Natural Hosts of *Xylella fastidiosa* in Florida

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ABSTRACT

Hopkins, D. L., and Adlerz, W. C. 1988. Natural hosts of *Xylella fastidiosa* in Florida. Plant Disease 72:429-431.

Fluorescence microscopy, enzyme-linked immunosorbent assay, and direct culturing were used to determine natural infection of various weeds and wild plants with *Xylella fastidiosa*, a xylem-limited bacterium. Strains of *X. fastidiosa* that could produce Pierce's disease symptoms in inoculated grapevine were cultured from American elder (*Sambucus canadensis*), Virginia creeper (*Parthenocissus quinquefolia*), peppervine (*Ampelopsis arborea*), American beautyberry (*Callicarpa americana*), and blackberry (*Rubus* sp.). These were cultured on PD3 medium, originally developed for the PD strain bacterium. Using PW medium, strains of *X. fastidiosa* that were not pathogenic to grapevine and would not grow on PD3 medium were cultured from eastern baccharis (*Baccharis halimifolia*), sumac (*Rhus* sp.), goldenrod (*Solidago fistulosa*), southern red oak (*Quercus falcata*), laurel oak (*Q. laurifolia*), water oak (*Q. nigra*), peach (*Prunus persica*), and sycamore (*Platanus occidentalis*). A large number of natural hosts of *X. fastidiosa* occur in Florida, and there is a need to study pathological and taxonomic relationships among the strains of the bacterium.

Pierce's disease (PD) of grapevine is endemic and limits grape production in the Gulf Coastal Plains of the United States. It is caused by strains of *Xylella fastidiosa* Wells et al (25), a gram-negative, xylem-limited bacterium. Pierce's disease has been studied more thoroughly than any other disease caused by *X. fastidiosa* and has a very wide host range (8,10,14). Known hosts include members of at least 28 families of monocotyledonous and dicotyledonous plants. Many of the host plants appear to be symptomless. The strains that cause Pierce's disease have been shown to also cause almond leaf scorch (17), alfalfa dwarf (10), and possibly citrus blight (12).

In California, *X. fastidiosa* kills susceptible grapevines in "hot spots" adjacent to permanent water sources where weed hosts of the bacterium and its sharpshooter leafhopper vectors occur (20,21). By means of enzyme-linked immunosorbent assay (ELISA), natural hosts of *X. fastidiosa* were identified in riparian weeds in the Napa Valley. The hosts included poison hemlock (*Conium maculatum* L.), umbrella sedge (*Cyperus eragrostis* Lam.), dallis grass (*Paspalum dilatatum* Poir.), wild strawberry (*Fragaria vesca* L.), miner's lettuce (*Montia linearis* (Dougl.) Greene), blackberry (*Rubus procerus* P. J. Muell.), and periwinkle (*Vinca minor* L.).

Florida Agricultural Experiment Station Journal Series Paper 8385.

Accepted for publication 11 December 1987 (submitted for electronic processing).

In addition to the Pierce's disease strains of *X. fastidiosa*, other strains are associated with diseases that cause large losses in many economically important crops and urban trees (7,11). These diseases include phony disease of peach, plum leaf scald, periwinkle wilt, oak leaf scorch, elm leaf scorch, mulberry leaf scorch, and sycamore leaf scorch (5,6,11,23). Diseases caused by *X. fastidiosa* are a serious problem primarily in areas with tropical or subtropical climates.

The primary goals of this study were to identify natural hosts of the strains of *X. fastidiosa* that cause Pierce's disease of grapes in Florida and may be important in the epidemiology of the disease and to identify natural hosts of other strains of *X. fastidiosa* in Florida. A preliminary report of part of this work has been made (3).

MATERIALS AND METHODS

Collection and preparation of plant samples. Leaves and small shoots were collected from wild plant species within 50 miles of the Central Florida Research and Education Center in Leesburg over a period of 6 yr. Plants were sampled either because they were located in or adjacent to citrus groves with blight or vineyards with Pierce's disease or because they had leaf scorch symptoms characteristic of diseases caused by *X. fastidiosa*.

Preparation of plant samples for immunofluorescence and ELISA. Petioles and leaf veins (1.0–2.5 g) were ground with a mortar and pestle with 8 ml of extraction buffer. The buffer consisted of phosphate-buffered saline (0.1 M, pH 7.0), Tween 20 (0.05%), polyvinyl-pyrrolidone (2%), and ovalbumin (2%). The homogenates were filtered through cheesecloth and centrifuged at 4,500 *g* for 15 min. The pellets were resuspended in 1.5–2.0 ml of extraction buffer and used for direct immunofluorescence. For ELISA, the extracts were further homogenized by adding 1 cm^3 of glass beads (0.17–0.18 mm diameter) to the extracts and swirling for 2 min at top speed on a Vortex-Genie mixer.

