BACTERIAL LEAF SCORCH *XYLELLA FASTIDIOSA* WELLS et al. AND ITS POTENTIAL INSECT VECTORS IN PIN AND RED OAKS IN CENTRAL NEW JERSEY

by

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ABSTRACT OF THE DISSERTATION

BACTERIAL LEAF SCORCH

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The bacterium *Xylella fastidiosa* and its potential insect vectors were studied in central New Jersey. Eighteen *X. fastidiosa* isolates were obtained from symptomatic oaks; one isolate from the treehopper *Ophiderma definita*. The New Jersey *X. fastidiosa* strain shared high levels of nucleotide sequence identity (87-99%) with other known strains in the NCBI database. Phylogenetic analysis based on 16S-23S intergenic spacer region rRNA revealed that the New Jersey strain is *X. fastidiosa* subsp. *multiplex* closely related to isolates from oak, plum, porcelain berry, wild grape, peach, and sycamore.

Xylem feeding insects were monitored in oak canopies using yellow sticky traps and fogging techniques. Thirty-seven Cicadomorpha (Order Hemiptera) insect species were collected from oak canopies. Of the 12,880 xylophages collected, 91.40% were membracids, 6.93% were cicadellids, and 1.67% were spittlebugs. More insect species

and individuals were collected using fogging compared to sticky card collections. Sticky card sampling in more locations over long periods provided similar insect species numbers as fogging. *Ophiderma definita* comprised 68.18% of the total insects collected and peaked in early June. Sticky card collections of *O. definita* were male biased when females were gravid. *Ophiderma definita* was more abundant in pin oaks than red oaks. The sharpshooter *Graphocephala versuta* comprised 6.2% of the total collection and peaked in mid-July. More xylophages were collected in the asymptomatic than in symptomatic oak canopies.

The *X. fastidiosa* DNA was detected in 21 xylophage species throughout the summer. The nucleotide sequences obtained from insects were identical to those obtained from host oaks. Fourteen percent (13.89%) of the 1618 insect specimens tested DNA positive for *X. fastidiosa*. Eleven percent (11.03%) of 934 membracids tested positive for *X. fastidiosa: Enchenopa binota*, *Archasia belfragei, Cyrtolobus discoidalis, C. fenestratus, Glossonotus acuminatus*, *Microcentrus perditus*, *Ophiderma definita*, *Similia fasciata*, *Telamona extrema, T. monticola*, and *T. tiliae*. Nineteen percent (18.79%) of 490 Cicadellidae insects tested DNA positive for *X. fastidiosa*: *Aulacizes irrorata*, *G. versuta*, *G. coccinea*, *Draeculacephala anguifera*, *D. portola*, and *Oncometaopia orbona*. Fifteen percent (15.08%) of 194 spittlebugs tested DNA positive for *X. fastidiosa*: *Aphrophora quadrinotata, Clastoptera obtuse, Philaenus pallidus,* and *P. spumarius*.

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DEDICATION

To my children Alden, Andrew, and Amy

and

my wife Zhihua

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Chapter 1

Xylella fastidiosa and its Transmission

Xylella fastidiosa Wells et al. is a pathogen of many plant diseases and is limited to the xylem vessels, a unique, nutritionally dilute habitat (Press and Whittaker 1993). It belongs to Phylum Proteobacteria, Class Proteobacteria Gamma, Order Xanthomonadales, Family Xanthomonadaceae. *Xylella fastidiosa* cells are small (0.25-0.5 x 1.0-4.0 μm), stain Gram negative, and have no flagella. They have a thick, rippled cell wall, do not form a spore, and do not grow on conventional bacteriological media (Wells et al. 1987). The bacterium is transmitted to new host plants during xylem sap feeding by insect vectors such as sharpshooter leafhoppers (Hemiptera: Cicadellidae), spittlebugs (Hemiptera: Aphrophoridae and Clastopteridae) (Purcell and Hopkins 1996), or treehoppers (Hemiptera, Membracidae) (Zhang et al. unpublished). *Xylella fastidiosa* multiplies and spreads from the site of infection to colonize the xylem, a water transport network of vessels composed of dead, lignified cells. Vessels are interconnected by channels, called bordered pits, which allow the passage of xylem sap but block the passage of larger objects due to the presence of a pit membrane (Tyree and Zimmermann 2002). *Xylella fastidiosa* passes through the pit by causing the degradation of the membrane (Newman et al. 2003). Bacterial cells attach to the vessel wall and multiply, forming biofilm-like colonies that can, when sufficiently large, completely occlude xylem vessels and block water transport (Tyson et al 1985).

Xylella fastidiosa host plants

Xylella fastidiosa has been associated with many leaf scorch diseases such as Pierce's disease (PD) of grapevines (Davis et al. 1978), alfalfa dwarf (AD) (Goheen et al. 1973), almond leaf scorch disease (ALS) (Mircetich et al. 1976), bacterial leaf scorch (BLS) of oak (Gould et al. 1991, Lashomb et al. 2002), and many other diseases in plants including peach, plum, elm, sycamore, maple, and citrus (Huang et al. 2003, Huang 2004, Huang and Sherald 2004, Lashomb et al. 2002). BLS of oak is widespread and severe in states of New York, Pennsylvania, and New Jersey (Lashomb et al. 2002). One of the first symptoms is the death of the leaf margin and development of a distinct yellow border separating the dead tissue from the living tissue. Symptoms then progress toward the base of the leaf in an undulating front. Defoliation is not usually a symptom on red oaks but water sprouts (epicormic shoots) often develop on this species as the disease progresses.

Xylella fastidiosa attacks a wide range of host plants from over 120 genera in more than 30 families, including many asymptomatic herbaceous and woody species. The following are examples of species that have been found in association with *X. fastidiosa* or in which it has been isolated:

almond (*Prunus dulcis* (Mill.) D. A. Webb.) (Mircetich et al. 1976);

American beautyberry (*Callicarpa americana* L.) (Hopkins 1988);

American elder (*Sambucus canadensis* L.) (Hopkins 1988);

avocado (*Persea americana* Mill.) (Montero-Astúa et. al 2008);

blackberry (*Rubus procerus* Muell.) (Hopkins 1988);

Boston ivy (*Parthenocissus tricuspidata* (Siebold & Zucc.) Planch.) (Raju and Wells 1986);

citrus (*Citrus sinensis* (L.) Osbeck) (Hopkins 1989; Chang et al. 1993);

coffee (*Coffea arabica* L.) (Chang et al. 1993);

dallis grass (*Paspalum dilatatum* Poir.) (Hopkins 1989);

eastern baccharis (*Baccharis halimifolia* L.) (Hopkins 1989);

American elm (*Ulmus americana*) (Wester and Jylkka 1959);

English ivy (*Hedera helix* L.) (Hopkins 1989);

goldenrod (*Solidago fistulosa* Mill.) (Hopkins 1988);

grapevine (*Vitis vinifera* L) (Davis et al. 1978);

Japanese beech (*Fagus crenata* Bl.) (Huang et al. 2003);

maple (*Acer* sp.) (Sherald et al. 1987);

miner's lettuce (*Montia linearis* (Dougl.) Greene) (Hopkins 1989);

mulberry (*Morus* sp.) (Kostka et al. 1986);

oleander (*Nerium oleander* L.) (Purcell et al. 1999);

peach (*Prunus persica* (L.) Batsch) (Hopkins et al 1973);

pear (*Pyrus pyrifolia* (Burm.f.) Nakai) (Leu et al. 1993);

peppervine (*Ampelopsis arborea* (L.) Koehne) (Hopkins 1989);

periwinkle (*Vinca minor* L.) (McCoy et al. 1978);

plum (*Prunus salicina* Lindl.) (Raju et al. 1982);

poison hemlock (*Conium maculatum* L.) (Hopkins 1989);

porcelain berry (*Ampelopsis brevipedunculata* (Maxim.) Trautv.) (Huang 2004);

sumac (*Rhus* sp.) (Hopkins 1988);

sycamore (*Platanus occidentalis* L.) (Sherald et al. 1983);

sweetgum (*Liquidambar styraciflua* L.) (J. Bentz, personal communication);

umbrella sedge (*Cyperus eragrostis* Lam.) (Hopkins 1989);

Virginia creeper (*Parthenocissus quinquefolia* (L.) Planch.) (Hopkins 1989);

wild strawberry (*Fragaria vesca* L.) (Hopkins 1989);

Oak species

black (*Quercus velutina* Lam.) (Lashomb et al. 2002);

bluejack (*Q. incana* Bartr) (Barnard et al. 1998);

bur *(Q. macrocarpa* Michx) (Hartman et al. 1995);

chestnut (*Q. prinus* L) (Long 1994);

laurel (*Q. laurifolia* Michx) (Hopkins 1988);

live (*Q. virginiana* P. Mill) (Barnard et al. 1988);

northern red (*Q. rubra* L.) (Hartman et al. 1995);

pin (*Q. palustri* Muenchh) (Hartman et al. 1995);

post (*Q. stellata* Wangenh) (Hopkins 1988);

southern red (*Q. falcate* Woodland) (Barnard et al. 1988);

scarlet *(Q. coccinea* Muenchh) (Freitag 1951);

shingle *(Q. imbricaria* Michx) (J. Bentz, personal communication);

shumard (*Q. shumardii* Buckl) (Mullen 1993);

swamp (*Q. lyrata* Walt) (Long 1994);

turkey (*Q. laevis* Walt) (Chen et al. 1995, Barnard et al. 1998);

water (*Q.nigra* L.) (J. Bentz, personal communication);

white (*Q. alba* L.) (Long 1994);

willow (*Q. phellos* L.) (Long 1994);

***Xylella fastidiosa* detection**

The sensitivity and reliability of methods for detecting *X. fastidiosa* in plants and insects, and for differentiating subspecies or strains, has important implications for research and management of the disease. This is particularly true because of the many reported (and undoubtedly more numerous, unreported) plant species that are symptomless hosts of the bacterium. Effective and economically feasible detection methods, especially for plants with low bacterial populations, are essential for quarantine and management of this disease.

Classical microscopy is an essential technique for the detection of the bacterium. However, this technique is not always sensitive or reliable enough for some bacterial pathogens. Over a period of 90 years, numerous investigators of PD failed to detect a candidate pathogen using microscopy (Esau 1948, Hewitt et al. 1949). Microscopy of alfalfa with AD disease revealed an association with "bacterialike bodies" in most, but not all, diseased plants examined (Weimer 1937). Many common botanical stains used on grapes with symptoms of PD or on alfalfa with symptoms of AD (Esau 1948) do not differentiate *X. fastidiosa* from the matrix material in which they are often embedded in plants. Hematoxylin (Fry and Milholland 1990), carbolfuschin (Weimer 1937), or Giemsa stains (Purcell et al. 1979) have been found to be satisfactory for differentially staining bacteria embedded in intraxylar matrixes in plants. Transmission electron microscopy and scanning electron microscopy (Hopkins 1989) have been used to locate *X. fastidiosa* in plant hosts and insect vectors (Purcell 1990).

Green fluorescent protein (Gfp) has been engineered and expressed in a strain of *X. fastidiosa* (Newman et al. 2003). Fluorescent *X. fastidiosa* cells were visualized

directly in the plant by confocal laser-scanning microscopy (CLSM). This type of microscopy captures images from a sample, allowing visualization of unperturbed *X. fastidiosa* cells in intact xylem vessels. Fixation, washing, and staining are not needed in this method, and sample dissection is minimal, eliminating the potential for artifacts that can affect other types of microscopy used for in-plant analysis of *X. fastidiosa.* Newman et al. (2003) limited their study to petioles, where high levels of colonization of the xylem occur in symptomatic plants (Hopkins 1981).

Fluorescent antibody (Brlansky et al. 1982, 1983, French et al. 1978) or immunogold (Brlansky et al. 1991) labeling has been useful in detecting *X. fastidiosa* in vectors and plants. Enzyme-linked immunosorbent assays (ELISA) have been used to identify or confirm new plant and insect hosts of *X. fastidiosa* (Chen et al. 1992, Garnier et al. 1993, Hopkins and Adlerz 1988, Raju et al. 1980, 1983, Sherald and Lei 1991, Yonce and Chang 1987, Zaccardelli et al. 1993). Important limitations of ELISA using polyclonal antibodies for *X. fastidiosa* are low sensitivities (Hill 1994, Hill and Purcell 1995, Nome et al. 1980, Raju et al. 1983) and false positive readings (Hill 1994, Hill and Purcell 1995). Cross absorption of antisera with extracts from plants free of *X. fastidiosa* reduced false positive readings (Hill 1994, Hill and Purcell 1995). However, when the bacterial population is high enough and when employing a proper control, ELISA is a quick and reliable method for the diagnosis, especially with the confirmation from molecular biological technique when there is a need.

Molecular techniques for *Xylella* detection include DNA-DNA hybridization and immunoblotting for *X. fastidiosa* in citrus (Beretta et al. 1993, Lee et al. 1992), and polymerase chain reaction (PCR) for grapevine (Firrao and Bazzi 1994, Minsavage et al.

1994) and other plants such as oak (Pooler and Hartung 1995, Pooler et al. 1997). Most studies of PCR employed cultured cells. However, inhibitors of PCR occur in grape (Minsavage et al. 1994) and probably other plants. PCR detected less than 100 *X. fastidiosa* cells per sample, which was similar to threshold values obtained by in vitro culture (Hill and Purcell 1995). Using cloned and sequence specific RAPPD-PCR (Random Amplification of Pooled Polymorphic DNA -PCR) products, a set of PCR primers was developed that are specific for the strains of *X. fastidiosa* that have been found to cause citrus variegated chlorosis (CVC) (Pooler and Hartung 1995). Pooler et al. (1997) employed immunomagnetic separation and nested PCR to detect *X. fastidiosa* from alternate host plants (McElrone et al. 1999) and the leafhoppers, *Aulacizes irrorata* (Fabricius), *Oncometopia undata* (Fabricius), *Graphocephala coccinea* (Forster), *G. versuta* (Say), *Draeculacephala mollipes* (Say), one *Erythroneura* species, one *Typhlocyba* species, and three different species of treehoppers (J. Bentz, personal communication). Detection of the bacterium in receptor plants and in single *O. nigricans* (Walker) was achieved using immunocapture-nested PCR with primers specific for the CVC strian (Brlansky et al. 1996). Huang et al. (2003) confirmed an isolate from a 76-year old Japanese beech bonsai by comparing the PCR product with the existing *X. fastidiosa* genome listed with GenBank. A 96-99% sequence identity was obtained for the 472 base pair PCR product. The major disadvantages of PCR are the inability to estimate the bacterial viability or population density. On the other hand, assays using cultures require extended time for colonies of the slow growing bacterial culture to appear and the constant threat of contamination.

Smart et al. (1998) compared several techniques for *X. fastidiosa* detection in grape. PCR and immunocapture PCR were found to be the most sensitive methods. Culturing with specific medium was nearly as sensitive as PCR for live cells, and ELISA was less sensitive than live culture. Results from all methods indicated that *X. fastidiosa* was irregularly distributed in dormant vines. The bacteria first colonized in new primary growth during mid- to late June, and became widely distributed new growth only during July. The increasing population of *X. fastidiosa* in new growth only after June, coupled with the hypothesis that only early season infections produce chronically infected vines, may explain why there is no evidence for vine to vine spread of PD in California.

When the bacterial titer is very low in the vector, however, *X. fastidiosa* detection has been inefficient with PCR (Sherald and Lei 1991, Minsavage et al. 1993, Pooler and Hartung 1995, Smart et al. 1998, and Chen et al. 1999, 2000). The reason may be due to the difficulty in extracting bacterial DNA from the insect foregut and mouthparts where PCR inhibitors exist. Bextine et al. (2004) compared 15 DNA extraction kits available and found that Qiagen DNeasy Tissue Kit (Cat 69504) was the most efficient in extracting bacterial DNA from insect vectors. *Xylella fastidiosa* DNA was extracted from four families of Hemipterans collected from pin and red oaks in New Jersey with a modified procedure of this DNeasy Tissue Kit (Zhang et al. unpublished).

Xylella occurrence and movement in plants

The conclusion that *X. fastidiosa* is restricted to xylem is based mainly on microscopic examinations of diseased plants such as grape (Mollenhauer and Hopkins 1976, Nyland et al. 1973), peach (French et al. 1978), citrus (Chagas et al. 1992, Rossetti et al. 1991), and other trees (Hearon et al. 1980). However, *X. fastidiosa* may

occasionally be found in the intercellular spaces of the xylem (Goheen et al. 1973). For *X. fastidios*a, graft transmission of the pathogen requires inclusion of xylem tissues (Hewitt 1953, Hutchins et al. 1953, Weimer 1937, Wester and Jylkka 1959), blocking access to grapevine xylem with metal foil beneath the bark prevents vector transmission (Houston et al. 1947).

Xylella fastidiosa is cell or tissue specific; moreover, the pathogen also tends to accumulate in specific plant parts. This may vary with host and may impact the symptoms expressed. In leaf scorch diseases, bacteria accumulate fastest and with the highest concentrations in symptomatic leaf veins and petioles (Hearon et al. 1980, Hopkins 1984). In diseases with die-back and general decline, such as caused by infection of live oak, bacteria tend to accumulate in the branches or trunk of the tree and often may be detected only in the sector of the tree with symptoms (McGovern and Hopkins 1994). In diseases with stunting as the primary symptom, such as in phony peach (Aldrich et al. 1992, French et al. 1977, Wells et al. 1980), bacteria accumulate primarily in either the roots or basal part of the main stem.

Xylella fastidiosa grows in xylem fluid, which contains the lowest concentration of organic compounds (sources of available energy) of any plant tissue. However, xylem fluid contains a diversity of amino acids, organic acids, and inorganic ions, predominantly in monomeric form that are essential nutrients for the bacterium (Press and Whittaker 1993, Raven 1984). Glutamine and asparagine occur in disproportionately high concentrations, consistent with the apparent growth media requirement of *X. fastidiosa* for high concentrations of glutamine and organic acids (Chang and Donaldson 1993, Davis et al. 1980). The quantity and composition of xylem sap varies with plant

age, season of the year, time of day, location in the plant, and general plant health (Andersen and Brodbeck 1991, Andersen et al. 1993, Press and Whittaker 1993, Raven 1984). Many stress factors (plant senescence, over production of fruit, other diseases, drought, and root pruning) also favor disease develoment (Hill 1994, Hopkins and Thompson 1984). A comparison of the xylem fluid profile in regards to amino acids and other nutrients between infected and non-infected host plants, as well as between susceptible host plants such as pin or red oak and a resistant host like white oak will provide valuable information. Manipulation of xylem chemistry could present new approaches to control of diseases caused by *X. fastidiosa*.

Symptoms in plants appear to depend on the rate and extent of colonization by *X. fastidiosa*. In susceptible plants, leaf scorching, fruit shriveling, and other symptoms result due to increased xylem blockage as colonization progress (Newman et al. 2003). However, within the majority of host plants, *X. fastidiosa* behaves as a harmless endophyte (Freitag 1951, Purcell and Saunders 1999). Bacterial population comparisons between grapevines that are resistant and susceptible to PD demonstrate a positive correlation between high bacterial populations and symptom expression. Leaf scorch symptoms in grapevines with PD required a threshold bacterial population of approximately 10^6 bacteria per cm of leaf vein for symptom development (Hopkins 1985). *Xylella fastidiosa* attains higher populations in susceptible cultivars than in more resistant species and cultivars of grapevines, although numbers of culturable bacteria were similar during early infection (<8 weeks) among all cultivars examined (Fry and Milholland 1990). Average populations of *X. fastidiosa* were approximately 10 times greater in petioles and 100 to 1000 times greater in the stems of the susceptible cultivar

French Colombard than in the more resistant cultivars Carlos and Noble. Frequency of vessels colonized and blocked by *X. fastidiosa* in grapes is positively correlated with symptom development over the growing season (Hopkins 1981) and within individual plants (Tyson et al. 1985). *Xylella fastidiosa* colonies in the xylem were reported to be different sizes (Tyson et al. 1985), and to be either distributed evenly throughout the vessel or appressed against the vessel wall (Mollenhauer and Hopkins. 1974). Colonies were found to sometimes be accompanied by a matrix presumed to be a gel of either plant or bacterial origin (Brlansky et al. 1982, Fry and Milholland 1990, Mollenhauer and Hopkins 1974, Tyson et al. 1985). During the early part of the infection process, an affected plant may not show a leaf scorch symptom of the disease.

Although *X. fastidiosa* cells likely move along individual xylem vessels with the sap flow, it is thought that *X. fastidiosa* moves from one vessel to another through the bordered pits (intervessel channels) after degrading the membranes that guard them (Newman et al. 2003, Purcell and Hopkins 1996). Xylem-feeding insects can acquire and transmit *X. fastidiosa*, which forms a biofilm of polarly attached cells inside the foregut (Brlansky et al. 1983, Purcell et al. 1979). Once infected with *X. fastidiosa*, insects remain infective with the pathogen as the bacterium can multiply in the foregut (Severin 1949, Hill and Purcell 1997). *Xylella fastidiosa* depends on interactions with both of these organisms for survival. A diffusible signal factor (DSF) is required for biofilm formation in the vector and for transmission to plants. In this signal factor, an α, β unsaturated fatty acid (Wang et al. 2004) directs the interaction of *X. fastidiosa* with both its insect vector and plant host. The gene *rpf*F encodes a protein similar to enoyl-CoA

hydratases that synthesizes DSF (Barber et al. 1997), and is actively expressed by *X. fastidiosa* (Newman et al. 2003).

Different plant species probably vary enormously in their importance as reservoirs for vector spread of *X. fastidios*a, depending upon whether or not the bacterium spreads systemically with the plant. Plants that support systemic movement can preserve and expand inoculum during periods of low vector abundance. For example, *X. fastidiosa* multiplied in California mugwort *(Artemisia douglasian*a Besser) and water grass *(Echinochloa cruzgalli*) at or near (<1 cm) the point of infection but did not move systemically to other parts of the plant (Hill and Purcell 1995). These localized concentrations of viable bacterial cells were adequate for vectors to acquire *X. fastidiosa* from the inoculated plants (Hill 1994).

The relationship of bacterial populations to disease symptoms suggests that bacterial toxins are probably not involved in disease developments (Goodwin et al. 1988, Hopkins 1989). Concentrations of plant hormones in grapes were influenced by infection with *X. fastidiosa* (Goodwin et al. 1988), and exogenous applications of plant growth hormones suppressed disease symptoms as well as bacterial populations in peach (French et al. 1978) and grape (Hopkins 1985). Bacterial populations can be very high long before symptoms appear in tissues, such as for young stems of clove with Sumatra disease (Bennett et al. 1985) or for grape with PD (Hill and Purcell 1995, Hopkins 1985, Hopkins and Thompson 1984). Symptoms of water stress in grapes with PD, such as marginal leaf scorch, appear to be closely related to cumulative chronic blockage of the xylem transport system from the occlusion of tracheary elements by tyloses produced by plants (Esau 1948), gums, or bacterial masses (Fry and Milholland 1990, Hopkins et al.

1974, Mollebhauer and Hopkins 1976). The cohesion model of xylem transport suggests that bacteria within a xylem stream under tension should precipitate the cavitation of colonized vessels (Davis 1989, Zimmermann 1983). However, the eventual formation of embolisms within colonized vessels rather than the direct blockage of water flow by occlusions may cause the water stress symptoms of leaf scorch diseases (McCoy et al. 1978).

Xylella strain relationships

Although all strains of *X. fastidiosa* are grouped as a single species (Wells et al. 1987), they differ greatly in host range (Hopkins 1989, Hopkins and Adlerz 1988, Sherald 1993), pathogenicity (Fry et al. 1994, Hopkins 1984, Huang et al. 1986), nutritional fastidiousness (Fry et al 1990, Hopkins and Adlerz 1988, Hopkins et al. 1989), and DNA relatedness (Chen et al. 1992, 1995, Davis et al. 1983, Kamper et al. 1985, Wells et al. 1981). Conversely, genetically different strains of the bacterium produce the same symptoms in a common host. Since host range studies are too costly in time and labor to be useful in determining whether a bacterial strain from a newly discovered host is identical to reference strains or is a new pathovars, molecular techniques, such as restriction endonuclease fingerprinting, restriction fragment-length polymorphisms (RFLPs), the randomly amplified polymorphic DNA (RAPD) technique, and rRNA sequence techniques, have been successfully used to differentiate strains of *X. fastidios*a.

In DNA similarity studies, PD strains were distinct from strains causing phony disease of peach (PPD), plum leaf scald (PLS), and periwinkle wilt (PW) (Kamper and French 1985). Pulsed field electrophoresis was used for restriction endonuclease fingerprinting of strains of *X. fastidiosa* from grape, plum, oak, sycamore, and goldenrod

(Leite et al. 1993). After restriction with *Not*I and *Sfi*I, similarity coefficients of fragments of strains associated with PD had a high genetic homogeneity. The DNA of the strains from other hosts had diverse restriction patterns, indicating genetic heterogeneity.

RFLP analysis with two restriction enzymes and 12 probes from a grape strain of *X. fastidiosa* and 12 from a plum strain confirmed that strains associated with PD, AD, and ALS comprise a closely related PD taxonomic group (Chen et al. 1992). Of the six other strains tested, the ragweed and two mulberry strains were closely related to the PD group. The remaining plum and elm strains were similar to one another but distinct from PD group and the periwinkle strain.

RAPD (Randomly amplified polymorphic DNA) has been used for strain analysis. No pre-known sequences are required, and it is the simplest and quickest way to study strain difference between microorganisms (Lawrence et al. 1993). DNA banding profiles of different bacterial strains are quantified and similarity indices are calculated to generate a multidimensional data set. The inter-relationships of the bacterial strains are then elucidated by cluster analysis with a discrete dendrogram and/or by principal component analysis with a two or three-dimensional diagram. RAPD is good for analysis of unknown strains and can be used to compare the known strains. It is also used to compare the DNA banding of an unknown strain to a known strain in order to confirm the unknown strain's identity. Three separate studies utilizing RAPDs suggested several similarity groups among the strains evaluated (Chang et al. 1995, Mehta et al. 2000, Pooler and Hartung 1995). Chen et al. (1995) found that all seven PD strains were highly similar ($Sxy > 0.93$), that the seven BLS of oak strains were similar ($Sxy > 0.96$) but that

the PD and BLS of oak groups were distinctly different from each other (Sxy = 0.67). Also of interest, in one RAPD study, PD strains were more closely related to a ragweed strain than to an almond strain (Pooler and Hartung 1995) and two CVC strains were only distantly related to the other *X. fastidiosa* strains. These two studies suggested several distinct strain groupings by plant and virulence: the oak group, the grape-alfalfa-almond-ragweed group, the mulberry group, the plum-elm group, and the citrus group.

Mehta et al. (2000) studied the genetic diversity among strains of *X. fastidiosa*. For RAPD-PCR, six primers were used to analyze 23 strains of *X. fastidiosa* isolated from citrus (*Citrus sinensis*) compared to the strains isolated from coffee (*Coffea arabica*), grapevine (*Vitis vinifera*), plum (*Prunnus salicina*) and pear (*Pirus pyrifolia*). The phylogenetic analysis of the PCR products revealed five major clusters at 65% similarity: grapevine, pear, citrus-coffee, citrus, and plum. The coffee strains were closely related to the citrus strains with a similarity of approximately 65%. On the other hand, the grapevine, plum, and pear strains showed themselves to be distantly related to citrus with similarity index below 30%. The grouping of the citrus strains showed a relationship based on the geographic location where they were isolated. Most strains isolated from the southern states of Brazil formed one group whereas strains from the states of Sao Paulo and Sergipe formed another group. Coletta-Filho and Machado (2002) used both RAPD and VNTR (variable number of tandem repeats) to analyze the genetic variation of *X. fastidiosa* strains from sweet orange trees. One hundred forty four strains were analyzed with 10 RAPD primers selected from 71 primers and 3 VNTR primers. RAPD showed a low genetic diversity across CVC *X. fastidiosa* strains; VNTR markers were more polymorphic as estimated by typing values.

DNA sequence techniques have been used to study *X. fastidiosa*. Chen et al. (1999, 2000) were able to identify *X. fastidiosa* using small subunit (16S) rRNA as signature character compared to other bacteria such as *Xanthomonas*. This 16S rRNA is highly homogeneous among *X. fastidiosa* strains. The 16S-23S spacer region, however, has a higher level of variation than the 16S rRNA and has been used successfully to differentiate strains of other bacteria in phylogenetic analysis (Leblond-Bourget et al. 1996, Yoon et al. 1997). Mehta and Rosato (2001) distinguished five groups of *X. fastidiosa* by using sequence comparison of the 16S-23S rRNA intergenic spacer region: the citrus and coffee group, the peach, plum, and oak group, the maple and grape group, the oleander group, and the pear group. Huang and Sherald (2004) found that when they employed *Xanthomonas campestris* as an outgroup during the phylogenic analysis based on 16S-23S rRNA intergenic spacer region, geographic location may not have contributed as much to differentiation of strain as did the difference in host species. They found that the porcelainberry is most closely related to the wild grape strain. Porcelainberry and wild grape strains are more closely related to the oak, peach, and plum strains than they are to the mulberry and oleander strains. Maple and cultivated grapes strains are more closely related to each other than to the porcelainberry, wild grape, oak, or mulberry strains. However, these results are preliminary and must be followed by comparisons with many more strains from each group and from more hosts.

Cross-pathogenicity data also need to be correlated with the molecular data. Nunes et al. (2003) developed a microarray assay to study the genetically distinct strains of *X. fastidiosa.* They used the genome of *X. fastidiosa* 9a5c strain, associated with CVC, as a reference and compared 12 *X. fastidiosa* isolates from citrus, coffee, plum, mulberry,

almond, elm, ragweed, and grape, providing a good assessment of the variation in genomic composition across the group. The results demonstrate that *X. fastidiosa* displays one of the largest flexible gene pools characterized to date, with several horizontally acquired elements, such as prophages, plasmids, and genomic islands (GIs), which contribute up to 18% of the final genome. Transcriptome analysis of bacteria grown under different conditions shows that most of these elements are transcriptionally active, and their expression can be influenced in a coordinated manner by environmental stimuli. Evaluation of the genetic composition of these laterally transferred elements identified differences that may help to explain the adaptability of *X. fastidiosa* strains to infect such a wide range of plant species.

Schaad et al. (2004) conducted DNA-DNA relatedness assays and sequenced the 16S-23S intergenic spacer (ITS) region using 26 strains from 10 hosts to determine the taxonomic relatedness among strains of *X. fastidiosa*. Under stringent conditions (T_m -15 °C), the DNA relatedness for most *X. fastidiosa* strains was 70%. However, at high stringency (T_m -8 °C), three distinct genotypes (A, B, and C) were revealed. Taxon A included strains from cultivated grape, alfalfa, almond, and maple that were interrelated by 85%; taxon B included strains from peach, elm, plum, pigeon grape, sycamore, and almond, interrelated by 84%; and taxon C included only strains from citrus, interrelated by 87%. The mean reciprocal relatedness between taxons A and B, A and C, and B and C, were 58%, 41%, and 45%, respectively. ITS results also indicated the same grouping; taxons A and B, A and C, and B and C had identities of 98.7%, 97.9%, and 99.2%, respectively. Taxon A strains grow faster on PD agar medium whereas B and C strains grow more slowly. Taxon B and C strains are susceptible to penicillin and resistant to

carbenicillin whereas the inverse is true for A strains. Each taxon can be differentiated serologically as well as by structural proteins. They proposed that taxon A be named *X. fastidiosa* subsp. *piercei*; taxon B named *X. fastidiosa* subsp. *multiplex*, and taxon C named *X. fastidiosa* subsp. *pauca*.

Vectors that transmit bacterium

Insect vector of *X. fastidiosa* can be grouped into three categories. Authentic vectors include species that have been implicated in transmission of diseases, either experimentally or naturally, and usually have been confirmed by subsequent tests, the results of which have been or are being published. Many leafhopper species are authentic vectors for bacterial plant diseases. Suspect vectors include species that have been reported as vectors in the literature, but have not yet been confirmed by subsequent transmission tests. It is likely that most of these species will be proven to be authentic vectors. Some leafhoppers are potential vectors but have not yet been confirmed. These implied vectors include species that have not been incriminated through results of transmission tests but have been associated with diseased plants and are presumed to be vectors. Treehoppers are implied bacteria vectors and might play an important role in the transmission of BLS of oak.

