EFFECTS OF LOW TEMPERATURE ON POPULATIONS OF *XYLELLA FASTIDIOSA* IN SYCAMORE

by

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(Under the Direction of Katherine L. Stevenson)

ABSTRACT

To determine the effects of low temperature on populations of the bacterial plant pathogen, *Xylella fastidiosa*, in American sycamore hosts, field and laboratory experiments were conducted to examine population dynamics in both naturally and mechanically infected host plants. Bacterial presence, population density estimates, and bacterial distribution in host tissue were analyzed in relation to the extraction method used, environmental air and soil temperature and rainfall, low temperature treatment, host resistance, and reciprocal transmissibility of three strains of *X. fastidiosa* from sycamore, oak, and grape. Results indicate that increasing hours below -5ºC were best associated with reductions in bacterial populations in field trees at two sites. No relationship was detected between bacterial populations and either low temperature treatment at 5ºC or putative host resistance. Host reciprocity tests suggest significant differences in density and distribution of the three bacterial strains within their reciprocal hosts.

INDEX WORDS: *Xylella fastidiosa*, *Platanus occidentalis*, American sycamore, Bacterial leaf scorch, Low temperature treatment, Xylem-limited bacteria

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DEDICATION

With nine kinds of love to my husband, Jim. Can’t stop the magic . . .

To Mom and Thomas, for all of your faith and support.

You are the wind beneath my wings.

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CHAPTER 1

INTRODUCTION

Introduction to *Xylella fastidiosa* and the Diseases It Causes

Xylella fastidiosa Wells et al. has intrigued researchers since 1892, when N.B. Pierce first reported the presence of "bacteria-like bodies" in grapevines with leaf scorch symptoms (57). Although isolation attempts were long unsuccessful (34, 38, 57) these endophytic microbes were proposed to be the causal organisms of what is now known as Pierce's disease of grape, which affects many *Vitis* species. In the century that followed the discovery of Pierce's disease, *X. fastidiosa* was implicated as the cause of a widening array of plant diseases on diverse hosts encompassing 30 or more families of monocotyledonous and dicotyledonous plants (69). Among these are economically important diseases, including Pierce's disease of grape (*Vitis vinifera* L., *V. labrusca* L., and *V. rotundifolia* Michaux) (14), phony peach disease (*Prunus persica* [L.] Batsch) (75), alfalfa dwarf (*Medicago sativa*) (23), periwinkle wilt (*Catharanthus roseus* [L.] G. Don) (51), citrus variegated chlorosis (*Citrus sinensis* [L.] Osb.) (7, 28), leaf scorch of Japanese plum (*Prunus salicina* Lindl.) (64), almond (*Prunus dulcis* [Mill.] D.A.Webb) (55), pear (*Pyrus* spp.) (47), coffee (*Coffea arabica* L.) (18), mulberry (*Morus* spp.) (43), and, recently, pecan (*Carya illinoensis* [Wang.] K. Koch) (66). These diseases are threatening to the agricultural and horticultural industries where they occur; phony peach and plum leaf scald cause severe damage to fruit production in the southeast; and it has been suggested that *X. fastidiosa* is a major factor limiting grape production in the southeastern United States (38). Leaf scorch diseases caused by *X. fastidiosa* afflict many species of ornamentals and forest trees including elm (*Ulmus* spp.) (45), sycamore (*Platanus* spp.) (68), oak (*Quercus* spp.) (8, 10, 26, 29), sweetgum (*Liquidambar styraciflua*), maple (*Acer* spp.) (26, 27, 71), oriental bittersweet (*Celastrus orbiculata*), English ivy (*Hedera helix*), flowering dogwood (*Cornus florida*), buckeye (*Aesculus* x hybrid) (52), oleander (*Nerium oleander*) (12), blackberry (*Rubus* spp), bermudagrass (*Cynodon dactylon*), and mugwort (32), as well as other ornamental and weedy *Artemisia* species (20). Economic losses on forest and ornamental hosts are more difficult to assess, given the less tangible nature of values attributed to such aesthetically pleasing, multifunctional, and often long-lived species. Direct economic losses caused by *X. fastidiosa* in these hosts include loss in pulp and lumber volume, stock losses for landscape plants infected before sale, and replacement value of plantation and landscape plants. Indirect losses include the high-priced and long-term costs of therapy and management strategies (if available), aesthetic devaluation of landscapes, parks, and forests, and overall decline in plant vigor.

The 80-year span between first observation of the causal bacterium by Pierce in 1892 (57) and the completion of Koch's postulates for *X. fastidiosa* in 1978 by Davis et al. (14) seems a long time, especially in relation to the extensive range of affected hosts, and the nonselectively feeding, efficient, and voracious vectors (33). Following Pierce's initial observations of "bacteria" in 1892, understanding of the pathogen was greatly limited until the late 1930s, when phony peach disease and alfalfa dwarf were attributed to the newly characterized viral plant pathogens, due to their graft and vector transmissibility and noncultivability in vitro (42). This view persisted until 1972, when discovery of mycoplasma-like organisms (MLOs) led to improved understanding that the causal organisms for these various diseases were, in fact, xylem-limited, rickettsia-like bacteria -- first observed ultrastructurally in almonds with leaf scorch in 1973 (49, 50). In the decade after in vitro culture of *X. fastidiosa* had been achieved (14), serological methods including immunogold, immunofluorescence, and enzyme linked immunosorbent assay (ELISA) techniques were first employed, greatly improving ability to detect the pathogen (3, 4, 70). More recently, molecular techniques including DNA-DNA hybridization and polymerase chain reaction (PCR) have served not only as tools for detection, but have also provided valuable information about the taxonomic relationships among strains of *X. fastidiosa* and between *X. fastidiosa* and other bacterial plant pathogens (2, 9, 10, 54). Perhaps the most significant advancement to date is the genomic mapping of the citrus variegated chlorosis strain of *X. fastidiosa* (72). The first such mapping of a plant pathogen, this project has begun to yield more detailed and potentially useful knowledge about specific genetic mechanisms of pathogenicity, the identity and location within the genome of individual genes, and the possible implications for management of diseases caused by this bacterium (22).

Sycamore Leaf Scorch, Dieback, and Decline Caused by *X. fastidiosa*

Historically, research priority has been limited almost exclusively to the economically threatened hosts of *X. fastidiosa*, while many of the other "minor" hosts of this pathogen, including forest and landscape species that suffer leaf scorch and decline symptoms, have received little or no attention (34, 38, 62). However, it has been suggested that the role played by *X. fastidiosa* in the untimely decline and death of many forest, shade, and plantation trees may, in fact, result in the greatest overall losses (38).

American sycamore (*Platanus occidentalis* L.) is grown commercially for lumber, pulp, and other valuable wood products due to its rapid growth rate, ease of culture, minimal site preparation requirements, and excellent fiber quality (5, 73). *P. occidentalis* is one of the largest hardwood species in eastern deciduous forests, with the largest diameter of any of the North American hardwoods, and has been referred to as "a behemoth in the world of trees" (5, 19). Coarse-grained and tough sycamore wood is used for furniture, boxes, crates, and butcher blocks (19). As a landscape ornamental, sycamore is valued for its striking bark that exfoliates on the upper trunk to reveal creamy-white inner layers, its broad crown and massive branches, and its overall magnitude.

Another advantage of this species is its broad native range which extends from Maine to Ontario and Minnesota south to Florida and Texas into northeastern Mexico (5, 19). *P. occidentalis* is also known for the high degree of geographic variation within the species; trees from southern points of origin grow more rapidly than do their northern counterparts when planted near or slightly north of their provenances (76). This geographic differentiation along a north-south gradient also plays a role in disease resistance, most notably to stem canker pathogens (11) and, it has been suggested, to *X. fastidiosa*.

Sycamore leaf scorch, decline, and dieback caused by *X. fastidiosa* is the most common pathogen of sycamore in the southeastern U.S. (46). Incidence of this disease seems to have increased, possibly due to increased ornamental planting, and a resurgence of interest by paper companies in growing plantation sycamore to meet growing demands for hardwood fiber. Sycamore demonstrates exciting growth potential in short-rotation fiber farming tests for both pulp and bioenergy feedstock fuel production (5, 73). Air pollutants, insects, and disease all threaten the health of sycamore trees in the landscape and in commercial production, but diseases are estimated to be the primary limiting factor in sycamore production, especially in large monoculture situations where losses can be rapid and potentially absolute (73). Although it is one of few hardwood species used in commercial plantations, the threat of severe losses to leaf scorch and decline diseases in sycamore has limited commercial sycamore planting in the southeastern U.S. until economically feasible disease management strategies are discovered (46).

Sycamores infected with *X. fastidiosa* often begin to show foliar symptoms during mid-to-late summer in the form of interveinal and marginal chlorosis. This yellowing usually precedes the onset of marginal necrosis, leaf curling, and complete death of affected leaves, which frequently remain attached to the branch (68). Wilting does not occur in relation to this or most other leaf scorch diseases caused by *X. fastidiosa* (25). Leaf scorch symptoms tend to appear first on basal leaves of seedlings or on older leaves of individual branches of larger trees in late summer or earlier, if water stress occurs (29, 43). Bacterial leaf scorch of sycamore is often misdiagnosed as sycamore anthracnose (*Apiognomonia veneta*) which also produces foliar necrosis and dieback symptoms (46); however, anthracnose occurs early in the season, on new foliage, under cool, moist spring conditions, and infected leaves are shed once the average temperature exceeds approximately 19ºC (43, 46, 56, 69).

Initially, symptoms of infection by *X. fastidiosa* may be limited to one or two branches in the canopy but, in subsequent growing seasons, the upper canopy declines and the disease then appears to progress downward through the crown in a basipetal pattern (46). Epicormic sprouts are also common on trees with sycamore decline, a symptom of disruption of the healthy flow of water and nutrients (43). Infected trees may exhibit delayed leaf flush, reduced seed set, reduced vigor in later years, and ultimately death. The length of time between initial infection by *X. fastidiosa* and tree death is variable, ranging from three to 10 years (43, 46). This variation may arise from differences in climactic factors, cultural conditions, pathogen strain virulence, host resistance, and other unknown factors.

Biology of *X. fastidiosa*

X. fastidiosa is a gram-negative, xylem-limited prokaryote which has been characterized as fastidious due to its slow growth and strict requirement for specialized culture medium. These mediums must incorporate starch, bovine serum albumin, or acid-washed activated charcoal to control inhibitors of host-tissue origin during primary isolation (6, 15, 74). The rod-shaped bacterial cells are approximately 0.25 to 0.35 μm by 0.9 to 3.5 μm, with a cell wall that is often heavily wrinkled or rippled -- a trait that led to their initial comparison with the Rickettsiae (38, 74). Cell walls can be smooth-textured or lightly rippled (intermediate), and it has been suggested that there is a correlation between rippled cell wall morphology and cell maturity (41). Cells of *X. fastidiosa* divide by binary fission and often remain attached to one another in short chains after division (41, 49, 74). Although this bacterium is aflagellate, observations with scanning and transmission electron microscopy (SEM and TEM, respectively) have revealed peritrichous fimbriae or "micro-fibrils" growing most densely around the cells' polar regions (41, 68); their function is not well established. On periwinkle wilt (PW) medium (8, 16), colonies are discrete and circular with a creamy translucent or opalescent color. There is no detectable odor associated with this bacterium in culture. Individual colony size can range from 0.5 mm after 10 days up to 1.5 mm after four or more weeks (74).

It is surprising that *X. fastidiosa* should cause so much damage in such a broad range of hosts, given its habitat constraints. It lacks motility and is only able to survive in host xylem, vector foreguts, or in vitro culture (14, 32, 33). In fact, this prokaryote has evolved elegant solutions to its limitations: for dissemination it relies on vector transmission and for transport it utilizes xylem translocation. *X. fastidiosa* is transmitted from host to host by xylem sap-feeding insects in the order Homoptera, including spittlebugs, sharpshooters, and leafhoppers, major vectors of *X. fastidiosa* in the United States (32, 33, 66). Specific vectors transmitting *X. fastidiosa* in sycamore are unknown. Bacteria must multiply within the host or alternative host until they reach a threshold population level, approximately 10^4 colony forming units (CFU) per gram of host tissue, before efficient vector acquisition can occur, with increasing acquisition efficiency as *X. fastidiosa* populations exceed 10^8 CFU/g (32, 33). Inside the vector, the bacteria are non-circulative and multiply only in the mouth parts and foregut (61). Vector infectivity is lost after molting, but mature adults who acquire *X. fastidiosa* retain the pathogen for life (31, 61).

After vectors have ingested the bacteria, they may transmit it immediately; that is, there is no latent period required for vector transmission (31). Bacteria are introduced into the xylem tissue of the host during vector feeding. Purcell and Saunders (63) posit three possible fates for *X. fastidiosa* inside the plant host. First, the host is either propagative or non propagative; if the cells do not multiply to the acquisition threshold, no further transmission will occur. Second, the bacteria either move systemically within the host or they do not. Hill and Purcell (32) estimate that cell concentrations must reach 10^7 to 10^9 CFU/g before systemic movement occurs. Systemic movement increases the capacity of infected plants to provide sources of inoculum and reduces dependency on vectors for spatio-temporal spread. Third, the host is pathological or non-pathological; that is, it either exhibits symptoms from pathogen infection or it does not (63). As a host for *X. fastidiosa*, sycamore is propagative, systemic, and highly pathological (29, 69, 70). Symptom development depends strongly on a number of factors including the cultural environment, host age and general health at the time of infection, host resistance traits, growth and respiration rates (24). However, estimates of the incubation period range from 6 weeks to 6 months for seedlings in greenhouse and field conditions respectively (68), and longer than 6 months in mature trees in field situations (29, 43, 46, 68). As symptoms progress, hosts become reservoirs of inoculum for secondary spread, which is most common late in the growing season from late July through the onset of dormancy at the end of October or the beginning of November (32, 68). A threshold population of approximately 10^6 CFU/cm of leaf vein was reported as the requirement for symptom development in grapevines (37).

Management of Sycamore Decline and Dieback Caused by *X. fastidiosa*

Disease management for *X. fastidiosa* creates as much of a dilemma for researchers today as the cultural limitations of this organism did in past decades. Overlooked or misdiagnosed until its relatively recent identification, *X. fastidiosa* has almost no labeled chemical control measures. Two products under current investigation are acibenzolar-S-methyl (marketed as Actigard, Syngenta Crop Protection, Greensboro, NC) and oxytetracycline antibiotics (marketed as MycoJect, J.J. Mauget Company, Arcadia, CA). Acibenzolar-S-methyl is a foliar-applied systemic acquired resistance (SAR) compound (44), a synthetic analogue of salicylic acid, which promotes host synthesis of a variety of defense related proteins. Oxytetracycline antibiotics injected into the host tissue inhibit pathogen protein synthesis by binding to bacterial ribosomes (53). Both compounds have, when used on sycamore, resulted in symptom remission, but not complete cure (56). Antibiotics and SAR materials are quite costly and time consuming to apply since they must be reapplied to the infected host plants at least twice annually (65). These chemical management tools can also create their own problems. Actigard is used a prophylactic measure and, therefore, requires four or more days to take effect. After application, translocation of Actigard within the plant may be difficult to achieve and phytotoxic effects have also been observed when hosts are stressed at application or the compound is applied too frequently (44). Additionally, research on Actigard treatments has not yielded sufficient data on efficacy and impact of this product over the long term. Oxytetracycline treatment is only successful as long as maintenance of the chemical therapy continues since eradication of the pathogen, once systemic, is impossible without using cost-prohibitive dosages likely to harm the host. If injections are discontinued, symptoms soon reappear and rapid decline follows. Injection boreholes are another drawback of oxytetracycline treatment, such deep wounds in the tree-host can create points of ingress for secondary pathogens (40). Further, the environmental and resistance issues associated with long term use of antibiotics on plants are still a dominant concern for growers and consumers alike.

Vector control has been considered as a management measure, but effectiveness is limited by the indiscriminant feeding patterns of the insect vectors. One of the vectors, *Homalodisca coagulata*, has been reported to feed on 75 species of plants in 35 families. Conventional insect control measures might actually exacerbate disease incidence by increasing vector feeding activity, altering flight patterns, and increasing inoculation of potential alternative host plants by causing insect migrations (14, 33, 44, 52). In addition to increased risks that might be encountered in attempting to control vector populations, these types of vectors -- leafhoppers, sharpshooters, and spittlebugs -- are ubiquitous in the environment and would be difficult, if not impossible, to eradicate (58). For example, assuming 95% of vectors could be killed, a perennial host exposed continuously to even 5% of normal leafhopper populations is still quite likely to be infected.

Because the causal bacterium is generally systemic within its sycamore host, sanitation is not a plausible control measure. Infected regions cannot be pruned out as readily as with other bacterial pathogens, like *Erwinia amylovora*, the causal agent of fire blight of Rosaceaeous trees and shrubs. By the time symptoms caused by *X. fastidiosa* appear, bacterial populations have already surpassed the threshold for systemic movement in the host (32, 34). Furthermore, leafhoppers generally feed where probing has indicated that sap is flowing (31), so removing leaf litter is unlikely to reduce incidence of sycamore leaf scorch. Another form of sanitation that has been considered is the elimination of alternative hosts in regions surrounding host plantings (63). However, the exceptionally wide range of feeding hosts for the vectors and the equally wide host range of *X. fastidiosa* render this approach unworkable. In practice, the only effective sanitation strategy for *X. fastidiosa* is removal and destruction of infected trees, taking care that the species not be replanted in the same location (8, 34, 69).

