Production of Diagnostic Symptoms of Blight in Citrus Inoculated with *Xylella fastidiosa*

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ABSTRACT

Hopkins, D. L. 1988. Production of diagnostic symptoms of blight in citrus inoculated with *Xylella fastidiosa*. Plant Disease 72: 432-435.

Strains of *Xylella fastidiosa*, a xylem-limited, gram-negative bacterium isolated from various natural hosts in Florida, were used to inoculate rooted cuttings of rough lemon (*Citrus jambhiri*) seedlings, the rootstock that is most susceptible to citrus blight. Eighteen to 24 mo later, stunting and dieback symptoms were observed in rooted cuttings inoculated with *X. fastidiosa* strains obtained from citrus, oak (*Quercus* spp.), grapevine (*Vitis* spp.), goldenrod (*Solidago fistulosa*), blackberry (*Rubus* sp.), and peach (*Prunus persica*). One *X. fastidiosa* strain obtained from citrus produced stunting and dieback symptoms in rough lemon, rangpur lime (*C. limonia*), and sweet orange (*C. sinensis*) seedlings. Compared with healthy controls, inoculated seedlings also had reduced water conductivity in stem sections and elevated zinc levels in trunk wood and bark, which are diagnostic tests for citrus blight. Five of nine trees of the sweet orange cultivar Pineapple on rough lemon rootstock developed visible blight symptoms and had reduced water conductivity in stem sections. One of these trees had the complete blight syndrome: stunting, smaller and more upright leaves, dieback to near the bud union, zinc deficiency in the leaves, xylem dysfunction, elevated water-soluble phenolic levels, and elevated zinc levels in the bark. Thus, *X. fastidiosa* can produce blight symptoms in citrus.

Additional keywords: Pierce's disease bacterium

Citrus blight has been present in Florida citrus groves for at least 100 yr and may be endemic to Florida. Losses from blight are extensive, annually affecting as many as 10–20% of the trees in individual groves and 500,000 trees in the state (21). Symptoms of blight are indicative of xylem dysfunction and include wilt, canopy thinning, small leaves with zinc deficiency symptoms, twig dieback, trunk sprouts, progressive decline, and death of the tree. Citrus blight is characterized by reduced water conductivity of the xylem vessels in trunks, limbs, and roots (5,23), by high levels of zinc in trunk wood and trunk phloem (2,22), and by a high frequency of amorphous occlusions in the xylem (4,6). Transmission of citrus blight has been accomplished by root grafts, which implicates a systemic pathogen (20). Etiology of the disease is unknown, however.

Citrus blight has many characteristics in common with diseases caused by strains of *Xylella fastidiosa* Wells et al, such as Pierce's disease of grapevine, phony disease of peach, and oak leaf scorch (11,14). These similarities have resulted in studies of *X. fastidiosa* as the possible causal agent of blight. For example, tetracycline antibiotics that suppress Pierce's disease symptoms in grapevines were reported to suppress visible symptoms of citrus blight (19) and zinc accumulation in trunk wood (17). *X. fastidiosa* was detected in rough lemon roots of blighted citrus by electron microscopy and immunofluorescent techniques (9,16), and a Pierce's disease strain was transmitted from trees with blight to grapevine using a common leafhopper vector of the bacterium (15). Strains that cause Pierce's disease in grapevine have also been cultured from vacuum extracts of citrus stems and roots (*unpublished*). In greenhouse experiments, these strains produced a wilt-type disease in rough lemon (*Citrus jambhiri* Lush.) seedlings; symptoms included twig dieback, leaf drop, and reduced water-flow rates in stem sections (12). However, the incidence of symptoms after inoculation with these strains was erratic in rough lemon and other susceptible rootstock seedlings. While Pierce's disease strains produced wilt-type symptoms in rough lemon, zinc accumulation in trunk wood and trunk phloem were not measured and symptoms were not produced in sweet orange (*C. sinensis* (L.) Osb.) grafted on rough lemon rootstock, as occurs in the grove in Florida.

The objectives of this study were to evaluate *X. fastidiosa* from various natural hosts for pathogenicity to citrus, to determine if *X. fastidiosa* strains obtained from citrus and grape produce visible and diagnostic symptoms of blight in rootstock seedlings and to cause the complete blight syndrome in sweet orange cultivar Pineapple grafted on rough lemon rootstock.