All members of the subfamily Cicadellinae are xylem-fluid feeders (Young 1968). This subfamily, which includes the most important known vectors of *X. fastidiosa*, is taxonomically diverse, containing approximately 1,950 species (Redak et al. 2004) and represents nearly 9% of the total number of known Cicadellidae species (Knight and Webb 1993). Frazier and Freitag (1946) and Frazier (1966) pointed out that all members of the subfamily Cicadellinae that had been properly tested were shown to be vectors of

X. fastidiosa. The PD bacterium can only be acquired from or transmitted to the xylem tissues of a plant (Hewitt et al. 1946). The bacterium is seen only in the xylem of affected plants (Goheen et al. 1973). Therefore, of all the xylem feeding auchenorrhychous Hemiptera adequately tested as vectors that were found to be capable of transmitting the *X. fastidiosa,* did so with widely varying degrees of efficiency (Frazier 1966, Purcell and Finlay 1979). Cicadellinae leafhoppers, commonly named sharpshooters, have an inflated clypeus that encloses the strong musculature connected to the pumping diaphragm, or cibarium, enabling these insects to feed on xylem at high negative tensions. Novotny and Wilson (1997) showed that xylem-feeding insects tend to be larger than phloem-feeding insects owing to the fact that the significant metabolic costs of extracting xylem fluid decrease inversely with body size. Below are some of the reported vectors of *X. fastidiosa*:

PD: *Amphigonalia severini* Delong (Severin 1949), *Aphrophora angulata* Ball, *A. permutata* Uhler, *Clastoptera brunnea* Ball (Severin 1950), *Draeculacephala minerva* (Berg) (Purcell 1980), *Friscanus friscanus* (Ball) (Severin 1949), *Graphocephala atropunctata* (Signoret) (Purcell 1980, Hill and Purcell 1995, Severin 1949), *Helochara delta* Oman (Severin 1949), *Homalodisca coagulata* (Say) (Almeida et al. 2003, Costa et al. 2000), *Paragonia confuse* Oman (Severin 1949), and *Xyphon fulgida* Nottingham (Severin 1949).

PPD and PW: *Cuerna coastalis* (F.), *C. costalis* (F.), *Graphocephala versuta* (Say), *Homalodisca coagulata* (Say), *H. insolita* (Walker), *Oncometopia nigricans* and *O. orbona* (F.) (previously *O. undata*) (Turner and Pollard 1959, Adlerz and Hopkins 1979, McCoy et al. 1978).

CVC: *Acrogonia citrine* Marucci & Cavichioli, *A. virescens* (Metcalf), *Bucephalogonia xanthophis* (Berg), *Dilobopterus costalimai* (Young), *Plesiommata corniculata* Young, *Homalodisca ignorata* Melichar, and *Oncometopia facialis* (Signoret) (Krugner et al. 2000, Turner and Pollard 1959), and *O. nigricans* (Brlansky et al. 1996).

ALS: *Draeculacephala minerva* Ball, *Graphocephala atropunctata* (Signoret), and *Philaenus spumarius* L. (Purcell 1980).

Oak (*Quercus* spp.) and elm (*Ulmus* spp.): *Aulacizes irrorata, Draeculacephala mollipes,* one *Erythroneura* species, *Graphocephala coccinea, G. versuta, Oncometopia undata,* one *Typhlocyba* species, and three different species of treehoppers (J. Bentz, personal communication).

Oleander (*Nerium oleander* L.) leaf scorch: *Homalodisca coagulata* (Say) (Costa et al. 2000).

Feeding behavior by the bacterial vectors

Feeding is the first step for vector transmission of *X. fastidiosa*. Potential vectors transmit the bacterium during the feeding process. After transmission these bacteria reside in the xylem of the plant. Xylem fluid-feeding leafhoppers, especially in the Cicadellini and Proconiini, are unique organisms in terms of their nutritional ecology. The structure of the mouthparts and digestive system of the vector is eminently suited for the transmission for this kind of pathogen. The process of the feeding characteristics is associated with phases of the disease transmission. The feeding behavior is also related to the xylem fluid chemistry and tension. These insects display an extremely high rate of feeding upon a difficult to access, nutritionally dilute food: xylem fluid (Raven 1983).

Host preference. Sharpshooters are the most studied group of leafhoppers associated with *X. fastidiosa* transmission. The glassywinged sharpshooter *Homalodisca coagulata* is highly polyphagous, feeding on over 100 species in 31 families (Bethke et al. 2001). It is extremely mobile (Turner and Pollard 1959) and occurs throughout the year in California (Blua et al. 1999). *Homalodisca coagulata* also feeds on the xylem of both herbaceous and woody plants. Preferred plants depend on the season and locality, but, in general, the preferred species include crape myrtle, citrus, and holly.

When provided with 19 different host plants infected and uninfected by bacteria, *H. coagulata* prefers crape myrtle over other species (Mizell and French 1987). Uninfected Japanese plum was the second most accepted host and uninfected trees were favored over infected trees. East baccharis was the third most accepted host. Sumac also ranked highly acceptable by *H. coagulata*. Interestingly, although peach was commonly accepted by *H. coagulata*, only 5% of *H. coagulata* were ever observed on peach when provided a choice (Mizell and French 1987). However, when the peach is the only host available in an orchard setting, especially when peach trees display PPD symptoms, a high *H. coagulata* population is hard to maintain. Peach is not a preferred host, especially when PPD symptoms are present.

Host preference may have an impact on probing behavior of the insect vectors. Probing behavior of nymphs of two treehoppers, *Vanduzeea arquata* Say and *Enchenopa binotata* (Say), varied between host and non-host plants (Kiss and Chau 1984). Nymphs of *V. arquata* were more host-specific in nature and showed a higher degree of selectivity than nymphs of *E. binotata*. All nymphs probed readily and for an extended period on both host and non-host twigs. *Enchenopa binotata* nymphs showed no consistent

differences in probing behavior on host versus non-hosts. *Vanduzeea arquata* nymphs were more likely to withdraw their stylets within 60 seconds when on non-host twigs and produced honeydew only when on their host species. Nymphs of *V. arquata* always rejected non-host plants, apparently in the course of probing and prior to encountering the phloem sap. Chemical compounds released from ruptured parenchyma cells may act as probing stimulants or inhibitors (Kiss and Chau 1984).

Nymphal age effect on aggregation feeding. Spittlebugs are another group of insects involved in the bacterial disease transmission. Spittlebug feeding behavior differs between young and old nymphs. *Deois flavopicta* Stål have a strong aggregated distribution in the early instars, but not as large nymphs. This first instar aggregation may be due to limited movement of the nymphs. However, because *D. flavopicta* females lay their eggs in the soil, the distribution of spittlebugs on individual host plants could also be a consequence of nymphal foraging behavior (Pires et al. 2000). This may be because females have evolved behaviors to select oviposition sites related to soil features such as moisture and texture (Hewitt 1958, 1968) rather than the best resources for their offspring. Consequently, nymphs are selected to have a generalized ability to forage for themselves and to use resources of variable quality and type, leading to relatively high population levels. The young nymphs, despite their weak motility and the fact that they will die if they are unable to start feeding, produce spittle masses rapidly, move from the hatching spot, and choose a preferred feeding host plant species. The nymphs tend to be less mobile where host plants are readily available. Nymphs feed gregariously, forming groups of individuals feeding inside one spittle mass on buds and young shoots as first and second instars, and in small groups of third, fourth, and fifth

instars on old shoots of different sizes. These patterns of aggregation feeding behavior may be related to differences in plant tissue hardness or amino acid content between young and old shoots (Pires et al. 2000).

Feeding site preference. Turner and Pollard (1959) suggested that leafhoppers prefer one-year-old wood for feeding based on their field observations. Mizell and French (1987) studied feeding site acceptance and adult survival of two leafhoppers, *Homalodisca coagulata* and *H. insolita* (Walker), vectors of PPD. Although all sections of branches were used, both species of leafhoppers accepted the succulent growth terminal for feeding more frequently than older tissue on both infected and uninfected peach trees. Maximum survival time for both *H. insolita* and *H. coagulata* in the cages on peach was approximately the same (12 days). Survival of field collected adults of the *H. insolita* was not different on infected or uninfected branches. However, percent survival of *H. coagulata* was higher on uninfected branches than on infected branches. Therefore, Mizell and French (1987) suggested that *H. coagulata* feeds more often on and survives better on succulent terminal growth, that is last-year's growth and meristematic growth. When *H. coagulata* and *H. insolita* feed on peach, they feed predominantly on trees which do not display disease symptoms. Diseased plant tissue may have some adverse impact on leafhoppers or deter the feeding on infected branches. This may facilitate disease transmission since *X. fastidiosa* would be transmitted from infected to uninfected branches by leafhopper feeding behavior, as they always move on to uninfected branches.

Feeding penetration. Scanning electron micrographs showed that stylets on the adult leafhopper *Homalodisca coagulata* are an average of 2 mm long with 12 μm

internal radius and feed on xylem tissue of the plant (Andersen et al. 1992). Houston et al. (1947) used a staining method developed by Esau (1944) to study the leafhopper feeding penetration. The adult *Draeculacephala minerva* can reach plant xylem in the young and old plant organs of grapevines and alfalfa. Nymphs feed on young plant organs where the xylem is nearer to the surface than in the old plant organs. By inserting their mouthparts into the plant organs, the insects may reach the xylem by cutting in through the phloem or a puncture may miss the phloem and reach the xylem through the medullary ray. Sometimes the insect will change the position of its mouthparts without moving to a new feeding place, leaving a much-branched feeding puncture. The mouthparts of the insects either pass between or through cells. They may crush some cells come in contact with tracheary elements or penetrate into their lumina. In feeding upon leaves, the insects manage to reach the xylem either from the upper or the lower side of the leaf blade. The presence or absence of the bacteria in the plant or in the insect appeared to have no effect upon the feeding habit of the insect.

Plant xylem fluid effect on feeding behavior. Xylem fluid has the most dilute concentrations of dietary nitrogen and carbon of any plant tissue and is unlikely to contain compounds of secondary metabolism (Raven 1983). The primary organic constituents in xylem fluid are amino acids, organic acids, and sugars. Typically, there are 19 amino acids, 7 organic acids, and 3 or 4 sugars detected in the xylem of most host plants. Amides (glutamine and asparagines) are the predominant nitrogenous solute in the xylem fluid of most woody species investigated and are pivotal in insect nutrition, as they can be converted to a wide array of other amino acids (Andersen and Brodbeck 1989, 1991, 1993). Studies with the glassy-winged sharpshooter *H. coagulata* revealed

that Cicadellinae leafhoppers have well developed suction chambers that allow fluid intake even under strong negative xylem pressure (Purcell 1989). To make up for the low concentration of amino acids in the xylem sap of the plants, these insects usually ingest a large amount of liquids (Raven 1983, Purcell 1989). *Homalodisca coagulata* assimilates at least 99% of the amino acids, organic acids, and sugars in xylem fluid (Andersen et al. 1989, 1992, 2003).

Andersen et al. (1989) calculated the ratio of nitrogen ingested to nitrogen excreted and estimated the amount of energy required to drive the leafhopper's cibarial feeding pump, which is responsible for allowing high rates of fluid movement through stylets. *Homalodisca coagualata* received a net gain in energy, carbon from all host plants upon which they fed. Therefore, the energy carbon, instead of nitrogen, may be the most important limiting factor for xylem-feeding leafhoppers (Andersen and Brodbeck 1989).

Net energy gain (*Xneg*) can be calculated as: $Xneg = 0.70\ (Xe) - Excoef\ (Xt - P)$ where 0.70 (*Xe*) = net energy content of xylem fluid, a 70% efficiency of energy retention in the form of ATP times gross energy content in xylem fluid (*Xe*); *Excoef* = extraction coefficient accounting for the amount of biochemical energy expended by the cibarial pump during xylem fluid extraction (i.e., *Excoef* = 2 for a 50% conversion of chemical to mechanical energy); *Xt* = xylem tension, the units for net energy gain (J/cm^3) and xylem tension MPa are equivalent; P = negative pressure required to account for volume flow through a stylet (Andersen et al. 1992).

Leafhopper feeding behavior was related to xylem fluid chemical profile. The concentration of the predominant amino acids and organic acids peaked during midday.

The concentration of total amino acids was higher than organic acids, except at night. *Homalodisca coagulata* fed only during daytime hours. Leafhopper feeding rate measured as excretion rate was best correlated with the concentration of the amides. Correlation coefficients of other amino acids were low. When host plants were subjected to a high level of water stress, leafhopper feeding rate during midday was associated with xylem tension, and not the concentration of chemical variables. Feeding rate decreased exponentially as a function of xylem tension; feeding ceased at a tension of about 2.1 MPa (Andersen et al. 1992).

Plant nutrient requirement varies according to the development stage of *H. coagulata*, which rarely completes its development on a single host (Andersen et al. 1989, Brodbeck et al. 1993, 1995). According to Gravena et al. (1998), there is a clear difference between leafhopper species occurring on citrus trees and those on invasive vegetation of orchards. Nevertheless, some sharpshooters that occur predominantly on the weeds are eventually trapped in the citrus canopy. Likewise, citrus sharpshooters have been found on a wide range of trees and shrubs in woody habitats adjacent to citrus orchards (Lopes et al. 1995).

Excretion by the feeding vectors. Excretion by the leafhoppers while feeding is very characteristic. Houston et al. (1947) reported that two leafhoppers, *D. minerva* and *Neokolla circellata* Baker gave off large quantities of excrement without withdrawing the mouthparts from the plant tissues for long periods of time. Excrement from a single *D. minerva* female amounted to as much as 2.5 ml in 24-h period. Glassy-winged sharpshooters, *H. coagulata,* tend to feed on previous year's growth and meristematic tissue and excrete copious amounts of liquid as they feed (Mizell and French 1987).

These sharpshooters ingest 100 to 300 times their dry body weight in xylem fluid per day, and in large populations, their high volume of excreta ("leafhopper rain") can become a problem, leaving white residue on leaves.

Due to the dilute nutrient content of xylem fluid, *H. coagulata* must have special adaptations to obtain the proper balance of nutrients. Feeding times are thus orchestrated to coincide with the period of peak nutrient content in the host plant (Redak et al. 2004). Additionally, a specialized structure of the digestive system known as the filter chamber recycles the ingested fluid and improves nutrient absorption, resulting in excreta that are 99.9% water, inorganic ions, and ammonia, the sharpshooter's excretory form of nitrogen. Because xylem-feeding insects are not limited by water, the primary nitrogenous waste product of xylophagous leafhoppers is ammonium (Andersen and Brodbeck 1989, Andersen et al. 2003). Uric acid and urea have rarely been found in detectable concentrations in the excreta. The large volume of water excreted with ammonium likely reduces ammonia toxicity. Phosphorous, potassium, calcium, magnesium, sodium, and chloride are the elements occurring in high concentrations in the excreta (Andersen et al. 1989, Cheung and Marshall 1973, Ponder et al. 2002). These leafhoppers also display extensive “grooming” behavior and often smear excrement over their wings and abdomen with their hind legs causing heavy contamination of the leafhoppers exterior by bacteria. In addition, bacteria could escape from the rectum of leafhoppers through excrement (Purcell and Finlay 1979).

Disease transmission mechanism

Transmission process. The transmission process, in general, involves several major components. Acquisition is the initial phase of the transmission process whereby

leafhoppers acquire the bacteria, generally by feeding on infected plants. When acquisition is completed a latent period may be necessary for bacteriferous leafhoppers to become inoculative. In semipersistent transmission a latent period is absent. In persistent transmission, the latent period is present and represents the time necessary for bacteria that have been acquired by leafhoppers to multiply in the vector's body. Inoculation is the final phase of transmission, when bacteria cells are introduced by inoculative hoppers to plants while feeding.

Latent period. The minimum latent period for transmission of PD by the leafhopper *Graphocephala atropunctata* (Say) is no more than 2 hours, and vectors retain inoculativity throughout adult life, which may be for several months (Severin 1949). In contrast, other prokaryotic plant pathogens persistently transmitted by leafhoppers have much longer latent periods, and histological investigations of transmitting vs. non-transmitting individuals indicate that these organisms are transmitted via a salivary route (Nasu et al. 1970).

PD can also be transmitted by spittlebugs and the minimum latent period for this group of insects range from 2-7 hours. Successful infection varies with feeding duration on the infected plants. Spittlebugs can transmit the diseases after one hour of feeding. Severin (1950) reported that the highest number of infections was produced when adults were kept on diseased plants for a period of four hours which may be related to the bacterial population size inside the host.

Non-circulative persistent transmission. Purcell (1979) hypothesized that transmission of the PD bacterium is via a non-circulative mechanism that involves egestion from the alimentary canal. That is reasonable considering the very short latent

period of the pathogen in the vector and the very broad but nonetheless well-defined vector group specificity. Simultaneous electronic and visual monitoring of *G. atropunctata*'s feeding behavior on artificial diets revealed infrequent but consistent ejection of fluid during feeding. This fluid consisted of ejected gut contents, perhaps in addition to saliva. Fluid egestion may result in the bacteria transmission.

By using related bacteria *Erwinia amylovora* and *Lactobacillus hordniae*, Purcell and Finlay (1979) found that: 1) bacteria were transmitted following feeding acquisition, but not after needle injection into the body cavity or after external inoculation; 2) bacteria were not isolated from leafhopper haemolymph; and 3) all bacteria transmitted to parafilm sachets were also isolated from gut samples or whole insect homogenates. Leafhoppers stopped bacteria transmission after molting but resumed transmission after feeding on an infected plant. This suggested that bacteria are transmitted from the external surfaces of the vector's foregut since the cuticle lining of the foregut and associated bacteria are shed with molting. Therefore, Purcell and Finlay (1979) concluded that transmitted bacteria must be acquired by feeding and are located in the alimentary tract. Houston et al. (1947) observed that when leafhopper vectors feed heavily on tracheary contents, bacteria can be readily transmitted by the vector into the xylem. A rapid rate of upward movement of the PD agent in grapevine stems may be associated with the movement of water in the xylem.

Bacterial acquisition. Purcell et al. (1979) further found that *X. fastidiosa* was not observed in insects for the first few days after feeding acquisition, but only during the time in which they were capable of transmitting. After a few days, small colonies of bacterial cells were visible in the cibarium and pre-cibarium, and after 2 weeks "carpets"

of bacteria were visible in many transmitting *G. atropunctata*. By using scanning electron microscopy, Brlansky et al. (1983) observed that bacterial cells colonized the cibarium, the apodemal groove of the diaphragm, and the precibarium of the sharpshooters *Oncometopia nigricans* and *H. coagulata*. Bacteria were attached to the floor of the cibarium, the apodemal groove of the diaphragm, and the walls of the precibarial area above and below the valve. Bacteria in both leafhoppers species were attached by one end to the walls of the cibarium and precibarium by means of extracellular material and possibly fimbrae (short, fine hairlike structures that are thinner than flagella). Dense, single-celled-thick carpets of bacteria colonized regions within the foregut with a fast flow of ingested sap. Purcell et al. (1979) observed that the average of cross-sectional velocities were 5-10 cm per second in the narrow portions of the precibarium of the leafhopper. The rod-shaped bacteria found in vector foreguts are attached at one narrow end, which maximizes their profile exposure to the currents of ingested fluid. In xylem tissues, *X. fastidiosa* is often embedded in a gum-like matrix (Mollenhauer and Hopkins 1974, 1976). *Xylella fastidiosa* cells attached to the vector foregut also appear to be within in a matrix. The combination of matrix materials and *X. fastidiosa* cells within the insect constitutes a biofilm that has been proposed to protect *X. fastidiosa* from the disruption of fast-moving currents of ingested fluid and to aid nutrient extraction from the dilute xylem sap (Davis 1989, Hopkins 1977).

Retention. Under laboratory conditions, bacterial retention was determined with single males of spittlebug *Philaenus leucophthalmus* which had completed the last nymphal stages on diseased grapevines. The combined feeding time of the nymphs and adults that had molted on diseased vines was 30 days. Males retained the bacteria from

29 to 76 days with an average successful transfer rate to the plants of 18% with a range of 12-28%. The period between the last infection and death of the adults was 2 to 4 days, which suggested that bacteria can be retained by the spittlebug during the entire adult life when provided grapevine seedlings as the host plant (Severin 1950). However, spittlebugs may live longer under natural condition. Hence, the lab setting or bacterial infection may have some adverse effect on the spittlebug health.

Factors that affect transmission

The bacterial survivorship in the vector mouthparts is the main determinant of whether or not the bacteria can be transmitted. The bacterial concentration as well as distribution inside plant and vector may also be important. There may also be a threshold for effective transmission. Other biological and ecological factors may also affect disease transmission.

Impact of bacterial population inside insect vector. Despite the high bacterial population that can be seen within the vector foregut, there is no evidence that high bacterial numbers are necessary for transmission to plants (Almeida et al. 2003, Hill and Purcell 1995). Transmission efficiency was not related to population sizes of *X. fastidiosa* in *G. atropunctata* heads, in which the minimum number of bacteria that ensured efficiency transmission was below the detection threshold of about 100 cultivable cells per insect sample (1000 culturable cells per gram of plant tissue) with PW medium (Hill and Purcell 1995). Less than an estimated 200 viable bacterial cells per insect seems to be adequate for a successful transmission. The absence of a relationship between transmission efficiency and populations of *X. fastidiosa* in the leafhopper foreguts suggested that bacteria in the cibarium are probably superfluous to vector

transmission. The occurrence of bacteria in aggregates suggested that occasional losses of aggregated cells from the surface of the insect foregut is responsible for inoculation of the plant, but such losses from vector may not be dependent on aggregated size. The bacteria that are transferred to the plant probably reside in the food canal of the stylets or the precibarium anterior to the precibarial valve. The number of cells in the stylets would be much less than the number in the cibarium because the surface area and cross-sectional dimensions of the feeding stylets would not support aggregations as large as those in the pump chamber (Hill and Purcell 1995).

Impact of bacterial populations in source plants. In contrast, very high bacterial concentration in source plant tissues is required for viable acquisition by leafhopper vectors. The efficiency of vector acquisition of *X. fastidiosa* from plants is directly related to the concentration of cells of the bacteria in plants with a threshold populations of approximately 10^4 cultivable cells per gram of the plant tissue required for acquisition. Efficient acquisition from grape occurs only with cell concentrations exceeding 10^{6-7} cells per gram (Hill and Purcell 1997). A reason for slow disease spread may be due to few sufficient acquisitions. Higher populations of bacteria in plants resulted in higher rates of transmission. In grapes, the rate of transmission increased over time, from 4.5% in the first 10 days to 55% after 20 days. The maximum number of viable CFU of *X. fastidiosa* recovered by culturing also increased from 5×10^5 CFU per gram during the first 10 days to 5×10^8 after 25 days. Bacterial multiplication varies with host plant species. The bacterium multiplied most rapidly in grapes, and the associated incubation period before transmission also was much shorter for grapes than for Himalayan blackberry, California mugwort, and watergrass (Hill and Purcell 1997).

Grapes and blackberry supported higher populations of *X. fastidiosa* (Hill and Purcell 1995), and both plants also had higher rates of transmission by vectors than did mugwort and watergrass, which had lower bacterial populations. The minimum incubation periods in hosts before *Graphocephala atropunctat* acquired *X. fastidiosa* were 4, 22, 29, and 25 days for grapes, blackberry, mugwort, and watergrass, respectively (Hill and Purcell 1997).

Vector dispersal behavior. Flight activity of the vectors is also very important for the disease's distribution. First, the plant is immobile and a mobile vector is what makes the transmission possible or enhances it. Second, many pathogens are not easily transmitted without assistance. Especially for xylem residents, surface contact between plants probably does not provide a path for the transmission. Therefore, mobility of the vector plays a crucial role in the xylem disease transmission and distribution. By comparing *H. coagulata* with native vectors of *X. fastidiosa*, Blua et al (1999) observed that *H. coagulata* seems to move further into the vineyards. *Homalodisca coagulata* might feed on the lower portions of grapevine, enhancing vine-to-vine spread of PD (Varela et al. 2001). However, by using an immunoglobulin (IgG) mark-recapture technique, Blackmer et al. (2004) observed that sharpshooter leafhoppers had similar a dispersal pattern. They can disperse more than 90 meters in a short of period when wind speed is relatively slow (<3 m/s).

Transmission occurs during the seasonal sharpshooter flights when the population is high. In California, the incidence of *X. fastidiosa* in fields with abundant leafhopper populations has been observed to be as high as 18.6% (Frietag and Frazier 1954). In Florida, however, natural infectivity of the glassy-winged sharpshooter, *Homalodisca*

coagulata, is very low (Alderz and Hopkins 1979). Unlike the glassy winged sharpshooter, Costa et al. (2000) found that, under ideal conditions, 83% of oleander plants each exposed to a single leafhopper carrying *X. fastidiosa* became infected with the bacterium. A high vector population from diseased plant standing promotes the disease transmission.

Multiple vector species? Transmission of a plant diseases or a disease complex may involve more than one vector species. Twenty-one hemipteran species collected from pin and red oaks in New Jersey tested positive for the pathogen *X. fastidiosa*. Treehopper *Ophiderma definita* was the most dominant implied vector species in New Jersey oak canopies followed by leafhopper *Graphocephala versuta* (Zhang et al. unpublished data). Most implied vector species in the oak canopies had low population abundance throughout the summer season. Contribution of each vector species in the disease transmission may be a function of the species distribution, feeding behavior, and seasonal dynamics.

Does vector gender matter? Eastman et al. (1988) studied the transmission characteristics of horseradish brittle root disease by the leafhopper, *Circulifer tenellus* (Baker) and found that males were generally more efficient vectors than were females. They found that the mean number of infected test plants was 2-12% higher when males were used as vectors. In some cases, males had a higher acquisition access period (AAP) or a higher incubation period (IP) than females. Longer acquisition and incubation periods may obtain and produce more plant pathogens. Adult male *C. tenellus* are also reported to be better vectors of curly top virus than females (Bennett 1971). In contrast, female *Macrosteles fascifrons* (Stål) are reported to be better vectors than males in the

transmission of the aster yellow mycoplasma-like organism (MLO) (Swenson 1971). Gender differences in vector capability have also been reported for transmission of corn stunt spiroplasma by *Dalbulus maidis* (DeLong and Wollott) where females transmitted earlier than males and were more likely transmit to consecutive test plants (Alivizatos and Markham 1986).

Conclusions

X. fastidiosa has a distinctive ability to multiply within xylem and move systemically within some plant species without causing obvious symptoms. The bacterium depends upon insect transmission to move from plant to plant and perhaps also depends upon an array of hosts to persist in natural ecosystems. Disease epidemics arise chiefly through encounters with new crops or plantings with endemic pathogens. Currently, no effective control for infected plants, and no strategy for prevention of infection is available. Control of diseases caused by *X. fastidiosa* in landscape trees is limited to pruning of infected shoots and removal of infected plants (A. Gould, personal communication). However, since it requires several years for the bacterium to kill trees; the bacteria may be so widespread that removal of infected trees may not prevent the spread of the bacteria to neighboring trees. The antibiotic tetracycline (Mycoject) when injected into an infected tree will temporarily alleviate the symptoms. However, as the material breaks down in the tree, symptoms reappear. The possibility of utilizing thermal or low temperature therapy (Feil and Purcell 2001; Goheen et al. 1973), as well as antibiotics, to control *X. fastidiosa* needs to be investigated. Quarantines directed against strains of *X. fastidiosa* virulent to grape focus on indexing or assaying grapevines to prevent introductions of this bacterium. It would seem far more likely that introductions

of *X. fastidiosa* would arrive in symptomless but systemic hosts. Hence, sensitive detection methods able to discriminate pathovars or strain differences would be necessary. A better understanding of the disease triangle will provide necessary information of disease management not only through pathogen, but also through vector and other disease components.

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Chapter 2

Characterization of *Xylella fastidiosa* Strain Isolated from Oaks and Potential Insect Vectors in New Jersey

ABSTRACT

Bacterial leaf scorch of oak is spreading throughout the Northeastern and mid-Atlantic states of the United States and affects as many as 40% of susceptible species in some central New Jersey communities. As part of a regional survey conducted for this disease, 18 isolates of the causal agent, *Xylella fastidiosa* Wells et al., were obtained from symptomatic pin and red oak trees and 1 isolate from a treehopper, *Ophiderma definita* Woodruff, using periwinkle wilt medium. All the bacterium isolates were confirmed using ELISA and PCR with 272 and RST31/33 primers. *Xylella fastidiosa* DNA was detected in 21 insect species in the Aphrophoridae, Clastopteridae, Cicadellidae, and Membracidae families. Nucleotide sequences of the PCR products obtained from these insects were identical to those from the 18 *X. fastidiosa* isolates collected from oaks. This strain of *X. fastidiosa* shared high nucleotide sequence identity with other strains in the NCBI database. The sequence showed 99% identity with the Japanese beech bonsai strain, 96% identity with the Pierce's disease strain, and 87% identity with the citrus variegated chlorosis strain. Phylogenetic analysis based on the 16S-23S intergenic spacer region ribosomal RNA with primers G1 and L1 revealed that the New Jersey oak strain was closely related to strains isolated from oak, plum, porcelain berry, wild grape, peach, and sycamore in other states.

INTRODUCTION

Xylella fastidiosa Wells et al. (Wells et al. 1987) is a fastidious, Gram-negative bacterial pathogen that causes many economically important plant diseases including bacterial leaf scorch (BLS) of shade trees such as oak, sycamore, elm, mulberry, and maple that occur throughout the Eastern United States (Hopkins and Purcell 2002, Sherald 2001). In New Jersey, *X. fastidiosa* causes leaf scorch and decline primarily on mature red (*Quercus rubra* L.) and pin *(Q. palustris* Münchh) oaks planted as landscape and street trees. The incidence of BLS in oak for some municipalities is as high as 20 to 40%. With no current cost-effective rescue technology, arborists and land managers are forced to remove trees to prevent substantial liabilities that may result from falling branches in declining trees (A. Gould, personal communication).

Although all strains of *X. fastidiosa* are grouped as a single species (Wells et al. 1987), they differ in host range (Hopkins 1989, Hopkins and Adlerz 1988, Sherald 1993), pathogenicity (Fry et al. 1994, Hopkins 1989, Huang et al. 1986), nutritional fastidiousness (Fry and Milholland 1990, Hopkins and Adlerz 1988, Hopkins et al. 1989), and DNA similarity (Chen et al. 1992, Davis et al. 1983, Kamper et al. 1985, Wells et al. 1981). *Xylella fastidiosa* consists of more than one pathovar or subspecies. Strain identity characterization is essential for progress in research on the ecology and epidemiology of these bacteria.

Molecular biology techniques have been widely used for the bacterial identification at the species or subspecies level. Pooler et al. (1997) amplified *X. fastidiosa* bacterium DNA from insect vectors with 272-1/2 primers. Minsavage et al.

(1994) detected the bacterial DNA from plant tissues with RST31/33 primers. The DNA region between the 16S and 23S ribosomal RNA genes in bacteria (the intergene region, IGR) has been used for phylogenic analysis between bacterial strains due to its higher level of variation. Huang and Sherald (2004) found that their porcelainberry strain was most closely related to the wild grape strain based on 16S-23S rRNA intergenic spacer region sequences. Martinati et al. (2005) revealed a cladogram with two major clades for *X. fastidiosa*. Considering the bacterium's microbiology properties, DNA-DNA relatedness, and 16S-23S spacer region variation, Schaad et al. (2004) grouped North American *X. fastidiosa* strains into 3 subspecies. Together with plum, peach, and other shade trees, oak isolates from Florida and Georgia were placed in the *X. fastidiosa* subsp. *multiplex* taxon. We report here findings on the characterization of *X. fastidiosa* strain isolated from oaks and insect vectors in central New Jersey with a comparison to the reported strains available in GenBank.

