# Evaluation of a Rapid ELISA Test Kit for Detection of *Xylella fastidiosa* in Landscape Trees

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**ABSTRACT**

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Leaf scorch symptoms on landscape trees infected with *Xylella fastidiosa* are often confused with similar symptoms caused by stress factors. Because the isolation of *X. fastidiosa* from trees is difficult and time-consuming, an ELISA test kit for *X. fastidiosa* was evaluated for reliability and feasibility. The pathogen was detected in all leaf and stem samples collected in June from asymptomatic American elms (*Ulmus americana*) and American sycamores (*Platanus occidentalis*) that had had leaf scorch in 26–100% of their canopy during the previous September. The kit was used in a survey of 47 elms. The pathogen was detected in extracts from 17 of 18 diseased trees—12 before and five after symptoms appeared. *X. fastidiosa* was also detected in extracts from red maples (*Acer rubrum*), red oaks (*Quercus rubra*), and red mulberries (*Morus rubra*) with characteristic leaf scorch symptoms but not in extracts from symptomless trees.

*Xylella fastidiosa* Wells et al (31) is the recently classified fastidious xylem-inhabiting bacterium that causes, or has been associated with, Pierce's disease of grape (3,8,14), almond leaf scorch (4,21), alfalfa dwarf (8,29), peach phony disease (15,23), plum leaf scald (16), periwinkle wilt (20), ragweed stunt (30), and citrus blight (12). In 1951, Freitag (6) reported leafhopper transmission of the causal agent of Pierce's disease from naturally infected plants of 36 species representing 18 families. Currently, over 30 families of monocotyledonous and dicotyledonous plants are known hosts. Many, however, do not develop symptoms when infected.

Chronic leaf scorch of several species of landscape trees also has been associated with systemic invasion by *X. fastidiosa*. Bacterial strains genotypically and phenotypically similar to *X. fastidiosa* have been isolated from the xylem tissue of American elms (*Ulmus americana* L.) (17), red oaks (*Quercus rubra* L.) (1,18), American sycamores (*Platanus occidentalis* L.) (25), and red mulberries (*Morus rubra* L.) (19) showing chronic leaf scorch symptoms (31). Another bacterial strain isolated from a red maple (*Acer rubrum* L.) with leaf scorch symptoms reacted positively in indirect ELISA with monoclonal antibodies specific to strains of *X. fastidiosa* from grape (27). Cultures of fastidious bacteria isolated from sycamores, mulberries, red oaks, and American elms with leaf scorch have been found to be pathogenic in their respective hosts and produce characteristic leaf scorch symptoms (1,19,25,26; J. L. Sherald, *unpublished*). The recent association of *X. fastidiosa* with leaf scorch in landscape trees further demonstrates the pathogenic versatility of this bacterium.

As with other diseases caused by *X. fastidiosa*, leaf scorch of landscape trees is most common in the South. Leaf scorch of red oak and mulberry, however, have been found as far north as New York State (18,19). Symptoms begin to develop in midsummer and intensify in severity throughout late summer and early fall (9). Leaves typically develop an irregular marginal or interveinal necrosis that is preceded by a border of chlorotic or reddish tissue. Necrosis progresses throughout the leaf, causing leaf curl and early defoliation. Symptoms in elm, sycamore, and mulberry progress acropetally, diminishing in intensity from older to younger leaves on a branch. Each year symptoms spread further throughout the tree. Dieback is common in scorch-affected elm, red oak, and sycamore and less common in red maple and mulberry. Chronic leaf scorch symptoms caused by *X. fastidiosa* can be confused with similar symptoms caused by other biotic and abiotic factors. This is particularly true of landscape trees affected by a variety of urban stress factors such as excess road salt, confined growing spaces, and drought (9).

Diagnosis of diseases caused by *X. fastidiosa* is hampered by the organism's fastidious growth requirements and generally slow growth in culture (5,31). The disease has been diagnosed in woody plants by direct observation of bacteria by phase-contrast microscopy in vacuum-extracts of stem sections (7,11). Strains have also been obtained by expressing sap from petioles onto semisolid media developed for *X. fastidiosa* or by incubating wood chip samples in broth formulations of the same media (5,19,25,27). However, sap is difficult to express from petioles of some woody plants, particularly from leaves with advanced symptom development, and wood chips typically require 2–4 wk of incubation before bacteria appear (25). With both techniques, contamination is a frequent problem, necessitating repeated isolation attempts. Once obtained, cultures must be identified by such conventional serological techniques as immunofluorescent antibody staining and enzyme-linked immunosorbent assay (ELISA).

