

ABSTRACT

Title of Document: Chemotherapeutic Treatment Options to Manage
Xylella fastidiosa in Shade Trees

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Master of Natural Resource Sciences
2005

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Architecture

Xylella fastidiosa is a fastidious, xylem-limited, broad spectrum, bacterial plant pathogen native to the Americas, causing substantial economic losses to the viticulture, citrus, and shade tree industries. In shade trees the disease is manifested as a chronic late season leaf scorch largely confined to urban areas of southeastern North America. Proposed treatments include antibiotics and growth regulators. Recently paclobutrazol, a diastereomeric triazole with fungistatic and growth regulation properties has been associated with symptom remission. Investigation into direct interaction of paclobutrazol with *X. fastidiosa* show no significant reduction in growth at the manufacturers recommended dosage of 20 $\mu\text{g ml}^{-1}$; however significant reductions in growth were observed at a dosage of 200 $\mu\text{g ml}^{-1}$. Therefore high levels of paclobutrazol may have a direct effect on *X. fastidiosa* while other plant physiological effects induced by paclobutrazol merit investigation for association in symptom mitigation of *X. fastidiosa*.

**CHEMOTHERPEUTIC TREATMENT OPTIONS to Manage *Xylella*
fastidiosa in SHADE TREES**

By

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Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Master of Science
2005

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This work is dedicated to
The dignified and graceful trees of Capitol Hill

I am greatly indebted to the knowledge and abilities of my mentors

Dr. Jim Sberald of the National Park Service Center for Urban Ecology

Dr. Qi Huang of the US Dept. of Agriculture, Agriculture Research Service

and

Dr. Jay Stipes of Virginia Polytechnic Institute and State University

Also

Syngenta, for the provision of Paclobutrazol

The Casey Tree Foundation

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Chapter 1: *Xylella fastidiosa*

History

Xylella fastidiosa (Wells et al. 1987) is currently recognized as an indigenous pathogen of the Americas and was first identified as an agronomic problem in the 1890's in the western United States (Pierce, 1892). A previously unknown disease affecting vineyards in California prompted Newton B. Pierce, the noted USDA plant pathologist, to investigate the disease in the Central Valley. The syndrome was dubbed the California vine disease and effectively halted vineyard establishment in the state south of Anaheim. Pierce provided the first scientific characterization of the syndrome resulting from the disease, thereafter known as Pierce's Disease in grape. Symptoms of the disease include irregular marginal necrosis or leaf scorch resulting in dieback and a slow systematic death of the vine (Pierce, 1892).

Around the turn of the 20th century across the country in Georgia a different syndrome known as Phony Disease was affecting peach production (Hutchins, 1933). Symptoms included dwarfing, distorted lateral branch growth, excessive green foliage, and reduction in size and number of fruit (Hutchins, 1933). Almost a century passed before these two distinct syndromes were positively linked.

Early diagnosis of the diseases favored a viral hypothesis in both syndromes which is reflected in the language of the early literature. Root grafting experiments with Phony disease demonstrated disease transmission between scion and plant (Hutchins, 1939) indicating that the causal agent resided in the "woody cylinder" or

vasculature of the plant system. In 1942, Hewitt confirmed insect transmission via xylem feeding arthropods; a more thorough analysis on the topic is presented by Houston et al. in 1947. While numerous insects are confirmed vectors of the pathogen, sharpshooters and spittlebugs as identified in these studies are the focus of dissemination (Houston et al. 1947).

The repeated failure of pathogen isolation attempts supported the viral hypothesis, however in 1971 tetracycline was found to suppress Pierce's Disease symptoms, effectively eliminating this diagnosis (Hopkins and Mortensen, 1971). Electron microscopy subsequently showed that "rickettsia like" prokaryotes with distinct rippled cell walls inhabited the xylem vessels of symptomatic Pierce's Disease and Phoney Disease specimens (Hopkins and Mollenhauer, 1972). The prokaryotes associated with each syndrome were phenotypically similar and closely resembled rickettsia (Hopkins and Mollenhauer, 1972), a prokaryote known from animal systems spread via arthropods.

The organism proved elusive and was first isolated *in vitro* in 1978 by Davis et al. at the University of California, Berkeley using a medium formulated to support the strict growth requirements of rickettsia organisms. Davis was the first to positively associate xylem limited prokaryotes with disease by fulfilling Koch's postulates thus proving a bacterium was the cause of the Pierce's Disease syndrome. Prior to its positive identification and proven pathogenicity the bacterium was already linked to several distinct syndromes. After publications on isolation and culture of the bacteria the list grew significantly (Table 1). Subsequent analysis of 25 strains of the pathogen from 10 distinct hosts available in 1987 allowed Wells et al. to describe

and dub the organism *Xylella* a new genus of plant bacteria containing a single species denoted as *Xylella fastidiosa* alluding to its fastidious nature.

Table 1 Agronomic Diseases Caused by *Xylella fastidiosa*

<i>Disease</i>	<i>Reference</i>
Pierce's Disease of Grape	Hopkins and Mollenhauer, 1973
Phony Disease in Peach	Hopkins and Mollenhauer, 1973
Alfalfa Dwarf	Goheen et al. 1973
Plum Leaf Scald	Kitajima et al. 1975
Almond Leaf Scorch	Mircetich et al. 1976
Periwinkle Wilt	McCoy et al. 1978
Pear Leaf Scorch	Leu et al. 1993
Citrus Variegated Leaf Scorch	Chang et al. 1993
Coffee Leaf Scorch	deLima et al. 1998

In 1993 Chang et al. identified *Xylella fastidiosa* as the causal agent of Citrus Variegated Chlorosis (CVC), which was first recognized in Brazilian citrus groves around Sao Paulo in 1987. Symptoms of the disease included interveinal chlorosis typical of zinc deficiency, brown lesions on the underside of mature leaves, and hard sugary fruit. In 1995, *Xylella* was confirmed in scorched coffee trees planted adjacent to a CVC infected citrus grove (Lima et al. 1998). Currently grape and citrus are the most important agronomic crops affected by *Xylella* (Brown et al. 2002).

Spurred by the economic importance of the Brazilian citrus industry and the severity of the problem, *Xylella* was the first plant pathogen to have its genome completely sequenced (Simpson et al. 2000). The availability of the complete sequence has made *Xylella* the subject of intensive molecular investigations around the world. In 2004 there were more than 70 publications on *Xylella*, the majority of which focused on the molecular aspects of the pathogen. Important practical

experimentation remains limited due to the fastidious nature of the pathogen and the complexity of the system. Currently there remains no practiced therapeutic treatment for *Xylella* although it has been recognized as a problem for over 100 years.

***Xylella* Biology**

The genus *Xylella* forms a distinct group currently recognized in the gamma subgroup of eubacteria which is defined by a single species denoted as *Xylella fastidiosa* (Wells et al. 1987). All strains, regardless of host, are phenotypically and genotypically similar. Electron microscopy shows that cells are single (occasionally filamentous), non-motile, aflagellate straight rods (0.25 to 0.35 by 0.9 to 3.5 μm) with rounded or tapered ends (French et al. 1977). Cell walls are typically furrowed and convoluted, often described as rippled, providing one of the phenotypic hallmarks of the genus when visualized with electron microscopy (Hopkins and Mollehauer 1972). When viewed with light microscopy bacterial bodies appear smooth and round displaying no characteristics reliable for identification.

Xylella is gram negative and aerobic with optimal growing conditions at a pH of 6.5-7.2 and temperatures of 26-28°C (Wells et al. 1987). Multiplication proceeds through the process of binary fission forming aggregations known as bio-films. Pathogenicity is hypothesized to result from bacterial aggregation in the xylem vessels effectively occluding the vessel and impeding xylem fluid movement. However this has not been proven and is often a point of discussion.

Significant differences in growth rate, aggregation, and robustness exist between *Xylella* strains which can be highly variable even within a single host range (Personal observation). Broth cultures can exhibit bacterial plaque formations with the significance of aggregation varying by strain (Personal Observation). Bacteria characteristically aggregate on the side of glass test tubes, and are easily dispersed upon agitation.

When grown on solid media colonies are discrete, circular, smooth, and rather translucent or opalescent depending on the chosen substrate (Wells et al. 1987). Colonization of substrate is variable however generally slow (0.6mm diameter after 10 days at 27°C) and limited to the region of inoculation (Wells et al. 1987). Colonization of solid media can take 2 weeks or may be as rapid as 4 days depending on the amount of inoculum present (Personal observation). Growth on solid media requires sterile conditions; the fastidious nature of the bacteria requires a rich media, which combined with a slow growth rate, increases the susceptibility of contamination. The difficulty in isolation is evident in the continued publication of isolation techniques employing new methods (Bextine and Miller 2004).

Xylella induces a wide variety of symptoms and is a chronic disease that is not typically lethal. In citrus, peach, and alfalfa it causes growth abnormalities while in grape, sycamore, oak, mulberry, maple, almond, coffee, pear, plum, and oleander a marginal necrosis of leaves is typical. Disease spread is often random within a population due to the variability of vector transmission and possible host susceptibility. The severity of symptom expression varies with prevailing annual

climatic conditions (McElrone et al. 2001) as well as the pathogenic variability of the strain.

Insects of the subfamily Cicadellinae (sharpshooters/leafhoppers) and Cercopidae (spittlebugs) are the primary vectors of disease dissemination (Houston et al. 1947). Pierce's Disease is confirmed to be spread by at least 24 species of leafhoppers (Houston et al. 1947). Vector efficiency is partially explained as a function of frequency in which a particular insect species reaches plant tissues susceptible to inoculation as well as length and manner of feeding on those susceptible tissues (Bentz and Sherald, 1999). Additionally, *Xylella* has been confirmed in more than 30 families of monocotyledonous and dicotyledonous plants in natural, cultivated, and urban landscapes (Hopkins and Alderz 1988) (Table 2). Many of the hosts have been recognized through molecular environmental sampling and are asymptomatic, indicating the recognized host range is likely to increase with further sampling (Hopkins and Alderz 1988). The combination of numerous vectors and hosts makes the pathogen difficult to control by host or vector management.

Symptoms of *X. fastidiosa* infection in shade trees are most evident in the late summer when bacterial populations in the host are likely to have reached a high. As the temperature drops it is believed *Xylella* populations are significantly reduced by cold stress, remaining viable bacterial cells are transported via negative xylem flow into the roots where they over winter. In the spring as xylem pressure builds *Xylella* is transported to the mature shoots of the plant where it adheres to wall of the xylem and multiplies. Insect vectors feeding on xylem fluid, uptake bacterial cells in their mouthparts from which it is disseminated to the next host.

Table 2 Asymptomatic and Economically Insignificant Hosts of *X. fastidiosa* in the Eastern United States

<i>Scientific name</i>	<i>Common name</i>
<i>Aesculus x hybrid</i>	Buckeye
<i>Ampelopsis arborea</i>	Peppervine
<i>Ampelopsis brevipedunculata</i>	Porcelain berry
<i>Artemisia spp.</i>	Mugwort
<i>Baccharis halimifolia</i>	Eastern baccharis
<i>Callicarpa americana</i>	American beautyberry
<i>Celastrus orbiculata</i>	Oriental bittersweet
<i>Cynodon dactylon</i>	Bermuda grass
<i>Fagus crenata</i>	Japanese beech bonsai
<i>Fragaria californica</i>	Wild strawberry
<i>Hedera helix</i>	English ivy
<i>Montia linearis</i>	Miner's lettuce
<i>Parthenocissus quinquefolia</i>	Virginia creeper
<i>Parthenocissus tricuspidata</i>	Boston ivy
<i>Paspalum dilatatum</i>	Dallis grass
<i>Rhus sp.</i>	Sumac
<i>Rubus procerus</i>	Blackberry
<i>Sambucus canadensis</i>	American elder
<i>Solidago fistulosa</i>	Goldenrod
<i>Sorghum halapense</i>	Johnson grass
<i>Trifolium repens var. latum</i>	Landino clover
<i>Vitis sp.</i>	Wild grape

Xylella Phylogenetics

Xylella fastidiosa (Wells et al. 1987) is currently considered a single species although it occurs in distinct geographic regions and within distinct host ranges. Phylogenetically it is placed in the gram negative proteobacteria gamma subgroup with Xanthamonas as its closest relative, and Pseudomonas also recognized in the group. Previous studies have attempted to differentiate isolates of *X. fastidiosa* through nutritional requirements (Hopkins, 1989), DNA homology (Kamper et al. 1985), restriction fragment length polymorphisms (RFLP) (Chen et al. 1992), and

random amplified fragment polymorphisms (RAPD) (Pooler and Hartung 1995) to the end of proposing speciation or pathovar designation. Currently speciation has not been accepted by the bacteriological community and pathovar designation is only sporadically used in the literature. If significant distinction within the group can be recognized it could impact the development of control strategies and further our understanding of the biological functioning of the organism.

Geographically *Xylella* isolates of Citrus Variegated Chlorosis (CVC) and coffee leaf scorch are isolated in Brazil, while isolates of almond, oleander, and maple are predominantly isolated from California specimens. The hypothesized native range of the southeastern United States is the source of the elm, oak, mulberry, sycamore, peach and plum isolates. Pierce's Disease is found in both California and the southeastern U.S. *Xylella* has also been identified in numerous asymptomatic plants within each geographic region through molecular sampling.

The 1,452 bp 16s rRNA gene is a standard sequence for bacterial phylogenetic positioning and provides clear distinction of *X. fastidiosa* from closely related species (Chen et al. 2000). Currently there are 73 accessions of this sequence in Genbank (www.ncbi.nlm.nih.gov). However this region does not contain enough variability to separate relationships within the group. Analysis using the neighbor joining method with the approximately 500 bp 16s-23s ITS can separate 26 *Xylella* accessions (excluding almond) into three distinct groups by hosts (i) CVC/coffee (ii) PD/oleander/maple (iii) peach/plum/oak (Mehta et al. 2001). The resulting two clades of the phylogenetic tree are dominated by host grouping and combine groups (i) and (ii) which are geographically divided. Another study using ITS in 8 North

American accessions (Huang and Sberald 2004) supports the host groupings and is unable to clearly separate the accessions geographically.

Combining various fragment analysis methods with 16S-23S ITS using 50 accessions from 8 distinct hosts (strictly North American) converged on the four groups (i) peach/plum (ii) oak (iii) oleander (iv) PD/maple with almond moving between groups (i) and (iv) depending on the technique analyzed (Hendson et al. 2001). Again the majority of the results group by host but follow geographic lines separating east and west, however PD is paraphyletic, grouped by host for both eastern and western isolates and almond is recovered as paraphyletic with 3 of 12 isolates grouping with PD.

Analysis of the DNA gyrase- β -subunit using 30 accessions from 9 distinct hosts results in phylogenetic grouping of (i) CVC/coffee (ii) PD (iii) Elm/plum/mulberry/periwinkle with almond moving between group (i) and (iii) (Rodrigues et al. 2003). Separation by geography was upheld with the exception of PD which formed a monophyletic group with both eastern and western strains, and almond which was paraphyletic grouping with North and South American isolates.

Unique Genetic Islands (GI) that have been found in *Xylella* isolates have also been gleaned for phylogenetic information. Primers developed to sequence these unique regions were used to scan the genomes of 30 accessions from 11 distinct hosts (Van Sluys et al 2003). This analysis clumped the accessions into 3 groups (i) CVC/coffee (ii) PD/oleander (iii) Oak/elm/plum/perwinkle again with almond accessions separating between groups (ii) and (iii). This analysis supports the results

of Rodriguez and Hendson grouping accessions geographically and by host with almond being unresolved.

Plasmids have been found in a substantial number of *Xylella* isolates numbering between 1 and 4 with a wide variance in size between ~50 - ~1 kb. Digests of 27 isolates from 6 distinct hosts formed 9 unique plasmid profiles that were host exclusive except for PD and almond (Hendson et al. 2001). Interestingly the plasmid profiles could separate strains of PD from the east and west coast of North America (no other method was able to), but did not distinguish between some western PD and the almond isolates. This method may have provided the most accurate results but is limited by the fact only 27 or the 44 sampled isolates have plasmids (Hendson et al. 2001).

The aforementioned studies have all based phylogenetic relationship on overall similarity, a simple paradigm which can be misleading. Employing the 16S-23S ITS region using the parsimony method both agreed with and contradicted previous results (Martinati et al. 2005). This method results in the formation of two large clades representing (i) elm/mulberry/PD/CVC/coffee and (ii) Almond/PD/oleander/ maple/peach/plum /oak. While these clades look distinctly different to previous results the disparity is reduced by the fact that parsimony was able to group most *Xylella* isolates into monophyletic groups by host however Pierce's Disease and Plum were found to be paraphyletic while almond was found to be monophyletic. The groups recovered from the analysis shared no geographic patterns. The published analysis does not provide the Bootstrap values that are

associated with parsimony analysis so that statistical support can be judged for the groupings.

While these phylogenetic analyses all trend toward host and/or regional separations, results remain ambiguous. All discussed studies represent single gene or fragment analysis which are inherently prone to inconsistency, combinations of datasets can possibly resolve some of the noise. Previous and current research has associated PD and almond leaf scorch (Davis 1978; Chen 2005) complicating the association by host (however not geographically), indeed this is most likely behind the inconsistency with the almond accessions in the phylogenetic analysis. Currently there is no consensus on if or how *Xylella* strains should be differentiated.

Knowing the phylogenetic relationships of *X. fastidiosa* strains will aid in the development of a control strategy for the pathogen. Understanding if virulent forms of the pathogen are host limited would have important implications on treatment recommendations.