Xylella fastidiosa is transmitted by insect vectors such as sharpshooters (Purcell et al. 1999) and spittlebugs (DeLong and Severin 1949, 1950) in agricultural systems. The vector transmission in non-agricultural systems, especially oaks planted as street trees is yet to be determined. Treehopper populations are much greater than that of sharpshooters in oaks. Zhang et al. (unpublished data) collected 37 xylem feeding Cicadomorpha insect species in pin and red oak canopies in New Jersey with the majority being treehoppers. Olszewski (1996) detected the pathogen *X. fastidiosa* by using ELISA technique from one treehopper *Telamona* species and one sharpshooter *Graphocephala* species collected from New Jersey oak canopies. This study reports on insect species collected from pin and red oaks in central New Jersey that tested positive for *X. fastidiosa* by testing

individual insect specimens using sensitive DNA PCR and confirmed by DNA sequencing techniques.

MATERIALS AND METHODS

***Xylella fastidiosa* bacterium isolation from oaks:** Pin and red oak leaves with scorch symptoms were collected at seven sites in four central New Jersey counties in the summers of 2004-2006 (Table 1). Leaf petioles were removed from the leaf blades and prepared for isolation as per Huang and Sherald (2004). Briefly, individual petioles were surface sterilized for 1.5 min in a 2% bleach solution (Clorox^{ultra}, Clorox Professional Products) followed by1.5 min in 70% ETOH, and then rinsed three times for 1 min each in sterile ddH_2O. Petioles were then cut into less than 1 mm slices and placed in 500 µl sterile ddH_2O where they remained for 30 minutes to allow bacterial release from the plant tissue. This was then mixed well by vertex-mixing. Fifty µl of this suspension was moved to 450 µl ddH_2O to create a 10^{-1} dilution. This was repeated to create a 10^{-2} dilution. Sixty µl of suspension from each of the 10^{0}, 10^{-1}, and, 10^{-2} dilutions per sample were placed into 3 drops (about 20 µl per drop) on periwinkle wilt (PW) selective medium, which were then tilted to allow the drops to run down along the medium surface to obtain 3 bands of inoculation. Each plate was then sealed with parafilm, inverted, and allowed to incubate in the dark at 28^{o}C. They were checked daily to remove undesirable organisms. The slow growing *X. fastidiosa* colonies took approximately 2 weeks to appear.

***Xylella fastidiosa* bacterium isolation from insects:** Fresh insects were used for the isolation of *X. fastidiosa* bacteria. Insects were collected from both symptomatic and asymptomatic oak trees at Duke Farms, Hillsborough, New Jersey and Mercer County Community College, West Windsor, New Jersey during the summers of 2005 and 2006.

Oak trees were fogged in the early morning before 7:00 am with 2 parts low odor base oil and 1 part Pyrethrins (3%) (Pretox® PyronyTM oil concentrate OR-3619-A, Prentiss Incorporated, Sandersville, GA) using a DYNA-FOG Golden Eagle fogger (model 2610E, series 3, DYNA_FOG® ltd., Westfield, IN) Fresh individual insects were surface sterilized for 1.5 min in 2% bleach (Clorox^{ultra}, Clorox Professional Products, Oakland, CA) followed by 1.5 min in 70% ETOH, and then rinsed three times for 1 min each in sterile ddH_2O. Insect eyes, wings, legs, and abdomen were removed from the body before homogenization in a sterilized 1.5 ml centrifuge tube with an autoclaved pestle. Five hundred µl ddH_2O was added into the tube by washing the pestle. The suspension was initially mixed by vertex-mixing and allowed to sit for 30 min to allow for bacterial release from within the insect tissue. The suspension was then plated on the PW media and observed in the same manner as the aforementioned isolation from oak petioles.

Bacterial DNA extraction from insects: *Xylella fastidiosa* bacterial DNA was extracted from insects which were collected by using the fogging method described above and from sticky card traps placed in oak canopies. Insects were collected at four research sites in 2003-2006: Duke Farms in Hillsborough, NJ; Mercer County Community College, in West Windsor, NJ; Riverview Cemetery in Trenton, NJ; and the Rutgers Garden in North Brunswick, NJ. Each individual insect was dissected to remove eyes, legs, and wings under a dissecting scope. The specimens were placed in a sterilized 2 ml screw top tube preloaded with two 5 mm glass beads. The tube was then submerged in liquid nitrogen for 20 seconds and ground the specimen on the Mini-bead Beater (BioSpec Products, Inc., Bartlesville, OK) for 20 seconds at low speed. DNA was extracted with Qiagen DNeasy Blood and Tissue Kit (Qiagen Inc., Valencia, CA) with

slight modification of the extraction procedure: the DNA was eluted twice with 50 µl AE buffer after incubating the membrane for 15 min.

Polymerase Chain Reaction (PCR): Pure *X. fastidiosa* bacterial cultures from 18 oak isolates and 1 treehopper isolate were collected with pipette tips to obtain a bacterial suspension. A nested PCR procedure (Pooler and Hartung 1995) was employed to confirm the identity of cultured *X. fastidiosa* on the plates, especially for the DNA extraction from insects due to the low bacterium titer. The first round of the nested PCR was performed by adding 5 µl of bacterial suspension or DNA extraction from insects to 15 µl of PCR master mix containing the following components: 1X PCR buffer, 45 nmol $MgCl_2$, 6 nmol dNTP, 7.5 pmol of each external primer 272-1-ext (5'-AGCGGGCCAATATTCAATTGC-3') and 272-2-ext (5'-AGCGGGCCAAAACGATGCGTG-3') (Pooler and Hartung 1995), and 0.6 unit of *Taq* polymerase (Qiagen Inc., Valencia, CA). For the second round of PCR, 2 µl of the first round PCR reaction mixture was added to 18 µl of the PCR master mix containing 1X PCR buffer, 27 nmol $MgCl_2$, 4 nmol dNTP, 10 pmol of each internal primer 272-1-int (5'-CTGCACTTACCCAATGCATCG-3') and 272-2-int (5'-GCCGCTTCGGAGAGCATTCCT-3') (Pooler and Hartung 1995), and 0.4 unit of *Taq* polymerase (Qiagen Inc., Valencia, CA). Amplification of the nested PCR was carried out in a Biometra UNO-Thermoblock (Biometra, Goettingen, Germany) using the following profile: 94^{o}C for 5 min., followed by 35 cycles of 94^{o}C for 30 seconds, 60^{o}C for 30 seconds, and 72^{o}C for 45 seconds. After a final extension at 72^{o}C for 7 min, the reaction was held at 4^{o}C.

DNA samples of *X. fastidiosa* were also amplified with two other sets of primers for further confirmation with the same equipment as in the nested PCR procedure using separate master mixes and PCR profiles. A 722-bp region of a *X. fastidiosa* DNA was amplified with the primer set RST31/RST33 (Minsavage et al. 1994) by adding 5 µl of the bacterial suspension to the 15 µl of PCR master mix containing the following components: 1X PCR buffer, 45 nmol $MgCl_2$, 6 nmol dNTP, 10 pmol of each primer RST31 (5'-GCGTTAATTTTCGAAGTGATTCGATTGC-3') and RST33 (5'-CACCATTCGTATCCCGGTG-3'), and 0.6 unit of *Taq* polymerase (Qiagen Inc., Valencia, CA). PCR was carried out using the following profile: 95°C for 5 min., followed by 40 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 45 seconds. After a final extension at 72°C for 5 min, reaction was held at 4°C.

The 16S-23S rRNA intergenic spacer regions of *X. fastidiosa* isolates were PCR amplified using primers G1 and L1, which are located in highly conserved regions within the 16S and 23S rRNA genes, respectively (Hendson et al. 2001, Jensen et al. 1993, Mehta and Rosato 2001). The primer sequence G1 (5'-GAAGTCGTAACAAGG-3') is located at the 3' end of the 16S rRNA gene 30 to 40 nucleotides upstream from the 16S-23S intervening space region, and primer L1 (5'-CAAGGCATCCACCGT-3') is located at the 5' end of the 23S rRNA gene, about 20 bases downstream from spacer boundary (Jensen et al. 1993). A 553-bp spacer region was PCR amplified by adding 5 µl of the bacterial suspension to the 15 µl of PCR master mix containing the following components: 1X PCR buffer, 45 nmol $MgCl_2$, 6 nmol dNTP, 7.5 pmol of each primer G1 and L1, and 0.6 unit of *Taq* polymerase (Qiagen Inc., Valencia, CA). PCR was carried out using the following profile: 94°C for 5 min., followed by 35 cycles of 94°C for 40

seconds, 55°C for 1 min., and 72°C for 2 min. After a final extension at 72°C for 10 min, reaction was held at 4°C.

PCR products were analyzed by 0.9% agarose gel electrophoresis, stained with SYBR Safe™ DNA gel stain by adding 10 µl of 10,000X concentrate in DMSO (Molecular Probes, Inc., Eugene, OR) per 100 ml gel solution right prior to pouring. The image was captured with Kodak Digital Science 1D LE software, version 3.02 with Kodak Electrophoresis Documentation and Analysis System 120 digital camera over a 312 nm UV transilluminator FBT1 88 (Fisher Biotech, Subiaco, WA). The gel image was then stored as a TIFF format file and processed with Adobe Photoshop CS2, version 9.02 (Adobe 2005).

***Xylella fastidiosa* species identity confirmation and DNA sequencing:** To confirm that the PCR amplified DNA originated from *X. fastidiosa*, the DNA for each isolate obtained from pin and red oak and each insect species was purified from the agarose gel and subjected to nucleotide sequencing. DNA was extracted from agarose gels using a QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA), and sent with their corresponding primers for direct PCR product sequencing (GeneWiz , South Plainfield, NJ). The resulting sequences (472 bp for 272-int primers, 722 bp for RST31/33 primers, and 533 bp for 16S-23S intergenic spacer region) were employed as a query sequence to GenBank database using BLASTN 2.2 (Basic local alignment search tool) (Altschul et al. 1997). Fresh PCR products of each National Center for Biotechnology Information (NCBI) confirmed *X. fastidiosa* isolates were then cloned into a plasmid vector, pCR®4-TOPO® using a TOPO TA Cloning® Kit (Invitrogen Corporation, Carlsbad, CA). Desired clones were purified with Qiagen's QIAprep spin miniprep kit (Qiagen Inc.,

Valencia, CA). Both strands of the inserts were sequenced at GeneWiz. Nucleotide sequences were assembled and edited using the Lasergene Sequence Analysis Software package (DNASTAR, Inc., Madison, WI). Seventy six DNA sequences were deposited in GenBank under the accession numbers EU334012-EU334087 (Table 2).

The 16S-23S spacer sequences of *X. fastidiosa* strains were aligned using the CLUSTAL W Method with MegAlign module in the Lasergene sequence analysis software developed by DNASTAR, Inc (Burland 2000). Phylogenetic analysis based on maximum parsimony of the 16S-23S rDNA sequence was resolved using PAUP*: Phylogenetic Analysis using Parsimony (*and other Methods), Version 4b10 (Swofford 1999). This method infers phylogenies by selecting the trees that minimize tree length (number of steps) and homoplasy. The trees were obtained by 1000 random addition heuristic searches using the TBR branch swapping algorithm. The strict consensus trees were obtained from the most parsimonious trees. The robustness of the tree was evaluated by applying 1000 bootstrap re-sampling through heuristic search. In order to root the tree, the pear strain (PE.PLS) of the *X. fastidiosa* and another bacterium species *Xanthomonas campestris* were used as outgroups. The cladogram was constructed with the TreeView program (Page and Holmes 1998).

RESULTS AND DISCUSSION

Xylella fastidiosa isolation

Eighteen *Xylella fastidiosa* bacteria isolates were obtained and maintained from red (*Q. rubra*) (8 isolates) and pin (*Q. palustris*) (10 isolates) oaks symptomatic for bacterial leaf scorch in 2004-2006 (Table 1). Only one isolate was obtained from a treehopper, *Ophiderma definita* Woodruff, adult collected from a symptomatic pin oak tree at Mercer County Community College on August 25, 2005. *Xylella fastidiosa* bacterial colonies began to appear 2 weeks after isolation or transfer. At first, colonies were very small and light-yellow but darkened to orange-yellow and turned slightly pearlescent with age (Fig.1). Colony morphology was consistent with that described in the literature for this species (Wells et al. 1987). *Xylella fastidiosa* bacteria were isolated when the first leaf scorch symptom appears in July until the end of the growing season in October. The earliest successful isolation was obtained from a previously ELISA confirmed (in 2003) on July 29, 2004 from a red oak tree in Riverview Cemetery, Trenton, NJ. The latest successful isolation in the summer season was obtained on Oct. 15, 2004 from West Windsor, NJ.

Confirmation of isolate identity

ELISA confirmation: All isolates obtained from this study were confirmed with ELISA. An ELISA test system for *X. fastidiosa* (Agdia, Elkhart, IN) was used on petiole material as well as bacterial colonies grown on PW medium; all samples tested positive.

DNA identity confirmation: *Xylella fastidiosa* DNA was extracted from all 18 oak bacterial isolates and an isolate from the treehopper *O. definita*. *Xylella fastidiosa*

DNA was detected and confirmed from four families of Cicadomorpha insects. Six Cicadellinae species were collected from oak canopies in this study: *Aulacizes irrorata* (Fabricius), *Draeculacephala anguifera* (Walker), *D. portola* Ball, *Graphocephala versuta*, *G. coccinea* (Forster), and *Oncometopia orbona* (Fabricius). *Xylella fastidiosa* bacterium DNA was detected in all six species. Leafhoppers have been considered as *X. fastidiosa* vectors in some crop systems. Purcell et al. (1999) found three insect vector species that transmitted the *X. fastidiosa* disease: the blue-green sharpshooter, *Graphocephala atropunctata* (Signoret), glassy-winged sharpshooter, *Homalodisca coagulata* (Say), and the smoke tree sharpshooter, *Homalodisca lacerta* (Fowler). Pooler et al. (1997) detected *X. fastidiosa* DNA from *G. coccinea,* and *G. versuta*.

Treehoppers (Hemiptera : Membracidae) were the most abundant group of the potential *X. fastidiosa* vectors in pin and red oak canopies in central New Jersey (Zhang et al. unpublished data). This family comprises 91.40% of the total potential vector collection, most of which from *Ophiderma* or *Telamona*. Seventy-five percent (75.31%) were *O. definita*, of which 15.49% tested DNA positive for *X. fastidiosa*. Live *X. fastidiosa* bacteria were isolated from this treehopper species. *Xylella fastidiosa* DNA were also detected in 10 other treehopper species: *Archasia belfragei* (Stål), *Enchenopa binota* (Say), *Cyrtolobus discoidalis* (Emmons), *C. fenestratus* (Fitch), *Glossonotus acuminatus* (Fabricius), *Microcentrus perditus* (Amyot & Serville), *Similia fasciata* (Amyot & Serville), *Telamona extrema* Ball, *T. monticola* (Fabricius), and *T. tiliae* Ball.

Xylella fastidiosa DNA was also detected from less abundant spittlebugs. Spittlebugs are known xylem feeders (Malone et al. 1999) and are able to transmit Pierce's disease in grape (DeLong and Severin 1949, 1950). Five spittle bug species in

the family Aphrophoridae were collected from oak canopies in this study. *Aphrophora quadrinotata* Say*, Philaenus pallidus* (L.)*,* and *P. spumarius* (L.) tested positive for the *X. fastidiosa* DNA. The spittlebug, *Clastoptera obtusa* (Say) was the only Clastopteridae species collected this study and tested positive for *X. fastidiosa* DNA.

PCR amplified sequences from DNA extracted directly from insect specimens were identical to those from 19 bacterial isolates from pin and red oaks and *O. definita*. A 649-bp PCR product using 272-1-ext and 272-2-ext primers was obtained from all 18 oak isolates (Fig.2) and one isolate from *O. definita*. It was not detectable from the DNA extracted from positive insects due to the low bacterium concentration. This sequence codes a hypothetical protein XF1100 described in the strain 9a5c (AE003849). No DNA was detected in control reactions where the bacterial suspension was replaced with distilled water. The DNA sequence of this product was identical for all isolates regardless of origin. A GenBank database search found significant nucleotide-sequence similarities between this sequence and regions of the reported genomic sequence of *X. fastidiosa*. Nucleotide identities of 632/646 (97%) was found with the Temecula1 strain of the *X. fastidiosa* isolated from grape. The identity of 608/689 (88%) with 59 (9%) gaps were found with 9a5c strain isolated from citrus in Spain. Nucleotide identities of 470/472 (99%), 416/431 (96%), 416/431(96%), and 398/405 (98%) were found with Japanese Beech Bonsai (JB-USNA), citrus (Found-5), coffee (Found-4), and chitalpa (NM-1), respectively. Relatively low nucleotide identities of 200/203 (98%) and 108/140 with 28 gaps (20%) were found with two segments of citrus variegated chlorosis CVC-1 strains. This sequence identity confirmed *X. fastidiosa* in oaks with bacterial leaf scorch in central New Jersey.

A 472-bp PCR product with 272-1-int and 272-2-int primers was detected after first PCR amplification with primers 272-1-ext and 272-2-ext from DNA extracted from 21 of the 37 tested insect species and from DNA extracted from the 19 bacterium isolates collected from oaks and treehopper in central New Jersey. The DNA sequence was identical between insect species and oaks, but differed from DNA sequences available at GenBank from other states. A GenBank database search found significant nucleotide-sequence similarities between this sequence and six other genomic sequences of *X. fastidiosa*. Nucleotide identities of 468/472 (99%), 459/472 (97%), 414/431 (96%), 414/431 (96%), 397/405 (98%), and 436/488 (89%) with 32 (6%) gaps were found with the Japanese Beech Bonsai (JB-USNA), grape (Temecula1), coffee (Found-4), citrus (Found-5), two sequences from chitalpa (NM-1), and 9a5c (citrus), respectively (Fig.3). High sequence identities ranges from 96% to 99% of *X. fastidiosa* strains have also been observed by others using the same 272-1-int and 272-2-int PCR primers (Huang et al. 2003, Pooler and Hartung 1995). This indicates that this DNA region is very conservative in *X. fastidiosa* strains. The high levels of DNA similarity (>85%) (Wells et al. 1987) among *X. fastidiosa* strains indicates that differences among strains would primarily lie in the linear arrangement of cistrons (a segment of DNA that is involved in producing a polypeptide chain; it can include regions preceding and following the coding DNA as well as introns between the exons; it is considered a unit of heredity) within the genome (Hendson et al. 2001).

A 722-bp PCR product was obtained from 19 isolate bacteria and DNA extracted from insect specimens with primers RST31 and RST33, which codes a RNA polymerase sigma-70 factor. DNA sequences from all isolates and extracted DNA were identical. A

GenBank database search found significant nucleotide-sequence similarities between this sequence and three other sequences available in the database. Nucleotide identities of 710/722 (98%) with gaps of 2/722 (0.3%), 705/736 (95%) with 17 gaps of (2%), and 559/586 (95%) with 14 gaps (2%) were found with the strains of *X. fastidiosa* isolated from grape (Temecula1, CA), citrus (9a5c, Spain), and citrus (Brazil), respectively (Fig. 4).

Phylogenetic relationship between strains: Bacterial ribosomes carry three types of rRNA (23S, 16S and 5S) encoded in genes organized in operons separated by intergenic spacer regions (IGR) containing one or more tRNA genes. The genetic information derived from the rRNA operon provides valuable taxonomic information, since IGR is, especially those located between the 16S and 23S regions of the rDNAs, suffer less evolutionary pressure allowing them to present greater genetic variation than the regions encoding for the rRNAs. The DNA region between the 16S and 23S ribosomal RNA genes in bacteria is variable in length, having highly conserved sequences on either side (the 5'-end of the 16S rRNA gene and the 3'-end of the 23S rRNA gene). There are also usually several copies of this region in a single bacterium (e.g., six in *Escherichia coli* Migula). Along with this, there can be size variability within the same organism.

A 553-bp DNA sequence of the 16S-23S spacer region for *X. fastidiosa* was obtained from the PCR product amplified with primers G1 and L1 from all the oak isolates and DNA extracted directly from insects in this study. The spacer sequences contained tRNAs for alanine (nucleotides 141-212 with UGC as the anticodon) and isoleucine (nucleotides 229-302, with GAU as the anticodon), and were the same as those

found in other strains of *X. fastidiosa* (Hendson et al. 2001, Mehta and Rosato 2001). DNA sequences from all isolates in this study were identical. A phylogenetic tree was constructed (Fig.5) using this New Jersey strain sequence and sequences from 46 other *X. fastidiosa* strains (Table 3) selected from 107 similar sequences available in GenBank. One sequence was presented from each identical strain group. These sequences represented strains from almond, grape, maple, mulberry, oak, oleander, peach, pear, plum, sycamore, and a variety of other host plant species. As observed by Huang and Sherald (2004), our phylogenetic tree shows that geographic location may not contribute as much as the host species in differentiating the strain phylogenetic relationships.

Our phylogeny revealed that two large groups (bootstrap 100%) contained all the strains analyzed except the pear strain from Taiwan, China. This agrees with the results of Martinati (2005) and Huang and Sherald (2004). Strains from plum, porcelainberry, wild grape, oaks, peach, sycamore, periwinkle, ragweed, and three foreign strains including citrus and coffee, are closely related and formed one of the large groups. Our oak strain is closely related to the oaks from other states along with almond, plum, and peach strains. This group was defined by Schaad et al. (2004) as *X. fastidiosa* subsp. *multiplex*. Interestingly, the citrus and coffee strains belonging to the subspecies *X. fastidiosa* subsp. *pauca*, according to Schaad et al. (2004), also lies within this large group forming a monophyletic clade with a 95% bootstrap value. Periwinkle and ragweed strains formed a monphyletic clade as well with a 64% bootstrap value. Although strains from plum, peach, oak, pecan, porcelain berry, and wild grape exist in the same large group, they are not monophyletic, instead showing a polyphyletic relationship (Fig. 5).

The second large group consists of oleander, mulberry, cultivated grape, elm, sage, Spanish broom, and *brassica* strains with a bootstrap value of 74%. This also agrees with the Schaad et al. (2004) subspecies grouping, *X. fastidiosa* subsp. *piercei*, as well as grouping by others (Martinati et al. 2005, Huang et al. 2004). Inside this group, oleander strains formed a monophyletic clade with a bootstrap value of 69%. Four California strains including grape, almond, brassica, and maple formed a clade with a bootstrap value of 70%. Although they are from different plant hosts, they may be related in some unknown ways such as history or locality. Almond strains appeared in both large groups indicating a paraphyletic host strain property.

In summary, we obtained 18 *X. fastidiosa* bacterial isolates from infected pin and red oaks and one isolate from treehopper *Ophiderma definita* in central New Jersey. DNA sequences amplified by three sets of PCR primers indicated that *X. fastidiosa* isolates were identical between pin and red oaks from various locations in central New Jersey. Comparison of our DNA sequences to those available in GenBank suggested that New Jersey strain is belong to *Xylella fastidiosa subspecies multiplex. Xylella fastidiosa* DNA were detected from 21 hemipteran species collected from pin and red oaks and identical to those from host oak species, suggesting that many insect species are involved in the disease transmission in the oak canopies.

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Table 1. *Xylella fastidiosa* isolates obtained from pin (*Q. palustris*) and red (*Q. rubra*) oaks and the treehopper *Ophiderma definita* in central New Jersey.

Isolate ID	Collection Date	County	Township	Host Species	Latitude (North)	Longitude (West)
NJPE1	September	Mercer	East Windsor	*Q. palustris*	40°15'19.05"	74°33'54.44"
NJRE1	September	Mercer	East Windsor	*Q. rubra*	40°15'36.56"	74°33'44.32"
NJRT1	July	Mercer	Trenton	*Q. rubra*	40°11'39.75"	74°45'20.16"
NJPW1	August	Mercer	West Windsor	*Q. palustris*	40°15'17.80"	74°38'48.86"
NJPW2	September	Mercer	West Windsor	*Q. palustris*	40°15'17.80"	74°38'48.86"
NJPW3	October	Mercer	West Windsor	*Q. palustris*	40°15'17.80"	74°38'48.86"
NJOW1	July	Mercer	West Windsor	*O. definita*	40°15'17.80"	74°38'48.86"
NJPC1	August	Middlesex	Cranbury	*Q. palustris*	40°18'51.05"	74°30'44.51"
NJPC2	August	Middlesex	Cranbury	*Q. palustris*	40°18'51.05"	74°30'44.51"
NJRC1	September	Middlesex	Cranbury	*Q. rubra*	40°19'02.20"	74°30'37.81"
NJRC2	September	Middlesex	Cranbury	*Q. rubra*	40°19'02.20"	74°30'37.81"
NJRC3	September	Middlesex	Cranbury	*Q. rubra*	40°19'02.20"	74°30'37.81"
NJRR1	July	Middlesex	New Brunswick	*Q. rubra*	40°28'26.05"	74°25'13.81"
NJRR2	August	Middlesex	New Brunswick	*Q. rubra*	40°28'26.05"	74°25'13.81"
NJRR3	August	Middlesex	New Brunswick	*Q. rubra*	40°28'26.05"	74°25'13.81"
NJPA1	August	Monmouth	Allentown	*Q. palustris*	40°10'56.31"	74°34'47.53"
NJPA2	August	Monmouth	Allentown	*Q. palustris*	40°10'56.31"	74°34'47.53"
NJPA3	August	Monmouth	Allentown	*Q. palustris*	40°10'56.31"	74°34'47.53"
NJPD1	September	Somerset	Hillsborough	*Q. palustris*	40°33'12.74"	74°37'18.79"

Table 2. GenBank accession numbers for the DNA sequences PCR amplified from *X. fastidiosa* isolates obtained in pin (*Q. palustris*) red (*Q. rubra*) oaks and treehopper *Ophiderma definita* in central New Jersey.

Primer	G1L1	272-int	272-ext	RST3331
Product size	553 bp	472 bp	649 bp	722 bp
NJPE1	EU334012	EU334031	EU334050	EU334069
NJRE1	EU334013	EU334032	EU334051	EU334070
NJRT1	EU334014	EU334033	EU334052	EU334071
NJPW1	EU334015	EU334034	EU334053	EU334072
NJPW2	EU334016	EU334035	EU334054	EU334073
NJPW3	EU334017	EU334036	EU334055	EU334074
NJPC1	EU334018	EU334037	EU334056	EU334075
NJPC2	EU334019	EU334038	EU334057	EU334076
NJRC1	EU334020	EU334039	EU334058	EU334077
NJRC2	EU334021	EU334040	EU334059	EU334078
NJRC3	EU334022	EU334041	EU334060	EU334079
NJRR1	EU334023	EU334042	EU334061	EU334080
NJRR2	EU334024	EU334043	EU334062	EU334081
NJRR3	EU334025	EU334044	EU334063	EU334082
NJPA1	EU334026	EU334045	EU334064	EU334083
NJPA2	EU334027	EU334046	EU334065	EU334084
NJPA3	EU334028	EU334047	EU334066	EU334085
NJPD1	EU334029	EU334048	EU334067	EU334086
NJOW1	EU334030	EU334049	EU334068	EU334087

Table 3. *Xylella fastidiosa* strains used for phylogenetic analysis based their 16S-23S rDNA sequences obtained from GenBank.

Strain	Host of origin	State from which strain was isolated	GenBank accession number
Manteca	almond	California	AF073241
ALS3	almond	California	AF073244
ALS4	almond	California	AF073245
ALS.BC	almond	California	AY388465
H51	almond	California	AY603080
276	almond	California	AY603084
H50	almond	California	AY604730
Fresno	almond	California	DQ011259
Same102	black sage	California	AY603092
G10	brassica	California	AY603091
9a5c	citrus	Macaubal, SP	AE003849
C1-11067	citrus	Brazil	AF237650
P3	coffee	Pindorama, SP	AY388464
ELM-1	elm	Washington, D.C.	AY388468
Temecula1	grape	California	AE009442
Douglass	grape	California	AF073237
GR-8935	grape	Florida	AF203397
ATCC35876	grape	Taiwan, CN	DQ991168
ATCC35879	grape	Taiwan, CN	DQ991169
Maple	maple	California	AF073219
Mul-Va	mulberry	Virginia	AY196794
MUL-1	mulberry	Massachusetts	AY388467
MLS012	mulberry	California	DQ415376
MLS024	mulberry	California	DQ415376
GHS505	mulberry	Taiwan, CN	DQ991170
Mu17	mulberry	Taiwan, CN	DQ991171
92-3	oak	Florida	AF073211
Stucky	oak	Georgia	AF073214
NJRR1	oak, red	New Jersey	EU334023
TR1	oleander	California	AF073218
H44	oleander	California	AY603088
Texas	oleander	Texas	DQ011263
GH-9	oleander	Taiwan, CN	DQ991172
5S2	peach	Georgia	AF073206
4-5	peach	Taiwan, CN	DQ991174
PE.PLS	pear	Taiwan, CN	AF203396
4BD7	pecan	Taiwan, CN	DQ991178

PWT-22	periwinkle	Florida	AY388470
2#4	plum	Georgia	AF073209
PL788	plum	Georgia	AF203395
2-4	plum	Taiwan, CN	DQ991175
2-5	plum	Taiwan, CN	DQ991176
PB-Va	porcelain berry	Virginia	AY196793
RGW-R	ragweed	Florida	AY388469
N10	Spanish broom	California	AY603081
SLS55	sycamore	Taiwan, CN	DQ991180
Saap110	white sage	California	AY603094
WG-Va	wild grape	Virginia	AY196795

Fig. 1. Bacterial colonies of *Xylella fastidiosa* isolate NJRR1on periwinkle (PW) medium.

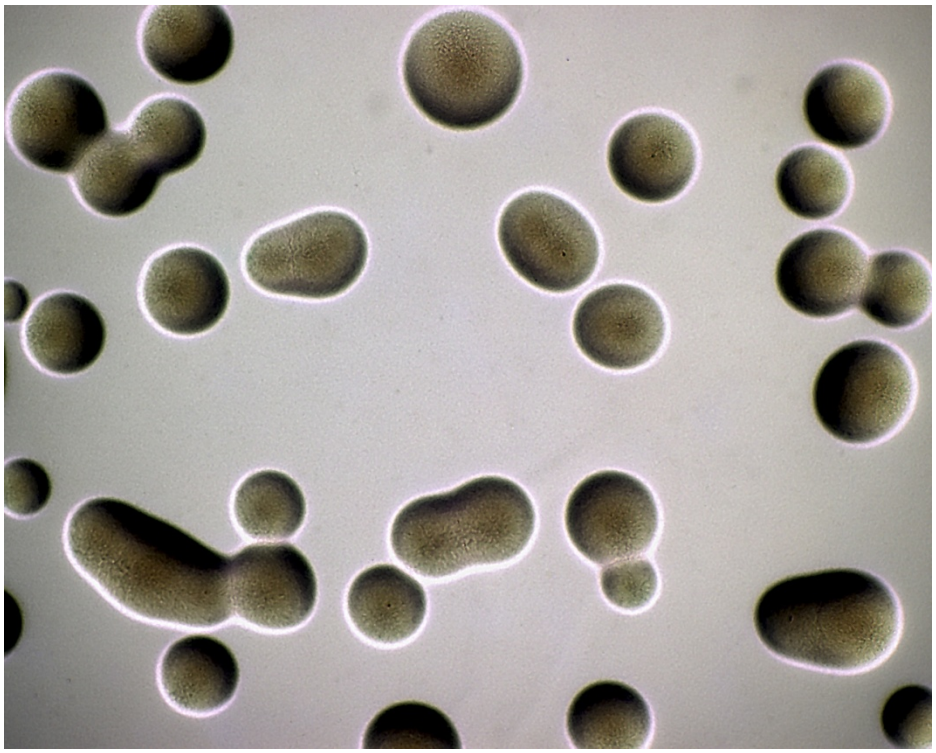


Fig. 2. Amplification of bacterial DNA for 18 *X. fastidiosa* isolates using primers 272-1-ext and 272-2-ext. Two marginal lanes contain 100-bp ladder DNA, center lanes from left to right are control, NJPE1, NJRE1, NJRT1, NJPW1, NJPW2, NJPW3, NJPC1, NJPC2, NJRC1, NJRC2, NJRR1, NJRR2, NJRR3, NJPA1, NJPA2, NJPA3, NJPD1.