The most promising management strategy for *X. fastidiosa* at this time is genetic engineering and breeding for improved host resistance. For many high value crop hosts, genetic engineering is preferred because of the length of time required for conventional breeding and seed production in long-lived perennial hosts such as grape, peach, and sweet orange (62). In sycamore, however, conventional breeding is the standard means of obtaining disease resistant plant lines. Westvaco Corporation, for example, has had some success with open crossings between families of various resistance levels and has produced at least five families with differing levels of resistance (R. J. Rousseau, *unpublished data*). Little access to information is available about the lineages of these putatively resistant families due to the proprietary nature of commercial breeding and the ever-shifting nature of cultivar copyright legislation. This putative resistance was evaluated by controlled inoculation studies and hypotheses regarding the mechanisms of resistance were tested as part of this thesis research.

A final strategy for management of *X. fastidiosa* in sycamore is that of "environmental therapy," so termed by Alexander Purcell (60), a pioneer of atmospheric modification as a management tool for this pathogen. Explored as a mechanism to reduce bacterial populations in diseased grapevines (23, 59, 60), the method entails subjecting infected plants to treatments of prolonged heat or cold exposure. In 1972, Goheen, Nyland, and Lowe discovered that the causal agent of Pierce's disease, then unnamed and not yet grown in axenic culture, could be inactivated in vivo by immersion of the host in hot water (23). Submersion of infected grapevines in water at 45°C for 3 h, 50°C for 20 min, or 55°C for 10 min resulted in remission of Pierce's disease symptoms after plants were reestablished and asymptomatic growth in subsequent seasons. Studies of low temperature effects on Pierce's disease symptom development were undertaken both in the lab in 1977 and in the field in 1980 by Purcell (59, 60). In his first study of low-temperature environmental therapy, Purcell found that multiple cold treatments of infected, dormant grapevines at -8 to -12°C resulted in failure of symptom development in a significant portion of these plants during the subsequent growing season (59). From the results of his study, he postulated that the therapeutic effect was caused by freezing of less cold-hardy xylem tissues inhabited by *X. fastidiosa* and resultant pathogen mortality. In 1980, Purcell tested his hypothesis in the field by monitoring infected plants at different locations and altitudes in conjunction with minimum winter temperatures (60). His results, however, were inconclusive. Some of the low altitude, warmer climate grown plants were "cured," while many infected plants from colder sites developed symptoms again the following season. He suggests that there may be a temperature/ moisture interaction causing variability from location to location, since cultural conditions were not identical at all sites.

There is evidence to support the hypothesis that environment is highly active in the selection process for host resistance to *X. fastidiosa* (23, 34, 59, 62). Pierce's disease and its causal organism are endemic to the southeastern United States and the northward expansion of *X. fastidiosa* diseases appears to be limited by winter low temperatures in association with host hardiness (30, 34). Sycamore and grape hosts taken from more southerly provenances and moved north seem to possess greater resistance to bacterial infection and leaf scorch than do those from more northerly provenances which have been relocated to the south of their point of origin (69). Comparison of their geographic ranges shows that the natural distribution of the leafhopper vectors far exceeds that of *X. fastidiosa*, which was limited to the southeastern and mid-Atlantic United States until human and vector activity aided its further spread (67, 69). The role of climate in limiting disease spread is further supported by reports that the northern boundaries for Pierce's disease and other diseases caused by *X. fastidiosa* appear to fluctuate in response to the severity of the preceding winter. This phenomenon may be because of direct temperature effects on overwinter survival of bacterial cells or to the potential presence of climate-mediated host resistance responses (34). Additional support for the importance of host environment is that heat stress and water stress have been found to exacerbate symptom development and hasten decline in many hosts infected with *X. fastidiosa* (23, 25, 59).

Survival of *X. fastidiosa* in adverse conditions has been studied at differing levels of plant physiology (23, 25, 36, 39, 59, 60), but few have examined bacterial movement within the entire host plant from fine roots to apical meristem in response to shifts in the cultural environment. The few studies in which this has been done have generally focused on crops of economic import such as grape and sweet orange (36, 39). In a study by Hopkins et al. (39), monthly extractions from roots and stems of infected sweet orange trees revealed a cyclical pattern in bacterial populations. Bacteria were cultured and detected in nearly equal numbers from both root and stem extracts. Bacterial populations were observed to have annual "peaks" in summer (July, August, or September) and in winter (December, January, or February). In grape, Hopkins found a similar peak in *X. fastidiosa* populations during the summer months, although his study was limited to bacterial populations within leaves, petioles, and stems (36). While these studies have provided much information about systems-level fluctuation in bacterial populations, they have overlooked the need for a more holistic understanding of these shifts. Greater utility may be found in discovering where the bacteria are moving within the infected plant, whether they are surviving within the host through winter temperatures, and if the hosts are generating compounds that might be influencing fluctuation of *X. fastidiosa* populations, in conjunction with seasonal shifts in the environment. In addition, each new year brings a new report of bacterial leaf scorch caused by *X. fastidiosa* on yet another species of forest or amenity hardwood. This growing number of affected species underscores the necessity for broadening the scope of study to incorporate more host types than those with the highest potential profit margin.

An improved understanding of interactions between the sycamore host, *X. fastidiosa*, and the environment could lead to improved management of sycamore leaf scorch, dieback, and decline over the long term. Research that yields strategies for improved selection for resistance, more advantageous site selection, enhanced crop cultural practices, or for reduction in the probability of stress-induced susceptibility, would be useful to sycamore growers, homeowners, and those working with this pathogen in other morphologically similar host-types.

Research Objectives

This research was undertaken with the primary goal of improved comprehension of the effects of environmental changes on bacterial population density and dynamics within sycamores infected by *X. fastidiosa* and the following specific objectives:

1. Identify the most effective and efficient method for extraction of *X. fastidiosa* from infected, lignified, sycamore tissues. Many methods have been used to remove bacterial cells from the host tissue. Each extraction technique employed has special requirements, such as needs for additional labor or expensive equipment. The necessity for utmost sterility further hinders extraction, because the slow growth rate of *X. fastidiosa* increases the possibility of isolate contamination. Reported extraction methods include vacuum infiltration of petioles or stems (1, 21, 39, 54, 68), syringe infiltration of petioles (18), squeeze extraction of sap from petioles, stems, or roots (4, 8, 13, 15, 17, 41, 70), petiole or leaf midvein maceration (6, 13, 18, 48, 54, 63), centrifugation of petioles, stems, or roots (15, 52), orbital shaking of stem or runner tissue (52), cultivation of infected chips of woody tissue in appropriate broth media (67, 68, 70), or a combination of methods, such as petiole/midvein maceration and centrifugation of the homogenate (14, 37). When preparing to isolate, culture, or quantify *X. fastidiosa* populations from host tissue, it is useful to know the method that is most efficient and which yields the greatest amount of bacterial cells per volume of host tissue sample. Six extraction methods were tested to determine which was best for extraction of *X. fastidiosa* from lignified sycamore tissue. These methods included petiole maceration as described in Chang and Donaldson (6), vacuum replacement extraction from woody tissue as described in French et al. (21), manual squeeze extraction from woody tissue as described by Davis et al. (13) and Chang and Walker (8), sap displacement from twig samples with a pressure bomb, similar to the method used by Goodwin et al. (25) for determination of xylem flow resistance alteration caused by *X. fastidiosa* in grapevine, and centrifugal extraction of sap from 1-year-old shoots. For centrifugal removal of sap, the bark on twig samples taken from confirmed *X. fastidiosa*-infected trees was either removed or left intact to determine the effect of bark presence and absence on frequency of culture contamination and colony growth.

2. Identify seasonal patterns of bacterial population dynamics in naturally infected sycamore trees within the landscape. Because of major physiological differences between seedlings and mature trees, including growth rate, photosynthetic capability, and stress-tolerance, full-grown trees were used in this study. Variations in the performance of greenhouse, container, and planted trees make knowledge of effects of seasonal environmental changes helpful in better understanding the relationship between *X. fastidiosa* and sycamore. To determine the effects of actual low temperature on bacterial populations within different host tissues, several mature *Xylella*-infected sycamore trees planted in the landscape were sampled at regular intervals and populations determined by culture and confirmed by ELISA. *X. fastidiosa* population dynamics were correlated with soil and air temperature and rainfall data.

3. Determination of effects of low temperature treatment on bacterial populations in host tissue. Prior research has suggested that therapeutic effects may be obtained by exposing infected plants to low temperature (59, 60); however, results were based on serological assay and visual observation of symptoms rather than isolation, culture, and quantification of bacterial populations. Visual and serological assessments should be validated with definitive culture and population counts before any judgment can be made about the utility of cold therapy for sycamore, grapevine, or any other host of this pathogen. The pin-prick method, described by Hopkins (35), was used for inoculation of all plants. Effects of cold treatment were evaluated on both sycamore seedlings from families putatively resistant or susceptible to *X. fastidiosa* and, for comparison of host responses, on grape cultivars reported as resistant or susceptible to systemic infection by *X. fastidiosa*.

4. Determination of degree of host reciprocity between different strains of *X. fastidiosa*. Research has shown that distinct differences exist between strains of *Xylella fastidiosa* isolated from different hosts (37, 38, 67). Different strains of the bacterium often have distinct host ranges, virulences, colony characteristics, infection rates, and multiplication rates. Differentiation of *X. fastidiosa* strains is difficult without use of molecular analysis. While molecular studies of strains from different hosts clearly indicate compositional variation amongst the various host-strains of *X. fastidiosa* (9, 10, 53) and even suggest groupings among specific strains, the effects of these variations on host specificity is not as well understood. Since some of this research involves both sycamore- and grape-derived strains of the bacterium, it is important to understand the degree of cross-transmissibility between strains and hosts. As bacterial infection of northern red oak has become a serious problem in New Jersey, Kentucky (56, 69), and has recently been reported in Georgia (8), oak was included in reciprocal transmission tests to assess host specificity, in a manner similar to the evaluation performed between sycamore and elm strains and hosts by Sherald (67). Susceptible varieties of grape, sycamore, and oaks with unknown resistance were inoculated with strains of *X. fastidiosa* isolated from grape, oak, and sycamore, grown in greenhouse for sufficient time to allow systemic infection of the hosts, and assayed to determine the amount of bacterial multiplication and distance of systemic spread within the hosts.

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CHAPTER 2

COMPARATIVE ANALYSIS OF FIVE METHODS FOR EXTRACTION OF *X. FASTIDIOSA* FROM INFECTED, LIGNIFIED SYCAMORE TISSUE

The xylem-limited bacterial plant pathogen, *Xylella fastidiosa* Wells et al. (34), has been implicated as the cause of diseases on various species from 30 or more families of monocotyledonous and dicotyledonous hosts (31). In ornamental and forest tree species, the pathogen causes leaf scorch, decline, and dieback diseases on a steadily increasing number of reported hosts (15, 16, 17, 33). While economic damage is difficult to quantify on many hosts, types of direct economic losses caused by *X. fastidiosa* in forest, shade, and ornamental hosts can include loss in pulp and lumber volume, stock losses for landscape plants infected before sale, and replacement value of plantation and landscape plants. Indirect losses can include expensive and extended therapy and management costs, aesthetic devaluation of landscapes, parks, and forests, and overall declines in plant vigor. It has been suggested that the role played by *X. fastidiosa* in the untimely decline and death of many forest, shade, and plantation trees may, in fact, result in the greatest overall losses of any disease caused by *X. fastidiosa* (18).

Sycamore leaf scorch, decline, and dieback caused by *X. fastidiosa* is the most common pathogen of sycamore in the southeastern U.S. (23). Sycamores infected with *X. fastidiosa* often begin to show interveinal and marginal foliar chlorosis during mid-to-late summer or earlier if water stress is a problem. This yellowing precedes onset of marginal necrosis, leaf curling, and death of affected leaves, which often fail to abscise (30). Wilting does not occur in relation to this or most other leaf scorch diseases caused by *X. fastidiosa* (14, 27). Leaf scorch symptoms first appear on basal leaves of seedlings or elder leaves of individual branches (17, 22). Initially, symptoms may be limited to one or two branches in the canopy, but the upper canopy declines in subsequent growing seasons and the disease then appears to progress downward through the crown in a basipetal pattern (23). Epicormic sprouts are observed frequently on trees with sycamore decline, a symptom of inadequate flow of water and nutrients to apical regions (22). Infected trees may exhibit delayed leaf flush, reduced seed set, reduced vigor in later years, and ultimately death. Dynamics of *X. fastidiosa* infections of sycamore and other forest and ornamental tree hosts are not as well understood as many diseases caused on other hosts (18) for two primary physiological reasons. First, leaf scorch diseases are often slow to progress in tree hosts and therefore are difficult to model experimentally (18). Second, given the size (≈ 0.25 to 0.35 μm by 0.9 to 3.5 μm) and discrete habitat of the pathogen (34) relative to the magnitude of the host, the bacterium is very difficult to locate and isolate from tree hosts, even with symptomatic tissue as a guide (2). For example, in a survey of Florida oaks, Barnard reported that an unexpectedly large proportion of symptomatic oaks failed to test positive for the presence of *X. fastidiosa*, either by culture or by enzyme linked immunosorbent assay (ELISA) (2).

Many methods have been used to remove bacterial cells from various host tissues. Each extraction technique employed has special requirements, such as needs for additional labor or expensive equipment. Necessity for utmost sterility is the primary hindrance to successful extraction when working with *X. fastidiosa,* because even small amounts of isolate contamination can overwhelm this slow-growing pathogen. It then follows that extraction methods with the most potential were those that could either be completed within the confines of a laminar flow hood or could be maintained within a closed, aseptic processing system. Effective extraction methods reported for removal of *X. fastidiosa* cells from host tissue include vacuum infiltration of petioles or stems (1, 13, 20, 26, 30), syringe infiltration of petioles (11), squeeze extraction of sap from petioles, stems, or roots (3, 7, 10, 21, 32), petiole or leaf midvein maceration (5, 8, 11, 24, 26, 28), centrifugation of petioles, stems, or roots (10, 25), orbital shaking of stem or runner tissue (25), cultivation of infected chips of woody tissue in appropriate broth media (29, 30, 32), or a combination of methods, such as petiole/midvein maceration and centrifugation of the homogenate (9, 19). Several of the methods above had to be eliminated because they were too easily contaminated, too time consuming, or required equipment that was cost prohibitive.

All methods, other than ultrastructural examination, for determining the presence of *X. fastidiosa* in host tissue require that the bacterium be extracted from the vascular cylinder where it proliferates. To perform both serological immunoassays to confirm bacterial presence and quantify bacterial populations in host tissue by serial dilution and isolate the pathogen in axenic culture, it was necessary to obtain a relatively large amount of xylem sap (>250 μl) under aseptic conditions. Requirements for sterile plant exudates in large volume led us to consider which methods would be used for this study and the experiments that follow. We needed to find the extraction method which would most efficiently yield the largest volume of uncontaminated xylem sap for our purposes. Indeed, when preparing to isolate, culture, or quantify any pathogen populations from numerous samples of host tissue, it is useful to know at the outset which method is most efficient and which yields the greatest amount of bacterial cells per volume of host tissue sample.

Initially, six extraction methods were tested to determine the best for extraction of *X. fastidiosa* from lignified sycamore tissue. The methods evaluated in this study included petiole maceration, vacuum extraction, squeeze extraction, centrifugation of both peeled and unpeeled stem tissue, and pressure-bomb expulsion.

Materials and Methods

Plant material selection. One symptomatic and one asymptomatic stem tissue sample from current and one-year growth was collected in late summer from each of 112 sycamores located on the University of Georgia Athens campus (n = 224). In the absence of symptoms, two shoot samples were chosen arbitrarily from each tree for vacuum extraction of xylem contents. Before vacuum extraction, samples were surface disinfested in a solution of 9.5% ethanol and 1.05% sodium hypochlorite and deionized water for 10 min, then rinsed three times for 3 min each in sterile, deionized water. Vacuum replacement extraction from woody tissue was then conducted under a laminar air flow hood (Model 1856, Forma Scientific, Marietta, OH), in a manner similar to that described by French et al. (13). Stem segments were trimmed to approximately 1 g and fitted into a sterile vacuum infiltration apparatus composed of two parts: i) a 65 mm autoclaveable funnel attached to approximately 20 cm of autoclaveable tubing and ii) 20 cm of tubing fitted onto a double- stoppered 250-ml collection flask connected to a vacuum pump (Model 5KH32EG550A, Curtin Matheson Scientific Inc., Hampton, NH). One gram stem samples were fitted into the loose ends of each tube to complete a line from funnel to pump. For each stem sample, 3-ml sterile polyvinylpyrrolidone (PVPP) extraction buffer (8.0 g sodium chloride, 1.15 g dibasic sodium phosphate (anhydrous), 0.2 g monobasic potassium phosphate (anhydrous), 0.2 g potassium chloride, 0.5 g Tween-20, and 2.0 g 2% acid-washed polyvinylpyrrolidone 40,000 [PVP-40], and 1000 ml of distilled water, pH adjusted to 7.4) was introduced into the funnel end of the apparatus, the pump activated, and vacuum pressure applied until all extraction buffer had been pulled through the stem segment and collected in the flask. The extract was collected into a sterile volumetric cylinder for measurement and 1.5 ml was retained for use in plating and ELISA tests. Extract from each sample was subjected to two plate replications of a commercially available ELISA immunoassay kit (PathoScreen Xf peroxidase, Agdia, Elkhart, IN) according to manufacturer instructions provided with the kit. After completion of the assay protocol, plates were evaluated on a microplate reader (Model 550, Biorad, Hercules, CA) at $A_{490\text{nm}}$. Microplate reader scores were confirmed using microplate analysis software (Biorad Microplate Manager, v.5.1 for Windows, Biorad, Hercules, CA).

