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Studies on bacterial leaf scorch of American elm and other tree species.

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STUDIES ON BACTERIAL LEAF SCORCH OF AMERICAN ELM AND OTHER TREE SPECIES

A Dissertation Presented

by

Stanley John Kostka, Jr.

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DEDICATION

To My Wife

Justine Foley Kostka

for without her I may never have attained this goal. Her patience, understanding, and support in our pursuit of this goal have been boundless. I have been very fortunate to have a friend like her.

and My Father

Stanley John Kostka

for teaching me to persevere and instilling in me the desire to learn and excel. I thank him and wish he was here to enjoy this achievement with me.

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ABSTRACT

STUDIES ON BACTERIAL LEAF SCORCH
OF AMERICAN ELM AND OTHER TREE SPECIES

(September, 1984)

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Fastidious, xylem-inhabiting bacteria (FXIB) were consistently isolated from leaf scorch-affected elms (Ulmus americana, U. pumila and U. glabra) and red mulberries (Morus rubra) in the Washington, DC, area. Elm leaf scorch (ELS) was distributed north to Baltimore, MD, and Lewes, DE, while mulberry leaf scorch ranged north to New Rochelle, NY. Neither disease was observed in Massachustts or Connecticut. Both diseases were at epiphytotic levels in the Washington, DC, area. Bacteria were cultured using a wood chip technique and modified PW broth medium and were gram-negative, catalase positive, oxidase negative, and ultrastructurally similar to the Pierce's disease (PD) bacterium. The ELS-bacterium was cultured from stems of affected trees throughout the year and was systemically distributed in the xylem of above and below ground portions of a severely affected tree. Both the ELS-bacterium and the MLS-bacterium were serologically related to the PD-bacterium and the ELS-bacterium, but not to any other genera of phytopathogenic bacteria tested. The MLS-bacterium was less fastidious than the ELS-bacterium, as demonstrated by its culture on nutrient agar.

Mulberry seedlings inoculated with the MLS-bacterium developed leaf scorch symptoms after six months, and the MLS-bacterium was recovered from all symptomatic seedlings. Koch's postulates were not fulfilled with the ELS-bacterium. However, the bacterium was graft-transmissible from ELS-affected to symptomless trees, was recovered from symptomatic trees, and ELS-symptoms were suppressed in symptomatic trees injected with oxytetracycline.

ELS caused a significant reduction (P=0.05) of terminal elongation, but had no affect on leaf size. Stem hydraulic conductivity and stem water potential in ELS-affected elms were significantly different (P=0.05) from stems of symptomless elms after the onset of symptoms. No significant difference in hydraulic conductivity occurred prior to symptom development. ELS-affected trees had fewer functional stem vessels (P=0.05) due to occlusion than did symptomless trees. November mean stem starch content was significantly lower (P=0.05) in ELS-affected trees than in symptomless trees.

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CHAPTER I

INTRODUCTION

American elm (*Ulmus americana* L.) is extensively planted as a street and landscape tree in the metropolitan Washington, DC, area. While American elm populations have been reduced drastically in the United States and Canada by Dutch elm disease (DED)(Sinclair and Campana, 1978), a large population has been maintained in Washington, DC by the National Park Service and the District of Columbia, Department of Transportation. Because these plantings are valuable in the design of parks, memorials, and city streets, extensive sanitation and therapeutic treatments have been applied for the control of the DED pathogen [*Ceratocystis ulmi* (Buisman) C. Moreau] and its insect vector, the European elm bark beetle (*Scolytus multistriatus* Marsham).

American elm is frequently affected by a potentially serious leaf scorch condition characterized by a marginal necrosis bordered by a chlorotic halo. Subsequent upward curling is common. The disorder is evident in the Washington, DC area by mid-summer; symptoms become more noticeable as the summer progresses. Symptoms may appear on a single branch or throughout a tree. Limbs with scorched leaves one year produce apparently healthy leaves the following spring which scorch by mid-July (Hearon *et al.*, 1980). Symptoms first appear on older leaves at the base of the stem then progress distally to younger leaves through the summer. Necrotic leaves remain attached or prematurely abscise with tufts of green, symptomless leaves at branch apices.

After the first reported observation of elm leaf scorch in the late 1940's severity and incidence increased over the next decade (Wester and Jyllka, 1963). Of additional concern was the discovery that leaf scorch-affected elms appeared to be more susceptible to beetle breeding attack than non-affected trees (Wester and Jyllka, 1963). In a five year study, 14.8% of scorch-affected elms were infected with C. ulmi versus 1.2% of the remainder of the elm population. Leaf scorch-affected trees were more heavily infested with S. multistriatus than non-scorched trees. Wester (unpublished data) in a 1958 survey of seven southern states (West Virginia, Virginia, Tennessee, Georgia, Alabama, South Carolina, and North Carolina) found elm leaf scorch widely distributed. Scorch was prominent in American elm but not observed in other elm species.

Similar leaf scorch symptoms occur in sycamore (Wester, 1968; Hearon et al., 1980, Sherald et al., 1983) and red oaks (Hearon et al., 1980; Chang and Walker, 1983). In sycamore, marginal, olive drab lesions appear along the leaf margin in mid-summer. Lesions expand into large, undulating, necrotic zones often covering much of the leaf surface. The chlorotic halo is less conspicuous than in elm. Symptoms first appear in older leaves, then progress to younger leaves. Necrotic tissues become brittle and readily crumble when leaves are folded. By late summer, the entire canopy of severely affected trees is brown and leaf abscission has begun. Branch dieback is followed by extensive development of water sprouts, stag-horning, and tree decline and in some cases tree death. Leaf scorch symptoms in sycamore have been attributed to early fall coloration or a late summer form of anthracnose (Cooper

et al., 1977). Symptoms of sycamore leaf scorch (SLS) are more severe in Texas and Louisana than in the Washington, DC area (Sherald et al., 1983).

Foliar symptoms in red oaks consist of an undulating marginal necrosis with a chlorotic halo (Hearon et al., 1980). A dark brown band can be found between the chlorotic halo and necrotic tissues (Hearon et al., 1980; Chang and Walker, 1983). Premature defoliation, branch dieback, and development of water sprouts commonly occurs in trees with leaf scorch. Symptoms progress through the canopy and tree death may occur after several seasons. Foliar symptoms are similar to the early symptoms of oak wilt. However, oak leaf scorch (OLS) symptoms develop later in the season (mid-summer) than oak wilt symptoms (spring), there is no vascular dis-coloration, and the oak wilt fungus is absent from scorch-affected trees (S. J. Kostka and J. L. Sherald, unpublished).

Because of the significance of trees in the urban environment, the etiology and control of any disease or disorder that may reduce their vitality or longevity must be understood. Bacterial leaf scorch is such a debilitating factor. Affected trees are less vigorous, may have increased branch dieback and are more susceptible to attack by other pathogens (Wester and Jyllka, 1959; Hearon et al., 1980; Sherald et al., 1983). A potential decrease in the aesthetic nature of the urban community or forest ecosystem and the debilitation of existing trees necessitates an understanding of bacterial leaf scorch. The objectives of this research are to develop a better understanding of the etiology, epidemiology, and abnormal physiology of bacterial leaf scorch diseases.

C H A P T E R II

LITERATURE REVIEW

Leaf Scorch Inducing Agents

The wide range of agents that can induce leaf scorch symptoms can be grouped into two categories: abiotic and biotic. Abiotic agents may be water stress, soil compaction, limited rooting space, mineral extremes, or toxic materials applied to foliage. Biotic leaf scorch-inducing agents are insects and pathogenic microorganisms. These agents may act singularly or in combination to cause the decline and death of leaf scorch-affected trees (Wester and Jyllka, 1959; Parker, 1965; Halliwell, 1966; Stipes and Davis, 1972; Filer et al., 1975; Lewis and Van Arsdel, 1975; Lewis, 1976; Lewis and Van Arsdel, 1978; Tattar, 1978).

Water Stress

Water stress can result from an inadequate supply of water to the roots (soil drought), interference in water transport, or excessive loss of water from the leaves (atmospheric drought). These aspects of drought are not entirely separable since leaf scorch may result from their combined action (Henckel, 1964; Linzon et al., 1972; Tattar, 1978).

Soil drought occurs when the available water in the soil is significantly below the field capacity and approaches the permanent wilting percentage (Henckel, 1964). Soil dryness does not necessarily refer to

an absolute or extreme dryness of the soil but to the condition of dryness in relation to its normal condition. Trees growing under normally wet conditions will suffer more in a drought (available water decreases for one to several years) than trees growing normally under conditions where water is more limited. Trees growing under wet or high moisture conditions, for example, near bodies of water or in regions with high water tables usually have a shallower, more reduced root system than trees growing under conditions substantially below field capacity which develop a wider, deeper reaching root system. For this reason, a reduction in soil water can severely affect trees growing under normally ample water conditions. The reduction of available water and drop in the water table cause root injury and death. This decrease in the functional absorptive structures causes xylem embolism and induction of water stress (Hepting, 1971; Van Arsdel, 1972).

Atmospheric drought commonly occurs on days with high temperatures and dry winds or on bright, windy days after and extended cloudy, rainy period (Henckel, 1964; Linzon et al., 1972; Tattar, 1978). Rapid transpiration of water from the leaves induces a water deficiency because water is removed from the plant more rapidly than the roots can supply it. This rapid water loss often causes foliar cell damage and collapse; first at the leaf margin then progressively inward to the midrib (Henckel, 1964). Numerous examples of these interactions exist. Leaves of young beech (Fagus grandifolia Ehrh.) in southern Ontario scorched under conditions of bright sunshine and high winds following a wet, cloudy period (Linzon et al., 1972). American elm, dogwood (Cornus sp.) and chestnut oak (Quercus muehlenbergii Engelm.)

scorched and prematurely defoliated under drought conditions (Tattar, 1978). Symptoms are similar in soil drought-affected trees.

Adverse climatic conditions combined with certain planting practices enhance development of leaf scorch symptoms. Trees between roadways and sidewalks, in raised or subsurface planters, or in areas with heavy pedestrian or vehicular traffic, often have reduced amounts of available soil water, restricted root space, and limited root penetration and water movement due to decreased soil pore space and increased bulk density (compaction) (Patterson and Mader, 1982; Ruark et al., 1982). The interaction of these factors often induce water stress in trees on such sites.

Stipes and Davis (1972) suggested that the decline of shade trees in Northern Virginia may be attributed to the rapid urbanization of forest land with the concommitant creation of less than optimal conditions for tree growth and survival. Maples (Acer sp.) grown as street trees scorch severely under water stress conditions (Holmes et al., 1966; Linzon et al., 1972; Walterscheidt, 1978).

Mineral Excesses and Deficiencies

Sodium chloride (NaCl) and calcium chloride ($CaCl_2$) are applied to roadways to prevent ice accumulation. Subsequently, these salts are washed as run-off from the roadway into the adjacent soils. Maples planted as street trees in the northern United States and Canada are particularly affected by deicing salt runoff and accumulation in the soil (Parker, 1965; Tattar, 1978; Walterscheidt, 1978). Salt accumulation in the soil may inhibit water movement into the roots or may be absorbed

and accumulate in the plant, particularly in the foliage (Parker, 1965). Although the concentration of salts is highest in the leaves and is decreased at leaf-fall, the tree continues to accumulate salt in the trunk, branches, and twigs after annual winter deicing salt applications (Henckel, 1964; Parker, 1965). Once salt concentrations reach a threshold level, usually over a period of several years, the tree is killed. Similar foliar injury and even tree death can occur due to excessive salt accumulations that occur when trees are over fertilized (Van Arsdel and Bush, 1970; Van Arsdel, 1972).

Mineral deficiencies can also produce leaf scorch symptoms (Kenaga, 1974; Tattar, 1978). In pecan [Carya illinoensis (Wang) K. Koch] fruiting was correlated with leaf scorch and premature defoliation (Sparks, 1977) It was suggested that fruiting suppressed nitrogen, phosphorus and zinc accumulation in the leaves which resulted in a net loss of potassium. Sparks (1977) proposed that potassium deficiency was responsible for the observed leaf scorch symptoms.

In red and silver maples (A. rubrum L. and A. saccarinum L.) grown in alkaline soils, the lack of manganese induced chlorosis and scorch-like symptoms (Kielbaso and Ottman, 1976; Smith and Mitchell, 1977). In citrus a zinc deficiency commonly produced a mottled chlorosis of the leaves (Anderson and Calvert, 1970). As the season progressed, leaves became extensively necrotic (Cohen, 1968; Kenaga, 1974). Chiu and Bould (1977) observed a marginal leaf scorch in apple (Malus sp.) deficient in calcium. When magnesium also became limiting, leaf scorch was acute.

Insects

Osburn et al. (1966) reported the association of "scorch-like" symptoms with Eriophid mite feeding on pecan [Carya illinoensis (Wang) K. Koch]. Leaf scorch symptoms produced by mite feeding differ from those caused by other agents. Mite-induced symptoms followed the mite feeding pattern while symptoms induced by other agents commonly originated at the leaf margin then moved inward. Mites began feeding near the midrib then advanced to the leaf margin, with scorch symptoms moving from the midrib to the margin as blotchy, necrotic patches.

Pathogenic Microorganisms

Microorganisms (fungi and bacteria) have been reported to cause leaf scorch either singularly or as members of a disease complex. Carter (1945), suggested that a single organism (Erwinia nimipressuralis = Enterobacter cloacae) caused wetwood of elm of which leaf scorch was an associated symptom. Carter (1945) first reported the disorder and suggested that Erwinia nimipressuralis Carter [Enterobacter cloacae (Jordan Horm and Edw.] was the causal agent. Affected species included U. americana, U. fulva Michx., U. procera Salisb., and U. pumila L. Wetwood was characterized by a persistent or intermittent flowing or oozing of fluids through cracks or injuries of the bark or wood. Affected xylem was water-soaked with brown bands or streaks of wetwood. Fluids exuding from the fissures appeared as a moistening of the bark or as a copious flow down the trunk. Cut branches exuded fluids and gaseous fermentation products.

Exuded fluids were under pressure and toxic to bark tissues, foliage, and turf (Carter, 1945). Callus formation around wounds was inhibited or retarded and the cambium at the base of the wound was killed. Young shoots directly below the fluxing site were observed to wilt. Carter (1945) described the leaf damage accompanying wetwood as a leaf browning with interveinal and marginal leaf necrosis. Symptoms in greenhouse grown seedlings inoculated with exuded fluids were similar to "midsummer scorch" symptoms of marginal curling, wilting, and defoliation.

Other workers have suggested that leaf scorch is a symptom of decline/dieback diseases which are of complex etiology involving one or more pathogens and influenced by insect injury as well as environmental and edaphic factors (Thompson, 1951; Halliwell, 1966; Van Arsdel and Halliwell, 1970; Stipes and Davis, 1972; Van Arsdel, 1972; Filer et al., 1975; Lewis and Van Arsdel, 1975; Ricketts, 1975; Lewis, 1976; Lewis and Van Arsdel, 1978). These diseases include declines of red oak, live oak, sycamore, and maple.

Decline of live oak (Quercus virginiana Mill.), a disease of complex etiology, was first reported near Austin, Texas, in 1934 (Taubenhaus, 1934). Symptoms were the production of small leaves in the spring, tan discoloration of the foliage (scorch) in hot weather, premature defoliation, twig dieback, resprouting from larger branches, and eventual death of the tree. Brown vascular streaking and cankers were often found in affected trees. Dothiorella sp. [D. quercina (Cke. and Ell.) Sacc.] was consistently isolated from cankers and was shown to be pathogenic. The disease also occurs on post oak (Q. stellata Wang.) and Spanish or scarlet oak (Q. coccinea Muench.)(Van Arsdel,

1972). Taubenhaus (1934) reported that trees were not water stressed and isolated an unidentified causal organism of live oak decline. Halliwell (1966) first believed the causal agent to be a virus but found that it was not graft transmissible. Cephalosporium sp. was ultimately isolated from affected trees and Koch's postulates satisfied (Halliwell, 1966). Van Arsdel and Halliwell (1970) subsequently associated Dothiorella, Ceratocystis, and Hypoxylon with the disease. Van Arsdel (1972) reported that Cephalosporium diospyri Crandell was the cause of the scorch symptoms and that Dothiorella and Ceratocystis are different stages of the same organism. Recent research (Lewis and Oliveria, 1979; Appel, 1983) reported that the disease was caused by the oak wilt fungus [Ceratocystis fagacearum (Bretz) Hunt].

Decline of red oaks initially appeared in the mid-1950's in New Jersey, southern New York, Pennsylvania, and West Virginia on scarlet oak (Q. coccinea Muench.), red oak (Q. rubra L.), and black oak (Q. velutina L.)(Fergus and Ibberson, 1956; Gillaspie, 1956). The disorder is now known to extend south to Florida and west to Arkansas and Texas (Staley, 1965; Skelly, 1974; Lewis, 1981; Tainter and Benson, 1983). Affected species include: water oak (Q. nigra L.) and willow oak (Q. phellos L.) in Arkansas and Mississippi, southern red oak (Q. falcata Michx.) in Texas, and laurel oak (Q. laurifolia Michx.) in Florida (Lewis, 1981).

An initial symptom of oak decline is foliar leaf scorch with subsequent branch dieback, crown deterioration, and tree death after several years. The disease was attributed to a number of abiotic and biotic stresses including insect attack, various root rotting fungi,

late spring frost, poor soils, and drought (Tyron and True, 1958; Skelly, 1974; Lewis, 1981; Tainter and Benson, 1983).

Viggars and Tarjan (1949) described a disease of pin oak in Delaware that they believed was due to root attack by nematodes, mainly Hoplolaimus coronatus. Symptoms were "hypersenstivity" to drought; chlorosis of foliage on individual limbs; marginal leaf necrosis, spreading inward; followed by premature leaf abscission and formation of water sprouts. In the fall, the affected foliage was a dull tan instead of reddish brown.

Sycamore decline, another disease of complex etiology, was first reported in Georgia in 1951 (Thompson, 1951). Leaf scorch was later described as the first symptom observed in affected trees (Ricketts, 1975; Cooper et al., 1977; Filer et al., 1977). Filer et al. (1975) found leaf scorch incidence ranging from 22 to 88 per cent in twenty-six sycamore plantations surveyed. Plantations were selected from throughout the geographic range where the disease was known to occur (Louisiana, Mississippi, Alabama, Tennessee). In each plantation 100 to 250 trees were selected randomly and examined in the fall for scorch symptoms. The disease ranged from east Texas (Lewis, 1976) to southwestern Illinois and southeastern Missouri (Ricketts, 1975). The causal agent of the leaf scorch portion of the disease was reported to be Cephalosporium diospyri, while Botryodiplodia theobromae Pat., which was consistently isolated from branch cankers, was reported to be responsible for cankers and rapid crown dieback (Lewis, 1976; Lewis and Van Arsdel, 1975; Lewis and Van Arsdel, 1978). Sycamore decline/dieback was reported to be enhanced by high temperature, water

stress, and Cephalosporium wilt ("scorch-like" symptoms). The widespread occurence of this disease in the southeastern United States is one of the limiting factors in the culture of sycamore in the region (Ross, 1971; Filer et al., 1975; Ricketts, 1975; Lewis, 1976; Filer et al., 1977). London plane tree (Platanus acerifolia Willd.) was also found susceptible (Ricketts, 1975).

Leaf Scorch Associated with Fastidious, Xylem-inhabiting Bacteria

Early investigations by Wester and Jyllka (1959) demonstrated that the causal agent of ELS was graft-transmissible using bud chip, scion, and root grafts. Symptoms in grafted trees were analagous to those in naturally affected elms. Plants with bark patch grafts did not develop symptoms. Based on symptomatology and the xylem-limited nature of the causal agent, they postulated that the agent was a "virus" similar to the "virus" causing Pierce's disease (PD) of grapevines (Vitis vinifera L.).

PD is analagous to vascular wilt diseases in symptom expression. Symptoms include a decline in vigor, marginal leaf necrosis (leaf scorch), decreased fruit size and yield, and death of the plant (Hewitt et al., 1942). The pathogen was transmitted by the leafhopper(s) Hordnia circellata in California and Homalodisca coagulata and Oncometopia nigricans in Florida (Hopkins, 1977). The disease, endemic to the Southeast and of epiphytotic proportions in southern California, is the limiting factor in grape cultivation in those regions (Hopkins, 1977).

Prior to 1971, the PD agent was believed to be a virus. In that year Hopkins and Mortensen (1971) found that symptoms could be suppressed using soil drenches of tetracycline antibiotics. Because a remission of symptoms was obtained using these materials, the prokaryotic nature of the causal agent was suggested. Goheen et al. (1973) and Hopkins and Mollenhauer (1973) subsequently observed a pleomorphic bacterium in the xylem of petioles, midveins, and small veins of grape leaves infected with PD. Bacteria were either sparsely distributed or formed dense matricies in the xylem. No bacteria were observed in transverse sections of tissue samples from healthy leaves (Goheen et al., 1973).

The bacterium was described as rod-shaped, 0.4-0.5 μm in width by 1-3 μm in length, with a defined wall-membrane complex. The wall was rippled, thin and trilaminar. Mollenhauer and Hopkins (1974) occasionally observed a four-layered wall in the PD-bacterium. Some forms had thicker, less defined walls (Lowe et al., 1976). Fimbriae were often associated with the surface of the bacteria (Hopkins, 1977). Ribosomes were most abundant at the periphery of the bacterial cells. Changes in wall structure of the organism characterized, by the multilayered wall structure becoming less obvious and the cells compressed, have been attributed to aging (Mollenhauer and Hopkins, 1974) or suggested to be resting or degenerative stages (Hopkins, 1977).

Similar fastidious, xylem-inhabiting bacteria (FXIB) were also associated with:

Alfalfa Dwarf (AD)	Goheen et al., 1973
Phony Peach Disease (PPD)	Hopkins et al., 1973 Nyland et al., 1973

Plum Leaf Scald (PLS)	Kitajima et al., 1975 French and Kitajima, 1978
Almond Leaf Scorch (ALS)	Lowe et al., 1976 Mircetich et al., 1976
Citrus Young Tree Decline (CYTD) (Blight)	Feldman et al, 1977 Hopkins et al., 1978
Periwinkle Wilt (PW)	McCoy et al., 1978

Of these diseases only two, PPD and AD do not express leaf scorch symptoms. The PD, PPD, ALS, and PW organisms were shown to be transmitted by leafhoppers (Turner and Pollard, 1959; Hopkins and Mortensen, 1971; Feldman et al., 1977; Hopkins, 1977; McCoy et al., 1978). Cross transmission using leafhoppers has been confirmed between PD-infected grape and alfalfa and PD-infected grape and almond. In addition, the PD-bacterium was transmitted from blight-affected citrus to healthy grape, which subsequently developed typical PD symptoms (Hopkins et al., 1978).

The rippled wall, intracellular (intra-xylem) nature of these bacteria, and their arthropod vectors led to their original designation as rickettsia-like bacteria. These early descriptions of the PD-bacterium suggested that the bacterium was "rickettsia-like" and led to the inclusion of hemin chloride and bovine serum albumin in culture media (PD-2 medium)(Davis et al., 1978c). Both hemin chloride and bovine serum albumin were components of the culture medium for Rochalimaea quintana, a member of the Rickettsiaceae (Myers et al., 1969). The role of hemin chloride is believed to be inhibition of peroxides during initial stages of bacterial growth (Davis et al., 1978c). Bovine serum albumin serves as a detoxicant, since activated charcoal or

soluble starch can be used as substitutes in the medium (Davis et al., 1981b).