The discovery of the susceptibility of landscape trees to *X. fastidiosa* has created an interest among diagnosticians, foresters, and arborists in the availability of rapid and reliable diagnostic techniques. Here we report an evaluation of the feasibility and reliability of a commercially available ELISA test kit for the diagnosis of *X. fastidiosa* in the five tree species previously determined to be infected with *X. fastidiosa*. ELISA has been used previously in detecting *X. fastidiosa* in grape, sycamore, and several natural hosts (10,13,22,24).

## MATERIALS AND METHODS

**Sample collection.** Five 1-yr-old American sycamore seedlings were inoculated in 1981 with a strain of *X. fastidiosa* isolated from a diseased tree (26). One year after inoculation, bacteria resembling *X. fastidiosa* were isolated from the inoculated trees but not from five control trees treated with buffer alone (26). Symptoms have appeared in the inoculated but not in the control trees every year since 1982. In 1986, 26–100% of their canopy showed leaf scorch, whereas control trees remained free from symptoms. Forty-seven American elm trees (30 cm mean diameter at 1.4 m above the ground) were also evaluated. These trees had been planted in four rows in a landscaped turf panel of the National

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Mall in Washington, D.C. Several of the trees were naturally infected, and their range of leaf scorch symptoms was rated for severity in September 1986.

Initially, samples were collected from sycamore trees on 5 May 1987 before symptoms had appeared. One branch with leaves expanded about 25% was collected from each of five trees that had been inoculated with *X. fastidiosa* or buffer. At the time of collection, the 7-yr-old trees were 5.4 cm in diameter at 1.4 m above the ground. Five naturally infected elm trees that had had 51–100% leaf scorch the previous fall and five symptomless trees were sampled similarly. A 30-cm branch with leaves almost fully expanded was collected at approximately 2–5 m above the ground from the four cardinal points of each elm tree. The four branches from each tree were combined for the test.

Subsequently, branches were collected from each of 47 elm trees on 6 June 1987 before symptoms had appeared. The trees were evaluated for symptom development in September, and samples were collected from each of the trees that had reacted negatively to ELISA in the June test. Whenever possible, samples collected in September included symptomatic branches.

Stem sections were collected from symptomatic limbs of five of the 47 elms for isolation of *X. fastidiosa*. Wood chips were removed and incubated at 28 C in 10 ml of periwinkle wilt (PW) broth medium (2) supplemented with 0.85 g of $(NH_4)_2HPO_4$, 2 g of potato starch, 1 g of L-histidine, and 25 mg of cyclohexamide per liter (25). Cultures were examined by phase-contrast microscopy (×1,000) for rod-shaped cells characteristic of *X. fastidiosa*. Strains isolated from two elms were subcultured on semisolid supplemented PW medium and retained for subsequent testing by ELISA.

Leaf samples were collected in September 1987 from red oaks, red maples, and red mulberries with symptoms characteristic of leaf scorch. Other samples were taken from symptomless trees. Immediately after all collections, branch and leaf samples were packaged in plastic bags and shipped by overnight mail to Agdia, Inc., Elkhart, Indiana, for ELISA.

**Sample preparation.** Leaf petioles and branch segments collected in May 1986 were ground in extraction buffer (10 mM sodium phosphate buffer [pH 7.4], 0.136 M NaCl, 2.6 mM KCl, 2% polyvinylpyrrolidone, 4% fresh egg white, 10 mM sodium sulfite, 0.02% sodium azide, 2% Tween 20) at the ratio of 1:5, w/v. In subsequent tests, a high-pressure press developed by Chester L. Sutula of Agdia, Inc., was used to extract the plant sap. The press was made of two stainless-steel blocks, one mounted on one side of a giant C clamp, the other on a hydraulic cylinder fixed on the other side of the clamp. Typically, 280–350 kg/cm² of pressure was needed to extract the sap. The sap samples were diluted 1:10, v/v, in extraction buffer. Extracts from healthy plant tissue were used as negative controls and suspensions of *X. fastidosa* (1 × 10⁷ cells per milliliter) were used as positive controls.