The two trees that had the highest mean absorbance scores were selected for use in this study to ensure that there would be large bacterial populations within the naturally infected tissue samples. Five, approximately 1-m-long (≈ 1.5 cm diameter) sections of 1-year-old stem tissue, with intact leaves for petiole maceration, were cut from each tree for a total of 10 branch samples. Each sample branch was subdivided into five segments of approximately 3 g each. Petioles were removed and collected into a sample of approximately 1 g. One stem segment (or petiole group) from each tree was then used for each extraction method.

Extraction of *X. fastidiosa* from sycamore tissue. Prior to extraction, all shoot and petiole samples were surface disinfested as described above for initial stem samples. Additionally, all tissue samples were agitated on a New Brunswick Model G-33 shaker (New Brunswick Scientific, Edison, NJ) at 1 to 2 *g* during surface disinfestation to ensure thorough coverage of sterilization and rinse solutions. After disinfestation, samples were removed to a laminar flow hood for bacterial extraction.

Removal of *X. fastidiosa* cells by petiole maceration was performed as described by Chang and Donaldson (5). For petiole maceration, 1 g petiole samples were finely minced in 3 ml of sterile PVPP extraction buffer in a sterile petri dish. Once minced, large particles were strained from the homogenate by squeezing against the walls of the petri plate. The remaining suspension was then transferred to a sterile graduated cylinder for measurement. After measurement of total extract volume, 1.5 ml was retained for use in dilution plating to determine cell concentration and ELISA for confirmation of presence of *X. fastidiosa*.

Vacuum replacement extraction from woody tissue was carried out as described above for initial stem samples, in a manner similar to that described by French et al. (13). Because more extract was needed to evaluate extraction methods, stem segments were trimmed to approximately 3 g before vacuum infiltration. Vacuum extraction was conducted under a laminar air flow hood. Extract was collected in a sterile 10-ml graduated cylinder to measure volume and 1.5 ml was retained for use in quantitative dilution plating and ELISA tests.

The protocol for manual squeeze extraction of sap from woody tissue was modified from the processes described by Davis et al. (8) and Chang and associates (6, 7). Flame-sterilized pliers were used to firmly grip stem tissue while flame-sterilized pruning shears were used to cut a fresh face on the stem end. Pressure was then applied and the sap was collected as it emerged from the cut surface with a sterile glass capillary tube. The sap was expelled from the capillary tube with air from a sterile syringe and collected in a graduated microcentrifuge tube for volume measurement, dilution plating, and ELISA.

For centrifugal removal of sap, twig samples were either peeled after surface disinfestation with a sterile scalpel, forceps, and gloves or spun with bark intact. Bark removal was used to determine the effect of bark presence on frequency of culture contamination and colony growth. After surface disinfestation and peeling (where applicable), samples were placed in sterile 15-ml centrifuge tubes into the bottom of which sterile, 3-cm^2 sections of plastic screening were crumpled to minimize contact between the stem section samples and sap after centrifugation. Stem sections were centrifuged in a Fisher Centrific Model 225 (Fisher Scientific, Pittsburgh, PA) at approximately 3300 *g* for 10 min to physically displace sap from woody tissue. In a laminar air flow hood, the stem tissue was removed from the tubes with sterile forceps and discarded. With a pipette, the sap was transferred to graduated microcentrifuge tubes for volume measurement, quantitative dilution plating, and ELISA.

The original protocol for sap extraction from twig samples with a pressure bomb was similar to that used by Goodwin et al. (14) for determination of xylem flow resistance alteration caused by *X. fastidiosa* in grapevine. Three-gram stem samples were fitted into the nitrogen pressure bomb orifice and sealed around the stem sides with surface sterilized modeling clay. Once securely fitted, compressed nitrogen gas was used to pressurize the interior of the chamber and expel fluid contained in the vascular cylinder of the sample. Aseptic collection of exudates was very difficult. Expelled from the sycamore xylem at high speed, the pressurized sap quickly formed a frothy foam at the cut surface of the stem. After a variety of collection methods including sterile syringes, squeeze bulbs, and capillary tubes was attempted, it became apparent that, while the pressure bomb was effective in displacing sap, it was not possible to aseptically recover the fluid as it emerged. This method was subsequently eliminated from the study.

Determination of bacterial presence. After extraction, 100 µl of sap from each sample was utilized for two replications of a double antibody sandwich ELISA (PathoScreen Xf peroxidase, Agdia, Elkhart, IN) according to manufacturer instructions to confirm presence of *X. fastidiosa* cells. An additional 100 µl was diluted into PVPP extraction buffer in a 5-fold dilution series and 10 µl from each dilution was plated onto periwinkle wilt (PW) agar (7). Cultures were incubated at approximately 28°C and examined weekly for 1 month until colony growth was observed. In plates exhibiting characteristic growth of *X. fastidiosa,* colonies were counted and numbers of bacterial colony forming units (CFU) per gram of host tissue were determined for each extraction method. A second ELISA was then performed on colonies to confirm the identity of the bacteria. Total volume of sap obtained by each extraction method, CFU/g obtained for each method, and frequency of uncontaminated culture on PW medium of *X. fastidiosa* per method were recorded to determine the most useful method for repeated use in further research of *X. fastidiosa* in sycamore hosts. CFU/g obtained by each extraction method were transformed as log (n + 1), where n = CFU/g of sample tissue, to stabilize variances. Percent of uncontaminated plate cultures was calculated as 100% - n, where n = % isolate contamination. The frequency of uncontaminated plates was calculated for all plated isolates, whether or not growth of *X. fastidiosa* was detected, to determine variations among contamination levels in extracts obtained by each method.

Data analysis. The entire experiment was conducted two more times for a total of 3 experimental trials. Although the experiment was run three times, excessive contamination in the dilution plate series of the first run prevented clear quantification of results from that trial. Remaining trial results were analyzed by analysis of variance (ANOVA) using the statistical package SAS (SAS Institute, Cary, NC). As a result of a significant interaction between trial run and extraction method, results of the second and third trials were analyzed separately. ANOVA and Fisher's protected least significant difference (LSD) tests were used to compare the five extraction methods.

Results

Quantitative data from the first experimental trial were not collected due to excessive contamination of dilution plate series. As a result, this trial was omitted from data analysis. In the second trial, the largest volumes of extract obtained were for those methods that required extraction buffer: vacuum pump extraction and petiole maceration. Mean extract volumes from manual squeeze extraction, peeled stem centrifugation, and unpeeled stem centrifugation were not significantly different from one another (Table 2.1). No characteristic colony growth was observed in the two centrifuge treatments after a month of weekly observation. Colony forming units obtained per gram of sample in the second run did not differ significantly between any of the remaining three treatments, which yielded between 110 and 1530 CFU/g of tissue (Table 2.1). No significant difference in the frequency of uncontaminated plate cultures was observed among any of the five extraction methods in the second experimental trial (Table 2.1).

Volumes of extract obtained by each extraction method in the third trial were similar to volumes obtained in the second trial. Vacuum pump extraction and petiole maceration yielded the highest volumes, and squeeze extracted and centrifuged samples, whether peeled or not, generated significantly lower volumes (Table 2.2). In the third experimental trial, very few of the centrifuge extract isolates from unpeeled stem samples grew and none grew from the peeled sample extracts (Table 2.2). Means comparison indicated a significant difference in CFU/g tissue from peeling/ centrifugation and the other methods included in the study, although the number of bacterial cells centrifugally extracted from unpeeled stem sections was not significantly different from numbers extracted from peeled stems or the remaining three treatments (Table 2.2). In the third trial, the treatments were separated into three groups when mean frequency of uncontaminated cultures was compared (Table 2.2). One-hundred percent of cultures obtained by vacuum pump extraction were uncontaminated. Contamination frequency in cultures obtained from peeled, centrifuged stem tissue was not significantly different from contamination levels in cultures obtained by vacuum extraction (Table 2.2). Centrifuged isolates from peeled tissue were not significantly different from petiole maceration and squeeze extraction in terms of uncontaminated cultures on solid PW medium. Contamination was most frequent in *X. fastidiosa* plate cultures obtained by centrifugation of stem tissue with intact bark, with less than 22% uncontaminated cultures.

Discussion

Of the five extraction methods examined in this study, no one method showed itself to be the definitive "best." When maximum volume of extract was considered, vacuum pump extraction (given its buffer input) yielded the greatest amount of sap solution. When contamination of cultures was of primary concern, vacuum pump extraction yielded the largest percentage of viable, contamination-free cultures. When the factor of interest was the number of bacterial cells that could be recovered from a given mass of tissue however, there was no difference between vacuum pump extraction, petiole maceration, and squeeze extraction of xylem sap.

While these results suggest that vacuum extraction might be the preferable method for bacterial extraction, this experiment did not include other important factors such as equipment needs, available processing time, and size of sterile work area. In these areas, vacuum replacement extraction was found to have numerous requirements. Although extraction could be accomplished by one individual, this method demanded advance sterilization of numerous, easily damaged, unwieldy apparatuses. A large sterile workspace, in which to set up the cumbersome vacuum replacement system, was also necessary. Finally, while some stem samples were readily infiltrated by buffer under suction pressure and took less than a minute, others were much slower to pass the buffer and took as long as 20 min to complete the extraction. Ultimately, vacuum extraction claimed large blocks of time – although unpredictably so, making it very difficult to budget time for processing. Although no studies have been published on the specific utility of vacuum extraction of *X. fastidiosa* from host tissue, it has been used to recover the pathogen for several decades. In 1977, before *X. fastidiosa* had been successfully grown in axenic culture, French et al. employed this method to extract bacteria from peach tissue for ultrastructural examination (13). Vacuum extraction was used to study *X. fastidiosa* from sycamore, again ultrastructurally, in 1983 by Kostka et al.; however, when they wished to extract bacteria for culture, they chose to cultivate infected wood chips in PW broth and later transfer isolates onto semisolid medium (30). Hopkins et al. successfully used vacuum-extracted *X. fastidiosa* isolates from blighted citrus for both culture and immunoassay, and additionally reported similar success with vacuum-extracted bacteria from grapes (20). More recently, vacuum extraction of *X. fastidiosa* has been used to obtain xylem contents of infected rough lemon (*Citrus jambhiri* Lush.) for use in culture, immunoassay, and PCR. The abovementioned, successful uses of vacuum-extracted cell suspensions suggests that vacuum infiltration may be maintained under aseptic conditions and yield viable cells and cultures; although these studies, similar to our own, fail to address logistical considerations (13, 20, 30).

Petiole maceration required few materials and was successfully performed by one individual. Only one sterile petri dish, a few milliliters of extraction buffer, a sterile razor blade or scalpel, and sterile forceps were needed. Since sycamore petioles were often as much as 1 cm thick and heavily lignified, mincing them to a fine pulp was often time consuming. Times required for maceration of 1 g of petiole tissue to a consistency suitable for dilution series, culture plating, or testing by ELISA ranged from 2 to 10 min. Space requirements were minimal, as were preparation time and labor. Because of buffer inputs, the volume of extract obtained by petiole maceration was larger than most methods excepting vacuum replacement extraction. Because of the presence of large amounts of particulate host tissue in the homogenate, frequency of uncontaminated cultures was slightly lower than for all other methods, except centrifugation of unpeeled tissue. These results suggest that when isolation is the objective, petiole maceration may not be the best extraction method to employ. Additionally, this method is only useful during those times of the year when host plants have intact petioles to sample, which rules out any experiment in which host tissue is to be sampled during dormancy. Petiole maceration has often been used in previous research to obtain initial isolates of *X. fastidiosa* because petioles and leaf midveins are closely associated with leaf tissue, which shows symptoms most readily when bacteria are present. For example, Davis et al. utilized homogenate from triturated, or macerated, peach petioles to obtain strains of *X. fastidiosa* to test a variety of media to determine the best for culture of the phony peach pathogen (8) and Chang and Donaldson macerated grape petioles to obtain bacteria to test their new chemically defined medium (5). De Lima et al. macerated petioles from scorched coffee plants in sterile, distilled water then centrifuged the entire sample to help release bacteria into the supernatant for ELISA, PCR, dot immunobinding assays (DIBA), and culture (11), although vacuum extracted cell suspensions were used exclusively for light and scanning electron microscopy to facilitate visualization of cells without interference from host tissue in the homogenate. Not surprisingly, petiole maceration seems limited to use when rapid or short-term (within the growing season) sampling is required. When dormant season sampling and sampling of non-leafy tissues is desired, petiole maceration is not an option.

Since there were no buffer inputs for squeeze extraction of xylem sap, extract yields were much lower than those obtained by vacuum replacement extraction and petiole maceration. Squeeze extraction did not require elaborate apparatus or expensive equipment, large workspaces, or unpredictably large amounts of time. Extraction with pliers was fairly rapid; it was usually possible to perform an extraction from one sample in under 2 min. It was, however, impossible for one individual to successfully perform extraction by this method. These findings indicate that where labor is limited or where large amounts of extract are needed, this method may not be preferred. However, when preparation and processing time and workspace are at a premium and there is assistance available, this method is most useful. Squeeze extraction has been used previously in research which demands long-term sampling and sampling of non-leafy tissues like stems and roots. Chang and Walker used squeeze extraction to recover *X. fastidiosa* from northern red oak stem tissue on a monthly basis for culture and immunoassay over a period of 17 months (7). Brlansky et al. used squeeze extraction to accommodate a variety of infected host types including sugarcane (*Saccharum officinarum* L. ‘L62-96’), periwinkle, plum, ragweed, and grape to test for specificity of fluorescein-conjugated antisera to a variety of strains of *X. fastidiosa* for their studies on in situ immunofluorescent detection of a variety of strains of the bacterium (3). Interestingly, Sherald and Lei, in their evaluations of an ELISA test kit for detection of *X. fastidiosa* in landscape trees, shifted from maceration of petioles and stem segments to a specially designed high-pressure press to obtain sap for ELISA sample preparation (32). Researchers have even chosen squeezing petioles over petiole maceration in several cases. For example, when Davis et al. performed the first reported in vitro culture of *X.*

fastidiosa, they obtained many of their inoculum strains by pressing sap out of petiole tissue of infected grapes with forceps (10) and Huang et al. procured inoculum for their ultrastructural study of Pierce's disease bacteria in resistant and susceptible *Vitis* species by plating sap squeezed from the petioles of symptomatic leaves (21).

Extract yields from peeled and unpeeled stem sections by centrifugation were comparable to those obtained by squeeze extraction. Growth of isolates extracted by centrifugation was almost nil; thus, it was nearly impossible to quantify bacterial densities obtained from tissue by this method. Positive ELISA scores for many samples, however, suggested that cells or cell fragments were present. Overall frequency of uncontaminated culture on PW medium was good for extracts from peeled stems obtained by this method; although frequency of uncontaminated culture was considerably lower when bark was left intact, possibly from contamination by bark endophytes which survived disinfestation and were removed from the bark during centrifugation. Centrifuges are fairly common equipment in most laboratories and all other supplies necessary for this method of extraction were inexpensive and easily obtained. Samples could be prepared and processed easily by one individual. A sterile environment could be maintained inside the sealed centrifuge tubes, even though centrifugation itself took place outside of the laminar flow console. The greatest benefit of centrifugal extraction over the other methods tested was the speed with which samples could be processed. After preparation and loading of samples into sterile tubes, centrifugation was accomplished in 10 min for as many as 24 samples at one time. Because isolates extracted by this method largely failed to grow, it was determined that this method was not useful for this research; however, efficiency and time saving features of this method suggest that further research into centrifuge extraction is warranted. Additionally, centrifugation has been successfully used to obtain isolates of *X. fastidiosa* from both petioles (10) and stems (25). In addition to using squeeze extraction with forceps for field-collected grapevine samples for the first in vitro culture of *X. fastidiosa*, Davis et al. centrifuged petiole samples from inoculated greenhouse grapevines for 2.5 min at 8000 *g* before blotting them onto their test media (10). Similarly, McElrone et al. used three types of centrifugal extraction to accommodate host variation in their nested PCR survey of 27 plant taxa to identify alternative hosts of *X. fastidiosa* in the Washington, D.C. area (25). For woody and rigid-stemmed taxa, bark or outer surfaces were removed and 3- to 4-cm samples were centrifuged at 13,500 *g* for 5 min to yield approximately 100 µl of xylem fluid. A second extraction method included an immunomagnetic capture protocol in addition to centrifugation as described above and the third method, for runners or less lignified stems, incorporated partial maceration followed by repeated vortexing and orbital shaking to release bacteria and separate plant tissue from supernatant (25). McElrone et al. did not attempt culture on media with these isolates, so no information is available about the cultivability of *X. fastidiosa* strains obtained by centrifugal extraction at such high forces. Since the bacterium has been reported to form aggregates and filaments in broth culture (34) as well as to adhere to walls of culture flasks until loosened by agitation or sonication (4, 12), centrifugal methods for extraction of *X. fastidiosa* from host tissue could be improved with further study of the aggregative and adherent properties of the bacterium in various media and on various surfaces, as well as by examination of growth curves for bacteria isolated by centrifugation at a variety of forces for various increments of time, to more clearly determine the effects of centrifugal force and duration on cultivability of bacterial cells.