Xylella Genomics

Xylella was the first plant pathogen to have its entire genome sequenced (Simpson et al. 2000). The effort was undertaken by a Brazilian team using an isolate of Citrus Variegated Chlorosis (strain 9a5c) isolated in 1992 in Bordeaux (France) from infected *citrus sinensis* ‘Valencia’ sampled in the environs of Sao Paulo Brazil. The genome is composed of a 2.7 Mb (2,679,305 bp) circular chromosome and two plasmids containing 51,158 bp and 1,285 bp respectively. Subsequently, draft sequences (gapped genome sequences covering 95% of the genome) were produced

by the US DOE Joint Genome Institute (www.jgi.doe.gov) for almond and oleander isolates (Bhattacharyya et al. 2002) composed of a 2.4 Mb and 2.6 Mb circular chromosomes respectively and large plasmid of 30,270 bp in almond while the oleander isolate lacked a plasmid. In 2003 a Pierce's Disease isolate collected in Temecula California was completely sequenced by the Brazilian group (Van Sluys et al. 2003), the PD isolate is composed of a 2.5 Mb (2,519,802 bp) chromosome and a small 1,345 bp plasmid, the large plasmid found in the 9a5c and almond isolates is absent. Plasmids have been found in 27 of 44 isolates of *Xylella* tested (Hendson et al. 2001).

Comparative analysis of the four *X. fastidiosa* genomes has shown little genomic variability, with >90% amino acid identity in equivalent regions (Van Sluys et al. 2003). The development and usage of microarrays has furthered insight into the genetic variation across the *Xylella* group. This technique first demonstrated the differences in genetic composition between the completely sequenced PD and CVC genomes, resulting in the identification of exclusive coding sequences between strains (Oliveira et al. 2002), despite these results the genomes showed little divergence. Further microarray analyses of 12 *X. fastidiosa* isolates revealed a highly conserved “core” gene pool which contains biochemical pathways and cellular function tailored to xylem limited existence (Nunes et al. 2003). Conversely there is also a large “flexible” gene pool composed of laterally transferred genomic elements including plasmids, prophages, and Genomic Islands (GI) which total up 18% of the entire genome, one of the largest “flexible” gene pools recorded to date. The acquisition of genes through horizontal gene transfer was proposed upon the first sequencing of

Xylella (Simpson et al. 2000) and elaborated on with the draft sequencing of two more isolates (Bhattacharyya et al. 2002); it is now accepted as the method of acquisition of this more amorphous portion of the genome. Essentially the differences between the sequenced genomes are limited to this “flexible” gene pool with its various phage related genes and insertion and deletion events.

Genomic islands are identified by typical characteristics including (i) high GC concentration (ii) altered codon bias (iii) insertion at the 3' end of a tRNA gene (iv) genes encoding an integrase at one end. Different GIs have been identified in both the completely sequenced isolates of *Xylella*, 67 kb and 15.7 kb in CVC and PD respectfully (Van Sluys et al. 2002). A superset of GI's including islets or much smaller laterally transferred elements in 12 isolates of *Xylella* was analyzed with a microarray and have been found to be transcriptionally active (Nunes et al. 2003) often coding nonessential functions thought to provide advantages in various environmental conditions (i.e. hosts). Although genes possibly linked to pathogenicity have been found to be active within GIs which might explain the diverse symptoms observed from *Xylella* (Van Sluys et al. 2003), it is much more likely that pathogenicity is multidimensional and resides in the highly conserved core sequence. The diseases caused by *Xylella fastidiosa* most likely rely on the expression of a common set of genes that facilitate the establishment of the bacteria *in planta*.

An interesting difference between the sequenced genomes is an in frame stop codon within the polygalacturonase gene in CVC that is absent in the other three sequences, leaving the gene intact. This gene is considered essential for the synthesis

of cell wall degrading enzymes that facilitate intervessel migration. This difference is hypothesized to result in the disparity between symptoms of CVC and the other scorch associated strains (Van Sluys et al. 2003) however this is a tenuous inference which is almost certainly more complex.

The establishment of bacterial infection typically depends on the successful interaction of products from an avirulence (avr) gene with its counterpart in the host which forms a complex initiating colonization. This interaction is often the factor limiting the breadth of host range. A Basic Logical Alignment Search Tool (BLAST) search with all known avr genes failed to find any genes encoding similar proteins in *Xylella*. The highly conserved nature of avr genes implies that it is simply absent in *Xylella* rather than modified which could explain its abnormally large host range. This argument is supplemented by the fact that the pathogen is xylem limited and insect transmitted possibly eliminating the requirement of host cell interaction to establish infection and that *Xylella* is the only sequenced plant pathogenic bacteria that does not have a recognized homolog of the SoxRS regulon which controls the expression of several genes related to bacterial defense. The SoxRS regulon is induced by oxidative stress resulting from a hypersensitive response expressed after the recognition of an avr gene by a plant.

Comparative analyses of complete genomic sequences from a variety of bacteria have also provided insight into the biology of *Xylella*. The average size of the *Xylella* genome at 2.5 Mb is relatively small when compared to other completely sequenced plant pathogenic bacteria, which includes *Xanthomonas* (www.lbm.fcav.unesp.br/) at 5 Mb and *Ralstonia*

(sequence.toulouse.inra.fr/R.solanacearun) at 5.8 Mb. This observation is theorized to be the result of xylem limited existence which provides relatively constant environmental conditions and is inherently nutrient limited, restricting metabolic options and promoting evolutionary specialization, thus resulting in the reduction of excess metabolic machinery (Van Sluys et al. 2002). This is expressed in the fastidious nature of the organism which cannot exploit excess nutrients even when provided in growth media and requires a relatively mesic temperature and a narrow pH range for growth.

Xylella in Shade Trees

In the late 1950's the American elm (*Ulmus americana*) was the dominant urban tree in North America. The outbreak of Dutch Elm Disease (DED) sparked intensive scrutiny of elm health in urban centers around the country. A previously undescribed chronic late season leaf scorch dubbed Elm Leaf Scorch was reported on the National Mall in 1959 which increased susceptibility to DED (Wester and Jylkka, 1959). Elm leaf scorch was shown to be transmitted from grafts, and showed similar symptoms to Pierce's Disease. Since no pathogen could be isolated from the trees at that time, the disease was thought to be viral, in line with the diagnosis for Pierce's Disease.

Observations from a variety of tree species showing scorch symptoms in urban environments where the symptoms were typically explained as the result of abiotic stress were investigated by Hearon et al. in 1980. Using electron microscopy,

Hearon associated a newly discovered xylem limited bacteria of peach and grape with oak (*Quercus*), elm (*Ulmus*), and sycamore (*Platanus*) showing leaf scorch symptoms. In 1983, Sherald et al. fulfilled Koch's postulates for Sycamore and demonstrated that the xylem limited bacteria is responsible for the leaf scorch symptoms. Isolated strains of *Xylella* from trees (Table 3) include Oak (Chang and Walker 1988), Elm (Kostka et al.1986), Sycamore (Sherald et al.1983), and Mulberry (Kostka et al.1986). In 1987 Sherald et al. also associated red maple (*Acer*) showing scorch symptoms with the xylem inhabiting bacterium. The genera *Acer*, *Quercus*, and *Platanus* comprise the majority of urban and landscape planted shade trees in the southeastern United States today.

In most cases the syndrome of *X. fastidiosa* in shade trees manifests itself as a chronic late season leaf scorch resulting in the reduction of growth and possible dieback; it is commonly referred to as Bacterial Leaf Scorch (BLS). *Xylella* is found irregularly distributed within the urban forest showing no characteristic pattern. The disease is not typically lethal, however, it severely debilitates infected hosts increasing susceptibility to other diseases and stresses (Sherald, 1999). Under good conditions infected specimens can persevere for decades however urban trees can become unsightly and are often removed on aesthetic and safety grounds.

Affected trees may foliate late in the spring and have sparse or uneven canopies when in full leaf and prematurely defoliate in the autumn. The extent of the symptomatic severity within the canopy is partially a function of the climatic conditions of the particular year, with drought years showing greater symptoms (McElrone et al. 2001). *Xylella* moves systematically through the xylem of the tree,

so symptomatic regions will appear in the crown adjacent to infected regions or dieback from previous seasons; however infection of the entire canopy is not uncommon. Identification of the disease in the field is aided by observing the entire individual from afar taking into account the trees position and overall health (Figures 1-2).

Table 3 Shade Tree Hosts of *X. fastidiosa*

<i>Scientific name</i>	<i>Common name</i>
<i>Acer rubrum</i>	Red maple
<i>A. negundo</i>	Boxelder
<i>A. saccharum</i>	Sugar maple
<i>Cornus florida</i>	Flowering dogwood
<i>Celtis occidentalis</i>	Hackberry
<i>Liquidambar styraciflua</i>	Sweet gum
<i>Morus alba</i>	White mulberry
<i>Platanus occidentalis</i>	American sycamore
<i>P. x acerifolia</i>	London plane
<i>Quercus velutina</i>	Black oak
<i>Q. incana</i>	Bluejack oak
<i>Q. macrocarpa</i>	Bur oak
<i>Q. prinus</i>	Chestnut oak
<i>Q. laurifolia</i>	Laurel oak
<i>Q. virginiana</i>	Live oak
<i>Q. rubra</i>	Northern red oak
<i>Q. palustris</i>	Pin oak
<i>Q. stellata</i>	Post oak
<i>Q. coccinea</i>	Scarlet oak
<i>Q. imbricaria</i>	Shingle oak
<i>Q. shumardii</i>	Shumard oak
<i>Q. falcata</i>	Southern red oak
<i>Q. bicolor</i>	Swamp white oak
<i>Q. laevis</i>	Turkey oak
<i>Q. nigra</i>	Water oak
<i>Q. alba</i>	White oak
<i>Q. phellos</i>	Willow oak
<i>Ulmus americana</i>	American elm

An irregular marginal leaf necrosis or scorch is visible in late summer, often there are several different regions of necrotic tissue on a single leaf (notably in elm,

Figures 3-4), and other times symptoms can be entirely marginal (notably in oak, Figure 5). The disease displays slightly different symptoms depending on the specific host including branch dieback and a chlorotic halo separating healthy tissue from the necrotic margin (Figure 6). Confirmation of BLS infection is best done with Enzyme Linked Immunosorbent Assay (ELISA) (Sherald and Lei, 1991) or PCR based identification methods (Pooler and Hartung, 1995).

Symptoms are difficult to associate with *X. fastidiosa* due to the similarity in appearance with abiotic stresses such as drought, salt/chemical damage or root disturbances and soil compaction. Furthermore disease symptoms are exacerbated by drought and stressful conditions (McElrone et al. 2001), both typical in urban settings where *Xylella* is primarily diagnosed in shade trees. Increasingly, BLS has been cited as a problem in the urban settings of southeastern North America (Sherald, 1999). This is due in part to the increased awareness of the pathogen in the region.

The economic impact of BLS is difficult to gauge since metrics for urban tree value are controversial largely due to the inherent difficulty of assigning monetary value. This problem is compounded by the fact that *Xylella* significantly reduces carbon sequestration, which is typically calculated by the diameter breast height of the specimen assuming a normal growth rate. Incorporation of *Xylella* into the algorithm is not a simple proposition due to the erratic spread and difficulty of diagnosis of the pathogen as well as the physiological alterations. *Xylella* reduces the productivity of trees within the urban canopy such that it is difficult to accurately gauge the productivity of an urban canopy suffering with the disease.

Management of *X. fastidiosa* has encompassed a variety of strategies, including host removal, vector management, and antibiotic treatment. While host removal and vector management are important components of an integrated management scheme their efficacy is limited by the high number of possible vectors and hosts. Treatment of individual specimens with antibiotics has been shown to be effective (Kostka et al. 1985), however not curative, and the continued usage of antibiotics for horticultural purposes is currently under question. Recently Paclobutrazol, a diastereomeric triazole possessing fungistatic and plant growth regulation properties has been associated with the remission of symptoms from the disease (Personal communication Bruce Frederich, Barlett Tree Labs Charlotte, North Carolina). The mechanism by which paclobutrazol is able to achieve symptom remission is currently hypothesized to result from plant physiological changes induced by the chemical (Personal Communciation Bruce Frederich).

Figure 1 *X. fastidiosa* symptoms in August 2005, on a mature *Ulmus americana* specimen located on the National Mall, Washington D.C. surrounding trees are also *Ulmus americana*



Figure 2 *X. fastidiosa* symptoms in late August 2005, on *Quercus palustris* in Washington D.C., the tree on the right is infected and symptomatic the tree on the left is not infected



Figure 3 *X. fastidiosa* symptoms on a leaf sampled from an *Ulmus americana* specimen in July 2005



Figure 4 *X. fastidiosa* symptoms on a twig sampled from an *Ulmus americana* specimen in July 2005



Figure 5 Marginal scorch symptoms of *X. fastidiosa* on a *Quercus palustris* leaf sampled in August 2005



Figure 6 Leaf scorch symptoms and associated chlorotic halo of *X. fastidiosa* infection on an *Ulmus americana* leaf sampled in July 2005



Research Goals

The goals of this research are

1. Locate *Ulmus americana* specimens infected with *X. fastidiosa* in Washington D.C. using GIS technology and molecular tools
2. Isolate the bacterium from *Ulmus americana* specimens using a recently adapted protocol, pressure chamber (Bextine and Miller, 2004).
3. Test the growth of *X. fastidiosa* isolates from shade trees on *Xylella* Defined Media (Alameida et al. 2004).
4. Determine whether paclobutrazol has a direct inhibitory effect on *X. fastidiosa* *in vitro*.

Chapter 2: Location and Isolation of *Xylella fastidiosa*

Introduction

Washington D.C. is home to a significant population of *Ulmus americana* specimens (~10,000, including the National Mall) and was the first region to identify *X. fastidiosa* as a problem within this population (Watson and Jylkka, 1959). In 2002, the Casey Tree Foundation (www.caseytrees.org) conducted an exhaustive Global Information System (GIS) inventory of the street trees of the city, compiling a database of more than 120,000 individual specimens. The inventory captured a variety of information including species, size and condition. This database is publicly available (www.dcgis.dc.gov), and is compatible with ESRI software systems ARCGIS and ArcView. The disease resulting from *X. fastidiosa* (Wells et al. 1987) is a chronic late season leaf scorch resembling abiotic stress that is primarily diagnosed on but not limited to *Quercus*, *Platanus*, and *Ulmus* genera (Sherald, 1999). The disease is essentially limited to urban and suburban communities where it has been increasingly diagnosed over the past decade (Sherald, 1999).

Initial isolation of *X. fastidiosa* was achieved by expressing sap from the petiole of symptomatic grape leaves and plating the fluid directly onto a media designed for culture of rickettsia organisms (Davis et al. 1978). Isolation from tree specimens was first achieved by excising xylem cells from symptomatic branches of sycamore specimens and incubating them in broth media (Sherald et al. 1983). Isolation of the bacteria remains difficult and a variety of techniques have been

published (Davis et al. 1978; Wells et al. 1981; Sherald et al. 1983; Bextine and Miller 2004). The most commonly used techniques for isolation of *X. fastidiosa* involve crushing or grinding plant tissue. This practice dependant on the ratio of plant cells to bacterial cells and can prove difficult in samples that contain a low bacterial titer. The process of isolation is further complicated by the slow growth of *X. fastidiosa* which makes culturing susceptible to contamination. A high yield isolation technique is needed to facilitate practical experimentation with the organism.

Materials and Methods

Sampling and Scouting Methodology

The Casey Trees database was queried using ARCGIS 9.0 (ESRI) to display the *Ulmus americana* population of the city. The city was broken into parcels by zip code, and a sampling region was randomly selected. This region was scouted twice monthly for the duration of the 2004 and 2005 growing seasons. Scouting was done on foot and canopies were examined from the sidewalk by eye. Sampling of symptomatic tissue was done using a pole pruner, symptomatic specimens that could not be reached with pole pruner were noted and skipped.

Molecular Identification

Collected samples were tested by an Enzyme Linked Immunosorbent Assay (ELISA) Pathoscreen kit specific for *X. fastidiosa* detection following the manufacturer's instructions (Sherald and Lei, 1993) (Agdia Inc., Elkhart, IN). Plates were read by eye and at 492 nm in FLUOstar Galaxy microplate reader (BMG Labtechnologies, Inc., Durham, NC). Collected samples were also subjected to *X. fastidiosa* DNA extraction using a DNeasy tissue kit according to the manufacturer's instructions (Qiagen Inc., Valencia CA). Specific detection of *X. fastidiosa* was achieved by Polymerase Chain Reaction (PCR) using specific primers 272-1-int (5'-CTGCACTTACCCAATGCATCG-3') and 272-2-int (5'-GCCGCTTCGGAGAGAGCATTCT-3') (Pooler and Hartung 1995).

Xylem Vessel Excision

Stem samples from symptomatic regions of confirmed *X. fastidiosa* hosts were taken with pole pruners. Samples were rinsed in 70% ethanol and flamed. The bark and phloem was removed and wood chips containing xylem vessels were excised with a sterile blade (Sherald et al. 1983). The wood chips containing the xylem vessels were placed in Periwinkle Wilt broth media (Table 4) and incubated for 20 days at 28°C.

Petiole Technique

Symptomatic leaves from confirmed hosts were sampled. Petioles were excised and surface sterilized in 10% bleach for 1 min and subsequently washed 3 times in deionized water. Petioles were squeezed with sterile forceps and pliers to express sap. Petioles were also macerated with a mortar and pestle and re-suspended in 1ml of PW broth. This suspension was vortexed and put through a 1/10 dilution series. Aliquots of 100 µl from each dilution were plated on PW, XfD, and BCYE media and incubated for 20 days at 28°C.

Pressure Chamber

Twig samples were taken from symptomatic regions of confirmed hosts with pole pruners. The cut end of the sample was immersed in water and cut with sterile pruners prior to processing. The cut end of the twig was surface sterilized in 10%

bleach for 1 min, then washed 3 times in deionized water. The bark and phloem was excised with a sterile blade from the cut end to 1 cm distal. The sample was then placed in a pressure chamber and pressure applied with nitrogen gas. The resulting xylem fluid expressed from the sample was drawn off with a micropipette and plated directly on PW, XfD, and BCYE media and incubated for 20 days at 20°C.