MATERIALS AND METHODS

Pathogenicity of *X. fastidiosa* from various hosts to rough lemon seedlings. Strains of *X. fastidiosa* used in this study were isolated as previously described (13) from petioles of leaves with marginal necrosis sampled from grapevine (*Vitis rotundifolia* Michx. and other *Vitis* spp.), oak (*Quercus* spp.), goldenrod (*Solidago fistulosa* Mill.), blackberry (*Rubus* sp.), peach (*Prunus persica* (L.) Batsch), and sumac (*Rhus* sp.) in central Florida. Three strains were from citrus (*C. jambhiri* and *C. sinensis*) with blight. Two of these citrus strains were isolated directly from root and stem segments. The segments were vacuum-infiltrated with succinate-citrate-phosphate (SCP) buffer (disodium succinate, 1.0 g/L; trisodium citrate, 1.0 g/L; K_2HPO_4, 1.5 g/L; and KH_2PO_4, 1.0 g/L; pH 7.0) to extract the bacteria. The vacuum extracts were streaked onto PD3 medium (8) and incubated for 5–10 days at 28 C. With the third citrus strain, a leafhopper vector was used to transmit *X. fastidiosa* from citrus trees with blight to Carignane grapevine, and the bacterium was then cultured from a petiole of the grapevine. All *X. fastidiosa* strains obtained from grapevine and citrus produced Pierce's disease symptoms in inoculated Carignane grapevine. None of the other strains produced symptoms in grapevine.

The citrus and grapevine strains were grown at 28 C on PD3 medium (8) and other strains were grown on PW medium (7). Bacterial suspensions were prepared in SCP buffer from 4- to 6-day-old cultures on PD3 and 6- to 10-day-old cultures on PW medium. Using a spectrophotometer, the suspensions were adjusted to A_{600nm} = 0.25 (10^7 to 10^8 cfu/ml) for inoculations (13).

Three or four rooted cuttings (minimum of eight nodes) of rough lemon citrus were inoculated with each *X. fastidiosa* strain in May 1983, May 1984, and October 1984. Inoculations were made with a pinprick technique. A drop (0.02 ml) of bacterial suspension was placed on each of the lower five internodes. A dissecting needle was used to pierce through the drops into the vascular tissue

Florida Agricultural Experiment Station Journal Series Paper 8220.

Accepted for publication 21 December 1987 (submitted for electronic processing).

of the plant stem. The suspensions were absorbed into the xylem vessels. In this manner, 10^6 to 10^7 cfu were inoculated into each rough lemon plant. Inoculated plants were maintained in 15-cm pots in the greenhouse at 30–37 C in the daytime and 20–28 C at night. Disease incidence, based on visible symptoms, was evaluated at 6-mo intervals.

Inoculation of seedlings of citrus rootstocks. One-year-old seedlings of rough lemon and rangpur lime (*C. limonia* Osb.), rootstocks, which have a high incidence of blight in the field, and of sweet orange, which has a low incidence of blight as a rootstock, were inoculated, as described above, with Pierce's disease strains. Four seedlings of each rootstock were inoculated with each bacterial strain. Five strains of the bacterium were used, two from citrus (CB-9, CB80-4) and three from grapevine (PD-15, PD82-19, PD82-20). The control was SCP buffer only. Seedling inoculation dates were March 1984, October 1984, May 1985, and October 1985. Symptoms were evaluated at 6-mo intervals.

Inoculation of Pineapple sweet orange grafted on rough lemon rootstock. Two different inoculation experiments were done with 1-yr-old trees of the sweet orange cultivar Pineapple on rough lemon rootstock. In the test initiated in 1979, one strain (CB-9) isolated from citrus and two strains (PD-1, PD-15) from grapevine were inoculated into the sweet orange scions in March 1979, March 1980, May 1981, May 1982, and May 1983. In addition, suckers from the rough lemon rootstocks were inoculated in July 1979. Inoculated plants were maintained in a screenhouse. All plants were killed back to the main trunk by a freeze in January 1981, and the experiment was terminated when most plants were killed by a December 1983 freeze. Plants were evaluated twice yearly for visible symptoms.

In November 1981, two different strains of *X. fastidiosa* obtained from citrus with blight and one strain obtained from peppervine (*Ampelopsis arborea* (L.) Koehne) were inoculated into Pineapple scion on rough lemon rootstock. These were reinoculated in October 1982 and October 1983. This experiment was also prematurely terminated by the December 1983 freeze.

Diagnostic tests for citrus blight. Xylem conductivity was evaluated by rate of water flow through sections of the main stem (11). A vacuum of 600 mm Hg was applied to stem sections 7 cm long and 7–12 mm in diameter. The time in seconds required to pull 1.0 ml of water from a 10-ml pipet (connected to the section with plastic tubing) into the stem section was determined.

Water-soluble phenolics were determined in extracts of wood chips from the main trunk of the plants (21). Phenolics were extracted from 100 mg of oven-dried wood chips into 20 ml of water by shaking for 2 hr on a reciprocal shaker. The extract was decanted into a cuvette and scanned in the 290- to 250-nm range on a spectrophotometer, using water as the reference. The height of the absorption peak at 277 nm was used to determine relative amounts of phenolics.

