the cell wall, and the bacteria had lost their motility. Although no cell counts were taken, the turbidity of the bacterial suspension remained visually static, suggesting little or no increase in titer. After 24 hour incubation in 15% sucrose, all cells of both ZO1 and HO2 moved more slowly than control cells suspended in LB, as determined by visual observation. They formed multiple cell clumps and individual cells appeared as short rods or spheres, but no plasmolysis occurred. They multiplied at a slow rate; nearly twice as many cells per field of view were observed after the 24 hour incubation period as at the time of inoculation into the medium. When 24 hour, 15% sucrose suspensions of *S. marcescens* ZO1 and HO2 were inoculated into Luria broth, the cells recovered their normal rod-shaped form and motility within 24 hours. They also appeared to resume their normal growth rate, estimated by the observation of approximately three times more cells per field of view after the incubation period than before.

### Aphid mortality

All live aphids were initially green in color; however, their color post mortem differed between treatments. Some control aphids remained green post mortem, while

others became tan in color within 24 hours of death (Fig. 2), both of which were interpreted as normal color change. The color of aphids fed non-pigmented *S. marcescens* strains RO2, ZO1, and HO2 ranged from tan to black within 24 hours of death (Fig. 3). However, more aphids exposed to RO2 became dark brown or black after death than did insects exposed to HO2 and ZO1. In contrast, aphids fed the red-pigmented strain, 73-1-1, changed to tan or various shades of red post mortem (Fig. 4); the latter color was likely due to the red pigment, prodigiosin, produced by the bacterium (Grimont and Grimont, 1978). The cast exoskeleton of 73-1-1 fed aphids was pink in color (Fig. 4) while cast exoskeletons of all other aphids were clear or white in color (Fig. 5).

Twenty-four hours after exposure to the treatment, 4.2% of control aphids, fed 15% sucrose alone, had died. Aphid groups exposed to *S. marcescens* treatments experienced higher mortality rates than the controls (Table 2). Only aphid groups exposed to RO2 ($10^8$ cells/ml) exhibited significant mortality (57.9%) after 24 hours, as indicated by SAS analysis. By 48 hours, aphid groups exposed to treatments 73-1-1 ($10^8$ cells/ml), HO2 ($10^8$ cells/ml), and RO2 ($10^8$ cells/ml) showed significant mortality (56.7%, 56.7%, and 83.4%, respectively) as compared to the control. All treatments, excluding ZO1 ($10^2$ cells/ml), were significantly different from the control 72 hours after exposure, at which time mortality of control insects was 27.9% and that of ZO1 ($10^2$ cells/ml) was 37.8%. At 96 hours only the aphids in the HO2 ($10^2$ cells/ml) and RO2 ($10^8$ cells/ml) treatments remained significantly different from the control. By 120 hours, there was no significant difference in mortality between any of the treatments and the control. However, there were significant differences between ZO1 ($10^2$ cells/ml) and

ZO1 ($10^8$ cells/ml), as well as between ZO1 ($10^2$ cells/ml) and RO2 ($10^8$ cells/ml). The overall mortality rates over a seven day period are depicted in Figure 6.

## Discussion

### Optimization of experimental conditions

A preliminary experiment determined sucrose concentrations supportive of both aphid feeding and bacterial survival. While 20-30% sucrose is commonly used as an artificial diet for aphids, the osmolarity of this solution was unsuitable for the bacteria. Use of lower sucrose concentrations would improve bacterial survival, but less than 10% sucrose would provide insufficient nutrition for the aphids (Wayadande, personal communication). Fifteen percent sucrose was selected as a compromise that allowed bacterial survival and was sufficient for aphid feeding. Although *S. marcescens* exhibited abnormal morphology in 15% sucrose, forming multiple cell clumps and shorter rods, and exhibiting reduced activity, the bacteria recovered after transfer to LB medium.

### Aphid mortality

Comparing all treatments, the proportion of surviving aphids was highest in the control treatment, in which the insects were exposed to 15% sucrose not containing *S. marcescens*. However, approximately half of the 15% sucrose-exposed control insects had died after five days, suggesting that 15% sucrose is not optimum for aphid survival and accounts for some level of aphid mortality. A previous study by Srivastava and Auclair (1971) identified 35% sucrose as the optimum concentration for development and reproduction of pea aphids, in which 25% mortality was recorded after 72 hours.

Interestingly, they also tested 15% sucrose, which resulted in only 12.5% mortality after 72 hours. However, 35% sucrose was identified as the optimum concentration because aphids were able to grow and reproduce on this concentration, while aphids fed 15% sucrose died before reproducing. In addition, the mean days of survival of pea aphids on 15% sucrose was 8 days, while mean days of survival on 35% sucrose was 22 days.

Though aphid age was not controlled in this study, we demonstrated in a separate experiment that the mortality rate of unknown age adult aphids was no different than that of 10 day old adult aphids under the conditions of this study. Therefore, differences in mortality could be attributed to the bacterial treatments and not to the age of the aphids.

The rice endophytic strain of *S. marcescens*, RO2-A, offered in high ($10^8$ cells/ml) concentrations, was the most lethal of the treatments, causing significant aphid mortality as compared to the control; after five days, 97% of RO2-A ($10^8$ cells/ml) exposed aphids had died. The CYVD strain, ZO1-A, offered in low ($10^2$ cells/ml) concentrations, was the least damaging treatment, resulting in mortality rates nearly identical to those in the control. In general, as expected, the higher concentrations of all bacterial strains resulted in higher aphid mortality.

These results, showing 63-99% mortality at the end of this study for aphids fed the bacterial treatments, were consistent with results of other studies of *S. marcescens* pathogenicity to insects. For example, apple maggot flies fed *S. marcescens* at a concentration of $4.7 \times 10^4$ cells/ml experienced 40% mortality within 24 hours of ingestion (Lauzon et al., 2003). Though the strain used in the apple maggot fly study was not the same as those used in this aphid study, the mortality rate in the fly experiment is similar to that caused in aphids by the rice endophytic strain RO2-A, which caused 25%

mortality in the low ($10^2$ cells/ml) concentration and 58% mortality in the high ($10^8$ cells/ml) concentration within 24 hours of aphid exposure to the bacterium.

The fact that low concentrations (as would be expected to occur naturally) of the CYVD strain ZO1-A, caused insignificant aphid mortality at any time during the experiment suggests that ZO1-A was less pathogenic to aphids than were strains 73-1-1, HO2, and RO2, all of which did cause significant mortality at both $10^8$ cells/ml and $10^2$ cells/ml concentrations. The rice endophyte, RO2, resulted in high and rapid mortality in the pea aphid. Both the insect isolate, 73-1-1, and the human isolate, HO2-A, also caused significantly higher mortality to aphids than did the CYVD strain. The latter two strains, ZO1 and HO2, are less virulent to aphids than are RO2 and 73-1-1 under the conditions of this test.

We do not know whether a similar pathogenic relationship occurs between *S. marcescens* strain ZO1-A and other insect species, including the natural vector of CYVD, the squash bug. However, the mild pathogenicity of CYVD strain ZO1-A to aphids, suggests adaptation of the CYVD strains to the insect environment. Attenuation of pathogenicity to the vector species, if it occurs, might allow greater opportunity for bacterial transmission to the host plant, since rapid vector death would limit the time available for transmission of the bacterium to a suitable plant.

Although they are well studied and understood insect models, aphids are not known to transmit *S. marcescens*, but do transmit several viruses, such as barley yellow dwarf virus and maize streak virus. Whether the degree of *S. marcescens* virulence on a vector species such as *A. tristis* could affect the insects' ability to transmit that pathogen is a question that warrants further study.

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Figure 1. Wilting of squash infected with *S. marcescens.*

Table 1: *S. marcescens* strains and titers used in the experiment

| Strain | Niche | Titer | Pigment | Source |
|---|---|---|---|---|
| ZO1 | CYVD-zucchini | Low ($10^2$ cells/ml) | - | Bextine & Wayadande |
| ZO1 | CYVD-zucchini | High ($10^8$ cells/ml) | - | Bextine & Wayadande |
| 73-1-1 | Insect pathogen | Low ($10^2$ cells/ml) | + | Purcell |
| 73-1-1 | Insect pathogen | High ($10^8$ cells/ml) | + | Purcell |
| HO2 | Human pathogen | Low ($10^2$ cells/ml) | - | Adamson |
| HO2 | Human pathogen | High ($10^8$ cells/ml) | - | Adamson |
| RO2 | Rice endophyte | Low ($10^2$ cells/ml) | - | Prasad |
| RO2 | Rice endophyte | High ($10^8$ cells/ml) | - | Prasad |

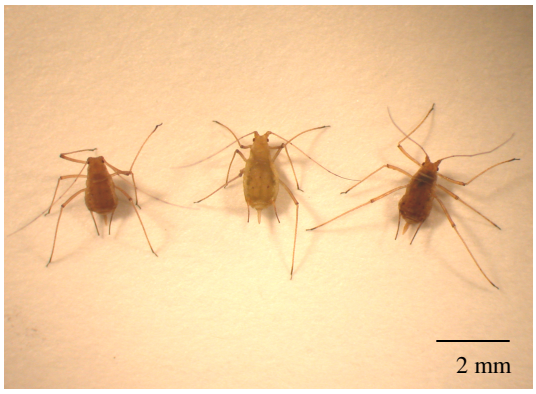


Figure 2. Control aphids post-mortem.