Both the SC medium and the S-8 medium developed for the coryneform bacterium causing ratoon stunting disease of sugarcane (Davis et al., 1980a) and the PW medium developed for the PW, PLS, and PPD bacteria (Davis et al., 1981a) are modifications of the PD-2 medium. For SC/S-8 media, tryptone was eliminated and glucose and L-cysteine HCl H_2O were substituted for citrate and succinate (Davis et al., 1980a). PW medium, besides some proportional changes in constituents, substitutes glutamine for citrate and succinate (Davis et al., 1981a). Wells et al. (1981) developed BCYE medium, a modification of the medium developed for Legionella sp., containing yeast extract, activated charcoal, L-cysteine HCl·H_2O, ferric pyrophosphate, and ACES buffer for the cultivation of the PPD and PLS-bacteria.

Serological analyses using indirect immunofluorescent antibody staining and/or Ouchterlony double gel diffusion demonstrated serological relatedness among FXIB (Table 1) (French et al., 1977b; Davis et al., 1978a, 1978c, 1978d; Lee et al., 1978; McCoy et al., 1978). No serological relatedness has been observed to any other genera of bacteria. Recently, bacteria serologically related to the PPD-bacterium were found in Johnsongrass (Sorgham halapense), ragweed, and numerous Prunus sp. adjacent to or in PPD-affected orchards (Weaver et al., 1980; Wells et al., 1981; Timmer et al., 1983). Similarly, bacteria serologically related to the PD-bacterium were cultured from weeds and woody plants adjacent to PD-infected vineyards (Adlerz and Hopkins, 1981; Raju et al., 1980). Indicator

TABLE 1. Serological relatedness between several fastidious, xylem-inhabiting bacteria

Bacterium	Antisera prepared against: PD-bacterium	ALS-bacterium	PPD-bacterium
PD	+	+	+
ALS	+	+	+
AD	+	+	+
CYTD	+		+
PPD	+	+	+
PLS			+
PW			+

grapevines developed typical PD symptoms after inoculation and the bacterium was readily recovered from infected grapes (Raju et al., 1980). Besides extending the host range of FXIB, these weeds may serve as inoculum reservoirs in or adjacent to plantings of highly susceptible, agriculturally important hosts. These studies support the early host range experiments of Frietag (1951) who demonstrated that the PD agent was leafhopper transmitted from infected grape to alfalfa. He found that the PD agent, at that time believed to be a virus, could be transmitted to 75 species of plants representing a total of 23 plant families. Many of the plants tested were plants commonly found in PD-affected vineyards.

Bacteria similar to FXLB were observed in xylem elements of midveins of leaf scorch-affected American elm, sycamore, and red oak (Hearon et al., 1980). Slight variations in size and morphology were found among the three bacteria. In elm, the bacteria were rod-shaped, 0.3-0.4 µm wide by 0.9-2.4 µm long, with a rippled wall structure which in some cells was composed of four layers. Internal structure consisted of ribosomes, a nuclear region with DNA-like strands, and round osmiophilic bodies. Fimbriae were occasionally observed. Small spindle-shaped bodies with dense cytoplasmic contents and cell walls occurred along xylem element walls or completely occluded the lumen of the vessel. Tubular structures were located along bacterial walls and throughout the matrix surrounding the cells.

In sycamore, the bacteria were pointed at one end in comparison to elm where both ends of the cell were rounded (Hearon et al., 1980; Sherald et al., 1983). Bacteria from sycamore measured 0.3-0.4 µm

by 1.0-1.8 μm. Tubular structures appeared in the matrix surrounding cells and fimbriae were common. In oak, bacteria were 0.3-0.4 μm by 1.0-2.0 μm with some cells appearing to be tapered at one end. Small dense bodies were observed in both sycamore and oak.

In all three species, bacteria were most commonly found in smaller tracheary elements of the collateral vascular bundles and associated with matricies or vascular occlusions analagous to those observed in PD-affected grapevines (Hopkins, 1979). No bacteria were observed in leaves from unaffected trees.

Using a modification of French's vacuum infiltration technique (French et al., 1977a; Hearon et al., 1980) and phase contrast microscopy, bacteria serologically related to the PD-bacterium were extracted from the vascular cylinder of leaf scorch-affected elm, sycamore, and red oak (Hearon et al., 1980; Sherald et al., 1983). Bacteria were not extracted from the majority of symptomless trees.

Bacteria associated with elm and sycamore leaf scorch were isolated in a S-8 broth medium (developed for the RSD bacterium) and subcultured on semi-solid PD-4 medium (Kostka, et al., 1981, Sherald et al., 1983). Although the ELS- and SLS-bacteria could be isolated on artificial media developed for the ratoon stunting disease (RSD) bacterium, incubation periods were excessive (circa 21 days)(Kostka et al., 1981; Sherald et al., 1983). The isolation of the SLS-bacterium using a modified PW broth medium (Sherald et al., 1983) enabled the fulfillment of Koch's postulates in inoculated sycamore seedlings and determination of its presence in Louisiana and Texas. Sherald et al. (1983) suggested that SLS may be a component of sycamore decline

in the southern United States. Early pathogenicity tests with the ELS-bacterium were unsuccessful (S. Kostka, unpublished data)

Recently, the oak leaf scorch-associated (OLS) bacterium was isolated through wood chip incubation in modified PW broth (Kostka et al., 1984) or by placing bacteria extracted from crushed stem segments on a newly developed, though unpublished culture medium (Chang and Walker, 1983).

Xylem Dysfunction in Diseases Caused by Fastidious, Xylem-inhabiting Bacteria

In Pierce's disease, similar occlusions caused by gums and tyloses were suggested to disrupt water transport and induce leaf scorch symptoms (Esau, 1948; Mollenhauer and Hopkins, 1974; Hopkins, 1977). Similar suggestions have been made for ELS, SLS, and OLS (Hearon et al., 1980). Esau (1948) found tyloses in uninfected older wood of grapevines, but not in uninfected younger wood. On the other hand, gums and tyloses were commonly found in younger PD-affected wood. Mircetich et al. (1976) observed the fastidious xylem-inhabiting bacterium (FXIB) associated with ALS in 15% of xylem elements in scorched almond leaves sampled. They did not believe that this proportion of vascular occlusions could sufficiently impede water movement to induce ALS symptoms. Hopkins (1977) questioned those findings because too few sections were examined. When considering the entire vascular system of a leaf, the 1-2 mm examined in the study by Mircetich et al. (1976) represented an inconsequential sample (Hopkins, 1977). Hopkins (1977)

suggested, instead, that serial sections of the entire midrib of a leaf may yield a more accurate picture of the extent of vascular occlusion in an affected leaf.

Hopkins (1981) found that in serial sections of 0.5 cm lengths of PD infected grape petioles and leaf veins, vessel plugging was 4-12 times greater than would be approximated from individual cross-sectional analysis. In leaves with marginal necrosis, vascular occlusion approached 80 per cent in 0.5 cm leaf mid-vein samples. Not only were vessels occluded with gums and gels, but also large masses of bacteria surrounded with a matrix like material occluded pits and vessel ends. He concluded that vascular occlusion was sufficient in mid-veins of PD-infected grape to cause marginal necrosis (Hopkins, 1981).

Lee *et al*. (1982) reported a toxin or toxins to be produced in culture by the Pierce's disease (PD) bacterium that induced scorch symptoms in excised grape and almond leaves. Vacuum infiltration of the toxin into rooted and dormant grape cuttings also produced symptoms and followed the same differential susceptibility among cultivars as demonstrated in pathogenicity experiments. Neither the structure of the toxin(s) nor its (their) mode of action are known.

Effects of Water Deficit on Plant Physiology

Water deficits inhibit various aspects of host growth and physiology (Kramer and Kozlowski, 1979), yield and viability (Durbin, 1978), and predisposition to disease. One of the first responses in plants

under water stress is a reduction in growth, even prior to measurable effects on metabolic systems (Boyer, 1973; Hsiao, 1973).

Plants under water stress, either climatic, edaphic, or pathogen induced, have higher levels of free sugars than non-stressed plants (Parker and Houston, 1971; Hsiao, 1973; Parker and Patton, 1975; Parker, 1979). Hsiao (1973) reported that water stressed plants produced increased levels of alpha-amylase which catalyzes the conversion of starch to sugars. The availability of free sugars may enhance infection by non-aggressive pathogens.

Reductions in stored starch reserves occurs in trees undergoing water stress, defoliation, and decline (Parker and Houston, 1971; Wargo et al., 1972; Wargo, 1976; Parker, 1979; Carroll et al., 1983). The status of starch reserves during the dormant season can provide an indication of tree health.

Xylem occlusion (Talboys, 1968, 1978; Beckman, 1980) or embolism (Zimmerman and McDonough, 1978) inhibit water movement and ultimately influence growth regulator levels and stomatal opening and closing. In vascular diseases, foliar wilting and necrosis are caused by a decrease in water availability rather than excessive water loss (MacHardy and Beckman, 1973; Hall and MacHardy, 1981). Cell integrity can not be maintained due to the inadequate water supply and desiccate. The symptoms associated with vascular disease are acute water stress symptoms characterized by an extensive wilting and necrosis of foliage of the diseased plant. If subacute water stress conditions occur, symptoms are foliar necrosis followed by premature leaf abscission (Talboys,

1968). Bacterial leaf scorch symptoms are analagous to symptoms produced in plants undergoing subacute water stress.

C H A P T E R III

SYMPTOM VARIATION, DISTRIBUTION, AND INCIDENCE OF ELM LEAF SCORCH IN THE NORTHEASTERN UNITED STATES

Introduction

Within the last five years leaf scorch diseases caused by or associated with an as yet un-named group of fastidious, xylem-inhabiting bacteria have been recognized as a threat to forest and shade trees, particularly in the southern half of the United States (Hearon *et al.*, 1980; Sherald *et al.*, 1983; Chang and Walker, 1983). Affected species include American elm (Hearon *et al.*, 1980), sycamore (Sherald *et al.*, 1983), red oak (Hearon *et al.*, 1980) and red mulberry (Kostka *et al.*, 1982, 1983) Pathogenicity of the cultured bacterium has been demonstrated in sycamore (Sherald *et al.*, 1983) and red mulberry (Kostka *et al.*, 1983). All leaf scorch-associated bacteria were serologically related to the Pierce's disease (PD) bacterium, which causes a similar leaf scorch disease of grapes and almond (Hearon *et al.*, 1980; Sherald *et al.*, 1983; Kostka, *et al*, 1983).

Although the pathogenicity of the elm leaf scorch (ELS) bacterium has yet to be demonstrated, the disorder and bacterium are graft-transmissible (Wester and Jyllka, 1959; Hearon *et al.*, 1980).

Symptoms of ELS develop by mid-July in the Washington, DC, area (Wester and Jyllka, 1959; Hearon *et al.*, 1980) and are characterized by an undulating, marginal necrosis bordered by a chlorotic halo. During the summer months symptoms progress acropetally, becoming more pro-

nounced. Necrosis extends inward towards the midrib and leaves curl. Early leaf abscission commonly leaves a tuft of unaffected leaves at the branch apex. Affected trees may exhibit branch dieback (Hearon et al., 1980) and have been reported more susceptible to Ceratocystis ulmi (Buisman) C. Moreau, the Dutch elm disease fungus, than are non-scorching trees (Wester and Jyllka, 1963).

The objectives of this study are: 1) to describe the most frequently occurring symptom types in American elms affected with ELS, 2) to determine the incidence of ELS and of each symptom type in an urban elm population in Washington, DC, 3) to determine the distribution of ELS in the northeastern United States and 4) to describe ELS symptoms in other elm species.

Materials and Methods

Symptom Variation

Symptom development was followed in ELS-affected American elms (Ulmus americana L.) from January 1981 to October 1983. All trees were located in the metropolitan Washington, DC area in park or roadside plantings under the jurisdiction of the National Park Service or the District of Columbia, Department of Transportation.

Disease Incidence

The sample population of American elms selected for disease incidence surveys was the planting of nearly 500 American elms (10-80 cm dbh) on the National Mall in Washington, DC. Surveys were conducted in

late August of 1982 and 1983 when symptoms were well advanced. Symptom type and per cent canopy affected were identified from the ground using field glasses and recorded in 1982. In 1983 only per cent leaf area affected was recorded. Newly transplanted trees were not included in the survey nor were subsequent transplants. The initial survey consisted of 473 trees.

Isolations were made from stem segments of 28 symptomatic trees and 10 symptomless trees in September 1982 by incubating aseptically excised wood chips in 10 ml of a modified PW broth (Sherald et al., 1983) and/or by vacuum extracting bacteria from stem segments and confirming their presence with phase contrast microscopy (French et al., 1977a; Hearon et al., 1980; Sherald et al., 1983). Isolated bacteria were reacted to antisera prepared against PD- and ELS-bacteria (S. Kostka, unpublished) using indirect immunofluorescent antibody staining (Goldman, 1968; French et al., 1978).

Disease Distribution

A survey of urban and rural roadside and park trees was conducted in August 1982 and 1983 along the east coast from the northern Virginia suburbs of Washington, DC, to Massachusetts. Surveyed areas included rural areas as well as the following major urban areas: Washington, DC; Baltimore, MD; Wilmington, DE; Philadelphia, PA; New York, NY; New Haven, CT; and Boston, MA. A local sampling was conducted in the metropolitan New Orleans, LA, area. Affected American elms as well as other symptomatic elm species were noted and symptoms recorded. Symptomatic

trees were sampled and isolations made as described above to confirm the presence of the ELS-bacterium.

Results

Symptom Variation

Three symptom types designated Type I, II, or III could be differentiated in the naturally ELS-affected population. The predominant and symptoms (Type I)(Fig. 1) were those previously described for ELS (Hearon *et al.*, 1980). Symptom Type I developed an undulating marginal necrosis which was separated from the inner green tissues of the leaf by a chlorotic halo. Affected leaves curled adaxially (upward) and scorched leaves remained attached until late summer when they abscised. Branch dieback could be observed in previously affected branches in the spring.

Initial symptoms of ELS appeared by mid-July as a buff, brown-green (olive drab), undulating discoloration along the leaf margin that resembled water-soaking (Fig. 2). A distinct brown band subsequently developed demarcating the healthy from the discolored tissues. By late summer leaves curled adaxially (upward). By August, leaves began to abscise. Dieback of scorch-affected branches was occasionally observed the following spring though no obvious effects on flowering, seed set and development, or leaf expansion were noted.

Early Type II symptoms were analagous to those in Type I trees. The notable differences in trees with advanced Type II symptoms were that the majority of leaves curled abaxially (downward), necrotic tis-

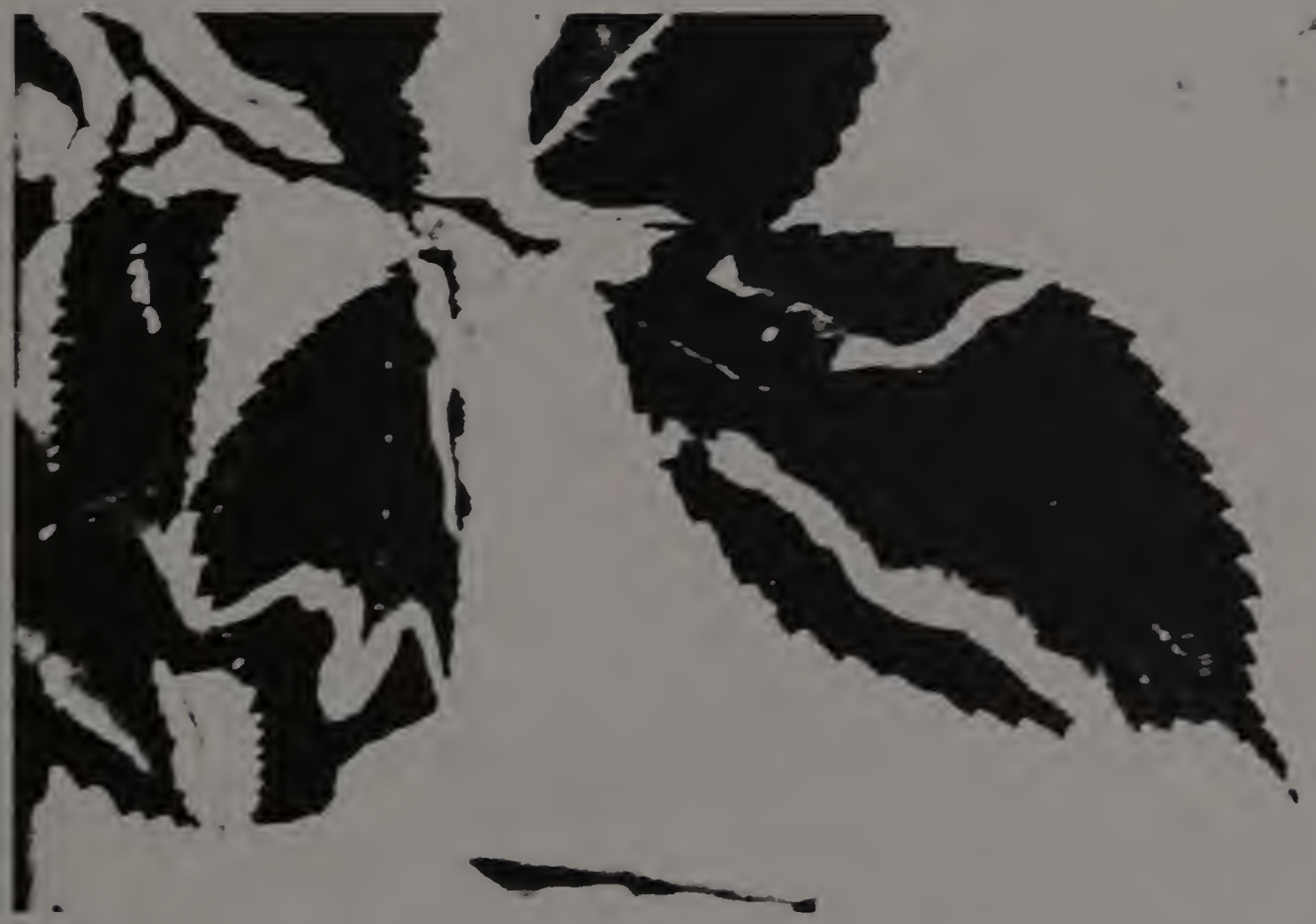

Fig. 1. Elm leaf scorch, symptom Type I. Note the undulating marginal necrosis, chlorotic halo, and adaxial upward leaf curl.

Fig. 2. Early water-soaked appearing (olive drab) lesions of elm leaf scorch. Arrows indicate the demarcation between early lesions and symptomless tissues.

sues were lighter in color, often had an ashen cast, and were bordered by a wide chlorotic halo (Fig. 3).

Type III symptoms were the most severe. In early June, foliage became epinastic. Typical early leaf scorch symptoms appeared by mid- to late June; up to a month prior to the appearence of symptoms in Type I or Type II trees. Symptoms developed rapidly in all Type III trees in comparison to symptom Type I or symptom Type II trees. Several days after symptom onset, necrotic marginal lesions expanded. Within two weeks extensive necrotic areas covered much of the leaf lamina in conjunction with adaxial leaf curl (Fig. 4). The chlorotic halo was inconspicuous or absent. By August, leaf curl was so severe that opposite leaf margins overlapped entirely (Fig. 5). The only chlorophyllous tissue remaining in affected leaves was adjacent to the midrib. Premature leaf abscission occurred and dieback was extensive (Fig. 6). The bark of symptomatic (Type III) branches became orange-gray in color.

Flower bud expansion, seed load, and leaf bud break and expansion did not appear to be affected in trees with Type I or Type II symptoms. However, in trees with Type III symptoms there was a notable absence or depression in the number of flower buds. Flower buds that were present often failed to open or aborted soon after bud break (Fig. 7). Leaf bud break and leaf expansion were suppressed in Type III trees in comparison to symptomless trees or trees with Type I or Type II symptoms (Fig. 7). Leaf bud expansion was also suppressed in symptomatic branches when compared to symptomless branches in the same tree.

Fig. 3. Abaxial (downward) leaf curl, wide chlorotic halo (arrows), and ashen necrotic tissues (N) characteristic of symptom Type II.

Fig. 4. Early Type III symptoms of elm leaf scorch. Single arrows indicate water-soaked appearing lesions; the double arrow indicates tissues that are becoming necrotic. Note the adaxial (upward) leaf curl.

Fig. 5. Advanced Type III symptoms; note the overlapping leaf margins. Chlorophyllous tissue remains limited to the leaf lamina adjacent to the midvein.

Fig. 6. Extensive branch dieback (arrows) that occurs in trees with the Type III symptoms of elm leaf scorch.

Fig. 7. Flower bud abortion and suppressed leaf bud break and expansion in a tree with the Type III symptoms of elm leaf scorch (left), in comparison to a non-affected tree (right).

Incidence

Of 473 American elms surveyed in Washington, DC, in 1982, 196 trees or 41.4% of the population exhibited ELS symptoms. In 1983, the elm population was reduced to 446 trees due to Dutch elm disease and construction activities. Of this remaining population, 181 trees (40.6%) were affected with ELS.

The majority of ELS-affected trees exhibited symptoms in <10% of the total leaf area of the canopy (Table 2). Symptomatic foliage was widely dispersed in the canopy, in a single branch, or even limited to a single shoot. Between 1982 and 1983, disease incidence remained stable, but symptom severity increased in those trees previously affected with ELS: from 28 trees with 10% or more of the canopy leaf area affected in 1982 to 69 trees with 10% or more of the canopy affected in 1983 (Table 2). Seven ELS-affected trees were lost to Dutch elm disease in each survey year.

Symptom Type I predominated in the population in 1982, affecting 106 trees. Type II symptoms appeared in 28 trees and Type III symptoms were at the lowest levels: 17 trees. Forty-one trees had symptoms that were undifferentiable. All trees with undifferentiable symptoms had <10% of the canopy leaf area affected. Symptoms could not be differentiated due to the limited quantity of affected foliage and its location in the upper canopy at the limits of the resolution of the field glasses used.

Fastidious, xylem-limited bacteria serologically related to the PD- and ELS-bacteria were isolated from 11 of 12 symptom Type I trees, 7 of 8 symptom type II trees, and 8 of 8 symptom Type III trees. No fastid-

TABLE 2. Degree of elm leaf scorch symptom development within the American elm population on the National Mall in Washington, DC

Year	Ratio (no. diseased/no. surveyed)	Percent canopy leaf area affected			
		<10	10-20	21-50	51-100
1982	194	152[a]	16	15	11
1983	172	112	27	18	14

[a] Number of trees affected at the indicated level within the surveyed population

ious, xylem-limited bacteria were isolated from the 10 symptomless trees.

Disease Distribution

Elm leaf scorch occurred in the Washington, DC area and ranged north to Baltimore, MD (Fig. 8). The disease was not observed in natural elm populations on the Delmarva penninsula (located in Maryland and Delaware), but did occur in street trees in Lewes, DE. The disease was not encountered in urban or rural elm populations north of Baltimore, MD or Lewes, DE.