Results

Sampling Region

The 2003 S.E. Capitol Hill/Lincoln Park parcel was selected as the sampling region, which represented 50 unique species with 5,790 individual specimens (Figure 7, trees represented in green), including 720 American Elm specimens (Figure 8, elms represented in brown). A focused population of 240 American Elms was found on North Carolina Ave, South Carolina Ave, Kentucky Ave, 11th St, and East Capitol Street.

Figure 7 GIS map representing the total street tree inventory (5,790 individual specimens) of the sampling region (Capitol Hill/Lincoln Park SE 20003 Washington DC), trees are represented by green dots

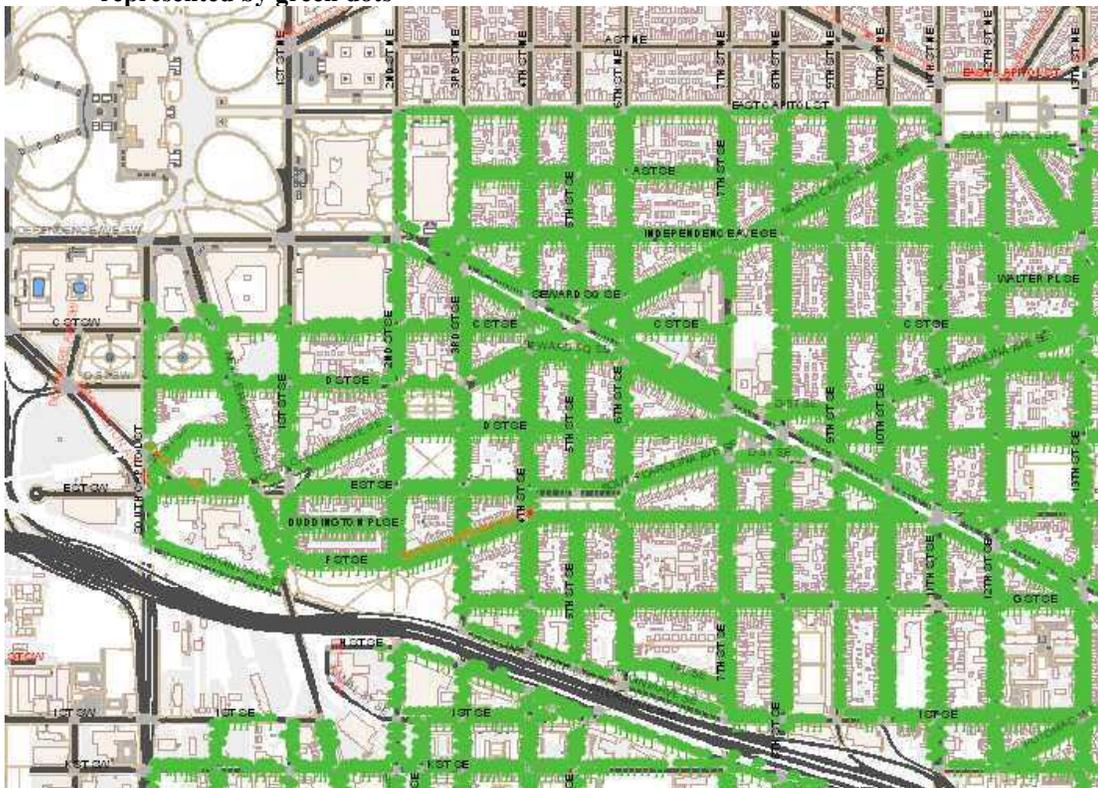


Figure 8 GIS map representing the *Ulmus americana* population (brown dots, 240) within the total tree inventory (green dots, 5,790) of the sampling region (Capitol Hill/Lincoln Park SE 20003 Washington DC)

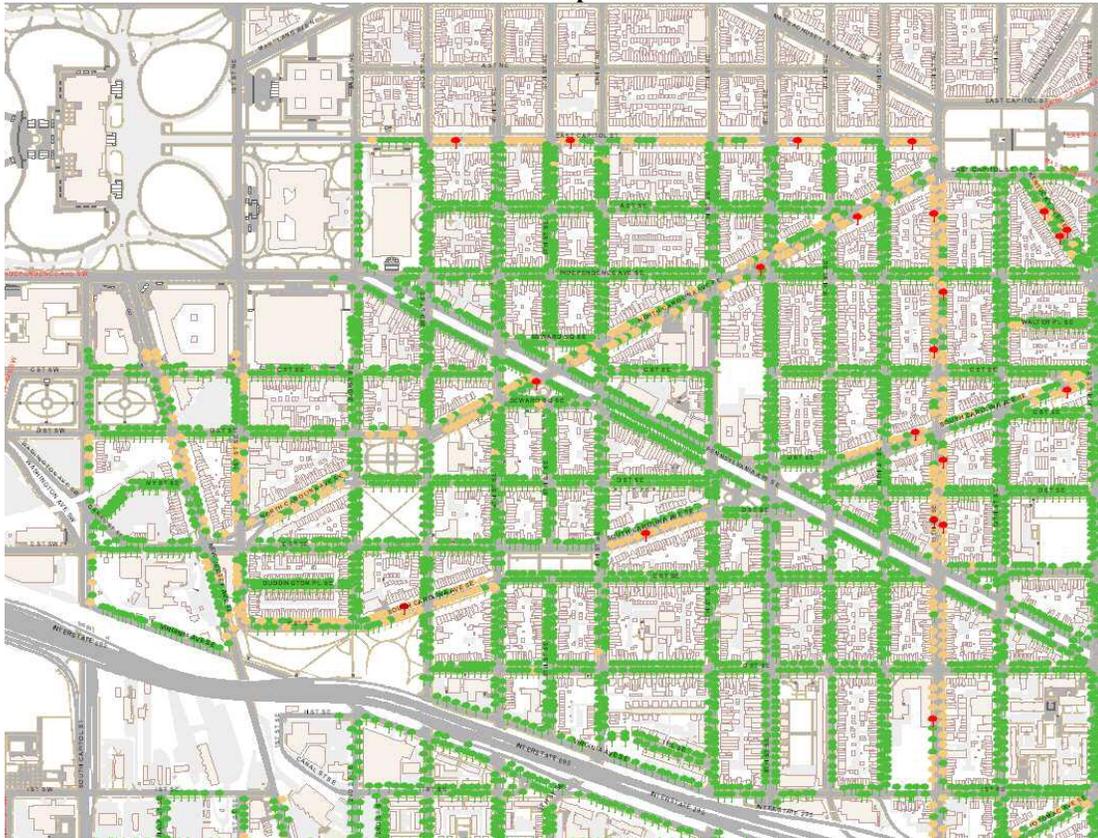


Disease Incidence

The scouting operation identified 120 symptomatic *Ulmus americana* trees in the sampling region. Identification of 23 (approximately 10%) of the 240 total specimens was confirmed. Condition of the trees found to be positive ranged from mild to severely symptomatic. These results were subsequently mapped using ArcGIS (ESRI, Redlands CA) software to visually assess disease incidence within the sampling region (Figure 9).

Figure 9 GIS map representing disease incidence within the sampling region (Capitol Hill/Lincoln Park SE 20003 Washington DC). Red dots (23) represent *Ulmus americana* trees that are confirmed to be infected with *X. fastidiosa*, brown dots (240) represent

Ulmus americana trees that are not confirmed to be infected, and green dots represent the remainder of the street trees that are species other than *Ulmus americana*



Xylem Vessel Excision

Isolation attempts using wood chips containing xylem vessels incubated in Periwinkle Wilt broth media proved successful on three separate hosts. This technique required high replication due to significant problems with contamination. Bacterial aggregation on the sides of glass test tubes was visible after 18 days of incubation, while contamination was visible within 3-5 days of attempted isolation.

Petiole Technique

Isolation attempts using the petiole did not provide any successful isolates. The petiole of *Ulmus americana* in general tends to be small and no fluid can be expressed with forceps or pliers. When the tissue was macerated and plated, plates were either contaminated or completely sterile; indicating surface sterilization of this tissue was problematic. Attempted to modifications of this procedure using ethanol instead of bleach and as well as varying the time of exposure did not change the results.

Pressure Chamber

Isolation attempts with the pressure chamber did not provide any successful isolates. Pressures as high as 40 Bars were applied resulting in limited xylem fluid expression. The cut end of the sample tended to bubble under pressure, with minimal fluid flow. Asymptomatic tissue used in the pressure chamber performed better, providing minimal accumulation of xylem fluid, however significantly less than reported by Bextine and Miller et al 2004.

Discussion

The sampling region used in this study contained 720 *Ulmus americana* specimens 83% of which were mature (>12" diameter breast height). Specimens ranged from 20-50 feet in height which complicated the sampling procedure that employed the usage of 20 foot pole pruner. The situation was exacerbated by the location of the trees on frequently traveled urban streets. Additionally, all the specimens had been pruned to a height of 15 feet to allow traffic to pass, making sampling more difficult. Symptomatic regions were at times high in the canopy and inaccessible with the available equipment. Therefore the 10% disease incidence reported in this study may underestimate the actual infection rate in the sampling region. Equipment such as a bucket truck would increase the ability to sample symptomatic regions within a specimen however this equipment would also significantly increase the cost of such a survey.

The identification of symptomatic specimens was done by eye which was at first subjective, however after repeated scouting and testing visual identification of *X. fastidiosa* became more accurate. The two year length of the study period aided in the identification of infected specimens, trees that were perennially scorched by early August were able to be identified as likely candidates. While the 2004 season saw adequate rain, the 2005 season suffered an acute drought beginning in early August which facilitated the identification of infected specimens since symptoms are exacerbated by drought conditions. The combination of all these factors increases the confidence that the reported 10% infection rate is a representative estimate.

The isolation procedure is largely dependent on the quality of the material that is being used. The petiole and pressure chamber techniques use small amounts of material, single leaves or twigs, and success is predicated on the material containing enough bacterial titer for isolation. The sampling issues previously described may have contributed to failure in isolation of *X. fastidiosa* using these two techniques. In contrast the xylem vessel excision technique employs the usage of large amounts of material (branches approximately 0.5-1” in diameter) which is more destructive to sample, however may provided higher bacterial titer. Further experimentation with both the petiole and pressure chamber technique should be pursued due to the advantages they present including the reduced destruction in sampling, and the possibility of higher yields of isolates.

Chapter 3: Evaluation of the Defined Medium XfD

Introduction

Fastidious is defined by the Oxford English Dictionary as ‘difficult to please in matters of taste’. This term is applied to bacteria that have evolved to survive in niche environments with restrictive dietary and cultural conditions which require specific media to grow *in vitro*. *Xylella fastidiosa* is a fastidious bacterial plant pathogen native to the Americas. While *X. fastidiosa* was recognized as a problem in the 1890’s and bacterial cells were visualized in infected tissue by electron microscopy in 1972 (Hopkins and Mollenhauer 1972) it was not isolated until 1977 (Davis et al. 1978). The initial difficulty in isolation and cultivation is attributed to the bacteria’s fastidious nature. Comparative genomic analysis has shown *Xylella* to have a relatively compact genome that is hypothesized to code for a limited set of metabolic pathways that manifest into the fastidious nature of the organism (Van Sluys et al. 2002).

The initial isolation medium formulation was named JD-1 and contained PPLO broth, Hemin Chloride, Bacto-Agar, and Bovine Serum Albumin (BSA) this media was reported to require two to three weeks in aerobic conditions before colonization was visible. A variant in the same series (JD-3) reduced the incubation period by half (Davis et al. 1978). This medium was modified further to support

consistent primary isolations and continuous sub-culturing of the bacteria, and became known as PD2 (Davis et al. 1980).

The Phony Disease pathogen of *Prunus persica*, which would later be found to be caused by *X. fastidiosa*, initially could not be isolated with the PD2. A rich medium originally intended to culture *Legionella pneumophila*, a fastidious bacterium causing Legionnaires disease in humans, was the first medium to be successful in the isolation of Phony Disease and Plum Leaf Scald (Wells et al. 1981). This is a distinct and simple media composed of five components, activated charcoal, L-cysteine, ferric pyrophosphate, yeast extract, and ACES buffer and is known as Buffered Charcoal Yeast Extract (BCYE) (Table 5). A different complex medium variant of PD2 was also developed and shown to work with Phony Disease in the same year (Davis et al. 1980) known as Periwinkle Wilt medium (PW) after the *Xylella* strain it was used to isolate (Table 4).

Variations of the original JD media series (Davis et al. 1978) include PD2 (Davis et al. 1980), PW (Davis et al. 1983), PWH (Lee et al. 1993), SPW (Hartung et al. 1994), and PWG (Hill et al. 1995) of which PW has become most accepted and has been shown to support the growth of all known *X. fastidiosa* strains. None of these media are fully autoclavable. They all employ peptone as the primary nutrient source, include an iron source (Hemin Chloride), and a supplemented amino acid (Glutamine). The BCYE medium, which is fully autoclavable, also supplements iron (Ferric pyrophosphate) and an amino acid (Cysteine) but uses yeast extract as the nutrient source.

All aforementioned media contain undefined constituents; phytone and yeast extract which serve as the primary nutrient source. A chemically defined medium required for the elucidation of nutritional requirements, metabolic pathways, and biosynthetic capabilities of *X. fastidiosa* was first formulated by Chang and Donaldson in 1992 and is known as XF-26. This formulation is comprised of 17 amino acids, 2 tricarboxylic acids, and inorganic salts. This defined media yielded growth rates of *X. fastidiosa* that were statistically similar to PD2. Through a systematic process of elimination this media formulation was truncated to components essential for growth (Chang and Donaldson, 2000). The sequencing of the *X. fastidiosa* genome by Simpson et al in 2000 invigorated interest in the pathogen and provided opportunities for new approaches to media development. The previously formulated defined media was re-examined considering the newly available genetic information and has been further truncated (Almeida et al 2004) and defined as minimal for the investigation of metabolic pathways.

Research on *X. fastidiosa* isolates affecting shade trees has traditionally used PW media, however all shade tree strains have been shown to grow on PW and BCYE. Early arguments proposing speciation focused on media specificity, noting a degree of selectivity among strains possibly corresponding to host (Hopkins, 1989). Studies have not been conducted to confirm the growth of shade tree isolates of *X. fastidiosa* on the recently published XfD medium.

Materials and Methods

Bacterial strains and conditions

All experiments were conducted using *X. fastidiosa* isolates Elm, Oak, Sycamore, Mulberry, and Pierces Disease on PW plates provided by Dr. Huang at the USDA ARS Beltsville MD. The Elm and Sycamore isolates were isolated by Dr. Jim Sherald in Washington D.C. in the 1980's and held in cryogenic storage at the USDA, while the Oak and Mulberry isolates were isolated by Dr. Huang in 2000 and held in cryogenic storage at the USDA ARS in Beltsville, MD. All strains were transferred to and maintained on BCYE solid media and in PW broth cultures at 28°C and sub-cultured every 30 days.

Media

Periwinkle Wilt (PW) media was prepared according to Davis et al. 1983 (Table 4). Ingredients were autoclaved and allowed to cool to 40°C in a heated water bath. When the media temperature stabilized Bovine Serum Albumin (BSA) was filter sterilized with .25 µm Millipore membrane and added to the media.

Table 4 Periwinkle Wilt Media Formulation, 1 Liter

Periwinkle Wilt Media	Grams
Phytone peptone	4
Trypticase peptone	1
KH ₂ PO ₄	1
K ₂ HPO ₄	1.2
MgSO ₂ *7H ₂ O	0.4
Bovine Serum Albumin	6
L-Glutamine	4
Hemin Chloride	10 ml
Phenol Red	10 ml

The BCYE media was prepared according to the formulation from Feeley et al. 1979 (Table 5) and autoclaved.

Table 5 Buffered Charcoal Yeast Extract Formulation, 1 Liter

Buffered Charcoal Yeast Extract	Grams
Yeast Extract	10
L-Cysteine	0.4
Activated Charcoal	2
Ferric iron pyrophosphate	0.25
ACES buffer	10

The XfD media was prepared according to Almeida et al. 2004 (Table 6).

Table 6 Xylella Defined Media Formulation, 1 Liter

Xylella Minimal Defined Medium	Grams
L-glutamine	3.0
L-asparagine	1.0
L-cysteine	0.5
Trisodium citrate	1.5
Disodium succinate	1.5
Potato Starch	3.0
KH ₂ PO ₄	1.0
K ₂ HPO ₄	1.5
MgSO ₂ *7H ₂ O	0.5
Hemin Chloride	10 ml
Phenol Red	10 ml

Media formulations were adjusted to pH 7.2 using 1M KOH and were stored at 28°C. Solid media preparations were prepared with the addition of 10g l⁻¹ Gelrite. No pH indicator was added to media preparations to be analyzed by spectrophotometry. Media was held for one day prior to inoculation and visually inspected for signs of contamination.

Spectrophotometer CFU Relationship

A spectrophotometer (Spectronic Genesis 5) was used to measure the absorption at 600nm of *X. fastidiosa* cells in PW broth cultures. Broth cultures with varying degrees of *X. fastidiosa* cell densities were measured in the spectrophotometer and subsequently put through a logarithmic series of dilutions to allow direct calculation of bacterial Colony Forming Units (CFU's) from each sample. Dilution series were executed by plating a 100 µl aliquot of each sample followed by a 10 fold dilution; this was repeated until the 1x10¹⁰ dilution was achieved. The resulting points were graphed and evaluated by regression analysis using Statistical Analysis Software (SAS) to determine the best fit model.