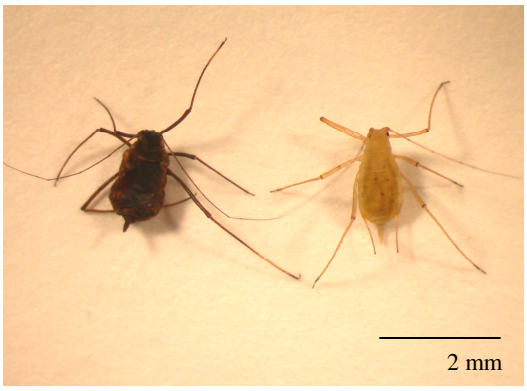


Figure 3. RO2-exposed aphids post-mortem.


Figure 4. 73-1-1 exposed aphids with cast skins.


Figure 5. RO2-exposed aphids with cast skins.

Table 2: Mortality of *Acyrthosiphon pisum* fed on different concentrations and strains of *S. marcescens*

| Treatment | Cumulative % Mortality | | | | |
|---|---|---|---|---|---|
| | **0-24 hr** | **24-48 hr** | **48-72 hr** | **72-96 hr** | **96-120 hr** |
| Control | 4.2±0.01 a[1] | 15.9±0.05 a | 27.9±0.06 a | 50.1±0.06 a | 69.5±0.05 ab |
| ZO1 ($10^2$ cells/ml) | 13.4±0.09 a | 28.9±0.11 ab | 37.8±0.04 ab | 52.3±0.06 a | 63.4±0.08 a |
| ZO1 ($10^8$ cells/ml) | 16.8±0.09 a | 32.3±0.1 ab | 68.9±0.2 cd | 78.9±0.15 ab | 93.4±0.04 b |
| 73-1-1 ($10^2$ cells/ml) | 14.5±0.07 a | 32.3±0.02 ab | 61.2±0.122 bc | 68.9±0.14 ab | 74.5±0.16 ab |
| 73-1-1 ($10^8$ cells/ml) | 22.3±0.04 a | 56.7±0.13 bc | 72.3±0.15 cd | 74.5±0.16 ab | 77.9±0.16 ab |
| HO2 ($10^2$ cells/ml) | 20.1±0.1 a | 42.3±0.15 ab | 65.6±0.1 bcd | 87.8±0.04 b | 91.2±0.06 ab |
| HO2 ($10^8$ cells/ml) | 24.5±0.13 a | 56.7±0.15 bc | 76.7±0.12 cd | 80.1±0.1 ab | 90.1±0.04 ab |
| RO2 ($10^2$ cells/ml) | 24.5±0.14 a | 40.1±0.16 ab | 65.6±0.06 bcd | 76.7±0.07 ab | 81.2±0.07 ab |
| RO2 ($10^8$ cells/ml) | 57.9±0.04 b | 83.4±0.05 c | 96.7±0 d | 96.7±0 b | 98.9±0.01 b |

[1] Numbers followed by different letters of the alphabet were significantly different at a 95% confidence level.

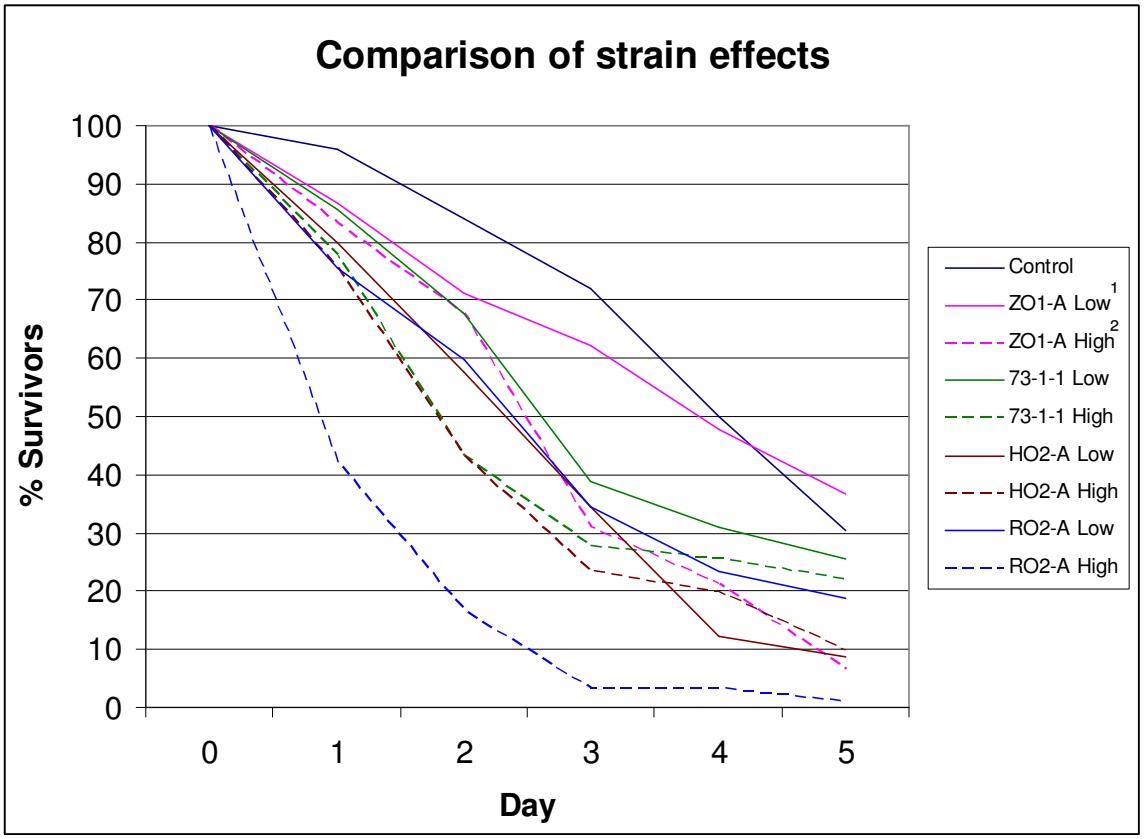


Figure 6. Comparison of aphid mortality over a 7 day period after exposure to *S. marcescens* strains in feeding sachets.

[1] Low equals $10^2$ cells/ml.

[2] High equals $10^8$ cells/ml.

CHAPTER III

# Pathogenicity of *Serratia marcescens* to the Squash Bug, *Anasa tristis*


**Abstract**

*Serratia marcescens* is the causal agent of cucurbit yellow vine disease (CYVD), which is transmitted in nature by the squash bug, *Anasa tristis.* Here, we tested for pathogenic effects of different *S. marcescens* strains on the squash bug. Squash bug nymphs were fed on *S. marcescens* suspensions at $10^8$ and $10^2$ cells/ml each of an insect isolate (73-1-1), a human isolate (HO2-A), a rice endophyte (RO2-A), or CYVD (ZO1-A) strain or a water control introduced by vacuum infiltration into squash fruit cubes. After a 24 hour acquisition access period, the nymphs were removed from the squash cubes and placed on squash seedlings. Mortality and the number of days between molts were recorded for 60 days.

Significant differences were found in total mean days required to complete development to the adult stage. The times to reach the adult stage after exposure to ZO1-A ($10^8$) as well as ZO1-A ($10^2$), 73-1-1 ($10^2$), and RO2-A ($10^2$) were all similar to the control while HO2-A ($10^8$ and $10^2$) and RO2-A ($10^8$) and 73-1-1 ($10^8$) required fewer days to complete development. Despite these differences in total mean days of

survival, the overall rates of nymph survival to the adult stage was not significantly different among the eight bacterial treatments and the control. These treatment data suggests that the CYVD strain of *S. marcescens* is not significantly pathogenic to the squash bug and that the other three strains (RO2-A, 73-1-1, and HO2-A) have no overall effect on the survivorship of nymphs to the adult stage. It also indicates that strains RO2-A, 73-1-1, and HO2-A can significantly shorten the life span of the squash bug.

**Introduction**

Cucurbit yellow vine disease (CYVD), which causes yellowing and rapid wilting of cucurbit species (Cartwright 1993) (Figure 1), is caused by a phloem-resident bacterium, *Serratia marcescens* (Bruton et al., 2003). The squash bug, a vector of the bacterium, spreads the pathogen through north and central Texas, central and eastern Oklahoma, and several other states (Bruton et al., 2003, Bruton et al., 1997, Bost et al., 1999, Pair et al., 2000, Nesmith 2002, Pair et al., 2003, Boucher 2005, Hausbeck 2004).