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Table 2.1. Trial 2. Variation in volume of extract per gram of sycamore tissue, $\log_{10}$-transformed values for colony forming units of *Xylella fastidiosa* per gram of sycamore host tissue, and percent uncontaminated isolates obtained by 5 different methods of extraction

Extraction method[y]	Mean extract vol. (ml)	Mean $\log_{10}$ CFU/g tissue	Frequency of uncontaminated culture (%)
Vacuum pump extraction	2.8058 a[z]	2.037 a	81.67 a
Petiole maceration	0.9925 b	3.185 a	80.00 a
Centrifuge (peeled)	0.0588 c	0 b	93.00 a
Centrifuge (unpeeled)	0.0929 c	0 b	86.67 a
Squeeze extraction	0.0378 c	2.697 a	98.33 a

[y] Means presented for each extraction method are calculated from 10 replications of extraction by each method with 3 g stem samples or 1 g samples of sycamore petiole tissue, previously determined to be infected with *X. fastidiosa* by confirmatory ELISA. Methods of extraction used in this study included vacuum infiltration of xylem tissue with PVPP extraction buffer and vacuum pressure, maceration of petioles in PVPP extraction buffer, squeeze extraction using pliers pressure, and centrifugal extraction of woody tissue both with and without bark at ≈3300 *g* for 10 min.

[z] Means in each column followed by the same letter are not significantly different based on Fisher's Protected LSD test ($P > 0.05$).

Table 2.2. Trial 3. Variation in volume of extract per gram of sycamore tissue, $\log_{10}$-transformed values for colony forming units of *Xylella fastidiosa* per gram of sycamore host tissue, and percent uncontaminated isolates obtained by 5 different methods of extraction

Extraction method[y]	Mean extract vol. (ml)	Mean $\log_{10}$ CFU/g tissue	Frequency of uncontaminated culture (%)
Vacuum pump extraction	2.7408 a[z]	3.025 a	100.00 a
Petiole maceration	0.9858 b	1.851 a	68.33 b
Centrifuge (peeled)	0.0713 c	0 b	86.00 ab
Centrifuge (unpeeled)	0.0451 c	0.229 ab	21.67 c
Squeeze extraction	0.0372 c	1.943 a	68.00 b

[y] Means presented for each extraction method are calculated from 10 replications of extraction by each method with 3 g stem samples or 1 g samples of sycamore petiole tissue, previously determined to be infected with *X. fastidiosa* by confirmatory ELISA. Methods of extraction used in this study included vacuum infiltration of xylem tissue with PVPP extraction buffer and vacuum pressure, maceration of petioles in PVPP extraction buffer, squeeze extraction using pliers pressure, and centrifugal extraction of woody tissue both with and without bark at ≈3300 *g* for 10 min.

[z] Means in each column followed by the same letter are not significantly different based on Fisher's Protected LSD test ($P > 0.05$).

CHAPTER 3

DISTRIBUTION AND POPULATION DENSITY OF *XYLELLA FASTIDIOSA* IN NATURALLY INFECTED SYCAMORE ASSOCIATED WITH LOW WINTER TEMPERATURES [1]

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[1] Henneberger, T. S., Stevenson, K. L., Britton, K. O., and Chang, C. J. To be submitted to Plant Disease.

Abstract

Henneberger, T. S., Stevenson, K. L., Britton, K. O., and Chang, C. J. 2003. Distribution and population density of *Xylella fastidiosa* in naturally infected sycamore associated with low winter temperatures. Plant Dis.

To determine the effect of low temperature on populations of *Xylella fastidiosa* in naturally infected American sycamore (*Platanus occidentalis* L.), root and shoot samples were collected monthly for 13 months from two locations in Georgia. To estimate bacterial populations, sap extracted from each sample was tested for *X. fastidiosa* by ELISA and diluted onto periwinkle wilt medium. Soil and air temperatures and rainfall were recorded at each site and used to generate cumulative hours below temperature thresholds ranging from -5ºC to 10ºC and cumulative rainfall. Bacterial populations in shoots were negatively correlated with cumulative hours below an air temperature of -5ºC ($r = -0.96$), but in roots, bacterial populations were only weakly correlated with cumulative hours below soil temperature thresholds ranging from 2.5ºC to 10ºC ($-0.61 < r < -0.25$). Rainfall data did not corellate well with bacterial growth variables. Our results indicate that air temperatures below -5ºC are best associated with limiting bacterial multiplication in sycamore shoots and that factors other than low soil temperature may influence multiplication of *X. fastidiosa* in sycamore roots.

The fastidious, xylem-inhabiting bacterium, *Xylella fastidiosa* Wells et al. (19) has been associated with a wide range of diseases on members of more than 30 families of mono- and dicotyledonous hosts. Although this pathogen causes a variety of symptoms on its many hosts, shade tree species like oak (*Quercus* spp.), elm (*Ulmus* spp.), maple (*Acer* spp.) and sycamore (*Platanus* spp.) respond similarly to infection (12, 17). Sycamore and other ornamental and forest trees infected with *X. fastidiosa* often begin to show interveinal and marginal foliar chlorosis during mid-to-late summer. Symptoms may appear earlier if water stress is a problem (12). This yellowing precedes onset of marginal necrosis, leaf curling, and death of affected leaves, which often fail to abscise (16, 17). Leaf scorch symptoms first appear on basal leaves of seedlings or elder leaves of individual branches (5, 9). Initially, symptoms may be limited to one or two branches but, in the southeastern U.S., the upper canopy declines in subsequent growing seasons and the disease then appears to progress downward through the crown in a basipetal pattern (10). Epicormic sprouts are observed frequently on trees infected with *X. fastidiosa*, a symptom of inadequate flow of water and nutrients to apical regions (9). Infected trees may exhibit delayed leaf flush, reduced seed set, reduced vigor in later years, and ultimately death. Diseases causes by *X. fastidiosa* on ornamental and forest tree hosts are less well understood than those diseases caused by the pathogen on more economically important hosts. It has been suggested, however, that leaf scorch, decline, and dieback caused by *X. fastidiosa* in forest, shade, and ornamental trees may result in the greatest overall losses (6).

Previous research on California grapevines has indicated that severity of infection can be significantly reduced by low temperature therapy (13, 14). Much of this research has involved use of artificially modified environments or has been conducted in climes where winter temperatures become low enough to cause considerable mortality in pathogen populations, either directly or indirectly (13, 14). However, research in citrus and grapevines grown in Florida suggests that winter temperatures in the southeast are not low enough to reduce populations of *X. fastidiosa* in these hosts (7, 8). In fact, some researchers have suggested that, in milder climates, bacteria that reach the root xylem of their hosts may be able to overwinter and provide a ready reservoir of inoculum in spring, when conditions again favor bacterial multiplication (3).

This research was conducted in order to better understand natural infection of sycamore by the pathogen, *X. fastidiosa*, which is becoming increasingly widespread through the southeastern United States. To determine the quantitative relationship between low temperature and distribution and population density of *X. fastidiosa* in naturally infected sycamores in landscape settings, we quantified populations of the bacterium in sycamore roots and shoots over a period of 13 months at two locations in Georgia and correlated these data with weather information collected near each site by the Georgia Automated Environmental Monitoring Network.

Materials and Methods

Sample collecting. This study was replicated at two locations, Athens and Griffin, Georgia. Shoot and root tissue was sampled on an approximate monthly basis, with sampling intervals ranging from 21 to 31 days, from September 5, 2001 to September 16, 2002. We collected three root and three shoot samples from each of three sycamore (*Platanus occidentalis* L.) trees at each location, for a total of 18 samples per month from each location (n = 36). Samples were collected for 13 intervals, covering a period of 409 days.

At the Athens location, trees were selected by evaluating the results of an earlier survey of trees on the University of Georgia campus. In the prior survey, 1 g symptomatic and 1 g asymptomatic stem tissue was sampled from current and 1-year growth was collected in late summer from each of 112 sycamores located on the University of Georgia Athens campus (n = 224). In the absence of symptoms, two shoot samples were chosen arbitrarily from each tree for vacuum extraction of xylem contents. After stem samples were surface disinfested, vacuum replacement extraction was then conducted for each sample under a laminar air flow hood (Model 1856, Forma Scientific, Marietta, OH) using a vacuum pump (Model 5KH32EG550A, Curtin Matheson Scientific Inc., Hampton, NH) to pull 3 ml polyvinylpyrrolidone (PVPP) extraction buffer (8.0 g sodium chloride, 1.15 g dibasic sodium phosphate (anhydrous), 0.2 g monobasic potassium phosphate (anhydrous), 0.2 g potassium chloride, 0.5 g Tween-20, and 2.0 g 2% acid-washed polyvinylpyrrolidone 40,000 [PVP-40], and 1000 ml distilled water, pH adjusted to 7.4) through the stem segment and into a collection flask. Extracts were tested by enzyme-linked immunosorbent assay (ELISA) to determine presence of *X. fastidiosa* in sample tissue. Each sample was subjected to two plate replications of a commercially available ELISA immunoassay kit (PathoScreen Xf peroxidase, Agdia, Elkhart, IN) according to manufacturer instructions. Finished plates were evaluated on a microtiter plate reader (Model 550, Biorad, Hercules, CA) at $A_{490\text{nm}}$ and scores were confirmed using microplate analysis software (Biorad Microplate Manager, v.5.1 for Windows, Biorad, Hercules, CA). To ensure that there would be large bacterial populations within the naturally infected tissue samples, three trees were selected on the basis of positive ELISA results with high absorbance readings (> 1.5), characteristic leaf scorch and marginal necrosis symptoms, and proximity to one another.

In Griffin, fewer sycamores were available from which to choose. Several trees were located, checked for visible symptoms, and 1 g of symptomatic stem tissue was sampled from current and 1-year growth on each tree. Vacuum extracted xylem sap from each sample was tested by ELISA as described above for confirmation of bacterial presence. Once presence of the pathogen was confirmed, three trees that had severe symptoms but not more than 40% dieback were selected. Trees with larger amounts of dieback were eliminated from the potential selections to ensure that the trees chosen were not in danger of dying as a result of disease during the projected year of sampling.

All trees sampled at both locations were approximately 10 years of age or older, with trunks ranging from 15 cm diameter at breast height (dbh) to 65 cm dbh. All six trees sampled were well established within their respective landscapes and had been installed in their respective plantings for 2 years or longer.

At the end of each sampling interval, three root and three shoot samples were collected from each of the three trees at each location on the same day. During the growing season, 10-cm or longer segments of current season or 1-year-old stem tissue (diameter ≤ 1.5 cm) were preferentially sampled on the basis of presence of characteristic symptoms. Subsequent samples were collected from nearby or adjacent stems. Ten-centimeter or longer root segments (0.5 to 2 cm diameter) were collected by arbitrary digging around the base of trees, approximately halfway between the trunk and canopy edge. Depths from which roots were collected ranged from 2 cm to 25 cm. Once roots of suitable size were located, they were marked to enable sampling from the same roots in subsequent months. This method was not infallible, however; in some months, grounds maintenance crews at both locations removed flagging and markers and new sample roots had to be located. Stem and root samples were placed in plastic bags, transported to the laboratory in an ice-packed cooler, and stored in a refrigerator at 5°C until processing. Samples were processed within 48 h after collection.

Pathogen detection and isolation. Leaves, if present, were manually removed from stem samples and root samples were rinsed and scrubbed under tap water to remove any excess soil from root surfaces. A portion (5.0 ± 0.25 g) of each sample was weighed out and mass of each sample was recorded. Root and stem samples were surface disinfested for 10 min in a solution of 9.5% ethanol and 1.05% sodium hypochlorite and deionized water and rinsed 3 times for 3 min in sterile, deionized water. Samples were agitated on a shaker during surface disinfestation to ensure thorough coverage of disinfestant and rinse solutions. After surface disinfestation, samples were transferred to a laminar flow hood for bacterial extraction.

Sap was extracted from surface disinfested stem and root tissues by squeezing each section with pliers. We collected sap from the cut face of the sample tissue with a sterile glass capillary tube and expelled the sap into a volumetric microcentrifuge tube with air from a sterile syringe. Total amounts of sap extracted were recorded and 100 μl of sap from each sample was diluted in sterile PVPP extraction buffer in a 5-fold dilution series. Ten microliters from each dilution was then spread onto periwinkle wilt (PW) agar (3). Cultures were maintained at 28°C and examined weekly until characteristic colony growth of *X. fastidiosa* was observed. Characteristic colonies of *X. fastidiosa* were counted and populations of bacterial colony forming units (CFU) per unit mass of host tissue were determined for each sample. Bacterial population densities (CFU/g) transformed as log (n + 1), where n = CFU/g of sample tissue, to stabilize variances.

An additional 100 μl of extracted sap was utilized for two replications of a commercially available ELISA kit (PathoScreen Xf peroxidase, Agdia, Elkhart, IN) according to manufacturer instructions to confirm presence of *X. fastidiosa* cells. Finished plates were evaluated on a microplate reader (Model 550, Biorad, Hercules, CA) at $A_{490\text{nm}}$ and scores were confirmed using microplate analysis software (Biorad Microplate Manager, v.5.1 for Windows, Biorad, Hercules, CA).

Weather data. Weather data from monitoring stations within 10 km of each location for the 13-month sampling interval were obtained from the database of the

Georgia Automated Environmental Monitoring Network (Gerrit Hoogenboom, University of Georgia Department of Biological and Agricultural Engineering, Griffin, GA). Air and 5-cm, 10-cm, and 20-cm deep soil temperature measurements and rainfall (mm) recorded every 15 min were collected for the entire 409-day sampling period. The minimum air and soil temperature over the entire sampling period at both locations never dipped below -10°C air temperature and 0°C soil temperature at any depth. Therefore, 5 degree temperature increments were constructed above these minima. The air temperature data were used to generate mean and cumulative hours below 10°C, 7°C, 5°C, 0°C, and -5°C for each sampling interval. In addition, for the three soil temperature measurement depths, mean and cumulative hours below 10°C, 7.5°C, 5°C, and 2.5°C were calculated. Months in which there was no appreciable accumulation of low temperature hours (< 25 hours during the sampling interval) were omitted from the analysis. Thus, mean and cumulative temperature data from 7 of the 13 sampling intervals (mid-October through mid-April) were used for correlations with bacterial growth. Rainfall data were used to generate both mean and cumulative rainfall measures for each sampling interval.

Data analysis. For each sampling interval, ELISA scores were used to determine the frequency of detection of *X. fastidiosa* in host tissue, which was calculated as 100% - *n*%, where *n* = % of samples testing negative by ELISA for presence of the bacterium. Frequency of isolation of *X. fastidiosa* from host tissue was calculated for each sampling interval by determining the percentage of cultures which exhibited typical colonies of *X. fastidiosa.* For each sampling interval, $\log_{10}$-transformed bacterial densities were expressed in two ways to determine which would correlate more strongly with the weather data. Log_{10}-transformed bacterial population density ($\log_{10}$ CFU/g) was calculated as the sum of all bacterial density measurements for a given sampling interval divided by the number (n = 18) of samples from which isolation was attempted during that interval. Censored $\log_{10}$-transformed bacterial population density ($\log_{10}$ CFU/g) was calculated as the sum of all bacterial density measurements for a given sampling interval divided by the number of cultures which showed characteristic growth of *X. fastidiosa* for that interval.

For both locations, bacterial growth variables frequency of detection, frequency of isolation, and censored and uncensored bacterial densities were analyzed by analysis of variance (ANOVA) using the statistical package SAS (SAS Institute, Cary, NC) with location and tissue type as main factors. If no significant differences were found between the two locations, then the data from both locations was pooled. If there were significant differences between tissue types for any of the growth variables evaluated, then Fisher's Protected LSD test was performed for each significant variable.

Linear correlations were performed using the statistical package SAS (SAS Institute, Cary, NC) to determine whether mean or cumulative rainfall and mean or cumulative hours below the designated temperature thresholds were more strongly associated with the growth variables measured in this study. The temperature variable which was most strongly correlated with the measured growth variables was used for final correlations with specific growth variables.

Simple linear regressions for each combination of growth variable and air and soil temperature interval and rainfall measure were evaluated separately for roots and shoots, respectively, using PROC GLM in SAS software (SAS Institute, Cary, NC). From the regression of each combination, correlation coefficients (r) and associated p-values were recorded.

To observe seasonal shifts in bacterial detectability, frequency of detection was plotted against sampling date for roots and shoots together. To observe seasonal shifts in bacterial cultivability and variation by tissue type, frequency of isolation from root samples and from shoot samples was plotted against sampling date.

Results

No significant difference was found between Athens and Griffin locations for any of the bacterial growth variables measured: frequency of detection, frequency of isolation, and uncensored and censored bacterial densities. Therefore, data from the Athens and Griffin locations were pooled. ANOVA indicated that censored and uncensored bacterial densities isolated from roots and shoots were not significantly different at $P = 0.05$. The mean, $\log_{10}$-transformed, censored bacterial density was estimated at 1.75 $\log_{10}$ CFU/g and uncensored density estimated at 1.41 $\log_{10}$ CFU/g for each month in which there was a considerable accumulation of below-threshold temperatures. There were, however, significant differences between shoots and roots in terms of frequency of detection ($P = 0.0019$) and frequency of isolation ($P = 0.0003$) of *X. fastidiosa*. Frequency of detection of the bacterium in shoot samples was 54.3%, in contrast to 22.1% in root samples. Similarly, frequency of isolation was significantly greater from shoot samples (56.0%) than from root samples (24.8%).

A much greater degree of correlation was found between bacterial population estimates and cumulative hours below selected temperature thresholds ($-0.96 < r < -0.25$)

than for mean hours below the selected temperature thresholds (-0.50 < r < -0.00). As a result, temperature effects on bacterial populations were further analyzed in relation to cumulative hours below the designated temperature thresholds.