Growth on Solid Media

To determine whether a media supported the growth of a particular *Xylella fastidiosa* isolate a randomized complete block design (in blocks of time) with a 5 x 3 factorial treatment structure was established. Solid plates of PW, BCYE and XfD were inoculated and monitored for colonization. The inoculum of *X. fastidiosa* was

prepared by scraping solid PW plates of the respective *X. fastidiosa* isolates (Oak, Sycamore, Elm, Mulberry, Pierce's Disease) and suspending cells in 5 ml of PW broth and incubating for 5 days at 28°C. Cultures were prepared in triplicate and inspected visually for signs of contamination. The broth cultures were agitated by hand and 100 µl aliquots of inoculum were dripped on plates of the three respective media types (BCYE, PW, XfD) in triplicate. Determination of Colony Forming Units (CFU) was done by dilution series on each respective media. The plates were incubated at 28°C for 10 days after which visible colonies were counted. Randomly selected colonies from randomly selected plates of each respective media were subjected to Enzyme Linked Immunosorbent Assay (ELISA) and Polymerase Chain Reaction (PCR) tests to confirm *X. fastidiosa* identity. Data were analyzed to determine significant effects of media and isolates.

Growth in Broth Media

To determine the efficacy of XfD and PW media in cultivation of *X. fastidiosa* a randomized complete block design in time with a 5 x 2 factorial treatment structure was established. Growth curves were produced by measuring absorbances of samples with a spectrophotometer and calculating the CFU's using the regression equation developed for the study (Figure 10). The nature of the BCYE medium is not conducive to usage as a broth due to the incorporation of activated charcoal which is insoluble and dark in color making visual analysis impossible and so was omitted from the study. Inoculum was prepared by scraping solid PW plates of respective *X. fastidiosa* isolates (Oak, Elm, Mulberry, Sycamore and Pierce's Disease) and

suspending cells in 3 ml of PW and XfD and incubated for 5 days at 28°C. The inoculum was vortexed for 10 sec. and added to 30 ml of its respective media. The resulting suspension was vortexed for 10 sec. and portioned into 1 ml aliquots in autoclaved microcentrifuge tubes. Duplicate reference tubes of pure media from the same batch were also portioned in microcentrifuge tubes and incubated.

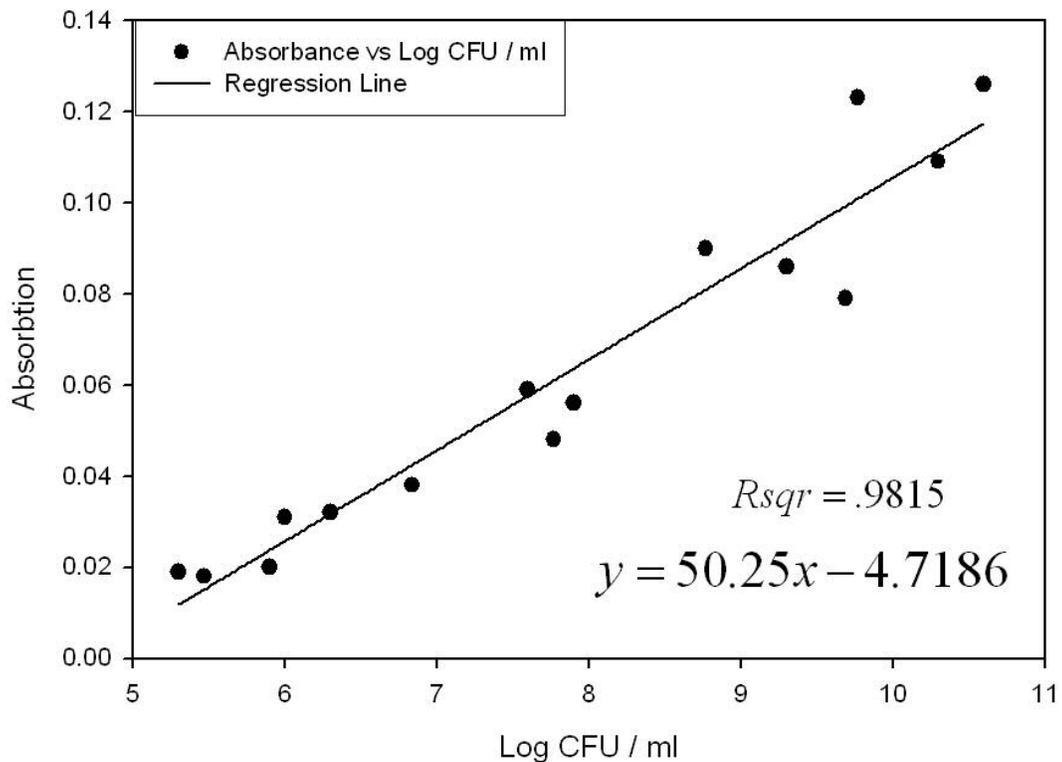
Three randomly selected tubes of each combination of media and isolate were analyzed in random order by a spectrophotometer at 600 nm at the same time each day for ten days to quantify bacterial colonization using two samples of the respective uninoculated media as references. References were used in duplicate to ensure consistency and accuracy. Randomly selected tubes from each isolate were sampled and plated to ensure cultures were not contaminated.

Results

Spectrophotometer CFU Relationship

A linear model, represented by the regression equation $[y=0.0199x - 0.0939]$ resulted from the calibration measurements. The absorption is dependent on the CFU's in the broth culture therefore inverse regression was employed to calculate CFU's from absorbance measurements. Inverse regression was done regressing on CFU's, resulting in the equation $[x=50.25y + 4.7186]$.

Figure 10 Regression analysis and equation of the relationship between absorbance of a Perwinkle Wilt broth sample measured by a spectrophotometer and amount of *X. fastidiosa* Colony Forming Units (CFU's) within the sample as determined by dilution plating.



Solid Media

All three solid growth media formulations were successful in cultivation for the *X. fastidiosa* isolates Oak, Elm, Sycamore, Pierce's Disease, Mulberry (Elm isolate in Figures 11-13). Data analyzed for colonization on solid media showed statistically significant difference between media ($P<0.01$) as well as between isolates ($P<0.01$). There were no differences between the undefined media, BCYE and PW ($P=0.59$). However, both undefined media were found to be significantly different from the minimal medium XfD ($P<0.01$) (Figure 14). Differences in calculated colonization of isolates on all media were found to be significantly different and are reported in Table 7.

Figure 11 Buffered Charcoal Yeast Extract Plate (BCYE) showing growth of *X. fastidiosa* isolated from an *Ulmus americana* specimen after 14 days of incubation



Figure 12 Perwinkle Wilt (PW) media showing growth of *X. fastidiosa* isolated from an *Ulmus americana* specimen after 14 days of incubation



Figure 13 *Xylella* Defined Medium (XfD) showing growth of *X. fastidiosa* isolated from an *Ulmus americana* specimen after 14 days of incubation

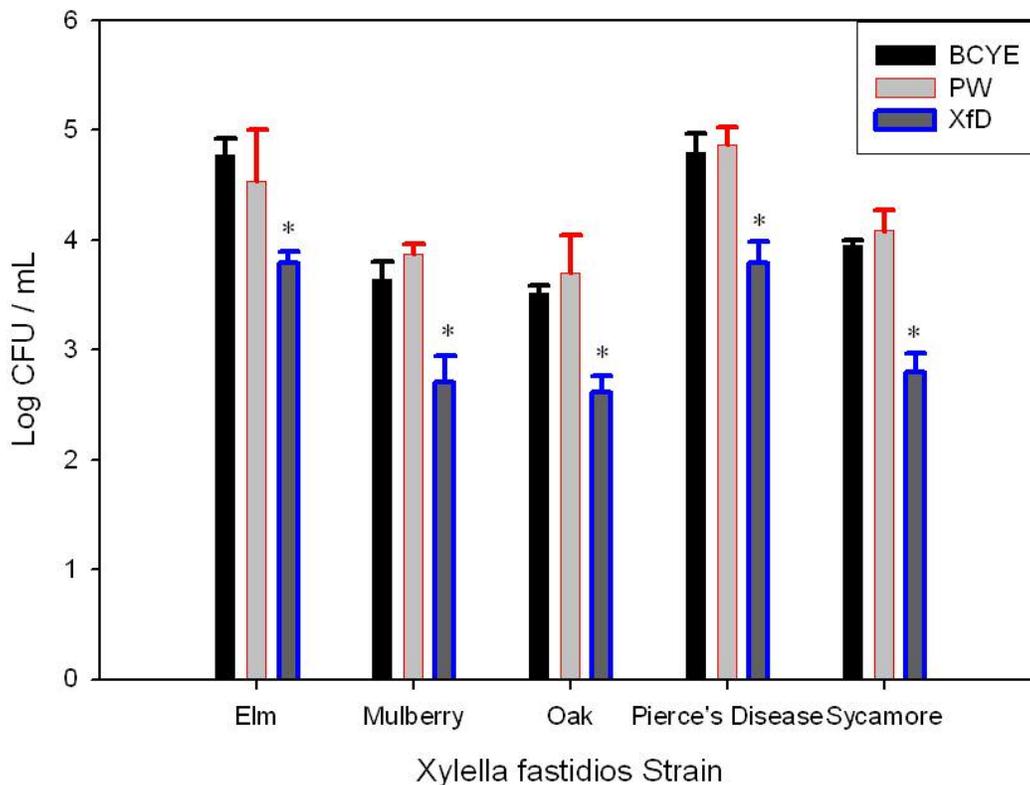


Table 7 Means and statistical significance of *X. fastidiosa* isolates Pierce's Disease, elm, oak, mulberry, and sycamore growth after 10 days on Solid PW Media. Colony Forming Units (CFU's) determined by dilution plating.

Strain	Mean Log CFU / ml
Pierce's Disease	4.48a
Elm	4.36a
Mulberry	3.40b
Sycamore	3.60b
Oak	3.27b

*means with identical letters are not significantly different at the .05 level by SED = .069

Figure 14 Growth of *X. fastidiosa* isolates Elm, Mulberry, Oak, Sycamore, and Pierce's Disease on Buffered Charcoal Yeast Extract (BCYE), Perwinkle wilt media (PW) and Xylella Defined Media (XfD) . Bars represent mean growth in Log of Colony Forming Units (CFU's) and their associated standard errors.



*means with symbol are significantly different at the .05 level by SED = .054

Broth Media

Broth media of PW and XfD were compared for efficacy of growth using 5 isolates of *X. fastidiosa* (Elm, Mulberry, Oak, Sycamore, and Pierce's Disease). Both tested broth media formulations were successful in cultivation of all the selected *X. fastidiosa* strains. No significant differences ($P=0.95$) in colonization could be determined between PW and XfD media formulations (Figures 15-16). Mean colonization of isolates was found to be significantly different and are reported in table 8.

Table 8 Means and statistical significance of *X. fastidiosa* isolates Pierce's Disease, elm, oak, sycamore, and mulberry growth measured with a spectrophotometer for 10 days. Absorbance readings were converted to Colony Forming Units (CFU's) using the regression equation determined in this study (Figure 10).

Strain	Mean Log CFU / ml
Pierce's Disease	8.79a
Elm	7.40b
Mulberry	7.04c
Oak	6.95cd
Sycamore	6.75d

*means with identical letters are not significantly different at the .05 level by SED = .041

Figure 15 Growth curve of *X. fastidiosa* isolates Pierce's Disease, Mulberry, Oak, Elm, and Sycamore in Perwinkle Wilt (PW) broth over a period of ten days measured with a spectrophotometer. Absorbance readings were converted to Colony Forming Units (CFU's) using the regression equation determined in this study (Figure 10). Means are plotted with associated standard errors.

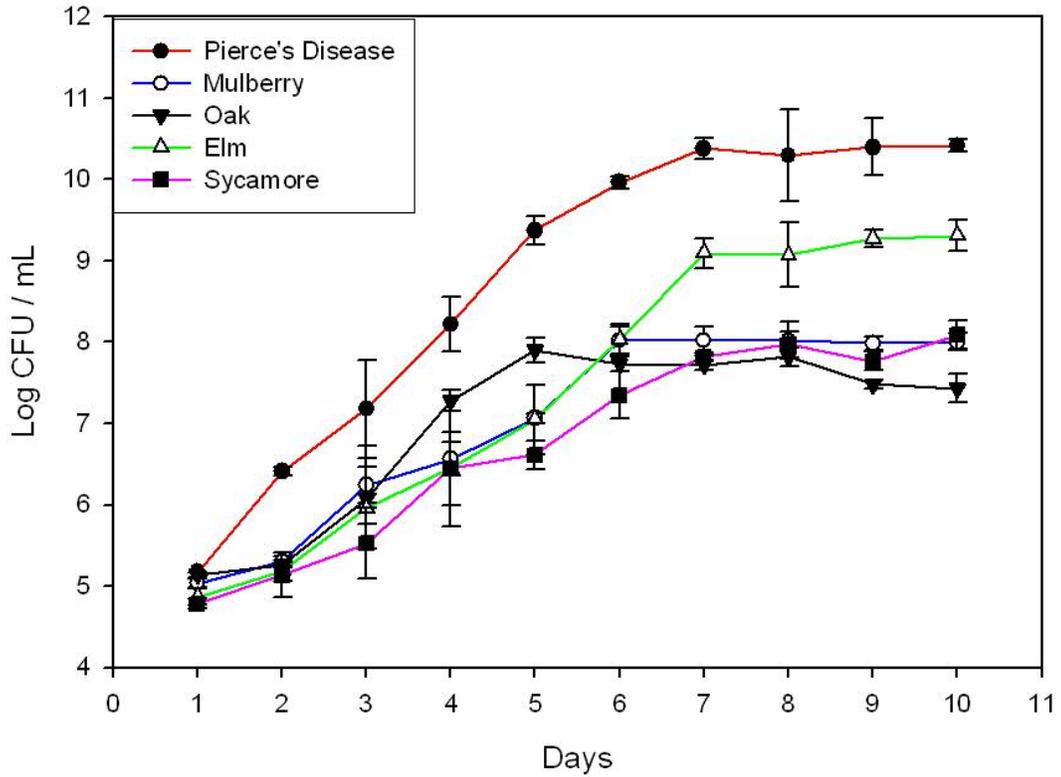
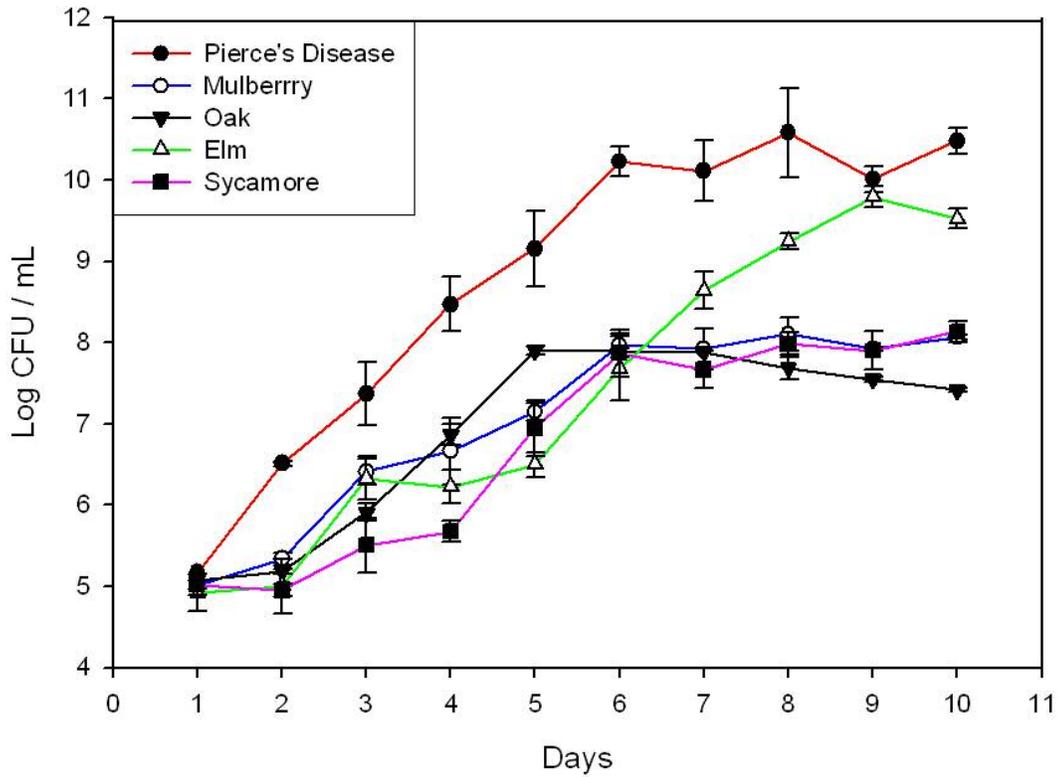


Figure 16 Growth curve of *X. fastidiosa* isolates Pierce's Disease, Oak, Elm Mulberry, and Sycamore in Xylella Defined Media (XfD) broth measured with a spectrophotomer over 10 days. Absorbance readings were converted to Colony Forming Units (CFU's) using the regression equation determined in this study (Figure 10). Means are plotted with associated standard errors.



Discussion

Xylella is fastidious and requires strict adherence to the pH range (6.8 – 7.2) and incubation temperature (28°C) for optimal growth (Wells 1987). The pH requirement varies slightly between strains of the pathogen and experimentation is required with each individual strain in order to achieve optimal growth (Personal Observation). There is significant variability in growth rate and plaque formation even within strains of the same host. The pathogen is quite resilient despite its fastidious nature; it appears to become latent in sub-optimal conditions, returning to robust growth once optimal conditions are restored (Personal Observation). Honing the skill of consistent optimal cultivation is essential before growth studies can be undertaken.

In the extensive usage of all the media during experimentation BCYE was preferred due to its simplicity and overall efficacy. The black color of the media also aided in the visualization of bacterial colonization. The PW media is difficult to work with due to the addition of the BSA protein which must be added after autoclave sterilization increasing the potential for contamination. Pouring plates of PW must be done in small batches (no more than 500 ml at a time) because plating must be done following the addition of BSA at temperatures below 40°C, close to the temperature at which the media will begin to solidify. Furthermore, the BSA tends to create “bubbles” in the media complicating the plating procedure.