*S. marcescens*, a Gram-negative bacterium in the family Enterobacteriaceae, occupies many diverse niches including those of plant endophyte, plant pathogen, human pathogen, animal pathogen, soil and water inhabitant and food contaminant (Grimont 1977, Grimont and Grimont, 1978, Hejazi et al., 1997, Gyaneshwar et al., 2001, Bruton et al., 2003). Certain strains of *S. marcescens* are pathogenic to more than 70 species of insects (El Sanousi et al., 1987, Matsumoto et al., 1998, Sikorowski et al., 2001, Lauzon et al. 2003). Preliminary evidence suggested that CYVD strains of *S. marcescens* have detrimental effects on squash bugs (A. Wayadande, personal communication), but whether those effects differ in nature or severity from those caused by known insect-

pathogenic *S. marcescens* strains, or by strains from other ecological niches, is not known. Information about differences in *S. marcescens* strain pathogenicity to both plant and insect hosts could help to explain the process by which the CYVD strains evolved into insect-transmitted plant pathogens. In this study, we assessed the pathogenicity of several strains of *S. marcescens* on squash bugs, *Anasa tristis*.

In previous experiments, the percent mortality of pea aphids (*Acyrthosiphon pisum*) exposed to the CYVD strain ZO1-A was higher than that of the control, but occurred later than in aphids exposed to the other three *S. marcescens* strains (HO2-A, RO2-A, and 73-1-1) tested (Heppler, unpublished data). These findings indicate low initial pathogenicity to the insect, a feature that could reflect adaptation of the ZO1-A strain to the insect environment. Minimal negative impact to the squash bug is especially important considering the circulative nature of *S. marcescens* in the squash bug, along with the fact that the bacterium must overwinter in the squash bug before being transmitted to new crops in the spring (Wayadande et al., 2005, Pair et al., 2004). These data could support the hypothesis that the pathogenicity of the CYVD strain is attenuated, facilitating transmission by insects. Alternatively, it is possible that the observed differences in *S. marcescens* pathogenicity to pea aphids in no way reflects the interaction of the pathogen with squash bugs or other insects. The objective of this study was to assess pathogenicity of *S. marcescens* strains from four different ecological niches to the squash bug, *A. tristis.*

## Materials and Methods

### Bacterial strains

*S. marcescens* strains used in this experiment were ZO1-A, HO2-A, RO2-A, and 73-1-1 (Table 1). ZO1-A was isolated originally from CYVD-diseased zucchini collected in Coyle, OK (Rascoe et al., 2003). HO2-A was isolated from a human patient by D. Adamson, Medical Arts Laboratory, Oklahoma City, OK. RO2-A, a non-pathogenic rice endophyte, was isolated from rice by G. Prasad, International Rice Research Institute, the Philippines. The red-pigmented 73-1-1 was obtained from an insect by Alexander Purcell, University of California, Berkeley. After isolation, culturing and triple cloning, all bacterial samples were suspended in 30% glycerol and stored at -80ºC. For experiments, a single loopful was streaked on LB-agar plates and incubated in the dark at room temperature for 24 hours.

### Growth of squash plant seedlings

Squash seedlings were planted singly in pots and placed in a greenhouse at ambient temperature (50-100°F). They were watered daily and fertilized weekly with Miracle Gro liquid fertilizer. Sticky traps were placed above the seedlings to control whiteflies and at no time were chemical pesticides used on or near the seedlings. Seedlings were used at the two-leaf stage.

### Sources and maintenance of insects

Squash bug adults, nymphs, and eggs were field-collected from cucurbit plants in Coyle, Perkins, and Stillwater, Oklahoma in the months of April, May, and June of 2006. The insects and eggs were placed in 21 x 12 x 20 inch aluminum-framed mesh cages and supplied with young squash plants as a food source. The temperature of the squash bug colony room was a constant 25ºC and the daylength was 14 hours with 10 hours of darkness. Eggs laid by the feral squash bugs were removed from the colony and placed in a new colony cage. All cages were washed prior to use with 10% sodium hypochlorite and allowed to dry to reduce the risk of pathogen spread from feral squash bugs to the newly hatched nymphs. The insects were provided squash plants and fruit, and squash bugs were removed as needed from the healthy colony for use in experiments.

Insect exposure to bacteria

First, second, and third instar squash bug nymphs, exposed to bacteria for a 24 hour acquisition access period, were monitored for mortality and sub-lethal effects on development. I attempted to start all treatments at the second instar stage, but because of the morphological similarities between second and third instars, the stage was sometimes misjudged, resulting in unequal numbers of insects analyzed in each instar group. Exposure was limited to a fixed time, so no cumulative effects were likely. To prepare the treatments, bacterial colonies collected from LB agar were suspended in sterile water. Serial dilutions were performed at a ratio of 1:10 and cell counts were taken to determine the titer of the original bacterial solution, which was then diluted to either $10^2$ or $10^8$ cells/ml. Because squash bugs will not feed on liquid diet-filled feeding sachets (Bextine et al., 2003), bacteria were vacuum-infiltrated into cubes of acorn squash fruit, which

were offered to the insects. The control treatment consisted of sterile water infiltrated cubes. Vacuum infiltration was achieved by placing 1 cm² squash fruit cubes in a vacuum flask and adding the treatment suspension until the cubes were immersed to half their height. House vacuum was applied for 10 seconds, then released, forcing the liquid into the squash cube. These steps were performed three times for each sample. Both a high titer ($10^8$ cells/ml) and a low titer ($10^2$ cells/ml) of each strain was tested, resulting in a total of 8 treatments plus the control. The experiment was carried out in a complete randomized block design with three replications and a total of 39 nymphs per treatment.

To administer the treatments, 10 squash bugs each were placed in Petri dishes containing 5 vacuum-infiltrated squash cubes per treatment/dish and allowed to feed for 24 hours. After acquisition, fed nymphs in the first replication were placed individually in Petri dishes and provided 1cm² squash fruit cubes to serve as the food source to maintain the nymphs throughout the duration of the experiment. Nymphs in all other replications following the first replication were placed individually on 2 to 4-leaf stage squash seedlings, each enclosed in a cylindrical plastic-and-mesh cage (10" high x 4" diameter). Percent mortality was recorded twice a day for 60 days, and the dates on which molting occurred were noted.

Data analysis

Data were analyzed using the GLM procedure of SAS followed by LSD means separation ($p=0.05$). A protected pairwise t-Test was performed to compare survivorship based on proportions among high and low concentrations of each treatment. Dunnett's t

tests were performed to compare the total number of squash bugs that reached the adult stage among the nine treatments at the end of the experiment.

## Results

Visual analysis

All squash bugs were alive and were grey to green in color at the beginning of all experiments. In the first replication only, some became physically deformed and abnormally colored within a few weeks. This effect was most evident in the legs, which were abnormally curved, twisted out from the body, and occasionally non-functional in live nymphs. Although the normal post mortem color is the same as that of the living insect, some squash bugs turned reddish-brown before death and post mortem. Deformation of the legs and reddish-brown coloration was found among all treatments, including the control, but only in the first replication, in which nymphs were maintained on squash fruit cubes as the food source throughout the experiment. Data from this replication were not utilized in data analysis.

In all subsequent replications, nymphs were maintained on squash plant seedlings rather than squash fruit cubes. No physical deformation or pre- and post mortem color changes were seen in any treatment at any time during these experiments. The presence or absence of *S. marcescens* strains in the bodies of nymphs was not tested. Data collected from these replications were utilized in data analysis.

Mean total days (mtd) of survival

"Mean total days" (mtd) is the mean total number of days that all nymphs survived, including those that did not survive long enough to collect data for mean duration of instar stages and nymphs that died between instars. A low concentration of the cucurbit strain, ZO1-A, had no significant effect on survival of squash bug nymphs; their survival after exposure to ZO1-A at $10^2$ cells/ml (40.7 mtd) was indistinguishable from that of nymphs exposed to water-infiltrated cubes (40.4 mtd) (Table 1). No survival rate differences were found among nymphs exposed to ZO1-A at a high concentration ($10^8$ cells/ml) (32.4 mtd), and those exposed to low concentrations of strains isolated from an insect (73-1-1 at $10^2$ cells/ml (38.4 mtd)), or a rice plant (RO2-A at $10^2$ cells/ml (30.5 mtd)). Survival rates also were indistinguishable among nymphs exposed to the water control, to ZO1-A at either concentration (40.7 and 32.4 mtd, respectively), and to the low concentrations of the insect- and rice-derived strains (38.4 and 30.5 mtd, respectively). However, nymphs exposed to high concentrations of the rice endophytic strain, and to both concentrations of the human pathogenic strain, survived fewer days than those exposed to water-infiltrated cubes as indicated by SAS analysis. In these experiments, survival rates were: RO2-A at $10^8$ cells/ml (28.6 mtd), HO2-A at $10^2$ cells/ml (26.4 mtd), HO2-A at $10^8$ cells/ml (29.5 mtd), and 73-1-1 at $10^8$ cells/ml (18.4 mtd) Figure 1 depicts the total mean days of survival for squash bugs in all treatments.

Mean duration of instar stages

No significant differences occurred in mean number of days spent in the second instar stage among squash bugs exposed to the control and any of the eight bacterial

strain treatments (Table 1). However, there were some significant differences among survival rates of insects receiving the eight strain treatments. Nymphs fed a low concentration of the yellow vine strain, ZO1-A, spent more days in the second instar stage, resulting in slower development, than did nymphs fed either concentration of the human strain, and the high concentration of the insect pathogenic strain, but had a developmental time similar to that of nymphs fed ZO1-A ($10^8$), 73-1-1 ($10^2$), or RO2-A ($10^2$). Squash bugs exposed to HO2-A ($10^2$) developed faster than those exposed to all other low bacterial concentration treatments and squash bugs fed ZO1-A ($10^8$) developed more slowly than those fed 73-1-1 ($10^8$). The sample size for determining mean days spent in the second instar was relatively small, 10 nymphs per treatment, because few nymphs were in the first instar at the beginning of the experiment. Those nymphs were observed until their first molt into the second instar, at which time it was possible to count the number of developmental days between the second and third instars. No data were collected on mean days spent in the second instar for insects in the RO2-A ($10^8$) treatment because all first instar nymphs in this treatment died before molting.