Symptoms of ELS were observed in 13 of 16 specimens of the American elm cultivar 'Augustine Ascending'(Fig. 9), as well as 2 of 24 Siberian elms (U. pumila L.)(Fig. 10), and 2 of 3 Wych elms (U. glabra Huds.)(Fig. 11) in Washington, DC. One symptomatic Siberian elm and 5 symptomatic 'Augustine Ascending' elms were observed in New Orleans, LA. Foliar symptoms in these three species were analagous to those observed in American elm. Leaf curl, premature defoliation, and branch dieback were observed in all species. Dieback was most severe in 'Augustine Ascending' elms in New Orleans. Affected trees had nearly 100% of the leaf area affected with ELS in late summer. Further, all trees showed extensive defoliation, branch diebach, and stag-horning. In Washington, the disease was less severe. Only 2 of the 13 ELS-affected 'Augustine Ascending' elms had more than 10% of the canopy leaf area affected (40% and 80% respectively). Due to the small number of symptomatic trees, no attempt was made to classify them according to symptom Type.

Fastidious, xylem-limited bacteria, serologically related to the ELS-

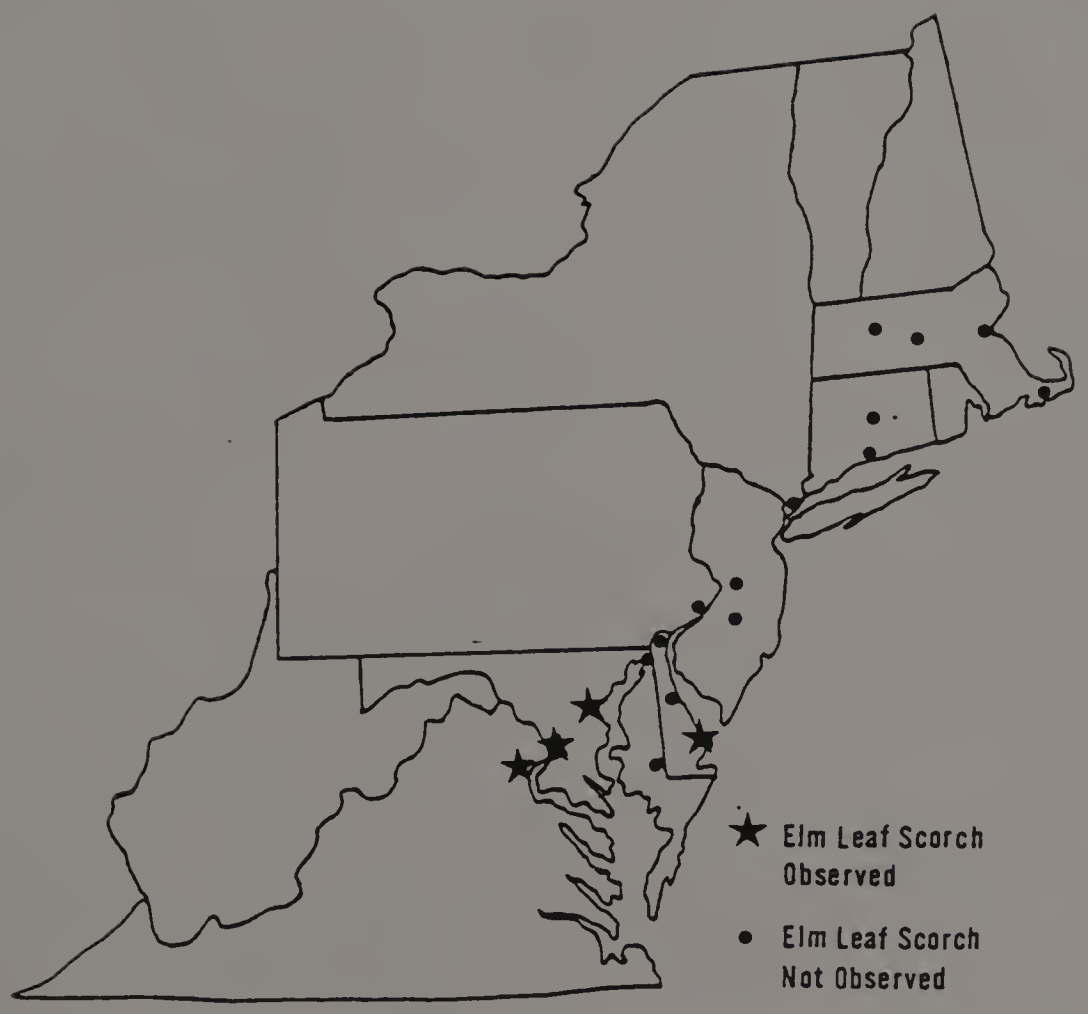

Fig. 8. Distribution of elm leaf scorch in the mid-atlantic and northeast coastal states.

Fig. 9. Foliar symptoms of elm leaf scorch in the American elm cultivar 'Augustine Ascending'.

Fig. 10. Symptoms of elm leaf scorch in Siberian elm. Necrotic tissues are bounded on the inner margin by a dark brown band and a chlorotic halo.

Fig. 11. Early symptoms of elm leaf scorch in Wych elm characterized by prominent water-soaked appearing lesions (arrows) and adaxial leaf curl.

and PD-bacteria were isolated from ELS-affected elms in all areas where the disease was present.

Discussion

Three distinct symptom types occur in American elms affected with bacterial leaf scorch. The observed symptom variation in American elm may be due to differences in the host (anatomical or physiological) or to differences in pathogen virulence. In addition, the observed symptoms may reflect disease progression or variations in bacterial populations within the tree. Similar symptoms, particularly water-soaking and the chlorotic halo, in Pierce's disease-affected grape have been attributed in part to pathogen produced toxins (Lee *et al.*, 1982). However, the role of toxins in ELS symptomatology are not known.

ELS appears to be limited to the southern states with its northern range in Maryland and Delaware. In comparison to mulberry leaf scorch and oak leaf scorch, ELS has a more limited range similar to that of sycamore leaf scorch (S. Kostka, unpublished). This decreased range may be due to climatic effects, differences in host susceptibility, or absence of the, as yet, unidentified insect vector(s). The role of soil water stress is not known, but in certain sites water stress may stimulate the subacute water stress symptoms (Talboys, 1968) of ELS.

The appearence of ELS in exotic elm species indicates that resistance may not be widespread in the genus. However, ELS has not been observed in most European elm cultivars planted in the Washington, DC area. Based upon the occurrence of ELS in Louisiana in 'Augustine Ascending' elm and Siberian elm and sycamore leaf scorch in Louisana and

Texas (Sherald *et al.*, 1983), it is likely that ELS occurs through much of the southern range of the genus *Ulmus*.

C H A P T E R IV

ELM LEAF SCORCH: EVIDENCE SUPPORTING THE ROLE OF A FASTIDIOUS, XYLEM-INHABITING BACTERIUM

Introduction

Leaf scorch is a common disorder in trees growing in impacted urban environments. The disorder has been commonly attributed to drought and/or physiological stresses, such as soil compaction, limited rooting space, and deicing salt toxicity (Tattar, 1978). Wester and Jyllka (1959) observed a mid-summer leaf scorch of American elms in the Washington, DC area with symptoms differing from those commonly attributed to drought stress and in sites not likely to be under severe physiological stress. Although no biotic pathogen was isolated, the graft transmissibility of the disease and the xylem-limited nature of the causal agent was demonstrated. Based on these observations, Wester and Jyllka (1959) postulated that the disease was caused by a "virus" similar to the "virus" that caused Pierce's disease (PD) of grape. Since the early studies of elm leaf scorch (ELS) the etiology of PD has been elucidated and the causal agent identified as a fastidious, xylem-inhabiting bacterium (FXIB)(Davis *et al.*, 1978b). A reinvestigation of ELS (Hearon *et al.*, 1980) confirmed the presence of a graft transmissible FXLB serologically related to the PD-bacterium in xylem tissues of ELS-affected elms.

Artificial media have been developed for the cultivation of the FXIB causing PD and almond leaf scorch (Davis *et al.*, 1978b, 1980b,

1980c), plum leaf scald and phony peach disease (Davis et al., 1981a; Wells et al., 1981, 1983), periwinkle wilt (Davis et al., 1983), and sycamore leaf scorch (Sherald et al., 1983). Limited success has been reported in the isolation of the ELS-bacterium (Kostka et al., 1981) using a culture medium developed for the coryneform bacterium causing ratoon stunting disease of sugarcane (Davis et al., 1980a).

This report describes the consistent isolation and culture of FXIB from ELS-affected trees, as well as, seasonal recovery and systemic distribution of bacteria within an affected tree. Bacteria are characterized and described physiologically, serologically, and morphologically. Pathogenicity and graft-transmissibility studies are reported.

Materials and Methods

Isolation and Culture

Isolations were made from American elms were located in the metropolitan Washington, DC area and primarily on lands under the jurisdiction of the National Capital Region of the National Park Service. Selected trees ranged from 25-80 cm dbh.

Stem sections (0.5-1.0 cm in dia. x 8 cm in length) were vacuum extracted (Fig. 12)(French et al., 1977a) with sterile 0.01 M phosphate-buffered saline (pH 7.0)(PBS) or a sterile phosphate-buffered citrate magnesium solution (PBCM)(Davis et al., 1980c). Approximately 1 cm of bark was removed from the ends of each stem section to facilitate connection of the vascular cylinder to the buffer reservoir (a

Fig. 12. Apparatus for vacuum extracting bacteria from elm stem segments.

pipette). Buffer volume increased proportionally with increased volume of the cylinder. Extracted fluids were examined for the presence of FXIB with phase contrast microscopy (1000X).

Three small branches were pruned from ELS-affected and symptomless trees, leaves were removed, then stem segments (0.5-1.0 cm in dia. x 15 cm in length) were excised. Samples were collected monthly from symptom onset (June/July) to October of 1982 and 1983. Eighty-two ELS-affected and 32 symptomless trees were sampled. ELS-affected trees were sampled only until the ELS-bacterium was isolated. All stem segments collected from ELS-affected trees were from symptomatic branches. Samples from symptomless trees were collected randomly within the canopy. Isolations were made from healthy trees repeatedly throughout the study period. Stem segments were transported to the laboratory in polyethylene bags. All isolations were made within 48 hours of collection.

A wood chip isolation technique was used for the isolation of the ELS-bacterium (Sherald et al., 1983). Stem sections measuring 0.5-1.5 cm in dia. x 15-20 cm in length were rinsed in 70% ethanol and flamed. Xylem chips approximately 0.5 x 2.5 cm in size were aseptically removed from surface disinfested, debarked stem segments and placed in tubes containing 10-25 ml of a broth culture medium and incubated in the dark at 28 C.

Three media were compared initially for isolation of the ELS-bacterium: PD-2 broth, developed for the PD-bacterium (Davis et al., 1980b); S-8 broth, developed for Clavibacter xyli, the causal agent of ratoon stunting disease of sugarcane (Davis et al., 1980a; 1984); and a modified formulation of PW broth, originally developed for the

periwinkle wilt bacterium (PW)(Davis et al.,1981a). PW broth was supplemented with 0.85g $(NH_4)_2HPO_4$, 2g potato starch, 1g L-histidine, and 25 mg cyclohexamide (optional) per liter. After initial media screening, all isolations were made in modified PW broth. Isolates were subcultured on semi-solid modified PW medium (1.2% Bacto-agar) then maintained through biweekly transfer on semi-solid PD-4 (Davis et al., 1980b).

Isolations from stem segments were also made by disinfesting stem segments as described above and squeezing xylem fluids aseptically from stem segments with a hand vice (Davis et al., 1980c). The droplet of xylem fluid was blotted onto the surface of semi-solid modified PW agar.

Petiole and midvein isolations were made using two techniques. Petioles and midveins were trimmed from sampled leaves, then cut into 1-2 cm segments. Segments were surface sterilized in 0.5% sodium hypochlorite for 2 min., followed by 3 rinses in sterile distilled water. Sap was expressed from each segment aseptically with a pliers then blotted onto semi-solid modified PW medium. Each segment was blotted onto the medium at six to eight sites. For the second technique, 5-8 surface sterilized petiole and midvein sections were placed in a sterile microcentrifuge tube (1.5 ml) with 0.5 ml of sterile PBCM and centrifuged for 2 min. at 15,600 x G in an Eppendorf Microfuge, Model 5412. Bacteria were resuspended by vortexing and aliquots were spot inoculated on the semi-solid modified PW medium.

Characterization

Isolates were gram stained and tested for catalase and oxidase reactions. Ability to grow on common microbiological media was tested by streaking pure cultures of the ELS-bacterium on potato dextrose agar, King's medium B agar, and nutrient agar. All cultures were incubated in the dark at 28 C.

Electron Microscopy

Ten to fourteen day old colonies of the ELS-bacterium grown on semi-solid PD-2 medium (Davis *et al.*, 1980b) were overlaid with 0.8% agarose. Sandwiched colonies were removed from the plates, fixed in glutaraldehyde, sectioned, stained, and examined by transmission electron microscopy as described by Sherald *et al.* (1983). Negative stains were made of preparations of the culture bacterium, as previously described (Sherald *et al.*, 1983).

Serology

Four New Zealand white rabbits weighing approximately 2 kg each were injected in pairs with either an isolate of the ELS-bacterium or the PD-bacterium (isolate VT1, provided by M. J. Davis, IFAS, University of Florida, Ft. Lauderdale, FL). Bacteria were grown in PD-2 broth (Davis *et al.*,1980b) for 10 days at 28 C, harvested by centrifugation (15,000 rpm for 15 min.) and fixed for 24 hours in 2% formalin. Fixed bacteria were pelleted by centrifugation, as above, washed 3 times with sterile PBS, and standardized at 1×10^9 cells per ml in PBS using a bacterial counting chamber (Hausser Scientific, Blue Ball, PA). Pre-

immune serum was collected via cardiac puncture prior to immunization. Each rabbit was intravenously injected with increasing dosages of 0.25, 0.5, 1.0, 2.0, and 3.0 ml of antigen over a five day period. Rabbits were bled 1 week after the final injection. Gamma globulins were purified by ammonium sulfate precipitation.

Serological relationships between ELS-isolates and the PD-bacterium were studied using microagglutination, indirect immunofluorescent antibody staining (IFAS), and agar gel double diffusion with antisera to an isolate of the ELS-bacterium and the PD-bacterium. Bacteria were prepared for microagglutination by centrifuging (15,000 rpm for 15 min.) 10 day old PD-2 broth cultures and reserving the pelletized cells. Cells were washed 3 times with PBS and standardized as above to 1×10^8 cells per ml. Microagglutination tests were performed for homologous and heterologous systems as described by Delatt (1976).

Indirect IFAS was performed as described by Hearon et al. (1980). Block titrations of standardized bacterial concentrations (1×10^8 cells per ml; either ELS- or PD-bacterium) were performed with homologous and heterologous antisera to determine the optimum rabbit gamma globulin concentration. Fluorescein-conjugated goat anti-rabbit globulin was used at a 1:20 dilution in PBS. Serological relatedness was determined both for vacuum extracted bacteria (Hearon et al., 1980) and for cultures (Sherald et al., 1983) of the PD-bacterium and isolates of the ELS-bacterium. Other bacteria tested included:

Erwinia caratovora pv. carotovora

Pseudomonas syringae pv. syringae

Clavibacter michiganense pv. sepedonicum

Clavibacter xyli subsp. xyli

Agrobacterium radiobacter pv. tumefaciens

Enterobacter cloacae

Controls were treated with normal (pre-immune) serum or antiserum to the PD-bacterium provided by M. J. Davis. Indirect IFAS was used for screening and identifying potential FXIB isolates.

Antigens for gel double diffusion were prepared by sonicating the respective bacterial isolates (100 mg/ml in sterile PBS) for 10 min. at maximum output on a Sonifier Cell Disruptor, Model W105C (Heat Systems Co., Melville, NY) equipped with a microtip. Cells were sonified for 5, 2 min., intervals in glass tubes containing glass beads (42 µm in dia.) to 1/3 the volume of the bacterial suspension. Tubes were immersed in a crushed ice/methanol bath during sonification. The ice-methanol bath was replaced after each 2 min. sonification treatment. Sonified preparations were heat-treated for 30 min. at 100 C. Glass beads and insoluble cell wall fragments were removed from the solubilized antigen preparation by centrifugation (15,000 rpm for 15 min.) Antigen preparations were stored frozen at -20 C.

Agarose gels (0.5% and 0.8%) prepared in PBS, 0.01M Tris (pH 7.4) and distilled water were compared. Antigen was added at 0, 8, and 24 hrs. prior to the undiluted homologous or heterologous antisera. Plates were incubated at room temperature in a moist chamber.

Systemic Distribution

Isolations were made throughout the canopy of an American elm (31 cm dbh) severely affected with ELS (>90% of the canopy leaf area affect-

ed in August 1983). Stem segments were collected from 11 locations within the canopy and isolations were made by incubating aseptically excised wood chips in modified PW broth. Bole isolations (1 m above the soil line) were made in September 1983 by surface disinfesting the sampling site with 70% ethanol, then excising wood-bark wedges (approximately 2 cm^2 x 1 cm deep) with a surface sterilized drywall hammer and mallet. Samples were removed with sterile forceps, placed in a sterile polyethylene bag, and transported to the laboratory (Ecological Services Laboratory, NPS, Washington, DC) where isolations were made immediately. Wood bark wedges were removed from the polyethylene bags and immersed for 15 sec. in 70% ethanol then flamed. Exposed wood and bark were trimmed aseptically to expose the unsterilized xylem beneath. Wood chips were excised from the xylem, placed in modified PW broth and incubated at 28 C. Tubes were examined at 4 to 7 day intervals for signs of bacterial growth. Root flare/soil line samples were collected and isolations made as described above.

Root samples were collected by exposing roots (approximately 1-1.5 cm in diameter x 30 cm in length), surface disinfesting the exposed roots with 70% ethanol and excising with disinfested pruning shears. Excised roots were transported to the laboratory (ESL, NPS, Washington, DC) in sterile polyethylene bags. Roots were immersed in 0.5% NaOCl for 10 min. then rinsed under running tap water for 30 min. After rinsing, root segments were cut into 15 cm long sections, placed in 70% ethanol for 30 sec., then flamed. Isolations were made aseptically from the vascular cylinder, as previously described (Sherald et al., 1983). One wood chip was placed in each tube of modified PW broth in order to limit

contamination. After isolations were completed, root segments were vacuum infiltrated with sterile PBCM and extracts were examined with phase contrast microscopy (1000X).

Seasonal Recovery

Six trees were selected for this study; 3 symptomless "healthy" trees and 3 ELS-affected trees. Each ELS-affected tree had more than 70% of the canopy leaf area affected with ELS in 1981. Presence or absence of the ELS-bacterium was established previously for each of the 6 trees (Hearon et al., 1980; Kostka et al., 1981).

Three stem segments were collected from each test tree on 11 sampling dates from February 1982 to July 1983. All trees were sampled on each sampling date. Stems that scorched the previous year were sampled in ELS-affected trees. Four wood chip isolations were made from each stem segment in modified PW broth from each of the stem samples for a total of 12 isolations per tree per sampling date. Occasionally an increased number of isolations were made. The ELS bacterium was identified from cultures using phase contrast microscopy and indirect IFAS.

Graft Transmission

Eight field grown American elms approximately 3 years old (ESL Nursery, Alexandria, VA) and free of ELS in 1981 were cleft grafted on March 22, 1982, with scion wood collected from ELS-affected trees representing each of the 3 symptom types. Three control trees were grafted with scion wood collected from a symptomless tree known to be free of the ELS-bacterium (Hearon et al., 1980; Kostka et al., 1981).

All scion wood was collected in January 1982 and stored at 5 C until March. Trees were pruned in March to reduce the number of leads. Scion wood segments approximately 15-25 cm in length with 4-6 buds were cut from collected stems. A tapered "V" was cut at the basal end of the segment and inserted into an incision into the stock branch with the cambium of the stock and scion in contact. The scion was secured to the stock with grafting tape and coated with pruning compound to prevent dessication. Each tree was grafted with 4-9 scions. Pruning shears and knife were surface sterilized with 70% ethanol between cuts. Scions were examined after 1 month, 2 months and 14 months to determine scion success and graft survival, respectively. Trees were monitored through the 1982 and 1983 growing seasons for symptom development. Date of initial symptom appearence and subsequent systemic development were recorded. Isolations were made from stem segments collected from symptomatic scions and systemically infected branches as previously described. In August and September 1983, isolations were made from all remaining viable scions.

Pathogenicity Tests

Five isolates representing each of the 3 ELS-symptom types, an isolate of the mulberry leaf scorch (MLS) bacterium, and an isolate of the PD bacterium were grown for 7-10 days on semi-solid PD-4 medium (Davis et al., 1980b) to serve as inoculum. Bacteria were washed from plates with sterile PBCM and standardized to a concentration of 10^8-10^9 cells per ml in PBCM using a bacterial counting chamber (Hausser Scientific, Blue Ball, PA). Control inoculum was prepared by washing un-

inoculated, sterile PD-4 plates with sterile PBCM for 2 min. The sterile PBCM served as the control inoculum.

Healthy, greenhouse grown elm seedlings (125 two-year-old plants in 3.8 L containers) were forced in the greenhouse in March 1982 (Florist and Nursery Crops Laboratory, USDA, Beltsville, MD). Seedlings were divided into 8 groups of 10 plants each and inoculated on May 18, 1982, with 1 of 5 isolates of the ELS-bacterium, the MLS-bacterium, the PD-bacterium, or sterile PBCM washings of uninoculated PD-4 plates. Seedlings were unpotted and a major root was disinfested with 70% ethanol then severed with sterile pruning shears. The severed end of the attached root was immediately connected with a segment of Tygon or latex tubing to a 10 ml pipette containing 10 ml of inoculum. Plants were repotted with the pipette attached, watered, then maintained under a reduced watering regime.

On June 15, 1982, 25 two-year-old, greenhouse grown American elm seedlings in 3.8 L containers (Florist and Nursery Crops Lab., USDA, Beltsville, MD) were inoculated using an adaptation of the technique developed by Gardner and Kageler (1982) for the introduction of systemic herbicides into a severed branch. Seedlings were divided into 5 groups of 5 plants each, with each group inoculated with one of the 3 symptom type isolates, or control inoculum. Inoculum was prepared as above. Stems were disinfested with 70% ethanol then the apical end removed with a sterile pruning shears. A segment of Tygon tubing was immediately attached to the stem end and 1 ml of inoculum was placed in the tubing reservoir. Each seedling was inoculated at 2 sites. Twenty-five 3 year old seedlings in 11.2 L containers were inoculated as above, using 1 of

3 ELS isolates, the PD-bacterium or control inoculum. All 3-year-old seedlings were grown outdoors at the ESL Nursery in Alexandria, VA.

Thirty field grown American elms, approximately 3 years old and 10-12 cm dbh, (ESL Nursery, Alexandria, VA) and free of ELS in 1981 were inoculated on June 16, 1982 at five sites per tree and in the same manner as above. Six trees were inoculated per symptom type isolate of the ELS-bacterium, the PD-bacterium, or control inoculum. All inoculated trees were monitored monthly for symptom development.