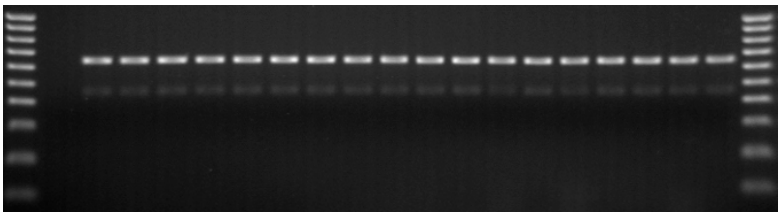


Fig. 3. DNA sequence of 272-int PCR product, hypothetical protein gene, comparing the New Jersey strain of *Xylella fastidiosa* to other strains available in GenBank (EU334023 = NJRR1 red oak NJ, AF344191 = Found 5 citrus BR, AF344190 = Found 4 coffee BR, AY196792 = JB-USN Japanese Beech MD, EF109936 = NM 1 chitalpa NM, AE009442 = Temecula1 grape CA, AE003849 = 9a5c sweet orange BR).

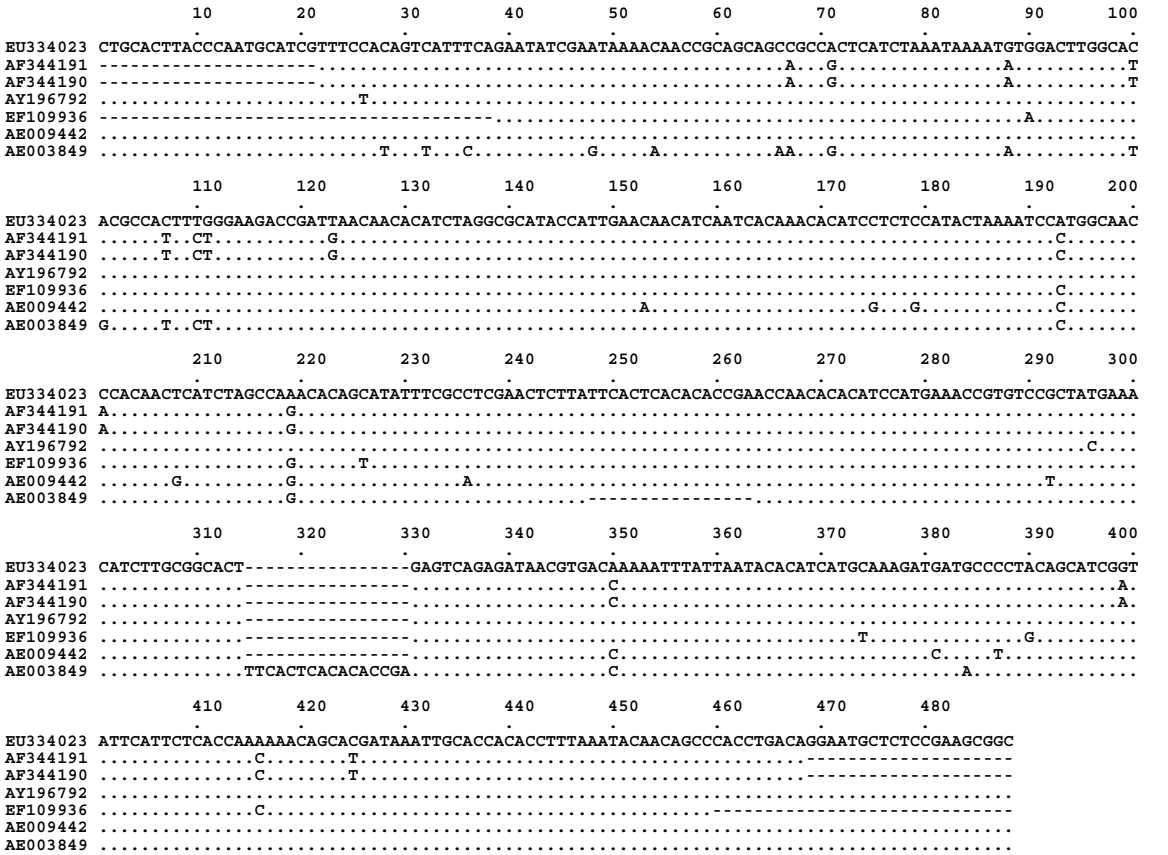


Fig. 4. DNA sequence of RST31/33 PCR product, RNA polymerase sigma-70 factor gene, comparing the New Jersey strain of *X. fastidiosa* to other strains available in GenBank (EU334023 = NJRR1 red oak NJ, AE009442 = Temecula1 grape CA, AE003849 = 9a5c sweet orange BR, AY039798 = citrus BR).

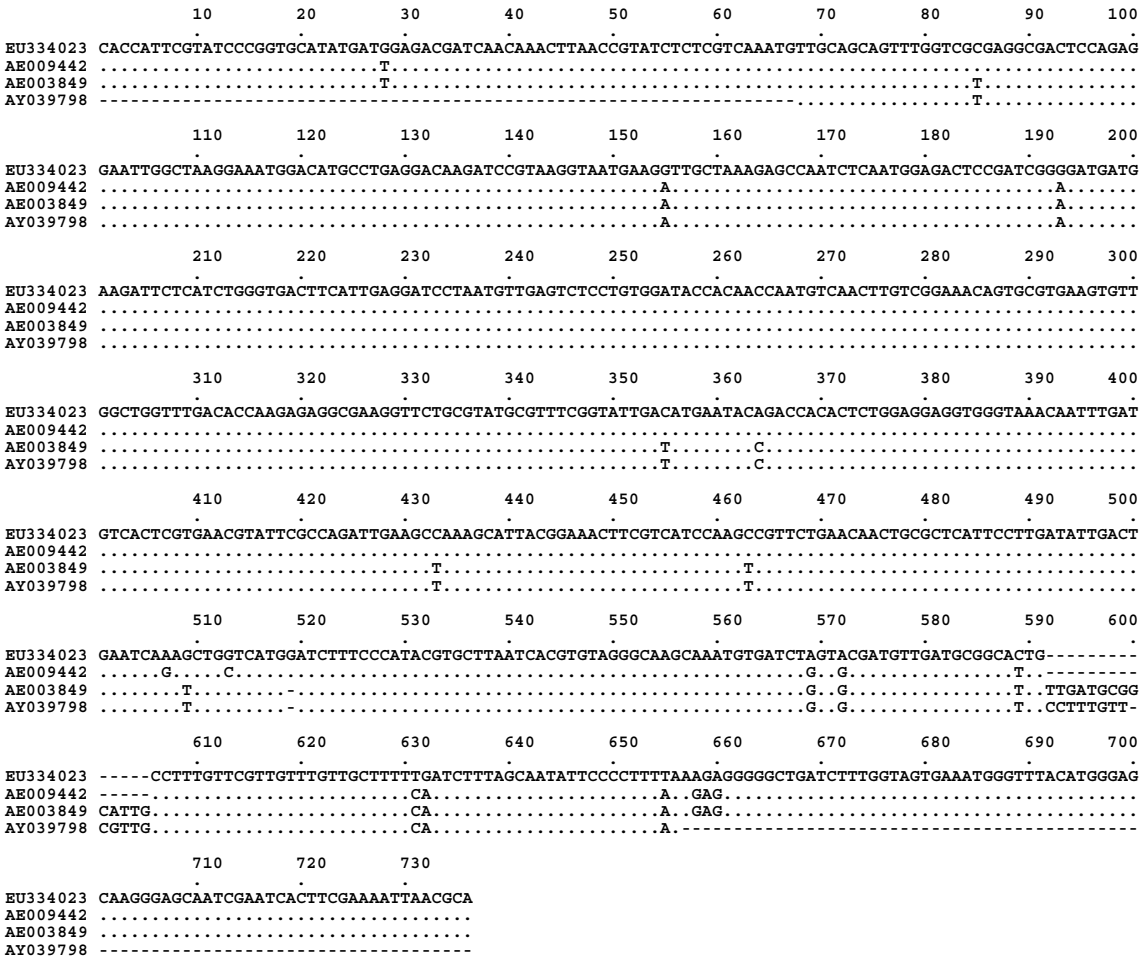
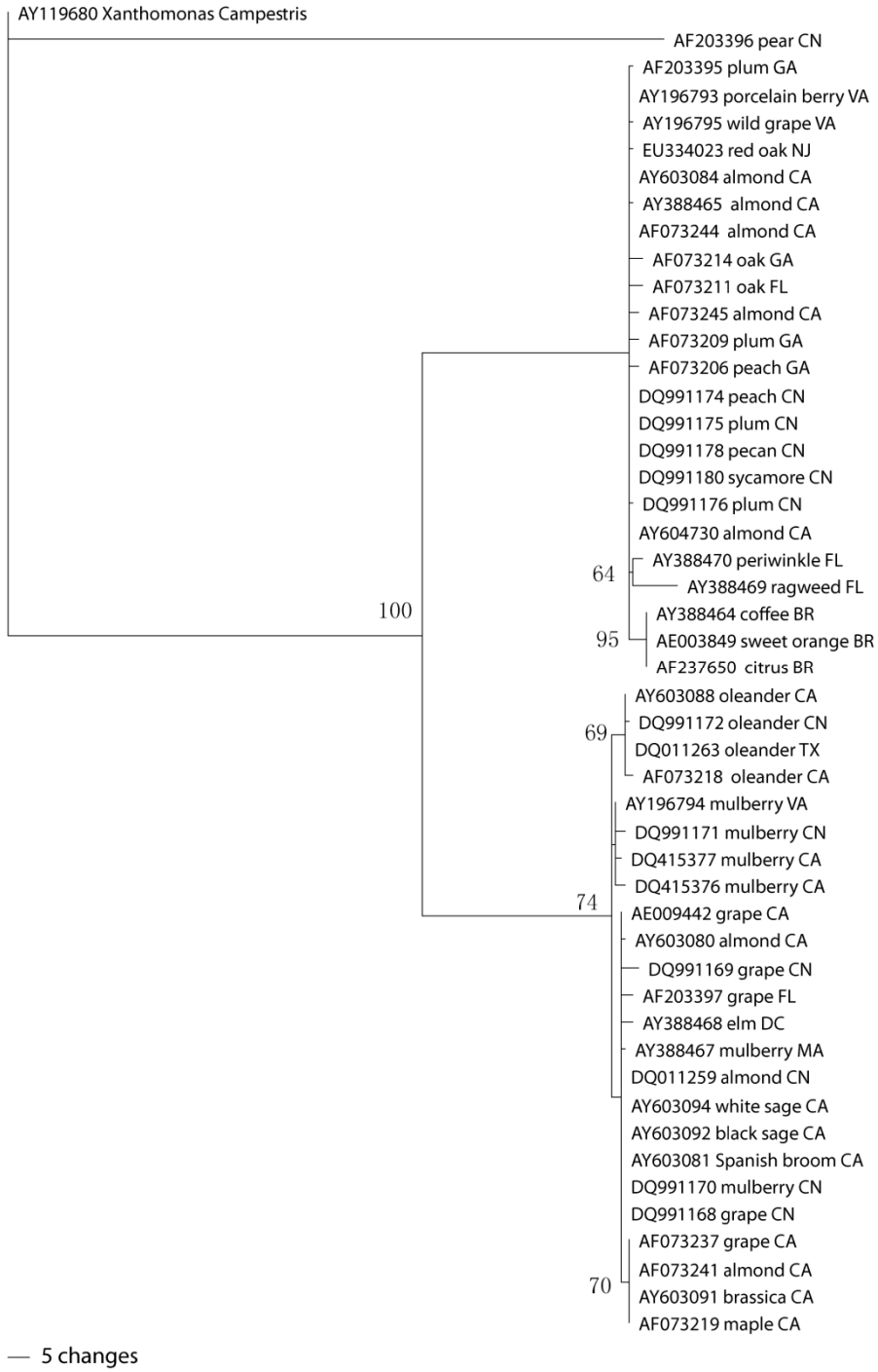


Fig. 5. Phylogeny constructed by the maximum parsimony method, based on 16S-23S spacer sequence data of *Xylella fastidiosa* strains with the pear strain and *Xanthomonas campestris* species as outgroups.



Appendix 1: Periwinkle Wilt (PW) Medium for *Xylella fastidiosa*

1) Phytone peptone 4.0 g

2) Trypticase peptone 1.0 g

3) KH_2PO_4 1.0 g

4) K_2HPO_4 1.2 g

5) $MgSO_4.7H_2O$ 0.4 g

6) Hemin chloride (0.1%)[a] 10 ml

[a] 0.1 g Hemin chloride + 0.2 g NaOH. Add water to a final volume of 100 ml, store at 2-8°C. Store original Sigma bottle of Hemin chloride in desiccator in 2-8°C.

7) Phenol red, sodium salt (0.2%)[b] 10 ml

[b] 0.2 g phenyl red + 10 drops (eye drop) of NaOH (store in desiccator) (10%). Add water to 100 ml.

Add water to a final volume of 940 ml. Adjust pH with 5M NaOH to 7.1 (working pH for *Xylella fastidiosa* is 6.9 – 7.1). Add 1% agar for solid medium. Autoclave the contents in a 2 L flask at 121°C for 30 min. After the medium is cooled to 50°C, add the following two ingredients:

8) Glutamine (4%) Dissolve 4 grams glutamine in 100 ml water with low heat (use heat setting of 3 or 4 on the stir-heat plate). Sterilize by filtering through Nalgene 0.22 µm filter. Store at room temperature. Add 100 ml of 4% glutamine per 940 ml of PW.

9) BSA (10%): Dissolve 6 g BSA in 60 ml water. Sterilize by filtering through Nalgene 0.22 μm filter. Store at room temperature. Add 60 ml of 10% BSA per 940 ml of PW.

Dispense 25 ml of medium per plate.

Appendix 2: Cryopreservation of *Xylella fastidiosa* Bacterial Stocks

The bacterial culture shall be at "healthy mid-log" growth when frozen. i.e. bacteria are at active growing stage. The basic principle of successful cryopreservation is a slow freeze and quick thaw. Although the precise requirement may vary with different cell lines as a general guide cells should be cooled at a rate of –1ºC to –3ºC per minute and thawed quickly by incubation in a 37ºC water bath for 3-5 minutes.

(1) Use PW protocol (do not add agar) to make liquid medium. Store at -4^{o}C.

(2) Make 80% glycerol by adding 20% ddH_2O and 80% glycerol. Autoclave. Store at -4^{o}C.

(3) Place PW liquid medium and 80% glycerol under the hood, warm up to room temperature.

(4) Prepare 4 Wheaton 1.8ml cryotubes for each isolate. Brief label the cap of the tube with cryomarker. Label the side of the tube with complete strain number, including designation. Number of cryotubes is dependent on the future usage of the stock. Each future usage or recovery will need 1 tube to avoid multi-thawing.

(5) Add 500µl PW liquid medium to each tube.

(6) Transfer some bacteria from clean culture plate into each cryrotube. Hand or gental vortex mix well.

Note: For liquid culture, spin 10 ml (for 1 ml cryopreservation volume = 500µl PW liquid medium + 500µl 80% glycerol, 1 ml freezing suspension for every 10ml liquid culture) overnight saturated liquid culture at 2500 rpm for 12 min at 4°C. Discard the supernatant. Then add 500µl PW liquid medium. For agar plates, you transfer bacteria from plate to cryotube with liquid medium. For liquid

culture, you spin liquid culture first, then add liquid medium to bacteria pellet, then re-suspend.

(7) Add 500µl 80% glycerol into each tube. Hand or gentle vortex mix well.

(8) Place all the tubes into a "mini freezing chamber", a container filled with isopropanol (C_3H_7OH) at the bottom. Cover the chamber. Make sure to fill with 100% isopropanol to the desired level. This mini freezing chamber will provide a step down cooling process.

(9) Next day: Transfer the cryotubes from mini freezing chamber to a permanent storage box in -80^oC for long term storage. Place replicate strains in separate freezer boxes.

(10) Segregate one box for emergency purposes only. If possible, store each isolate in 2 different -80^oC freezers in case there is a problem. When the emergency vials are used, make a copy immediately.

(11) Thaw quickly by incubation in a 37ºC water bath for 3-5 minutes. Alternatively, thaw them at room temperature. Make 3 plates without dilution, 20µl/drop.

(12) When isolate is established and complete described, make an ATCC deposition.

Appendix 3: Purification of *Xylella fastidiosa* Genomic DNA from Insect Vector

Qiagen DNeasy Blood and Tissue Kit, catalog number 69506 for 250 samples.

Specimen Preparation:

Place two 5mm glass beads in each 2.0 ml conical screw cap microtube, cat.# 522-S-Q, BioSpec Products, Inc.. Leave the tube capes loosely, not tight. Autoclave.

Carefully removing leafhopper/treehopper eyes, wings, legs, and abdomen under microscope. If it is too sticky with the sticky card, you may want to dip the forceps in 95% ETOH before picking up the insect so that it's easy to transfer the specimen into the 2 ml tube.

Place specimen into 2.0 ml conical screw cap microtube. Store the specimens at -20^{o}C before DNA extraction.

Place the specimen tube in a wire holder and submerge it in liquid nitrogen for about 20 seconds till it doesn't make any more bubbling noise.

Place the tube on to arm holder of the Mini-BeadBeater-1 (Cat. No. 3110BX, BioSpec Products, Inc.). Grind the insect tissue on the machine at the speed of 2500 rpm (25 on display) for 20 seconds. If there is more big pieces, repeat this step again to make sure a fine powder is achieved.

1. Add 180 ATL buffer immediately after each grinding so that DNAase will not be released to avoid DNA degrading. Shake well for 1 minute after it warms up from liquid nitrogen.

Spin at 5000 rpm briefly to settle down the solution. Do not spin too long, otherwise pellet will form. If pellet is formed, use the vortex to break it down.

2. Add 20 µl proteinase K, mix by light vortexing, and incubate at 55°C in water bath for 2 hours. Tap the tube occasionally (every 30 min.) during incubation to disperse the sample.

3. Mixing by tapping. Add 200 µl Buffer AL to the sample, mix each tube immediately and thoroughly by brief vortexing or pipetting, and incubate at 70°C for 10 min. It is essential that the sample and Buffer AL are mixed immediately and thoroughly by vortexing or pipetting to yield a homogeneous solution.

4. Add 200 µl ethanol (96–100%) (Pharmco™, USP Ethyl Alcohol) to the sample, and mix thoroughly by brief vortexing. It is important that the sample and the ethanol are mixed thoroughly to yield a homogeneous solution.

It is essential to apply all of the precipitate to the DNeasy Mini spin column.

5. Open the sealed plastic to get the DNeasy Mini spin column and label them according to the above 2 ml specimen tubes.

Pipette the mixture from step 4 into the DNeasy Mini spin column placed in a 2 ml collection tube (it is attached to each column sealed with a plastic cover). Centrifuge at 6000 x *g* (8000 rpm) for 1 min. Liquid will be collected outside the collection tube. Remove each inner column from its collection tube and place the column into a new collection tube (they are in a bag of 50 loose collection tubes). Discard old collection tube with flow-through.

6. Check the Buffer AW1 tube, make sure to add 100% ethanol to dilute the concentrate before you use it.

Add 500 µl Buffer AW1, and centrifuge for 1 min at 6000 x *g* (8000 rpm). Place the DNeasy Mini spin column into a new 2 ml collection tube (tubes are in a bag, if you

don't have enough new collection tubes, you may empty the old tube and reuse that tube again, but don't reuse the collection tube for different sample to avoid sample cross contamination). Discard old collection tube with flow-through.

7. Check the Buffer AW2 tube, make sure to add 100% ethanol to dilute the concentrate before you use it.

Add 500 µl Buffer AW2, and centrifuge for 3 min at 20,000 x *g* (14,000 rpm) to dry the DNeasy membrane. Discard the collection tube together with flow-through. This centrifugation step ensures that no residual ethanol is carried over during the following elution.

Following the centrifugation step, remove the DNeasy Mini spin column carefully so that the column does not come into contact with the flow-through, since this will result in carryover of ethanol. If carryover of ethanol occurs, empty the collection tube and reuse it in another centrifugation step for 1 min at 20,000 x *g* (14,000 rpm).

8. Label (if not done yet) autoclaved 1.5 ml centrifuge tubes according to above sample IDs. Place the DNeasy Mini spin column in a clean 1.5 ml centrifuge tube.

Pipette 50 µl Buffer AE directly onto the DNeasy membrane. Incubate at room temperature for 15 min, and then centrifuge the 1.5 ml centrifuge tube with column in it for 1 min at 6000 x *g* (8000 rpm) to elute the DNA.

9. Repeat elution once as described in step 8: add another 50 µl to the membrane, incubate 15 min. Then centrifuge 1 min. The total DNA is 100 µl.

Appendix 4: *Xylella fastidiosa* 16S-23S PCR Log sheet

Date: _____ Number of reactions (tubes): 10

PCR Start time: _____ End time:_____

Gel starting time: _____ Gel ending time:_____

	Volume ratio	MasterMix (µl)	lot number
10X PCR Buffer II	1.00	10.70	
25 mM $MgCl_2$	1.00	10.70	
10 mM dNTPs (each)	0.20	2.14	
5 µM upper-Primer (G1)	0.50	5.35	
5 µM lower-Primer (L1)	0.50	5.35	
HPLC ddH_2O	1.75	18.73	
Tag polymerase (5u/µl)	0.05	0.54	
Total volume (µl)	5.00	53.50	

Lane Number	Sample ID	Sample Description	Sample Volume (µl)	Master Mix (µl)	10X Loading buffer (µl)	Present of Fragment	Note
1			5	5	1		
2			5	5	1		
3			5	5	1		
4			5	5	1		
5			5	5	1		
6			5	5	1		
7			5	5	1		
8			5	5	1		
9			5	5	1		
10			5	5	1		
11			5	5	1		
12			5	5	1		
13			5	5	1		
14			5	5	1		
15			5	5	1		
16			5	5	1		
17			5	5	1		
18			5	5	1		
19			5	5	1		
20			5	5	1		

Appendix 5: *Xylella fastidiosa* isolate NJRR1 16S-23S ribosomal RNA intergenic spacer, partial sequence; and tRNA-Ala and tRNA-Ile genes, complete sequence.

1: EU334023. Reports Xylella fastidiosa [gi:164415472]

```
LOCUS       EU334023                553 bp    DNA     linear   BCT 07-JAN-2008
DEFINITION  Xylella fastidiosa isolate NJRR1 16S-23S ribosomal RNA intergenic
            spacer, partial sequence; and tRNA-Ala and tRNA-Ile genes, complete
            sequence.
ACCESSION   EU334023
VERSION     EU334023.1  GI:164415472
KEYWORDS    .
SOURCE      Xylella fastidiosa
  ORGANISM  Xylella fastidiosa
            Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales;
            Xanthomonadaceae; Xylella.
REFERENCE   1  (bases 1 to 553)
  AUTHORS   Zhang,J., Gould,A.B., Lashomb,J.H., Huang,Q.,
            Staniszewska-Goraczniak,H., Goraczniak,R. and Kjer,K.
  TITLE     Characterization of Xylella fastidiosa strain isolated from oak and
            insect vectors in New Jersey
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 553)
  AUTHORS   Zhang,J., Gould,A.B., Lashomb,J.H., Huang,Q.,
            Staniszewska-Goraczniak,H., Goraczniak,R. and Kjer,K.
  TITLE     Direct Submission
  JOURNAL   Submitted (10-DEC-2007) Entomology Department, Rutgers University,
            93 Lipman Dr., New Brunswick, NJ 08901, USA
FEATURES             Location/Qualifiers
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                     /country="USA"
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     tRNA            141..212
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     tRNA            229..302
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      301 ggttcgagtc ctcccagacc caccaatgtt atatcaatta ttctgaatgt ggtttgcgca
      361 ttttttatgc ttattagcct tggagctgtg aagcgttctt ttataatttg atgatgtagc
      421 aagcgtttga acttttttatt aataatttct cattggaagc cttaagtgac aatgtttatc
      481 cattgtcttg tagattttga ggcgactttg ggttatatgg tcaagcgaat aagcgcacac
      541 ggtggatgcc ttg
//
```

Appendix 6: *Xylella fastidiosa* isolate NJRR1 hypothetical protein gene, complete cds.