Immunofluorescence and ELISA. Tissue extracts from wild plant species were examined for the presence of *X. fastidiosa* using direct immunofluorescence (DIF) (22). Indirect immunofluorescence was used to determine if suspect cultures were strains of *X. fastidiosa* (9,22). The antiserum used in the immunofluorescence test and ELISA was against a strain isolated from grapevine with Pierce's disease. Antiserum preparation was as previously described (13), and the agglutination titer was greater than 1:1,280.

For ELISA, gamma globulin was purified from antiserum and conjugated with alkaline phosphatase (4). The ELISA procedure was as previously described (18) with slight modifications. Flat-bottom microtiter plates were coated with gamma globulin by incubation for 2–4 hr at 37 C. The tissue extracts were incubated overnight in wells at 6 C. The enzyme substrate (*p*-nitrophenyl phosphate, 1 mg/ml) was allowed to react for 30 min, then the reaction was terminated by adding 30 μl of 5 N NaOH per well. Plates were kept on ice until readings were made at A_{405nm} with a Bausch & Lomb Spectronic 20 spectrophotometer. For positive controls, 10^5 cfu/ml of a PD strain were added to tissue extracts before grinding the extracts with glass beads.

Isolation of *X. fastidiosa* from natural hosts. Leaves with marginal necrosis were selected from wild plant species for isolation attempts. If the plants were symptomless, the older leaves were sampled. Isolations were made from leaf petioles and leaf veins. The petioles and veins were surface-sterilized in 1% sodium hypochlorite for 3 min and rinsed four times in sterile water. They were aseptically cut into 0.5- to 1.0-cm sections, which were squeezed with forceps. The sap exuding from each section was blotted onto PD3 medium (7) or PW medium (6).

Any bacterial colony visible to the unaided eye within 3 days was discarded

as a contaminant. Colonies visible after 3 days were streaked onto nutrient agar and PD3 and PW media. If the bacterium did not grow on nutrient agar but grew on one or both of the other media, it was tested in an indirect immunofluorescence test (9) with antisera to the PD bacterium. Reactive strains were considered to be *X. fastidiosa*.

Pure cultures were obtained by dilution plating and transfer of isolated individual colonies. These were tested immediately for pathogenicity to grapevine or were lyophilized for later testing.

Pathogenicity to grapevine. Strains of *X. fastidiosa* were cultured at 28 C on PD3 medium or, if they did not grow on PD3, on PW medium. From PD3 cultures 4–6 days old or PW cultures 6–10 days old, bacterial suspensions were prepared in succinate-citrate-phosphate buffer (13). Using a Bausch & Lomb Spectronic 2000, inocula were adjusted to $A_{600nm} = 0.25$ (10^7 to 10^8 cfu/ml) (13).

For each *X. fastidiosa* strain, three rooted cuttings of Carignane bunch grape were inoculated by a pinpricking technique. Drops (0.02 ml) of inoculum were placed on the first and third internode from the base of the rooted cuttings. A dissecting needle was used to pierce the grapevine stem three to five times through the drop; the inoculum was pulled into the plant by the transpiration stream. Inoculated plants were kept in the greenhouse at 28–33 C in the daytime and 20–25 C at night. Disease occurrence, based on symptoms, was recorded every 2 wk. Reisolation of *X. fastidiosa* strains from petioles confirmed visual diagnosis of disease. Inoculated plants were observed for 4 mo.

RESULTS

In the first year of the study, ELISA, DIF, and culture on PD3 medium were used to identify important alternate hosts of *X. fastidiosa*. In addition to abundant wild grapevines infected with *X. fastidiosa* (13), the bacterium was isolated from American elder (*Sambucus canadensis* L.), Virginia creeper (*Parthenocissus quinquefolia* (L.) Planch.), peppervine (*Ampelopsis arborea* (L.) Koehne), and American beautyberry (*Callicarpa americana* L.) (Table 1). Extracts from eastern baccharis (*Baccharis halimifolia* L.), sumac (*Rhus* sp.), goldenrod (*Solidago fistulosa* Mill.), and peach (*Prunus persica* (L.) Batsch) were positive in ELISA and DIF serological tests, but the bacterium could not be cultured on the PD3 medium. Thirteen other plant species were negative in all three tests.