A combination of both root and branch inoculation techniques was used on potted greenhouse forced seedlings in May 1983. Bacterial inoculum was prepared as above, except that all ELS-isolates had been in culture through no more than 2 passages on PD-4 agar to limit any loss of virulence. Forty seedlings were divided into 4 groups of 10 seedlings each; each group inoculated with 1 of the 3 respective ELS symptom type isolates or with control inoculum. One ml of inoculum was introduced into the severed root of each plant, followed 4 days later by two cut branch inoculations (1 ml each) per plant. Plants were maintained in the greenhouse under reduced watering conditions. Root uptake of inoculum was complete in 24 hours, while stem inoculum uptake required only 6-8 hours.

Elm seedlings (15-30 cm in length) and rooted grape cuttings were inoculated with suspensions of 1 of 5 isolates of the ELS-bacterium, the PD-bacterium, or control inoculum. Three methods were used with elm seedlings: syringe injection and two forms of wounded root inoculation. Grape seedlings were inoculated using syringe injection. Syringe injected seedlings were inoculated at 3-4 sites along the stem by inserting a

28 gauge hypodermic syringe into the xylem and injecting the inoculum while withdrawing the syringe. For root inoculations, elm seedlings were removed from the potting mix, soil washed from the roots, and roots severed and immersed in inoculum for 24 hrs., then plants were repotted. For the second technique, soil was washed from the roots of unpotted plants, a major root severed, a segment of tubing attaced to the vascular system, and 0.05-0.1 ml of inoculum placed in the tubing reservoir. Exposed roots were wrapped in wet paper towelling to prevent desiccation. After 4 hrs., plants were repotted and maintained under a reduced watering regime.

All plants were monitored for symptom development for up to 16 months. Isolations were made from apical stem segments of all inoculated trees using modified PW broth and wood chip isolation. Sequential stem isolations were made from 20 trees root-inoculated in May 1982 and all of the 1983 root- and shoot-inoculated trees. Grape isolations were made using sap from crushed or centrifuged petiole/midrib sections.

Results

Isolation and Culture

Under phase contrast microscopy (1000X), small rod-shaped bacteria (0.4-0.5 um x 1-3 um in size) were observed in buffer extracts of 34 of 48 ELS-affected trees. Darkened polar regions, as previously reported (Hearon *et al.*, 1980), were observed at one or both ends of the organism. Bacteria appeared singly, in pairs, or in clumps. Bacteria were not observed in multiple extracts from 21 symptomless trees.

The ELS-bacterium was isolated in modified PW broth, from stem sections of 78 of 82 (95%) ELS-affected trees sampled over a 2 year period in the Washington, DC, area. Repeated isolations from 38 symptomless trees did not yield the ELS-bacterium. Isolations on modified PW agar of expressed sap from stem segments or petiole/midveins of affected trees did not yield the bacterium even after 8 weeks incubation.

Faint turbidity developed in modified PW broth cultures after 7-12 days incubation and in S-8 medium after 10-14 days. in excess of 28 days incubation time required for cultivation of the ELS-bacterium in PD-2 broth. Cultured bacteria examined under phase contrast microscopy (1000X) were approximately 0.4-0.5 μm in dia. and 1-10 μm in length, with longest forms appearing in S-8 medium and PD-2 broth. Primary isolations in both media contained multiple, intertwined chains or aggregates of elongated cells, while in modified PW broth, cells were no longer than 6 um and appeared singly, in pairs, or small aggregate clumps. Isolation effeciency was similar in both modified PW broth and S-8 medium. However, because of the elongate forms in S-8 medium, modified PW broth was selected as the isolation medium.

Bacterial films developed from droplets of broth cultures placed on modified PW agar after 7-10 days incubation. Subcultures of bacterial films streaked on modified PW agar produced circular, buff white to yellow, appressed colonies, 0.5-0.8 mm in diameter after 2 weeks incubation. Once bacteria were established on modified PW agar, isolates were streaked onto PD-4 agar and produced discrete white, convex colonies with entire margins. Maximum colony size was 0.8 mm after 2 weeks incub-

ation. Cell size on modified PW agar or PD-4 agar as determined with phase contrast microscopy was 0.4-0.5 μm in dia. x 1-6 μm in length. Elongate forms were absent. Colonies on PD-4 left a slight indentation or pit in the medium when disloged, analagous to that produced by the PD-bacterium (Davis et al., 1980b).

Characterization

Isolates of the ELS-bacterium were gram-negative, catalase positve, and oxidase negative, in agreement with results of other FXIB (Davis et al., 1981b, Wells et al., 1983; Sherald et al., 1983; Davis et al., 1983). None of the ELS-isolates tested grew on common microbiological media (nutrient agar, King's medium B agar, or potato dextrose agar) after up to 8 weeks incubation.

Electron Microscopy

Cultured ELS-bacteria strongly resembled the bacteria previously observed in ELS-affected elms (Hearon et al., 1980). Bacteria were rod-shaped, ranging in size from 0.3-0.4 μm in width x 1-6 μm in length with elongate forms commonly observed (Fig. 13). Multinuclear areas were evident in longitudinal sections of elongate forms. The presence of elongate, multinuclear forms suggests a repression of cell division. Nodulated, microtubule-like structures occurred in the interbacterial spaces of the colony (Fig. 13). Microtubules were identical to those observed surrounding bacteria within the xylem of affected tissues (Hearon et al., 1980). The presence of these structures in cultures of the ELS-bacterium indicates bacterial origin rather than host origin.

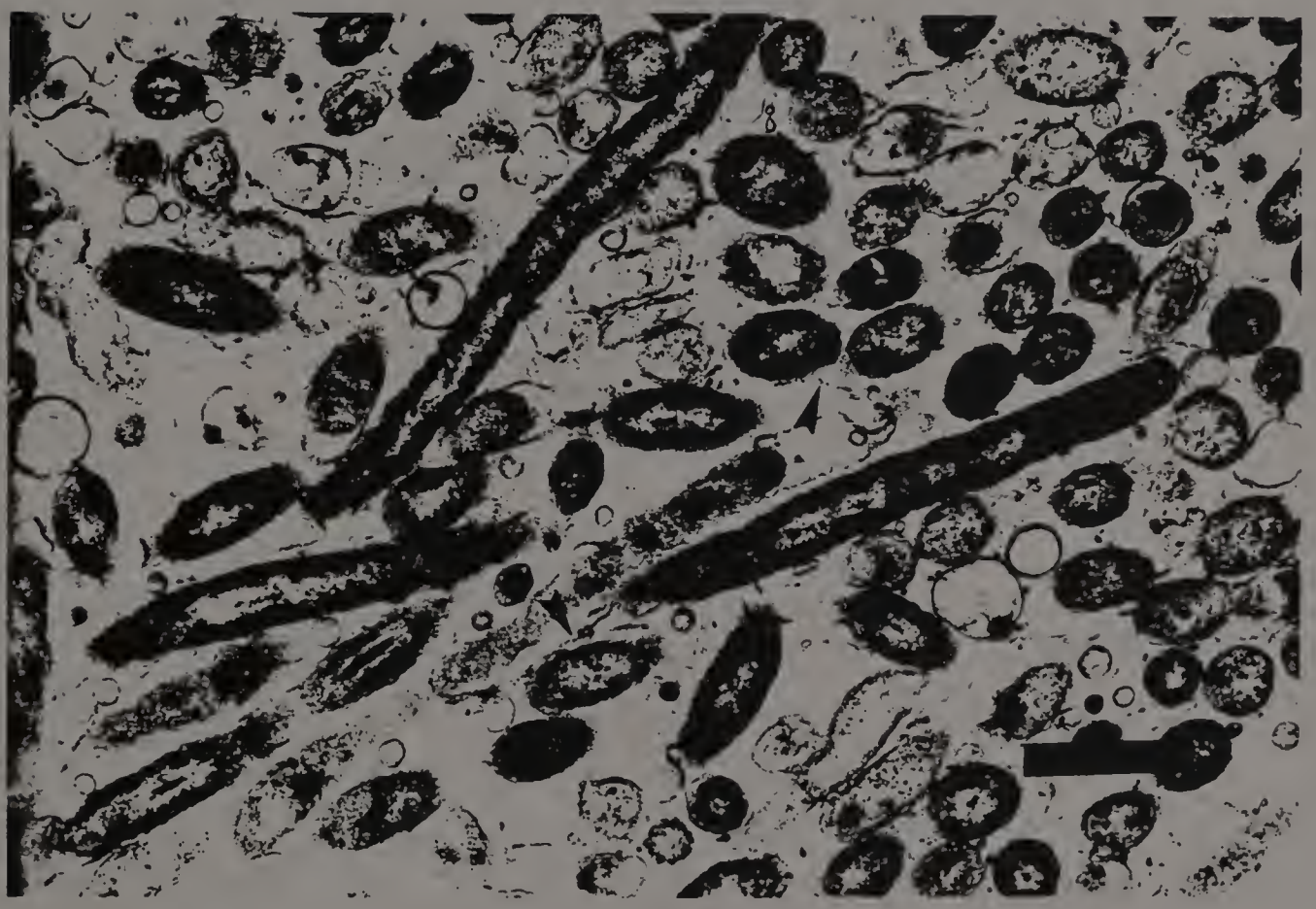

Fig. 13. Transmission electron micrograph of the elm leaf scorch bacterium from culture; note the microtubules in the matrix surrounding the cells. Scale bar = 1 μm.

Similar structures were observed in cultures of the sycamore leaf scorch bacterium (Sherald et al., 1983).

Both ultrathin sections and negative stained bacteria demonstrated a rippled, extensively convoluted wall characteristic of gram negative bacteria (Fig. 13, 14). Individual cells with smooth walls were occasionally observed (Fig. 15).

Serology

Vacuum extracted and cultured bacteria were reacted initially with antiserum to the PD-bacterium (provided by M. J. Davis) in the immunofluorescent antibody staining test (IFAS) confirming that the extracted an cultured bacteria were FXIB and serologically related to the PD-bacterium (Fig. 16, Table 3). Rabbit antiserum prepared against cultured isolates of the ELS-bacterium and the PD-bacterium agglutinated pure culture preparations (1×10^8 cells/ml in PBS) of the ELS-bacterium or the PD-bacterium at a titer of 640 for both the homologous and heterologous systems (Table 3). No reaction was obtained when either antiserum was reacted with E. cloacae or C. xyli. No reaction was obtained when pre-immune serum was reacted with any of the above antigens.

Indirect IFAS confirmed that the extracted bacteria and primary isolates were serologically related to the ELS-bacterium, to each other, and to the PD-bacterium (Table 3). No reaction was obtained when cells were reacted with pre-immune serum. All other species of phytopathogenic bacteria tested were immunofluorescent negative to antiserum against the ELS-bacterium, the PD-bacterium, or pre-immune serum.

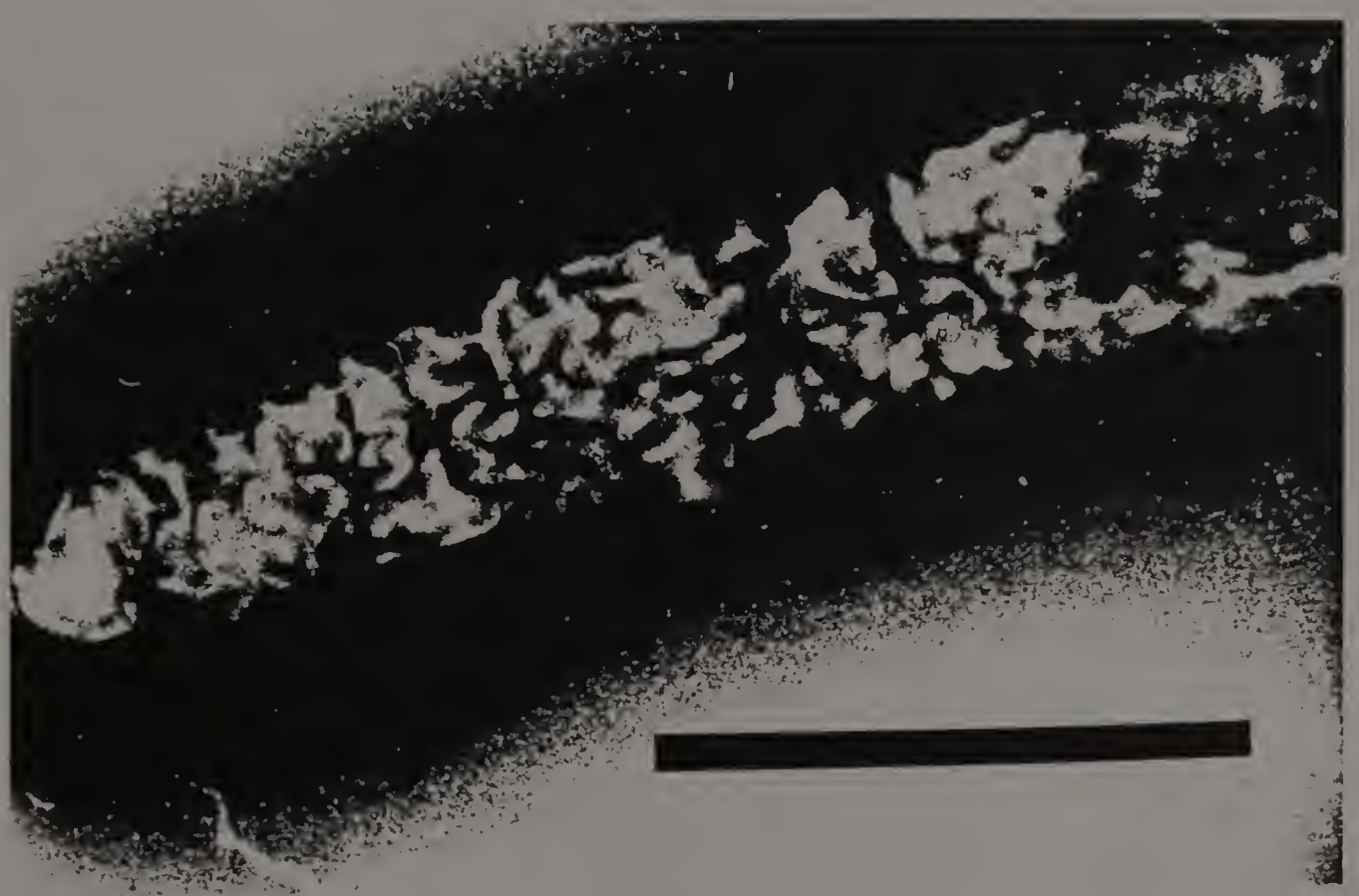

Fig. 14. Transmission electron micrograph of a negative stained cell of the elm leaf scorch bacterium from culture; note the extensive convolutions of the cell wall. Scale bar = 1 μm.

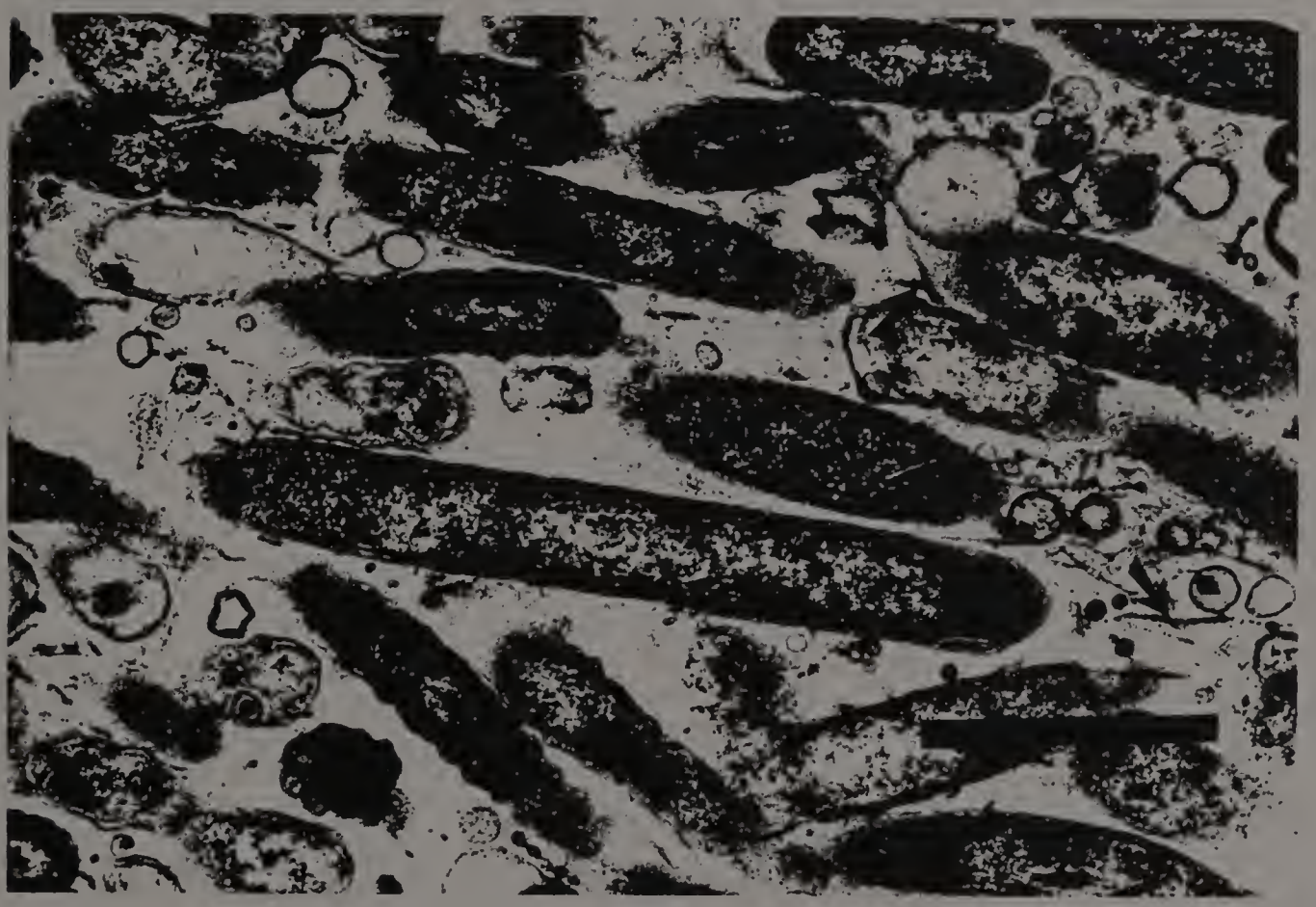

Fig. 15. Transmission electron micrograph of the smooth wall variant of the elm leaf scorch bacterium occasionally observed in cultures. Arrows indicate microtubules. Scale bar = 1 μm.

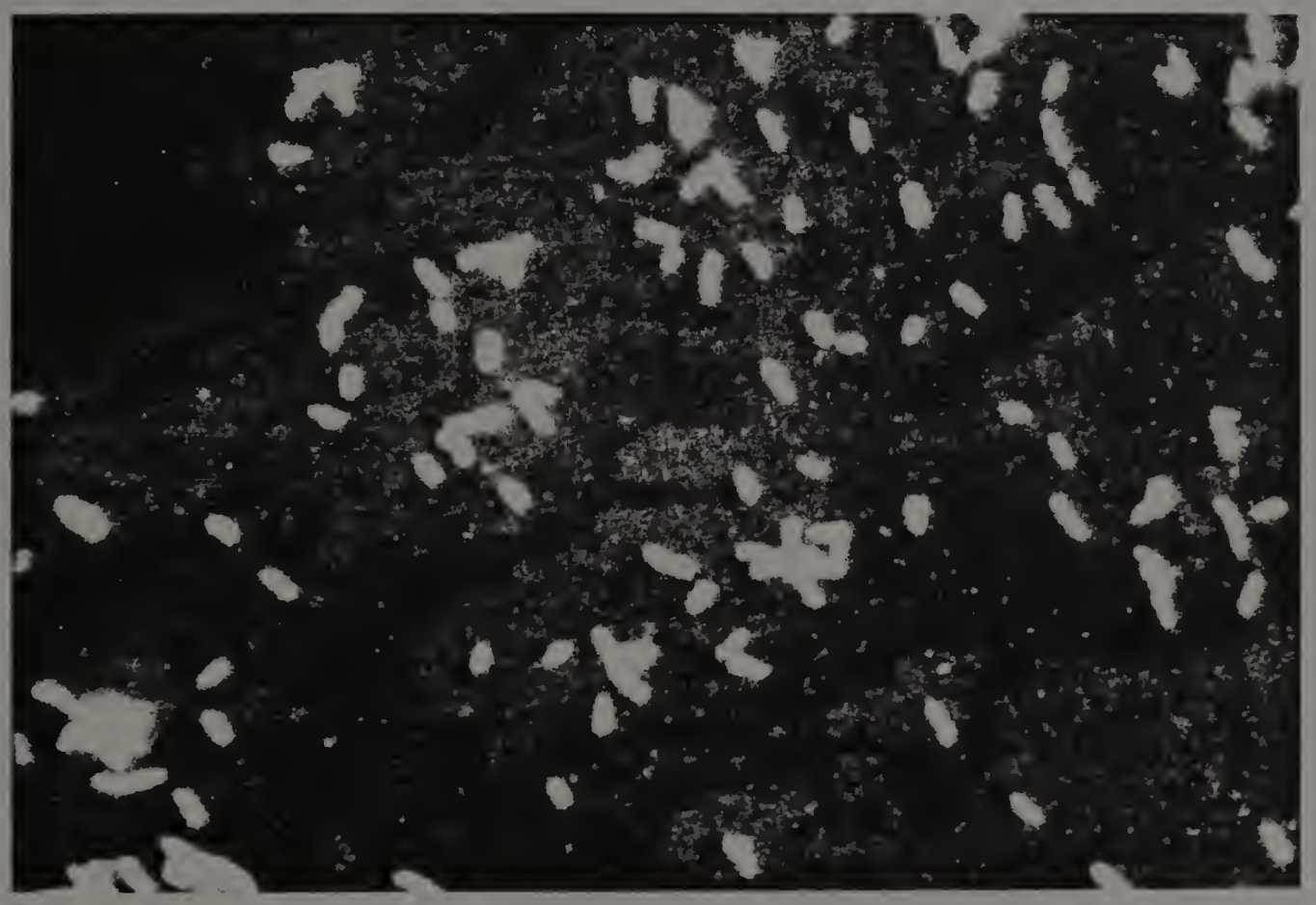

Fig. 16. Positive indirect immunofluorescent antibody staining reaction of the elm leaf scorch bacterium reacted with the homologous antiserum.