1: EU334042. Reports Xylella fastidiosa [gi:164415502]

```
LOCUS       EU334042                472 bp    DNA     linear   BCT 07-JAN-2008
DEFINITION  Xylella fastidiosa isolate NJRR1 hypothetical protein gene,
            complete cds.
ACCESSION   EU334042
VERSION     EU334042.1  GI:164415502
KEYWORDS    .
SOURCE      Xylella fastidiosa
  ORGANISM  Xylella fastidiosa
            Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales;
            Xanthomonadaceae; Xylella.
REFERENCE   1  (bases 1 to 472)
  AUTHORS   Zhang,J., Gould,A.B., Lashomb,J.H., Huang,Q.,
            Staniszewska-Goraczniak,H., Goraczniak,R. and Kjer,K.
  TITLE     Characterization of Xylella fastidiosa strain isolated from oak and
            insect vectors in New Jersey
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 472)
  AUTHORS   Zhang,J., Gould,A.B., Lashomb,J.H., Huang,Q.,
            Staniszewska-Goraczniak,H., Goraczniak,R. and Kjer,K.
  TITLE     Direct Submission
  JOURNAL   Submitted (10-DEC-2007) Entomology Department, Rutgers University,
            93 Lipman Dr., New Brunswick, NJ 08901, USA
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Appendix 7: *Xylella fastidiosa* isolate NJRR1 hypothetical protein gene, complete cds.

1: EU334061. Reports Xylella fastidiosa [gi:164415540]

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DEFINITION  Xylella fastidiosa isolate NJRR1 hypothetical protein gene,
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ACCESSION   EU334061
VERSION     EU334061.1  GI:164415540
KEYWORDS    .
SOURCE      Xylella fastidiosa
  ORGANISM  Xylella fastidiosa
            Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales;
            Xanthomonadaceae; Xylella.
REFERENCE   1  (bases 1 to 649)
  AUTHORS   Zhang,J., Gould,A.B., Lashomb,J.H., Huang,Q.,
            Staniszewska-Goraczniak,H., Goraczniak,R. and Kjer,K.
  TITLE     Characterization of Xylella fastidiosa strain isolated from oak and
            insect vectors in New Jersey
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 649)
  AUTHORS   Zhang,J., Gould,A.B., Lashomb,J.H., Huang,Q.,
            Staniszewska-Goraczniak,H., Goraczniak,R. and Kjer,K.
  TITLE     Direct Submission
  JOURNAL   Submitted (10-DEC-2007) Entomology Department, Rutgers University,
            93 Lipman Dr., New Brunswick, NJ 08901, USA
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Appendix 8: *Xylella fastidiosa* isolate NJRR1 RNA polymerase sigma-70 factor gene, partial cds.

1: EU334080. Reports Xylella fastidiosa [gi:164415578]

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LOCUS       EU334080                722 bp    DNA     linear   BCT 07-JAN-2008
DEFINITION  Xylella fastidiosa isolate NJRR1 RNA polymerase sigma-70 factor
            gene, partial cds.
ACCESSION   EU334080
VERSION     EU334080.1  GI:164415578
KEYWORDS    .
SOURCE      Xylella fastidiosa
  ORGANISM  Xylella fastidiosa
            Bacteria; Proteobacteria; Gammaproteobacteria; Xanthomonadales;
            Xanthomonadaceae; Xylella.
REFERENCE   1  (bases 1 to 722)
  AUTHORS   Zhang,J., Gould,A.B., Lashomb,J.H., Huang,Q.,
            Staniszewska-Goraczniak,H., Goraczniak,R. and Kjer,K.
  TITLE     Characterization of Xylella fastidiosa strain isolated from oak and
            insect vectors in New Jersey
  JOURNAL   Unpublished
REFERENCE   2  (bases 1 to 722)
  AUTHORS   Zhang,J., Gould,A.B., Lashomb,J.H., Huang,Q.,
            Staniszewska-Goraczniak,H., Goraczniak,R. and Kjer,K.
  TITLE     Direct Submission
  JOURNAL   Submitted (10-DEC-2007) Entomology Department, Rutgers University,
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Chapter 3

Relationship between Cicadomorpha Insect Populations and Bacterial Leaf Scorch of Oak in Central New Jersey

ABSTRACT

Oak leaf scorch is a disease caused by the bacterium *Xylella fastidiosa* Wells et al. and is transmitted by insect vectors. Potential insect vector population biology in pin and red oaks was determined by placing yellow sticky card traps in tree canopies and fogging with heated pyrethrin insecticide at four sites in Mercer, Middlesex, and Somerset Counties in New Jersey during 2002-2006. Thirty-seven Cicadomorpha (Suborder Auchenorrhyncha, Order Hemiptera) insect species were collected from 20 genera in the families Cicadellidae, Membracidae, Aphrophoridae, and Clastopteridae. Of the 12,880 potential vectors collected from sticky traps and fogging, 91.40% were from 25 Membracidae treehopper species, 6.93% were from six Cicadellinae, Cicadellidae leafhopper species, and 1.67% belonged to six Aphrophoridae and Clastopteridae spittlebug species.

Fogging collected more insect species and individuals compared to sticky card collections done on a similar time and space sampling scales. Sticky card sampling at a greater frequency and a larger number of locations provided similar community structure information as that from fogging. Sampling efficiency varied with insect behavior and distribution in oak canopies. Sticky card collections of the dominant treehopper species *Ophiderma definita* Woodruff was male biased when females were gravid. *Ophiderma definita* population peaked in early June and comprised 68.18% of the total collection.

Graphocephala versuta (Say), a proven vector of *X. fastidiosa* in grapes, peaked in mid-July and was the second most dominant species this study, comprising 6.2% of the total collection. Insect populations varied between years and oak species. *Ophiderma definita* was more abundant in pin oaks than red oaks. Insect population abundance and species composition were affected by plant community structure and the presence of leaf scorch disease. Higher Cicadormorpha populations were observed in asymptomatic oak canopies than in the neighboring *X. fastidiosa* infected oaks.

Individual insect specimens collected from oaks were subjected to a *Xylella fastidiosa* DNA screening by PCR amplification with 272-int/ext primers and confirmed using additional PCR with G1/L1 and RST31/33 primers. *Xylella fastidiosa* DNA was detected in 21 of 32 tested insect species of the Aphrophoridae, Clastopteridae, Cicadellidae, and Membracidae. The nucleotide sequences of the PCR products obtained from these insects were identical to those obtained from host oaks. The average *X. fastidiosa* DNA positive rate was 13.89% for all specimens tested. Membracidae comprised 91.40% of the total potential vector, of which 11.03% tested positive for *X. fastidiosa* DNA. Most of the treehoppers were from *Ophiderma* or *Telamona*. Seventy-five percent (75.31%) of the Membracidae collected were *O. definita*, of which 15.49% tested DNA positive for *X. fastidiosa*. Live *X. fastidiosa* bacteria were isolated from this treehopper species. *Xylella fastidiosa* DNA was also detected in 10 other treehopper species: *Archasia belfragei* (Stål), *Enchenopa binota* (Say), *Cyrtolobus discoidalis* (Emmons), *C. fenestratus* (Fitch), *Glossonotus acuminatus* (Fabricius), *Microcentrus perditus* (Amyot & Serville), *Similia fasciata* (Amyot & Serville), *Telamona extrema* Ball, *T. monticola* (Fabricius), and *T. tiliae* Ball.

Six species of Cicadellidae were collected from pin and red oaks. *Graphocephala versuta* was the dominant species comprising 91.58% of all leafhoppers collected. *Xylella fastidiosa* DNA was detected in all six species: *Aulacizes irrorata* (Fabricius), *Graphocephala coccinea* (Forster), *G. versuta*, *Draeculacephala anguifera* (Walker), *D. portola* Ball, and *Oncometopia orbona* (Fabricius). Nineteen percent (18.79%), mostly *G. versuta,* carried *X. fastidiosa* DNA.

Xylella fastidiosa DNA was also detected from spittlebugs. Five spittlebug species in the family Aphrophoridae were collected from pin and red oaks. *Aphrophora quadrinotata* Say*, Philaenus pallidus* (L.)*,* and *P. spumarius* (L.) tested DNA positive for *X. fastidiosa*. The spittlebug *Clastoptera obtusa* (Say) was the only Clastopteridae species collected this study and tested DNA positive for *X. fastidiosa*. Fifteen percent (15.08%) of the spittlebugs tested were positive for the presence of the bacterium DNA.

Key words: *Xylella fastidiosa*, Cicadellidae, Membracidae, Aphrophoridae, and Clastopteridae, vector

INTRODUCTION

Xylella fastidiosa Wells et al. is a gram negative bacterium limited to the plant xylem vessels, a unique, nutritionally dilute habitat (Press and Whittaker 1993). It belongs to the Phylum Proteobacteria, Class Proteobacteria Gamma, Order Xanthomonadales, Family Xanthomonadaceae. *Xylella fastidiosa* cells are small (0.25-0.5 x 1.0-4.0 μm), stain gram negative, and have no flagella. They do not form a spore, have a thick rippled cell wall, and do not grow on conventional bacteriological media (Wells et al. 1987). The bacterium is transmitted to new host plants during xylem sap feeding by Hemipteran insect vectors (Purcell and Hopkins 1996). *Xylella fastidiosa* multiplies and spreads from the site of infection to colonize the xylem, a water transport network of vessels composed of dead, lignified cells. Vessels are interconnected by channels, called bordered pits, which allow the passage of xylem sap but block the passage of larger objects due to the presence of a pit membrane (Tyree and Zimmermann 2002). *Xylella fastidiosa* can pass through the pit by causing the degradation of the membrane (Newman et al. 2003). Bacterial cells attach to the vessel wall and multiply, forming biofilm-like colonies that can, when sufficiently large, completely occlude xylem vessels and block water transport (Tyson et al. 1985).

Xylella fastidiosa is the causal agent of oak bacterial leaf scorch (BLS) (Hearon et al. 1980) as well as many other plant diseases such as almond leaf scorch (Mircetich et al. 1976), citrus variegated chlorosis (CVC) (Lee et al. 1993), grape Pierce's disease (PD) (Hopkins and Mollenhauer 1973), maple leaf scorch (Sherald et al. 1987), oleander leaf scorch (Huang et al. 2003), periwinkle wilt (McCoy et al. 1978), phony peach disease (PPD) (Hopkins et al. 1973), plum leaf scald (Kitajima et al. 1975), and sycamore leaf

scorch (Sherald et al. 1983). Spittlebugs and sharpshooter leafhoppers are known *X. fastidiosa* vectors of the almond leaf scorch (Purcell 1980a), CVC (Brlansky et al. 1996, Krugner et al. 2000), PPD (Turner and Pollard 1959), periwinkle wilt (McCoy et al. 1978), PD (Severin 1949, 1950, Purcell 1980b, Adlerz and Hopkins 1979, Almeida and Purcell 2003), oleander leaf scorch (Costa et al. 2000), and possibly BLS of shade trees (oak and elm) (J. Bentz, personal communication). Sharpshooters and spittlebugs usually are larger in size (Novotny and Wilson 1997) with the ability to feed from negatively pressured xylem fluid. Little is known about the insect vectors of bacterial leaf scorch, especially in oaks in the northeastern United State.

Northern red oak *Quercus rubra* L. and pin oak *Q. palustris* Muenchh are major shade trees in New Jersey and are susceptible to *X. fastidiosa* infection. During the past 20 years, bacterial leaf scorch disease of oak has spread throughout the state with infections being observed in every county (Gould et al. unpublished data). Although, live bacterium and DNA of *X. fastidiosa* have been detected in many xylophage insects (Zhang et al. unpublished data), this seasonal appearance and relationship to the disease is yet to be determined. This study was done to determine which insect species harbored *X. fastidiosa* and the infection rate for each when collected from the pin and red oak species in New Jersey.

Knowledge of seasonal history through appropriate sampling of *X. fastidiosa* vectors can provide important insights about disease progression. Fogging has been used to study arthropods within tropical forests (Basset et al. 2003) and insect food web structures (Barbosa et al. 2000). Stork and Hammond (1997) compared various sampling

techniques from tree crowns and found that fogging the canopy with a knockdown insecticide provided the most accurate insect population estimates in oak canopies.

The sticky-trap method is a relative estimator of insect populations and is widely used in agricultural ecosystems (Southwood and Henderson 2000), as well as forest arthropod studies (Basset et al. 1997). Sticky card sampling is inexpensive, flexible in time and space, enables a large number of replicates to be taken, and is highly suitable for the study of insect spatial distributions and stratification. A "complete" sampling of the insect vector population in the oak canopy is essential to understanding to the oak bacterial leaf scorch transmission. We used both fogging and extensive sticky card sampling to investigate the Cicadomorpha insect seasonality and community structure, and the degree of *X. fastidiosa* infection in insects in various landscapes containing oak plantings.

MATERIALS AND METHODS

Sampling of insects by use of yellow sticky traps placed in the oak canopy.

Sample sites: This study was conducted at four sites in central New Jersey, based on their oak species and landscape setting.

Site 1: Duke Farms is located in Hillsborough, New Jersey with 1,100 ha of farmland and landscaped gardens. Pin oak trees (*Quercus palustris* Muenchh) were planted and maintained in double row alleys lining the streets with a height of 35 m and a mean crown diameter of 13 m. Twenty-five pin oaks on the north side of the road were used for sticky card trap sampling during 2002-2004.

Site 2: Mercer County Community College (MCCC) is located in West Windsor, New Jersey. Pin oak is the major shade tree lining the college's parking lots. Pin oaks planted in rows around student parking lots were uniform in size with an average height of 14 m and diameter of 10 m. Forty pin oaks outlining parking lot 1 (2.5 ha) were sampled using yellow sticky traps.

Site 3: Riverview Cemetery is located on the east bank of the Delaware River, in Trenton, New Jersey. It is a 20 ha historical cemetery founded in 1699 and is shaded by various trees. Eight northern red oaks (*Quercus rubra* L.) and five pin oaks randomly planted with other shade trees at the southwest section of the cemetery were sampled by sticky card traps during 2003, 2004, and 2006. The sampled oaks had an average height of 26 m and an average of 23 m in crown diameter.

Site 4: Rutgers Garden is a botanical collection located in North Brunswick, New Jersey and is estimated to be 20 ha. Northern red oaks are the major shade trees lining

the roads. Average oak tree height was 22 m with a 12 m crown diameter. Twenty red oak trees were used for sticky card trap sampling during 2002-2004.

Sampling procedure: All sampled oaks were identified as either symptomatic for bacterial leaf scorch (BLS) or asymptomatic. BLS infected symptomatic oaks were identified if over 25% of branches showed dieback in May and tested positive for *X. fastidiosa* by ELISA using an Agdia "DAS ELISA for *Xylella fastidiosa"* kit (Elkhart, IN) in September. Asymptomatic trees were classified as such if they had less than 5% branch dieback in May and *X. fastidiosa* was undetectable in September using ELISA tests.

In 2004 and 2006, 20 symptomatic and 20 asymptomatic pin oaks from MCCC were sampled with yellow Sticky Strips™ Insect Traps (7.5 cm x 12.5 cm, Olson Products, Medina, OH). Three sticky traps were placed 120 degrees apart, 30 cm from the edge of each pin oak canopy. During 2003 and 2004, 2-7 sticky traps, depending on the tree size, were placed in 25 pin oak trees at Duke Farms and 20 northern red oak trees at Rutgers Gardens. Two to seven sticky trap cards were placed in 8 northern red oaks and 5 pin oaks in Riverview Cemetery during 2003, 2004, and 2006. Different numbers of sticky cards per tree at these sites were used to obtain an approximately uniform sample volume/coverage for each card. Because the canopy at Duke Farms and Rutgers Gardens was too high to reach with a step ladder, sticky traps at these two sites were placed in the canopy using a bucket truck during 2002, 2003, and 2004 respectively. A sticky card was attached to 2 clips, one at the top and one at the bottom. Both clips were attached to free spinning fishing swivels. The bottom swivel was fastened to a 2 oz fishing weight while the top swivel was fastened to a fishing line that passed through

several screw eyes placed directly above the trap and along the branch towards the main trunk, in order to hold the line tightly against the tree, thus avoiding line snags. The other end of the fishing line was connected to a fishing leader which was hooked onto a screw eye placed on the trunk of the tree at eye level. A fishing line from a spinning reel was hooked to the leader of the fishing line on the oak trunk when the sticky card was replaced. The sticky card trap could then be raised and lowered by reeling the fishing line while standing on the ground. All the sticky cards were replaced weekly from May to August each sample year. The cards were stored in the freezer at -20^{o}C. All Cicadamorpha insects were carefully removed from sticky cards and were placed in 0.6 ml or 1.5 ml centrifuge tube according to the specimen size and stored at -20^{o}C for species identification, sex determination, and *X. fastidiosa* DNA detection. For those that could not be easily removed, a razor blade was used to cut around the insect, yielding a very small piece of sticky card with the insect still attached.

Insect sampling by fogging pin oak canopy.

Fogging was carried out on the south side the Lake Road at the Duke Farms and student parking lot 2 (2 ha) at MCCC described above to obtain an absolute insect population estimate of Cicadomorpha insects harboring *X. fastidiosa*. Pin oaks were divided into symptomatic, possible *X. fastidiosa* infection (more than 25% of branch were dead in May) and asymptomatic, healthy without an infection (less than 5% branch were dead in May). All sampled trees were tested for the presence of *X. fastidiosa* in September by using ELISA tests. None of the experimental trees had been sampled for insects prior to this study. On three different dates, a set of three BLS infected pin oaks

and three asymptomatic pin oaks were fogged (June 16, July 22, and August 23, 2004). Fogging was done 4 times in 2006: May 24, June 21, July 24, and August 23, 2006.

Equipment and material: A gas generated DYNA-FOG Golden Eagle fogger, model 2610E, series 3 (Curtis DYNA-FOG® Ltd., Westfield, IN) that contained one part Pyrethrin (3%) (Prentox® Pyronyl™ oil concentrate, Prentiss Incorporated, Floral Park, NY) mixed with two parts of a low odor base oil (Rockland® Corporation, Roanoke, VA).

Procedure: All fog sampling was conducted between 6:00-7:00 AM EDT prior to onset of air turbulence. Plastic drop sheets were placed under the canopy of each oak to be sampled. At MCCC, where pin oaks were less than 14 m high, each tree was fogged while standing on the ground until fog enveloped each tree canopy. At Duke Farms, oak trees approximately 35 m tall were fogged from a bucket truck. The bucket was extended to the canopy level and the fog was released until it enveloped the canopy. In all cases, insects began dropping from the canopy about 10 minutes after fogging had ceased and were collected from the drop sheets 15 minutes after fogging and continued for 90 minutes. Preliminary studies showed that no more insects were dropping by then. All the specimens were kept in freezer at -20^{o}C until species identification and *X. fastidiosa* DNA detection was done.

Insect specimen identification All potential *X. fastidiosa* vectors inhabiting pin and red oak canopy i.e., larger leafhoppers, treehoppers, and spittlebugs in the Order Hemiptera: Infraorder Cicadamorpha were identified and analyzed this study.

Taxonomic terminology and phylogenetic systems to the family level used in this study are from Cryan (2005) and Dietrich (2005). Species identification was based on

literature available for identifying leafhoppers: DeLong 1948, Nielson 1968, Dietrich 2005; treehoppers: Osborn 1940, Dennis 1952, 1965, Kopp and Yonke 1973a, 1973b, 1973c, 1974, Deitz 1975, Dietrich et al. 2001, Cryan et al. 2004; and spittlebugs: Doering 1930, 1941, Hanna and Moore 1966, Hamilton 1982, Dietrich 2005. Pinned voucher specimens of each insect species collected during this study are deposited at the Insect Museum, Department of Entomology, Rutgers University, New Brunswick, New Jersey.

***Xylella fastidiosa* DNA detection of insects**

Each individual insect was dissected to remove eyes, legs, abdomen, and wings under Stereo Star (Reichert Scientific Instruments) dissecting scope to prevent potential PCR inhibition in the following procedures. We placed each specimen in 2 ml screw top sterilized tubes preloaded with two 5 mm glass beads. We then submerged the tube in liquid nitrogen for 20 seconds; and ground the specimen in the Mini-bead Beater (BioSpec Products, Inc., Bartlesville, OK) for 20 seconds at low speed (2500 rpm) (Bextine 2004). *X. fastidiosa* DNA was immediately extracted with Qiagen DNeasy Blood and Tissue Kit with slight modification of the extraction procedure. Briefly, we added 180µl ATL buffer immediately after grinding, shook well 1 minute later when the tube was thawed from liquid nitrogen. We centrifuged tube briefly to bring down the solution to avoid cross contamination. Then 20 µl proteinase K was added to the tube, mixed by vortex-mixing, and incubated at 55°C in a water bath for 2 hours. We tapped the tube every 30 min. during incubation to disperse the sample. After incubation 200 µl Buffer AL was added to the sample, each tube was mixed immediately by brief vortex,