Bacterial populations in shoots were negatively correlated with cumulative hours below several of the designated temperature thresholds (Table 3.1), except for the regression of frequency of detection of *X. fastidiosa*, which showed no significant correlation. Regressions of bacterial populations in shoots on the five shoot-associated temperature increments revealed that frequency of isolation, censored bacterial density estimates, and uncensored bacterial densities were strongly correlated with cumulative hours below -5ºC (-0.79, $P = 0.0348$; -0.96, $P = 0.0005$; -0.91, $P = 0.0049$, respectively). Correlations between bacterial populations and cumulative hours below the other 4 temperature thresholds were weaker and less consistent (Table 3.1). Of the four variables evaluated, censored bacterial population density was most strongly correlated with cumulative hours below all temperature thresholds and had the greatest correlation of all comparisons ($r = -0.96$, $P = 0.0005$) with cumulative hours below -5ºC. In contrast to results obtained from shoot sample analysis, bacterial growth in roots did not show significant correlation with any of the temperature thresholds evaluated (Table 3.1).

Frequency of detection of the pathogen plotted over the 13 months of sampling revealed two peaks in the frequency of detection, one from December to February and another from July to September (Fig. 3.1). Differences in detection of the pathogen in root and shoot samples reveals that the two distinct peaks are more closely associated with bacterial presence in the stems than in roots (Fig. 3.2).

Bacterial growth variables did not corellate with mean rainfall for any sampling interval. Cumulative rainfall for each sampling interval was only weakly corellated with frequency of isolation ($r^2 = 0.571$, $P = 0.049$) and did not show a significant relationship to any other bacterial growth variable.

Discussion

Seasonal patterns of bacterial population growth have been observed in similar studies of sweet orange (*Citrus sinensis* (L.) Osbeck), grape (*Vitis* spp.), and red oak (*Quercus rubra* L.) infected with *X. fastidiosa* (3, 7, 8). In grapevines, bacterial populations were undetectable until May or early June, then peaked in late summer, and remained detectable at declining levels until early winter (8). Chang and Walker found that *X. fastidiosa* could be successfully isolated from northern red oaks in Griffin, Georgia only from August until January (3) with maximum frequency of isolation during November. Frequency of detection of the bacterium in Florida citrus reached a peak in late summer similar to grape, but declined in the fall and increased again, attaining a second peak in December, January, and February (7). Our results show that the bacterial populations in sycamores sampled in Athens and Griffin, Georgia exhibit two distinct peaks like the pattern reported by Hopkins in citrus, with a peak from December to February and a second from July to September. Hopkins suggested that host senescence may be a primary factor triggering the peak bacterial presence in fall, but this fails to explain the second, winter peak which occurs after hosts are dormant. Chang and Walker suggested that bacterial populations may increase in January and be carried upward from underground tissues at the end of winter (3). In another study of sharpshooter vectors in

Florida citrus, Timmer and Lee showed that there are peaks in vector populations in Florida groves which are concurrent with peaks in populations of *X. fastidiosa* within citrus hosts (18). The springtime decline in detection frequency may also be related to accelerated host growth in spring; perhaps bacterial titer is reduced as the spring growth flush reduces the concentration of bacteria within the xylem sap.

Diseases caused by *X. fastidiosa* vary by host and are often classified by the types of symptoms they cause (15). Symptom expression has been linked to both host type and tissues in which bacterial multiplication occurs. In stunt diseases like phony peach, *X. fastidiosa* cells commonly accumulate in roots (1); while leaf scorch diseases like Pierce's disease of grape are most often characterized by bacterial accumulation in petioles and veins of symptomatic leaves (8). In dieback and decline diseases of forest and ornamental trees like sycamore and oak, *X. fastidiosa* has been reported to accumulate mainly in the trunk and stems, especially in those sectors showing typical symptoms (2, 11, 13, 15). In our study, we detected the pathogen more frequently in stem tissues than roots for most of the year, even during winter months when the pathogen population was most likely to survive and accumulate in soil-insulated root xylem, as suggested by Chang and Walker (3). Reduced frequency of bacterial detection within root tissues may also be explained by arbitrary collection of root samples; stems were initially selected during active growth on the basis of symptom presence, while roots were selected by meeting criteria of sufficient diameter and accessibility. In their study, Aldrich et al. provide evidence that the bacteria are not uniformly distributed within the roots of infected peach trees and suggest that likelihood of detection in roots may be increased by selecting sampling size on the basis of probability of host infection in the sampling area (1). In the case of our research, sampling a larger number of roots might have increased the frequency of detection of *X. fastidiosa*.

A conservative approach was taken in assessing positive detection by ELISA in our research. Only those samples which gave strong positive reactions to serological assay (readings of 0.95 or greater at 490 nm absorbance) were considered positive. While this heightens the certainty of accurate positives, it may also have resulted in underestimation of the presence of *X. fastidiosa* within the trees tested. Research relating ELISA absorbance readings to growth curves for *X. fastidiosa* would be most useful in helping to resolve questions of bacterial presence and population density, particularly in situations where latent infections can contribute significantly to the reservoir of inoculum (15).

Results of our analysis of low temperature thresholds indicate that air temperatures below -5ºC are associated with reduced bacterial multiplication in sycamore shoots. This finding is consistent with previous reports of therapeutic effects of low temperatures on infected grapevines (13). In his research, Purcell found that multiple exposures of 1.5 to 24 h to temperatures ranging from -12ºC to 0ºC greatly enhanced the likelihood of survival of inoculated, symptomatic grapevines. Both sets of results suggest that *X. fastidiosa* populations may be reduced directly by exposure to suboptimal temperatures or indirectly by freezing or dehydration of the infected xylem tissues (13). These findings may also explain the lack of correlation between root tissue and soil temperatures in this study. Soil temperatures below 2.5ºC were seldom observed at 5 cm and 10 cm below the soil surface at either location; while at 20 cm depth, the temperature never reached below 2.5ºC. In fact, at 20 cm depth, only 347 h accumulated below the

5ºC threshold during the 13 months in which temperature was monitored. These warm soils may have insulated bacteria within roots or failed to reach temperatures low enough for long enough to have a significantly deleterious effect on populations of viable bacterial cells. This hypothesis is supported by a recent study of temperature-dependent growth and survival of *X. fastidiosa* both in vitro and in infected, containerized grapevines (4). While in vitro populations did not grow below 12ºC and minimum threshold temperature for bacterial growth in planta was determined to be between 17ºC and 25ºC, populations of *X. fastidiosa* in plants held at 5ºC declined slowly and still exceeded 6 $\log_{10}$ CFU/g after 18 days. Bacterial populations in infected grapevines held at 10ºC declined very slightly (- 1 $\log_{10}$ CFU/g) over the first 9 days, but stabilized over the next 9 days to a level comparable to that present before temperature treatment was initiated (4). These results suggest that, while bacterial multiplication may be reduced or retarded at suboptimal temperatures, enough viable cells may remain for populations to remain stationary or suffer only mild reductions (4). Lack of correlation between recovery of *X. fastidiosa* from sycamore roots and cumulative hours below any of the temperature thresholds examined indicates that factors other than low soil temperature probably influence multiplication of *X. fastidiosa* in sycamore roots. Successful detection by ELISA and recovery of the pathogen in culture indicates that *X. fastidiosa* cells may be present in host root systems throughout periods of dormancy and active growth. Presence of cultivable cells during the coldest times of the year at both locations provides evidence that this pathogen may be able to overwinter within the root xylem of infected hosts and suggests that cells may be moved to distal limbs in spring via the translocation stream.

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Table 3.1. Correlation coefficients (r) and associated p-values (in parentheses) for linear correlations between detection and recovery of *Xylella fastidiosa* from sycamore shoot and root tissue sampled monthly and cumulative hours below air and soil temperature thresholds

Shoot tissue					Root tissue				
Air temp. (ºC)	Frequency of detection (%)[x]	Frequency of isolation (%)[y]	Censored $\log_{10}$ CFU/g host tissue[z]	Uncensored $\log_{10}$ CFU/g host tissue[z]	Soil temp. (ºC)	Frequency of detection (%)	Frequency of isolation (%)	Censored $\log_{10}$ CFU/g host tissue	Uncensored $\log_{10}$ CFU/g host tissue
10	-0.45 (0.3156)	-0.76 (0.0463)	-0.94 (0.0014)	-0.88 (0.0085)	10	-0.33 (0.4667)	-0.34 (0.4557)	-0.60 (0.1530)	-0.60 (0.1523)
7	-0.42 (0.3454)	-0.75 (0.0542)	-0.94 (0.0019)	-0.88 (0.0095)	7.5	-0.31 (0.4990)	-0.31 (0.4994)	-0.59 (0.1582)	-0.60 (0.1650)
5	-0.44 (0.3283)	-0.75 (0.0542)	-0.94 (0.0016)	-0.88 (0.0082)	5	-0.29 (0.5309)	-0.29 (0.5345)	-0.58 (0.1612)	-0.59 (0.1750)
0	-0.44 (0.3222)	-0.76 (0.0487)	-0.94 (0.0016)	-0.90 (0.0064)	2.5	-0.25 (0.5860)	-0.25 (0.5862)	-0.55 (0.1932)	-0.56 (0.1967)
-5	-0.60 (0.1510)	-0.79 (0.0348)	-0.96 (0.0005)	-0.91 (0.0049)					

[x] Percentage of samples that tested positive for *X. fastidiosa* based on 2 replications of a commercially available DAS-ELISA (PathoScreen Xf peroxidase, Agdia, Elkhart, IN). Test was performed according to manufacturer instructions provided with the kit.

[y] Percentage of samples from which *X. fastidiosa* was isolated on PW medium.

[z] Uncensored $\log_{10}$-transformed bacterial population density ($\log_{10}$ CFU/g) values were calculated as the sum of all bacterial density measurements for a given sampling interval divided by the number (n = 18) of samples from which isolation was attempted during that interval. Censored $\log_{10}$-transformed bacterial population density ($\log_{10}$ CFU/g) values were calculated as the sum of all bacterial density measurements for a given sampling interval divided by the number of samples from which successful isolations were grown on PW medium.

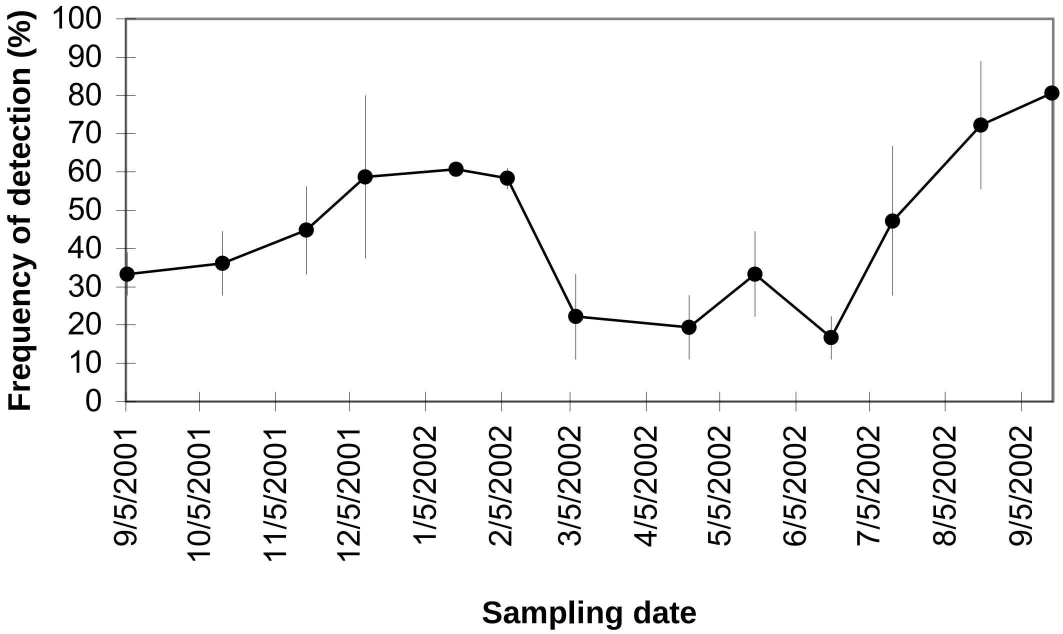

Figure 3.1. Monthly fluctuation in detectability of *Xylella fastidiosa* in naturally infected sycamore from 2 locations in Georgia. Bacterial presence in stem and root extracts was determined by ELISA. Each data point represents 18 root and 18 shoot samples pooled from Athens and Griffin, GA sampling sites. Vertical lines indicate one standard deviation above and below the mean.

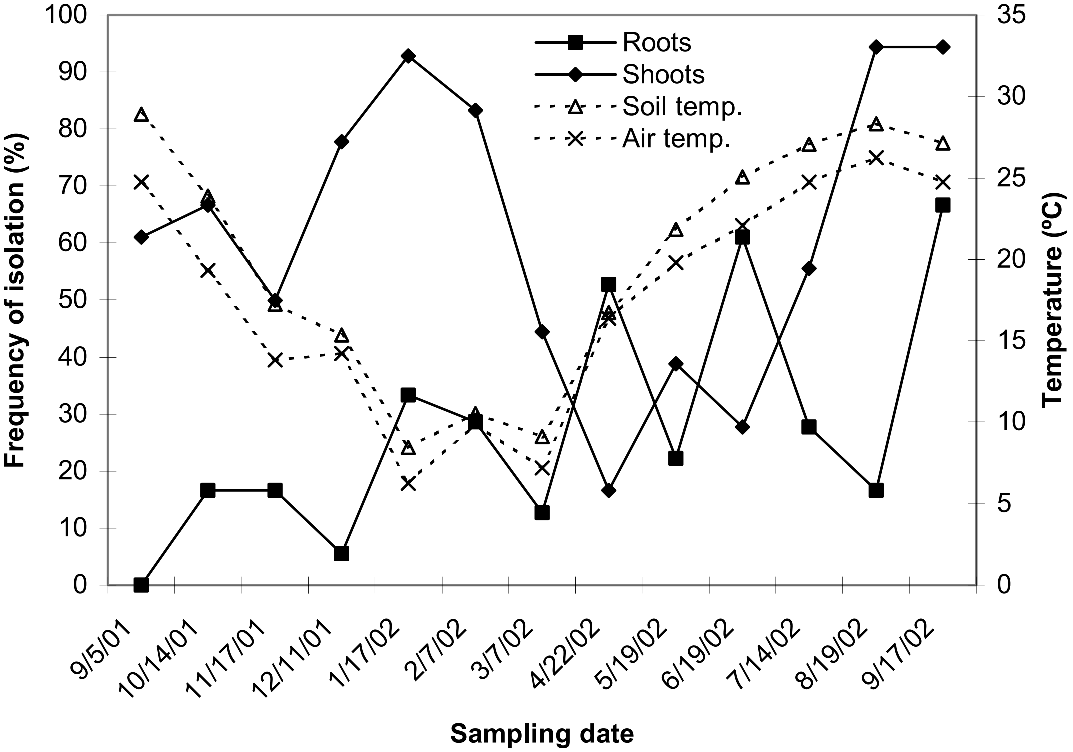

Figure 3.2. Frequency of isolation of *Xylella fastidiosa* in naturally infected sycamore stems and shoots and mean minimum air and soil temperatures. Bacterial presence in stem and root extracts was determined by plating xylem sap samples onto PW agar medium. For roots and shoots, each data point represents the percentage of successful isolations from 18 root or 18 shoot samples from the pooled Athens and Griffin sampling sites. Air and soil temperature minima are pooled from Athens and Griffin sampling sites.

CHAPTER 4

DISTRIBUTION AND POPULATION DENSITY OF *XYLELLA FASTIDIOSA* IN MECHANICALLY INOCULATED SYCAMORE ASSOCIATED WITH LOW TEMPERATURE TREATMENT AND PUTATIVE HOST RESISTANCE [2]

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[2] Henneberger, T. S., Stevenson, K. L., Britton, K. O., and Chang, C. J. To be submitted to Plant Disease.

Abstract

Henneberger, T. S., Stevenson, K. L., Britton, K. O., and Chang, C. J. 2003. Distribution and population density of *Xylella fastidiosa* in mechanically inoculated sycamore associated with low temperature treatment and putative host resistance. Plant Dis.

To determine the effect of low temperature treatment on populations of *Xylella fastidiosa* in naturally infected American sycamore (*Platanus occidentalis* L.), trees resistant and susceptible to infection by *X. fastidiosa* were inoculated with the pathogen and held for 12 weeks in dark chambers at either 5ºC or 22ºC. After temperature treatment, stem sections containing the inoculation point were collected and sap was extracted. To estimate bacterial populations, sap from each sample was tested for *X. fastidiosa* by ELISA and diluted onto periwinkle wilt medium. Trees with inoculation point segments that tested positive for presence of *X. fastidiosa* were divided into five additional tissue samples based on position relative to the inoculation point and assessed by ELISA and dilution plating. Our results indicate no significant effects for low temperature treatment at 5ºC or for putative host resistance. Bacteria were detected most frequently and were most densely populated in stem sections containing the inoculation point. Analysis of bacterial population distribution in trees that tested positive for infection by *X. fastidiosa* indicates that the primary direction of bacterial spread is acropetal, in the direction of the transpiration stream.