TABLE 3. Serological reactions between fastidious, xylem-inhabiting bacteria and other genera of phytopathogenic bacteria

Test antigen	Immunofluorescence with antiserum prepared against:			Agglutination with antiserum prepared against:	
	Pierce's disease bacterium (MJD[a])	Elm leaf scorch bacterium	Pierce's disease bacterium	Elm leaf scorch bacterium	Pierce's disease bacterium
Extracts from elm leaf scorch (ELS)-affected stems	+[b]	+	+	ND[c]	ND
Extracts from healthy elm stems	-	-	-	ND	ND
Cultured ELS bacterium	+	+	+	640[d]	640
Agrobacterium radiobacter pv. *tumefaciens*	ND	-	-	ND	ND
Pseudomonas syringae pv. *syringae*	ND	-	-	ND	ND
Erwinia carotovora pv. *carotovora*	ND	-	-	ND	ND
Clavibacter xyli subsp. *xyli*	-	-	-	NR[e]	NR
Clavibacter michiganense sepedonicum	ND	-	-	ND	ND
Enterobacter cloacae	ND	-	-	NR	NR

[a] Antiserum to the Pierce's disease bacterium provided by M. J. Davis
[b] Fluorescence was rated as negative (-) or positive (+)
[c] ND = not determined
[d] Values represent the highest antiserum dilution producing visible cell agglutination
[e] NR = no reaction

Endpoint indirect IFAS reactions with isolates of the ELS-bacterium or the PD-bacterium (1×10^8 cell/ml) were obtained at antiserum dilutions of 1:32, for both the homologous and heterologous systems. Optimal fluorescence was obtained between 1:8 and 1:16 antiserum dilutions in sterile 0.01 M PBS. An antiserum dilution of 1:10 was used for the majority of the indirect IFAS tests.

Sharpest precipitan bands in gel double diffusion were obtained in 0.5% agarose to which solubilized antigens were added 24 hours prior to the undiluted antiserum. In 0.8% gels (antigen added 0, 8, or 24 hours prior to the antiserum) or 0.5% gels (antigen added 0 or 8 hours prior to the antiserum), precipitan bands developed immediately adjacent to the antigen wells indicating that the movement of soluble antigens was limited by the small gel pore space or that there was an antibody excess. Antiserum dilutions of 1/2, 1/4, and 1/8 had no affect on the position of the precipitan band, although band intensity decreased with increasing dilution. At 1/8 dilution, precipitan bands were only marginally discernable.

Isolates of the ELS-bacterium were serologically identical to each other and to the PD-bacterium based on precipitan reactions obtained in the homologous and heterologous antigen/antibody combinations. A single, confluent precipitan band developed with solubilized antigens (ELS-isolates and the PD-bacterium) in 0.01 M Tris-buffered (pH 7.4) 0.5% agarose. No differences in precipitan reaction occurred among or between isolates when reacted with the homologous or heterologous antisera (Fig. 17, 18). Precipitan bands in PBS-buffered and unbuffered agarose were analagous to reractions in Tris. However, a second, broad,

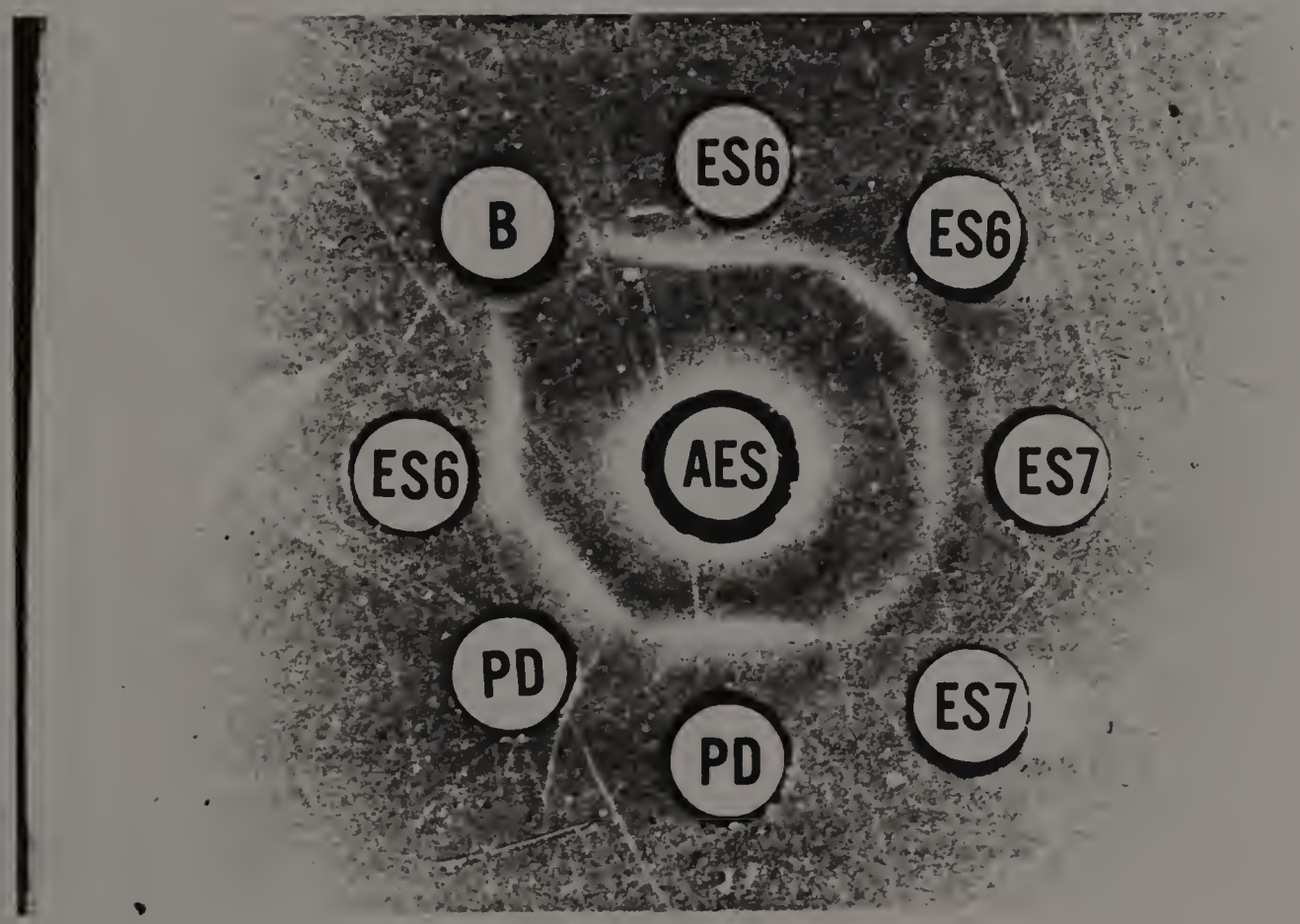

Fig. 17. Gel double diffusion reaction of soluble antigens of isolates of the elm leaf scorch bacterium (ES6, ES7) and the Pierce's disease bacterium (PD) with antiserum against the ES6 isolate of the elm leaf scorch bacterium (AES). The well labelled "B" indicates a control well containing sterile buffer.

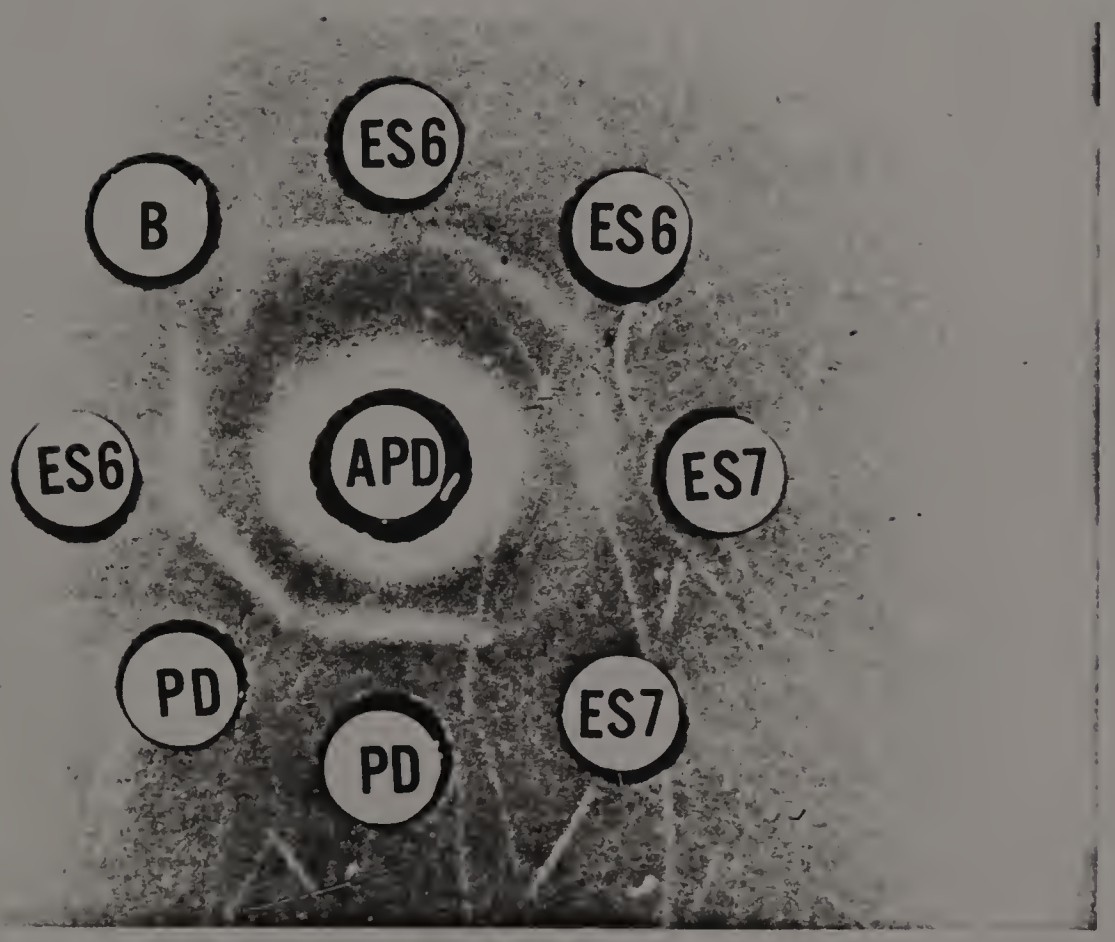

Fig. 18. Gel double diffusion reaction of soluble antigens of isolates of the elm leaf scorch bacterium (ES6, ES7) and the Pierce's disease bacterium (PD) with antiserum against the VT1 isolate of the Pierce's disease bacterium (APD). The well labelled "B" indicates a control well containing sterile buffer.

diffuse non-specific band was also present. Occassionally, the non-specific band over-lapped or obscured the inner specific precipitan band.

Systemic Distribution

The ELS-bacterium was recovered from 8 of 10 branches sampled within the canopy. Vascular discoloration indicative of Dutch elm disease was observed in 1 of 6 bole samples, 1 of 3 root flare/soil line samples, and 1 of 4 roots sampled. Discolored tissues were confined to the southern quadrant of the tree. The ELS-bacterium was isolated from 3 of 6 bole samples and 1 of 3 samples collected at the root flare/soil line interface. Of 16 isolations from the 4 root samples, none yielded the ELS-bacterium, due to contamination by other bacteria. The ELS- bacterium was recovered in buffer extracts of each of the 4 roots and extracted bacteria were identified using indirect IFAS.

Seasonal Recovery

The ELS-bacterium was isolated from branches of symptomatic trees throughout the study period (Table 4). Bacterial isolation success was low in February and March 1982. However, all isolation attempts were successful in January and March of 1983, on a per tree basis. Over the 11 sampling dates, a total of 57 isolation attempts were made from each tree. The ELS-bacterium was isolated from 21 of 57, 28 of 57, and 12 of 57 isolation attempts from each of the respective ELS-affected trees (35.6% success overall. Isolations from symptomless trees were negative for the ELS-bacterium.

TABLE 4. Seasonal isolation (1982-1983) of fastidious, xylem-inhabiting bacteria from leaf scorch-affected and symptomless American elms[a] growing in Washington, DC

Sampling date[b]		Bacterial recovery	
		scorch-affected	symptomless
1982	February 23	1/3	0/3
	March 8	1/3	0/3
	June 15	3/3	0/3
	July 14	2/3	0/3
	August 13	3/3	0/3
	September 21	1/3	0/3
	November 21	3/3	0/3
	December 6	3/3	0/3
1983	January 26	3/3	0/3
	March 23	3/3	0/3
	July 12	3/3	0/3

[a] Six American elms were sampled throughout the course of the study; 3 leaf scorch-affectd and 3 symptomless. The same 6 trees were sampled on each sampling date.

[b] At least 4 isolation attempts were made per tree per sampling date.

Graft Transmission

Scion survival was high 2 months after grafting in symptom Type I, Type II, and control scions (15 of 16, 4 of 5, and 11 of 14, respectively). Only 6 of 26 of the Type III scions survived. By July 1982, symptoms appeared in 2 trees (#2,#7) grafted with scions from the symptom Type III tree (Table 5). Symptoms were well developed in the scions and several scorched leaves occurred on the stems beneath the grafts. Symptoms were similar, but not identical to Type III symptoms in the source tree. The necrotic tissues appeared water-soaked and the necrosis on the adaxial surface was not a prominent brown color as in the source tree. Rather, affected tissues were a dull green-brown. The necrotic tissue on the underside of the leaf (abaxial surface) was a subdued, dark brown. The chlorotic halo between green and necrotic tissues was diffuse and often inconspicuous, appearing as a light green zone rather than a distinct yellow band. Affected leaves exhibited adaxial leaf curl. The ELS-bacterium was isolated from the symptomatic scions on both trees.

By August 1982, more than 80% of the canopy leaf area of tree #2 and 20% of the canopy leaf area of tree #7 were affected; necrosis in affected leaves extended to the midvein, and some of the affected leaves had abscised. Symptoms also appeared in 2 scions (symptom Type III source tree) grafted onto tree #3 (Table 5). Symptoms in tree #3 were limited to the scion and were the same as symptoms in tree #2 and #7. No symptoms occurred during 1982 in controls or trees grafted with scions from symptom Type I or symptom Type II trees.

TABLE 5 Graft survival and development of leaf scorch symptoms in American elms grafted with scions from scorch-affected and symptomless trees in Washington, DC

Tree #	scion wood symptom type	no grafts/ tree (3/82)	Ratio (no. grafts viable/ no. grafts made) 5/82	6/82	1982 symptom observation dates 7/12/82	8/23/82	Ratio (no. grafts viable/ no. grafts made) 5/83	1983 symptom observation dates 7/11/83	8/23/83
1	II	5	5/5	1/5	0/1[a]	0/1	1/5	0/1	0/1
2	III	7	4/7	2/7	1/2**	1/2**	2/7	2/2**	2/2**
3	III	5	4/5	2/5	0/2	2/2*	1/5	0/1	1/1**
4	CONTROL	6	6/6	4/6	0/4	0/4	2/6	0/2	0/2
5	II	5	5/5	5/5	0/5	0/5	3/5	0/3	1/3*
6	CONTROL	5	5/5	5/5	0/5	0/5	3/5	0/3	0/3
7	III	6	2/6	2/6	2/2*	2/2*	1/6	1/1*	1/1**
8	CONTROL	4	4/4	2/4	0/2	0/2	1/4	0/1	0/1
9	II	9	7/9	6/9	0/6	0/6	3/9	0/3	0/3
10	II	5	5/5	4/5	0/4	0/4	2/5	0/2	0/2
11	I	5	5/5	4/5	0/4	0/4	4/5	0/4	0/4

[a] Ratio indicates the number of scions with elm leaf scorch symptoms/ number of viable scions
* Symptoms only appear in the source scion
** Symptoms appear in the scion and systemically in adjacent branches

In July 1983, systemic symptoms appeared in tree #2 (Type III scion-wood). ELS-affected foliage on both surviving scions abscised. Symptoms in tree #7 were limited to the 1 remaining viable scion, while the single remaining viable scion on tree #7 was symptomless (Table 5). By August 23, nearly 100% of the canopy leaf area in tree #2 was affected with ELS. Systemic symptoms had developed in both tree #3 and tree #7 (50% and 40% of the canopy leaf area, respectively). One symptom Type II scion grafted on tree #5 developed ELS symptoms. Symptoms were analogous to those observed in the source tree. Necrotic tissue was undulating and separated from green tissue by a chlorotic halo. Leaf curl was both abaxial and adaxial and limited to 7 leaves on a single scion. The ELS-bacterium wes isolated from all symptomatic scions representing symptom Type I and symptom Type II as well as from systemically infected branches where present. Bacteria were not isolated from symptomless branches in trees where symptoms did not expand beyond the scion. No further symptoms were observed in September 1983.

Pathogenicity

Root or stem inoculation of 2 or 3 year old potted elm seedlings with isolates of the ELS-bacterium, the MLS-bacterium, or the PD-bacterium did not induce leaf scorch symptoms up to 16 months after inoculation (Table 6). Bacteria could not be reisolated from inoculated seedlings. Sequential stem isolation attempts made to 10 cm above the soil line of 20 inoculated seedlings also failed to yield FXIB.

In July 1982, slightly more than 1 month after branch inoculation, leaf scorch symptoms appeared in 2 inoculated branches of a tree that

TABLE 6. Response of greenhouse grown American elm seedlings to inoculation with isolates of fastidious, xylem-inhabiting bacteria

Inoculation Method	Isolate/Symptom Type	Ratio (diseased/inoculated)
Severed root 2 yr. old seedlings	J98/I	0/10
	ES134/I	0/10
	ES6/II	0/10
	J357/III	0/10
	ES7/III	0/10
	VT1/PD-bacterium[a]	0/10
	MS1/MLS-bacterium[b]	0/10
Cut branch 2 yr. old seedlings	ES134/I	0/5
	ES6/II	0/5
	ES7/III	0/5
Cut branch 3 yr. old seedlings	ES134/I	0/5
	ES6/II	0/5
	ES7/III	0/5
	VT1/PD-bacterium	0/5
Severed root/cut branch 2 yr. old seedlings	Ana28/I	0/10
	ES6/II	0/10
	ES7/III	0/10

[a] VT1 is an isolate of the Pierce's disease bacterium (provided by M. J. Davis)
[b] MS1 is an isolate of the mulberry leaf scorch bacterium

had been inoculated with the PD-bacterium (Table 7). Each of the symptomatic branches had been inoculated with the PD bacterium. No symptoms appeared in uninoculated branches. Affected foliage was necrotic (brown on the lower surface of the leaf and a gray, water-soaked appearing color on the upper leaf surface), leaves curled, and a faint chlorotic halo occurred on the uppper leaf surface separating the necrotic from green tissues. By August, symptoms extended into adjacent uninoculated branches and affected leaves abscised. Isolation from affected stems yielded a FXIB serologically related to the PD-bacterium and the ELS-bacterium by indirect IFAS. No other symptoms developed in inoculated, field-grown trees in 1982. In 1983, symptoms were more severe in the PD-inoculated tree, by August affecting nearly 100% of the canopy. FXIB were readily isolated from all stems sampled.

Symptoms also appeared in August in 1 of 6 trees inoculated with isolate ES6, a symptom Type II isolate of the ELS-bacterium. Symptoms, however, were not in the inoculated branch, but rather, were in the next most adjacent branch. Symptoms were approximately 2 m from the inoculation site. FXIB were isolated from the affected branch, but not from the inoculated branch. None of the remaining 26 trees developed symptoms by the end of the second growing season (1983) nor were FXIB isolated from inoculated branches.

Two year old elm seedlings inoculated in May 1983 with newly cultured isolates of the ELS-bacterium and by means of both cut branch and root inoculation, did not develop symptoms by January 1984 (Table 6). All trees were sacrificed in January and isolations made from sequential stem sections did not yield the ELS-bacterium. Control seedlings

TABLE 7. Symptom development and bacterial reisolation in 3 yr. old field grown American elms inoculated with isolates of the elm leaf scorch bacterium or the Pierce's disease (PD) bacterium

Inoculation Technique	Isolate	Symptom Type	Ratio (diseased/healthy)	Bacterial Reisolation
Branch inoculation 3 yr. old field grown trees	ES134	I	0/6	0/6
	ES6	II	1/6	1/6
	ES7	III	0/6	0/6
	VT1	PD-bacterium	1/6	1/6
	Buffer controls		0/6	0/6

TABLE 8. Symptom development in young American elm seedlings or rooted grape cuttings inoculated with 1 of 3 symptom type isolates of the elm leaf scorch bacterium or the Pierce's disease bacterium (PDB)

Inoculation Method	Isolate/Symptom Type	Plant Inoculated	Ratio (diseased/inoculated)
Hypodermic	ES134/I	elm	0/10
2 yr. old seedlings	ES134/I	grape	0/14
	ES6/II	elm	0/10
	ES6/II	grape	0/14
	ES7/III	elm	0/10
	ES7/III	grape	0/14
	VT1/PDB	elm	0/10
	VT1/PDB	grape	5/14
Wounded Root	ES134/I	elm	0/10
Immersion	ES6/II	elm	0/10
	ES7/III	elm	0/10
	VT1/PDB	elm	0/10
Wounded Root	ES134/I	elm	0/10
small seedlings/	ES6/II	elm	0/10
inoculum reservoir	ES7/III	elm	0/10
	VT1/PDB	elm	0/10

remained symptomless and free of FXIB.

None of the 4-5 month old American elm seedlings inoculated by hypodermic injection, root immersion in bacterial suspensions, or with a small inoculum reservoir attached to a severed root developed leaf scorch symptoms (Table 8). FXIB were not recovered from any of the inoculated seedlings. Rooted grape cuttings inoculated with isolates of the ELS-bacterium also failed to develop leaf scorch symptoms (Table 8). The ELS-bacterium was not isolated from inoculated elm seedlings six months or more after inoculation. The PD-bacterium was recovered from 5 of 14 PD-inoculated grape plants (Table 8). However, none of the 5 plants had symptoms that could be differentiated from natural senesence symptoms that appeared in buffer inoculated controls or even uninoculated plants.

Discussion

Bacteria were consistently isolated from American elms naturally infected with elm leaf scorch, and cultured on an artificial medium developed for FXIB. Initial isolation of the ELS-bacterium was achieved on all broth formulations of the culture media tested using the wood chip isolation technique (Sherald *et al.*, 1983). However, bacteria isolated in modified PW broth grew more rapidily and were similar in size to bacteria in the xylem of ELS-affected leaves or in buffer extracts of stem segments than bacteria isolated in PD-2 or S-8 broth. The prevalence of elongate bacterial cells in PD-2 and S-8 suggests that one or more necessary growth factors may be limiting in the medium. Similar elongate bacteria were encountered in early media developed for the isol-

ation of the PD-bacterium (Davis et al., 1978b) and in isolation of the plum leaf scald and phony peach bacteria on BCYE medium (Wells et al., 1981). Isolation techniques and semi-solid media used for isolation of other FXLB were unsuitable for isolation of the ELS-bacterium. The same isolation difficulties were observed with the sycamore leaf scorch bacterium (Sherald et al., 1983).

Cultured bacteria were morphologically and ultrastructurally similar to FXLB isolated from PD-infected grapes and other hosts (Davis et al., 1978a, 1980b, 1980c, 1981a; Wells et al., 1981; Davis et al., 1983; Wells et al., 1983). Serological studies confirmed the relatedness among ELS isolates extracted from host tissues and in culture, as well as the relatedness of the isolated bacteria to the PD-bacterium. Indirect immunofluorescent antibody staining provided a rapid means of screening and identifying bacterial isolates as FXLB. Based on double gel diffusion studies, both the ELS isolates and the PD-bacterium are serologically identical. Although these studies indicate identity, other workers (Davis et al., 1983, Wells et al., 1983) have found that serological differences occur between FXIB when studies are conducted using the enzyme-linked immunosorbent assay (ELIZA). Analysis of cell wall fatty acids also have demonstrated differences among these organisms (Wells et al., 1982). Based on fatty acid profiles, Wells et al.(1982) grouped the ELS-bacterium with the PD-bacterium, which was distinguishable from the PLS- and PPD-bacteria.