Results from the broth media trial showed no difference between XfD and PW ($P < 0.95$), while results from the solid media trials showed greater colonization on the

undefined BCYE and PW as compared to the minimal XfD media ($P < 0.01$). The results of the solid media trial are in contrast to Almedia 2004, who found no differences between *X. fastidiosa* colonization of PW or XfD using Pierce's Disease isolates. This may be due in part to the inherent differences in the parameters of broth and solid media cultivation that often affect bacterial growth (i.e. aeration).

Although the statistical results show highly significant differences, looking at the raw data puts the differences in context (Figure 14); the minimal media lags behind the two rich media by a single log unit in almost every case. Additionally the broth study required the usage of the CFU regression equation which increases the expected experimental error; it is possible that the spectrophotometer was not able to detect the differences in growth considering the magnitude of the difference and the sensitivity of the equipment combined with the experimental error.

The study was halted at 10 days for consistency among all tests, however when let grow indefinitely *X. fastidiosa* will completely colonize each media formulation (Figures 11-13). This point highlights the difficulty in development of effective media for *Xylella* and explains the impetus behind the high number of media variations that have been developed (more than a dozen). The slow growth of the pathogen is a major obstacle to practical experimentation with the *Xylella* and while a variety of media have been shown to support growth, there have been limited to no improvement of colonization rate since the initial isolation media formulation. The small disparity between undefined and defined media shown in this study is not surprising due to the fact that *Xylella* seems unable to exploit excess nutrients when

provided; therefore the minimal media is essentially no different from the undefined media.

In separate isolation tests conducted using broth media during experimentation no successful isolations were ever obtained using XfD broth, while PW broth proved successful. Possibly, this is due to the inability of the media to sufficiently support the growth of a low cell density of *X. fastidiosa*. The availability of all essential components is critical for supporting growth of the organism when bacterial titer is low. Alternatively, this could be simply due to poor execution of the isolation procedure; however the high number of replicates required for isolation reduces this possibility. However, this result is not surprising when the history of *X. fastidiosa* isolation is considered. The initial isolation of Plum leaf scald was accomplished using BCYE after the PD2 media developed for Pierce's Disease isolation was found ineffective (Wells et al. 1981), subsequently all isolated strains of *X. fastidiosa* have been found to grow on PD2 (Wells et al. 1987). Furthermore, nutritional requirements were considered a possibility for separation in the group (Hopkins, 1989) essentially based on the fact that different isolates require different media, however significant variation has not been able to be demonstrated.

It is possible that this is due to the genetics of the bacteria, which can change after continual sub-culturing, adjusting to their current growth environment. The plasticity of the genomic sequence of *X. fastidiosa* is evident from examining the available sequence data which reveal a "flexible" gene pool due to horizontally transferred elements (Nunes et al. 2003). This is apparent in the variations of "minimal" media produced (Chang and Donalson, 2000; Almeida et al. 2004), which

demonstrate the inability to clearly define what is minimally required by the organism for growth. All *X. fastidiosa* strains tested in this evaluation were thoroughly sub-cultured on PW media, which is close in composition to XfD, while isolations from the environment are plated on XfD directly from the xylem of the host. The variation in previous environmental conditions might explain the failure of XfD as an isolation media, but success when used in sub-culturing.

While the XfD medium is considered “minimal” it might be more appropriate to simply designate it as defined. It contains 5 of the 8 ingredients of the PW media (Tables 4 and 6), essentially substituting a defined nutrient (Trisodium citrate and Disodium succinate) for an undefined (Phytone peptone and Trypticase peptone), supplementing more amino acids (Asparagine and Cystiene) and eliminating the BSA protein making it autoclavable. Evaluations into the efficacy of this media in isolation or growing freshly isolated material would substantiate the minimal claim.

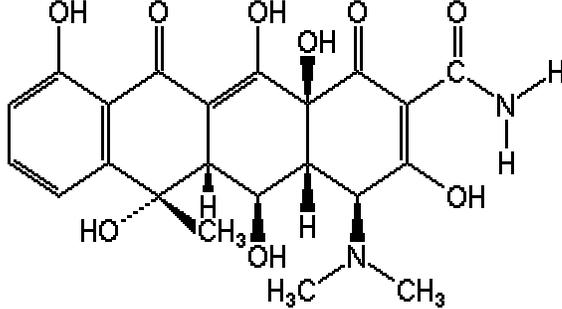
Clearly a medium designed to optimize the growth of *Xylella* is needed as well as formulations that are designed to elucidate the biochemical pathways of the organism. The recently available sequence data for the pathogen has renewed interest in media development (Campanharo et al. 2003; Lemos et al. 2003; Almeida et al. 2004) and will certainly lead to further advancement in the effective cultivation of the organism.

Chapter 4: Effect of Paclobutrazol on Bacterial Colonization

Introduction

In the Mid Atlantic and Southeastern region of the United States the syndrome resulting from *X. fastidiosa* is commonly referred to as Bacterial Leaf Scorch (BLS)(Hearon et al.1980). Bacterial Leaf Scorch is a debilitating disorder that primarily affects urban specimens of *Quercus*, *Platanus*, and *Ulmus* genera (Sherald, 1999). Recognition and diagnosis of the disorder has increased in urban localities across the Southeast of the United States over the past decade (Sherald, 1999).

Figure 17 Chemical structure of Oxytetracycline



Researchers initially investigated treatment of the disorder with an antibiotic, oxytetracycline, via microinjections directly into the xylem

of the tree (Kostka 1985). Oxytetracycline was discovered in the 1940's and was among the first tetracyclines to be described, it is a naturally occurring product of *Streptomyces rimosus*. Due to its relatively early discovery and its low cost this antibiotic has been extensively used for human, animal and plant disorders. Oxytetracycline molecules are composed of a tetracyclic nucleus with both dimethylamino and carboxamide functional groups (Figure 17).

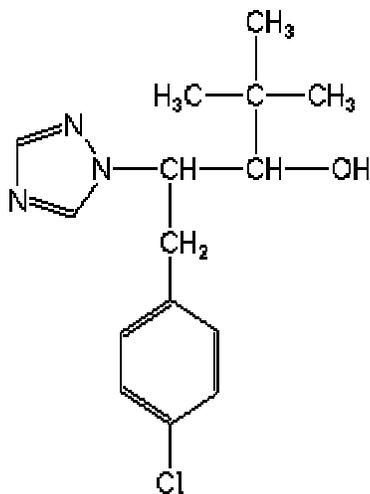
It has been established that tetracyclines inhibit bacterial protein synthesis by preventing the association of aminoacyl-tRNA with the bacterial ribosome (Schnappinger et al. 1996). Oxytetracycline must enter the cytoplasm via the single membrane of the gram-negative *Xylella* bacterium in order to disrupt protein synthesis (Schnappinger et al.1996). Entry is achieved as a positively charged cation-tetracycline complex (Schnappinger et al. 1996). This chemical has been tested in *Ulmus americana* infected with *Xylella* achieving a variable degree of symptom remission (Kostka et al.1985). There is speculation that the efficacy of the chemical is highly dependant on the severity of symptoms and the size of the specimen treated. The treatment is not curative and must be repeated annually (Kostka et al. 1985). Furthermore, the associated problems with the over usage of antibiotics is increasing pressure to discontinue their use in the environment and seek alternatives.

Plant growth regulators were first employed to overcome Phony Disease in peach, a stunting disorder induced by *Xylella* (French and Stassi, 1978). The application of gibberellic acid, a plant growth regulator responsible for cell elongation, was effective in reversing the stunting induced by the pathogen; however the treatment did not affect the bacterial population of the treated plants when assessed by light microscopy (French and Stassi, 1978). A separate study investigated a series of growth regulators applied to grape varieties with varying degrees of resistance to Pierce's Disease, a leaf scorching disorder caused by *X. fastidiosa*, yielding mixed results (Hopkins et al. 1985). The foliar application of growth regulators Kinetin (synthetic cell division regulator) and Indole-3-acetic acid (naturally produced auxin) improved resistance to Pierce's Disease in *Vitis*

rotundifolia ‘Carlos’ a moderately resistant variety, reducing bacterial concentrations measured in the petiole and reducing visible symptoms. However these growth regulators were not effective when used on *Vitis vinifera* varieties susceptible to Pierce’s Disease (Hopkins et al. 1985).

Paclobutrazol, a gibberellin biosynthesis inhibitor, was first used by arborists in the 1970’s to control the height of trees under power line right-of-ways and was initially applied as a trunk injection (Chaney, 2004). The low solubility of paclobutrazol in water favored formulations that employed alcohol based solvents which with repeated usage proved problematic causing cambial cracks, weeping injection wounds, and wood discoloration (Chaney, 2004). These issues favored the reformulation of the chemical for usage as a root drench.

Figure 18 Chemical Structure of paclobutrazol



Paclobutrazol is structurally classified in the triazole group of chemical compounds (Sugavanam, 1984), which includes members widely used for their fungistatic properties (Radenmacher, 2000). It was initially classified as a multi-protector due to its ability to inhibit growth

from five distinct fungi as well as confer tolerance to drought, chilling, and other stresses (Fletcher and Hofstra, 1986). Paclobutrazol disrupts terpenoid biosynthesis, inhibiting the catalytic activity of cytochrome P450 dependent monooxygenases (Miki et al. 1990). The molecule has two chiral centers, asymmetric carbon atoms, thus it can exist as two pairs of enantiomers in two optically inactive

diastereoisomeric forms (Burden et al. 1987). The name paclobutrazol specifically applies to the 2RS,3RS diastereoisomer. The 2R,3R enantiomer is active in the disruption of sterol biosynthesis and is similar in structure to lanosterol (Sugavanam, 1984). The 2S,3S enantiomer is active in the disruption of gibberellin biosynthesis and is similar in structure to ent-kaurene (Sugavanam, 1984). These enantiomers exist as a racemic mix in the 2RS,3RS diastereoisomer that is commercially available. In general, relatively high rates of the chemical which induce extreme growth reductions are required to achieve fungistatic effects, however this is species specific (Radenmacher et al. 1987).

The fungistatic effect of paclobutrazol is due to its disruption of sterol metabolism, specifically by blocking the oxidation of 14 α -demethylation during ergosterol biosynthesis (Miki et al. 1990). The growth regulation and subsequent conferment of stress tolerance is due to the inhibition of oxidative steps from ent-kaurene to ent-kaurenoic acid, which is hypothesized to shunt resources to the production of abscisic acid (Chaney, 2004). Additionally the requisite oxidative steps for abscisic acid breakdown are also disrupted by the chemical (Chaney, 2004).

The properties of triazoles and their horticultural applications have been intensively examined resulting in the proposal that modulations of Gibberellin (GA) levels, initiate a cascade of processes subsequently leading to stress protection (Vettakkaormakankav et al. 1999). This sequence of events includes increased biosynthesis of photosynthetic pigments, altered root to shoot ratios, and increased levels of antioxidant enzymes (Kraus et al. 1994). It has been demonstrated that the stress protective and morphological effects of triazoles can be reversed by the

application of GA3 (Gilley et al. 1998), demonstrating the relationship between PBZ and GA.

Experimentation with *Olea europaea* treated with this chemical and exposed to drought, demonstrated an increase in leaf water potential, decreased stomatal conductance, and a reduction in number of xylem vessels elements as compared with controls (Frakulli and Voyiatzis, 1999). Paclobutrazol has also been found to increase the density of stomata on leaves of treated *Prunus persica*, however leaf gas exchange appears unaffected presumably due to induction of stomatal closure (Blanco et al. 1998). There are conflicting reports on the effects of paclobutrazol to xylem vessels Frankulli and Voyiatzis 1999, reports no change in diameter of the vessels but rather a reduction in the total number in *Olea europaea*, while Prioetti et al. 1998, reports distinct alterations in vessels shape and function in total xylem vessel area compared to control in *Castanea sativa*.

Paclobutrazol has been marketed in North America by the Rainbow Tree Company under the name Cambistat essentially as a multi-protector although it is registered as a Plant Growth Regulator. Paclobutrazol has been associated with symptom remission of Bacterial Leaf Scorch at the Barlett Tree Laboratories, Charlotte N.C. There is no data on any direct effect of paclobutrazol on *X. fastidiosa*. Establishing a direct effect would represent the first logical step in elucidating the mechanism(s) by which symptom reduction is achieved. This study investigates the effects of paclobutrazol on *X. fastidiosa* growth in vitro.

Materials and Methods

Bacterial strains and conditions

All experiments were conducted using *X. fastidiosa* isolates Elm, Oak, Sycamore, Mulberry, and Pierces Disease on PW plates provided by Dr. Huang at the USDA ARS Beltsville MD. The Elm and Sycamore isolates were isolated by Dr. Jim Sherald in Washington D.C. in the 1980's and held in cryogenic storage at the USDA, while the Oak and Mulberry isolates were isolated by Dr. Huang in 2000 and held in cryogenic storage at the USDA ARS in Beltsville, MD. All strains were transferred to and maintained on BCYE solid media and in PW broth cultures at 28°C and sub-cultured every 30 days.

Broth Media

Periwinkle Wilt (PW) media was prepared according to Davis et al 1981 (Table 4) excluding the indicator Phenol Red. Ingredients were autoclaved and allowed to cool to 40°C in a water bath. When the temperature stabilized Bovine Serum Albumin was filter sterilized with 0.25 µm Millipore membrane and added to the media. Liquid media was inspected the day following production for contamination. One liter of PW broth was partitioned into 10-100 ml aliquots to which treatments were randomly assigned.

Inoculum

Cells of *X. fastidiosa* strains Elm and Pierces Disease grown for 10 days on BCYE medium were scraped and suspended in PW broth. The cultures were incubated for 5 days at 28°C and standardized to $\sim 1 \times 10^4$ CFU's using a spectrophotometer (Spectronic Genesis 5).

Treatments

A 10 ml aliquot of PW broth was drawn from a randomly selected 100 ml PW stock broth and used to dissolve 5 mg of oxytetracycline. The solution was filter sterilized through a .25 μ M Millipore membrane and added back to the PW broth from which it was drawn resulting in a 50 μ g ml⁻¹ oxytetracycline treatment.

Three 10 ml stock solutions of paclobutrazol (PBZ) dissolved in 95% ethanol, 2000 μ g ml⁻¹, 200 μ g ml⁻¹, and 20 μ g ml⁻¹, were produced and stored at room temperature in the laboratory. Aliquots of 100ul from each respective stock were randomly applied to 100ml PW broth stock solutions creating 200 μ g ml⁻¹, 20 μ g ml⁻¹ and 2 μ g ml⁻¹ paclobutrazol treatments respectively.

Growth studies were conducted using 1.5 ml sterile microcentrifuge tubes. Nine hundred μ L aliquots of each treatment (Control PW, 50 μ g ml⁻¹ oxytetracycline, 200 μ g ml⁻¹ PBZ, 20 μ g ml⁻¹ PBZ, 2 μ g ml⁻¹ PBZ) were added to 40 tubes each. Subsequently all treatments received 100 μ L of *X. fastidiosa* inoculum, were sealed,

labeled, and placed in an incubator at 28°C. Thirty 1ml standards from each respective treatment were also produced to serve as references in spectrophotometry.

Spectrophotometer CFU Relationship

A spectrophotometer (Spectronic Genesis 5) was used to measure the absorption at 600nm of *X. fastidiosa* cells in PW broth cultures. Broth cultures with varying degrees of *X. fastidiosa* cell densities were measured in the spectrophotometer and subsequently put through a logarithmic series of dilutions to allow direct calculation of bacterial Colony Forming Units (CFU's) from each sample. Dilution series were executed by plating a 100 µl aliquot of each sample followed by a 10 fold dilution; this was repeated until the 1×10^{10} dilution was achieved. The resulting points were graphed and evaluated by regression analysis using Statistical Analysis Software (SAS) to determine the best fit model.

Growth Study

Growth studies were executed in autoclaved microcentrifuge tubes for ten days. A logarithmic series of dilutions in autoclaved water was prepared for a randomly selected sample from each treatment every day. Dilution plating served to directly quantify bacterial colony forming units (CFU's). Dilutions were plated each day for 5 days on BCYE, incubated for 10 days and resulting colonies counted. Additionally three randomly selected microcentrifuge tubes of each respective treatment were removed and measured for bacterial growth in a spectrophotometer

each day for 10 days. References of each respective media from the same media batch were used in duplicate, to ensure consistency and accuracy. During the course of the study randomly selected tubes were sampled and plated to ensure purity of the cultures.

Results

Spectrophotometer CFU Relationship

A linear model, represented by the regression equation $[y=0.0199x - 0.0939]$ resulted from the calibration measurements. The absorption is dependent on the CFU's in the broth culture therefore inverse regression was employed to calculate CFU's from absorbance measurements. Inverse regression was done regressing on CFU's, resulting in the equation $[x=50.25y + 4.7186]$ (Figure 10).

Evaluation of Paclobutrazol with Spectrophotometry

Analysis of variance (ANOVA) performed on the data for the growth of *X. fastidiosa* in broth media treated with oxytetracycline at $50\mu\text{g ml}^{-1}$ and paclobutrazol at $200\mu\text{g ml}^{-1}$, $20\mu\text{g ml}^{-1}$ and $2\mu\text{g ml}^{-1}$ analyzed by a spectrophotometer showed significant interaction between strain and treatment ($P<0.01$) indicating one strain responded differently to the treatment(s).

Although data trends were similar (Figures 19 and 20) the interaction term implies only simple means for each treatment may be confidently reported and discussed. When the data sets for *X. fastidiosa* Elm and Pierce's Disease are analyzed separately they display a similar pattern in the simple means (Table 10). The analysis showed no differences ($P=0.99$) between the oxytetracycline (Figure 21) at $50\mu\text{g ml}^{-1}$ and paclobutrazol at $200\mu\text{g ml}^{-1}$ (Figure 22) in Elm or Pierce's Disease strains. Both treatments effectively controlled bacterial growth, however no reduction in bacterial

colonization is observed with the spectrophotometer. There were no differences in growth when *X. fastidiosa* was exposed to paclobutrazol at 2 µg ml⁻¹ (Figure 23) and 20 µg ml⁻¹ (Figure 24) from the control (Figure 25) in either Elm or Pierce's ($P=0.76$, $P=0.89$) and ($P=0.84$, $P=0.88$) respectively, showing the treatments provided no reduction in bacterial growth.