ELISA was more efficient than DIF for detecting *X. fastidiosa* (Table 1). With DIF, nonspecific-background fluorescence associated with tissue debris in the plant extracts reduced our ability to detect low numbers of fluorescing cells of *X. fastidiosa*. The Pierce's disease strains of *X. fastidiosa* could be cultured from every plant of American elder, Virginia creeper, peppervine, and beautyberry that tested positive by ELISA. These four hosts also had leaf necrosis or chlorosis symptoms.

Subsequent to the preceding tests, PW medium for the isolation of *X. fastidiosa* strains from periwinkle was reported (6). Using PW medium, we found that *X. fastidiosa* could be cultured from some of the plants that had tested positive by ELISA but negative by isolation on PD3 medium. In later surveys, therefore, hosts of strains that cause Pierce's disease and natural hosts of other strains were detected by culturing on PW medium, primarily from plants with some leaf chlorosis or necrosis symptoms.

With PW medium, *X. fastidiosa* strains were isolated from American elder, Virginia creeper, eastern baccharis, sumac, goldenrod, peach, blackberry, southern red oak (*Quercus falcata* Michx.), laurel oak (*Q. laurifolia* Michx.), water oak (*Q. nigra* L.), and sycamore (*Platanus occidentalis* L.) (Table 2). All of these plants had either leaf symptoms or dieback of branches. Similar symptoms were observed in mulberry (*Morus rubra* L.) and black cherry (*Prunus serotina* Ehrh.), but *X. fastidiosa* could not be isolated. Other plants with leaf chlorosis or necrosis from which *X. fastidiosa* could not be isolated included persimmon (*Diospyros* sp.), blueberry (*Vaccinium pennsylvanicum* Lam.), primrose willow (*Ludwigia peruviana* (L.) Hara), golden raintree (*Koelreuteria paniculata* Laxm.), and lantana (*Lantana camara* L.).

Strains of *X. fastidiosa* isolated from various naturally infected hosts consisted of at least two distinct types, based on ability to grow on PD3 or PW medium (Table 3). Those that grew on PD3 medium caused symptoms typical of Pierce's disease in Carignane grapevine. With one exception, those that grew on PW, but not on PD3, medium did not cause Pierce's disease in grapevine. One isolate from sycamore was the exception in that it did not grow on PD3 medium but did cause Pierce's disease.

Table 1. Assay of wild plants as natural hosts of *Xylella fastidiosa*

Plant species[a]	Number of plants assayed	Positive by:[b] ELISA	DIF	Culture
Sambucus canadensis L. (American elder)	5	5	3	5
Parthenocissus quinquefolia (L.) Planch. (Virginia creeper)	3	1	1	1
Ampelopsis arborea (L.) Koehne (peppervine)	3	3	2	3
Callicarpa americana L. (American beautyberry)	2	1	0	1
Baccharis halimifolia L. (eastern baccharis)	1	1	1	0
Rhus sp. (sumac)	4	2	0	0
Solidago fistulosa Mill. (goldenrod)	1	1	0	0
Prunus persica (L.) Batsch (peach)	2	0	1	0

[a] Species that were negative in all tests were *Rubus* sp. (blackberry), *Paspalum* sp., *Chenopodium ambrosioides* L. (mexicantea), *Eupatorium capillifolium* (Lam.) Small (dog fennel), *Myrica cerifera* L. (southern waxmyrtle), *Cotoneaster pyracantha* (L.) Spach, *Bidens leucantha* L. (beggarticks), *Prunus serotina* Ehrh. (black cherry), *Cynodon dactylon* (L.) Pers. (bermudagrass), *Panicum* sp., *Commelina* sp., *Lantana camara* L., and *Ulmus alata* Michx. (winged elm).

[b] ELISA = enzyme-linked immunosorbent assay, DIF = direct immunofluorescence. Culturing was done on PD3 medium, developed for the Pierce's disease of grapevine bacterium.

Table 2. Isolation of *Xyllela fastidiosa* from various natural hosts

Plant host	Positive isolations/attempted isolations[a] 1982	1983	1986
Sambucus canadensis L. (American elder)	9/9	···	3/3
Parthenocissus quinquefolia (L.) Planch. (Virginia creeper)	2/2	1/2	1/1
Baccharis halimifolia L. (eastern baccharis)	···	0/2	2/2
Rhus sp. (sumac)	1/1	4/7	2/3
Solidago fistulosa Mill. (goldenrod)	···	1/2	1/4
Prunus persica (L.) Batsch (peach)	2/2	4/7	1/4
Rubus sp. (blackberry)	1/1	0/4	0/2
Prunus serotina Ehrh. (black cherry)	···	0/1	0/1
Quercus falcata Michx. (southern red oak)	3/6	1/3	2/4
Q. laurifolia Michx. (laurel oak)	2/4	2/3	2/4
Q. nigra L. (water oak)	2/4	2/3	2/4
Platanus occidentalis L. (sycamore)	···	2/4	1/4
Morus rubra L. (mulberry)	0/4	0/2	0/2

[a] Isolations were on PW medium, developed for the periwinkle wilt bacterium.