and incubated at 70°C for 10 min. DNA was eluted with 50 µl AE buffer after incubation of the membrane for 15 min. Elution was repeated once.

PCR: A nested PCR procedure (Pooler and Hartung 1995) was employed to detect *X. fastidiosa* presence from the insect samples. The first round of the nested PCR was performed by adding 5 µl of the extracted DNA to 15 µl of PCR master mix containing the following components: 1X PCR buffer, 45 nmol $MgCl_2$, 6 nmol dNTP, 7.5 pmol of each external primer 272-1-ext (5'AGCGGGCCAATATTCAATTGC3') and 272-2-ext (5'-AGCGGGCCAAAACGATGCGTG3') (Pooler and Hartung 1995), and 0.6 unit of Taq polymerase (Qiagen Inc., Valencia, CA). For the second round of the PCR, 2 µl of the first round PCR reaction mixture was added to 18 µl of the PCR master mix containing 1X PCR buffer, 27 nmol $MgCl_2$, 4 nmol dNTP, 10 pmol of each internal primer 272-1-int (5'-CTGCACTTACCCAATGCATCG-3') and 272-2-int (5'-GCCGCTTCGGAGAGCATTCCT-3') (Pooler and Hartung 1995), and 0.4 unit of Taq polymerase (Qiagen Inc., Valencia, CA). Amplification of the nested PCR was carried out in a Biometra UNO-Thermoblock (Biometra, Goettingen, Germany) using the following profile: 94^{o}C for 5 min., followed by 35 cycles of 94^{o}C for 30 seconds, 60^{o}C for 30 seconds, and 72^{o}C for 45 seconds. After a final extension at 72^{o}C for 7 min, reaction was held at 4^{o}C. PCR products were analyzed by 0.9% agarose gel electrophoresis, stained with SYBR Safe^{TM} DNA gel stain by adding 10 µl of 10,000X concentrate in DMSO (Molecular Probes, Inc., Eugene, OR) per 100 ml gel solution right before pouring and the image was captured with Kodak Digital Science 1D LE software, version 3.02 with Kodak Electrophoresis Documentation and Analysis System 120 digital camera over a 312 nm UV transilluminator FBT1 88 (Fisher Biotech, Subiaco, WA).

The gel image was stored as a TIFF format file and processed with Adobe Photoshop CS2, version 9.02 (Adobe 2005).

Xylella fastidiosa DNA samples were also amplified with two other primer sets for further confirmation with the same equipment previously described for the nested PCR procedure using their own master mixes and PCR profiles. A 722-bp region of *X. fastidiosa* genome was amplified with the primer set RST31/RST33 (Minsavage et al. 1994) by adding 5 µl of the extracted DNA to the 15 µl of PCR master mix containing the following components: 1X PCR buffer, 45 nmol $MgCl_2$, 6 nmol dNTP, 10 pmol of each primer RST31 (5'-GCGTTAATTTTCGAAGTGATTCGATTGC-3') and RST33 (5'-CACCATTCGTATCCCGGTG-3'), and 0.6 unit of *Taq* polymerase (Qiagen Inc., Valencia, CA). PCR was carried out using the following profile: 95^{o}C for 5 min., followed by 40 cycles of 95^{o}C for 30 seconds, 55^{o}C for 30 seconds, and 72^{o}C for 45 seconds. After a final extension at 72^{o}C for 5 min, reaction was held at 4^{o}C.

The 16S-23S rRNA intergenic spacer regions of *X. fastidiosa* isolates were PCR amplified with primers G1 and L1 (Hendson et al. 2001, Mehta and Rosato 2001). The primer sequence G1 (5'-GAAGTCGTAACAAGG-3') is located at the 3' end of the 16S rRNA gene 30 to 40 nucleotides upstream from the 16S-23S intervening space region, and primer L1 (5'-CAAGGCATCCACCGT-3') is located at the 5' end of the 23S rRNA gene, about 20 bases downstream from the spacer boundary (Jensen et al. 1993). A 553-bp spacer region was PCR amplified by adding 5 µl of the extracted DNA to the 15 µl of PCR master mix containing the following components: 1X PCR buffer, 45 nmol $MgCl_2$, 6 nmol dNTP, 7.5 pmol of each primer G1 and L1, and 0.6 unit of *Taq* polymerase (Qiagen Inc., Valencia, CA). PCR was carried out using the following profile: 94^{o}C for 5

min., followed by 35 cycles of 94°C for 40 seconds, 55°C for 1 min., and 72°C for 2 min. After a final extension at 72°C for 10 min, reaction was held at 4°C.

***Xylella fastidiosa* DNA sequencing and species identity:** *Xylella fastidiosa* PCR products were purified with QIAquick gel extraction kit (Qiagen Inc., Valencia, CA) and sent with their corresponding primers for direct PCR product sequencing (GeneWiz, South Plainfield, NJ). The sequences of the PCR products, 472 bp for 272-int primers, 553 bp for 16S-23S intervening spacer region, and 722 bp for RST31/33 primers, were employed as a query sequence for a GenBank database searched by BLASTn (Basic local alignment search tool) (Altschul et al. 1997). Fresh PCR products of each NCBI confirmed *X. fastidiosa* isolates were then cloned into a plasmid vector, pCR®4-TOPO® using the TOPO TA Cloning® Kit (Invitrogen Corporation, Carlsbad, CA). Desired clones were purified with Qiagen's QIAprep spin miniprep kit. Both strands of the inserts were sequenced at the GeneWiz. DNA sequences were analyzed with DNASTAR's Lasergene sequence analysis software (DNASTAR, Inc., Madison, WI) (Burland 2000).

RESULTS

Cicadomorpha species diversity

The potential insect vectors for transmitting *X. fastidiosa* to pin and red oaks collected were from the Order Hemiptera: Suborder Auchenorrhyncha, Infraorder Cicadomorpha. Four families from this group were collected: Aphrophoridae and Clastopteridae from Cercopoidea superfamily, Cicadellidae, and Membracidae from Membracoidea superfamily (Table 1).

Treehoppers: A total of 11,662 treehoppers were collected from sticky cards and fogging during the 4 years of sampling (Table 1). Many were known oak feeders (Wood 1983, 1993). Among them, 15 adult *Platycotis vittata* (Fabricius) collected were in the Hoplophorinae subfamily, 26 adult *Enchenopa binotata* (Say) collected are from the Membracinae subfamily, and 111 adult eared hoppers *Microcentrus perditus* (Amyot & Serville) were collected from Stegaspidinae subfamily. The other 22 Membracidae species collected were from the Smiliinae subfamily (Dietrich 2005). The predominant treehopper species was *Ophiderma definita* Woodruff, comprising 75.30% of the total Membracidae collection, followed by *Telamona monticola* (Fitch) 7.71%, *Archasia belfragei* (Fabricius) 5.43%, *T. tiliae* Ball 3.63%, *Glossonotus acuminatus* (Fabricius) 2.91%, and *Cyrtolobus fenestratus* (Fitch) 1.00%. Rest of the other 19 Membracid species comprised less than 4.03% of the total treehopper collection (Table 1).

Leafhoppers: Most Cicadellidae collected were in the subfamilies Athysaninae, Neocoelidiinae, and Typhlocybinae. They are not xylem feeding insects and may not be important for BLS disease transmission, thus the life history of these leafhoppers will be discussed in a separate paper. Known *X. fastidiosa* vectors are the relatively large

leafhoppers from the Cicadellinae subfamily. Cicadellinae leafhoppers have an inflated clypeus with enlarged pump that enables leafhoppers to feed on the xylem fluid of the host plant. Eight hundred ninety three Cicadellinae leafhoppers from six Cicadellinae species were collected from this four year sampling period. *Graphocephala versuta* (Say) was the most dominant Cicadellinae species (90.15%) followed by *G. coccinea* (Forster) (4.93%), *Draeculacephala portola* Ball (1.57%), *Oncometopia orbona* (Fabricius) (1.46%), *Aulacizes irrorata* (Fabricius) (1.23%), and *Draeculacephala anguilfera* (Walker) (0.67%).

Spittlebugs: Spittlebugs belong to two families: Aphrophoridae and Clastopteridae. Insects in these groups also have an inflated clypeus with enlarged feeding pump that allows xylem feeding and are thus all are potential *xylella* vectors. Despite being collected during the entire season, spittlebug populations were relatively low. Of 214 specimens collected from both fogging and sticky card traps, 32.71% (70) were *Clastoptera obtusa* (Say), 31.87% (68) were *Philaenus spumarius* (L.), and 24.30% (52) were *Philaenus pallidus* (L.).

Sampling method comparison

Fogging collected more insect specimens than sticky cards when both methods were employed at the same time (Table 2). Six thousand one hundred twenty one xylophage hemipteran insects from 29 species of 19 genera were collected from 84 pin oaks by fogging. At the same time, only 299 xylem feeding hemipteran insects from 13 species of 11 genera were collected from 1004 sticky trap cards placed in 105 pin oaks. Each species collected from sticky cards were also collected from fogging samples except for *G. coccinea,* which was found only on sticky cards (Table 1). Seventeen species were

present only in fogging samples, in addition to the 12 common species that presented in both sampling methods (Table 1) indicating that the sampling efficiency of sticky cards is low when compared to the fogging method.

In addition to fogging at Duke Farms and MCCC, weekly sticky card sampling was also conducted at all four sites during 2002, 2003, 2004, and 2006. By sampling every week and at multiple locations over several years, sticky card sampling collected similar numbers of individuals, 6759 over the four years at four sites compared to the 6121 collected from fogging over two years at two sites, and similar numbers of species were collected. Thirty three species were collected from all sticky cards compared to 29 species for fogging. There were 25 common species present in both fogging and sticky card samples, eight unique species present only in sticky card collections, and four unique species present only in fogging collections. All unique species were collected at levels less than 44 adults on either method (Tables 1, 2).

Sampling efficiency can also be measured by using insect community indices. Comparing community species richness (number of species) and diversity using Shannon-Weiner index in this study. Monthly fogging and extended weekly sticky trap card sampling showed similar community structure. Shannon-Weiner H′ values ranged between 0.4 – 0.6 for MCCC and around 0.8 for Duke Farms, except for a low H′ value for fogging in 2006 at both MCCC and Duke Farms, as well as the whole 2006 season of card collection at MCCC. Increased population abundance due to high numbers of *O. definita* in 2006 at both sites or reduced species numbers for fogging in 2006 at MCCC resulted in low H′ values (Table 3).

In addition to the sample size and frequency, sampling efficiency also varied with insect mobility. *Ophiderma definita* was the most dominant species from both fogging and sticky card collections comprising 79.39% of the total fogging collection. However, only 58.35% of the total sticky card collection was *O. definita*. This 21% reduction in the sticky card collection from fogging collection was due to the low female sex ratio (female / total adults) in the sticky card collection compared with that in the fogging collection (Fig. 3). Fewer gravid female adults were collected on the sticky cards when they were carrying eggs. Therefore, the male biased sticky card collection underestimated the *O. definita* population.

For the leafhopper species *G. versuta*, only 5 adults were collected by fogging compared to 97 collected from sticky traps on fogging days. *Graphocephala versuta* made up 32.44% of the total sticky card collection on fogging days. Eight hundred *G. versuta* were collected from sticky cards during the entire study period, comprising 11.84% of the total sticky card collections. No *G. coccinea* was collected from fogging indicating that this species were hard to collect using fogging method. On the other hand, the eared hoppers, *Microcentrus perditus,* were almost exclusively collected by fogging except for a single individual collected on a sticky card. Low sticky card collections for this species may be due a lack of adult mobility. Due to lack of flight ability, immature treehoppers were collected only from fogging (Table 1).

Seasonal dynamics

Xylophage insect seasonal profiles were monitored by yellow sticky traps at the each research sites. Although xylophage abundance varied with locations and years,

insect seasonal dynamics (peak time) was similar between sites with a small variation between years.

Xylophages were present on sticky cards from May to September each year. However, most trap catches occurred from the end of May to mid July. An initial peak appeared during the first 3 weeks of June and was followed by a second small peak from the end of June to the second week of the July.

Ophiderma definita:

Ophiderma definita was the predominant xylophage species making up 68.18% of the total collection. The largest number of adults caught on one sticky card was 174 during the week of June 5, 2002 at Duke Farms. The second highest *O. definita* catch was 94 adults during the week of May 31, 2006 at MCCC. Nine percent (9.05%) of the 7922 sticky cards during this study caught at least one *O. definita* adult, of which, 36.68% cards had 1 adult, 11.28% cards had 2 adults, 37.40% cards had 3-10 adults, 14.64% of the cards caught more than 10 adults in a week.

Ophiderma definita had one adult peak throughout the summer season (Fig.1). Adults were collected on sticky cards from the end of May to the end of June with a clear peak in the first week of June. *Ophiderma definita* population abundance varied between locations. The highest numbers observed were on pin oaks at Duke Farms and MCCC. Low populations were observed each year for both Riverview Cemetery and Rutgers Garden and may be due to the oak species composition. Riverview Cemetery had 61% red oaks and 39% pin oaks while Rutgers Garden had only red oaks. Comparison of the *O. definita* adults on sticky cards collected at Riverview Cemetery indicated that there was significantly more *O. definita* adults trapped in pin oak canopies than in red oaks (F

$_{1, 1644}$ = 19.26, $p < 0.001$) (Fig.2). Adult catches also varied between years. At Duke Farms, high populations appeared only in 2002. Similarly at MCCC, high populations appeared only in 2006.

Adult *O. definita* numbers differed between males and females in both fogging and sticky card collection. Female sex ratios (female : total) from the first fogging on May 24, 2006 was 43.44% and reached 85.72% on June 21, 2006 (Fig. 3). By the end of the season, only females were collected. However, a much lower percentage of the female *O. definita* was observed in the sticky card collections. *Ophiderma definita* female sex ratio increased from 2.66% on May 31 to 19.80% when the population was at its peak and then declined to 6.20% on June 14, and 0.75% on June 28, 2006. A high male ratio, 10:7 male/female seasonal sex ratio, from sticky card collections was also found in three cornered alfalfa hoppers, from samples collected with a sweep net in alfalfa (Jordan 1952). Johnson and Mueller (1989) also collected significantly more males than females from the yellow sticky cards. Low female catches on sticky cards indicates low female mobility in oak canopies, especially when females were carrying eggs or during the oviposition period. Thirty one percent (31.11%) females carried eggs on June 7 during the population peak. The percentage of egg carrying female reached 75% around June 28 when the lowest female adult catch (indicating a low flight activity) on the sticky card. Female sex ratio reached 100% and most of the females were carrying eggs after mid July. This may indicate that females live longer than males or males left the canopies in late season.

***Graphocephala versuta*:**

Adult *G. versuta* had 1 major peak that extended from late June to late July which followed immediately after *O. definita* (Fig.4). They appeared on the sticky cards right after the *O. definita* peak. Nearly seven percent (6.83%) of the entire 7922 sticky cards contained *G. versuta* adults. Seventy two percent (72.46%) of the cards with *G. versuta* collected only one adult on each card; the highest *G. versuta* single card trap catch was seven. Unlike *O. definita*, adult *G. versuta* were active until the end of August. Since there were few catches of *G. versuta* from fogging, it is difficult to determine the *G. versuta* sex ratio; however, a high percentage of females appeared on the sticky cards. Female sex ratio (female : total) from sticky cards was ranged between 0.60 – 0.66 during the peak period of June 16-July 14 (Fig. 5). A small portion (6-10%) of females carried eggs during June 16-July 28 compared to the egg-carrying percentage of *O. definita*. This may allow females to have greater mobility, leading to a higher female sex ratio in the sticky card trap collection.

Graphocephala versuta adult population abundance varied between sites and years. The highest population occurred in 2004 for all sites. As in *O. definita*, the population in 2003 was the lowest for all 3 sites sampled. Although *G. versuta* adult population was significantly higher in red oak canopies than in pin oaks in Trenton ($F_{1, 1648} = 12.36$, $p = 0.0005$), the highest adult catches were observed at MCCC where only pin oaks existed in 2004 and 2006. The red oaks at Rutgers Garden had high populations in both 2002 and 2004, whereas, the *G. versuta* adult population at Duke Farms was the lowest of all four sites in 2004. This indicates that *G. versuta* may prefer red oak to pin oak.

Telamona monticola:

Six hundred ninety seven adult *T. monticola* were collected from sticky card traps and appeared throughout the summer season from June to September (Fig. 6). The female sex ratio was 43.94% of the 132 adults sexed across the entire collection season. A relatively high adult population was observed at Duke Farms and Rutgers Gardens all three years (2002-2004), while few adults were collected from Riverview Cemetery and MCCC.

***Telamona tiliae*:**

Three hundred forty eight adult *T. tiliae* were collected on sticky cards from June to September and peaked at the end of August, which is right after the *T. monticola* population peak (Fig. 7). Morphologically, *T. monticola* and *T. tiliae* are similar in shape and size about 7-10 mm long. The female sex ratio of the 161 adults was 90.68% across the entire collection season. The population abundance at Duke was much higher than other sites.

***Archasia belfragei*:**

Three hundred forty one adult *Archasia belfragei* were collected using sticky cards. As shown in Figure 8, the adults appeared throughout June to August with a major peak in early July. Ninety two percent (91.58%) of the adult *A. belfragei* were collected from Duke Farms during 2002-2004. Few *A. belfragei* adults were collected at the other 3 sites. The female sex ratio was 47.27% (110 adults sexed) across the entire collection season.

***Glossonutus acuminatus*:**

Two hundred thirty four *G. acuminatus* adults were collected from 205 of 7922 sticky cards. The female sex ratio was 50.46% (109 adults) across the entire collection season. They appeared from June until August at densities between 1 or 2 adults per card per week (Fig. 9).

***Cyrtolobus fenestratus*:**

One hundred and six adult treehopper *C. fenestratus* were collected (Fig. 10). The adult of this species is about the size of *O. definita*, approximately seven mm in length and can be confused with *O. definita* by unskilled collectors. It also appears at the same time of year as *O. definita:* from late May to late June. However, the population size was much smaller than *O. definita*. *Cyrtolobus fenestratus* was collected from 47 out of 7922 the sticky cards with a total 106 adults. Forty cards had only one adult. The highest catch was 22 adults on one card on May 31, 2006 at MCCC. The female sex ratio was 20% (94 adults) across the entire collection season.

Other Cicadomorpha species:

In addition to the species collected in large numbers described above, many other Cicadomorpha species with low numbers were also collected in this study. Seasonal abundances of these minor species with less than 100 individuals collected with both fogging and sticky card methods combined is summarized in Table 4. The scattered appearance of these adults might be the result of low population abundance and may not reflect the actual seasonal life cycle of each species.

***Xylella fastidiosa* infection of hemipteran xylophages**

Fourteen percent (13.89%) of 1618 insect specimens tested in this study were *X. fastidiosa* DNA positive (Table 1). Twenty one of the 32 potential vector species tested were *X. fastidiosa* DNA positive. Treehoppers were the largest potential insect vector group from oak canopies in this study. Eleven percent (11.03%) of the tested treehopper specimens from 19 tested species was positive for *X. fastidiosa* DNA. These X. *fastidiosa* DNA carrying treehoppers were from 11 species. All six tested Cicadellinae species tested *X. fastidiosa* positive with 18.78% of the 490 tested testing DNA positive. Fifteen percent (15.49%) of the most dominant species, *Ophiderma definita* tested *X. fastidiosa* DNA positive. Twenty percent (20.19%) of the tested *G. versuta* carried *X. fastidiosa* DNA while a 3.57% positive rate was detected for a similar species, *G. coccinea*. Although spittlebug populations were not very high, 4 of 6 tested Cercopoidea species were *X. fastidiosa* positive with a 15.08% DNA positive rate for the 194 individuals tested.

Although the *X. fastidiosa* DNA positive rate of the treehoppers was lower than that of the leafhoppers or spittlebugs, the total treehopper vector numbers were substantially greater. Based on the *X. fastidiosa* DNA positive rate of each vector group, an estimate of 1311 treehoppers, 168 leafhoppers, and 32 spittlebugs from this collection pool of 12880 might vector *X. fastidiosa* bacterium. Prorated *X. fastidiosa* carrying treehopper numbers were 655.5% of the combined number of leafhoppers and spittlebugs. Therefore, treehoppers may play a major role in the bacterial leaf scorch disease transmission.