Significant differences in the duration of the third instar stage (Table 1) were noted. Nymphs fed the control treatment spent significantly more time in the third instar stage than did nymphs fed ZO1-A ($10^2$), 73-1-1 ($10^8$), HO2-A ($10^2$), and HO2-A ($10^8$). ZO1-A ($10^2$) exposed third instar nymphs developed faster than those exposed to the control, but did not differ significantly from nymphs fed any of the other bacterial strain treatments. There were no significant differences between third instar nymphs fed HO2-A ($10^2$) and HO2-A ($10^8$), nor were there differences among third instar nymphs fed RO2-A ($10^2$) and RO2-A ($10^8$) or ZO1-A ($10^2$) and ZO1-A ($10^8$). The only difference

found among squash bugs exposed to high and low concentrations of a single strain was in third instar nymphs fed strain 73-1-1; those exposed to the low concentration had a longer developmental time than did those receiving the high concentration.

No significant differences in days spent in the fourth or fifth instar stage were found among the nine treatments.

Number of squash bugs reaching adult stage

The total number of squash bugs surviving to the adult stage did not differ significantly between the water control and any of the eight bacterial treatments (Fig. 2). A total of 10 of the original 39 nymphs fed the water control survived to the adult stage. Ten nymphs fed ZO1-A ($10^2$) and 10 nymphs fed 73-1-1 ($10^2$) also did so. The fewest survivors were found in the 73-1-1 ($10^8$) treatment, with only 3 squash bugs reaching the adult stage, but this survival rate was not significantly different from that of the control insects.

**Discussion**

Exposure of *A. tristis* nymphs to *S. marcescens* caused no significant reduction in the total number of insects surviving and completing development to the adult stage regardless of the *S. marcescens* strain to which they were exposed. Bacterial concentration did not affect the result; for example, rates of insect survival after exposure to CYVD strain ZO1-A were the same at both high ($10^8$ cells/ml) and low ($10^2$ cells/ml) bacterial concentrations. In this experiment, pathogenic effects of the treatments were assessed by evaluating the number of total days of survival and the percentage of nymphs

that developed into adults. These data suggest that none of the strains of *S. marcescens* tested, ZO1-A, RO2-A, 73-1-1, or HO2-A, significantly impacted the survivorship of nymphs to the adult stage.

There were, however, significant differences in the nymphal life span between insects treated with water and those exposed to high concentrations of the three non-CYVD *S. marcescens* strains, HO2-A, RO2-A, and 73-1-1, and to low concentrations of HO2-A. Insects exposed to these treatments lived fewer total days than did nymphs fed the water control, ZO1-A at either concentration, and the low concentration of the insect isolate 73-1-1. The amount of time spent in a given life stage can indicate pathogenic effects of the treatments on the nymphs. In general, morbid nymphs would be expected to spend more time in an instar than a healthy nymph, but this may not always be true. High concentrations of HO2-A, RO2-A, and 73-1-1 and low concentrations of HO2-A significantly shortened nymphal life span, suggesting that these strains are more pathogenic to squash bugs than ZO1-A is. Bacterial strains that are highly pathogenic to a vector insect may not be well adapted for insect transmission because rapid death of the insect vector results in a shorter time for pathogen dissemination. The time allowed for transmission is important for bacteria transmitted in a circulative manner, which require a longer latency period than bacteria transmitted in a non-circulative manner.

Interesting results were found when evaluating the duration of nymphal instars. Squash bugs exposed to high concentrations of 73-1-1 and both concentrations of HO2-A spent significantly fewer days in the third instar than did the water control treatment, which was not expected. These same treatments also resulted in a smaller mean number of days spent in the second instar than that found in the control, though these differences

were not statistically significant. The cause of the shorter instar durations is uncertain, but could be attributed to some physiological effect of the bacterial treatments on the squash bug nymphs that resulted in faster development.

Nymphs exposed to either high or low concentrations of the CYVD strain, ZO1-A, lived as long as did nymphs exposed to water. The fact that ZO1-A did not cause significant mortality at any time during the experiment, while each of the other three strains did cause deleterious effects on nymphs, could reflect an adaptation of the ZO1-A strain and the insect vector to one another. Attenuation of pathogenicity to the vector species could allow greater opportunity for bacterial transmission to the host plant. In contrast, exposure to the human isolate, HO2-A, shortened the life span of the squash bugs, with most dying within a month, reducing the likelihood of transmission to another host plant. Although no tests were performed to verify infection by *S. marcescens,* there were significant differences in survivorship among the treatments. Survival of vector nymphs to adulthood, as well as a longer life span and ability to overwinter, would facilitate spread of the CYVD pathogen over a longer period of time than if the infected nymphs died before completing development. This work offers insights about how the plant pathogen-vector relationship affects pathogen dissemination and disease impact.

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Table 1: Mean number of days between molts and total days of survivorship of *Anasa tristis* fed on different concentrations and strains of *S. marcescens*

| Treatment | Mean Duration (Days) of Instar Stages | | | | Total mean days[3] |
|---|---|---|---|---|---|
| | 2nd instar | 3rd instar | 4th instar | 5th instar | |
| Control[1] | 13.7±1.9 abc[2] | 15.8±4.4 c | 12.6±1.2 a | 31.4±2.1 a | 40.4±3.5 cd |
| ZO1 ($10^2$) | 17.0±0.7 c | 9.8±1.0 ab | 15.4±1.5 a | 32.2±4.5 a | 40.7±3.4 d |
| ZO1 ($10^8$) | 16.0±1.0 bc | 14.7±3.7 abc | 12.5±2.0 a | 27.0±3.0 a | 32.4±3.8 bcd |
| 73-1-1 ($10^2$) | 12.0±2.0 abc | 14.9±1.7 bc | 16.2±2.0 a | 29.5±3.6 a | 38.4±3.6 cd |
| 73-1-1 ($10^8$) | 9.0 a | 8.5±1.7 a | 11.3±1.2 a | 27.0±6.7 a | 18.4±2.7 a |
| HO2 ($10^2$) | 10.4±1.0 a | 8.6±0.9 a | 11.9±1.4 a | 24.8±3.8 a | 26.4±3.3 ab |
| HO2 ($10^8$) | 11.0 ab | 9.0±1.3 a | 11.4±1.1 a | 28.1±1.5 a | 29.5±3.7 b |
| RO2 ($10^2$) | 13.5±1.4 abc | 15.6±2.0 bc | 15.6±2.5 a | 32.1±2.5 a | 30.5±3.6 bc |
| RO2 ($10^8$) | ND | 13.0±1.5 abc | 12.2±1.1 a | 31.8±3.4 a | 28.6±3.6 ab |

[1] Treatments include the mean data from all replications except the first replication.

[2] Numbers followed by different letters of the alphabet were significantly different at a 95% confidence level.

[3] Total mean days is the mean total number of days that all nymphs survived, including nymphs that did not survive long enough to collect data for mean duration of instar stages and nymphs that died between instars.