Table 3. Media requirements and pathogenicity to grapevine of strains of *Xylella fastidiosa* from various natural hosts

Strain source	Growth on:[a] PD3	PW	Pathogenic to grapevine
American elder	+	+	+
Virginia creeper	+	+	+
Peppervine	+	+	+
American beautyberry	+	+	+
Blackberry	+	+	+
Oak	−	+	−
Eastern baccharis	−	+	−
Sumac	−	+	−
Goldenrod	−	+	−
Peach	−	+	−
Sycamore	−	+	+−

[a] + = Positive for growth or pathogenicity to grapevine, − = negative for growth or pathogenicity to grapevine, and +− = some isolates positive and some negative.

DISCUSSION

In this current study and in many previous reports (5–8,11,20,21,24), a large number of natural hosts of *X. fastidiosa* have been identified. In Florida, strains of *X. fastidiosa* have been shown to cause Pierce's disease of grapevine, phony disease of peach, plum leaf scald, periwinkle wilt, and ragweed stunt. Strains of *X. fastidiosa* have also been shown to produce diagnostic symptoms of blight in citrus seedlings (Hopkins, *unpublished*). This study was the first time that goldenrod, eastern baccharis, and sumac had been identified as hosts of *X. fastidiosa*. Since the strains isolated in this study came from leaves with symptoms typical of diseases caused by *X. fastidiosa*, we assumed that the bacteria were producing the observed symptoms. Koch's postulates were not fulfilled, however.

The list of natural hosts of strains of *X. fastidiosa* in Florida identified in this report is not intended to be complete. After the first year, only plants with visible symptoms similar to those known to be caused by *X. fastidiosa* were sampled. Symptomless hosts of the bacterium would not have been detected, and most hosts of the Pierce's disease strains are thought to be symptomless (8).

In a leafhopper ecology study in Florida, leafhopper vectors had been observed on 71 different plant species (1). Certain plants from this leafhopper study were listed as potential natural hosts of *X. fastidiosa*, since the vectors were abundant on these plants. Of 13 plants on the list, goldenrod, eastern baccharis, American elder, and blackberry yielded strains of *X. fastidiosa*; the bacterium had previously been obtained from ragweed (24) and citrus (12). Because of the very wide host range of *X. fastidiosa*, the wide host range of the leafhopper vectors, and the warm Florida winters that are favorable (19), it is probable that many of the perennial plants in Florida are hosts of the bacterium.

Taxonomically, the gram-negative, xylem-limited bacteria from various hosts have been described as consisting of a single species, *X. fastidiosa* (25). There is considerable strain variability, however. The *X. fastidiosa* strains obtained from the natural hosts in Florida separated into two distinct groups: those that grew on PD3 and PW media and those that grew only on PW medium. These two groups have been called the Pierce's disease group and the phony disease of peach group, respectively (16). Very little is known about the pathological relationships among the strains of *X. fastidiosa* within these groups. Clearly, pathogenic relationships are more complex than suggested by these two groupings. For example, phony disease and plum leaf scald apparently are caused by the same strains (5). But both the periwinkle wilt and Pierce's disease strains were pathogenic to periwinkle, with the periwinkle wilt bacterium being more virulent. However, only the Pierce's disease strains were pathogenic on grapevine (6).

We report that American elder, Virginia creeper, peppervine, American beautyberry, and blackberry were natural hosts of the Pierce's disease strains of *X. fastidiosa*. The role of these natural hosts in the epidemiology of Pierce's disease of grapevine in Florida is not known. Whereas inoculum for Pierce's disease in California comes primarily from sources outside the vineyard, spread is almost always by secondary transmission within vineyards in Florida (2). In the spring, leafhopper vectors from alternate hosts are not infective and they do not become infective in the vineyard until early summer, when bacterial titer is high in grapevine (15).

ACKNOWLEDGMENTS

This research was supported in part by the U.S. Department of Agriculture under CSRS special grants 83-CRSR-2-2136 and 85-CRSR-2-2575 managed by the Caribbean Basin Advisory Group (CBAG). The technical assistance of Connie Thompson and Richard Roof is gratefully acknowledged.

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