The xylem-limited, endophytic bacterium, *Xylella fastidiosa* Wells et al. (24) has been reported to infect hosts from more than 30 families of monocots and dicots. The pathogen has been implicated as the cause of leaf scorch, decline, and dieback diseases of an increasing number of forest and ornamental trees including elm (*Ulmus* spp.), maple (*Acer* spp.), oak (*Quercus* spp.), and sycamore (*Platanus* spp.) (3, 9, 14, 23). Diseases caused by *X. fastidiosa* in landscape and forest trees are characterized by development of foliar marginal necrosis in late summer and, in subsequent growing seasons in milder climates, by progressive basipetal decline and dieback (9).

Limited options are available for management of leaf scorch, decline, and dieback diseases of forest and ornamental trees. At present, the recommended strategies for managing diseases caused by *X. fastidiosa* on trees are use of resistant plants where available, maintenance of stress-free plants, and removal of plants that become infected (18). Ubiquitousness of the sharpshooter leafhopper vectors (Homoptera, Cicadellidae) as well as their long season of activity negates the possibility of vector management (10, 11); no antibiotics or bactericides are commercially available for control of *X. fastidiosa* in landscape and forest trees (18); and, while a new breed of systemic-resistance-inducing chemicals have been labeled for this use, their effectiveness remains unproven.

In addition, previous forestry research on American sycamore has suggested that it may be a likely candidate for genetic improvement by selective breeding (8, 15, 25). In a study examining optimization of production practices and breeding for mature biomass in *P. occidentalis* (15), Land determined that sycamores grown from seed from provenances south of the growing sites studied had significantly less disease incidence than those grown from seed collected from origins north of the test site after five to seven growing seasons under high disease pressure. Similarly, Wells and Toliver (25) reported sycamore height variations attributable to latitude of origin among a set of provenances from along the hot and droughty Gulf coast but they failed to detect similar differences from a set of provenances located along the more temperate and rainy Mississippi River valley. Greene and Lowe (8) made similar correlations between increasing latitude of origin and increasing disease incidence and severity and decreasing height in their 15-year study of *P. occidentalis* from seed sources within the western Gulf region. Both sets of researchers attribute negative height correlations to top dieback and crown loss resulting from stem canker infections caused by *Ceratocystis fimbriata* f. *platani* (Ellis and Halst) and *Botryosphaeria rhodina* (Cooke) Arx; although, as understanding of sycamore decline and dieback improves, it has become clear that *X. fastidiosa* is a frequent precursor to these opportunistic stem canker diseases and causes considerable apical dieback and crown loss even before cankers appear (16). These various results suggest that either decreased susceptibility due to heavy selection in more disease-pressured southern latitudes or increased direct or indirect damage to less cold-hardy host tissues of southern origin in northern latitudes may reduce disease in sycamore grown from seed from sources south of intended biomass planting sites.

Additionally, previous research on grapes (*Vitis* spp.) has suggested the possible amelioration of infections caused by *X. fastidiosa* by exposure of plants to low temperatures sublethal to hosts, but lethal to the bacterium (7, 19, 20). Purcell reported that multiple exposures of 1.5 to 24 h to temperatures ranging from -12ºC to 0ºC greatly enhanced the likelihood of survival of inoculated, symptomatic grapevines (19). In another study of vector-inoculated grapevines infected with *X. fastidiosa*, reported recovery rates for vines exposed to winter conditions at a variety of cold climates reached 100% in some more extreme environments (20). Studies of the temperature-dependent growth and survival of the pathogen both in vitro and in planta indicated that populations of *X. fastidiosa* decline at “cool but above-freezing temperatures” (7). Feil and Purcell reported that in vitro populations grew at 18ºC but not 12ºC and determined that the in planta minimum threshold temperature for growth in containerized grapevines was between 17ºC and 25ºC (7). Our objectives were to determine whether cold-treatment of infected sycamores would yield reductions in disease similar to those observed in grape (19, 20) and to determine whether there was any difference between the effects of cold treatment on *X. fastidiosa*-resistant and susceptible sycamore cultivars and those reported on resistant and susceptible varieties of grape (20).

Materials and Methods

Plant Material Selection. In three independent experimental trials, trees from putatively *X. fastidiosa*-resistant and susceptible sycamore families were grown from cuttings in vector-free greenhouses from seed provided by Westvaco Corporation (Stamford, CT) (R. J. Rousseau, *personal communication*). These trees were approximately 2 years of age at the beginning of the experiment. In the first trial, 36 resistant and 36 susceptible sycamores were treated (n=72). In the second trial, 33 resistant and 33 susceptible sycamores were treated (n=66). In the third experimental trial, 30 resistant and 30 susceptible sycamores were treated (n=60). In the third trial, a separate, parallel experiment was performed in which 30 grapevines, *Vitis vinifera* L. 'Cabernet Sauvignon,' which are moderately resistant to *X. fastidiosa*, and 30 vines of the highly susceptible cultivar, Pinot Noir, were treated as described for resistant and susceptible sycamore. To ensure that grapes did not contain any latent infection, 2 year-old dormant vines were obtained from Double A Vineyards (Fredonia, NY) north of the reported range of *X. fastidiosa* in grape.

To prevent transmission by insect vectors during the study, all 3 experimental trials were conducted on host trees grown and maintained after inoculation within screened cages on greenhouse benches. Trees and grapevines were placed in the screen cages in a randomized fashion after transplanting and were re-randomized into the cages after inoculation.

Inoculum Preparation and Inoculation. For all three runs of this study, the sycamore strain of *X. fastidiosa* was isolated from a naturally infected sycamore on the University of Georgia campus in Athens, GA. The grape strain of *X. fastidiosa* used in the third, parallel trial of this experiment was originally isolated from an infected grapevine in Georgia and contributed by Chang and Donaldson (2). Both strains were maintained at 28ºC and subcultured on periwinkle wilt (PW) agar medium (3) every 7 to 10 days, as soon as visible colony development was detected (4). Before inoculation, subcultured isolates from sycamore and grape were grown on PW medium for 14 days. Plates were flooded with 3 ml of sterile PW broth, lightly scraped to loosen colonies, collected into a sterile test tube, vortexed for 1 min, and the concentration adjusted by spectrophotometer to 10^8 to 10^9 colony forming units (CFU) per milliliter. At the time of each inoculation, inoculum density was confirmed by a series of 5 quantitative 10-fold dilutions in filtered PW broth and spread plating on PW medium. Confirmatory cultures were maintained at 28ºC and counted for bacterial density estimates as soon as visible colony growth was observed, approximately 10 to 14 days after plating.

Inoculations were performed during mid to late summer while trees were actively growing, using the pinprick method described by Hopkins (12). Trees and vines were not watered for 1 week before inoculation to ensure optimal uptake of inoculum. At inoculation, trees and vines were inspected for visible symptoms and all were found to be asymptomatic, although they were not tested for presence of the bacterium prior to inoculation. A 0.02-ml drop of inoculum suspension or sterile, filtered PW broth was placed on a petiole attached to the main leader midway between the soil line and the tree's apex, approximately 60 cm above the soil line. An insulin needle (25 × 0.7 mm) was used to pierce through the droplet and into the stem tissue of the main leader. As soon as the inoculum droplets were completely absorbed into the stems, inoculation points and a segment extending 2 cm above and below the inoculation point were marked with indelible pen for later use. After inoculation, trees and grapevines were maintained in the greenhouse screen cages for approximately 12 weeks after inoculation to allow time for systemic spread of *X. fastidiosa*.

Temperature Treatment. In all experimental trials, half of the controls and half of the inoculated trees were arbitrarily selected near or after dormancy onset and placed in a dark cooler at 5ºC to induce dormancy and to mimic low winter temperature conditions. Remaining trees were placed in a dark chamber within the greenhouse at 22ºC to induce dormancy with no added low temperature stimulus. Resistant and susceptible cultivars were divided evenly between the two temperature treatments. Trees were then maintained under these conditions with biweekly watering for approximately 12 weeks.

Pathogen Detection and Isolation. After 3 months of temperature treatment, a 4-cm, approximately 5-g segment of the stem tissue containing each marked inoculation point was collected, plants were removed from the pots, soil was shaken from the roots, and plants were placed in large bags and temporarily stored at 5ºC. Stem sections containing inoculation points were surface disinfested in a solution of 9.5% ethanol and 1.05% sodium hypochlorite for 10 min, then rinsed three times for 3 min in sterile, deionized water. After surface disinfestation, stem sections were squeezed with sterile pliers to extract approximately 0.3 ml of sap from each stem section. One-hundred microliters of extract was immediately assayed by ELISA (PathoScreen Xf peroxidase, Agdia, Elkhart, IN) according to manufacturer instructions in order to determine bacterial presence. An additional 100 μl was used in a five serial 10-fold dilutions in polyvinylpyrrolidone (PVPP) extraction buffer (8.0 g sodium chloride, 1.15 g dibasic sodium phosphate (anhydrous), 0.2 g monobasic potassium phosphate (anhydrous), 0.2 g potassium chloride, 0.5 g Tween-20, and 2.0 g 2% acid-washed polyvinylpyrrolidone 40,000 [PVP-40], and 1000 ml distilled water, pH adjusted to 7.4). To estimate actual bacterial density within host tissue, 5 µl from each dilution was spread onto PW medium and plates were maintained at 28ºC for 10 to 14 days, until visible colony growth was observed.

If the stem section containing the inoculation point tested positive for presence of *X. fastidiosa* by ELISA, then the tree was retrieved from storage for further sampling. Each plant was divided into five parts based on position relative to the inoculation point: ≈20 cm above the inoculation point, ≈20 cm below the inoculation point, plant crown (≈5 cm above and below the soil line), coarse roots (5 to 10 mm diameter), and fine roots (≤2 mm diameter). Three grams of tissue from each of these portions were prepared as described above for the inoculation points except fine root samples. To obtain a representative sample, root balls were divided into quadrants and 1 to 3 g fine roots were collected from each quadrant. After soil was rinsed from the fine root surfaces with tap water, they were placed in a petri dish, chopped into approximately 2-cm segments, stirred lightly, and then approximately 1 g of the fine roots was collected and surface disinfested as described above for stem sections. The disinfested fine roots were then finely macerated (segments ≤ 2 mm) in 3 ml of sterile PVPP extraction buffer with a sterile razor blade. To prevent cross-contamination between samples, a fresh blade was used for each sample. The fine root suspension and extracted sap samples from larger stem and root tissues were assayed by ELISA and serial dilution plating, as previously described for stem sections.

Bacterial presence was confirmed both by ELISA and culture on PW medium. A conservative approach was taken when assessing ELISA results. Only those samples with absorbance values of 0.95 or greater when read with a microtiter plate reader (Model 550, Biorad, Hercules, CA) at 490 nm were considered to be positive. Culture assays were considered to be positive when characteristic *X. fastidiosa* colonies appeared on the PW medium after approximately 10 to 14 days. Once colonies grew to visible size (0.25 to 1 mm), they were counted and bacterial density within the sample tissue was estimated. Bacterial densities were $\log_{10}$-transformed prior to analysis.

Data Analysis. Frequency of detection and population density of *X. fastidiosa* in stem sections that included the inoculation point were analyzed separately. For each experimental trial, and for each combination of temperature treatment and host resistance, the frequency of detection in stem sections including the inoculation point was expressed as the percentage of samples (reps) that tested positive for the pathogen. The influence of treatment factors on the frequency of detection was determined by analysis of variance (ANOVA) using SAS software (SAS Institute, Cary, NC) with experimental trial, temperature treatment, and host resistance as main factors. Main effects and two-way treatment interactions were tested using the trial × temperature × host resistance interaction as an error term. Log_{10}-transformed bacterial population densities ($\log_{10}$ CFU/g) were analyzed by ANOVA with experimental trial, temperature, and host resistance as main factors. If interactions between experimental trial and treatment factors were significant, then an ANOVA of bacterial population densities was performed separately for each experimental trial, with temperature and host resistance as main factors.

Frequency of detection and population density of *X. fastidiosa* in the different tissue samples from plants that had stem sections containing inoculation points which tested positive for the bacterium were subjected to further analysis. The influence of treatment factors on the frequency of detection was determined by ANOVA, with experimental trial, temperature treatment, host resistance, and plant part as main factors. Main effects and two- and three-way interactions were tested using the trial × temperature × host resistance × plant part interaction as an error term. Linear contrasts and Fisher's protected LSD were used to determine significant differences among plant parts. Log_{10}-transformed bacterial population densities ($\log_{10}$ CFU/g) were analyzed by ANOVA with experimental trial, temperature, host resistance, and plant part as main factors. If interactions between experimental trial and treatment factors were significant, then an ANOVA of bacterial population densities was performed separately for each experimental trial, with temperature, host resistance, and plant part as main factors. Linear contrasts and Fisher's protected LSD were used to determine significant differences among plant parts.

The parallel experiment utilizing grapevines in the third experimental trial was analyzed separately. For grapes, the frequency of detection and population density of the bacterium in vine sections that contained the inoculation point were analyzed separately. For each combination of temperature treatment and host resistance, the frequency of detection in vine samples that included the inoculation point was expressed as the percentage of samples (reps) that tested positive for the bacterium. Additionally, logistic regression (PROC LOGISTIC) was used to model the probability of a positive detection by ELISA as a function of temperature treatment, host resistance, and their interaction.

Log_{10}-transformed bacterial population densities (log_{10} CFU/g) were analyzed by ANOVA with temperature treatment and host resistance as main factors.

Grapevine inoculation point segments that tested positive for presence of *X. fastidiosa* and their different associated tissue samples were subjected to further analysis. For binary ELISA score data, logistic regression was used to model the probability of a positive detection by ELISA as a function of plant part, temperature treatment, host resistance, and their interactions. Frequency of detection in grapevine sections including the inoculation point was expressed as the percentage of samples (reps) that tested positive for the pathogen. Log_{10}-transformed bacterial population densities (log_{10} CFU/g) were analyzed by ANOVA with plant part, temperature treatment, and host resistance as main factors.

Results

No spread of *X. fastidiosa* to uninoculated control trees occurred during any run of this experiment; therefore data from controls were eliminated from further analysis.

There were no significant interactive effects between experimental trial and treatment factors on the frequency of detection of the bacterium in stem sections that included the inoculation point. Neither were there any significant differences between temperature treatments or host resistances in terms of frequency of detection in stem sections that included the inoculation point. Because no significant interactions were detected for either cold treatment or host resistance, results were pooled for further analysis. The mean frequency of detection of the bacterium in sycamore stem sections that included the inoculation point was 39.8% over all treatments and experimental trials.

Bacterial population densities in stem sections that included the inoculation point differed significantly among experimental trials, but were not affected by either temperature treatment or host. In Trials 1 and 3, mean bacterial population densities in stem sections that included the inoculation point were 5.15 and 6.00 $\log_{10}$ CFU/g, respectively, and were significantly greater than the mean bacterial population density of 3.23 $\log_{10}$ CFU/g recorded in Trial 2.

In those plants from which stem sections that included the inoculation point tested positive for the bacterium, the frequency of detection in tissue samples was not significantly affected by either temperature treatment or host resistance. However, the frequency of detection differed significantly among plant parts (Table 4.1). *X. fastidiosa* was detected most frequently in the apical stem portions (66.3%) and least frequently in the fine roots (1.3%). Frequency of detection of the bacterium was significantly greater in above-ground samples than in roots in all three trials ($P<0.0001$, $P=0.0069$, $P<0.0001$, respectively). Bacterial population density in portions of plants that tested positive for the bacterium at the inoculation point differed significantly among the three experimental trials; therefore, distributions of bacterial population densities within various host tissue samples were evaluated individually for each run of the study (Table 4.2). Bacterial densities were determined to be greatest in stem segments containing the inoculation point in all three trials; although, in Trial 2, bacterial densities in apical and basal stem sections were not significantly different from the inoculation point. No *X. fastidiosa* was recovered from below-ground samples in Trial 2. In Trial 1, bacterial densities in apical and basal stem samples were significantly lower than in the stem section containing the inoculation point (2.16 and 2.40 $\log_{10}$ CFU/g, respectively) but were not significantly different from one another. In Trial 3, after the stem segment containing the inoculation point, bacterial density was greatest in the apical stem segment (1.28 $\log_{10}$ CFU/g). In the third trial, however, bacterial density in basal stem and coarse root samples were not significantly different (Table 4.2).

Putatively resistant and susceptible grapevines used in the third run of this study were included to provide a physiological comparison with sycamores, since *X. fastidiosa* has been studied more extensively in grape than any other host. However, of 40 inoculated vines, only 2 non-cold-treated 'Pinot Noir' vines had confirmed infection by ELISA at the inoculation points. The results of the logistic regression analysis of the detection of *X. fastidiosa* by ELISA indicated no significant differences in the probability of detection of the bacterium in vine segments that included the inoculation point between temperature treatment and host. Mean frequency of detection in grapevines inoculated with *X. fastidiosa* was determined to be 5%. No significant differences in bacterial density were found between temperature treatments or host resistance. Mean bacterial density in grapevine segments containing the inoculation point was 5.53 $\log_{10}$ CFU/g. In vines that tested positive for the bacterium, further analysis of the 5 additional plant tissue samples failed to detect the presence of *X. fastidiosa* beyond the inoculation point. Further, the bacterium could not be recovered in culture from additional plant tissue samples.