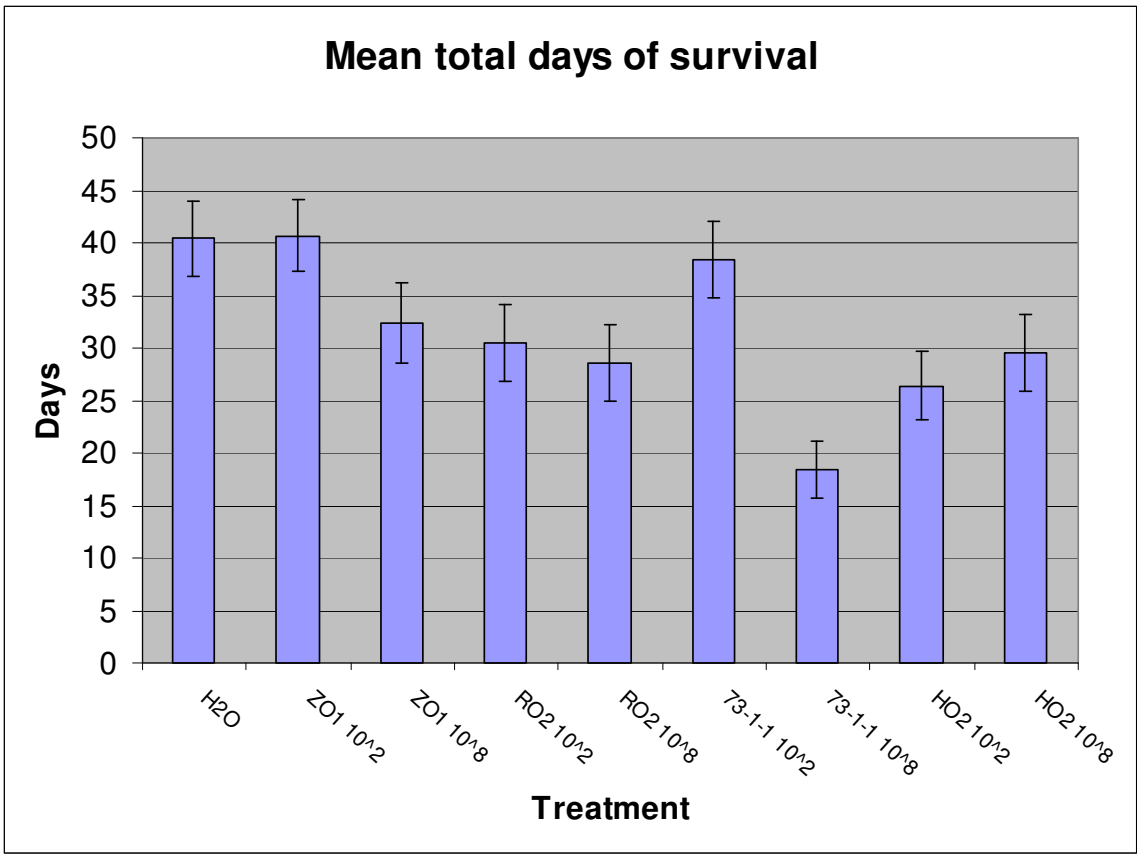


Figure 1. Mean total days of squash bug survival (out of 39 bugs per treatment) over a 60 day period after access to *S. marcescens* strains provided in vacuum-infiltrated squash cubes. Error bars represent standard error.

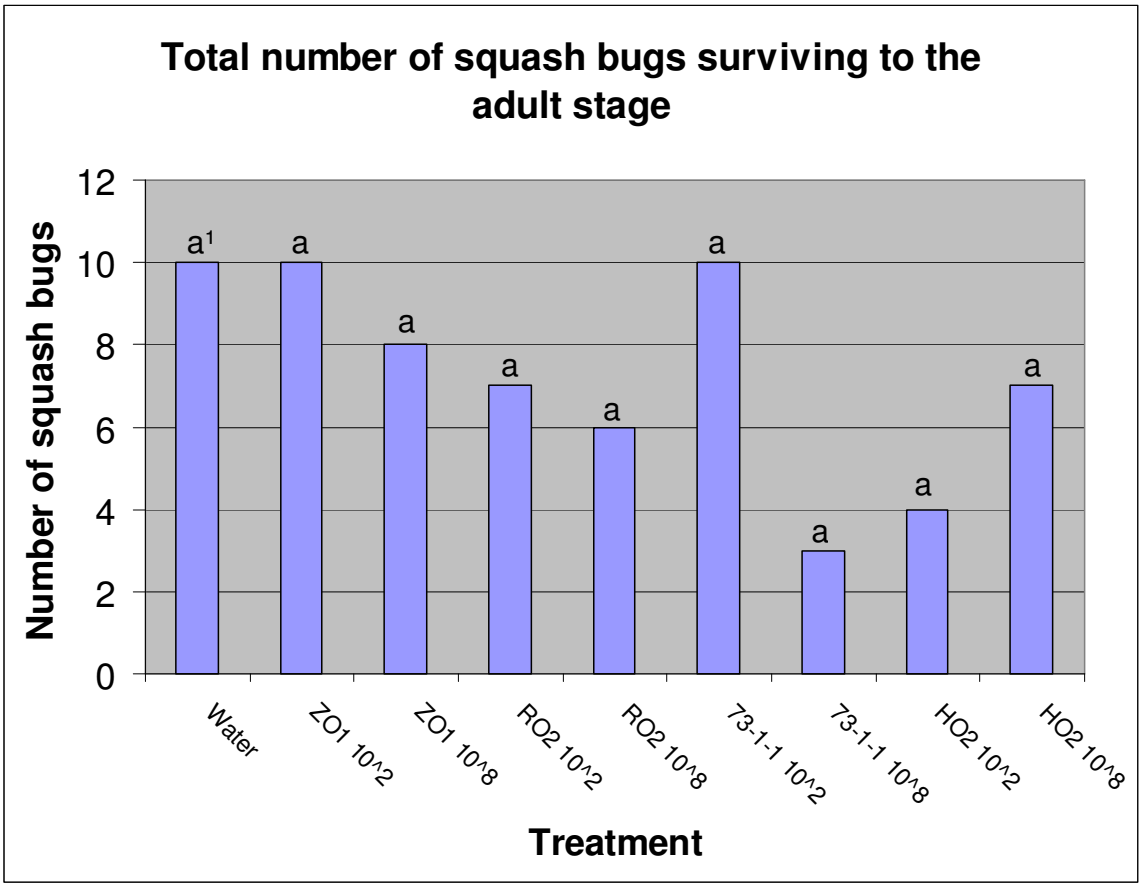


Figure 2. Comparison of total number of squash bug nymphs (out of 39) surviving to adulthood.

[1] The letter of the alphabet indicates no significant difference at a 95% confidence level.

CHAPTER IV

# Discovery of a Trypanosomatid in the Squash Bug, *Anasa tristis*


## Abstract

Trypanosome-like protozoans (TLP) were observed in the abdomen contents of adult squash bugs, *Anasa tristis*. Feral insects collected in Stillwater, OK and colony-reared insects were dissected and examined by light microscopy. TLPs were elongate and narrow (40-50 by 4-6µm) with a single, polar flagellum (20-28µm), consistent with features of described trypanosomatids. Wild and colony-reared insects were subjected to polymerase chain reaction using primers that amplify a highly conserved 5S rRNA gene repeat region of trypanosomatids. The resulting sequence most closely matched that of an unclassified member of the family Trypanosomatidae, confirming that the squash bug protozoan is a trypanosomatid.


## Introduction

Trypanosomes, eukaryotic protozoa in the order Kinetoplastida and Kingdom Protista, include the genera *Endotrypanum, Trypanosoma, Leptomonas, Herpetomonas, Crithidia, Blastocrithidia*, *Rhyncoidomonas,* and *Phytomonas.* Trypanosomes occur worldwide and

reside in a wide range of animals and plants, in which they may or may not cause disease. These organisms have a complex life cycle, existing in different morphological forms (amastigote, promastigote, trypanomastigote, epimastigote, and ophistomastigote) that may be associated with different hosts or environments (McGhee et al., 1971a). The promastigote, ophistomastigote, epimastigote and trypomastigote forms consist of an elongate body with a single long flagellum. The spherical amastigote form may or may not have flagella. Transformation from one form to another is generally triggered by environmental cues; for example, *Trypanosoma cruzi* exists as an epimastigote in the gut of its insect vector and as an amastigote in the blood of its vertebrate host (Chagas 1909).

Trypanosomes infect both animal and plant hosts. Some of the pathogenic trypanosomes are transmitted by insects of the Hemipteran families Coreidae, Lygaeidae, Pentatomidae, and Reduviidae (Dollet et al., 1982, Ayala et al., 1975, McGhee et al., 1971a, McGhee et al., 1982, McGhee et al., 1971b, Kastelein 1985, Louise et al., 1986). The best known trypanosomes that cause human disease are *Trypanosoma cruzi*, causal agent of Chagas' disease, and *Trypanosoma brucei*, causal agent of African sleeping sickness (Chagas 1909, Bruce 1903), which are transmitted by a reduviid, *Rhodnius prolixus*, and the tsetse fly, *Glossina* sp., respectively. Other trypanosomatids in the genera *Blastocrithidia, Leptomonas, Herpetomonas, Crithidia* and *Rhyncoidomonas* infect only insects. Three important plant diseases caused by trypanosomes transmitted by Heteropteran true bugs are coffee phloem necrosis, hartrot of coconut, and oil palm marchitez (Stahel 1931, Vermeulen 1963, Parthasarathy et al., 1976, Dollet et al., 1977). The causal agents of hartrot of coconut and oil palm marchitez, in the genus *Phytomonas*, are both transmitted by true bugs (Hemiptera: Pentatomidae).

During experimental microscopic examination for the presence of *S. marcescens* in abdomen samples of colony-reared squash bugs, I discovered organisms having morphology similar to that of trypanosomatids. In this work, I described and identified the trypanosome-like protozoan.

**Materials and Methods**

Adult squash bugs were collected from a laboratory-maintained colony and from pumpkin and squash fields near Stillwater, OK. The colony, established in 2005, was started using feral insects from the Stillwater, OK area. Hemolymph and gut samples were collected after immobilizing single insects, dorsal side down, in wax. With the aid of a dissecting microscope, an abdominal incision was made through the intersegmental membrane between the dorsal and ventral sclerites. A micropet tube (Becton, Dickinson and Company, Parsippany, NJ) (20µl volume with 0.5µm diameter bore) was inserted into the abdominal cavity to collect hemolymph. The incision was then enlarged and the entire alimentary canal removed. Insect hemolymph or tissues were mounted without added liquid on glass slides and observed using bright field optics with an Olympus BH-2 microscope (Tokyo, Japan) at 120x, 240x, 480x, and 1200x. Micrographs were taken using an Olympus BX51 microscope (Tokyo, Japan) with digital imaging. A total of 69 colony-raised and feral bugs were tested using visual observation and 41 of those were subjected to PCR over the duration of this study.