Table 9 Means and statistical significance of *X. fastidiosa* isolates elm and Pierce's Disease growth in Perwinkle Wilt (PW) broth supplemented with chemical treatments of oxytetracycline (antibiotic) and paclobutrazol measured with a spectrophotometer and converted to Colony Forming Units (CFU's) using the regression equation determined in this study (Figure 10)

Treatment and Dosage	Mean Log CFU / ml (Elm, PD)
Oxytetracycline 50 µg ml ⁻¹	4.97, 4.95a
Paclobutrazol 200 µg ml ⁻¹	5.00, 4.99a
Paclobutrazol 20 µg ml ⁻¹	7.23, 8.31b
Paclobutrazol 2 µg ml ⁻¹	7.18, 8.27b
Periwinkle Wilt Media	7.38, 8.61b

*means with identical letters are not significantly different at the .05 level by SED = .028, 0.52 SED

Figure 19 Growth curve of *X. fastidiosa* isolate elm in Perwinkle Wilt (PW) broth supplemented with chemical treatments of oxytetracycline (antibiotic) and paclobutrazol measured over ten days with a spectrophotometer. Absorbance readings were converted to Colony Forming Units (CFU's) using the regression equation (Figure 10) determined for this study. Means and associated standard errors are plotted.

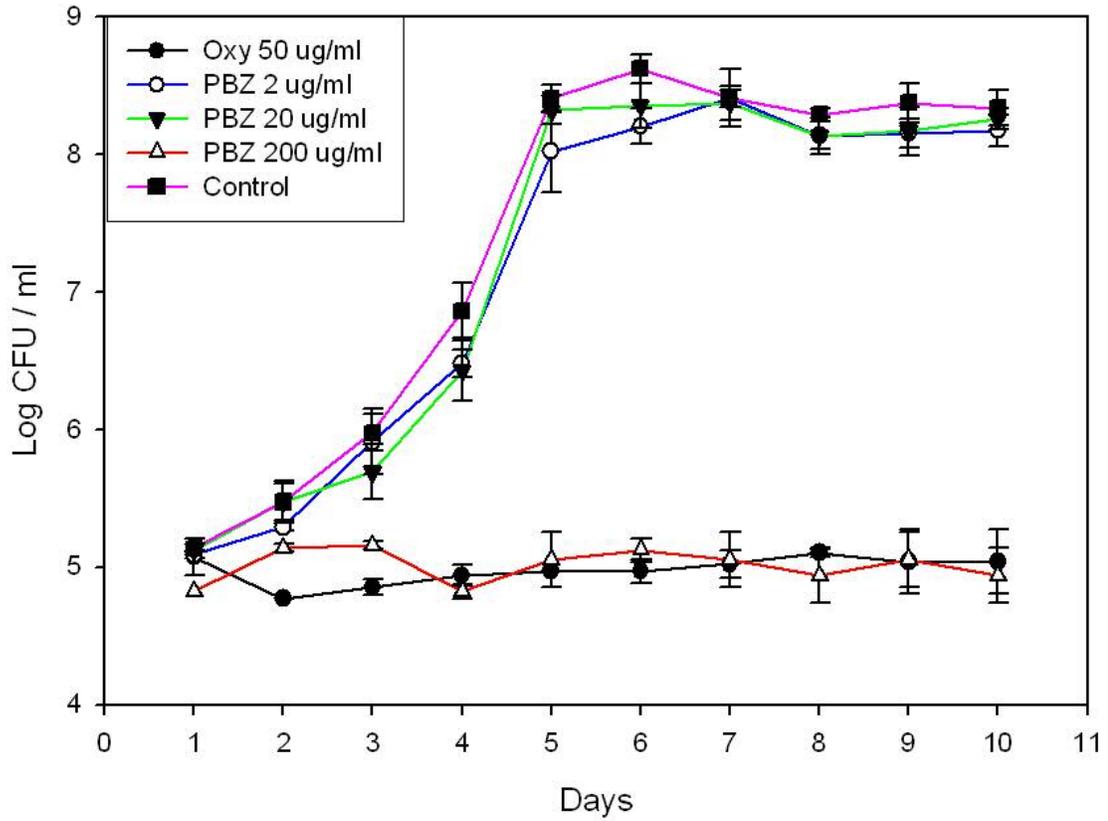


Figure 20 Growth curve of *X. fastidiosa* isolate Pierce's Disease in Perwinkle Wilt (PW) broth supplemented with chemical treatments of oxytetracycline (antibiotic) and paclobutrazol measured over ten days with a spectrophotometer. Absorbance readings were converted to Colony Forming Units (CFU's) using the regression equation (Figure 10) determined for this study. Means and associated standard errors are plotted

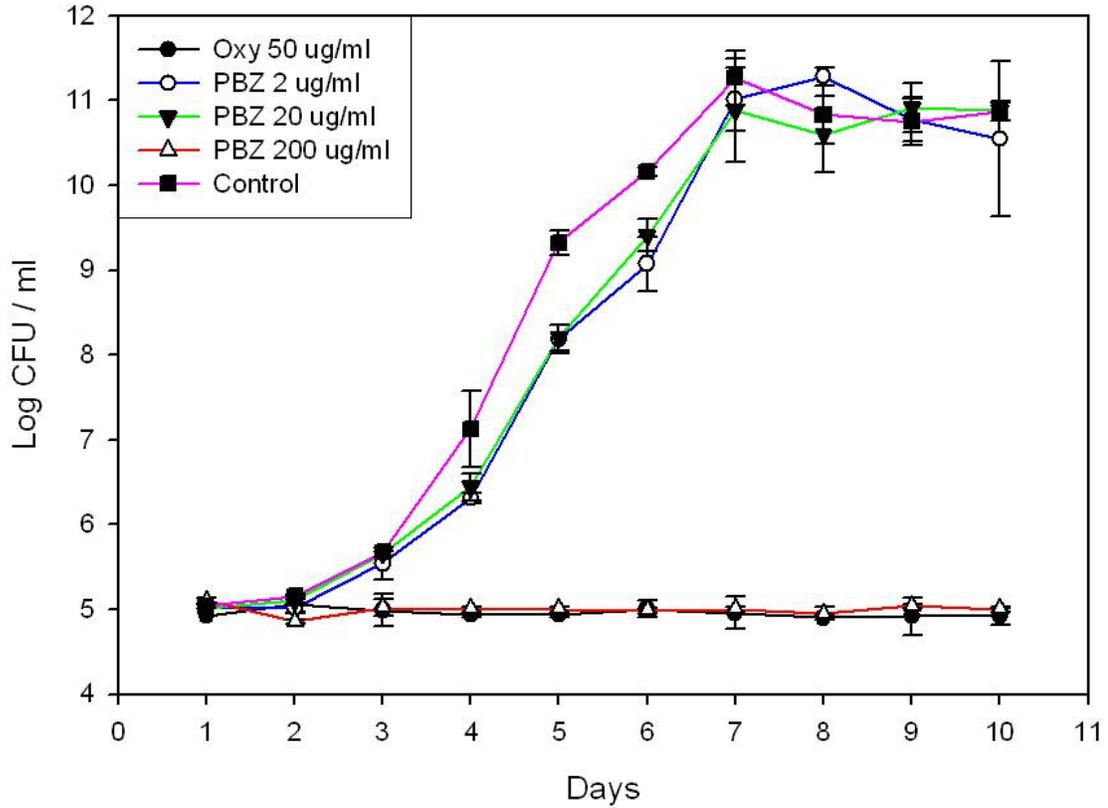


Figure 21 Growth of *X. fastidiosa* isolates elm and Pierce's Disease in Periwinkle Wilt (PW) broth supplemented with oxytetracycline at a dosage of $50 \mu\text{g ml}^{-1}$ measured with a spectrophotometer. Absorbance readings were converted to Colony Forming Units (CFU's) using the regression equation (Figure 10) determined for this study. Means are plotted with associated standard errors.

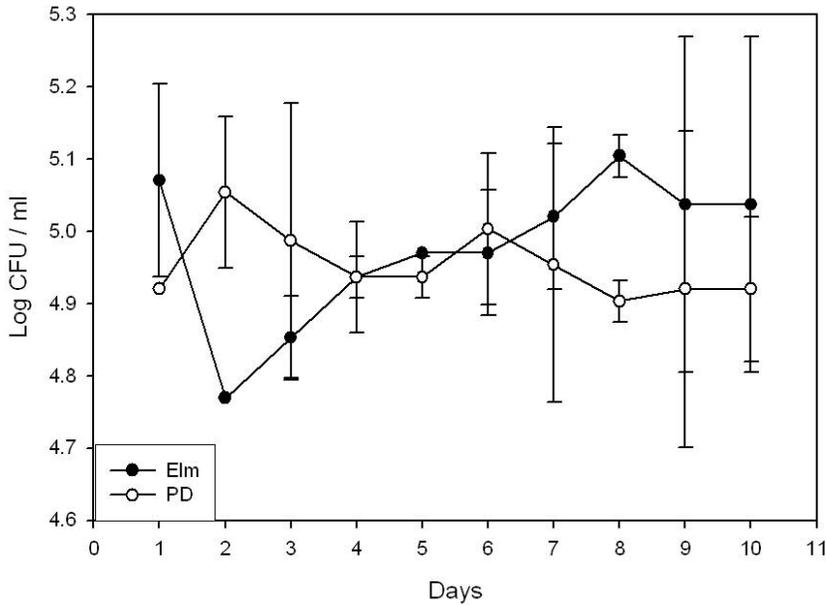


Figure 22 Growth of *X. fastidiosa* isolates elm and Pierce's Disease in Periwinkle Wilt (PW) broth supplemented with paclobutrazol at a dosage of $200 \mu\text{g ml}^{-1}$ measured with a spectrophotometer. Absorbance readings were converted to Colony Forming Units (CFU's) using the regression equation (Figure 10) determined for this study. Means are plotted with associated standard errors.

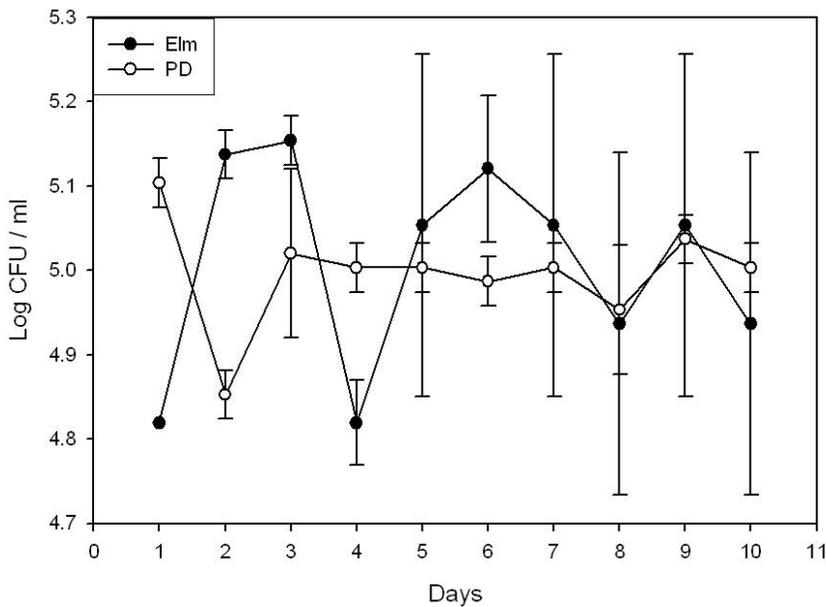


Figure 23 Growth of *X. fastidiosa* isolates elm and Pierce's Disease in Periwinkle Wilt (PW) broth supplemented with paclobutrazol at a dosage of $2 \mu\text{g ml}^{-1}$ measured with a spectrophotometer. Absorbance readings were converted to Colony Forming Units (CFU's) using the regression equation (Figure 10) determined for this study. Means are plotted with associated standard errors

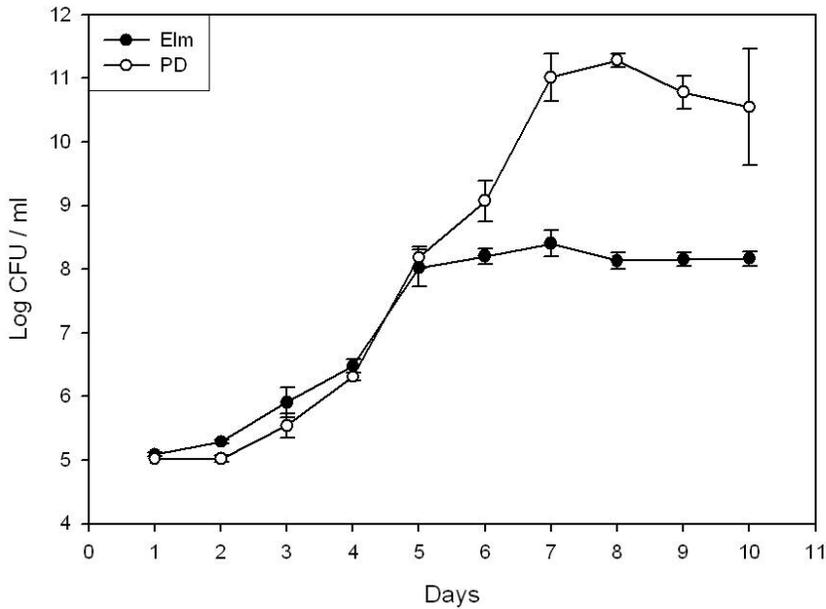


Figure 24 Growth of *X. fastidiosa* isolates elm and Pierce's Disease in Periwinkle Wilt (PW) broth supplemented with to paclobutrazol at a dosage of $20 \mu\text{g ml}^{-1}$ with a spectrophotometer. Absorbance readings were converted to Colony Forming Units (CFU's) using the regression equation (Figure 10) determined for this study. Means are plotted with associated standard errors

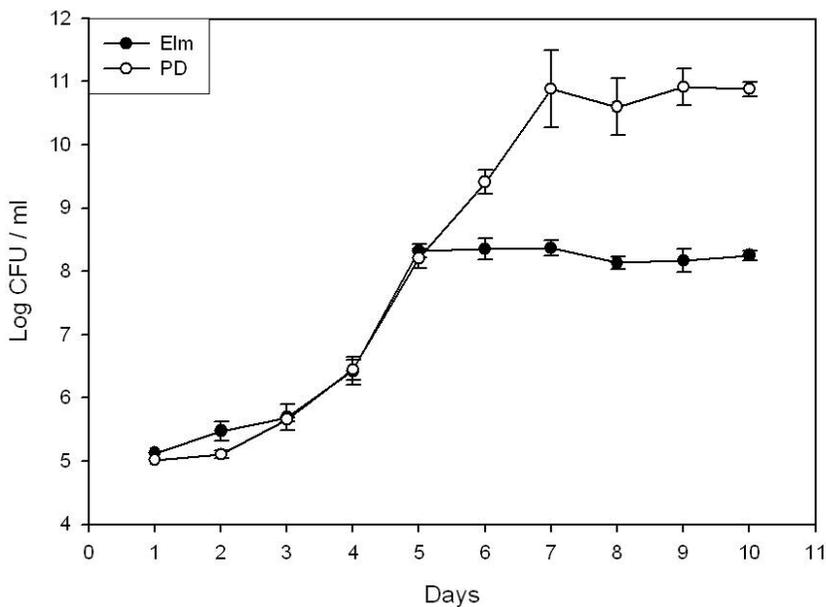
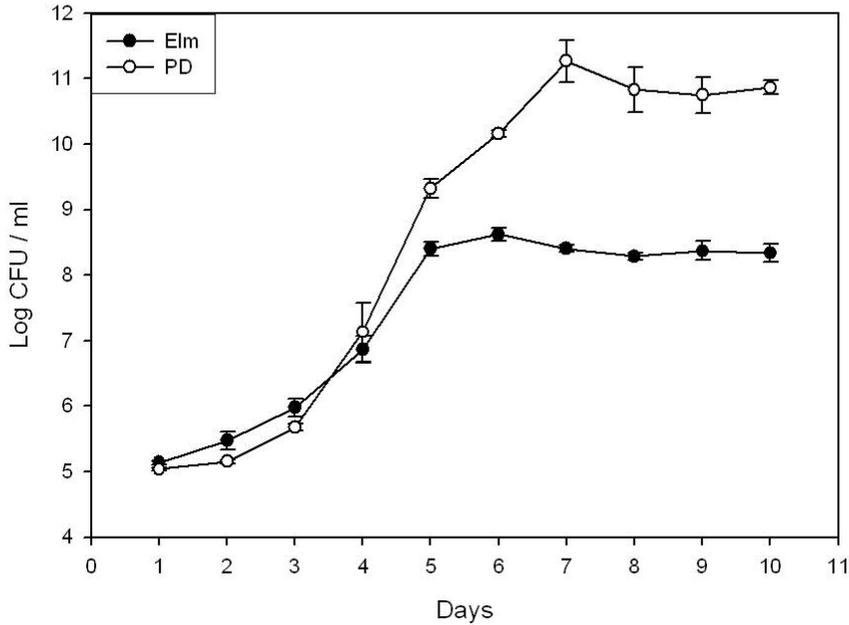


Figure 25 Growth of *X. fastidiosa* isolates elm and Pierce's Disease in Periwinkle Wilt (PW) broth measured with a spectrophotometer. Absorbance readings were converted to Colony Forming Units (CFU's) using the regression equation (Figure 10) determined for this study. Means are plotted with associated standard errors



Broth Media Evaluation by Dilution Plating

Analysis of Variance performed on the resulting colony counts from the executed treatments showed a significant interaction between treatments and strains of *X. fastidiosa* ($P=0.02$). The mean estimates show a magnitude interaction indicating differing sensitivities of the strains to the treatment(s).