The systemic distribution of the ELS-bacterium within the host supports the hypothesis that FXIB affect xylem transport. Extensive

colonization of the xylem throughout the canopy, bole, and roots of the tree will cause a reduction in xylem flow rates, vessel occlusion (Talboys, 1968, 1978; Beckman, 1980), and potentially embolism of invaded vessels (Zimmerman and McDonough, 1978). Colonization of the bole and roots also provides the bacterium with protected environments during periods of adverse climactic conditions, particularly extremely low temperatures. The recovery of viable bacteria from the canopy (branches <1 cm in dia.) throughout the dormant season indicates that the xylem environment suitably buffers low temperatures encountered during the winter months or that the bacteria are in a hypobiotic state. In either case, distribution throughout the tree and survival of the bacteria in the canopy facilitates potential sites for bacterial migration into newly formed xylem in the spring.

The success of graft transmission studies supports the role of FXIB as the causal agent of ELS and suggests that differences may exist between symptom type isolates. The rapid development of ELS symptoms in scions from the most severe symptom type tree (Type III) and the subsequent development of symptoms in adjacent branches can be attributed to the Type III organism being more virulent than the Type I or Type II organisms or to the bacterial population in the Type III scions being at higher levels than bacteria in scions from trees with Type I or Type II symptoms.

Pathogenicity studies failed to demonstrate conclusively the pathogenicity of the ELS-bacterium. In only 1 test did ELS symptoms develop in a tree inoculated with the ELS-bacterium. Symptoms occurred more than one year after inoculation in a field grown tree inoculated

bt the cut branch technique. Symptoms in the tree did not develop in the inoculated branch, but in a branch adjacent to the inoculated branch and more than 2 m from the inoculation site. The absence of symptoms and the ELS-bacterium in the inoculated branch brings the source of the observed infection into question. Rather than the observed symptoms being caused by the inoculated bacterium it is possible that the symptoms were due to natural infection.

Manipulation of plant age, symptom type isolate, age of culture, and environmental conditions under which plants were maintained did not affect pathogenicity in any of the studies conducted. Failure to cause disease may be due to a juvenile resistance analogous to that encountered in young American elm seedlings inoculated with Ceratocystis ulmi (Heybroek, 1957), avirulence of the isolates used, poor inoculation route, or absence of predisposing climatic or edaphic stresses.

Although none of the ELS-isolates were pathogenic or recoverable from inoculated seedlings and trees, one isolate of the PD-bacterium did cause the development of leaf scorch symptoms in a branch inoculated field grown tree. The development of leaf scorch symptoms in this single tree suggests that the PD-bacterium can cause ELS. However, none of the other trees inoculated with the PD-bacterium in this test or in greenhouse tests developed symptoms. Although pathogenicity of the ELS-bacterium has not been conclusively demonstrated, the consistent association of the FXIB with ELS, its relatedness to other FXIB, and the observed symptoms, isolate-related, graft-transmitted symptom variation have been established.

C H A P T E R V

REMISSION OF BACTERIAL LEAF SCORCH SYMPTOMS THROUGH OXYTETRACYCLINE MICROINJECTION

Introduction

Tetracycline antibiotics block protein synthesis in prokaryotes and have been used to control diseases caused by mycoplasma-like organisms (McCoy, 1982). These materials, applied as a soil drench, have been used to demonstrate the involvement of fastidious, xylem-limited bacteria in Pierce's disease of grape (Hopkins and Mortensen, 1971; Hopkins and Mollenhauer, 1973) and citrus young tree decline (Tucker et al., 1974). Nyland (1979) demonstrated that oxytetracycline injected into the xylem of leaf scorch-affected almonds could serve as a therapeutic control.

Fastidious, xylem-limited bacteria have been associated with elm and oak leaf scorch (Hearon et al., 1980) and have been shown to be the cause of sycamore leaf scorch (Sherald et al., 1983) and mulberry leaf scorch (Kostka et al., 1983). Although the elm leaf scorch-associated bacterium has been cultured on artificial media, pathogenicity has not been demonstrated (Kostka et al., 1981; S. Kostka, unpublished data.)

The objective of this study was to determine if injection of oxytetracycline (OTC) into the xylem of leaf scorch-affected American elms would cause a remission of symptoms.

Materials and Methods

Naturally infected American elms (Ulmus americana L.), 2 to 6 m in height, were selected for treatment. All trees were growing in naturalized sites in Washington, DC. Symptom expression was initially evaluated in August 1982. Isolations were made from all selected trees by incubating aseptically excised wood chips in a modified PW broth (Sherald et al., 1983) or by vacuum extracting bacteria from stem segments and confirming their presence using phase contrast microscopy (French et al.,1977a; Hearon et al., 1980; Sherald et al., 1983).

Two microinjection techniques were selected for introduction of oxytetracycline (OTC)(Terramycin Tree Injection Formula, Pfizer Chemical Company): 1) Mauget capsules (McIntyre et al, 1978; McCoy, 1982) or 2) the pipette injection technique (Lacy and McIntyre, 1978) developed for the introduction of antibiotics into pears with pear decline. Basal oxytetracycline levels were established from the rates developed for control of elm phloem necrosis (Filer, 1976). Trees were treated in August 1982 and June 1983.

Eleven trees (approx. 12 cm dbh) were selected in 1982 for treatment using Mauget capsules (provided by J. J. Mauget Co.). OTC (Terramycin Tree Injection Formula) was solubilized in distilled water to a concentration of 50 mg a.i./ml. Capsules were manually filled so that each tree would receive 500 mg a.i. OTC (3 capsules per tree). Trees were treated in August 1982 using recommended Mauget injection techniques. Capsules containing 4 ml of a 2% a.i. OTC suspension were sup-

plied by J. J. Mauget Co. for June 1983 treatments. Trees were injected in 1983 with 600 mg a.i. OTC.

Two treatment levels (40 mg a.i./cm dbh and 80 mg a.i./cm dbh) were used with the pipette injection technique. Antibiotic suspensions were prepared in distilled water as above. Trees ranged from 10-25 cm dbh. Fourteen trees were treated at the 40 mg a.i./cm level and 8 at the 80 mg a.i./cm level. Mauget capsules containing 4 ml of 2% a.i. OTC were used in all June 1983 treatments. Use of prefilled Mauget capsules caused an adjustment of OTC levels in 1983. Ten leaf scorch-affected American elms served as untreated controls. The success or failure of treatments was based on the following observations:

1) Presence or absence of symptoms in August 1983

2) Date on which first symptoms appeared

3) Degree of symptom remission

Results

Initial symptom development was observable in the sites in June of 1982. By August, symptoms were well developed. Symptoms in selected trees ranged from 10 to 90 percent of the total leaf area of the canopy. The presence of bacteria in symptomatic trees was confirmed by isolation and/or buffer extraction of stem segments and microscopic observation of bacteria in extracts.

Uptake times for antibiotic solutions was approximately 24 hours for the August 1982 treatments and less than 4 hours for the June 1983 treatments. The pipette injection technique was eliminated in 1983 for

several reasons: 1) leakage around the pipette and 2) the excessive depth required for the injection hole to securely hold the pipette.

By mid-July 1983, 7 of 10 control trees were exhibiting leaf scorch symptoms (Table 9). Symptoms were extensive in two of the trees and limited in the remaining five. Symptoms were observed in 1 of 24 trees injected at the low OTC levels (1X) and 1 of 6 trees injected at the high OTC level (2X). Although symptoms developed in three of the treated trees, the majority were symptomless in July.

In August, all trees were rated for presence and severity of symptoms (Table 10). Percent total leaf area affected in untreated, control trees was equivalent to or increased above 1982 levels. Fourteen of the scorch-affected elms treated at the 1X levels showed a complete remission of symptoms. Of the remaining 11 trees, a reduction in symptoms was observed in 8, while increased symptoms were observed in 1. Two of the 11 trees died from Dutch elm disease prior to the final rating and were dropped from the study. A complete or near complete remission of symptoms was observed in treated trees which had 20 percent or less of the total leaf area exhibiting leaf scorch in 1982 (Table 11). Results from trees treated at the 2X level were inconsistent. Three trees had substantially reduced symptoms and three were unchanged from 1982. Two trees were accidently removed and were dropped from the study.

Discussion

When the rules of proof (Koch's postulates) can not be fulfilled with a fastidious prokaryote (i.e., the fastidious, xylem-inhabiting bac-

TABLE 9. Antibiotic injection dosage and host response in leaf scorch-affected American elms in Washington, DC, one year after treatment

Mauget Injection			Pipette/Mauget Injection						Controls		
			Low Level			High Level					
Tree #	Treatment 1982/1983	Symptoms[a] 7/83	Tree #	Treatment 1982/1983	Symptoms 7/83	Tree #	Treatment 1982/1983	Symptoms 7/83	Tree #	Treatment 1982/1983	Symptoms 7/83
1	500/600[b]	0	5	600/600	0	26	1600/1680	0	6A	none	50%
2	500/600	0	8	500/600	0	27	1000/960	0	10A	none	10%
3	500/600	0	9	500/600	<10%	28	2000/2040	0	10B	none	10%
4	500/600	0	9A	500/600	0	29	1000/960	0	17A	none	10%
6	500/600	0	10	500/600	0	30	1400/removed[c]		23A	none	10%
7	500/DED[d]	0	12	750/840	0	31	2000/removed		28A	none	10%
11	500/600	0	13	600/600	0	33	1000/960	0	30A	none	80%
17	500/600	0	14	1000/1080	0	34	1000/960	30%	32	none	0
18	500/600	0	15	650/720	0				35	none	0
21	500/600	0	16	550/600	0				36	none	0
23	500/600	0	19	500/600	0						
			22	500/600	0						
			24	500/600	0						
			25	600/600	0						

[a] Percent canopy leaf area affected with elm leaf scorch
[b] mg a.i. oxytetracycline injected per tree at each treatment date
[c] Trees removed inadvertantly by right of way clearence crews
[d] Tree severely affected by Dutch elm disease; second treatment not applied

TABLE 10. Pre- and post-treatment comparison of percent elm leaf scorch-affected canopy area in American elms injected with oxytetracycline in August 1982 and June 1983

Mauget Injection			Pipette/Mauget Injection						Controls		
			Low Level			High Level					
	Symptoms			Symptoms			Symptoms			Symptoms	
Tree #	8/82	8/83	Tree #	8/82	8/83	Tree #	8/82	8/83	Tree #	8/82	8/83
1	10	0	5	>30	DED[b]	26	25	10	6A	>90	90
2	10	<<10[a]	8	20	0	27	<10	<10	10A	<10	>10
3	50	DED[b]	9	>25	<10	28	>50	30	10B	<10	40
4	<10	0	9A	20	0	29	50	50	17A	<10	10
6	10	0	10	>10	<10	30	>90	removed[c]	23A	40	100
7	10	0	12	>10	<<10	31	>50	removed	28A	>50	80
11	10	0	13	>10	0	33	75	<10	30A	75	100
17	<10	0	14	<10	0	34	>30	>30	32	>20	50
18	>95	<<10	15	10	0				35	<10	10
21	>20	<10	16	10	0				36	80	100
23	>80	30	19	>20	70						
			22	<10	0						
			24	20	<<10						
			25	20	0						

[a] <<10 indicates negligible symptoms
[b] Tree killed by Dutch elm disease
[c] Tree indvertantly removed by right of way crews

TABLE 11. Percent leaf scorch symptoms in oxytetracycline-treated (1X) American elms before treatment (1982) and after treatment (1983)

	0-10% TLA[a] Symptoms			11-20% TLA Symptoms			21-50% TLA Symptoms			51-100% TLA Symptoms	
TREE #	1982	1983	TREE #	1982	1983	TREE #	1982	1983	TREE #	1982	1983
1	10	0	8	20	0	3	50	DED[b]	18	>95	<<10
2	10	<<10[c]	9A	20	0	5	>30	DED	23	>80	30
4	<10	0	10	>10	<10	9	>25	<10			
6	10	0	12	>10	<<10	19	>20	70			
7	10	0	13	>10	0	20	>30	70			
11	10	0	24	20	<<10	21	>20	<10			
14	<10	0	25	20	0						
15	10	0									
16	10	0									
17	<10	0									
22	<10	0									

[a] TLA = Total Leaf Area
[b] Tree killed by Dutch elm disease
[c] <<10 = Negligible symptoms

terium), an important step in confirming the involvement of the organism in disease is the remission of disease symptoms through antibiotic treatment (Hopkins and Mortensen, 1971; Nyland, 1979; McCoy, 1982) Oxytetracycline injected into the xylem of American elms affected with bacterial leaf scorch delayed symptom onset and suppressed symptom development. Optimal results were obtained when less than 20% of the canopy leaf area was affected prior to treatment. Results in trees with more than 21% of the canopy leaf area affected were inconsistent at either OTC dosage. The development of symptoms in treated trees was not unexpected based upon previous OTC injection studies of almond trees affected with almond leaf scorch (Nyland, 1979). Inconsistent symptom remission may be due to inadequate distribution or final concentration of the antibiotic in the xylem to inhibit bacterial growth.

The observed delay in the onset of symptoms and the complete or partial symptom remission that occurred in treated trees supports the role of an OTC-sensitive organism (i.e., the fastidious, intra-xylem bacterium) as the causal agent of elm leaf scorch. Although OTC induced symptom remission, its applicability as a therapeutic control requires further study to determine long-term control and potential phytotoxicity of the antibiotic in treated trees.

C H A P T E R VI

EFFECTS OF ELM LEAF SCORCH ON HOST PHYSIOLOGY

Introduction

Few studies have been conducted to define the effects of fastidious, xylem-inhabiting bacteria on the physiology of infected plants. Those studies that have been conducted have dealt with general observations on growth respones, such as plant height or yield (Pierce, 1892; Hutchins, 1933), cold injury (Daniell and Krewer, 1981), rooting (Krewer et al., 1981), and effects on elemental concentrations in diseased plants (Smith, 1974; Wutscher et al., 1977; Wutscher and Hardesty, 1979; Evert et al., 1981). Water uptake and dye distribution have been shown to be restricted in the bole, roots, and small stems of citrus trees affected with citrus blight (Cohen, 1974; Young et al., 1979; Young, 1980).

Vascular diseases are a group in which the pathogen exerts its primary effect on water relations in the suscept. The pathogen is located in the vascular elements at least during the early stages of pathogenesis, but may invade other tissues at later stages (Kenaga, 1974). Blockage of the xylem and resultant water deficits are characteristic of wilt diseases and are responsible for most if not all of the observed symptoms (Kenaga, 1974; Agrios, 1978).

Symptoms of vascular wilt diseases are manifested by a sudden wilting and collapse of leaves while still green and a failure of the affected leaves to abscise (Talboys, 1968; Kenaga, 1974; Agrios, 1978) Sub-

sequent symptoms include shoot and branch dieback and commonly culminate in plant death. Pathogens that induce subacute water stress induce foliar chlorosis and necrosis and premature leaf abscission (Talboys, 1968). Bacterial leaf scorch symptoms are similar to symptoms of plants undergoing subacute water stress.

This report describes the effects of elm leaf scorch (ELS) on the following parameters: stem elongation, leaf size, stem hydraulic conductivity, xylem dysfunction and occlusion, stem water potential, and stem starch content.

Materials and Methods

Stem Elongation

Effects of ELS on stem terminal elongation were determined in March 1983 by measuring 1982 shoot lengths in 10 ELS-affected and 10 symptomless American elms in Washington, DC. Trees ranged in size from 20-80 cm dbh. Ten shoots were measured at branch apices in the upper canopy using an aerial platform. Elongation measurements were made in the upper canopy in order to limit the effects of light competition on stem elongation.

Leaf Size

Leaf sizes were compared in the same trees in June 1983. Five branches were sampled randomly per tree and 5 fully expanded leaves from the lower portion of each shoot were collected. Leaf area measurements

were made using a portable area meter (Lambda Instruments Corp., Lincoln, NE).

Stem Hydraulic Conductivity

Comparisons of stem hydraulic conductivity were made monthly between ELS-affected and symptomless trees from June to September of 1983. Three stems were collected randomly from within the canopy of each of 13 ELS-affeceted and 10 symptomless trees in Washington, DC. In September, only 12 ELS-affected trees were sampled due to the removal of 1 tree. Stems were cut into segments (approximately 15 cm in length), brought into the laboratory, and vacuum infiltrated with a sterile phosphate-buffered citrate magnesium solution (Davis *et al.*, 1980c) at 2 atmospheres vacuum for a maximum of 2 min. (120 sec.), as previously described (Hearon *et al.*, 1980). Buffer volumes were proportional to the volume of the vascular cylinder.

Xylem Dysfunction/Occlusion

Stem segments (0.5 cm in dia. x 10 cm in length) from 10 ELS-affected and 10 symptomless elms were vacuum infiltrated with a 0.01% aqueous crystal violet solution. Three stems were collected randomly from selected trees growing in Washington, DC, in September 1983 and stored under refrigeration in polyethylene bags. All measurements were made within 4 days of sampling. Stem segments were connected to a dye reservoir in normal sap flow orientation and infiltrated under 3/4 atmosphere vacuum (Young, 1980) for a maximum of 3 min. Three segments were measure per tree. After infiltration, stems were cut at 0.5, 2.5,

5.0, 7.5, and 9.5 cm and the number of stained vessels counted. Dye infiltration rates were measured for each stem segment. The mean of the infiltration rates and the number of dye stained vessels at each stem level were determined for the 3 stems and each mean was assigned to the respective tree sampled.

Stem Water Potential

Stem water potential was measured monthly from June to September 1983 using a pressure bomb and techniques described by Scholander et al. (1965). Three randomly selected apical shoots with 3-5 leaves attached were measured within 2 min. of collection per tree per sampling date. From June to August, stem water potentials were measured on each sampling date in 13 ELS-affected and 10 symptomless trees. In September, stem water potential was measured in 7 ELS-affected and 7 symptomless trees.

Stem Starch Analysis

Ten ELS-affected and 9 symptomless American elms were selected for analysis of stem starch content. In November 1982, stem samples were collected randomly from within the canopy, cut into 15-20 cm lengths and transported to Amherst, MA, at 0 C. Stems were stored at -20 C.

One stem from each of the 3 sampled branches was thawed and sectioned with a pruning shears into segments (approximately 0.5 cm thick) and stained with an iodine-potassium iodide solution (Wargo, 1975; Carroll et al., 1983). The surface to be stained was trimmed

with a razor prior to sectioning to provide a smooth surface for uniform stain penetration. Three segments from each stem were stained; a total of 9 segments per tree. After staining, stem segments were coated with glycerin to prevent drying. Each stained segment was examined with a dissecting microscope and visually scored for starch content as high = 3, medium = 2, low = 1, or depleted = 0. All segments were rated by 3 individuals to avoid in ratings. The mean of the ratings was the numerical starch value given to each tree.

Results

Terminal Elongation and Leaf Size

Elm leaf scorch caused a reduction in terminal elongation in affected trees in comaprison to symptomless trees. The mean stem elongation in the 10 ELS-affected trees was 27.7 cm while in symptomless trees was 42.9 cm. Statistical analysis of data using analysis of variance (ANOVA) indicated a significant difference (P=.05) in stem elongation between ELS-affected and symptomless trees. Elm leaf scorch did not cause a significant reduction in leaf size between diseased and symptomless trees (40.5 cm^2 versus 44.5 cm^2).

Stem Hydraulic Conductivity

Stem hydraulic conductivity in ELS-affected trees decreased over the 4 monthly measurement dates (0.077 ml/min/mm^2 to 0.039 ml/min/mm^2) while hydraulic conductivity for symptomless trees increased over the same period (0.112 ml/min/mm^2 to 0.14 ml/min/mm^2) (Fig. 19).

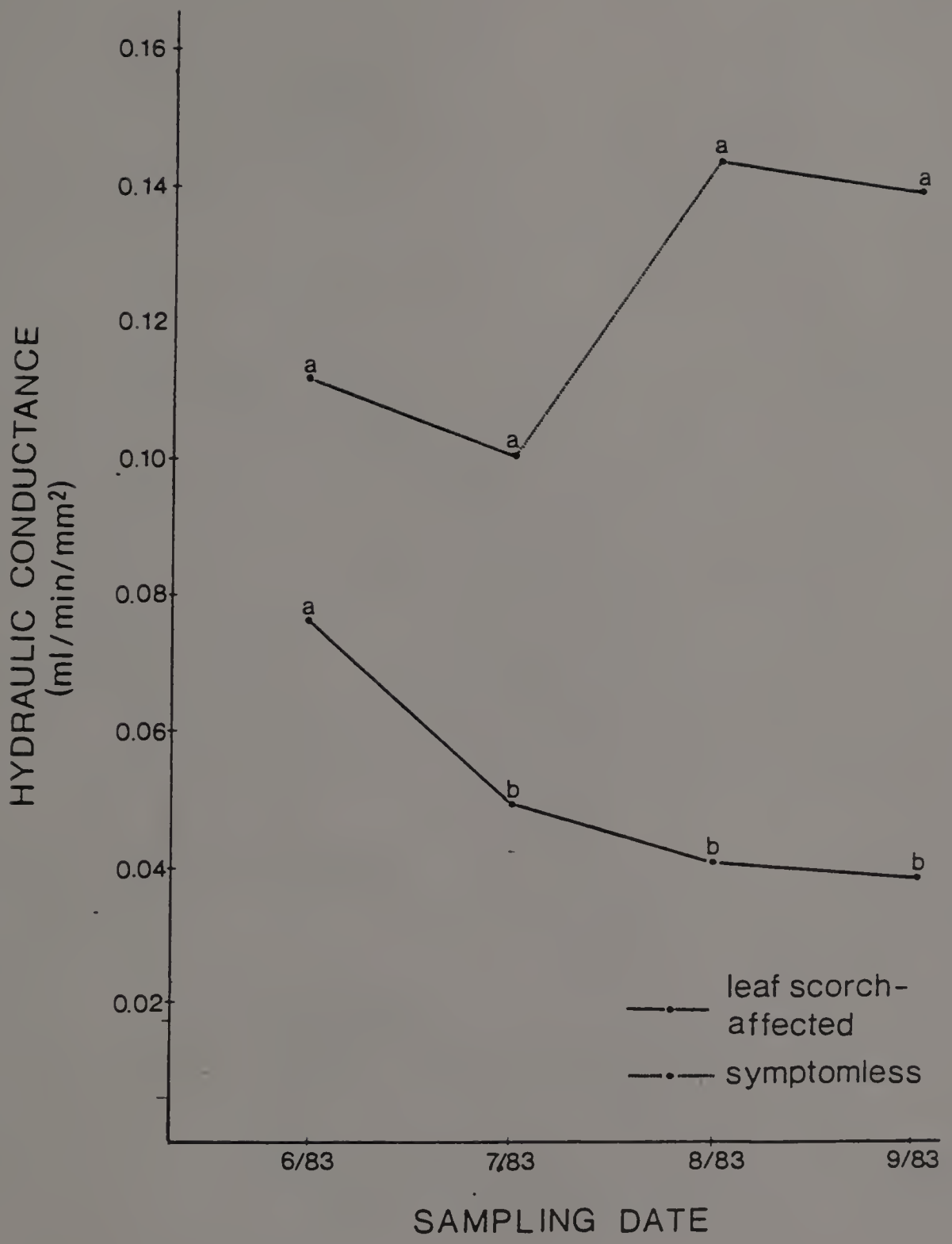

Fig. 19. Mean stem hydraulic conductivity in leaf scorch-affected and symptomless American elms sampled in Washington, DC, in 1983. Values with the same letter are not significantly different according to analysis of variance (P=0.05). Each data point represents the mean of the number of stained vessels in segments from 10 trees.