DNA was extracted from individual squash bug samples (consisting of a mixture of hemolymph and internal organs suspended in phosphate buffer solution) using a DNeasy tissue kit (Qiagen Inc.; Valencia, CA), using the manufacturer's protocol for

purification of genomic DNA. 5S and SL RNA genes were amplified using the trypanosome-specific primers Tr5s-L (5´-TAACTTCACAAATCGGACGGGAT-3´) and Tr5s-R (5´-CCGTCCGATTTGTGAAGTTAAGC-3´) (Podlipaev et al., 2004, Dollet et al., 2000). PCR products were separated electrophoretically on a 1% agarose gel and amplicons were recovered using the GENECLEAN SPIN kit (BIO 101; Carlsbad, CA). Sequencing was performed at the Oklahoma State University Recombinant DNA/Protein Resource Facility. A BLAST search (Altschul 1990) was performed on the resulting sequences.

**Results**

Two morphological forms of the trypanosome-like protozoan (TLP) were observed in the squash bug. One, which most closely resembled a promastigote form (Fig. 1), was the more common form in visually positive gut samples. No trypanosomatids were found in hemolymph-only samples. These organisms had body dimensions of 20-25µm x 4-6µm, with a longitudinal flagellar pocket and a single polar flagellum approximately 20-28µm long. The second morphological type, a spherical body that resembled an amastigote, was 10µm in diameter and possessed a flagellum 30µm long. This type was seen in only two squash bug samples, and 15-20 individual TLPs were observed. Numerous promastigotes were also observed in the same samples.

On the day of the initial TLP discovery, the promastigote form was present in 6 out of 10 of the colony-raised squash bugs sampled (Table 1). Their presence and prevalence were not consistent over time, however, with the percentage of colony insects infested varying considerably over the dates of examination (Table 1).

Colony-reared squash bugs with promastigote-like forms tested positive by PCR using the trypanosome primers Tr5S-L and Tr5S-R (Table 1). Two feral squash bugs that were negative for visual confirmation of trypanosomes tested PCR positive with the same primers. PCR amplified a 700 bp DNA fragment, of which 500-600 bp were useful in sequencing. The two closest sequence matches revealed by the BLAST search were identical for all squash bugs, whether feral or colony-reared. The best sequence match, with 95% identity over 69% of the total sequence, was to unclassified Trypanosomatidae sp. 129SI (e-value $6x10^{-98}$), which was isolated from an *Althos* sp. (Coreidae) collected in Ecuador. The second closest match, with 89% identity over 76% of the total sequence, was to unclassified Trypanosomatidae sp. 119YS-A (e-value $2x10^{-87}$), which was isolated from a *Leptopetalops* sp. (Coreidae) also from Ecuador. The third closest match varied among the samples; four were similar to *Blastocrithidia triatoma* (e-value approximately $8x10^{-42}$), two were similar to unclassified Trypanosomatidae sp. 80MV-C (e-value approximately $2x10^{-42}$), two were similar to unclassified Trypanosomatidae sp. 71CN-D (e-value $5x10^{-14}$), one was similar to unclassified Trypanosomatidae sp. 117YS (e-value $2x10^{-32}$), and two were similar to unclassified Trypanosomatidae sp. 33EC (e-value approximately $1x10^{-39}$).

**Discussion**

The detection of trypanosomatids in both colony-reared and feral squash bugs did not appear to be consistent over time. Recent work has indicated a possible explanation. Most samples collected in 2006 were visually negative for TLPs at 120x, 240x, and 480x magnification and were not tested by PCR. It was not until 2007 that samples were

observed at 1200x magnification and more positive samples were identified. Small promastigote forms were difficult to see at 480x magnification, but were easily identified at 1200x. Therefore, some visually negative samples collected in 2006 may have actually contained TLPs, but due to inadequate magnification, they were not observable.

The results of this study indicate that the TLPs seen in colony-reared *A. tristis*, and detected by PCR in both colony-reared and feral squash bugs, are trypanosomes. Comparison of nucleotide sequences using the BLAST database indicated that the protozoan belongs to the Family Trypanosomatidae. As the closest sequence match with a classified species, *Blastocrithidia triatoma,* was only the third-best match in a few samples, the classification of the squash bug trypanosomatid remains unclear. The genus of the squash bug resident Trypanosomatidae sp. is not certain, and no conclusion can be made as to its impact, beneficial, detrimental, or neutral, on the insects. The possibility that the unclassified Trypanosomatid is an insect transmitted plant pathogen cannot be ruled out.

Described plant-infecting trypanosomes colonize within the vascular phloem, one of several plant tissues in which squash bugs feed (Stahel 1931, Parthasarathy et al., 1976, Dollet et al., 1977, Dollet 1984). If the *A. tristis* trypanosomes are capable of colonizing within cucurbits, they could be acquired by the bugs when they feed in infected tissues. In addition, there is some evidence that insect trypanosomes can be transmitted to various types of fruits, but do not infect other parts of the plant (M. Dollet, personal communication). Squash bugs readily feed on cucurbit fruits, possibly facilitating horizontal transmission of the trypanosome. The colony-raised squash bugs were fed on both whole squash plants and fruit, therefore they may have acquired the

trypanosomes from these food sources. Further work will be needed to determine any effect the trypanosome may have on the squash bugs or their plant hosts.

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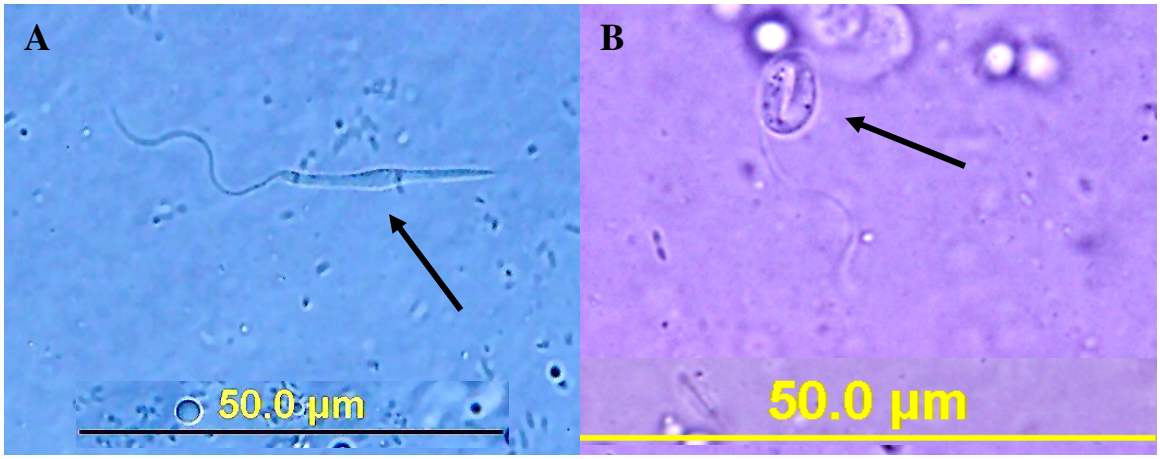


Fig. 1. Promastigote form trypanosome (A) in squash bug abdominal contents. Insert: (B) amastigote form.

Table 1. Squash bug samples[a] testing positive for TLOs by visual observation or by PCR.

| Date | # colony bugs with TLOs/ total # bugs examined | PCR positive colony bugs | # feral bugs with TLOs/ total # bugs examined | PCR positive feral bugs |
|---|---|---|---|---|
| 2-18-06 | 6/10 [1] | ND | --- | --- |
| 4-27-06 | 1/3 | 1 | --- | --- |
| 7-18-06 | --- | --- | 0/12 | 2 |
| 9-25-06 | 0/4 | 0 | 0/8 | 0 |
| 10-16-06 | 1/4 | 1 | 0/10 | 0 |
| 7-11-07 | --- | --- | 1/1 [2] | ND |
| 7-12-07 | --- | --- | 2/3 | ND |
| 7-20-07 | 1/1 | ND | 0/1 | ND |
| 8-14-07 | --- | --- | 1/1 | ND |
| 10-2-07 | 11/15 | 8/11 | --- | --- |

[1] Samples collected in 2006 consisted of mixed hemolymph and excised gut tissue.

[2] Hemolymph and gut tissue samples collected in 2007 were observed separately.

# Final Summary

Aphids, as a model organism, were fed concentrations of $10^8$ or $10^2$ cells/ml each of either an insect isolate (73-1-1), a human isolate (HO2-A), a rice endophyte (RO2-A), or a CYVD strain (ZO1-A) of *S. marcescens*. The CYVD strain, ZO1, offered in low ($10^2$ cells/ml) concentrations, was the least damaging treatment, resulting in mortality rates nearly identical to those in the control. In general, as expected, the higher concentrations of all bacterial strains resulted in higher aphid mortality. The fact that low concentrations (as would be expected to occur naturally) of the CYVD strain ZO1-A, caused insignificant aphid mortality at any time during the experiment suggests that ZO1-A was less pathogenic to aphids than were strains 73-1-1, HO2, and RO2, all of which did cause significant mortality at both $10^8$ cells/ml and $10^2$ cells/ml concentrations. The rice endophyte, RO2, resulted in high and rapid mortality in the pea aphid. Both the insect isolate, 73-1-1, and the human isolate, HO2-A, also caused significantly higher mortality to aphids than did the CYVD strain. The mild pathogenicity of CYVD strain ZO1-A to aphids suggests attenuation of the CYVD strains to the insect environment.