Zinc concentrations were determined in the trunk bark and wood of inoculated trees. Sections 5 cm long were cut from the main stem and the bark removed. Wood shavings from the sections and bark pieces were dried in an oven at 70 C for 24 hr. A sample of wood shavings (2 g dry wt) and bark (1 g dry wt) were ashed overnight at 550 C in a muffle furnace. Each ashed sample was dissolved in 25 ml of 5% HCl. Atomic absorption spectrophotometry was used to make zinc determinations.

RESULTS

Production of visible symptoms in rough lemon seedlings by *X. fastidiosa* from various hosts. Strains of *X. fastidiosa* obtained from six different hosts produced visible symptoms in rough lemon citrus seedlings (Table 1). Stunting of shoot growth and smaller leaf size were first observed 6–8 mo after inoculation. By 12–18 mo, dieback of shoots from the tip and severe stunting were observed. Two grapevine strains and a sumac strain failed to produce symptoms in the rough lemon seedlings. Two oak strains and one citrus, one grapevine, and one goldenrod strain produced the most severe dieback symptoms in the rough lemon seedlings.

Table 1. Inoculation of rough lemon seedlings with strains of *Xylella fastidiosa* obtained from various natural hosts

Source of strain	No. of strains[z]	Diseased seedlings/total seedlings
Citrus	3	7/10
Oak	5	13/18
Grapevine	5	5/15
Goldenrod	2	5/5
Blackberry	1	3/3
Peach	1	1/3
Sumac	1	0/3
Buffer control	···	0/4

[z]Each strain was used to inoculate three or four seedlings.

Production of diagnostic symptoms of citrus blight in rootstock seedlings. In the rough lemon seedlings, stunting was first observed in two plants 12 mo after inoculation with strain CB-9, originally obtained from citrus with blight. By 24 mo after inoculation, all four plants inoculated with CB-9 were stunted and three had dieback symptoms. In this 2-yr test, none of the other four isolates produced visible symptoms in rough lemon (Table 2). The plants inoculated with CB-9 also had reduced water-flow rates in the xylem and elevated zinc concentrations in the wood and bark of the main trunk. Some of the plants inoculated with other strains of *X. fastidiosa* had slightly elevated zinc levels in the wood or bark, but not significantly greater than the control seedlings.

During the 2-yr test, visible symptoms developed in only one rangpur lime seedling inoculated with strain CB-9. Symptoms included stunting and dieback, as occurred on rough lemon. In the symptomatic seedling, water-flow rate was 24 sec/ml, compared with 12 sec/ml in the uninoculated seedlings, and the bark zinc level was 106 μg/g, compared with 59 μg/g. Wood zinc level was not affected. There was a tendency toward reduced water flow in the xylem and elevated zinc levels in the bark of inoculated asymptomatic seedings.

In sweet orange seedlings, three of four plants inoculated with isolate CB-9 developed visible symptoms similar to those on rough lemon. Again the water-flow rates were 52 sec/ml in the symptomatic seedlings, compared with 23 sec/ml in uninoculated seedlings, and zinc levels in the bark were 103 μg/g, compared with 80 μg/g.

Production of symptoms of citrus blight in Pineapple sweet orange grafted

Table 2. Production of diagnostic blight symptoms in rough lemon seedlings with strains of *Xylella fastidiosa*

Strain	Seedlings with visible symptoms	Average trunk diameter (mm)	Water-flow rate (sec./ml)[x]	Mean zinc concentration (dry wt., g/g)[y]	
				Wood	Bark
CB-9	4/4	8.5	29 a[z]	19 a	66 a
CB80-4	0/4	10.3	11 b	13 ab	44 b
PD-15	0/4	10.8	12 b	17 a	43 b
PD82-19	0/4	10.0	10 b	16 a	40 b
PD82-20	0/4	9.3	16 b	15 ab	51 ab
Buffer control	0/4	10.8	12 b	10 b	38 b

[x]Data represent time in seconds required to pull 1.0 ml of water into a 7-cm-long stem section with 600 mm Hg vacuum.

[y]Samples for zinc determinations were taken from the main trunk of the seedling.

[z]Means in columns followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test.

on rough lemon rootstock. Visible symptoms were first observed in tree No. 1, inoculated with strain CB-9, at 24 mo after inoculation (Table 3). First symptom in this and other grafted trees was stunted growth, including smaller leaves that were more upright than normal and curled at the margins. Tree No. 1 inoculated with CB-9 died back to within 10 cm of the bud union during the summer of 1983, 4 yr after inoculation, and the new shoot growth had severe zinc deficiency. When this tree began to develop dieback, samples were collected for diagnostic tests. Tree No. 1 inoculated with CB-9 was positive for all three diagnostic tests for citrus blight. One of the trees inoculated with PD-1 had increased zinc concentration in the bark and a small reduction in water-flow rate. Unfortunately, the December 1983 freeze terminated this test before there was an opportunity for other symptoms to develop and further tests to be run.