**ELISA.** Double-sandwich ELISA was performed according to the manufacturer's specifications. All reagents (except distilled water) were provided in the kit (Agdia, Inc.). All procedures were done at room temperature. The prepared tissue extracts (100 μl) were applied to antibody-coated microwells in the plates, the plates were incubated for 2 hr, the extracts were discarded, and the plates were washed (flooded) five times with phosphate-buffered saline plus 0.5% Tween 20. Next, peroxidase-conjugated antibody (100 μl) was added to the microwells, and the plates were incubated for 2 hr, then washed as before. Peroxidase substrate was prepared by dipping one *o*-phenylenediamine dihydrochloride stick (10 mg) in 10 ml of citrate buffer (0.012% $H_2O_2$, 20 mM citric acid, 50 mM $Na_2HPO_4$ [pH 5.0]). Then, 100 μl of the substrate was applied to each well and the plates were

**Table 1.** Leaf scorch ratings and reactions to enzyme-linked immunosorbent assay (ELISA) for *Xylella fastidiosa* in extracts from sycamore and elm trees with and without symptoms of leaf scorch

| Species / Symptoms / Tree number | Leaf scorch rating[a] 1986 | Leaf scorch rating[a] 1987 | ELISA ($OD_{495}$)[b] 1986 | ELISA ($OD_{495}$)[b] 1987 |
|---|---|---|---|---|
| Sycamore | | | | |
| Leaf scorch | | | | |
| SS 13 | 5 | 5 | (+) 2.000 | (+) 0.618 |
| SS 10 | 3 | 4 | (+) 2.000 | (+) 0.642 |
| SS 12 | 3 | 5 | (+) 2.000 | (+) 0.967 |
| SS 4 | 4 | 5 | (+) 2.000 | (+) 0.698 |
| SS 5 | 4 | 5 | (+) 2.000 | (+) 1.285 |
| Symptomless | | | | |
| SC 21 | 0 | 0 | (−) 0.030 | (−) 0.046 |
| SC 19 | 0 | 0 | (−) 0.042 | (−) 0.043 |
| SC 23 | 0 | 0 | (−) 0.079 | (−) 0.035 |
| SC 25 | 0 | 0 | (−) 0.025 | (−) 0.039 |
| SC 27 | 0 | 0 | (−) 0.036 | (−) 0.044 |
| Elm | | | | |
| Leaf scorch | | | | |
| ES 215 | 4 | 4 | (+) 2.000 | (+) 0.553 |
| ES 205 | 5 | 5 | (+) 2.000 | (+) 0.099 |
| ES 206 | 5 | 5 | (+) 0.610 | (+) 0.496 |
| ES 208 | 5 | 5 | (+) 2.000 | (+) 0.084 |
| ES 197 | 5 | 5 | (+) 2.000 | (+) 0.210 |
| Symptomless | | | | |
| EC 207 | 0 | 0 | (−) 0.032 | (−) 0.043 |
| EC 200 | 0 | 0 | (−) 0.029 | (−) 0.033 |
| EC 203 | 0 | 0 | (−) 0.030 | (−) 0.035 |
| EC 190 | 0 | 0 | (−) 0.027 | (−) 0.038 |
| EC 189 | 0 | 1 | (−) 0.068 | (−) 0.035 |
| Negative control[c] | | | (−) 0.027 | (−) 0.034 |
| Positive control[d] | | | (+) 2.000 | (+) 2.000 |
| Negative mean + 4 SD[e] | | | 0.111 | 0.057 |

[a] Ratings in September 1986 and 1987 based on percentage of tree canopy affected by leaf scorch: 0 = 0%, 1 = trace to 5%, 2 = 6–25%, 3 = 26–50%, 4 = 51–75%, 5 = 76–100%.
[b] 1986 = Extracts taken from 1986 stem tissue collected 5 May 1987; 1987 = extracts taken from petioles collected 5 May 1987. (+) = Positive reaction, (−) = negative reaction.
[c] Extract taken from healthy plant tissue.
[d] Suspension of *X. fastidiosa* at 1 × 10⁷ cells per milliliter.
[e] Positive values greater than mean of negative values plus four times the standard deviation.