***Xylella fastidiosa* infection seasonal profile**

Xylella fastidiosa bacteria were detected from collected insects throughout the summer from May to August (Fig. 11-13). Treehoppers were the largest potential vector group compared to leafhoppers and spittlebugs. Throughout the summer, *X. fastidiosa* occurred in the treehoppers at a consistent level: 15.69% in May, 9.11% in June, 12.11% in July, and 13.33% in August, respectively. Cicadellinae populations, mostly *G. versuta,* peaked in early July (Fig. 4, Fig.20). Twenty percent (20.42%) of Cicadellinae insects from 6 leafhoppers species collected in July (when populations peaked) tested *X. fastidiosa* DNA positive, whereas 15.28% and 12.90% of the tested Cicadellinae specimens collected in June and August (when population was very low) were positive, respectively. Although spittlebug populations were relative low compared to leafhoppers or treehoppers, the percentage of the tested specimens carrying *X. fastidiosa* was similar to that of the Cicadellinae. They were 11.00%, 15.87%, and 17.95% for June, July, and August respectively.

Impact of BLS infection on hemipteran xylophage abundance and diversity in pin and red oak

The total number of xylem feeders and the sum of Cicadomorpha leafhoppers, treehoppers, and spittlebugs was significantly higher at Duke Farms than that at MCCC ($F_{1, 74} = 26.04$, $p < 0.0001$). Total number of xylem feeders collected in 2006 was greater than that in 2004 ($F_{1, 74} = 3.47$, $p = 0.0665$, Fig. 14), which was due to the significantly greater vector population density in May rather than in later months ($F_{3, 74} = 81.10$, $p < 0.0001$). There was a significant interaction between the location of Duke Farms vs. MCCC and the time (month) of the year on the total xylem feeder population ($F_{3, 74} =$

9.03, $p < 0.0001$). Xylem feeders peaked in the early season and decreased in July and August in both 2004 and 2006 at MCCC. The same trend occurred at Duke Farms in 2006. However, the total xylem feeder number was higher in August than in June and July at Duke Farms in 2004. The highest xylem feeder abundance of 575 individuals was observed from a single asymptomatic pin oak tree in the month of May at Duke Farms. The average number of xylem feeders collected from asymptomatic pin oaks was 90.05 ± 20.31 per tree, which was 60.53% significantly greater than 56.10 ± 17.06 per tree collected from symptomatic pin oaks ($F_{1, 74} = 8.65$, $p = 0.0044$) (Fig. 14).

The majority (95.97%) of the xylem feeders collected were membracids. Among all treehoppers, 82.13% were *O. definita*, the most abundant species in pin oaks at Duke Farms and MCCC. Membracidae population abundance also varied with location and time of year ($F_{3, 74} = 8.99$, $p < 0.0001$) (Fig.15) which is the same trend described for total xylem feeders. Duke Farms had the highest Membracidae population in May 2006 when compared to all other times at MCCC or Duke Farms. Membracidae population abundance (86.86 ± 20.30 per pin oak) in asymptomatic pin oaks was 62.64% significantly greater than that found in BLS symptomatic oaks (53.40 ± 17.04 per oak) ($F_{1, 74} = 8.57$, $p = 0.0045$) (Fig. 15).

Significantly more Cicadellinae leafhoppers, 0.29 ± 0.10 per tree found in the asymptomatic tree samples than 0.21 ± 0.13 per tree in BLS symptomatic trees ($F_{1, 74} = 8.57$, $p = 0.0045$) (Fig. 16). Also, there were more leafhoppers found in early season at MCCC, while more leafhoppers were found during late season at Duke Farms ($F_{3, 74} = 8.99$, $p < 0.0001$).

Few spittlebugs were found in the pin oak canopies using either sticky card (98) or fogging (116). Although they can be found throughout the summer season from June to August, significantly more spittlebugs were found in June than in other months ($F_{3, 74} = 6.55$, $p = 0.0005$) (Fig.17). Interestingly, few spittlebugs were found in 2006 at Duke Farms or in 2004 at MCCC compared to those found in 2004 Duke Farms and in 2006 at MCCC (Fig. 17). *Xylella fastidiosa* infection showed no significant impact on the spittlebug population density ($F_{1, 74} = 1$, $p = 0.3216$).

The impact of BLS infection in pin and red oaks on insect populations varied with each vector species. Adult *O. definita* collected in asymptomatic pin oaks was 71.34% greater than in BLS infected tree canopies ($F_{1, 74} = 7.49$, $p = 0.0078$). For the remaining xylem feeding species, only *T. tilae* showed significant population differences between infection status. Eighty-five percent (84.62%) more *T. tilae* were collected in asymptomatic pin oaks than in BLS infected pin oaks ($F_{1, 74} = 7.21$, $p = 0.0089$). No significant impact of BLS infection on other xylophage species between asymptomatic oaks and BLS infected oaks. There were 24.49% more *M. perditus* ($F_{1, 74} = 2.30$, $p = 0.1334$), 68.92% more *T. monticola* ($F_{1, 74} = 1.42$, $p = 0.2378$), 80.00% more *Smilia fasciata* ($F_{1, 74} = 0.45$, $p = 0.5064$), and 121.52% more treehopper nymphs ($F_{1, 74} = 1.6$, $p = 0.2095$) found in asymptomatic canopies than that in the BLS infected pin oak canopies. Conversely, 25.40% more *A. belfragei* ($F_{1, 74} = 0.13$, $p = 0.7209$) and 62.50% more *G. acuminatus* ($F_{1, 74} = 0.56$, $p = 0.4557$) adults were found in the BLS infected trees than in the asymptomatic pin oaks (Table 5).

Impact of BLS infection in pin and red oaks on relative xylophages population abundance

Analysis of insect abundance based on fogging during 2004 and 2006 indicated significantly more hemipteran xylem feeding insects in non-infected pin oaks than in nearby BLS infected oaks (Fig. 14-17). At the same time, insect populations sampled using sticky cards during 2002, 2003, 2004, and 2006 showed no significant impact of *X. fastidiosa* infection on vector populations (Fig. 18-23).

MCCC sticky card catches in pin oaks during 2004 and 2006 indicated no significant difference between symptomatic oaks and asymptomatic oaks was detected for total xylem feeding insect populations ($F_{1, 3164} = 0.32$, $p = 0.5723$) (Fig. 18) or vector family ($F_{1, 3164} = 0.34$, $p = 0.5576$ for Membracidae; $F_{1, 3164} = 0.16$, $p = 0.6901$ for Cicadellinae; $F_{1, 3164} = 2.30$, $p = 0.1296$ for Cercopoidea). Total sticky card catches were significantly higher in 2006 when compared to that of 2004 ($F_{1, 3164} = 626.65$, $p < 0.0001$). This was explained by higher Membracidae catches observed in 2006 ($F_{1, 3164} = 669.16$, $p < 0.0001$), due to the large numbers of *O. definita* ($F_{1, 3164} = 708.31$, $p < 0.0001$) (Fig. 19). The average *O. definita* catch per card was 0.5323 in 2006 and 0.0283 in 2004 at MCCC. *Ophiderma definita* catches comprised 96.08% of the Membracidae, or 89.58% of all xylem feeding insects collected in 2006. Similarly, no significant difference for the leafhopper *G. versuta*, the second most dominant vector species, was seen between BLS symptomatic pin oaks and asymptomatic oaks at MCCC ($F_{1, 3164} = 0.28$, $p = 0.5976$) (Fig.20).

Compared to pin oaks lining the MCCC parking lot, the pin oaks at Duke Farms were planted in double rows 10 m apart, along the many roads of the estate. As seen at

MCCC, no significant differences in sticky card trap catches were detected between BLS symptomatic and asymptomatic pin oaks at Duke Farms ($F_{1, 1840} = 0.70$, $p = 0.4012$ for total vectors; $F_{1, 1840} = 0.70$, $p = 0.4023$ for Membracidae; $F_{1, 1840} = 0.59$, $p = 0.4439$ for Cicadellinae; and $F_{1, 1840} = 0.21$, $p = 0.6455$ for Cercopoidea) (Fig. 21). Among the vectors, *O. definita* and *G. versuta* were the dominant species; making up 69.63% and 10.57% of the total vector population respectively. No significant impact of the BLS infection was detected on the *O. definita* ($F_{1, 1840} = 0.00$, $p = 0.9842$) (Fig. 22) and *G. versuta* ($F_{1, 1840} = 0.04$, $p = 0.8447$) (Fig.23).

DISCUSSION

Bacterial leaf scorch of oak caused by *Xylella fastidiosa* is transmitted by hemipteran xylophages. Frazier (1946, 1966) pointed out that all members of the subfamily Cicadellinae that had been properly tested were shown to be positive for *X. fastidiosa*. Spittlebugs are also known xylem feeders (Crews 1998, Malone et al. 1999) and are responsible for plant disease transmission (Purcell 1980). Therefore, of all the auchenorrhychous Hemiptera adequately tested as vectors, all that are xylem feeders were found to be capable of transmitting the *X. fastidiosa* with varying degrees of efficiency (Frazier 1966, Purcell and Finlay 1979, Purcell 1989). Spittlebugs and sharpshooter leafhoppers are known vectors of almond leaf scorch (Purcell 1980), citrus variegated chlorosis (CVC) (Turner and Pollard 1959, Brlansky et al. 1996, Krugner et al. 2000), oleander leaf scorch (Costa et al. 2000), phony peach disease (PPD) and periwinkle wilt (Turner and Pollard 1959, McCoy et al. 1978, Adlerz and Hopkins 1979), Pierce's disease (PD) (Severin 1949, 1950, Purcell 1980, Hill and Purcell 1995, Costa et al. 2000, Almeida and Purcell 2003), and possible leaf scorch of shade trees (oak and elm) (J. Bentz, personal communication). Hemipteran xylophage species composition varies with location and host plant. Most of the known *X. fastidiosa* vectors from grape are not present in the oak canopies in New Jersey. Of the known vector species from other studies, spittlebug *Philaenus spumarius* L. (Almond leaf scorch), and three sharpshooter leafhopper species *Oncometopia orbona* (F.) (Phony peach disease), *Graphocephala coccinea, G. versuta* (shade tree), also tested positive for *X. fastidiosa* DNA this study. In addition, two additional spittlebug species, *Philaenus pallidus* L. and *Clastoptera obtuse* (Say), and the leafhopper *Draeculacephala anguilfera* (Walker) tested positive for

X.fastidiosa DNA this study. Among the *X. fastidiosa* positive leafhoppers and spittlebugs, only *G. versuta* had a detectable population. All other species had low populations and may not play a critical role in the disease transmission.

Treehoppers utilize a variety of plants as hosts, including many species of oak (Kopp and Yonke, 1973a, b, c, 1974) and were the most abundant xylem feeding Cicadomorpha insects found in pin and red oak in central New Jersey. Twenty-seven treehopper species in thirteen genera were found, compared to twelve genera/species reported in Kentucky (Johnson and Freytag, 1997), of which *Ophiderma definita* was the most frequent treehopper collected. This species extensively uses red oak (Kopp and Yonke 1973c, Woodruff 1919), black oak, bur oak, white oak (Kopp and Yonke 1973c), and pin oak (Johnson and Freytag, 1997) as hosts. Adult *O. definita* peaks in the first week of the June, which is about one week later than the combined *Ophiderma / Cyrtolobus* peak observed in Kentucky (Johnson and Freytag, 1997). Considering New Jersey is approximately 300 km north of Kentucky, *O. definita's* seasonal population profile is similar to Kentucky. A similar seasonal profile in New Jersey and Kentucky is also observed for *Archasia, Glossonotus, Microcentrus*, and *Telamona* species.

From the twenty-five species found in New Jersey, eleven treehopper species tested positive for *X. fastidiosa* DNA this study. This is the first time a treehopper has been officially reported as a carrier of *X. fastidiosa*. Most *X. fastidiosa* disease vector studies were conducted in southern agriculture systems such as grape, peach, and citrus where leafhoppers are most abundant and few treehoppers are present. Obstacles in the bacterium DNA analysis from treehoppers also contributes to the deficiency in reporting. We were able to isolate the live *X. fastidiosa* bacteria and detect its DNA from treehopper

samples. DNA sequencing analysis indicated that *X. fastidiosa* DNA from treehoppers is identical to the DNA extracted from host oak petioles (Zhang et al. unpublished data). Treehoppers appear in the canopy throughout the summer season. *Ophiderma definita* nymphs hatch as oak buds break in late April / early May. Adult populations peak in the first week of June. *Xylella fastidiosa* bacteria present in oak stems grow into the petiole and leaf veins as the leaf emerges and grows. Hill and Purcell (1997) found that the minimum incubation periods in hosts before blue-green sharpshooter acquired *X. fastidiosa* were 4, 22, 29, and 25 days for grapevine, blackberry, mugwort, and watergrass, respectively. Efficient acquisition from grape only occurs with cell concentrations exceeding 10^{6-7} cells per gram (Hill and Purcell 1997). Although the cell concentration in the oak petioles or other feeding sites has yet to be determined, by the time *O. fastidiosa* populations peak in early June, bacterial populations should be large enough for efficient acquisition. If the bacterial population is not high enough in early June for infection to occur, it must be high enough when populations *G. versuta,* the second major vector, peak in July. We were able to isolate live bacteria in late July from treehoppers and extract *X. fastidiosa* DNA in June (Zhang et al. unpublished data), indicating the bacteria had been present in the insect for some period of time.

Vector population determination in the oak canopy is very important to understand BLS disease transmission and its capability for spread. There are many methods available for insect sampling (Southwood and Henderson 2000). Sweep netting has been used for treehopper population studies in the alfalfa field (Johnson and Freytag 1997), where host plant height is within arm's reach. Sticky cards are inexpensive and very flexible in time and space, enabling a higher number of replicates to be taken, and is

highly suitable for the studying insect spatial distribution and stratification. However, trap color, can introduce bias into census data. Yellow sticky cards have been successful in attracting and catching adult treehoppers (Johnson and Mueller 1988). Johnson and Freytag (1997) collected twelve genera / species of the treehoppers in oaks in Kentucky with yellow sticky traps: *Archasia, Cyrtolobus, Enchenopa binotata* (Say), *Entylia, Glossonotus, Microcentrus caryae* (Fitch), *Mircutalis calva* (Say), *Ophiderma, Platycotis vittata* (Fab.), *Smilia, Stictocephala*, and *Telamona*. Bentz (personal communication) used sticky traps to study the xylophage populations in elm canopies in Washington D.C. Olszewski (1996) collected Cicadellinae leafhoppers in the genera *Graphocephala* and *Oncometopia* and Membracidae treehoppers in the genera of *Archasia, Cyrtolobus, Enchinopa, Glossonotus, Ophiderma, Smilia,* and *Telamona* by using yellow sticky traps hung in oak canopies Moorestown, New Jersey.

During the last decade, fogging with knock-down insecticides has received attention for sampling tree canopies, especially in locations where trees were too high for deploying sticky cards, sweep netting, or other sampling methods. Pyrethrum producing a rapid knock-down with no side-effects on birds or mammals (Casida 1973, Jackson 1985) (Southwood and Henderson 2000) is commonly used when employing this method. Fog generated by harmless mineral oil can penetrate the canopy and reach all parts of the sample tree and provides an absolute population estimate on per tree basis. Fogging has been used to examine arboreal arthropod communities with respect to a wide range of factors including taxonomic group, guild, biomass, body size, and species abundance patterns (Morse et al. 1988, Stork 1988, 1991, Basset 1990, 1992, Stork and Hammond 1997, Tagger 2000, Barbosa et al. 2000). Trieff et al. (2001) surveyed Great Smokey

Mountains National Park insect fauna using fogging to compare community diversity between different habitats, using the Shannon-Weiner diversity index. Stork and Hammond (1997) surveyed beetle assemblage in oak crowns in Richmond Park, UK using fogging. The most important advantage of fogging using a knock-down insecticide is the independence with insect activity and the lack of any "attractants", so the samples reflect the standing insect population present on host surfaces such as branches, leaves, and fruit present in the canopy. Therefore, sample results are comparable between trees or treatments. However, there are limitations of fogging compared to other sampling methods. Fogging requires intensive labor, calm weather conditions, and availability of fogging machines. In addition, when tall oak trees are involved, a bucket truck is required to dispense the fog into the canopy. It is also difficult to standardize insect abundance per unit volume, it is not possible to re-sample the same tree until time has been allowed for re-colonization, and there is potential for insecticide drift. A significant liability is the fear generated by the public when fogging is used near houses.

We used both fogging and yellow sticky cards to study the hemipteran insect population in pin and red oaks. When the data from fogging and sticky cards is combined, 37 species of Cicadomorpha insect species from 21 genera were collected from oak canopies in central New Jersey. Fogging in this study collected many more species and individuals compared to sticky card. However, the disadvantages of sticky card sampling can be compensated by extended sampling with more locations, and by weekly sampling over multiple years. As pointed by Southwood and Henderson (2000), there is no hard fast line between relative and absolute methods, for absolute methods of sampling are seldom 100% efficient and relative methods can sometimes be corrected in

various ways to give density estimates. Combination of fogging and the extended sticky card sampling provided valuable information on insect community structure such as species diversity and species richness in this study.

At the species level, collections from fogging and sticky card sampling did not provide similar results. Treehoppers were collected in both fog and sticky card sampling, but few Cicadellinae insects were caught using fogging in this study. Most of the Cicadellinae leafhoppers collected in this study were from sticky card collections. Possible reasons for this difference between fogging and sticky card trapping may be variation in leafhopper distribution through time and space, flight activity, or the timing of the fogging. In this study leafhopper adult population peaks determined from fogging; for *G. versuta* tracked collections from sticky cards the same week as the fogging. However, the population in oak canopy may have been low on the days of fogging. Leafhoppers peaked around July 6 (ranging from June 21 to August 9) for both 2004 and 2006 according to the sticky card trap collections. Fogging was conducted on 6/16/04, 7/22/04, 6/21/06, and 7/24/06 when leafhopper populations were at the beginning or end of the July peak. Secondly, Cicadellinae leafhoppers may have a patchy spatial distribution pattern, in which case fogging may have simply missed oaks that had leafhoppers. Thirdly, sharpshooters are extremely vagile (Turner and Pollard 1959). They are active flyers in the oak canopy so sticky cards may overestimate the actual leafhopper population when compared to the fogging. Lastly, the leafhopper catches on sticky cards may have been due to dispersal flights, since the sticky card catch insects throughout the day while fogging only catch those insects present in the canopy at the time of sampling.

Xylophage species abundance varied between years. *Ophiderma definita* populations were highest in 2002, followed by those found in 2006. Very low *O. definita* populations were observed in 2003 and 2004. Meanwhile *G. versuta* populations were highest in 2002 and 2004, and moderately abundant in 2006. This was also observed in Kentucky where treehopper populations in 1994 was less than half of that in 1993 (Johnson and Freytag 1997). One possible explanation might be temperature variation between the four years sampled. 2002 and 2006 were relatively warm in early spring compared to 2003 and 2004. By comparing different temperature parameters over the 4 years of the study in central New Jersey, we found that the cumulative degree (>10^{o}C) since first day of the year during April and May were much higher in 2002 and 2006 compared to 2003 and 2004 (Fig.24). This was similar to that of the *O. definita* population abundance, which peaked in early June (Fig. 1). However, the mid-summer species, *G. versuta,* which peaked in July (Fig. 4) was not in line with the temperature variation.

Xylophagous insect population abundance in this study was determined by an absolute sampling method using pyrethrin fogging that caused the standing insect population in the oak canopy to fall to the ground. Therefore, the fogging technique used here provides a reliable insect population estimate and can be used for comparing treatments. We found more Cicadomorpha insects in the oak canopies where no BLS symptoms were observed when compared to infected oaks.

One characteristic of BLS disease in oaks is the apparent randomness of infected branches within infected trees. Within a locality it is common to find an apparent healthy oak tree next to a severely infected one. This distribution matrix provides insect vectors