Table 10 Means and statistical significance of *X. fastidiosa* isolates elm and Pierce's Disease growth in Perwinkle Wilt (PW) broth supplemented with chemical treatments of oxytetracycline (antibiotic) and paclobutrazol, Colony Forming Units (CFU's) determined by dilution plating

Treatment and Dosage	Mean Log CFU / ml (Elm, PD)
Oxytetracycline at 50 $\mu\text{g ml}^{-1}$	0.15, 2.19a
Paclobutrazol at 200 $\mu\text{g ml}^{-1}$	2.66, 3.64b
Paclobutrazol at 20 $\mu\text{g ml}^{-1}$	7.35, 7.48c
Paclobutrazol at 2 $\mu\text{g ml}^{-1}$	7.33, 7.67c
Perwinkle Wilt Media	7.61, 7.98c

*means with identical letters are not significantly different at the .05 level by Tukey SED = .040, 0.37 SED

When the data sets for *X. fastidiosa* Elm and Pierce's Disease are analyzed separately they display a similar pattern (Table 11) in the simple means. The data shows that oxytetracycline at 50 $\mu\text{g ml}^{-1}$ (Figure 28) severely reduced the growth of the Pierce's Disease isolate and effectively eliminated growth of the Elm isolate. Paclobutrazol at a 200 $\mu\text{g ml}^{-1}$ (Figure 29) dose reduced growth of the pathogen on both isolates however was not as effective as oxytetracycline. The other treatments of paclobutrazol at 2 $\mu\text{g ml}^{-1}$ (Figure 30) and 20 $\mu\text{g ml}^{-1}$ (Figure 31) could not be differentiated from the control (Figure 32) ($P=0.9$, $P=0.9$) respectively in elm and ($P=0.6$, $P=0.9$) respectively in Pierce's Disease and were ineffective in the control of *X. fastidiosa* growth.

Figure 26 Growth curve of *X. fastidiosa* isolate elm in Perwinkle Wilt (PW) broth supplemented with Chemical treatments of oxytetracycline (antibiotic) and paclobutrazol measured over five days. Colony Forming Units (CFU's) were determined by dilution plating. Means and associated standard errors are plotted

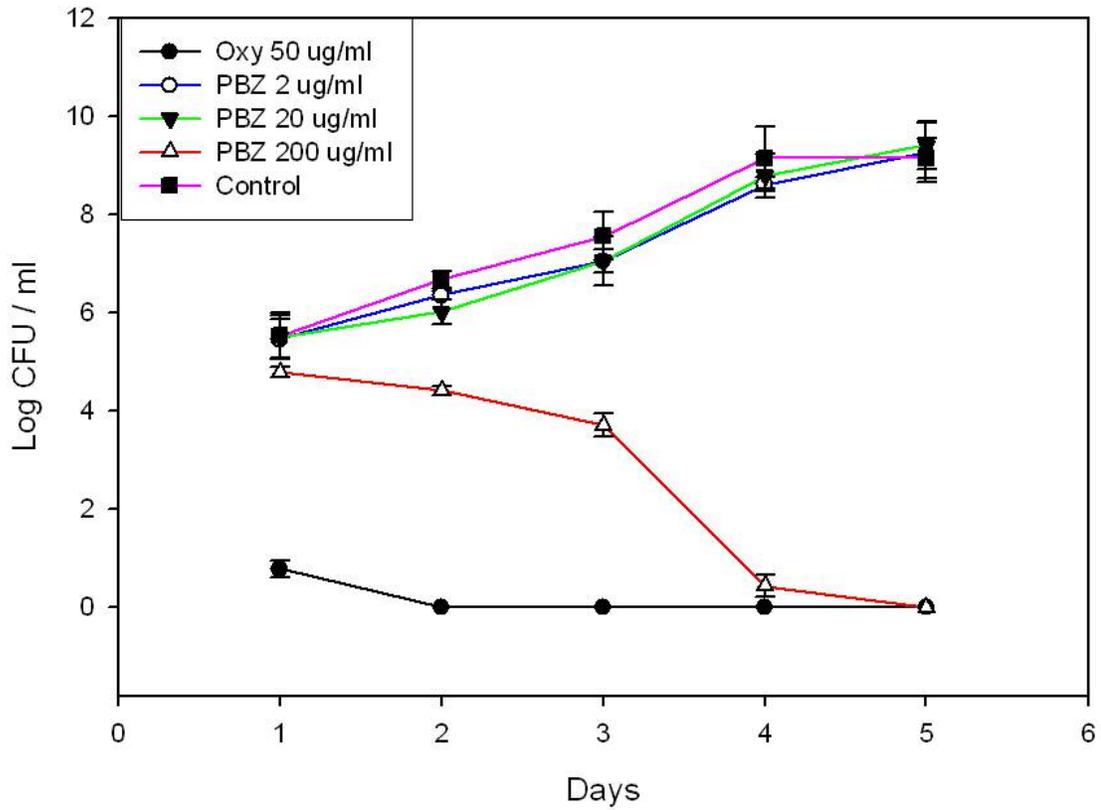


Figure 27 Growth curve of *X. fastidiosa* isolate Pierce's Disease in Perwinkle Wilt (PW) broth supplemented with Chemical treatments of oxytetracycline (antibiotic) and paclobutrazol measured over five days. Colony Forming Units (CFU's) were determined by dilution plating. Means and associated standard errors are plotted

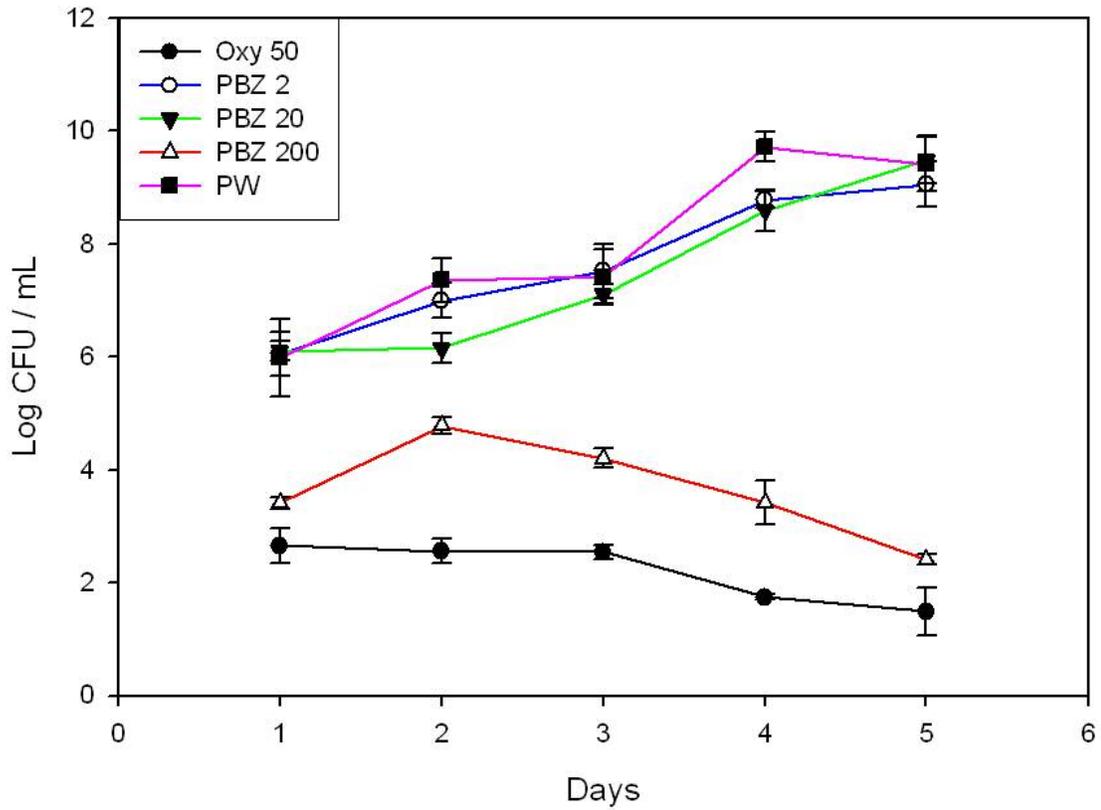


Figure 28 Growth of *X. fastidiosa* isolates elm and Pierce's Disease in Periwinkle Wilt (PW) broth supplemented with oxytetracycline at a dosage of $50 \mu\text{g ml}^{-1}$. Colony Forming Units (CFU's) were determined by dilution plating. Means are plotted with associated standard errors.

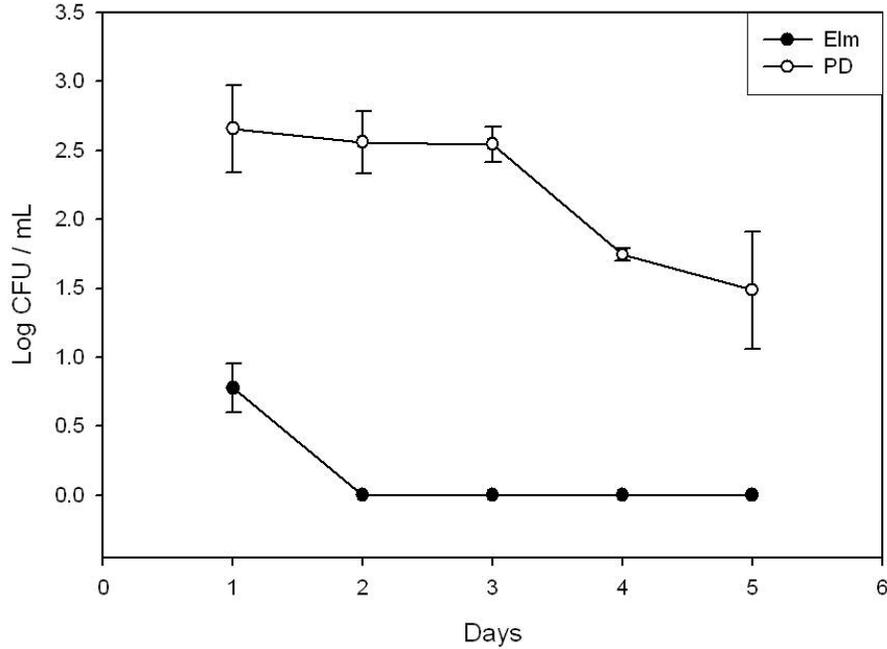


Figure 29 Growth of *X. fastidiosa* isolates elm and Pierce's Disease in Periwinkle Wilt (PW) broth supplemented with paclobutrazol at a dosage of $200 \mu\text{g ml}^{-1}$. Colony Forming Units (CFU's) were determined by dilution plating. Means are plotted with associated standard errors

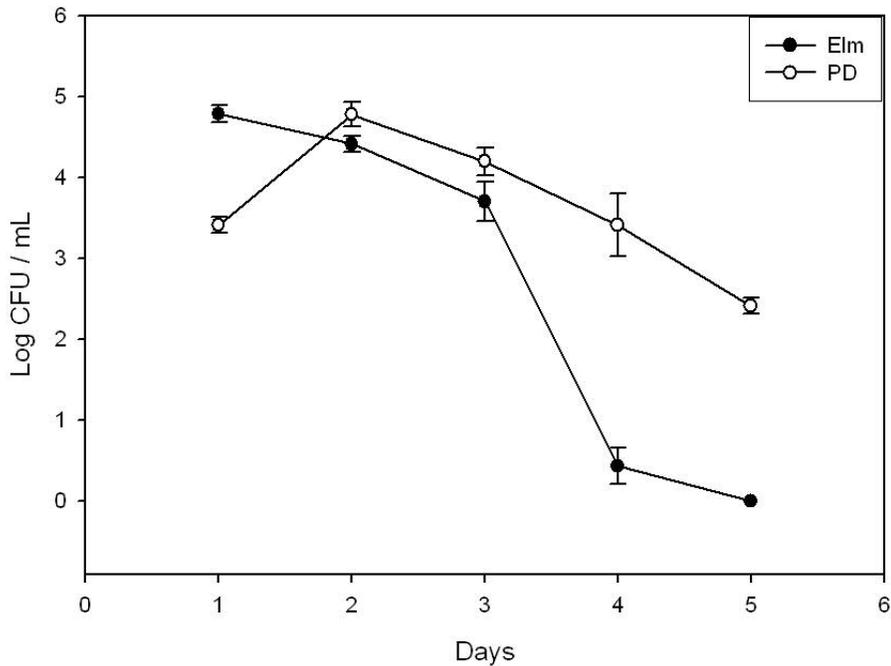


Figure 30 Growth of *X. fastidiosa* isolates elm and Pierce's Disease in Periwinkle Wilt (PW) broth supplemented with paclobutrazol at a dosage of $2 \mu\text{g ml}^{-1}$. Colony Forming Units (CFU's) were determined by dilution plating. Means are plotted with associated standard errors

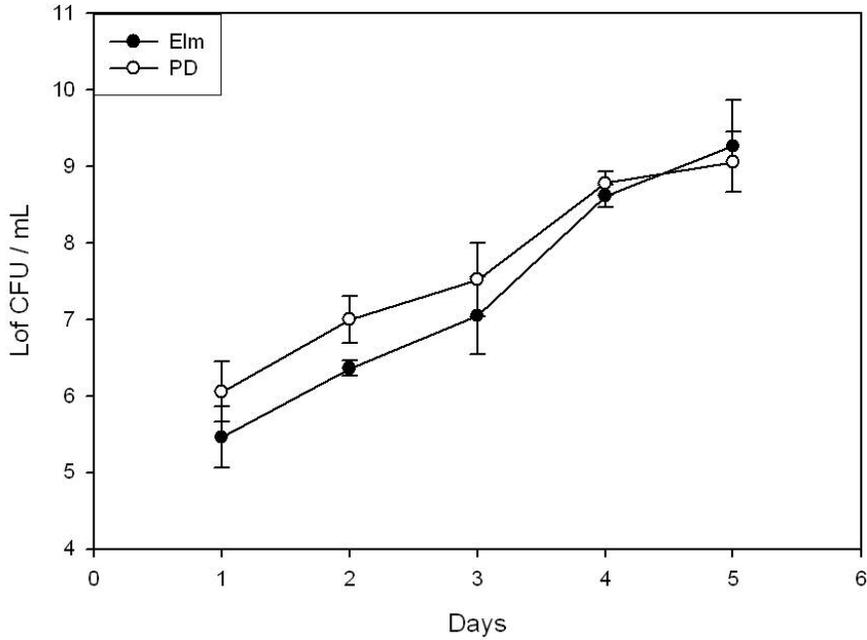


Figure 31 Growth of *X. fastidiosa* isolates elm and Pierce's Disease in Periwinkle Wilt (PW) broth supplemented with paclobutrazol at a dosage of $20 \mu\text{g ml}^{-1}$. Colony Forming Units (CFU's) were determined by dilution plating. Means are plotted with associated standard errors

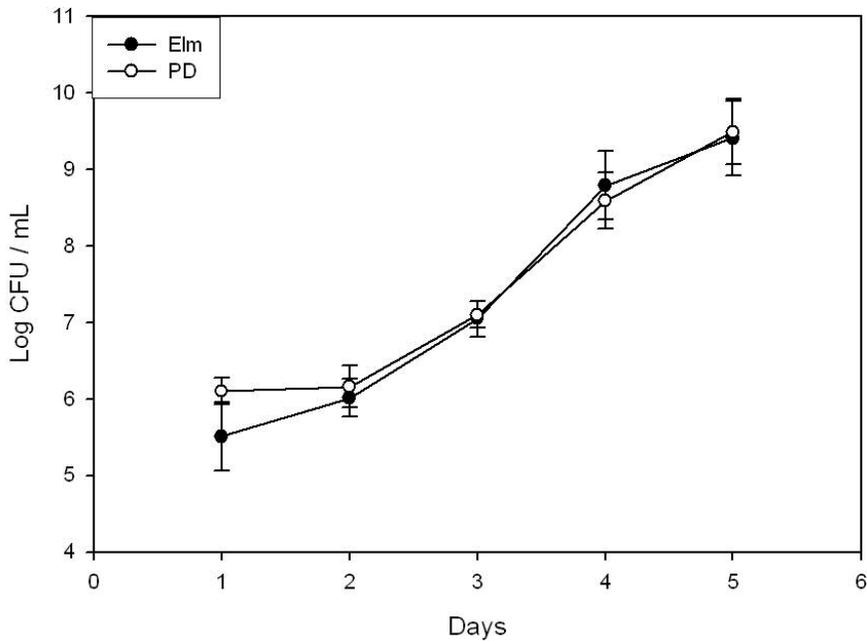
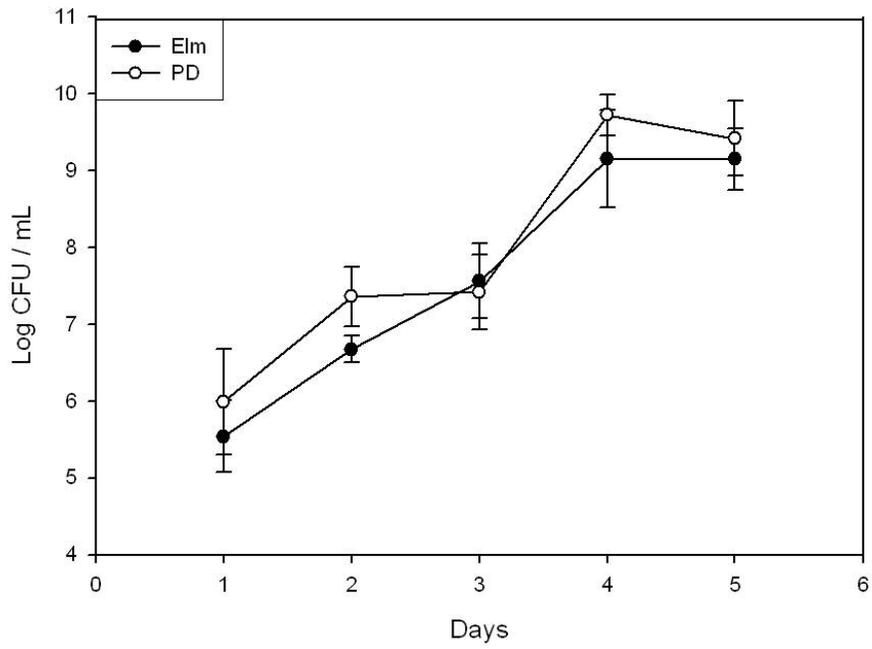


Figure 32 Growth of *X. fastidiosa* isolates elm and Pierce's Disease in Periwinkle Wilt (PW) broth. Colony Forming Units (CFU's) were determined by dilution plating. Means are plotted with associated standard errors



Discussion

Industry data show that paclobutrazol can be effective in reducing symptom development at a dosage of 20 mg ml⁻¹ on mature shade trees (Barlett Tree Lab). Currently symptom remission is hypothesized to be the result of induced physiological changes within the tree induced by paclobutrazol such as the alteration in the size and/or confirmation of the xylem vessel elements or reduced stomatal conductance rather than a bacteriostatic or bacteriocidal effects. These physiological alterations could ease or reduce the necessity of water movement through the xylem resulting in less acute drought stress. Indeed, if the full suite of physiological changes including increased root/shoot ratio, increased spongy leaf mesophyl, and increased diameter of xylem vessels associated with paclobutrazol were realized the treated specimen would have a distinct advantage over untreated specimens, especially in drought periods when *X. fastidiosa* symptoms are most evident.