Discussion

No significant response to low temperature treatment at 5°C was observed in *X. fastidiosa*-resistant and -susceptible plants. While it is generally accepted that the geographical distribution of *X. fastidiosa* is limited at the northern end of its range by low winter temperatures (19, 21), formal studies of seasonal and temperature-driven fluctuation in populations of *X. fastidiosa* within host plants have yielded variable results. In a study of grapevines infected with *X. fastidiosa* and exposed to 5 months of winter conditions at a variety of cold climates in California, researchers found that vines maintained in more extreme environments exhibited near 100% reduction in symptoms and had no recoverable populations of *X. fastidiosa* after 6 months (20). However, Purcell indicated that the weak correlation between average minimum temperature and the percentage of plants "cured" of disease suggested that factors additional to average minimum temperature might be at work in limiting disease incidence (20). Similar growth chamber research on cold-treated bare-root and containerized grapevines indicated that infection by *X. fastidiosa* might be ameliorated by exposing hosts to low temperatures (7, 19). Purcell reported that multiple exposures of container grown and bare-root grapevines for 1.5 to 24 h to temperatures ranging from -12ºC to 0ºC greatly enhanced the likelihood of symptom remission in inoculated, symptomatic grapevines as the number and severity of cold treatments increased (19). In a study of temperature-dependent growth and survival of *X. fastidiosa* in cold-treated, containerized grapevines (7), Feil and Purcell determined that the in planta minimum threshold temperature for growth was between 17ºC and 25ºC. In another study (6), Feil and Purcell discovered a significant correlation between lateness in the growing season of host inoculation and reduced multiplication and survival of bacterial populations in current and subsequent seasons. Since our inoculation dates tended to be later in the growing season, it is possible that bacterial populations did not establish well due to pre-dormancy alterations in host xylem chemistry such as increases in levels of dormancy-driven bacterial growth inhibitors.

Although a response to cold treatment was not observed in our study, the lack of response was not entirely unexpected. In a study of *X. fastidiosa* in naturally infected citrus groves in Florida, frequency of detection of bacteria within host plants was greatest from June to September and from December to February (13). It is likely that, in the milder Florida climate, winter temperature minima are not low enough to cause significant pathogen mortality, unlike the response observed by Purcell in the colder climes of the Pacific northwest (20). Additionally, in a study of naturally infected sycamore at two locations in Georgia, we found that air temperatures below -5ºC were best associated with limiting bacterial multiplication in host tissue (Chapter 3). These field study findings are consistent with reports that "bacterial leaf scorch diseases on ornamental and forest trees appear to extend into cooler winter climates than [does] Pierce's disease or phony peach disease" (21). Kostka et al. (14) successfully isolated the causal bacterium from oak trees as far north as New York and Pennsylvania and Sherald et al. (23) described leaf scorch on red maple in northern Virginia. Mechanisms for survival of *X. fastidiosa* in forest and ornamental trees in cooler winter climates are not well understood. It has been suggested, however, that during winter months pathogen populations may survive and accumulate in soil-insulated root xylem (3) and it is also possible that strains of *X. fastidiosa* infecting more northerly forest and ornamental trees have become adapted to lower temperatures. Whatever the survival mechanism(s) may be, there is strong evidence to suggest that the low temperature treatment we imposed may not have been cold enough to elicit a significant decline in bacterial titer within the sycamore host tissue; although more expanded trials are needed to determine the exact mechanisms of bacterial survival in planta.

No significant host resistance response was observed in either grape or sycamore. While failure to observe a significant resistance response suggests that putative resistance may be an artifact, it is important to note that the putative resistance of sycamores used in this study was based on 17 years of observations of ortets and parental families, which should not be discounted without more detailed examination (R. J. Rousseau, *personal communication*). Similarly, relative resistance and susceptibility of grapevines used in the parallel experiment in Trial 3, cultivars Cabernet Sauvignon and Pinot Noir, to infection by *X. fastidiosa* have been well documented. Hill and Purcell reported that the cultivar Pinot Noir is "a very susceptible host that supports rapid and extensive bacterial multiplication" (10) and Purcell found better symptom remission and reduction in bacterial presence for the cultivar Cabernet Sauvignon than seven other *Vitis* cultivars after 6 months of winter conditions at 5 sites in California and Washington (20). In another study on in planta populations of *X. fastidiosa* required for transmission by efficient vectors, Hill and Purcell determined that the grape cultivar Pinot Noir, along with blackberry (*Rubus discolor* Weihe & Nees), supported higher populations of *X. fastidiosa*, that bacteria multiplied most rapidly within these hosts, and that vectors feeding on these hosts transmitted the pathogen more frequently than did vectors feeding on the three other hosts in the study (11). Although specific mechanisms for host resistance in these hosts are not well understood, resistance may be based in tolerance of bacterial infection, differential physiology, or biochemical factors for which our examination was not structured. To determine the existence and nature of host resistance, it is necessary to construct a study wherein symptoms can be observed in conjunction with assays for presence and populations of *X. fastidiosa*.

Examinations of bacterial presence and distribution through the host indicate that *X. fastidiosa* infections of sycamore tend to remain localized in aerial portions of the plant, but that some spread to crown and root xylem does occur. This is consistent with reports that, in dieback and decline diseases of ornamental and forest trees, *X. fastidiosa* accumulates mainly in trunks and stems, around the point of inoculation or vector feeding. For example, Barnard et al. reported greater frequency of detection of the pathogen in symptomatic leaf, petiole, and stem tissues than in similar asymptomatic tissues (1). Further, Hill and Purcell in two of their *X. fastidiosa* population and vector transmission studies (11, 22), suggested that efficiency of acquisition of the bacterium by insect vectors might be reduced as distance of vector feeding from the site of original mechanical inoculation increased and that, in some non-proliferative hosts, bacteria may fail to spread beyond xylem immediately adjacent to the inoculation point. Additionally, bacteria may have either not had sufficient time to reach populations large enough for full systemic movement before cold treatment was imposed or may simply have lacked the strong virulence traits necessary for extensive host colonization.

In our results, the primary direction for bacterial spread was acropetal. This type of distribution is expected in a xylem-mobile bacterium, where spread should be greatest in the direction of the transpiration stream. Mechanisms of spread to distal downstream regions are not well understood, although lateral movement through breached pit membranes has been observed ultrastructurally (E. Alves et al., *unpublished data*). Additionally, a functional genetic analysis of the *X. fastidiosa* type II secretion system has suggested the potential to produce cell-degrading gene products including endoglucanase, polygalacturonase, and several proteases (5) which might break down pit membranes and facilitate bacterial movement into adjoining vessels. It is also possible that bacteria were present in roots at the time of inoculation, as health assessments for host plants made during inoculation were based on visual symptoms only. Presence of bacteria in roots such as we observed, regardless of transport mechanism, provides evidence that roots may provide a sheltered environment where bacteria may survive and develop into early season inoculum reservoirs even before vector populations become active. Further research should be aimed at identifying the degree to which bacterial populations overwintering in roots contribute to early season infection, which has been determined to lead to greatest losses in subsequent seasons (7).

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Table 4.1. Position relative to the inoculation point and mean frequency of detection of *Xylella fastidiosa* by ELISA in various tissues of 2-year old sycamore trees with confirmed infection at inoculation points

Plant part	Relative sample position	Frequency of detection (%)[y]
Leader apex	≈20 cm above inoculation point	66.3 b[z]
Inoculation point	≈60 cm above soil line	100.0 a
Basal stem	≈20 cm below inoculation point	39.0 c
Crown	soil line ± 5 cm	11.3 d
Coarse roots	5-10 mm diameter	41.1 c
Fine roots	<2 mm diameter	1.3 d

[y] Means are based on data pooled over treatments and experimental trials.

[z] Means followed by the same letter are not significantly different based on Fisher's Protected LSD test ($P > 0.05$).

Table 4.2. Population density of *Xylella fastidiosa* in 2-year-old sycamore trees inoculated with the pathogen and maintained at 5ºC or 22ºC for 12 weeks[y]

	Bacterial population density ($\log_{10}$ CFU/g)		
Plant part	Trial 1	Trial 2	Trial 3
Leader apex	2.16 b[z]	1.41 a	1.28 b
Inoculation point	5.15 a	3.23 a	6.00 a
Basal stem	2.40 b	1.41 a	0.36 cd
Crown	0.25 c	0.00 b	0.00 d
Coarse roots	0.00 c	0.00 b	0.61 c
Fine roots	0.00 c	0.00 b	0.00 d

[y] Results for both temperature treatment and host resistance groups are pooled, as no significant differences were detected among them.

[z] Bacterial population densities in each column followed by the same letter are not significantly different based on Fisher's Protected LSD test ($P > 0.05$).

CHAPTER 5

PATHOGENICITY AND HOST SPECIFICTY OF STRAINS OF *XYLELLA FASTIDIOSA* FROM OAK, SYCAMORE, AND GRAPE

The bacterium, *Xylella fastidiosa* Wells et al. (35) is a xylem-limited endophyte which has been reported to infect members of more than 30 families of monocotyledonous and dicotyledonous hosts (13, 32). While many host plants are asymptomatic (27), susceptible hosts may suffer severe losses in quality and yield. Among the many economically important diseases caused by *X. fastidiosa* are Pierce's disease of grapevine (7), phony peach disease (6, 36), citrus variegated chlorosis (3), and leaf scorch of plum (30), pear (25), almond (28), coffee (12), mulberry (24), elm, sycamore, oak (3, 17), and maple (33). Although classified as a single species (35), variations in host susceptibility, symptomology, and pathogenicity by various host strains of the bacterium indicate a complex relationship between hosts and strains of *X. fastidiosa* (22).

Symptoms of infection by *X. fastidiosa* are similar on grape (*Vitis vinifera* L.), oak (*Quercus* spp.), and sycamore (*Platanus* spp.) hosts. In all three hosts, early season infection may result in foliar marginal necrosis late in the growing season, especially in association with water-stress (16). Where climate does not limit the range of the bacterium, *X. fastidiosa* spreads systemically within host xylem, inducing formation of gums, gels, and tyloses by the host (29). In subsequent seasons, hosts suffer gradual declines in yield and vigor and increases in water-stress due to occlusion of the xylem vessels (26).

Thus far, taxonomic separation of *X. fastidiosa* at the pathovar level has proven difficult. Reciprocal inoculations performed with isolates from grape (*Vitis* spp.), almond (*Prunus dulcis* [Mill.] D.A.Webb), and alfalfa (*Medicago sativa*) indicate that diseases in these hosts are caused by the same strain of *X. fastidiosa* (10). In a similar pathogenicity study, Pierce's disease- and periwinkle wilt-causing strains of the bacterium were proven to be pathologically distinct (9). In another reciprocal inoculation study, the ragweed stunt-causing strain of *X. fastidiosa* multiplied within plum (*Prunus saliciliana* Lindl.) and periwinkle (*Catharanthus roseus* [L.] G. Don) asymptomatically and did not infect grape, peach (*Prunus persica* [L.] Batsch), or citrus (*Citrus* spp.). All strains from the abovementioned hosts failed to infect ragweed (*Ambrosia artemisiifolia* L.) except for the phony peach strain, which only infected a small percentage of inoculated plants (34). Thus, the ragweed stunt strain of *X. fastidiosa* is pathologically distinct; but it may be more closely related to phony peach disease strains than the others evaluated. While the above studies provide hints as to the relationships between hosts and strains of *X. fastidiosa*, they also demonstrate a need for more detailed understanding of *X. fastidiosa* at the pathovar level. This study was undertaken to examine the pathogenicity and host specificity of strains of *X. fastidiosa* isolated from grape, oak, and sycamore in same species and reciprocal hosts by evaluating bacterial presence, density, and systemic movement through mechanically inoculated host plants.

Materials and Methods

Plant material preparation. Sycamores used to test for reciprocal pathogenicity of *X. fastidiosa* were selected from half-sib seedlings of a family of American sycamore (*Platanus occidentalis* L.) putatively susceptible to infection by *X. fastidiosa.* Seeds were provided by breeders at Westvaco Corporation (Stamford, CT) (R. J. Rousseau, *personal communication*) and seedlings were grown in containers for 1 year before inoculation. Forty sycamore seedlings were used for this study. Grapes used to test for reciprocal pathogenicity of *X. fastidiosa* were *Vitis vinifera* cv. Pinot Noir (n=40), a cultivar reportedly susceptible to infection by *X. fastidiosa*. To ensure that grapes did not contain latent infection, 2-year-old dormant vines were obtained from Double A Vineyards (Fredonia, NY), north of the reported range of *X. fastidiosa* in grape. Dormant vines were planted in containers and grown in the greenhouse for 6 months prior to inoculation. Northern red oaks (*Quercus rubra* L.) used to test for reciprocal pathogenicity of *X. fastidiosa* were grown from purchased seed (Sheffield Seed Company, Locke, NY) in the greenhouse for 2 years before inoculation (n=40). Plants were maintained in the greenhouse with biweekly fertilization and regular watering. To prevent transmission by insect vectors during the study, oaks, sycamores, and grapevines were grown in screened cages in randomized blocks after transplanting and were re-randomized into the cages after inoculation.

Inoculum preparation and inoculation. Sycamore and oak strains of *X. fastidiosa* used to test for reciprocal pathogenicity were isolated from naturally infected sycamore and pin oak (*Quercus palustris* Münch.) trees on the University of Georgia campus in Athens, GA. Presence of *X. fastidiosa* in symptomatic tissue was verified by a commercially available enzyme-linked immunosorbent assay (ELISA) prior to isolation (PathoScreen Xf peroxidase, Agdia, Elkhart, IN) according to manufacturer instructions. The grape strain of *X. fastidiosa* was isolated from infected vines in a Georgia vineyard and contributed by Chang and Donaldson (2). All strains used in this study were maintained on periwinkle wilt (PW) agar at 28ºC and subcultured every 7 to 10 days, as soon as colonies were visible (8). Before inoculation, subcultured isolates from all three strains of *X. fastidiosa* were grown on PW agar medium for 10 days at 28ºC. Plates were flooded with 3 ml of sterile PW broth medium, lightly scraped to loosen colonies, collected into a sterile test tube, agitated with a vortex mixer for 1 min to obtain a uniform cell suspension, and the concentration adjusted by spectrophotometer to 10^8 to 10^9 colony forming units (CFU) per milliliter. At the time of inoculation, inoculum density was confirmed by a series of 5 quantitative 10-fold dilutions in filtered PW broth and spread plating on PW medium. Confirmatory cultures were maintained at 28ºC and counted for bacterial density estimates as soon as visible colony growth was observed, approximately 10 to 14 days after plating. Cultures from all three strains of the bacterium, once grown and quantified, were found to be consistent with the calculated inoculum density and exhibited characteristic colony morphology for *X. fastidiosa*.

Inoculations were performed in late August, while trees were actively growing, using the pinprick method described by Hopkins (11, 20). Trees and vines were not watered for 1 week before inoculation to ensure optimal uptake of inoculum. At inoculation, trees and vines were inspected for visible symptoms and all were found to be asymptomatic, although they were not tested for presence of the bacterium prior to inoculation. A 0.02-ml drop of inoculum suspension or sterile, filtered PW broth was placed on a petiole attached to the main leader midway between the soil line and the tree's apex, approximately 60 cm above the soil line. An insulin needle (25 × 0.7 mm) was used to pierce through the droplet and into the stem tissue of the main leader. As soon as the inoculum droplets were completely absorbed into the stems, inoculation points and a segment extending 2 cm above and below the inoculation point were marked with indelible pen for later use. After inoculation, trees and grapevines were maintained in the greenhouse screen cages for approximately 12 weeks to allow time for growth and systemic movement of *X. fastidiosa*.

Sample collection, pathogen detection, and isolation. Trees and grapevines were harvested while dormant; therefore, symptoms of infection by *X. fastidiosa* could not be observed. A 4-cm, approximately 3-g segment of the stem tissue containing the marked inoculation point was collected from each plant, plants were removed from the pots, soil was shaken from the roots, and plants were placed in large bags and temporarily stored at 5ºC. Stem sections containing inoculation points were surface disinfested in a solution of 9.5% ethanol and 1.05% sodium hypochlorite for 10 min, then rinsed three times for 3 min in sterile, deionized water. After surface disinfestation, stem sections were squeezed with sterile pliers to extract approximately 0.3 ml of sap from each stem section. One-hundred microliters of extract was immediately assayed by ELISA (PathoScreen Xf peroxidase, Agdia, Elkhart, IN) according to manufacturer instructions in order to determine bacterial presence. An additional 100 µl was used for 5 serial 10-fold dilutions in polyvinylpyrrolidone (PVPP) extraction buffer (8.0 g sodium chloride, 1.15 g dibasic sodium phosphate (anhydrous), 0.2 g monobasic potassium phosphate (anhydrous), 0.2 g potassium chloride, 0.5 g Tween-20, and 2.0 g 2% acid-washed polyvinylpyrrolidone 40,000 [PVP-40], and 1000 ml distilled water, pH adjusted to 7.4). To estimate actual bacterial density within host tissue, 5 µl from each dilution was spread onto PW medium and plates were maintained at 28ºC for 10 to 14 days, until visible colony growth was observed.

If the stem section containing the inoculation point tested positive for presence of *X. fastidiosa* by ELISA, then the tree or grapevine was retrieved from storage for further sampling. Each plant was divided into five parts based on position relative to the inoculation point: ≈20 cm above the inoculation point, ≈20 cm below the inoculation point, plant crown (≈5 cm above and below the soil line), coarse roots (5 to 10 mm diameter), and fine roots (≤2 mm diameter). Three grams of tissue from each of these portions were prepared as described above for the inoculation points except fine root samples. To obtain a representative sample, root balls were divided into quadrants and 1 to 3 g fine roots were collected from each quadrant. After soil was rinsed from the fine root surfaces with tap water, they were placed in a petri dish, chopped into approximately 2-cm segments, stirred lightly, and then approximately 1 g of the fine roots was collected and surface disinfested as described above for stem sections. The disinfested fine roots were then finely macerated (segments ≤ 2 mm) in 3 ml of sterile PVPP extraction buffer with a sterile razor blade. To prevent cross-contamination between samples, a fresh blade was used for each sample. The fine root suspension and extracted sap samples from larger stem and root tissues were assayed by ELISA and serial dilution plating, as previously described for stem sections.