with the option to easily move between oaks of different quality. Oak morphology, phenology, and physiology change when attacked by the disease. In spring, BLS infected branches usually foliate later than uninfected trees in the same community. Oak leaves are usually smaller and branches tend to dieback after serious BLS infection (personal observation). *Xylella fastidiosa* bacteria reside in the oak xylem cells and block water flow to leaves resulting in stressed oaks (Tyree and Zimmermann 2002). Our oak xylem fluid study showed that the BLS infected oak branches had different amino acid profiles than non-infected branches in the early summer (June), when treehoppers were active and before visual BLS symptoms were evident. Several amino acid concentrations were significantly higher in BLS infected branches (Zhang et al. unpublished data). The high proportion of a single amino acid in the diet of insects may be making it unusable or even toxic to insect feeders (Brodbeck and Strong 1987). Xylophagous leafhoppers need to locate optimal host plants for development, maintenance, and reproduction due to the dynamic nature of their diet (Blackmer et al. 2004, Tipping et al. 2004, Park et al. 2005, Mizell et al. 2007). Xylem feeding insects are often large and can quickly assess the quality of a potential host plant (Northfield et al. 2007). Organisms which require highly variable diets have an increased foraging needs relative to organisms that have static diets (Real and Caraco 1986). Because xylem fluid is often a nutrient poor resource in time and in space some xylem feeders disperse to find optimal host plants (Mizell et al. 2007). *Homalodisca coagulata* feeds more often on and survives better on succulent terminal growth, *i.e.* last-year's growth and meristematic growth. Mizell and French (1987) reported that *H. coagulata* feeds predominantly on trees which do not display *X. fastidiosa* disease symptoms when provided a choice. The xylem nutritional quality of

the BLS infected oak trees or branches may have some adverse impact on leafhoppers or deter them from feeding on infected branches, hence promoting insect foraging behavior resulting in the dispersal of individuals to uninfected oak trees in the vicinity of infected trees. This would cause higher Cicadomorpha populations on the asymptomatic oaks compared to the BLS infected ones which may facilitate disease transmission to uninfected trees by the leafhopper feeding.

Higher vector populations in asymptomatic oaks may also result from female adult ovipositional behavior. Treehoppers commonly overwinter in the egg stage in the northern states (Knowles et al. 1999, Lin 2006). Treehopper eggs hatch at the same time as host oak buds burst in late April, nymphs feed on new oak shoots, develop as the oak leaves do, and most to adults in early June when oak leaves are fully grown. In this study females started carrying eggs around mid June and were less likely to move after egg development had occurred. Compared to leafhoppers, treehoppers have been known to be "motionless" insects; "actual records" of the resting time have not been reported (Funkhouser 1917). However, based on the comparison of fog sampling and sticky card catches in this study, treehoppers were active flyers before females became gravid. Females flight, prior to selecting preferred oviposition sites, has been shown in asymptomatic oak. Female ovarian development is delayed before migration or dispersal when environmental conditions are deteriorating (Johnson 1969, Kennedy 1960). Ovaries may not develop until the female finds a suitable host, or until after feeding on healthy oak xylem and can be tested by examining the ovary development of females collected simultaneously on both symptomatic and asymptomatic oaks. Pre-oviposition

flights may result in higher actual population in the asymptomatic oaks and may also appear as high sticky card catches in the BLS infected oaks.

Oak leaves begin undergoing physical changes before the appearance of visible scorch symptoms. An oak leaf reflectance study showed that pre-visual BLS symptoms can be detected by using hyperspectral reflectance indices in mid July, 4-5 weeks before visual BLS symptoms are evident (Lashomb et al. unpublished data). This physical change can be used by early season treehoppers and leafhoppers to trigger movement to preferred hosts and transmission of the bacteria during feeding. Subsequent treehopper generations may stay on the same oak tree until the host quality declines as a result of the BLS disease progression. Since effective bacteria acquisition by a vector requires a high level of the bacteria accumulated in the host plant tissue (Hill and Purcell 1997), vector dispersal before *X. fastidiosa* population establishment reaches a certain threshold level may actually contribute little the spread of BLS.

Unlike what has been demonstrated by the fogging method, data from sticky cards did not show any significant insect population difference in abundance between BLS infected oaks and asymptomatic oaks. This might be because sticky card trapping is a relative sampling method that indicates insect flight activity, in addition to the abundance. As discussed above, insect vectors may increase their flight activity and move to healthier asymptomatic oaks for feeding and oviposition when BLS infected oak host quality declines. High mobility of these insects may result in high sticky card catches in the symptomatic oak trees. On the other hand, a healthy oak tree provides not only a better food resource that will reduce the necessity for dispersal flights, but also a

high density of leaf coverage which can block or reduce insect mobility leading lower numbers on sticky card in asymptomatic oak trees.

In conclusion, the fogging method indicates the actual insect vector population abundance residing in each oak canopy, which represents the numerical consequence of the BLS impact. Sticky card trapping represents the vector activity, which is a process of the impact of BLS infection. A high catch using sticky cards represents a high mobility of the insect vector. Whereas a low population in BLS infected oaks in the neighborhood of the asymptomatic oaks would be over-estimated by using the sticky cards and appears similar to the catches from uninfected oaks. By the integration of these two sampling methods, one can envision two dimensions of the entire vector population and their behavioral dynamics when BLS infection occurs.

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Table 1. Xylem feeding Cicadomorpha insect collection by sticky trap cards and fogging methods in pin and red oak canopies in central New Jersey during 2002-2006 and their *Xylella fastidiosa* DNA detection.

Species	Sticky Cards	Fogging	Total	Card on fog days	Total Tested	*X. fastidiosa* positive (%)
Total	6758	6122	12880	299	1618	13.89
Cercopoidea: Aphrophoridae: Aphrophorinae						
Aphrophora quadrinotata Say		19	19		15	33.33
Aphrophora cribrata (Walker)		2	2		1	0.00
Philaenus marginellus (Linnaeus)	2	1	3		7	0.00
Philaenus pallidus (Linnaeus)	34	18	52	3	51	12.28
Philaenus spumarius (Linnaeus)	49	19	68	10	78	19.23
Cercopoidea: Clastopteridae: Clastopterinae						
Clastoptera obtusa (Say)	13	57	70	5	42	7.14
Membracoidea: Cicadellidae: Cicadellinae						
Draeculacephala anguilfera (Walker)	5	1	6		5	20.00
Draeculacephala portola Ball		14	14		9	11.11
Graphocephala coccinea (Forster)	44		44	6	28	3.57
Graphocephala versuta (Say)	800	5	805	97	431	20.19
Aulacizes irrorata (Fabricius)	10	1	11	2	10	10.00
Oncometopia orbona (Fabricius)	13		13		7	14.29
Membracoidea: Membracidae: Hoplophorinae						
Platycotis vittata (Fabricius)	13	2	15		4	0.00
Membracoidea: Membracidae: Membracinae						
Enchenopa binota (Say)	24	2	26	2	17	11.76
Membracoidea: Membracidae: Smiliinae						
Acutalis tartarea (Say)	1	2	3	1		
Archasia belfragei Stål	341	292	633	81	120	3.33
Atymna querci Fitch	4	21	25			
Carynota mera (Say)	1		1			
Cyrtolobus maculifrontis (Emmons)	1		1		1	0.00
Cyrtolobus discoidalis (Emmons)	1	1	2		1	100.00
Cyrtolobus fenestratus (Fitch)	106	9	115		45	4.44
Cyrtolobus fuscipennis (Van Duzee)	1		1		4	0.00
Cyrtolobus gratiosus Woodruff	11		11		5	0.00
Cyrtolobus puritanus Woodruff	4		4		3	0.00
Entylia carinata Forster	1	1	2			
Glossonotus acuminatus (Fabricius)	234	105	339	33	114	11.40
Helonica excelsa Fairmaire	6	2	8	1	1	0.00
Ophiderma definita Woodruff	3944	4838	8782	54	226	15.49
Ophiderma evelyna Woodruff		9	9			

Ophiderma flava Goding	8	10	18		1	0.00
Smilia fasciata (Amyot & Serville)	11	44	55		22	4.55
Telamona concava Fitch	2	2	4		2	0.00
Telamona extrema Ball	4		4		1	100.00
Telamona monticola (Fabricius)	697	202	899		134	15.67
Telamona tiliae Ball	348	75	423		121	9.92
Telamona unicolor Fitch	25	4	29	4	22	0.00
Membracoidea: Membracidae: Stegaspidinae						
Microcentrus perditus (Amyot & Serville)	1	110	111		90	12.22
Nymphs		254	254			

Table 2. Number of species and genera of xylem feeding Cicadomorpha insects collected by sticky card traps and fogging methods in the pin and red oak canopies during 2002-2006 in central New Jersey.

Family	Fogging		Sticky Traps When Fogging		Sticky Traps Whole Season	
	Genera	Species	Genera	Species	Genera	Species
Aphrophoridae	3	5	1	2	1	3
Clastopteridae	1	1	1	1	1	1
Cicadellidae	3	4	2	3	4	5
Membracidae	13	19	7	7	15	24

Table 3. Insect community structure from fogging and sticky trap sampling at Mercer County Community College (MCCC) and Duke Farms during 2004 and 2006.

Site	Year	Fogging		Sticky Traps when Fogging		Sticky Traps Whole Season	
		Species (individual)	Diversity H'	Species (individual)	Diversity H'	Species (individual)	Diversity H'
MCCC	2004	18 (504)	0.4513	6 (72)	0.4071	12 (413)	0.4615
	2006	6 (1741)	0.114	8 (95)	0.5783	13 (2660)	0.1605
Duke	2004	17 (759)	0.7969	15 (236)	0.8227	20 (836)	0.8862
	2006	20 (3119)	0.2566	*	*	*	*

*Sticky trap sampling was not carried out at Duke Farms in 2006.

Table 4. Seasonal appearance of the insect species with less than 100 individuals of total collection from both fogging and sticky card sampling methods at Mercer County Community College (MCCC), Duke Farms (Duke), Rutgers Garden (Rutgers), and Riverview Cemetery (Trenton) during 2002-2006.

Species	May			June			July			August		
	Early	Mid	Late	Early	Mid	Late	Early	Mid	Late	Early	Mid	Late
Aphrophora quadrinotata Say					■			■				■
Aphrophora cribrata (Walker)				■				■				
Philaenus marginellus (Linnaeus)					■	■			■			
Philaenus pallidus (Linnaeus)				■	■	■	■	■	■	■	■	■
Philaenus spumarius (Linnaeus)					■	■	■	■	■	■	■	■
Clastoptera obtusa (Say)				■	■	■	■	■		■	■	■
Draeculacephala anguilfera (Walker)										■	■	■
Draeculacephala portola Ball					■			■				■
Graphocephala coccinea (Forster)					■	■	■	■	■	■	■	■
Aulacizes irrorata (Fabricius)			■			■				■		■
Oncometopia orbona (Fabricius)					■					■	■	■
Acutalis tartarea (Say)					■							
Archasia belfragei Stål			■	■	■	■	■	■	■	■	■	■
Atymna querci Fitch			■		■							
Carynota mera (Say)										■		
Cyrtolobus maculifrontis (Emmons)											■	
Cyrtolobus discoidalis (Emmons)			■	■								
Cyrtolobus fenestratus (Fitch)			■	■	■	■	■				■	
Cyrtolobus fuscipennis (Van Duzee)				■								
Cyrtolobus gratiosus Woodruff				■	■	■	■					
Cyrtolobus puritanus Woodruff				■		■	■		■			
Enchenopa binota (Say)					■	■	■		■	■	■	■
Entylia carinata Forster	■							■				
Helonica excelsa Fairmaire			■		■					■	■	■
Microcentrus perditus (Amyot & Serville)											■	■
Ophiderma evelyna Woodruff			■					■				■
Ophiderma flava Goding			■	■				■				
Platycotis vittata (Fabricius)				■		■	■	■	■		■	■
Smilia fasciata (Amyot & Serville)			■	■	■							
Telamona concava Fitch				■	■			■			■	
Telamona extrema Ball								■		■	■	
Telamona unicolor Fitch				■	■	■	■	■	■		■	■

Table 5. Impact of BLS infection on xylem feeding insect abundance in pin oak canopies sampled by fogging method at Duke Farms (Duke) and Mercer County Community College (MCCC) in 2004 and 2006.

Species	Asymptomatic (X ± Se)	Symptomatic (X ± Se)
Cercopoidea: Aphrophoridae: Aphrophorinae		
Aphrophora quadrinotata Say	0.24 ± 0.14	0.21 ± 0.12
Aphrophora cribrata (Walker)	0.02 ± 0.02	0.02 ± 0.02
Philaenus marginellus (Linnaeus)	0.00 ± 0.00	0.02 ± 0.02
Philaenus pallidus (Linnaeus)	0.14 ± 0.08	0.29 ± 0.12
Philaenus spumarius (Linnaeus)	0.21 ± 0.13	0.24 ± 0.14
Cercopoidea: Clastopteridae: Clastopterinae		
Clastoptera obtusa (Say)	0.83 ± 0.49	0.52 ± 0.41
Membracoidea: Cicadellidae: Cicadellinae		
Draeculacephala anguilfera (Walker)	0.02 ± 0.02	0.00 ± 0.00
Draeculacephala portola Ball	0.17 ± 0.08	0.17 ± 0.13
Graphocephala versuta (Say)	0.10 ± 0.07	0.02 ± 0.02
Aulacizes irrorata (Fabricius)	0.00 ± 0.00	0.02 ± 0.02
Membracoidea: Membracidae: Hoplophorinae		
Platycotis vittata (Fabricius)	0.00 ± 0.00	0.05 ± 0.05
Membracoidea: Membracidae: Membracinae		
Enchenopa binotata (Say)	0.02 ± 0.02	0.02 ± 0.02
Membracoidea: Membracidae: Smiliinae		
Acutalis tartarea (Say)	0.02 ± 0.02	0.02 ± 0.02
Archasia belfragei Stål	0.05 ± 0.05	0.02 ± 0.02
Archasia galeata (Fabricius)	3.00 ± 0.88	3.76 ± 0.90
Atymna querci Fitch	0.24 ± 0.14	0.02 ± 0.02
Cyrtolobus discoidalis (Emmons)	0.02 ± 0.02	0.00 ± 0.00
Cyrtolobus fenestratus (Fitch)	0.07 ± 0.05	0.14 ± 0.12
Entylia carinata (Germar)	0.02 ± 0.02	0.00 ± 0.00
Glossonotus acuminatus (Fabricius)	0.95 ± 0.25	1.55 ± 0.36
Helonica excelsa Fairmaire	0.02 ± 0.02	0.02 ± 0.02
Ophiderma definita Woodruff	72.74 ± 18.76	42.45 ± 15.57
Ophiderma evelyna Woodruff	0.12 ± 0.07	0.10 ± 0.06
Ophiderma flava Goding	0.12 ± 0.06	0.12 ± 0.07
Smilia fasciata (Amyot & Serville)	0.64 ± 0.42	0.36 ± 0.16
Telamona concava Fitch	0.02 ± 0.02	0.02 ± 0.02
Telamona monticola (Fabricius)	0.05 ± 0.03	0.02 ± 0.02
Telamona querci Fitch	2.98 ± 2.27	1.76 ± 0.68
Telamona tiliae Ball	1.14 ± 0.33	0.62 ± 0.24
Telamona unicolor Fitch	0.02 ± 0.02	0.05 ± 0.05
Membracoidea: Membracidae: Stegaspidinae		
Microcentrus perditus (Amyot & Serville)	1.45 ± 1.02	1.17 ± 0.63
Nymphs	4.17 ± 1.85	1.88 ± 0.64

Fig. 1. *Ophiderma definita* adult seasonal profile on yellow sticky cards in pin (*Quercus palustris* Muenchh) and red (*Q. rubra* L.) oak canopies adult seasonal profile at Mercer County Community College (MCCC), Duke Farms (Duke), Rutgers Garden (Rutgers), and Riverview Cemetery (Trenton) during 2002, 2003, 2004, and 2006.

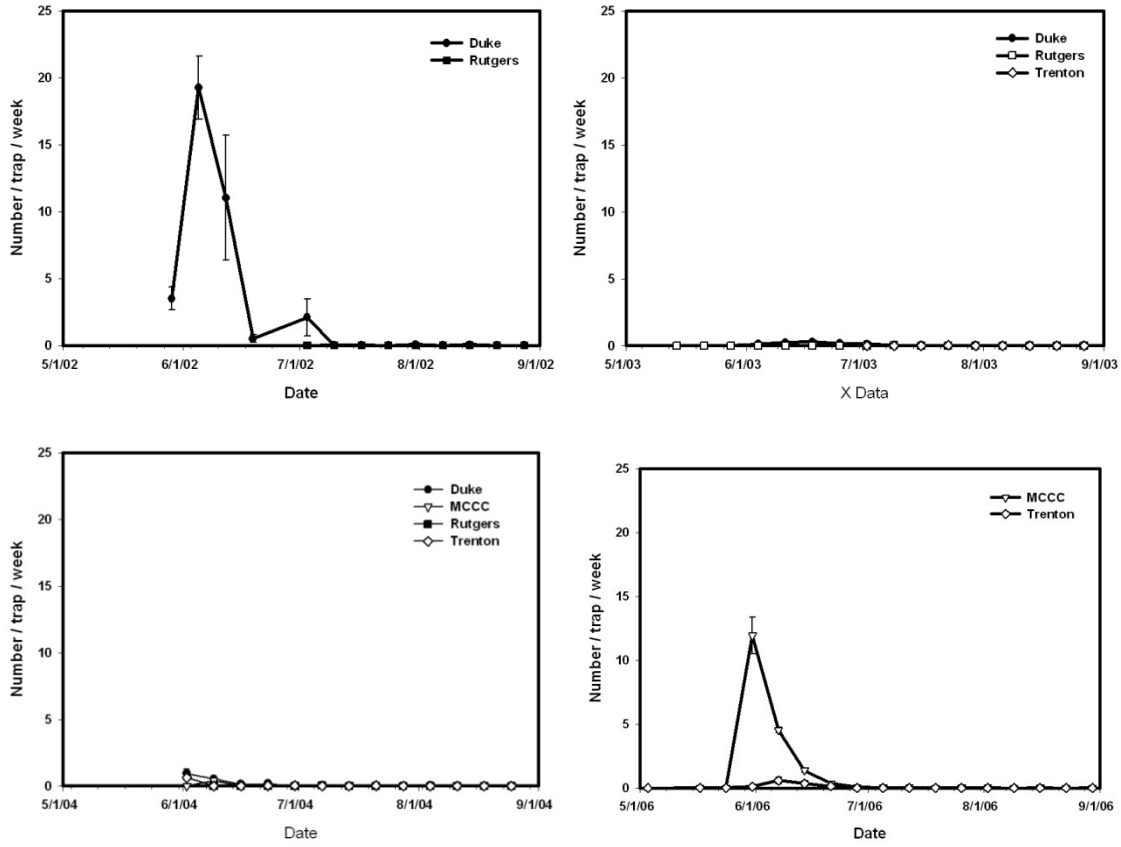


Fig.2. *Ophiderma definita* adult catches on sticky card traps in the pin (*Quercus palustris* Muenchh) and red (*Q. rubra* L.) oak canopies in Riverview Cemetery, Trenton during 2004 and 2006.

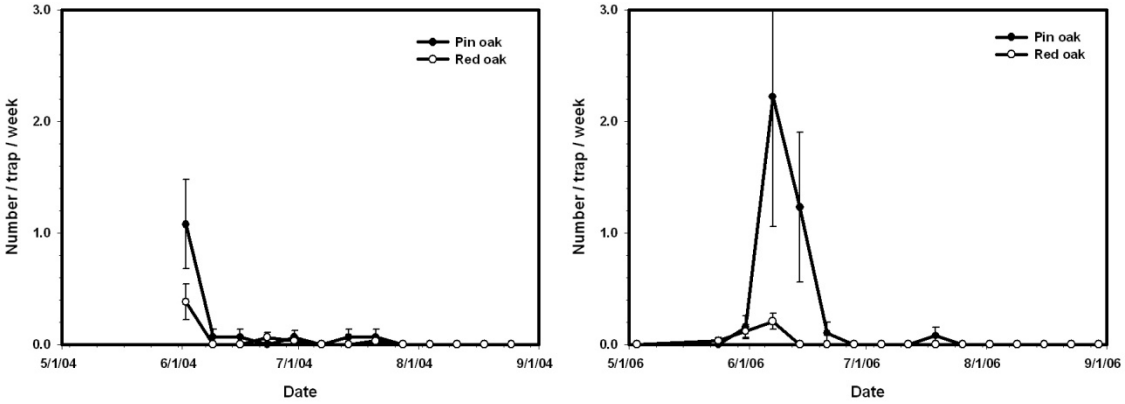


Fig.3. *Ophiderma definita* female adult ratio (females : total adults) from sticky card trapping and fogging methods and female with eggs ratio (females with eggs : total females) during 2006.

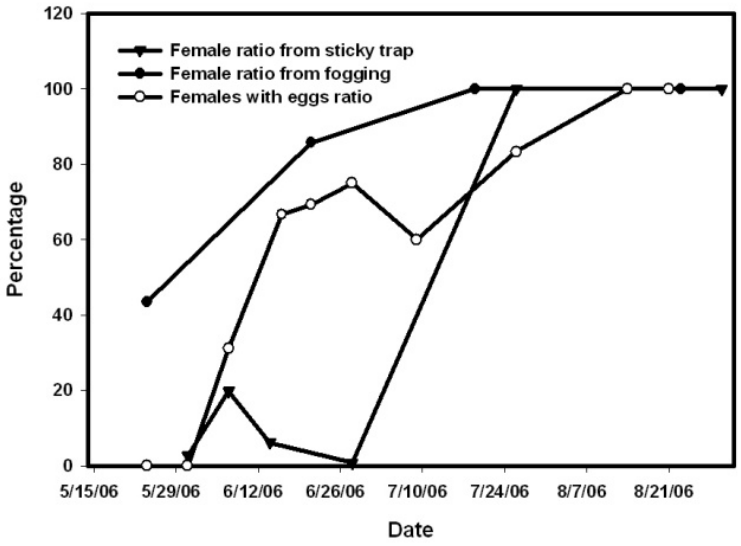


Fig.4. *Graphocephala versuta* adult seasonal profile presented on sticky cards at Mercer County Community College (MCCC), Duke Farms (Duke), Rutgers Garden (Rutgers), and Riverview Cemetery (Trenton) during 2002, 2003, 2004, and 2006.

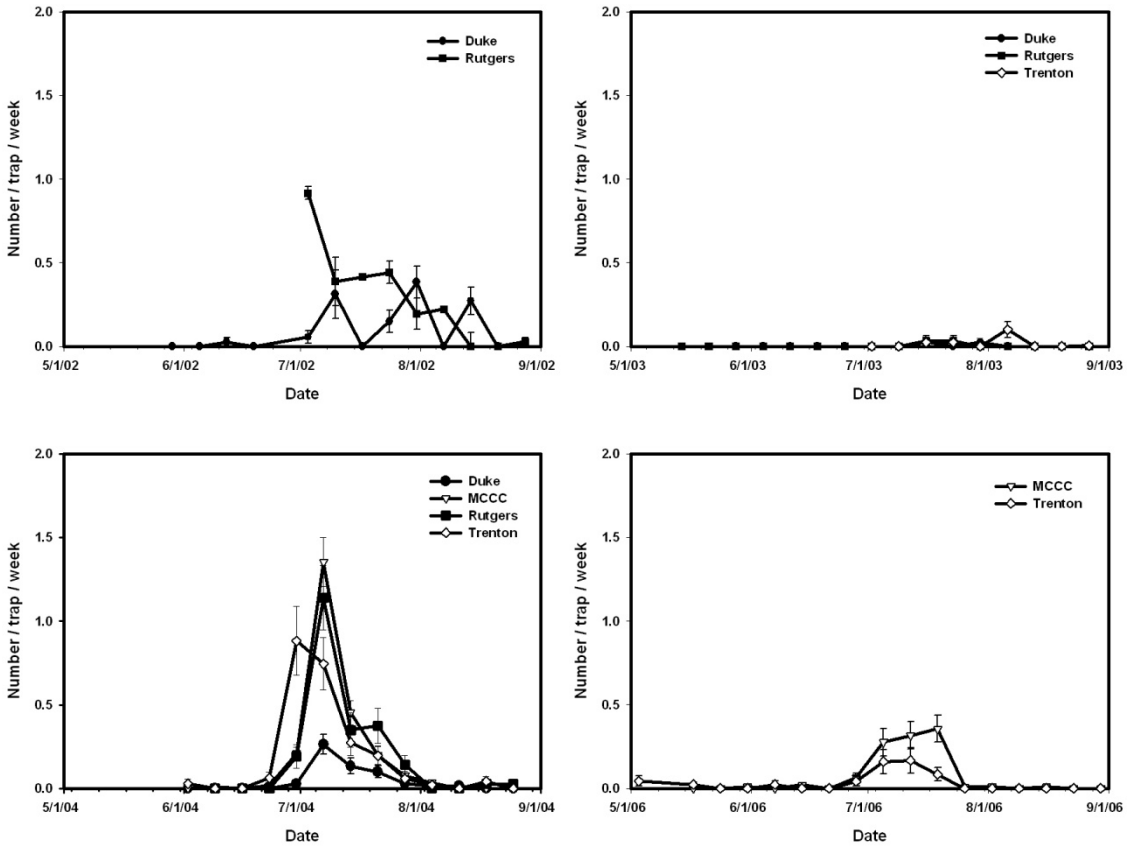


Fig.5. Seasonal dynamics of *Graphocephala versuta* adult female ratio (females : total adults) and the ratio of females carrying eggs during the summer in central New Jersey.

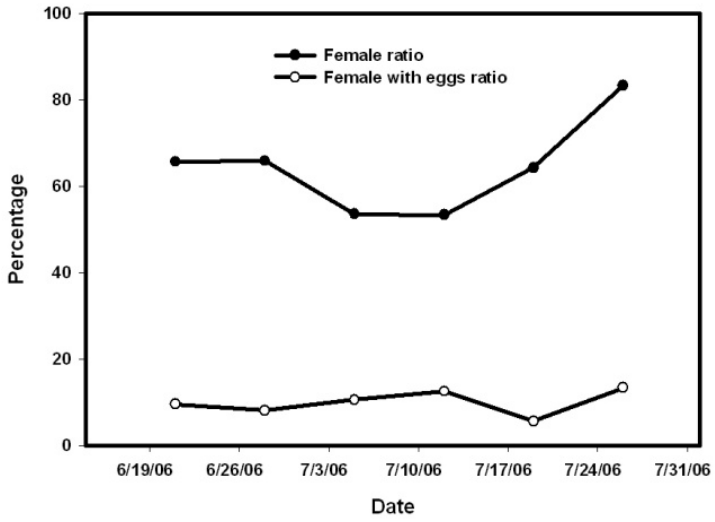


Fig.6. *Telamona monticola* adult seasonal profile presented on sticky cards at Mercer County Community College (MCCC), Duke Farms (Duke), Rutgers Garden (Rutgers), and Riverview Cemetery (Trenton) during 2002-2006.

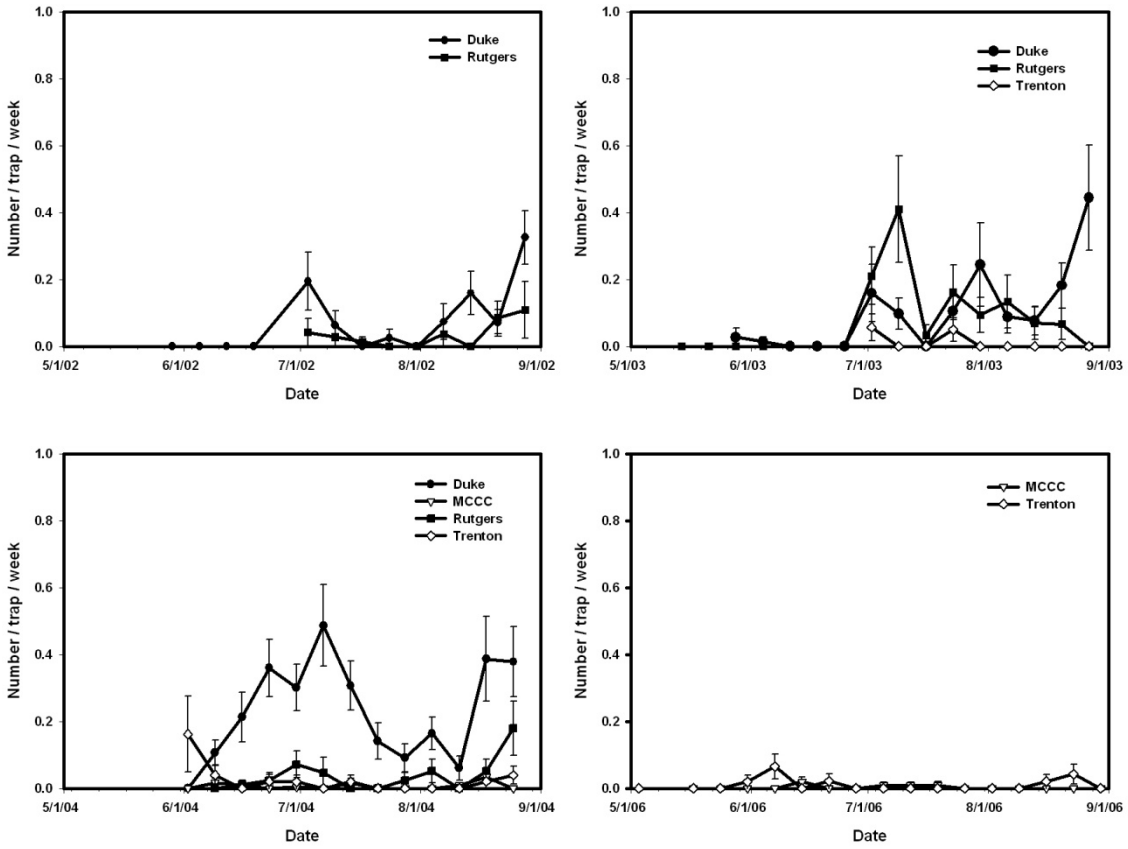


Fig.7. *Telamona tiliae* adult seasonal profile presented on sticky cards at Mercer County Community College (MCCC), Duke Farms (Duke), Rutgers Garden (Rutgers), and Riverview Cemetery (Trenton) during 2002-2006.

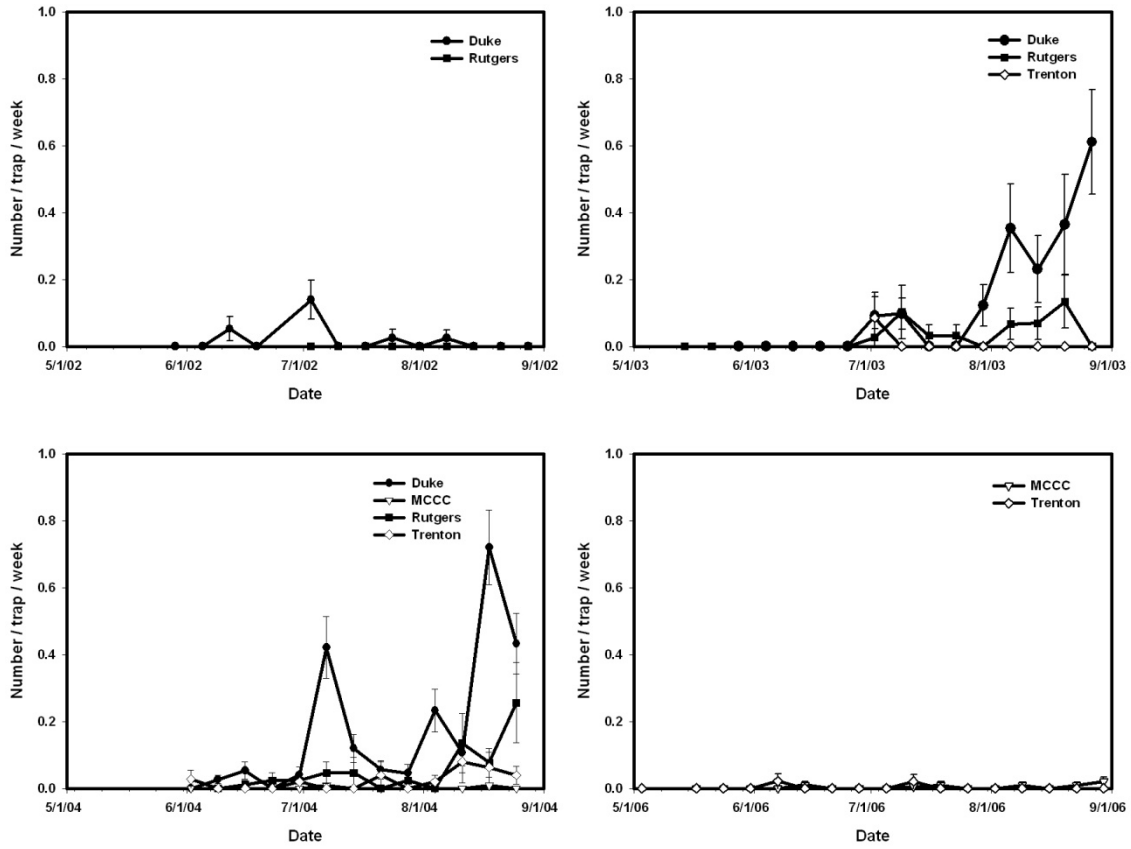


Fig.8. *Archasia belfragei* adult seasonal profile presented on sticky cards at Mercer County Community College (MCCC), Duke Farms (Duke), Rutgers Garden (Rutgers), and Riverview Cemetery (Trenton) during 2002-2006.

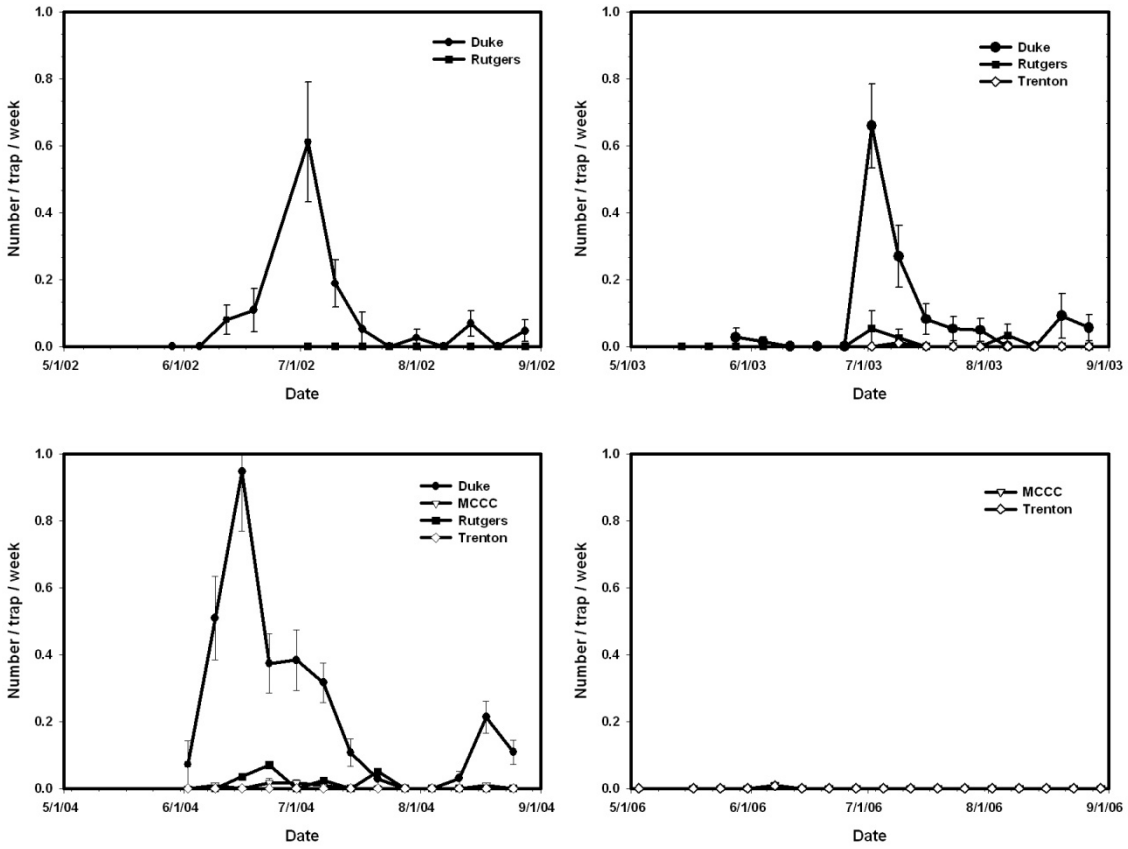


Fig.9. *Glossonutus acuminatus* adult seasonal profile presented on sticky cards at Mercer County Community College (MCCC), Duke Farms (Duke), Rutgers Garden (Rutgers), and Riverview Cemetery (Trenton) during 2002-2006.

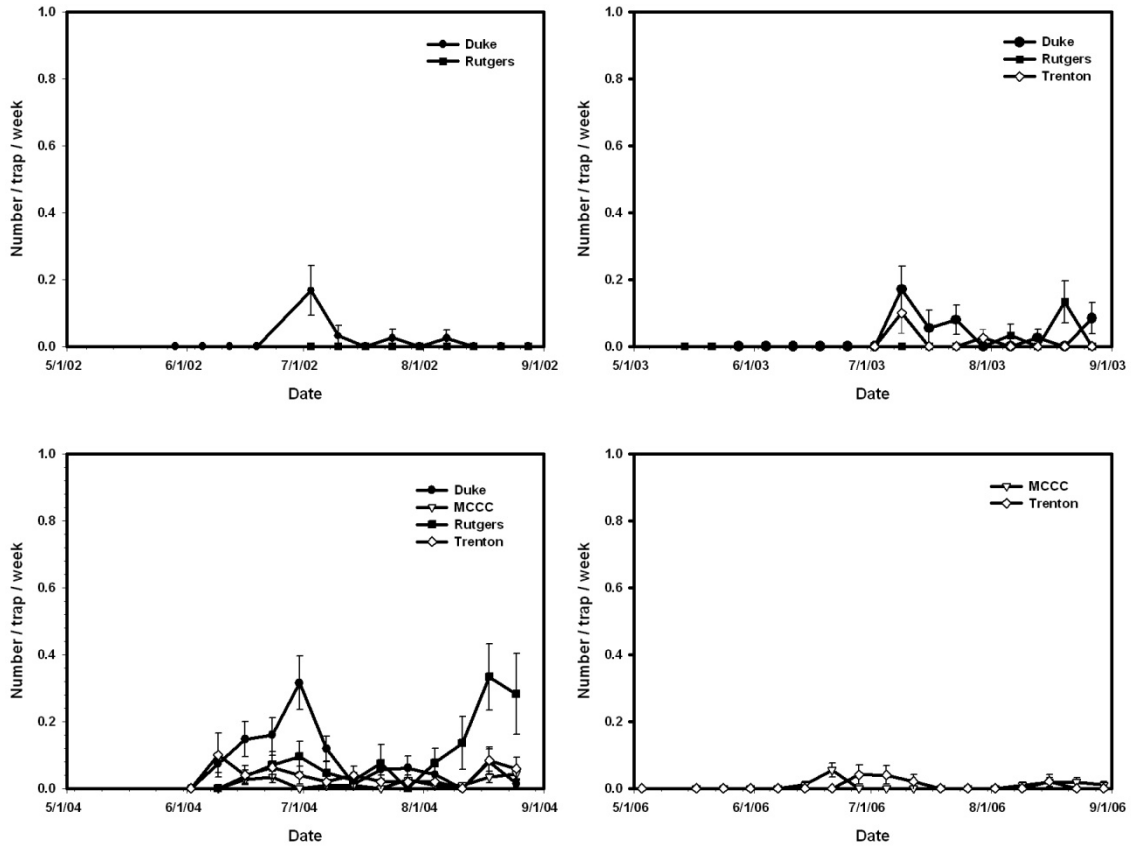


Fig.10. *Cyrtolobus fenestratus* adult seasonal profile presented on sticky cards at Mercer County Community College (MCCC), Duke Farms (Duke), Rutgers Garden (Rutgers), and Riverview Cemetery (Trenton) during 2002-2006.

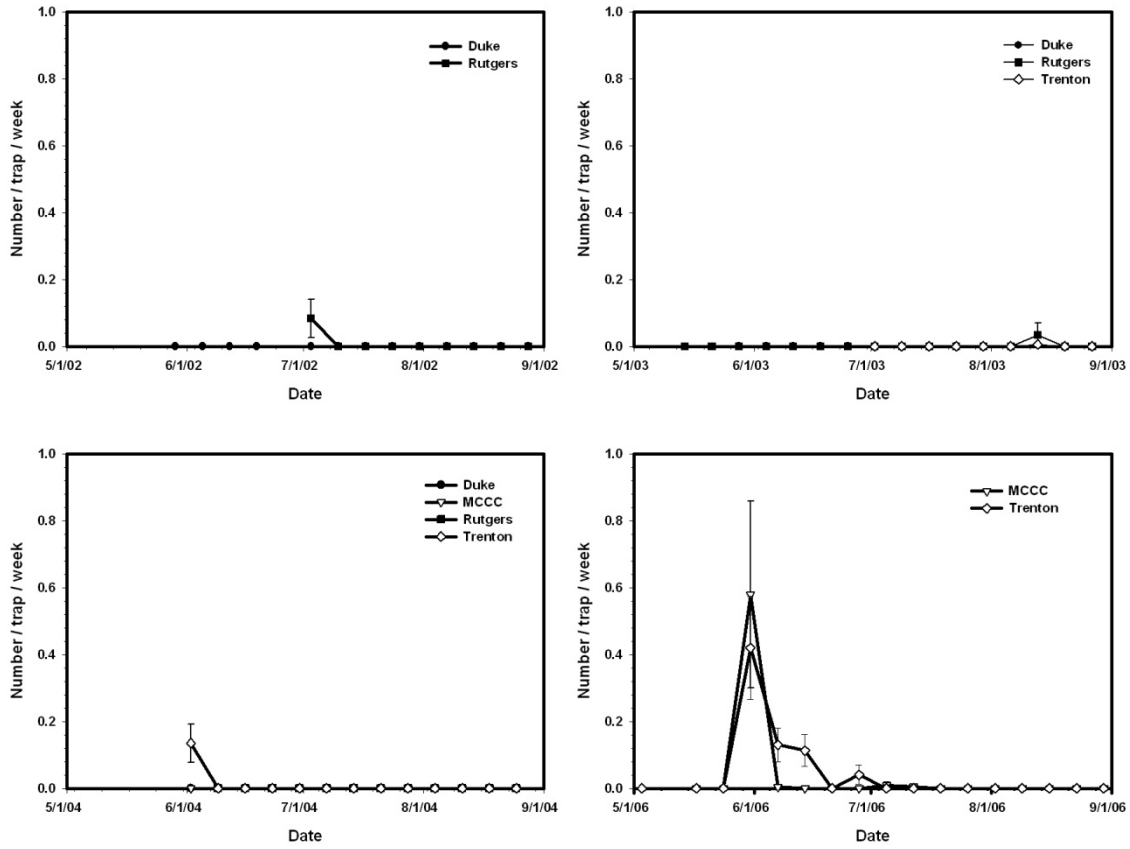


Fig.11. *Xylella fastidiosa* DNA detection from Cicadellinae leafhoppers collected in the summer season in oak canopies in central New Jersey during 2002-2006.

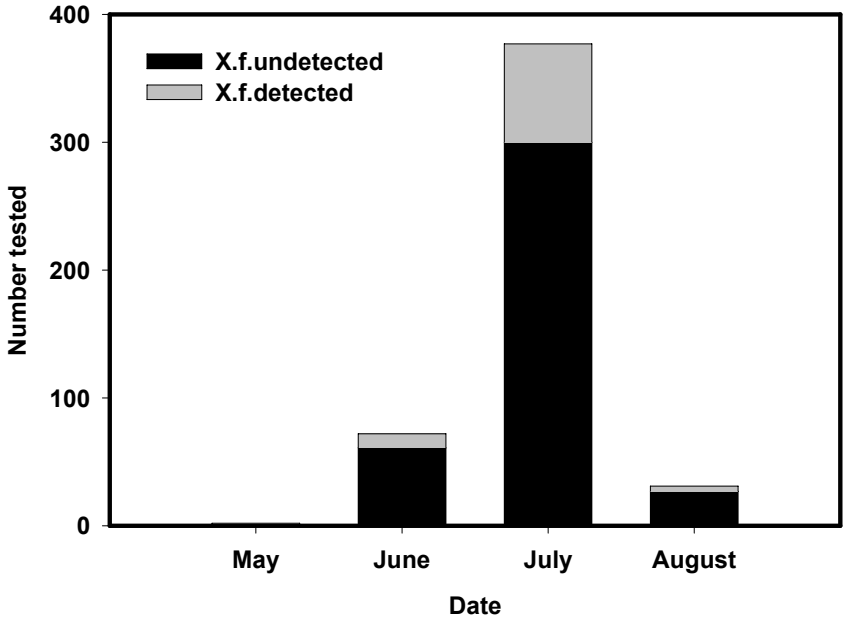


Fig.12. *Xylella fastidiosa* DNA detection from Cercopoidea spittlebugs collected in summer from oak canopies in central New Jersey during 2002-2006.

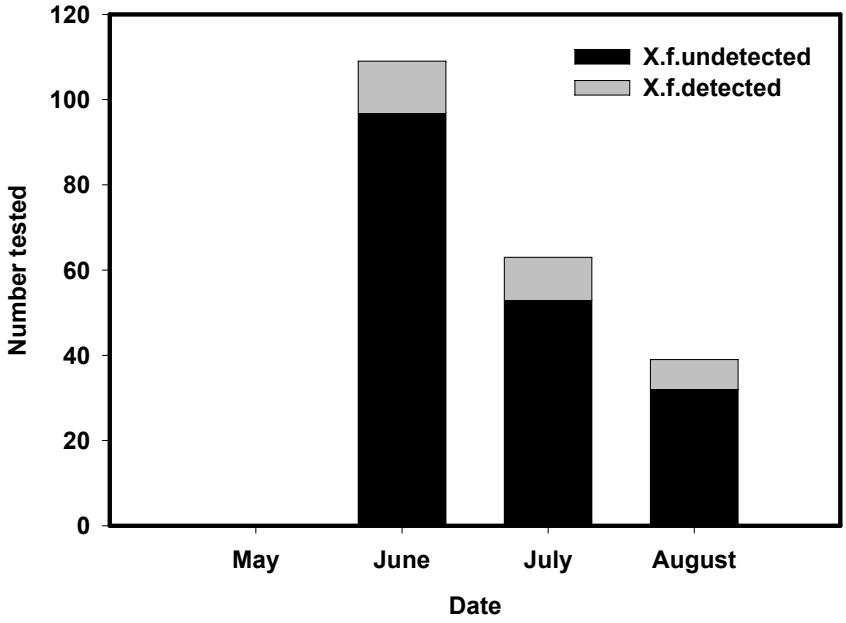


Fig.13. *Xylella fastidiosa* DNA detection from Membracoidea treehoppers collected in summer from oak canopies in central New Jersey during 2002-2006.

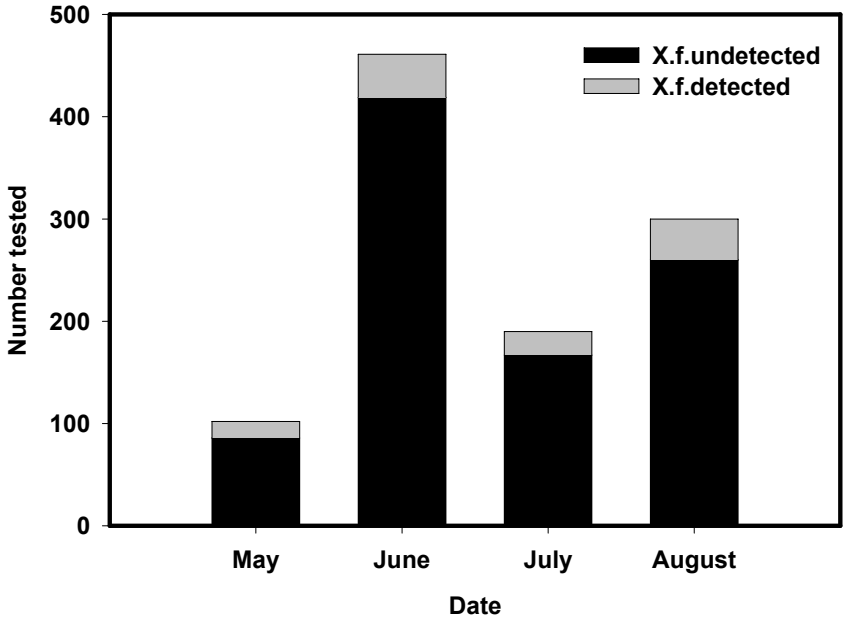


Fig.14. Total xylem feeding Cicadomorpha insect population abundance in pin oak canopies sampled by fogging method at Duke Farms (Duke) and Mercer County Community College (MCCC) in 2004 and 2006. The total number of xylem feeders and the sum of Cicadomorpha leafhoppers, treehoppers, and spittlebugs was significantly higher at Duke Farms than that at MCCC ($F_{1, 74} = 26.04$, $p < 0.0001$). Total number of xylem feeders collected in 2006 was greater than that in 2004 ($F_{1, 74} = 3.47$, $p = 0.0665$), which was due to the significantly greater vector population density in May rather than in later months ($F_{3, 74} = 81.10$, $p < 0.0001$). There was a significant interaction between the location of Duke Farms vs. MCCC and the time (month) of the year on the total xylem feeder population ($F_{3, 74} = 9.03$, $p < 0.0001$). The average number of xylem feeders collected from asymptomatic pin oaks was 90.05 ± 20.31 per tree, which was 60.53% significantly greater than 56.10 ± 17.06 per tree collected from symptomatic pin oaks ($F_{1, 74} = 8.65$, $p = 0.0044$).

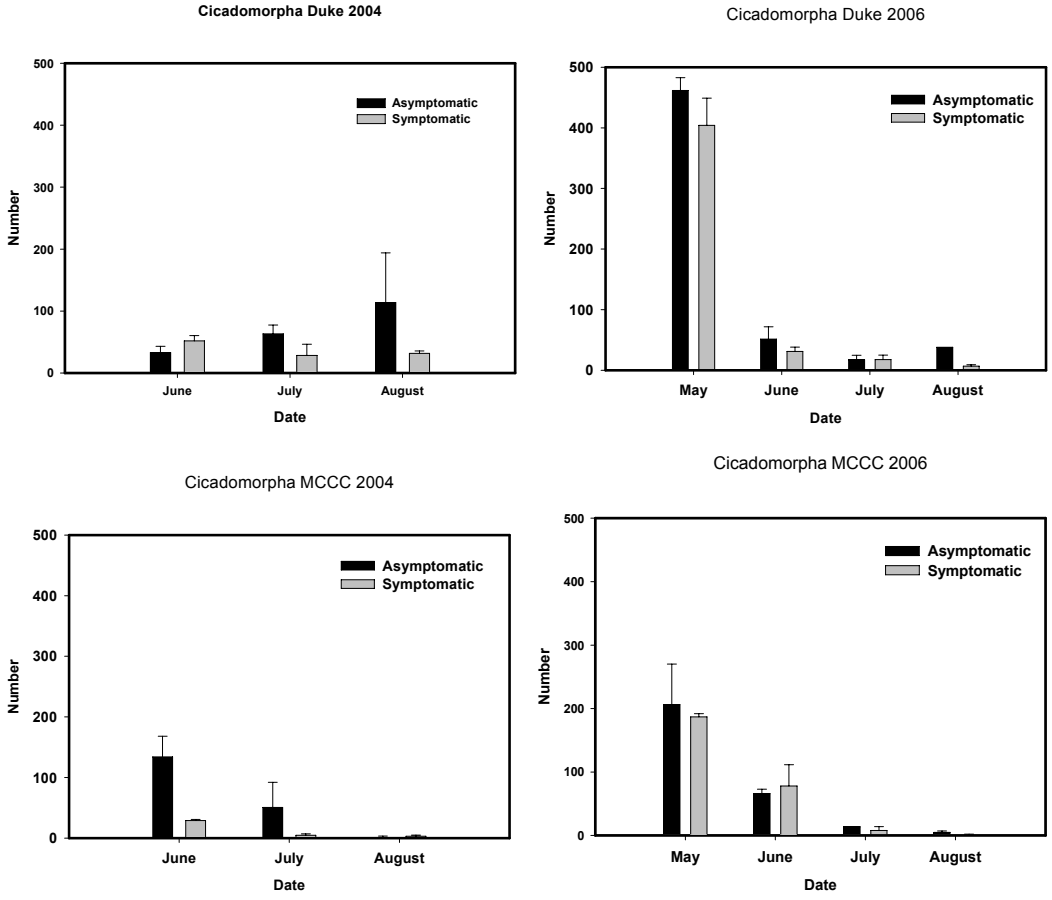


Fig.15. Membracidae population abundance in pin oak canopies sampled by fogging method at Duke Farms (Duke) and Mercer County Community College (MCCC) in 2004 and 2006. Membracidae population abundance varied with location and time of year ($F_{3, 74} = 8.99$, $p < 0.0001$). Duke Farms had the highest Membracidae population in May 2006 when compared to all other times at MCCC or Duke Farms. Membracidae population abundance (86.86 ± 20.30 per pin oak) in asymptomatic pin oaks was 62.64% significantly greater than that found in BLS symptomatic oaks (53.40 ± 17.04 per oak) ($F_{1, 74} = 8.57$, $p = 0.0045$).

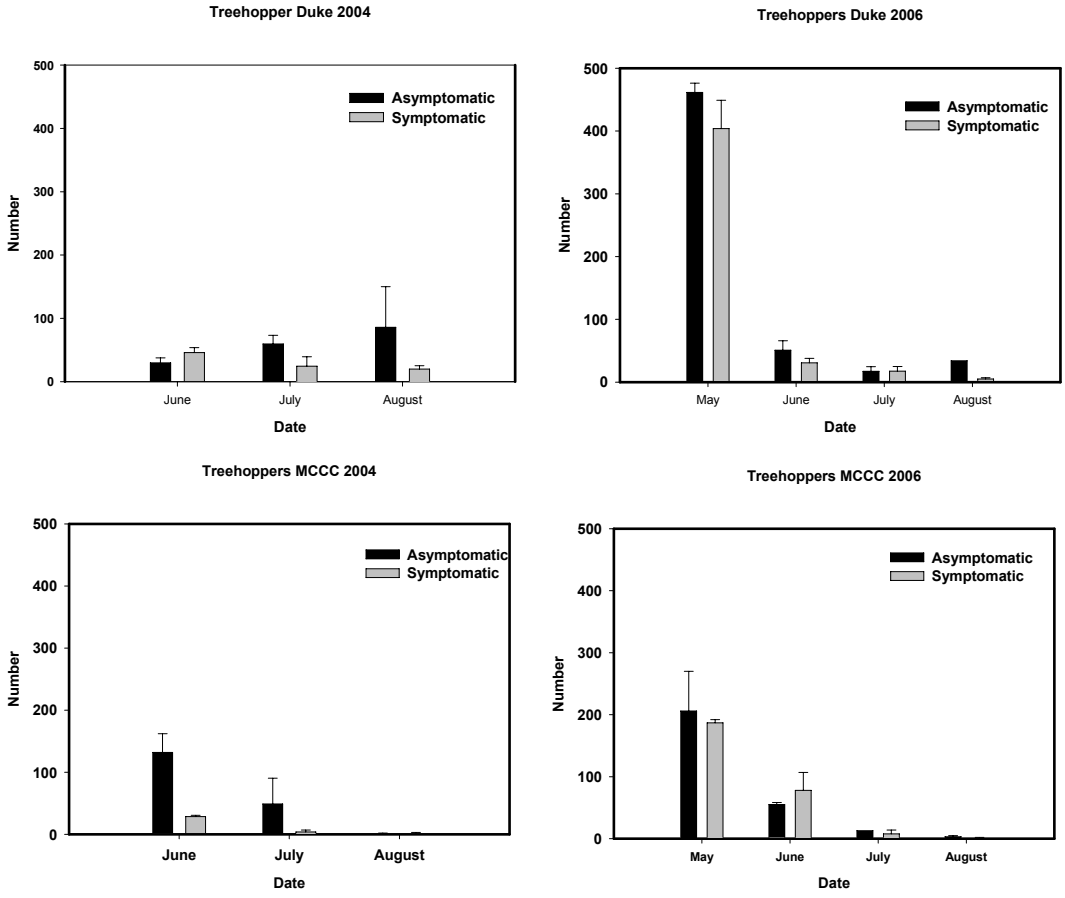


Fig.16. Cicadellinae population abundance in pin oak canopies sampled by fogging method at Duke Farms (Duke) and Mercer County Community College (MCCC) in 2004 and 2006. Significantly more Cicadellinae leafhoppers, 0.29 ± 0.10 per tree found in the asymptomatic tree samples than 0.21 ± 0.13 per tree in BLS symptomatic trees ($F_{1, 74}$ = 8.57, p = 0.0045). Also, there were more leafhoppers found in early season at MCCC, while more leafhoppers were found during late season at Duke Farms ($F_{3, 74}$= 8.99, p < 0.0001).

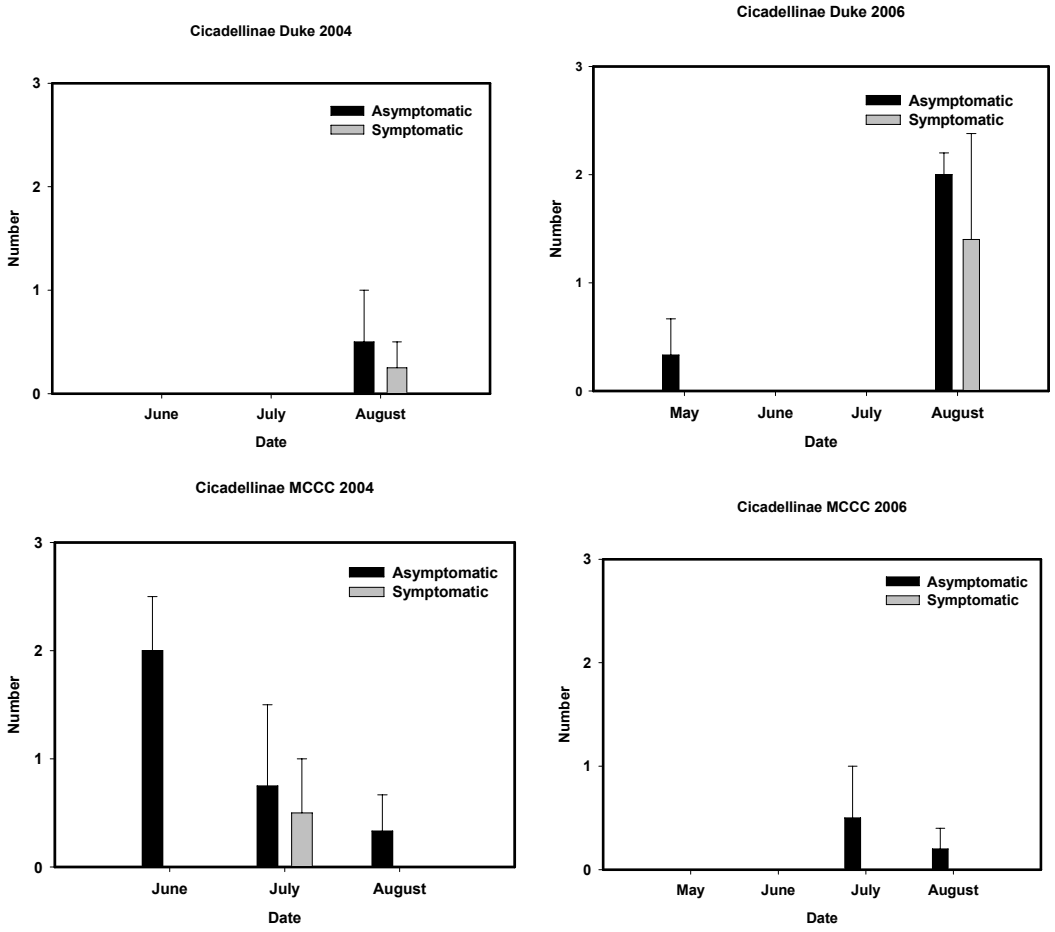


Fig.17. Spittlebug population abundance in pin oak canopies sampled by fogging method at Duke Farms (Duke) and Mercer County Community College (MCCC) in 2004 and 2006. Significantly more spittlebugs were found in June than in other months ($F_{3, 74} = 6.55$, $p = 0.0005$). Few spittlebugs were found in 2006 at Duke Farms or in 2004 at MCCC compared to those found in 2004 Duke Farms and in 2006 at MCCC. *Xylella fastidiosa* infection showed no significant impact on the spittlebug population density ($F_{1, 74} = 1$, $p = 0.3216$).

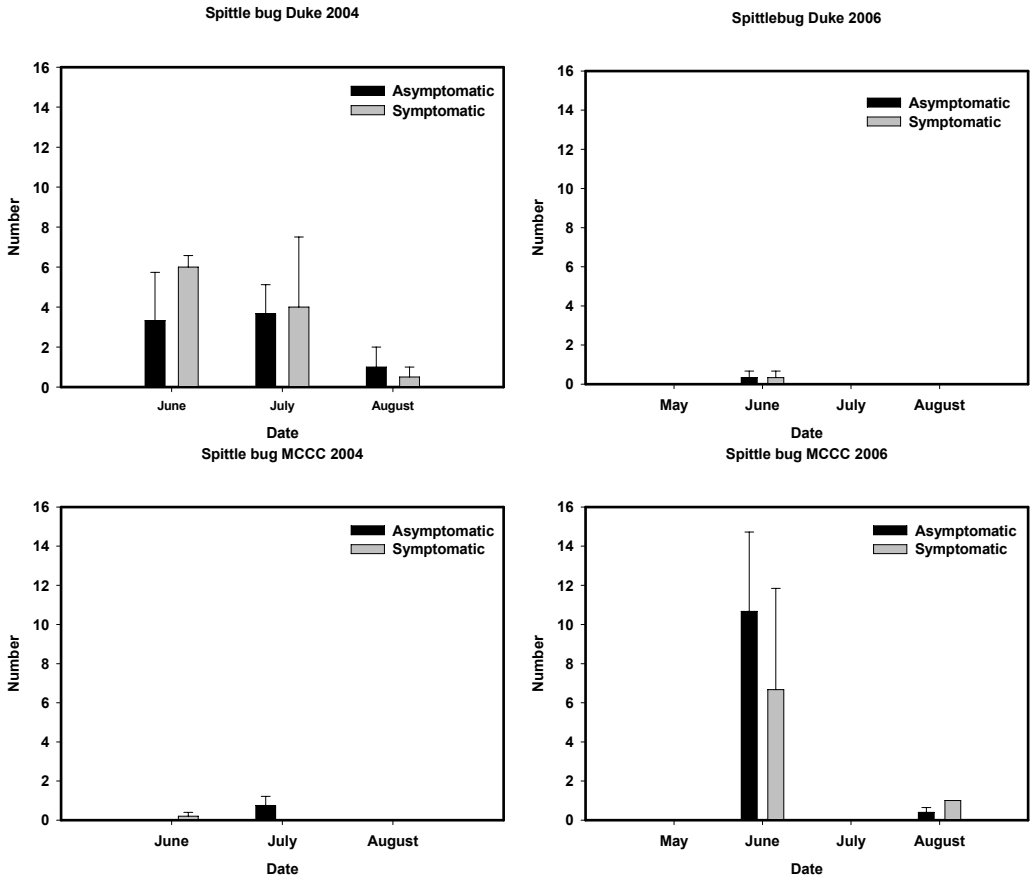


Fig.18. Total xylem feeder population seasonal dynamics from BLS infected and asymptomatic pin oaks at Mercer County Community College in 2004 and 2006. There were no significant impact of BLS infection on total xylem feeding insect populations ($F_{1, 3164} = 0.32$, $p = 0.5723$).

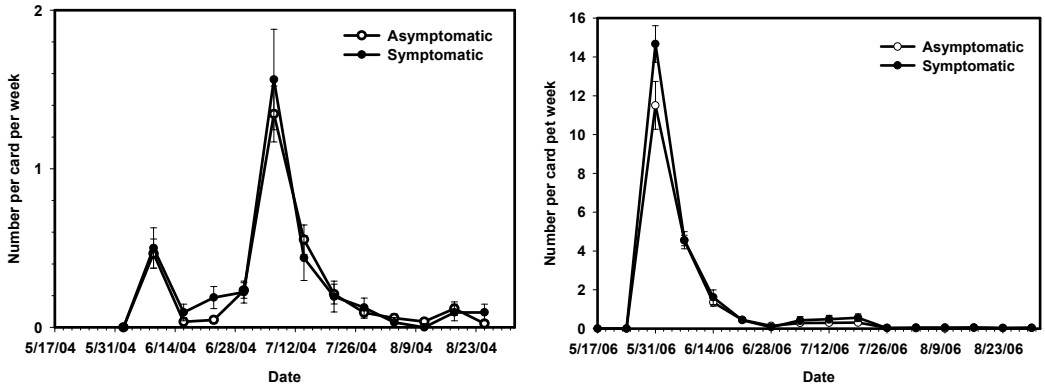


Fig.19. *Ophiderma definita* population seasonal dynamics from BLS infected and asymptomatic pin oaks at Mercer County Community College in 2004 and 2006. *O. definita* sticky card catches were significantly higher in 2006 when compared to that of 2004 ($F_{1, 3164} = 708.31$, $p < 0.0001$).

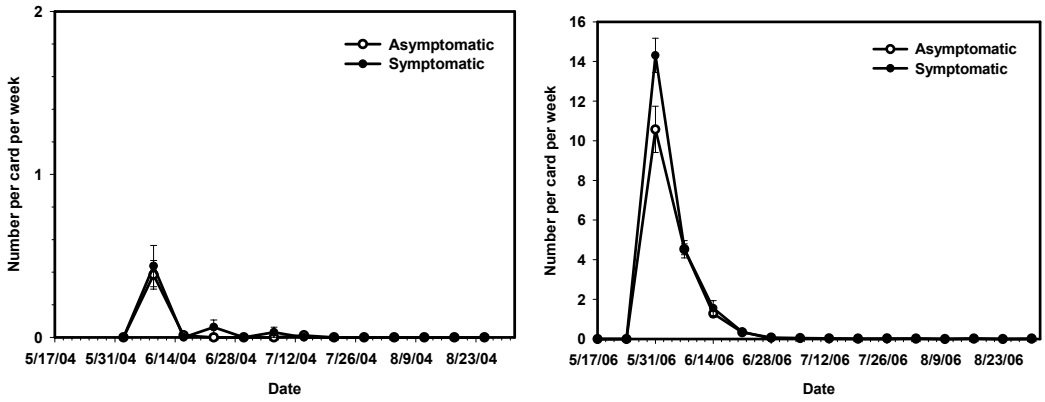


Fig. 20. *Graphocephala versuta* population seasonal dynamics from BLS infected and asymptomatic pin oaks at Mercer County Community College in 2004 and 2006. No significant difference for the leafhopper *G. versuta* was seen between BLS symptomatic pin oaks and asymptomatic oaks at MCCC ($F_{1, 3164} = 0.28$, $p = 0.5976$).

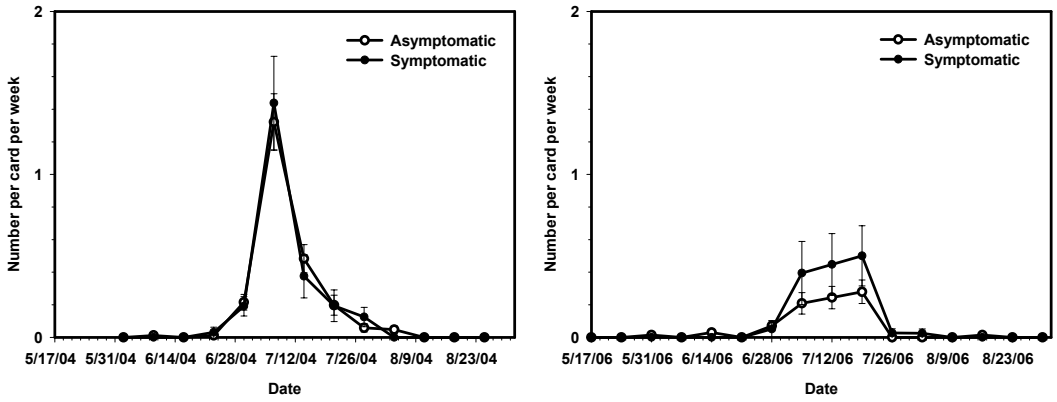


Fig. 21. Total xylem feeder population seasonal dynamics from BLS infected and asymptomatic pin oaks at Duke Farms in 2003 and 2004. No significant differences in sticky card trap catches were detected between BLS symptomatic and asymptomatic pin oaks at Duke Farms ($F_{1, 1840} = 0.70$, $p = 0.4012$).

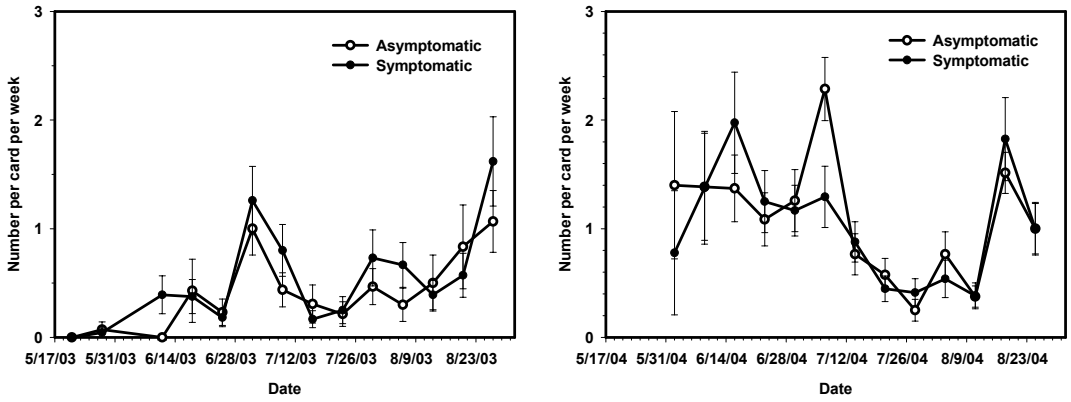


Fig. 22. *Ophiderma definita* population seasonal dynamics from BLS infected and asymptomatic pin oaks at Duke Farms in 2003 and 2004. No significant impact of the BLS infection was detected on the *O. definita* ($F_{1, 1840} = 0.00$, $p = 0.9842$)

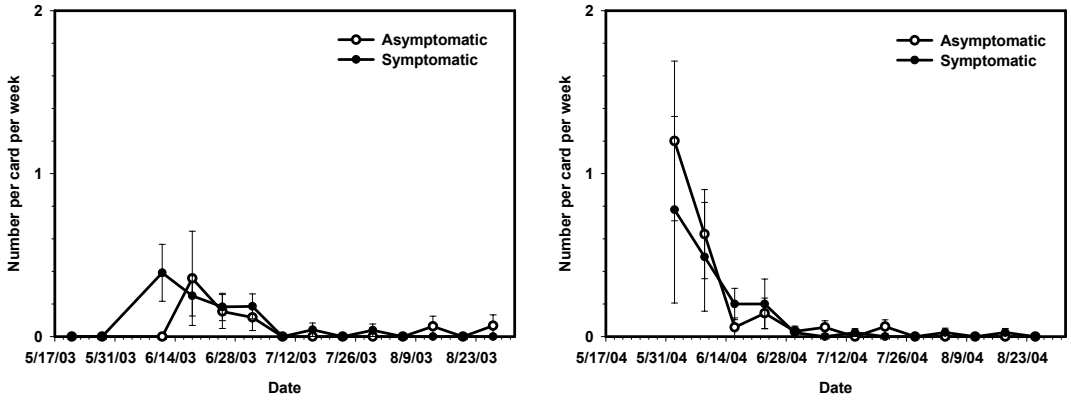


Fig. 23. *Graphocephala versuta* population seasonal dynamics from BLS infected and asymptomatic pin oaks at Duke Farms in 2003 and 2004. No significant impact of the BLS infection was detected on the *G. versuta* ($F_{1, 1840} = 0.04$, $p = 0.8447$).

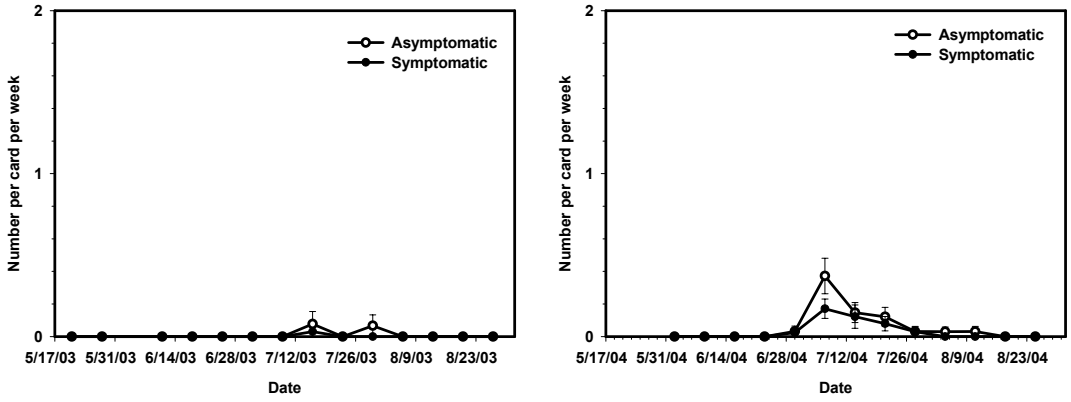
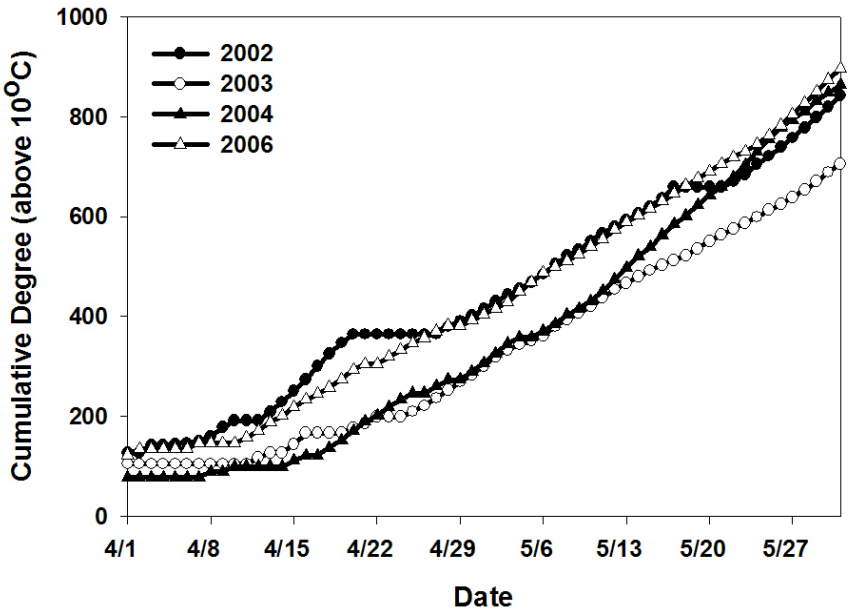


Fig. 24. Cumulative effective temperature (>10^{o}C since first day of the year) in central New Jersey between April and May during 2003-2006.



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