In this study the data clearly shows that oxytetracycline and paclobutrazol are effective *in vitro* at suppressing *X. fastidiosa* growth when applied in high enough dosage. The diastereomeric nature of paclobutrazol explains its dual function as both a growth regulator and fungicide; however this is the first report of possible bacteriostatic effects. The enantiomeric pair found in the commercially available paclobutrazol is the 2RS, 3RS diastereomer which exists as a racemic mixture including the 2R,3R enantiomer which has been found to disrupt sterol biosynthesis (Sugavanam, 1984; Burden et al. 1989). This enantiomer is currently not isolated and

exploited for its fungicidal properties, possibly due to the added expense and availabilities of alternatives.

Analysis of proposed biosynthetic pathways inferred from genomic data of *X. fastidiosa* shows a complete pathway for sterol biosynthesis including lanosterol, the intermediary identified as disrupted by the presence of paclobutrazol (www.genome.ad.jp/dbget-bin/get_pathway?org_name=xf&mapno=00100). The most simplistic explanation for the observed results of this study would be to implicate paclobutrazol in the disruption of this pathway in line with previous studies of alternate systems (Radenmacher, 2000). When this is considered with the general observance of a relatively high dosage of the compound to disrupt sterol biosynthesis as compared to growth regulation (Radenmacher et al. 1987) the results of the tests are predictable.

Alternatively, the observed results may be explained by the high dosage of Paclobutrazol altering the environmental conditions of the media such that the fastidious bacteria were unable to thrive. This could be achieved by the sequestration of a vital element, such as iron, required for *X. fastidiosa* growth. Paclobutrazol is an active molecule with electron pairs situated at the periphery facilitating the establishment of complexes with other elements, especially those which bond readily such as iron. There is also the remote possibility that the ethanol used to dissolve the chemical could remain in abundance sufficient to cause bacterial dieback, however this was considered in the experimental design and mitigated as much as possible by allowing the ethanol to volatilize with standing in a laminar flow hood for 24 hours. Furthermore, the relatively slow reduction of bacterial colonization observed in the

study does not support a dieback scenario, which would likely take effect immediately resulting in no bacterial growth. However, the fastidious and therefore extremely sensitive nature of the pathogen must be considered at all times, and caution must be exercised when interpreting any experimental results.

When taking into consideration the dual nature of paclobutrazol and the possible synergistic effects of its enantiomers on *X. fastidiosa* infected specimens, it may not be desirable to separate the enantiomers of the diastereomer for treatment of *X. fastidiosa*. It is possible that a dosage of the racemic mixture could be identified where bacteriostatic effects are observed as well as possible beneficial physiological changes within the specimen. This would prove to be the ideal circumstance for usage of the chemical and certainly merits further investigation. However, the dosage issue is not easily resolved, it is likely a species specific effect as well as dependent on bacterial concentration within the plant which is difficult to gauge. Furthermore, estimating the ultimate dilution of the chemical *in planta* is also not easily achieved. The establishment of dosage guidelines will require significant field investigation on mature trees, a resource which is scarce in the research field.

The differing statistical results from the two methods used to determine the efficacy of each treatment is explained by the inherent inability of the spectrophotometer to detect reduction in viability of bacterial cells compounded by the sensitivity of detection limitations of the instrument. While the spectrophotometer readings for the oxytetracycline at 50 $\mu\text{g ml}^{-1}$ and paclobutrazol at 200 $\mu\text{g ml}^{-1}$ appear to be stable (Figure 19-20), inspection of the standard deviation of the readings (Figure 21 and 22) reveal the readings were simply within the error range of the

instrument. Comparison with the dilution plating data (Figure 26-27) shows the inability of the spectrophotometer to account for the loss of cell viability. While the spectrophotometer readings indicated a complete secession in growth of *X. fastidiosa* in the two respective treatments, the dilution plating method showed even the direct effect of oxytetracycline at $50\mu\text{g ml}^{-1}$ was ineffective at completely stopping growth of *X. fastidiosa* Pierce's Disease. Additionally, the dilution plating method clearly displayed that although paclobutrazol at $200\ \mu\text{g ml}^{-1}$ was effective at reducing the growth of *X. fastidiosa* it was not as effective as oxytetracycline and did not result in complete control of Pierce's Disease.

Chapter 5: Conclusions

Introduction

Xylella fastidiosa (Wells et al. 1987) is a broad spectrum fastidious bacterial pathogen that significantly affects viticulture, citrus, and shade tree industries in the Americas (Brown et al. 2002). The bacterium is xylem limited and is disseminated by a variety of suctorial insects (i.e. leafhoppers, sharpshooters) (Houston et al. 1947) and is harbored by a wide range of hosts in more than 30 families including both monocots and dicots (Hopkins and Alderz, 1988). The disease of *Xylella fastidiosa* in shade trees is referred to as Bacterial Leaf Scorch (Hearon et al. 1980); it is a chronic late season leaf scorch concentrated in urban environments that debilitates infected specimens, however it is rarely if ever lethal. While the pathogen has been recovered from a variety of shade tree species the most severely affected genera include *Quercus*, *Platanus*, and *Ulmus* (Sherald, 1999). Urban trees infected with the disease become unsightly and lose the aesthetic qualities they were intended to provide, often leading to their removal. Bacterial Leaf Scorch incidence has been increasingly identified and reported as a significant problem in landscapes across the mid-Atlantic and southeastern United States (Sherald, 1999). Recently, the plant growth regulator, paclobutrazol has been associated with disease symptom remission in shade trees infected with *X. fastidiosa*.

The goals of this research were

1. Locate *Ulmus americana* specimens infected with *X. fastidiosa* in Washington D.C. using GIS technology and molecular tools
2. Isolate the bacterium from *Ulmus americana* specimens using a recently adapted protocol, pressure chamber (Bextine and Miller, 2004).
3. Test the growth of *X. fastidiosa* isolates from shade trees on *Xylella fastidiosa* Defined Media (Alameida et al. 2004).
4. Investigate any direct effects of paclobutrazol on *X. fastidiosa* growth *in vitro* as compared to oxytetracycline.

Location

In this study a sampling area for *Ulmus americana* specimens was identified and defined using the publicly available GIS database (www.dcgis.dc.gov) of street trees in Washington D.C. *Ulmus americana* specimens were surveyed for infection of *X. fastidiosa* using the ELISA (Sherald and Lei, 1991) and PCR (Pooler and Hartung 1995) molecular identification techniques. The sampling region contained 240 *Ulmus americana* specimens of which 23 (approximately 10%) were found to be infected with *X. fastidiosa*. Confirmed hosts within the sampling region were mapped resulting in an irregular disease distribution. This study represents the first usage of GIS technology to visually assess *X. fastidiosa* distribution.

Replicating this technique in several randomly selected regions within an urban center would provide a citywide assessment of *X. fastidiosa* infection, an important piece of data that is currently missing. Data collected over successive years

would show the disease dissemination within the urban forest allowing for predictive modeling as well as mitigation planning. Additionally, confirmed hosts could be assessed annually with digital photographs and condition ratings which could be incorporated into the database providing a tool to assess symptom severity over a long term. There is great potential for using GIS to track and manage a variety of pest and disease problems that affect urban forests including Dutch Elm Disease, the Emerald Ash Borer, and the Japanese Long Horn Beetle among others. This study has demonstrated how this can be executed.

Isolation

The inherent difficulty of isolating a fastidious organism such as *X. fastidiosa* limits practical experimentation on the organism. I attempted to use three distinct methods to isolate *X. fastidiosa* from *Ulmus americana*, a pressure chamber (Bextine and Miller 2004), a petiole technique (Chang et al. 1988), and a xylem vessel excision technique (Sherald et al. 1983). I was able to isolate *X. fastidiosa* from three *Ulmus americana* specimens using the xylem vessel excision technique, however the other techniques were unsuccessful.

The pressure chamber is an attractive technique since it results in the expression of xylem fluid, which *X. fastidiosa* is immersed in. This method requires an unbroken water column, thus cutting samples is best done underwater, which is not easily achieved when sampling mature trees. While there are difficulties with this method, I believe further experimentation can overcome these challenges, possibly

resulting in a high yield isolation technique. The petiole technique seems more appropriately used with other species such as oak and grape which have more significant petioles; the petiole of elm is small in comparison and difficult tissue to work with when macerated. While the xylem excision technique provided the only successful isolates in the study it required large amounts of material and high replication. Further experimentation in this area is needed in the future.

***Xylella* Defined Growth Media**

Currently the genus *Xylella* is composed of a single species (*X. fastidiosa*) lacking pathovar designation despite four completely sequenced genomes from distinct hosts; Grape (Van Sluys et al. 2003), Oleander (Bhattacharyya et al. 2002), Citrus (Simpson et al. 2000) and Almond (Bhattacharyya et al. 2002) and reports of failure in reciprocal transmission (Sherald, 1993). A variety of diversity studies employing environmental sampling show that the amount of variability in *X. fastidiosa* is surprisingly low despite its wide host range (Van Sluys et al. 2002; Rodrigues et al. 2003; Van Sluys et al. 2003; Koide et al. 2004). Initial attempts to separate groups were based on nutritional requirements (Hopkins, 1989). In this study *X. fastidiosa* isolates from oak, elm, mulberry, and sycamore were shown to grow on a minimal defined medium developed for Pierce's Disease (Alameida et al. 2004)

The development of defined media that support the growth of *X. fastidiosa* is a positive step in understanding the biology of the organism. The ability of *X. fastidiosa* isolates from shade trees to grow a defined media developed for Pierce's

Disease demonstrates the similarities between the organisms further complicating efforts to break up the *Xylella* group. Future studies will likely employ the XfD medium since it has no undefined constituents and has now been shown to support the growth of 5 isolates from 5 distinct hosts. The XfD media is particularly attractive for usage in chemical remitting agent tests due to its defined nature.

Paclobutrazol Evaluation

While *X. fastidiosa* has been recognized as a problem for over a century there are currently no practiced therapeutic treatments for the disease. This research focused on the usage of paclobutrazol (Sugavanam, 1984) a gibberellin biosynthesis inhibitor with demonstrated antifungal properties (Jacobs and Berg, 2000) as a chemotherapeutic treatment. This study investigated the effect of paclobutrazol on *X. fastidiosa in vitro* and demonstrated that at a relatively high dose of 200 $\mu\text{g ml}^{-1}$ of the chemical resulted in significant suppression of bacterial growth. However, doses of 20 $\mu\text{g ml}^{-1}$ (the manufacturer's recommended dose) and below proved ineffective and could not be differentiated from the control. Paclobutrazol exists as a racemic mixture of two enantiomers, the 2R,3R enantiomers is structurally similar to lanosterol and is associated with the disruption of sterol biosynthesis while the 2S,3S enantiomer is structurally similar to ent-kaurene and is associated with the disruption of gibberellin biosynthesis (Rademacher, 2000). This study is the first to propose a hypothesis that paclobutrazol has bacteriostatic effects via disruption of sterol biosynthesis of *X. fastidiosa* in line with the fungistatic mode of action.

While the separation of the enantiomers followed by investigation into their individual effect on the pathogen would test this hypothesis, and possibly result in a reformulation of the chemical mixture to be more effective, it should also be considered that optimal disease suppression could be achieved by exploiting both the growth regulating properties as well as the bacteriostatic properties of paclobutrazol. An alteration in the ratio of enantiomers may result in synergistic effects between the enantiomers; however simply increasing the dosage given to trees may also achieve this end. Limitations of this approach include the low solubility of paclobutrazol in water, which initially forced the reformulation of the product as a root drench rather than an injectible due to the associated issues of injecting alcohol based solvents into trees.

An increase in the recommended dosage of the chemical will require reconsideration of the environmental impact and therefore chemical application, possibly returning to an injectible format. The dosage question presents a significant problem for treatment of *X. fastidiosa* in general, the level of infection and volume of xylem fluid within a tree are two factors that are difficult to accurately estimate. The development of a representative estimate of infection is needed if guidelines are to be set. This could be achieved by the sampling multiple regions within the canopy, both symptomatic and asymptomatic and analyzing the samples with ELISA followed an optical density assessment to determine severity of infection.

If the mode of action proposed in the study is correct, other members of the triazole group currently registered as fungicides may also provide bacterial growth suppression based on their similar chemical structure and association with sterol

biosynthesis. While the particular chemical structure of paclobutrazol may be suited to the disruption of the sterol biosynthesis pathway in *X. fastidiosa* it is also possible that any compound containing a tetracyclic ring with an sp² hybridized nitrogen atom on the periphery of the molecule may sufficiently interfere with the function of monooxygenase and reduce bacterial growth.

However if the alternative hypothesis of an indirect effect altering the environment such that *X. fastidiosa* ceases to thrive (i.e. iron sequestration or pH change) proves to be the mode of action novel treatments could be devised to exploit that fact. It has been suggested that the alteration of xylem pH via the injection of a conjugate acid or conjugate base at a precise moment in the disease cycle could effectively reduce viable bacterial populations (Personal communication Jay Stipes, Virginia Polytechnic Institute). The possibility of limiting iron availability with a chelating agent injected into the xylem is also a possibility; however the requirement of the iron by the plant counters the logic of such an approach. It is likely that such treatments will not be curative however it may mitigate the severity of the infection and are environmentally benign. The extreme balance in environmental conditions required by the organism, favors future experimentation with this approach.

While no treatments tested to date are likely curative the chronic nature of the organism provides ample time for treatment, which may employ multiple strategies. Recommended treatments may be altered by disease severity as well as overall health of the specimen. Under the current paradigm of integrated pest management it is likely that a single treatment will not be found to be effective but rather a concerted

approach of vector control, host removal, as well as chemical treatment will provide the best results.

While the potential for paclobutrazol usage as a chemotherapeutic treatment for *X. fastidiosa* was shown in this study, several key questions remain. Some of these include:

1. Does paclobutrazol affect bacterial concentration *in planta*? At what dose?
2. Will trees tolerate a higher dosage of the chemical?
3. Can synergistic effects be achieved by increasing the dosage?
4. Does the 2R3R enantiomer of Paclobutrazol show a high degree of activity in direction interaction with *X. fastidiosa*?
5. Does the 2R3R enantiomer have deleterious effects on the specimen being treated?

Finding the answers to these questions would help clarify the direct effect of paclobutrazol on *X. fastidiosa* and the operative mechanism(s) for its effect. This could lead to more efficient use of paclobutrazol as a chemotherapeutic agent for management of *X. fastidiosa* in shade trees.

Appendices

SAS Code used for statistical analysis

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Title XfD vs. PW broth study;

proc print data=broth;
quit;

proc mixed data=broth;
class media strain date;
model CFU=media strain media*strain;
random date;
lsmeans strain / pdiff adjust=tukey;
quit;

Title PW vs. XfD vs. BCYE solid media;

proc print data=solid;
quit;

proc mixed data=solid;
class date media strain;
model CFU=media strain media*strain;
random date;
lsmeans media strain / pdiff adjust=tukey;
quit;

Title Paclobutrazol Broth;

proc print data=pbzborth;
quit;

proc mixed data=pbzbroth;
class date strain trt;
model CFU=strain trt strain*trt;
random date;
lsmeans strain*trt / pdiff adjust=tukey;
quit;

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quit;

proc mixed data=pbzsolid;
class strain trt date;
model CFU=strain trt strain*trt;
random date;
lsmeans strain*trt / pdiff adjust=tukey;
quit;

Title Elm Paclobutrazol Broth;
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quit;

proc mixed data=elmpbzbrot;
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model CFU=trt;
random date;
lsmeans trt / pdiff adjust=tukey;
quit;

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quit;

proc mixed data=elmpbzsolid;
class date trt;
model CFU=trt;
random date;
lsmeans trt / pdiff adjust=tukey;
quit;

Title PD Paclobutrazol Broth;

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quit;

proc mixed data=pdpbzbrot;
class date trt;
model CFU=trt;
random date;
lsmeans trt / pdiff adjust=tukey;
quit;

Title PD Paclobutrazol Solid;

proc print data=pdpbzsolid;
quit;

proc mixed data=pdpbzsolid;
class date trt;
model CFU=trt;
random date;
lsmeans trt / pdiff adjust=tukey;
quit;

```

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