Squash bugs, a natural vector of CYVD, were exposed to concentrations of $10^8$ or $10^2$ cells/ml each of either an insect isolate (73-1-1), a human isolate (HO2-A), a rice endophyte (RO2-A), or a CYVD strain (ZO1-A) of *S. marcescens*. The overall effects of

the bacterial treatments were different, but they caused no significant reduction in the total number of insects surviving and completing development to the adult stage regardless of the *S. marcescens* strain to which they were exposed. Thus ingestion of, or exposure to, the bacteria did not significantly alter survival or development of the insect. There were, however, significant differences in total mean days of nymphal survival between insects treated with water and those exposed to high ($10^8$ cells/ml) concentrations of three *S. marcescens* strains, HO2-A, RO2-A, and 73-1-1, and to low ($10^2$ cells/ml) concentrations of HO2-A. These treatments significantly shortened nymphal life span, suggesting that these strains are more pathogenic to squash bugs than ZO1-A is. Survival of nymphs to adulthood, as well as a longer life span, would facilitate spread of the CYVD pathogen over a longer period of time than would be allowed if the infected nymphs died before completing development.

Data collected from the aphid pathogenicity study suggest that the CYVD strain of *S. marcescens* is not significantly pathogenic to the pea aphid in low concentrations. Further data suggest that the CYVD strain of *S. marcescens* is not significantly pathogenic to the squash bug and that the other three strains (RO2-A, 73-1-1, and HO2-A) significantly shorten the life span of both squash bugs and aphids. The fact that the CYVD strain ZO1-A displays only mild pathogenicity to both aphids and squash bugs may reflect adaptation of the ZO1-A strain to the insect environment. Highly pathogenic strains are not well adapted for insect transmission due to the rapid death of the insect vector and shorter time for pathogen dissemination. The fact that the CYVD strain, ZO1-A, did not cause significant mortality at any time during these experiments while the other strains did cause deleterious effects on the insects could reflect an adaptation of the

ZO1-A strain and the insect to one another. Attenuation of pathogenicity to the vector species could allow greater opportunity for bacterial transmission to the host plant. In contrast, isolates that shortened the life span of the insects reduced the likelihood of transmission to another host plant. This work offers insights about how the plant pathogen-vector relationship affects pathogen dissemination and disease impact.

During the course of this work, trypanosomatid-like protozoans were seen in colony-reared *A. tristis.* Polymerase chain reaction was conducted on both colony-reared and feral squash bugs and comparison of nucleotide sequences using the BLAST database indicated that the protozoan belongs to the Family Trypanosomatidae. As the closest sequence match with a classified species, *Blastocrithidia triatoma,* was only the third-best match, the classification of the squash bug trypanosomatid remains unclear. The genus of the squash bug resident Trypanosomatidae sp. is not certain, and no conclusion can be made as to its impact, beneficial, detrimental, or neutral, on the insects. The possibility that the unclassified Trypanosomatid is an insect-transmitted plant pathogen cannot be ruled out and the effect, if any, that this organism has on the squash bug and/or on the colonization of the insect by *S. marcescens* remains unclear.

# Appendix

## Fate of GFP-Tagged *Serratia marcescens* After Feeding and Injection to the Squash Bug

**Abstract**

Squash bugs, *Anasa tristis*, which acquire the cucurbit yellow vine disease (CYVD) bacterium, *Serratia marcescens,* as nymphs retain and transmit the CYVD pathogen after molting to the adult stage. In this study, we investigated the mode of transmission of *S. marcescens* by examining the migration of the CYVD bacterium through the body of the vector. The objective was to determine organs invaded by *S. marcescens* in order to elucidate the mode of transmission.

The hemocoel of adult squash bugs was injected with GFP-tagged *S. marcescens* ZO1 (ZO1:GFP) to determine the usefulness of ZO1:GFP in monitoring the movement of *S. marcescens* cells inside the insect body. Further, squash bug nymphs and adults were allowed to feed upon a suspension of ZO1:GFP in vacuum-infiltrated acorn squash cubes. In each experiment internal organs, tissues, and hemolymph were excised from bugs and observed using epifluorescence microscopy.

Fluorescent cells were found in the hemocoel of 26% of the ZO1:GFP-injected squash bugs, but not in any of the tissues of squash bugs fed ZO1:GFP infiltrated squash cubes. Enzymes or symbionts in the alimentary canal may kill the bacterium or suppress fluorescence. Without being able to demonstrate whether or not the CYVD strain ZO1 crosses the gut barrier, little can be proven as to the mode of transmission of the pathogen.

**Introduction**

Cucurbit yellow vine disease (CYVD), which causes yellowing and rapid wilting of cucurbit species (Cartwright 1993), is caused by a phloem-resident bacterium, *Serratia marcescens* (Bruton et al., 2003). *S. marcescens* is a Gram-negative bacterium in the family Enterobacteriaceae and occupies many diverse niches, including those of plant endophyte, plant pathogen, human pathogen, animal pathogen, soil and water inhabitant and food contaminant (Grimont 1977, Grimont and Grimont, 1978, Hejazi et al., 1997, Gyaneshwar et al., 2001, Bruton et al., 2003). Certain strains of *S. marcescens* are pathogenic to more than 70 species of insects (El Sanousi et al., 1987, Matsumoto et al., 1998, Sikorowski et al., 2001, Lauzon et al. 2003). The squash bug, a vector of the bacterium, spreads the pathogen to cucurbits throughout north and central Texas, central and eastern Oklahoma, and several other states (Bruton et al., 2003, Bruton et al., 1997, Bost et al., 1999, Pair et al., 2000, Nesmith 2002, Pair et al., 2003, Boucher 2005, Hausbeck 2004).

Squash bugs that acquire the bacterium as nymphs are able to retain and transmit the CYVD pathogen after molting to the adult stage (Wayadande et al., 2005), indicating

a circulative mode of transmission, in which the pathogen enters the insect alimentary canal lumen and crosses the gut barrier into the hemocoel. The pathogen may or may not replicate in the hemocoel and then migrates to the salivary glands. The pathogen invades the cells of the salivary glands and is inoculated into the plant through the saliva of the insect vector during feeding. No other data has been collected to support the hypothesis of circulative transmission of the CYVD pathogen. In this study, we sought additional evidence to support or refute the hypothesis that *S. marcescens* is transmitted by squash bugs in a circulative manner by attempting to monitor the migration of the CYVD bacterium through the body of the vector and to identify in which tissues, if any, the bacterium localized. Emphasis was placed on determining if *S. marcescens* is capable of crossing the gut barrier into the hemocoel. Information about the location of the bacterium inside the squash bug could help to explain the transmission process of the CYVD strain of *S. marcescens* inside the insect vector.

**Materials and Methods**

*S. marcescens* ZO1-A was isolated from CYV-diseased zucchini collected in Coyle, Oklahoma by Blake Bextine and Astri Wayadande and was tagged with a construct containing the green fluorescent protein (GFP) gene and genes for resistance to ampicillin and Kanamycin by Luo to facilitate identification in insect tissues (Luo 2006). After isolation and culturing, all bacterial samples were stored in 30% glycerol at -80ºC (Sambrook et al., 1989). For use in experiments, a loopful of the stored sample was streaked on LB-agar plates (Sambrook et al., 1989) containing ampicillin (50µg/ml) and kanamycin (30µg/ml) and grown in the dark at room temperature for 48 hours.

Squash bug adults, nymphs, and eggs were field-collected from cucurbit plants in Coyle, Perkins, and Stillwater, Oklahoma in the months of April, May, and June of 2006. The insects and eggs were placed in mesh cages and supplied with young squash plants as a food source. The temperature of the squash bug colony room was 25ºC and the daylength was 14 hours with 10 hours of darkness. The insect colony was maintained on squash plants until populations were sufficient for use in experiments.

ZO1:GFP injection study

Adult squash bugs were exposed to GFP-tagged *S. marcescens* ZO1 (ZO1:GFP) through direct injections into the hemocoel to evaluate the usefulness of ZO1:GFP in identifying *S. marcescens* cells inside the insect body. In the first experiment, 48 hour cultures of ZO1:GFP were suspended in Luria broth (LB) at a concentration of $10^5$ cells/ml. The negative control treatment consisted of sterile LB. In the second experiment, 48 hour cultures of ZO1:GFP were suspended at a concentration of $10^5$ cells/ml in LB containing the antibiotics ampicillin (50µg/ml) and kanamycin (30µg/ml). The negative control treatment consisted of sterile LB containing ampicillin (50µg/ml) and kanamycin (30µg/ml). Needles were made from glass micropet capillary tubes (Becton, Dickinson and Company, Parsippany, NJ) (20µl volume with 0.5µm diameter bore) pulled over a flame to produce two needles of very small bore. The squash bugs were immobilized, ventral side up, by placing pieces of tape over the head and abdomen. One leg from either the mesothorax or metathorax was gently pulled back to expose the intersegmental membrane between the coxa and the trochanter. The needle was inserted into this membrane and a small amount (approximately 3-6µl) of the treatment was

injected into the hemolymph by applying air pressure from the mouth through rubber tubing and into the needle. The exact amount of fluid administered could not be measured due to bleeding of the wound after removal of the needle, possibly flushing out a portion of the treatment. After injection, the squash bugs were placed on squash plants that served as a food source for the insects throughout the duration of the experiment. A total of 42 control-injected squash bugs and 42 ZO1:GFP-injected squash bugs were tested in two replications.