Statistical analysis (ANOVA) showed no significant difference in June hydraulic conductivity between (pre-symptomatic) ELS-affected trees and symptomless trees. The means for stem hydraulic conductivity were significantly different between ELS-affected and symptomless trees in July (P=.05), August (P=.01), and September (P=.01)(Fig. 19). The decrease in hydraulic conductivity in diseased trees over the summer months indicates a reduced efficiency of the xylem in diseased trees. The restricted flow through the xylem supports the hypothesis that bacterial infection causes xylem occlusion in trees with ELS. (Hopkins, 1977, 1981).

Xylem Dysfunction/Occlusion

Dye conductance rates of 0.14 ml/min in stems of ELS-affected trees were significantly different (ANOVA, P=0.05) from conductance rates in stem segments from symptomless trees (Table 12). Numbers of stained vessels, representing functional conducting vessels, were consistently higher in symptomless elms than in ELS-affected trees (Table 12). Statistical analysis of data (ANOVA) demonstrated a highly significant difference (P=0.01) between the number of functional vessels in ELS-affected and symptomless trees in the stem sections cut at 0.5, 5.0, 7.5, and 9.5 cm, but not significant at 2.5 cm.(Table 12). The observed decrease in functional vessels over the length of the stem and the significant difference in stained vessels between diseased and symptomless trees was anticipated if tissues were extensively inhabited or occluded by the ELS-bacterium or products of pathogenesis.

TABLE 12. Differences in dye conductance and vessel staining in elm leaf scorch-affected and symptomless American elms in Washington, DC

Crown Condition	Dye Conductance ml/min. (P=.05)	Number stained vessels/segment level*				
		0.5	2.5	5.0	7.5	9.5
Leaf scorch-affected	0.14 a**	211 a	70 a	22 a	13 a	10 a
Symptomless	0.40 b	380 b	132 a	69 b	52 b	41 b

* Five sections were cut from the infiltrated stem at 0.5, 2.5, 5.0, 7.5, and 9.5 cm and number of stained vessels counted. Each value is the mean of 10 trees. For each variable, values with a common letter are not significant according to analysis of variance (P = 0.01).

** Each value is the mean of 10 trees. For each variable, values with a common letter are not significant according to analysis of variance (P = 0.05).

Stem Water Potential

Stem water potential patterns paralleled xylem infiltration results (Fig. 20). Analysis of data using ANOVA failed to show any significant difference between means of ELS-affected and symptomless trees prior to symptom development (June). Once symptoms developed in July, a significant difference (P=.05) was observed between ELS-affected and symptomless trees. As symptoms became more severe in August and September, the water potential became lower (more negative) in ELS-affected trees and highly significant differences between stem water potentials in diseased and symptomless trees became more apparent (P=.01)(Fig. 20).

Stem Starch Analysis

Mean stem starch levels were higher in the canopy of symptomless American elms than in ELS-affected trees (Table 13). Statistical analysis (ANOVA) of data indicated that mean stem starch levels in ELS-affected trees were significantly different from starch levels in symptomless trees. Based on the 27 ratings per tree, stem starch means ranged from 1.67 to 2.89 in symptomless trees and from 0.55 to 2.67 in ELS-affected trees (Table 13). Stem starch levels in ELS-affected trees did not correspond consistently with the crown condition. ELS symptoms were extensive in tree B215 and tree J26 (75% and 90+% of the canopy leaf area affected in each tree, respectively) but stem starch ratings were high (2.56 and 2.0, respectively)(Table 13).

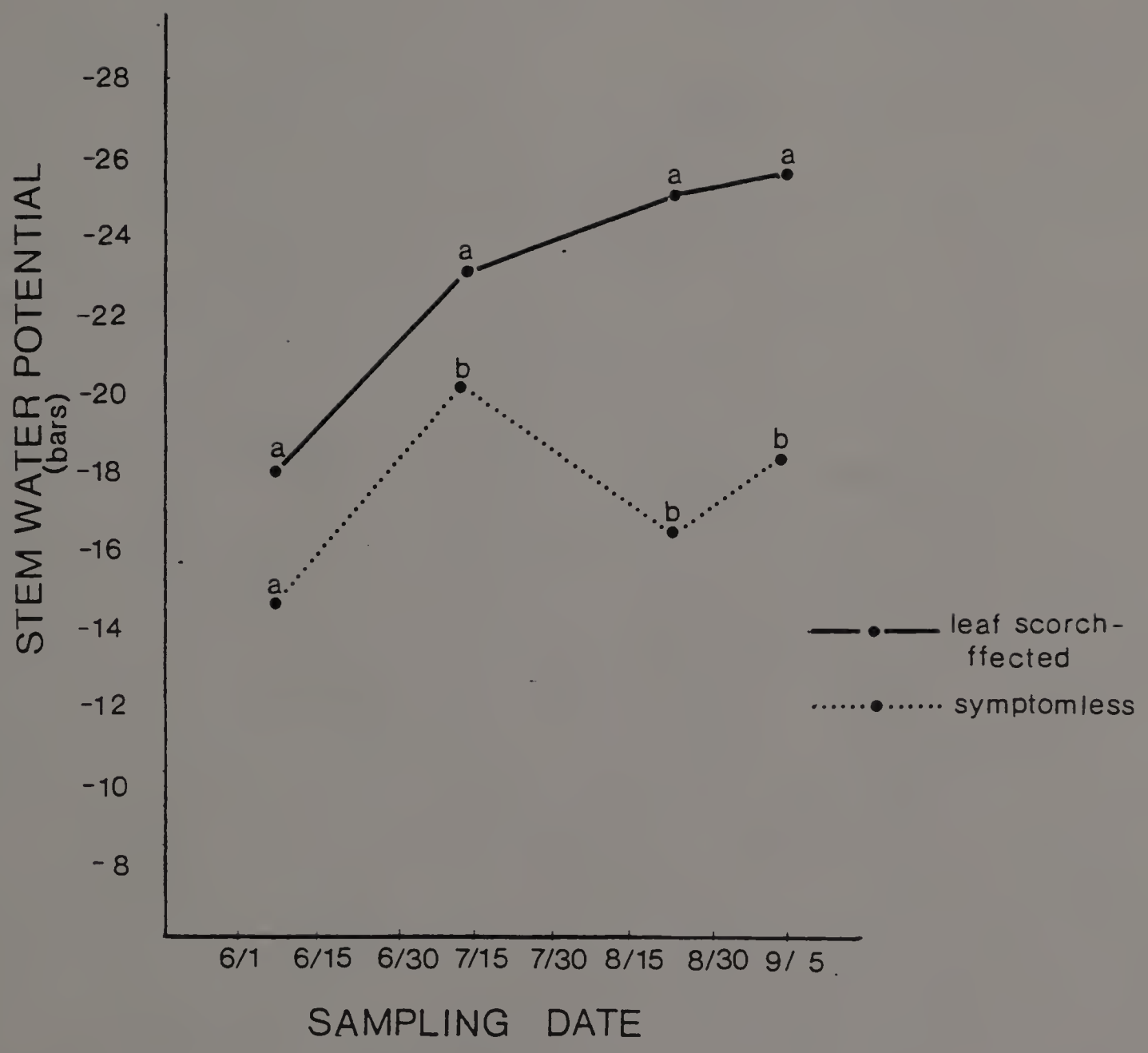

Fig. 20. Mean stem water potentials in leaf scorch-affected and symptomless American elms sampled in Washington, DC, in 1983. Values with the same letter are not significantly different according to analysis of variance (P=0.05). Each data point represents the mean of 3 readings from each of at least 7 trees.

TABLE 13. Crown condition, canopy leaf area with elm leaf scorch symptoms, and mean stem starch ratings in symptomless and elm leaf scorch-affected American elms[a] in Washington, DC, in 1983

Tree number	Crown condition	Percent Canopy Leaf Area Affected	Stem Starch Rating
EC4	symptomless	0	2.33
EC139	"	0	2.67
J21	"	0	1.67
J27	"	0	2.11
J181	"	0	2.33
J200	"	0	2.44
J211	"	0	2.89
J213	"	0	2.89
J217	"	0	2.22
ES6	scorching	90+	0.55
ES7	"	100	0.66
ES134	"	80	2.67
B169	"	80	2.22
B215	"	75	2.56
I47	"	75	0.78
J26	"	80+	2.00
J28	"	10	2.22
J174	"	20	1.33
J208	"	90	1.67

[a] All sampled trees were 20-80 cm dbh and located in turfed areas in a park or adjacent to roadways.

Discussion

Elm leaf scorch deleteriously affects stem growth, xylem function, water relations, and stem starch reserves in affected trees. All of the symptoms and growth responses observed in ELS-affected trees can be attributed, at least in part, to water stress. The stem water potential data presented confirm that diseased plants were growing under a water deficit which could be attributed to xylem dysfunction and occlusion.

Xylem dysfunction and occlusion may be due to physical occlusion of the xylem by bacterial cells (Wallis and Truter, 1978), accumulation of bacterial produced polysaccharides (Strobel, 1967; Strobel *et al.*, 1972; Van Alfen and Allard-Turner, 1978), or host production of gels, gums, and tyloses in response to infection (Beckman and Halmos, 1962; Talboys, 1968; Beckman, 1969; Robb *et al.*, 1975; Vander Molen *et al.*, 1977; Wallis, 1977). Zimmerman and McDonough (1978) have suggested that host-produced occlusive materials may be produced in response to pathogen-induced xylem embolism. Hearon *et al.* (1980) observed bacterial occlusions associated with gels or gums and tyloses in vessels of American elms, sycamores, and red oaks with leaf scorch.

The site of xylem occlusion in the plant vascular system determines the degree of effects on xylem water relations. If an infection occurred in the stem of a suscept, a relatively small change in water potential would result (Dimond, 1966; Duniway, 1973). However if the occlusion occurred in the petiole, there would be no alternative pathways for water translocation and water potentials low enough to cause wilting and death could be induced. Should massive occlusion of the xylem in the

stem occur, the outcome would be the death of the entire plant. If sub-acute water stress as described by Talboys (1968) is occurring in trees with ELS, it would account for the observed leaf scorch symptoms.

Plants growing under stress conditions have reduced starch reserves (Wargo et al., 1972; Carroll et al., 1983) and decreased growth (Boyer, 1973; Hsiao, 1973; Kramer and Kozlowski, 1979; Carroll et al., 1983). This study demonstrates a reduction in stem starch content in ELS-affected trees in comparison to symptomless elms as well as a reduction in stem elongation. Decreased stem growth can cause a decrease in the total number of leaves produced on the stem. Leaf scorch decreases the total functional photosynthetic area of affected leaves and due to premature abscision and decreased stem elongation, there are fewer photosynthetic units (leaves), thus causing a reduction in the net seasonal photosynthate (sugars) produced. A reduction in the photosynthetic capacity causes a reduction in the net photosynthate available for conversion to starch for storage.

The reduction in stored root starch in stressed trees deleteriously affects plant growth and survival (Wargo et al., 1972; Carroll et al., 1983). Once plants reach a depleted or near depleted level of stored root starch, stored energy for bud development and expansion and stem elongation becomes limiting and may lead to tree death. Although root starch levels have not been monitored in ELS-affected elms, a similar situation appears to exist with regard to stem starch content and branch dieback.

C H A P T E R VII

MULBERRY LEAF SCORCH: A NEW DISEASE CAUSED BY A FASTIDIOUS, XYLEM-INHABITING BACTERIUM

Introduction

Mid-summer leaf scorch is a common problem of deciduous trees in the mid-Atlantic and southern states. Leaf scorch disorders have been attributed commonly to a number of abiotic stresses, particularly drought (Tattar, 1978). In 1980, a novel group of fastidious, xylem-limited bacteria (FXLB) were associated with leaf scorch diseases of American elm, sycamore, and red oak (Hearon *et al.*, 1980). The associated bacteria were morphologically similar to and serologically related to the FXLB that causes almond leaf scorch and Pierce's disease (PD) of bunch grapes (Hearon *et al.*, 1980; Sherald *et al.*, 1983). Recently, Koch's postulates were satisfied for the bacterium cultured from leaf scorch-affected sycamores (Sherald *et al.*, 1983).

While surveying shade trees for the above mentioned diseases, numerous red mulberries (*Morus rubra* L.) were observed that exhibited leaf scorch symptoms. In this paper, we describe: 1) the symptoms and distribution of mulberry leaf scorch (MLS) in the eastern United States, 2) the isolation and culture of a bacterium from diseased trees, 3) the pathogenicity of three isolates of the bacterium, and 4) morphological similarities and serological relatedness of the MLS-bacterium to other FXLB. Preliminary reports have been published (Kostka *et al.*, 1982, 1983).

Materials and Methods

Symptomatology and Distribution

Leaf scorch-affected mulberries in the Washington, DC area were monitored monthly throughout the 1982 and 1983 growing seasons to follow symptom development. In August 1983, a natural population of red mulberries along 3 km of the George Washington Memorial Parkway in Alexandria, VA (a Washington, DC suburb) was surveyed to determine the incidence of MLS.

In August 1983, mulberries growing in both rural and urban roadside and natural sites were surveyed from northern Virginia through the eastern mid-Atlantic States to the northern range of red mulberry in southern New England to determine disease distribution.

Isolation and Culture

Stem samples were collected from 22 leaf scorch-affected and 19 symptomless red mulberries in Washington, DC and Arlington, VA. Sampled plants ranged from seedlings (1 m in height) to mature trees. Presence of xylem-inhabiting bacteria in collected stem segments was determined by infiltration of stem segments (0.5-1.0 cm X 8.0 cm) with sterile distilled water and examination of the extracts by phase contrast microscopy (French et al., 1977a).

For bacterial isolation, stem segments (1 cm X 15-20 cm) were rinsed in 70% ethanol and flamed. Bark was removed aseptically and 2-3 wood chips (0.5 cm X 1-2 cm) were excised and placed in 10 ml of a broth

formulation of periwinkle wilt medium (PW) (Davis et al., 1981a) supplemented with 0.85 g $(NH_4)_2HPO_4$, 2 g soluble potato starch, 1 g L-histidine, and 25 mg cyclohexamide per liter (Sherald et al., 1983). Cultures were incubated in the dark at 28 C and examined for turbidity daily for up to 7 days then at 4-7 day intervals.

Isolations from petioles were made on semi-solid PD-4 (Davis et al., 1980b) and modified PW agar plates using a petiole centrifugation technique or a crushed petiole technique modified from studies on PD and almond leaf scorch (Davis et al., 1980b, 1980c). Leaves were collected from symptomatic and symptomless trees, petioles excised and disinfested for 2 min. in a 0.5% sodium hypochlorite solution, then rinsed twice in sterile distilled water. Sap was extracted by placing 1.0-1.5 cm petiole segments in 0.5 ml of a sterile, phosphate-buffered citrate-magnesium solution (PBCM)(Davis et al., 1980b) and centrifuged for 2 min. at 15,600 x G in an Eppendorf Microfuge Model 5412. Bacteria were resuspended by vortexing and 0.05 ml aliquots were placed as droplets on semi-solid media. For crushed petiole extraction, petioles were disinfested as above and aseptically cut into segments. Segments were crushed aseptically with a pliers and the droplet adhering to the petiole segment was blotted onto the semi-solid medium. After initial isolation, all isolates were maintained through biweekly transfer on semi-solid PD-4 medium.

Isolates were tested for their ability to grow on the following common microbiological media: nutrient agar, King's medium B agar, potato dextrose agar, yeast dextrose calcium carbonate agar (YDC), D-1 agar, and crystal violet polypectate agar (CVP). All cultures were

incubated in the dark at 28 C. Preliminary screening of all isolates was made using phase contrast microscopy (1000X) for the presence of rod-shaped cells similar to other FXLB. Selected isolates were tested for Gram stain and catalase and oxidase reactions.

Serology

The serological relatedness of isolated bacteria to other FXLB was determined using indirect immunofluorescent antibody staining (IFAS) (Goldman, 1968; Hearon *et al.*, 1980) and antisera prepared against whole cell preparations of the PD- and ELS-bacteria (S. Kostka, unpublished). Cultures of the PD-bacterium and the ELS-bacterium were treated in the same manner. Control slides were reacted with rabbit pre-immune serum.

Pathogenicity Tests

Three isolates obtained in September 1982 from scorch-affected mulberries in the Washington, DC area were selected for pathogenicity tests. Bacterial colonies grown for 7-10 days on semi-solid PD-4, were washed from the agar, suspended by vortexing in sterile PBCM. Cells were standardized to 1 X 10^8-10^9 cells per milliliter using a bacterial counting chamber (Hausser Scientific, Blue Ball, PA). In January 1983, 12 greenhouse grown, potted mulberry seedlings, approximately 50 cm tall were inoculated. Each isolate was inoculated into four seedlings using three inoculation techniques.

Seedlings were unpotted and a major root was surface disinfested with 70% ethanol then severed with a sterile pruning shears. The

severed root was immediately connected via a segment of latex tubing to a 1 ml pipette containing 1 ml of bacterial inoculum. Seedlings were repotted with the pipette connected to the root and watered. After repotting, a surface disinfested stem approximately 0.4-0.5 cm in diameter was excised from near the apical point of the plant and a latex tubing reservoir attached. Approximately 0.5 ml of inoculum was placed in the reservoir and allowed to be taken up by the receding embolism. In addition, two sites along the stem were surface disinfested with 70% ethanol and two incisions made to the xylem with a sterile scalpel. Inoculum (0.02-0.03 ml) was placed in each of the two stem incisions with a hypodermic syringe. Four control seedlings were inoculated using the same techniques. Control inoculum consisted of sterile PBCM washings of uninoculated semi-solid PD-4 plates. Inoculum uptake was rapid via all inoculation routes; minutes for the xylem incision and cut stem techniques, and within 14 hours via the severed root. Plants were maintained under greenhouse conditions and watered sparingly through April 1983.

Electron microscopy

For ultrastructural studies, symptomatic and symptomless leaves were collected from an inoculated mulberry seedling. Primary and secondary veins were excised from non-necrotic tissue adjacent to scorched tissues and from analagous areas in symptomless leaves. Samples were prepared and examined as previously described (Hearon et al., 1980).

In conjunction with isolation studies, negative stain preparations were made of cultured bacteria (Sherald et al., 19834).

Results

Symptomatology and Distribution

Symptoms of MLS appeared in mid-July (1982 and 1983) in the Washington, DC area as a marginal leaf desiccation and curl. Although a major portion of or the entire leaf lamina may have been desiccated, only slight discoloration occured initially, (Fig. 21). Once tissues became desiccated, the lamina took on a water-soaked appearence and necrosis developed, first in the desiccated tissues adjacent to the unaffected tissues then outward in the leaf lamina to the margin.

Advanced symptoms were characterized by a marginal, undulating leaf necrosis bordered by a distinctive chlorotic halo (Fig. 22). Symptoms progressed apically and leaves in all stages of symptom development were observed on the same branch. Severely scorched leaves abscised prematurely, leaving tufts of symptomless leaves at the branch apex. Branch dieback was observed in affected trees, but no mortality was noted.

One hundred and sixty mulberries ranging in height from 2 m tall saplings to mature trees were surveyed along 3 km of the George Wasington Memorial Parkway in Alexandria, VA. Mulberries were common understory plants in sites containing leaf scorch-affected sycamores and red oaks. Of this population, 125 trees or 78 percent of the population

Fig. 21. Initial symptoms of mulberry leaf scorch; note the marginal tissue desiccation of the leaf on the right (indicated by the pointer) and the severe leaf curl on the left.

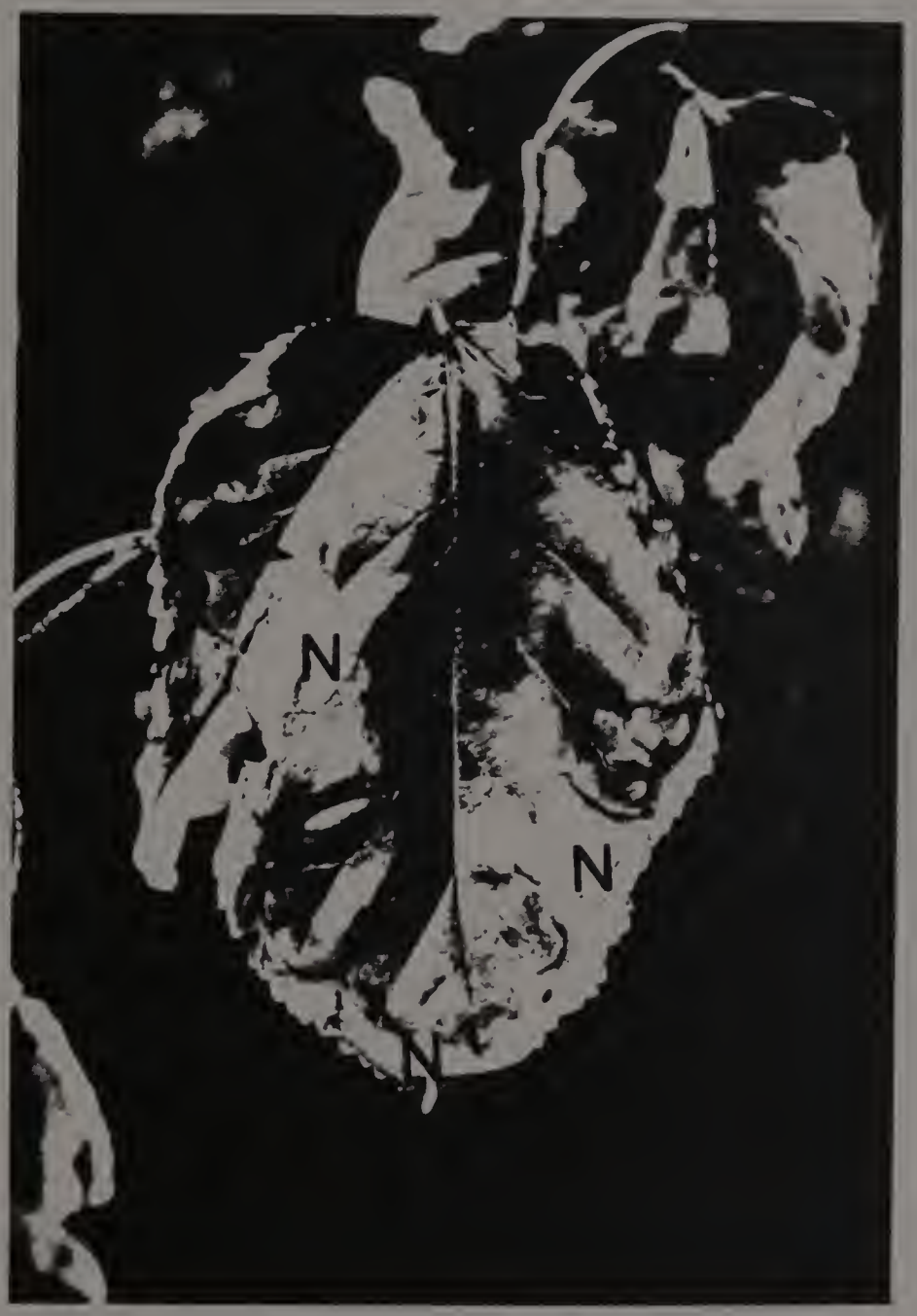

Fig. 22. Marginal necrosis (N) and bounding chlorotic halo (arrows) of advanced symptoms of mulberry leaf scorch.

expressed characteristic MLS symptoms. Symptoms were severe in all affected plants.