Bacterial presence was confirmed both by ELISA and culture on PW medium. A conservative approach was taken when assessing ELISA results. Only those samples with absorbance values of 0.95 or greater when read with a microtiter plate reader (Model 550, Biorad, Hercules, CA) at 490 nm were considered to be positive. Absorbance readings at 490 nm were based on a mean negative control (PVPP extraction buffer) reading of 0.004 and a mean positive control (solution of lyophilized *X. fastidiosa* suspended in PVPP extraction buffer at $\approx 1 \times 10^7$ cells per ml) reading of 1.049. Culture assays were considered to be positive when characteristic *X. fastidiosa* colonies appeared on the PW medium after approximately 10 to 14 days. Once colonies grew to visible size (0.25 to 1 mm), they were counted and bacterial density within the sample tissue was estimated. Bacterial densities were $\log_{10}$-transformed prior to analysis.

Data analysis. This experiment was a balanced two-way factorial design, with host and inoculum as main factors. Each host × inoculum combination was replicated 10 times during the single trial for this study. The statistical package SAS was used to analyze the data (SAS Institute, Cary, NC).

Separate analyses were performed for probability of detection by ELISA and population density of *X. fastidiosa* in stem samples containing inoculation points. For binary ELISA score data, logistic regression (PROC LOGISTIC) was used to model the probability of a positive detection by ELISA as a function of host, inoculum, and their interaction. Continuous response variables, ELISA absorbance reading and $\log_{10}$-transformed bacterial population density ($\log_{10}$ CFU/g), were analyzed by ANOVA with host and inoculum as main factors. Linear contrasts were used to determine significant differences among host responses to inoculum strains.

Population density measures from inoculation point stem sections which tested positive for the *X. fastidiosa* and their five associated plant part samples were subjected to further analysis. For binary ELISA score data, logistic regression (PROC LOGISTIC) was used to model the probability of a positive detection by ELISA as a function of host, inoculum, plant part, and their interactions. ELISA absorbance reading and $\log_{10}$-transformed CFU/g were analyzed by ANOVA with host, inoculum strain, and plant part as main factors. Main effects and two-way treatment interactions were tested using rep-to-rep variation as an error term. If there were no significant interactions between variables, significant differences among plant parts were determined by means separation using Fisher's protected LSD test at $P = 0.05$.

A linear correlation analysis (PROC CORR) was performed to investigate the relationship between the response variables ELISA absorbance reading at 490 nm and CFU/g in tissue samples that yielded measurable populations of *X. fastidiosa*.

Results

No spread of *X. fastidiosa* to uninoculated control trees occurred during this experiment; therefore, they were omitted from analysis.

The results of the logistic regression analysis of the detection of *X. fastidiosa* by ELISA indicated no significant differences were found in probability of detection of the bacterium in stem segments that included the inoculation point between host species or strains of *X. fastidiosa* inoculum based on non-significant Wald chi-square statistics at $P = 0.05$. The mean frequency of detection of *X. fastidiosa* in stem sections that included the inoculation point was 10% over all host species and inoculum strains. The host × inoculum strain interaction was highly significant ($P = 0.0034$) indicating that bacterial densities in stem sections that included the inoculation point differed significantly among host species and inoculum strains (Table 5.1). In sycamores, there was no significant difference between bacterial densities following inoculation with the sycamore or oak inoculum strains (*P* = 0.1039) and the grape strain could not be recovered in culture for quantification. In oak hosts, there was no significant difference between bacterial densities generated by sycamore and oak inoculum strains (*P* = 0.3824) and the grape strain could not be recovered in culture for quantification. None of the three strains were detected in or reisolated from grape stem sections containing the inoculation point; therefore bacterial populations were presumed to be below limits of detection or absent in this host.

ELISA absorbance readings in stem sections that included the inoculation point differed significantly among host species and inoculum strains (*P* < 0.0001). Sycamore stem sections including the inoculation point had significantly higher absorbance readings for *X. fastidiosa* after inoculation with the oak strain than with the sycamore strain (*P* < 0.0001). For oak, no significant difference was detected between absorbance readings for the sycamore and oak strains of the bacterium within oak stem sections including the inoculation point (*P* = 0.6025). Absorbance readings for *X. fastidiosa* detected by ELISA in grape stem sections including the inoculation point did not differ significantly from negative controls for any of the inoculum strains used in this study.

The results of the logistic regression analysis of the detection of *X. fastidiosa* by ELISA indicated no significant differences in probability of detection of the bacterium in the five additional tissue samples from plants that had inoculation points that tested positive for presence of *X. fastidiosa* among host species or strains of *X. fastidiosa* inoculum or plant parts. Wald chi-square statistics for variables and interactions were not significant ($P > 0.05$), indicating that bacterial densities in tissue samples representing ELISA-positive hosts did not differ significantly among host species or inoculum strains. Bacterial density differed significantly among plant parts ($P < 0.0001$). Stem segments containing inoculation points had the highest bacterial density (5.96 $\log_{10}$ CFU/g) and fine roots, from which no *X. fastidiosa* could be reisolated, had the lowest bacterial density (Table 5.2).

Absorbance readings in ELISA-positive stem sections that included the inoculation point and their associated tissue samples differed significantly among inoculum strains ($P = 0.0113$) and plant parts ($P < 0.0001$), while host species and all interactions were non-significant in relation to absorbance reading. Tissue samples infected by the oak strain produced a significantly higher mean absorbance score ($A_{490\text{nm}} = 0.06$) than tissue samples infected by the sycamore strain which had a mean score of 0.04. These were, as expected, intermediate relative to the mean negative control ($A_{490\text{nm}} = 0.004$) and mean positive control ($A_{490\text{nm}} = 1.049$). Grape inoculum was not significantly different from negative controls in terms of absorbance scores. Mean absorbance readings by plant part are presented in Table 5.2.

The linear correlation analysis of the relationship between bacterial density in samples that yielded estimable bacterial populations and ELISA absorbance scores indicated a significant ($P = 0.0400$) but not very meaningful positive correlation ($r = 0.4745$) between absorbance reading at 490 nm and untransformed bacterial density (CFU/g). Correlation was slightly stronger ($r = 0.5184$, $P = 0.0230$) (Fig. 5.1) after bacterial densities were $\log_{10}$-transformed as described above. However, the strongest correlation between the two variables was observed when both were $\log_{10}$-transformed ($r = 0.6192$, $P = 0.0047$).

Discussion

In this study, we discovered significant differences among hosts for infections caused by pin oak, sycamore, and grape strains of *X. fastidiosa*, both in bacterial density and absorbance readings. These findings may be explained, in part, by the wide host range of *X. fastidiosa*, which encompasses at least 30 families of monocotyledonous and dicotyledonous plants (32), and the difficulty of its isolation and culture, which have made clear relationships between host strains and host ranges difficult to assess for this bacterium. While early serological, cultural, and ultrastructural investigations of *X. fastidiosa* failed to clearly distinguish strains of the bacterium (6, 10, 18), more recent work with various host strains has indicated that variations do indeed exist among strains of *X. fastidiosa* from different host species (4, 31, 36). For example, when sycamore and elm were inoculated with two strains of *X. fastidiosa* isolated from naturally infected American sycamore and American elm (*Ulmus americana*), respectively, they were found to be pathogenic; however, they did not cause symptoms nor could they be recovered in culture when inoculated into the reciprocal hosts (31). Similarly, a series of Ouchterlony double-diffusion serological tests for interrelationships among oak leaf scorch (OLS), phony peach disease, and Pierce's disease strains of *X. fastidiosa*, revealed that the OLS strain was partially related to the Pierce's disease strain and unrelated to the phony peach disease strain (4). Even the first taxonomic description of *X. fastidiosa* as a new genus and species reported highest growth rates for strains isolated from grape, almond (*Prunus amygdalus* L.), elm, and mulberry (*Morus* spp.) and lowest growth rates for strains of *X. fastidiosa* isolated from peach (*Prunus persica* [L.] Batsch) and plum (*Prunus saliciliana* Lindl.)(36). In light of this array of variations and associations between host strains of *X. fastidiosa* and various hosts, our finding of significant differences in bacterial density and absorbance readings among hosts infected with strains from pin oak, sycamore, and grape are not unexpected.

While failure of the grape isolate of *X. fastidiosa* to infect any host, including grape, suggests that loss of virulence, similar to that observed by Hopkins in various grape strains of *X. fastidiosa* (21), may have occurred during weekly serial transfers; failure of oak and sycamore isolates to infect grapevines may be better explained by the recent analyses of bacterium DNA. In their analysis of 16S rDNA sequences from seven *X. fastidiosa* pathotypes, Chen et al. (5) reported a cytosine/thymine shift at sequence position 145 which effectively separates the seven pathotypes examined into two groups; grape and mulberry strains are in one group and citrus, oleander, periwinkle, plum, and peach strains in the other. A similar division of pathotypes has been reported by Kamper et al. (23), who described Pierce's disease strains as genetically uniform and those strains from the various tree hosts examined as diverse based on DNA homology. This division of pathotypes may explain the failure of infective transmission of oak and sycamore strains in grapevine, which falls into a different genetic host/strain grouping according to Chen et al. and Kamper et al. The reported genetic diversity of tree host strains of *X. fastidiosa* may further explain the variation in infection levels between oak and sycamore strains of the bacterium as quantified by bacterial density estimates and absorbance scores. More extensive evaluation of variations between strains of *X. fastidiosa* is needed to better understand differences and relationships among hosts and pathotypes. Given the inherent difficulty of establishing representative, successful host range and reciprocal inoculation studies in planta, future understanding of variations among strains of this fastidious endophyte may rest in the use of more rapid and sensitive molecular techniques such as restriction endonuclease fingerprinting and restriction fragment-length polymorphisms (RFLPs) or DNA homology tests similar to those described above (5, 23).

The failure of northern red oak to become infected by a strain of *X. fastidiosa* isolated from pin oak was unexpected; although, successful reciprocal infection of sycamores indicates that the oak strain was, at least, virulent. Although host-specific resistance mechanisms against *X. fastidiosa* are not well understood, host resistance has been observed in various *Vitis* species as both reduced symptom expression and reduced bacterial density in host tissue (14) and has been attributed to such diverse causes as structural barriers (including gels, gums, and tyloses), inhibitory chemicals and substances produced by host response to infection, and differential vascular anatomy between resistant and susceptible host species (14, 15) and in sycamore as delayed and reduced symptom expression (R. J. Rousseau, *personal communication*). While no specific resistance has been observed in oaks, it is possible that one or more of the abovementioned resistance mechanisms is at work in *Quercus* species as well, although more detailed cytological and ultrastructural examination is necessary to determine the nature of host resistance, if any exists.

X. fastidiosa was both detected in and recovered from plant tissues above and below the inoculation point, although bacterial densities and absorbance scores were greatest in stem sections that included the inoculation point. This is consistent with findings that bacterial cell concentrations at the inoculation point must reach 10^7 to 10^9 CFU/g before systemic movement of *X. fastidiosa* can occur (19). Interestingly, after inoculation point segments, the bacterium was found at highest densities in apical stem segments and coarse root samples from infected host plants. The primary direction of movement of xylem-limited bacteria is acropetal, in the direction of the transpiration stream, as supported by accumulation in apical portions of infected trees. Accumulation in coarse roots at levels similar to those in apical tips may have been induced by shortening daylengths during the greenhouse incubation period; although mechanisms for movement of *X. fastidiosa* downward through the xylem apoplast have not been well studied. Alternatively, xylem elements may be more susceptible to cavitation as bacterial by-products and gels, gums, and tyloses formed by infection upstream clog vessels and create emboli. A break in the column of xylem fluid and resultant gravity-driven drop may facilitate rapidly move bacterial cells along with xylem contents downstream to the next pit wall, where pit membrane degradation can begin anew. Lower bacterial densities in apical stem sections, stem sections taken from below the inoculation point, and crown samples may also be a function of the relative volume and orientation of vascular tissues in these sections as compared to coarse roots. Greater quantities of bacteria may be more readily extracted from secondary, coarse roots, which have small volume, centrally located vascular bundles, than from tissues at or above the soil surface, which have greater and more widely dispersed volumes of vascular tissue (1). In order to better understand mechanisms for growth and host colonization by *X. fastidiosa*, more detailed study of water relations and bacterial transport in host plants infected by the bacterium is needed.

The weak correlation between ELISA absorbance scores and bacterial density indicates that absorbance score may not be a useful tool for rapid estimation of bacterial populations within host tissue extracts. Although the regression was significant, an analysis of residuals indicated that the linear model was not appropriate for untransformed data. Log_{10}-transformation of bacterial densities stabilized variances as absorbance reading increased, but residuals still indicated the need for higher order terms. Even after both variables had been $\log_{10}$-transformed and regressed to obtain the best linear fit, correlation was still poor. These results indicate that, while ELISA is useful for detection, use of absorbance readings from the assay as a quantitative tool is inadvisable.

Results of this experiment are based on a single experimental trial. It is important to note that this is a discussion of preliminary results only. This experiment must be replicated in additional trials before valid conclusions can be based upon it.

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Table 5.1. Mean bacterial density ($\log_{10}$ CFU/g) in 4 ± 0.5 cm (3 g) stem sections containing inoculation points by host species and *X. fastidiosa* host strain

	***Xylella fastidiosa* host strain**		
Host species	American sycamore	Pin oak	Grape
American sycamore	2.378	3.552	ND
Northern red oak	0.627	ND	ND
Grape cv. Pinot Noir	ND[z]	ND	ND

[z] ND = no bacterial growth detected.

Table 5.2. Bacterial density expressed as mean $\log_{10}$-transformed CFU of *Xylella fastidiosa* per gram of host tissue by plant part and as ELISA absorbance reading at 490 nm

Plant part	Relative sample position	Mean bacterial density ($\log_{10}$ CFU/g)	ELISA absorbance reading at 490 nm[x]
Leader apex	≈20 cm above inoculation point	0.502 bc[y]	0.027 c
Inoculation point	≈60 cm above soil line	5.961 a	0.133 a
Basal stem	≈20 cm below inoculation point	0.915 bc	0.032 bc
Crown	soil line ± 5 cm	0.387 bc	0.025 c
Coarse roots	5-10 mm diameter	1.610 b	0.052 b
Fine roots	<2 mm diameter	ND c[z]	0.012 c

[x] Absorbance readings at 490 nm are based on mean negative control (PVPP extraction buffer) reading of 0.004 and mean positive control (solution of lyophilized *X. fastidiosa* suspended in PVPP extraction buffer at ≈ 1×10^7 cells per ml) reading of 1.049.

[y] Means are based on data pooled over host species and inoculum strains, values within a column followed by the same letter are not significantly different based on Fisher's Protected LSD test ($P > 0.05$).

[z] ND = no bacterial growth detected.

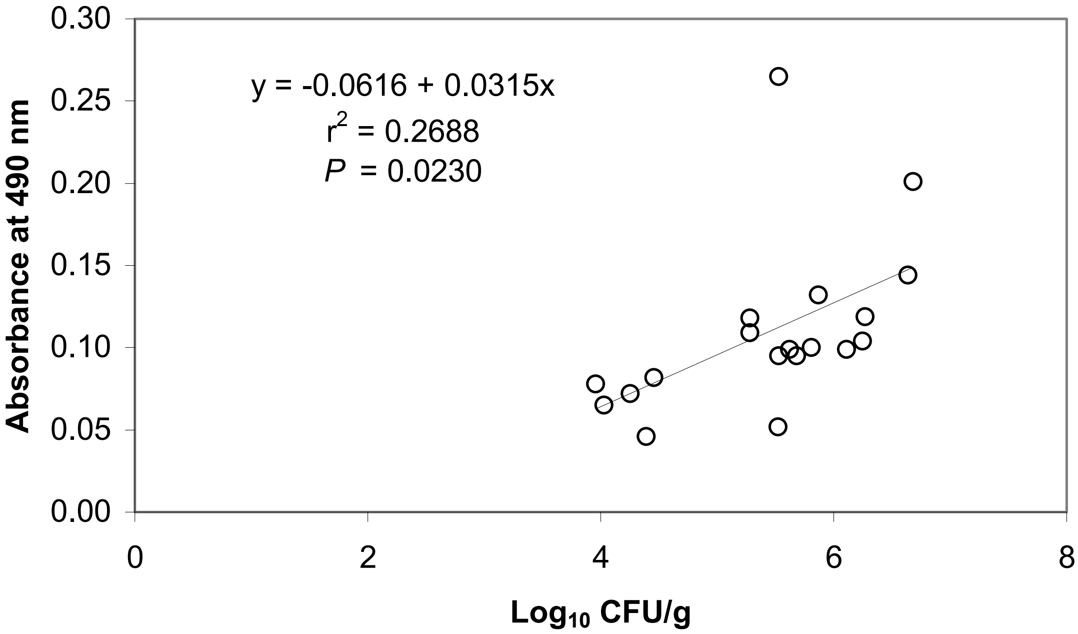

Figure 5.1. Relationship between log$_{10}$-transformed bacterial density (log$_{10}$ CFU/g) of *Xylella fastidiosa* and ELISA absorbance reading at 490 nm. Each point (n = 19) represents one tissue sample.