Salivary glands, midgut, muscle, reproductive organs, nerve tissue, fat body, and hemolymph were excised from 1-3 bugs in each treatment every day for up to 10 days post exposure. Tissues were either observed immediately after excision or suspended individually in 0.1M phosphate buffer solution (PBS) and stored in 0.5 ml microfuge tubes at -20ºC for later observation. Insect hemolymph or tissues were mounted in 0.1M PBS on glass slides and micrographs were taken at 100x, 200x, and 400x using an Olympus BX51 microscope (Tokyo, Japan) with digital imaging.

In addition to the injection treatments, a positive control for the microscopy step consisted of ZO1:GFP cells suspended in 5 ml each of antibiotic-free LB and LB containing ampicillin (50µg/ml) and kanamycin (30µg/ml) incubated at room temperature. Two hundred microliters were removed from each of these cultures on days of squash bug dissection and observed to confirm the fluorescence of the bacterium on each day.

ZO1:GFP feeding study

ZO1:GFP grown to log phase in LB, pelleted (14000 x g, 25°C, 10 min.), and resuspended in sterile water with ampicillin (50µg/ml) and kanamycin (30µg/ml) at a concentration of 10^5 cells/ml was introduced to squash bug nymphs and adults in vacuum-infiltrated acorn squash cubes, because squash bugs will not feed on liquid diet-filled feeding sachets (Bextine et al., 2003). The control consisted of squash cubes infiltrated with sterile water also treated with ampicillin (50µg/ml) and kanamycin (30µg/ml). To administer the treatments, 5 squash bugs each were placed in Petri dishes containing 5 vacuum-infiltrated squash cubes per treatment/dish and allowed to feed for 48 hours at 25°C with 14 hours of daylength and 10 hours of darkness. Vacuum infiltration was achieved by placing 1 $cm^2$ squash cubes in a vacuum flask and adding the treatment suspension until the cubes were immersed to half their height. Vacuum was applied for 10 seconds, then released forcing the liquid into the squash cube. These steps were performed three times for each sample. After the 48 hour acquisition access period, the squash bugs were removed from the Petri dishes and placed on squash plants, one plant for each treatment, to serve as a food source to maintain the insects. The experiment consisted of two treatments with a total of 15 adults and 24 nymphs ($2^{nd}$-$4^{th}$ instar) per treatment tested during four replications.

Salivary glands, midgut, muscle, reproductive organs, nerve tissue, fat body, and hemolymph were excised from 1-6 bugs in each treatment every day for up to 6 days post exposure, and were observed immediately. Insect hemolymph or tissues were mounted in 0.1M PBS on glass slides and micrographs were taken at 100x, 200x, and 400x using an Olympus BX51 microscope (Tokyo, Japan) with digital imaging.

The positive control was the original ZO1:GFP grown in 5 ml of LB containing ampicillin (50µg/ml) and kanamycin (30µg/ml) incubated at room temperature. This culture, which was used to make the bacterial solution for vacuum infiltration, was retained to verify the ability of the bacterium to fluorescence on each day of the squash bug dissections.

**Results**

ZO1:GFP injection study

Post-injection survival rate of squash bugs in both ZO1:GFP and LB control treatments was 100% for up to 10 days. The color and perceived integrity of the squash bug internal organs was the same for both treatments. None of the 30 LB-injected control insects in the first experiment displayed GFP fluorescence in any of the tissue or hemolymph samples on any day of the experiment, though faint non-specific fluorescence was seen in most tissues. Similarly, none of the 30 ZO1:GFP-injected insects in the first experiment displayed GFP fluorescence in any sample on any day. However, when the ZO1:GFP and LB control injections were repeated in the second experiment with a new group of insects in which antibiotics were included in the treatment, fluorescence was visible in the hemolymph of 11 of the 12 (90%) ZO1:GFP-injected squash bugs up to 10 days after injection. Most fluorescing cells were either free in the hemolymph or attached to the outer surfaces of tracheae or the alimentary canal. A few GFP-expressing cells were attached to the outer surface of the salivary glands and testes. No fluorescent cells were found in the interior of organs or in muscle tissue sampled. Fluorescence was seen up to 10 days post-injection. The only squash bug in

the second replication injected with ZO1:GFP not to exhibit fluorescent cells was dissected and observed 11 days post-injection and the internal tissues appeared identical to those of LB-injected control squash bugs. GFP fluorescence was visible in none of the 12 LB-injected control squash bugs.

ZO1:GFP feeding study

All 15 adult squash bugs fed on water-infiltrated squash cubes and all 15 adult squash bugs fed on ZO1:GFP infiltrated squash cubes survived the acquisition access period and, upon dissection and microscopic examination, were negative for GFP fluorescent bacterial cells. There were no differences in the visual appearance of the internal organs between the treatments.

Four of the 24 (17%) squash bug nymphs fed ZO1:GFP treated squash cubes died during the 48 hour acquisition access period. Of those, none were positive by microscopy for GFP fluorescent bacterial cells. The remaining 20 nymphs were also negative for GFP fluorescence. All of the 24 water control-fed nymphs survived the acquisition access period and were ZO1:GFP negative. The internal organs of the four ZO1:GFP-fed nymphs that died during the experiment were brown, while those of surviving insects were white, yellow, and green. The organ discoloration could have been the result of pre-mortem *S. marcescens* infection, or to colonization by various bacteria after the death of the insects.

**Discussion**

GFP-tagged *S. marcescens* strain ZO1 survived and fluoresced up to 10 days within the insect hemocoel. However, the fluorescent bacteria were visible only after ZO1:GFP cells were injected directly into the hemocoel, bypassing the alimentary canal. The failure to see fluorescent bacteria in insects exposed to them by feeding on infiltrated squash cubes could have resulted from the presence of some element, enzymatic or cellular, that is either bactericidal in nature or suppresses expression of GFP fluorescence. When the bacterial treatment was ingested from vacuum-infiltrated squash cubes and entered into the alimentary canal, no fluorescence was found in the gut lumen, gut contents, or any internal tissues. Even if the bacterium was unable to cross the gut wall, the GFP fluorescence should have still been visible in the gut contents if the bacterium was present and the GFP gene expressed. Enzymes or microbes in the alimentary canal may kill the bacterium or suppress fluorescence.

Demonstration of the ability of the CYVD strain ZO1 to cross the gut barrier is needed to prove the mode of transmission of the pathogen. If fluorescence were found in the gut, but not in the hemocoel, then the mode of transmission could be inferred to be non-circulative. If fluorescence were found in both the gut and the hemocoel, or any other tissues such as the salivary glands, such data would support circulative transmission of *S. marcescens.* To resolve the problem of losing GFP fluorescence in ZO1:GFP-fed squash bugs, we would first need to determine whether the bacterium was destroyed inside the insect gut or if the bacterium was still alive, but GFP expression was silenced. A plate culture could be made of squash bug gut contents (after feeding for 48 hours on ZO1:GFP-infiltrated squash cubes) and resulting colonies could be sequenced to

determine their identity and tested for fluorescence. If *S. marcescens* was not found on the plate, then it is possible that the bacterium was not acquired all; these experiments were carried out during the winter months and most colony insects had entered into diapause, during which they do not feed regularly. Though vacuum-infiltrated squash cubes were confirmed, by staining of probe sites, to have been probed by squash bugs, and visual confirmation of insect probing behavior was also made, these do not constitute proof that the squash bugs ingested the treatment. Since squash bugs are vectors of *S. marcescens*, it is not likely that the bacterium was destroyed inside the insect. Other experiments could be designed to elucidate the cause of GFP fluorescence loss by testing the effects of squash bug gut-inhabiting microbes, in culture, on ZO1:GFP. Similarly, the effects of enzymes found in the gut could be tested on ZO1:GFP by inoculating the bacterium into a liquid culture containing the enzymes.

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VITA

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Scope and Method of Study:

*Serratia marcescens*, the causal agent of cucurbit yellow vine disease (CYVD), is transmitted by the squash bug, *Anasa tristis*. It is unclear whether a *S. marcescens* strain from one niche can colonize another niche. We tested the ability of four *S. marcescens* strains to produce pathogenic effects on aphids and squash bugs and evaluated differences in the effects of these strains on the insects.

Findings and Conclusions:

These data suggest that the CYVD strain of *S. marcescens* is not pathogenic to the pea aphid in low concentrations, while low concentrations of the non-CYVD strains increased the mortality rate of pea aphids. Additional data suggests that the CYVD strain is not pathogenic to the squash bug and that the non-CYVD strains shorten its life span. The trypanosome-like protozoans observed in the abdomen contents of squash bugs were confirmed by DNA sequencing to belong to the family Trypanosomatidae.

ADVISER'S APPROVAL: Dr. Jacqueline Fletcher