The disease ranged from northern Virginia and the District of Columbia, east throughout the Delmarva penninsula and north through Maryland, Delaware, eastern Pennsylvania, and New Jersey to New York City and New Rochelle, NY (Fig. 23). The disease was widespread throughout much of the northeastern range of red mulberry and occurred in both urban and non-urban trees. The disease was not apparent in escape populations of red mulberry in the southern New England states.

Isolation and Culture

Vacuum infiltration of stem segments proved inadequate for determining the presence of xylem-inhabiting bacteria in stem segments collected from scorching trees. Bacteria were difficult to identify in extracts examined by phase contrast microscopy due to the presence of excessive crystalline, cellular debris. Bacteria were identified by repeated examination from 9 of 12 samples. Because of the difficulty in observing bacteria in the extracts, the vacuum infiltration technique was discontinued.

Bacteria were readily isolated from stem segments of affected trees when using modified PW broth and wood chip isolation. Turbidity commonly developed in cultures within in 5-7 days. Bacteria were isolated from 19 of 22 leaf scorch-affected mulberries and from 3 of 19 symptomless trees in the Washington, DC area. The MLS-bacterium was cultured from stems of affected trees in 7 days in simultaneously inoculated broth cultures of modified PW medium and PD-2 medium.

Bacterial growth was detected in crushed or centifuged petiole isolations after 18 days incubation on both semi-solid PD-4 and modified PW. Colonies on PD-4 agar were white, convex, with entire margins and approximately 0.5-0.8 mm in diameter. Darkened areas were observed at one or both ends of the organism under phase contrast microscopy. On modified PW agar, colonies were also 0.5-0.8 mm in diameter, but were buff white in color and more appressed to the medium. Bacteria stained Gram negative, and were catalase positive and oxidase negative. Bacterial cells from agar and broth culture measured 0.5 µm wide by 1-3 µm im length using phase contrast microscopy and an eyepiece micrometer. Growth was not observed on potato dextrose agar, King's medium B agar, D-1 agar, or YDC agar. Bacterial growth was obtained on nutrient agar after 2 weeks incubation and was maintained through 3 serial transfers. Isolates subcultured on PD-4 from nutrient agar cultures produced bacterial colonies and cells indistinguishible to those in the original PD-4 cultures. The bacterium was gram negative, catalase positive, and oxidase negative.

Isolations from leaf scorch-affected mulberries in all localities where the disease was observed (Fig. 23) consistently yielded fastidious, xylem-inhabiting bacteria (Table 14). In addition to isolation of the bacterium in the metropolitan Washington, DC area (northern Virginia and Maryland suburbs, the bacterium was also isolated from all of the 26 MLS-affected trees sampled in Maryland, Delaware, Pennsylvania, New Jersey, and New York (Fig. 23, Table 14).

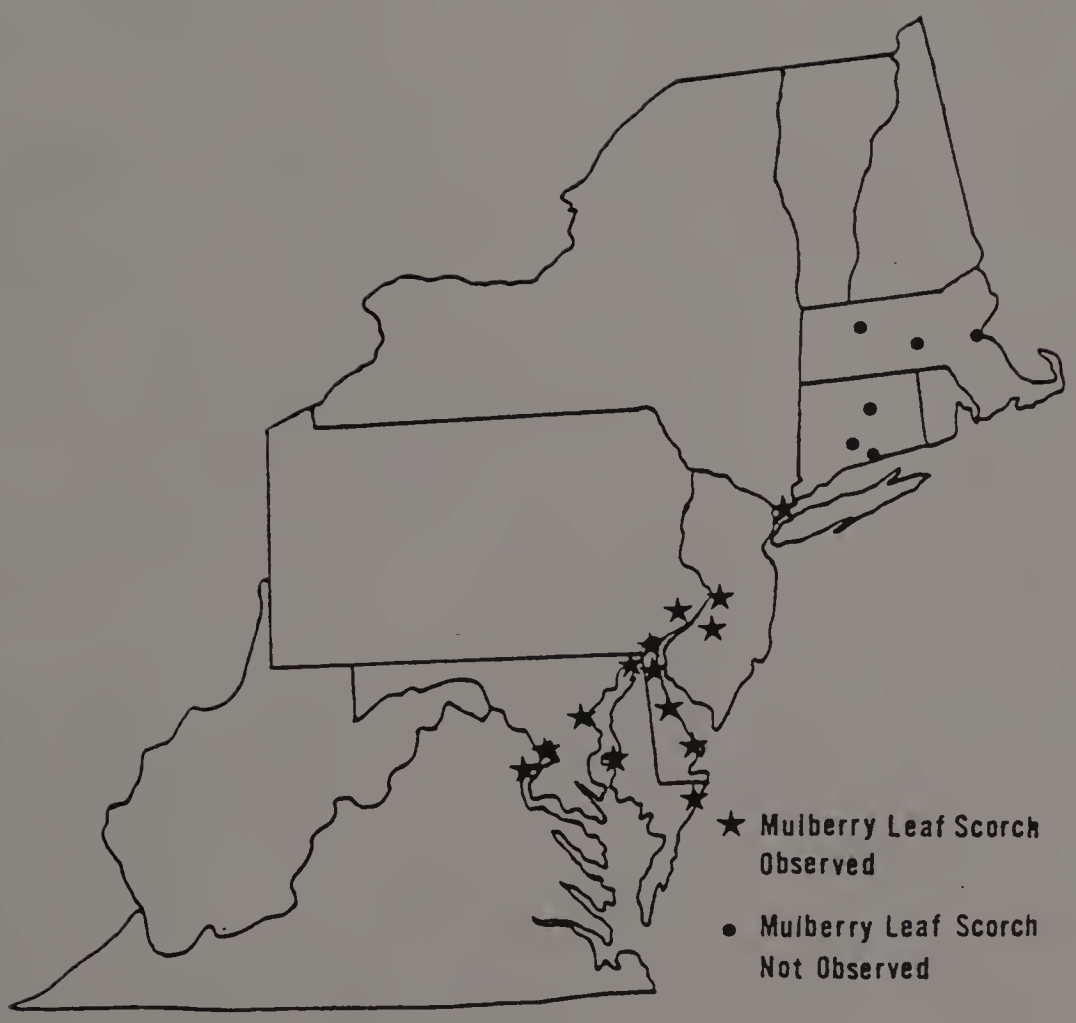

Fig. 23. Distribution of mulberry leaf scorch in the mid-atlantic and northeast coastal states.

TABLE 14. Isolation of fastidious, xylem-inhabiting bacteria from leaf scorch-affected mulberries in localities surveyed outside the Washington, DC area in 1982 and 1983

Locality	Ratio (number isolations/number trees sampled)
Baltimore, MD	1/1
Kent Island, MD	1/1
Ocean City, MD	1/1
Elkton, MD	2/2
Lewes, DE	2/2
Dover, DE	4/4
New Castle, DE	1/1
Wilmington, DE	3/3
Berwyn, PA	2/2
Masonville, NJ	1/1
Hedding, NJ	2/2
New York, NY	2/2
New Rochelle, NY	3/3
TOTAL	26/26

Serology

Bacterial isolates fluoresced strongly when reacted with antisera to the ELS- or PD-bacteria in the indirect IFAS test. Similarily, the ELS- and PD-bacteria reacted strongly with either antiserum. No reaction was obtained when isolates were reacted with preimmune sera. Isolates subcultured on nutrient agar produced the same results with indirect IFAS.

Pathogenicity Tests

Characteristic MLS symptoms developed in 3 of 12 inoculated seedlings 3 months after inoculation (Table 15). Isolations were made from the 3 symptomatic seedlings and the MLS-bacterium was recovered from each and its identity confirmed using indirect IFAS and antisera to the ELS- and PD-bacteria. Plants defoliated soon after and were fertilized to force bud break. Six months after inoculation symptoms developed in 10 of the 12 inoculated seedlings (Table 15). The MLS-bacterium was reisolated from the 10 symptomatic seedlings and from 1 symptomless bacteria-inoculated seedling. No bacteria were recovered from the 4 control seedlings.

Electron Microscopy

The MLS-bacterium was observed in the xylem vessels of symptomatic leaves collected from the inoculated mulberry seedling (Fig. 24, 25). Cells measured 0.3-0.4 μm in width by 1-2 μm in length and had a rippled cell wall. Bacteria were embedded in a lightly staining matrix (Fig. 24, 25) which contained nodulated strands surrounding the bacteria and

TABLE 15. Symptom development and recovery of bacteria from mulberry seedlings inoculated with pure cultures of the mulberry leaf scorch-associated bacterium

		April 1983		July 1983	
Treatment	No. Trees Treated	Symptomatic Trees	Bacterial Recovery	Symptomatic Trees	Bacterial Recovery
Isolate MS1	4	1	1/1	4	4/4
Isolate M3	4	2	2/2	4	4/4
Isolate M4	4	0	0/0	2	3/4
Sterile Buffer	4	0	0/0	0	0/4

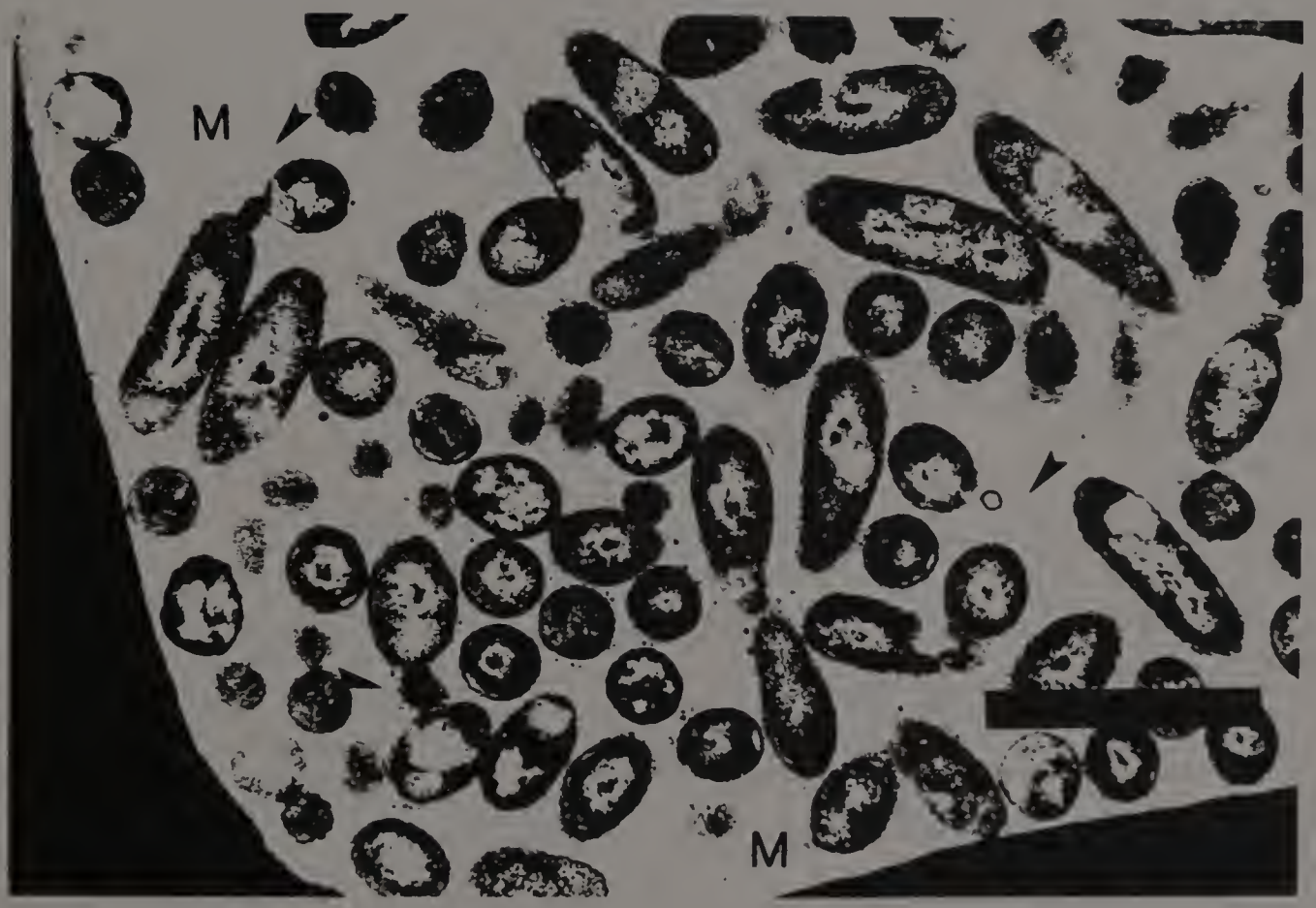

Fig. 24. Transmission electron micrograph of transverse and longitudinal sections of the mulberry leaf scorch bacterium in a vessel of a symptomatic leaf from an inoculated plant. Bacteria appear embedded in a lightly staining matrix (M). Nodulated tubular structures surround the bacteria and extend into the matrix (Arrows). Scale bar = 1 μm.

Fig. 25. Transmission electron micrograph of a transverse section xylem vessel with bacteria lodged in the pit (P) and an appressed, potentially degenerative bacterial cell (Arrow). Note the variation in matrix staining (M). Scale bar = 1 μm.

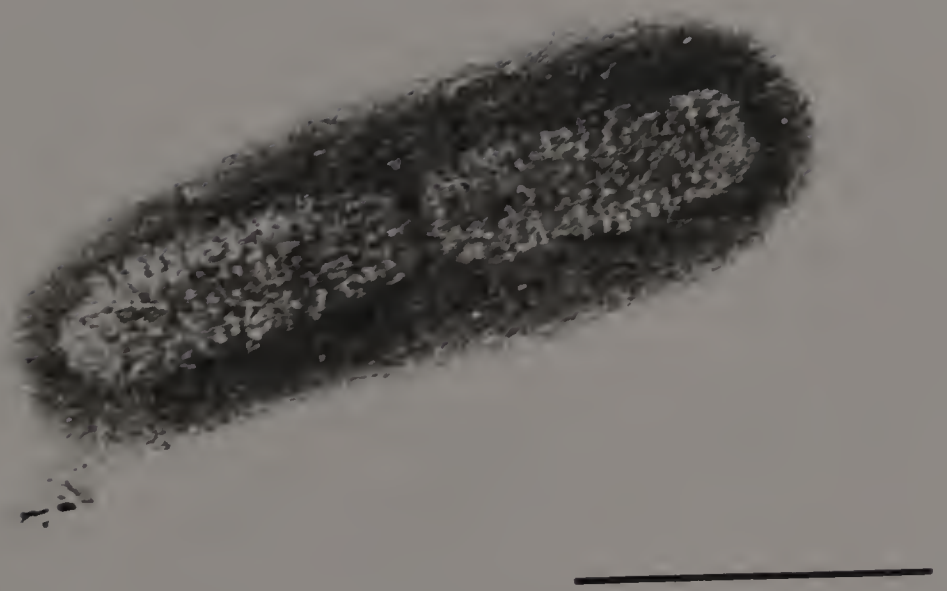

Fig. 26. Transmission electron micrograph of negatively stained mulberry leaf scorch bacteria from cultures showing typical "rippled" cell wall topography. Scale bar = 1 μm.

extending into the matrix (Fig. 24). Variations in matrix staining occurred between adjacent vessels (Fig. 25). Appressed, more densely staining cells, possibly degenerative forms of the bacterium, were observed (Fig 25). Cells lodged in pits were compressed and surrounded by granular material and fibrous strands.

Negatively stained bacteria from cultures had the characteristic "rippled" cell wall topography of fastidious, xylem-inhabiting bacteria. Cultured bacteria were morphologically similar to cells found in xylem vessels (Fig. 26).

Discussion

This is the first report of mulberry leaf scorch (MLS), a newly observed disease caused by a fastidious, xylem-inhabiting bacterium. The bacterium is morphologically similar to and serologically related to two other FXLB; the ELS-bacterium, associated with elm leaf scorch, and the PD-bacterium, the causal agent of Pierce's disease of grapevines. Although the MLS-bacterium was isolated in/on both modified PW or PD-2/PD-4 media and using several isolation techniques, growth was more rapid when wood chips were incubated in broth media (5-7 days) than when bacteria from crushed petioles or centrifuged petioles were used as inoculum on semi-solid medium (18 days). The wood chip/broth isolation technique is rapid and suitable for making isolations from large numbers of samples. Contamination of cultures by other wood-inhabiting or epiphytic bacteria was not considered a problem. Less than 20% of all isolations became contaminated with other bacteria.

Based growth studies, the MLS-bacterium is less fastidious than the sycamore leaf scorch bacterium (Sherald et al., 1983), the oak leaf scorch bacterium (Chang and Walker, 1983; Kostka et al., 1984), or the ELS-bacterium (S. Kostka, unpublished), but comparable to the PD-bacterium which to date is the least fastidious of this group of bacteria (Davis et al., 1981b). The MLS-bacterium is distinct from other cultured fastidious, xylem-limited bacteria because of its ability to grow on a common microbiological medium. This ability indicates a less fastidious nature of the MLS-bacterium quite unlike that of other fastidious, xylem-inhabiting bacteria.

In 1983, MLS occurred at epiphytotic levels in northern Virginia and the District of Columbia. Disease incidence was high in all localities where the disease was observed. In addition to the northern range, similar symptoms were observed in New Orleans, LA, though isolation of the bacterium was not attempted (S. Kostka, unpublished).

Bacteria serologically related to the ELS- and PD-bacteria were consistently isolated from symptomatic trees throughout the range of the disease. The recovery of bacteria from 3 symptomless trees in northern Virginia and the District of Columbia is not surprising in view of the epiphytotic in that locale. The presence of the bacterium in these trees may indicate presymptomatic infection. Leaf scorch symptoms caused by FXLB may be due to bacterial restriction of water translocation (Hopkins, 1977) and/or pathogen produced toxins (Lee et al., 1982). It is not known whether one or both of these factors are involved in MLS.

This study shows that MLS is the most widespread disease caused by a fastidious, xylem-inhabiting bacterium in the eastern United States. Further, this is the northern-most report of a disease caused by a member of this group of phytopathogenic bacteria.

C H A P T E R VIII

DISCUSSION AND CONCLUSIONS

Fastidious, xylem-inhabiting bacteria (FXIB) were consistently associated with a mid-summer leaf scorch of American elm and red mulberry, with Koch's postulates being fulfilled in mulberry. All isolated bacteria were morphlogically similar and serologically related to the ELS- and PD-bacteria. Although Koch's postulates were not fulfilled with the ELS-bacterium, evidence was accumulated to support the role of the bacterium as the causal agent; 1. the consistent isolation of FXIB from leaf scorch-affected, but not symptomless elms; 2. the graft-transmissibility of the bacterium and induction of appropriate leaf scorch symptoms in the grafted scions and systemically in the adjacent branches; 3. the serological relatedness of the ELS-bacterium to the PD-bacterium which causes similar symptoms in grape; and 4. the remission of symptoms in ELS-affected trees injected with oxytetracycline.

The inability to fulfill Koch's postulates with the ELS-bacterium may be due to a variety of factors. Isolate virulence may have been lost through repeated passages on artifical media. However, when newly cultured isolates were used in one study, no symptoms developed in the inoculated plants. In mulberry, loss of virulence did not occur even after 5 months in culture. Perhaps more significant may be as yet undefined environmental conditions required for successful inoculation, infection, and symptom development. In addition, juvenile resistance as found in young elms inoculated with the Dutch elm disease fungus

Heybroek, 1957) may occur to the ELS-bacterium. Inoculation of the ELS- or PD-bacterium into different age elms (4 months to 3+ yrs.) both under greenhouse and field conditions with failed to incite symptoms consistently. Of the 2 trees (field grown and branch inoculated) that did develop symptoms, 1 was inoculated with the PD-bacterium and the other with an isolate of the ELS-bacterium. Symptoms in the tree inoculated with the PD-bacterium initially developed in the inoculated stems then progressed to adjacent stems and systemically throughout the tree. Symptoms in the tree inoculated with an isolate of the ELS-bacterium, did not appear in the inoculated branch, but in an adjacent branch more than 2 m from the inoculation site. In the inoculated tree, the symptoms could be attributed to the inoculated bacterium, while in the tree inoculated with the ELS-bacterium isolate were likely caused by natural infection. The tree inoculated with the PD-bacterium was the only individual in which the involvement of a FXIB was confirmed as the causal agent and in which cross pathogenicity was demonstrated.

Inoculation route and conditions under which the trees were inoculated and maintained may be important factors in successful infection and symptom development. Well et al. (1983) found that peaches were infected successfully and developed symptoms after repeated root inoculation with the phony peach bacterium. Much of the root system was destroyed for each inoculation and plants were maintained under a reduced watering regime. In pathogenicity tests with the ELS-bacterium, American elm seedlings did not tolerate this manipulation. Root removal alone induced sufficient water stress to

cause development of drought scorch symptoms in plants that were not watered heavily after inoculation and repotting. Identification of the insect vector(s) of the ELS-bacterium should aid in identifying the time of year and conditions under which elms are susceptible.

Both ELS and MLS occurred in the Washington, DC, area and as far south as Louisiana. The northern distribution of the 2 diseases in the coastal eastern states differed. Elm leaf scorch was observed as far north as Baltimore, MD, and Lewes, DE, a range similar to that encountered with sycamore leaf scorch (S. Kostka, unpublished). Mulberry leaf scorch had a much greater northern distribution occurring as far north as New Rochelle, NY. Another leaf scorch disease associated with a FXIB, oak leaf scorch, has a range similar to that of MLS (Kostka, et al., 1984). Neither ELS nor MLS were observed in the southern New England states. Differences in disease distribution may be attributed to climatic differences, host susceptibility difference, absence of an as yet unidentified insect vector(s), or presence of other hosts which are the preferred hosts of the vector.

The symptoms observed in ELS-affected elms were analagous to those described by Talboys (1968) for plants under water stress. ELS-affected trees had a decreased stem hydraulic conductivity, a decreased number of functional vessels, and a higher (more negative) stem water potential than symptomless trees. Concommittant to this water deficiency was a decrease in stem elongation and a decrease in stem starch. The decreased growth and decreased stem starch indicate a plant under decline and dieback conditions (Carroll et al., 1983).

Based on the ability of the MLS-bacterium to grow on nutrient agar,

it was less fastidious than the ELS-bacterium, and is the least fastidious member of this group of bacteria. Different symptom types in leaf scorch-affected elms in addition to being related potentially to variation in the host, variation in the bacterium, or symptom progression, may be due to infection of the plant by different FXIB which each have a different genetic potential for causing disease in elms. Not until Koch's postulates are demonstrated in elm and the necessary environmental conditions for disease development defined can extensive comparisons between isolates of the ELS-bacterium and other FXIB be made.

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