**Table 2.** Leaf scorch ratings and reactions to enzyme-linked immunosorbent assay (ELISA) for *Xylella fastidiosa* in extracts from petioles of 47 elms sampled in June (before symptom development) and September (after symptom development) 1987

| | ELISA reaction according to leaf scorch rating[a] | | | | | |
|---|---|---|---|---|---|---|
| Month | 0 | 1 | 2 | 3 | 4 | 5 |
| June | 0/29[b] | 3/6 | 0/1 | 2/3 | 1/2 | 6/6 |
| September | 2/29 | 5/6 | 1/1 | 3/3 | 2/2 | 6/6 |

[a] Ratings based on percentage of tree canopy affected by leaf scorch: 0 = 0%, 1 = trace to 5%, 2 = 6–25%, 3 = 26–50%, 4 = 51–75%, 5 = 76–100%.
[b] Number of trees with positive reaction to ELISA/number of trees examined.

incubated for 30 min. A positive reaction was determined to be greater than the mean of the absorbance of the negative control wells plus four times the standard deviation (28).

## RESULTS AND DISCUSSION

The extracts from the samples collected in May 1987, before symptom development, from five American sycamores and American elms that were known to be diseased reacted positively with ELISA (Table 1). Positive reactions were observed with extracts of both 1-yr-old stem tissue and leaf petioles that had just developed. Two petiole samples that yielded $OD_{495}$ values of 0.099 and 0.084 were interpreted as positive because the values were larger than the mean of the negative values plus four standard deviations; in a real diagnostic situation, trees yielding samples with borderline positive values should be resampled and tested again. Optical density readings were consistently lower with petiole extracts than with stem extracts, possibly because pathogen populations are lower in newly developed, current-season tissue than in older tissue. All symptomless control trees yielded negative ELISA readings.

Symptoms characteristic of elm leaf scorch were observed in September 1987 in 18 of the 47 elm trees examined (Table 2). Bacteria resembling *X. fastidiosa* were isolated from the five symptomatic trees sampled. The two cultures tested yielded positive ELISA readings (>2.0 at $OD_{495}$). Among the 18 diseased trees, symptoms appeared in ≤50% of the canopy in 10 trees and in >50% in eight trees. When samples were collected in June 1987 before symptom development, 12 of the 18 trees had positive ELISA readings, including five of 10 and seven of eight trees with ≤50% or >50% of the canopy affected, respectively (Table 2). *X. fastidiosa* was not detected in extracts from 29 symptomless trees. When the trees were resampled in September, positive ELISA reactions occurred with extracts from all but one tree with leaf scorch. Two of the 29 symptomless trees yielded extracts that were weakly positive (Table 2). Symptoms did not develop in these two trees in 1988 and 1989, and attempts to isolate *X. fastidiosa* have been unsuccessful.

All extracts collected from five red maples, five red oaks, and two red mulberries with leaf scorch symptoms tested positive, with absorbance readings of 1.86–2.00. Extracts from four, four, and two symptomless maples, oaks, and mulberries, respectively, reacted negatively, with readings of 0.02–0.10 (negative control, 0.03; positive control, 2.00; negative mean plus four times standard deviation, 0.123).

ELISA was consistently effective for detecting *X. fastidiosa* in extracts from elm, sycamore, red oak, red maple, and red mulberry trees with leaf scorch. All but one of 30 samples collected from trees with symptoms yielded a positive ELISA reaction for *X. fastidiosa.*

Leaf and stem samples collected before symptom development from elms and sycamores known to be severely affected by *X. fastidiosa* also yielded extracts that were ELISA-positive. Reactions were positive with extracts from samples collected before symptom development from one-half of the elm trees that developed symptoms in ≤50% of their crowns, including three of six trees with only a trace to 5% of the crown affected. A positive ELISA reaction for trees with minimum symptom expression may indicate a more systemic infection than indicated by leaf symptoms.

In our preliminary evaluation, the ELISA test kit was simple to use and highly reliable for the five tree species examined. We used fresh tissue, leaf or stem, collected from May through September. We subsequently found that dormant stem tissue from infected trees is not as likely to yield extracts that test positive (J. L. Sherald, *unpublished*). Further studies of each host are necessary to determine the optimum time for sample collection and the most reliable tissue for analysis. Extraction procedures and extraction buffers may have to be modified according to the host, type of tissue, and period when the samples are collected in order to maximize the detection capability of the test. ELISA analyses will be a valuable tool for tree-care professionals and diagnosticians in the detection of *X. fastidiosa* in landscape trees as well as in other hosts. The ELISA kit will also be useful in confirming new hosts, in examining the systemic movement of the pathogen, and possibly in the screening of potential insect vectors.


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