In the test started in 1981, stunting was observed after 2 yr in two of five plants inoculated with citrus strains of *X. fastidiosa* but not in either of the two plants inoculated with the peppervine strains. No diagnostic tests were run before loss of the experiment to the freeze.

DISCUSSION

In a previous study, a Pierce's disease strain was transmitted from citrus with blight to grapevine and then cultured from the grapevine (15). Pierce's disease strains also caused a wilt-type disease in rough lemon citrus in the greenhouse (12). There are many strains of *X. fastidiosa* from hosts other than grapevine (3,7,11) whose relationship to blight have not been thoroughly investigated. When the strains were cultured from citrus, the media for many of the strains from other hosts had not been developed yet. In one attempt to recover *X. fastidiosa* from the sharpshooter vectors collected in blight-affected groves, only a ragweed stunt strain was detected (18). In this study, we demonstrated that the Pierce's disease strains of *X. fastidiosa* are not the only strains that can produce the wilt-type symptoms in rough lemon (Table 1). Strains obtained from citrus, oak, goldenrod, and blackberry caused a higher incidence of symptoms on rough lemon than strains from grapevine, peach, and sumac. This could indicate some host specificity among strains, or it could be merely a chance result of the natural variability in virulence that occurs among strains of *X. fastidiosa* (13).

The pathological relationship among *X. fastidiosa* strains from various hosts is not well understood. However, the Pierce's disease strains have a host range that includes members of at least 28 families of monocotyledonous and dicotyledonous plants (10), and the phony disease of peach strains also have a wide host range. With the apparent lack of host specificity in *X. fastidiosa*, it is not surprising that more than one type is capable of producing symptoms in citrus. With the lack of knowledge of taxonomic relationships among strains of *X. fastidiosa*, it is not possible to know how many different types were represented by the strains used in this study. There were, however, at least two distinguishable types: those that produced Pierce's disease symptoms in grapevine and could be cultured on PD3 medium and those that did not produce symptoms and did not grow on PD3 medium, whereas both produced wilt-type symptoms in rough lemon seedlings.

During the 2-yr inoculation study involving the rootstocks rough lemon, rangpur lime, and sweet orange, only one of the five Pierce's disease strains produced visible symptoms in the rootstock seedlings (Tables 2 and 3). In the test of strains of *X. fastidiosa* from various hosts, 15 of 18 strains produced visible symptoms in rough lemon. The reason for this apparent difference in virulence of strains between the two tests is not known, but the five Pierce's disease strains had been in culture for a year or more and may have lost virulence (13); these strains were still pathogenic to grapevine, however. Perhaps if the test had run longer, symptoms would have been produced by some of the other four strains, since some of the seedlings had an elevated bark zinc level but no visible symptoms, a condition previously described as predecline (1).

Strain CB-9 produced typical blight symptoms in inoculated seedling rootstocks, including elevated zinc levels in the wood and bark. Elevated zinc in the bark was more consistently associated with visible symptoms than elevated zinc in the wood. This has also been observed in seedling trees in the grove in Florida (2). Rough lemon and rangpur lime are classified as rootstocks highly susceptible to blight, but rough lemon appeared more susceptible than rangpur lime to CB-9. Sweet orange, which is considered a more resistant rootstock, is the scion on which blight symptoms usually occur in Florida. It was nearly as susceptible to strain CB-9 as rough lemon was.

Citrus blight occurs most frequently in Florida in mature trees of sweet orange grafted on rough lemon rootstock. In this study, five of nine Pineapple sweet orange trees budded on rough lemon and inoculated with Pierce's disease strains obtained from citrus developed visible symptoms of blight and positive diagnostic tests. However, only one of these trees was positive in all the diagnostic tests for citrus blight. At least in this one plant, the complete blight syndrome was produced by a strain of *X. fastidiosa*. In the grove, blight normally does not develop in trees less than 5–8 yr old. The oldest trees in our experiments were only 4 yr old when killed by the freeze in 1983.

In summary, strains of *X. fastidiosa* originally isolated from citrus with blight produced the visible symptoms of blight in susceptible rootstock seedlings and in sweet orange on rough lemon rootstock citrus trees. The diagnostic tests for blight also were positive in these inoculated seedlings and grafted trees. Although blight was not produced in a mature tree, the evidence is strong for the involvement of *X. fastidiosa* in the blight syndrome.

Table 3. Visible and diagnostic symptoms of blight in citrus trees of sweet orange cultivar Pineapple grafted on rough lemon rootstock inoculated with *Xylella fastidiosa*

Strain	Tree no.	Visible symptoms	Water-flow rate (sec./ml)[y]	Phenolics[z] (A_{277nm})	Zinc concentration in bark[z] (g/g)
CB-9	1	++	56	2.24	128
	2	−	8	0.98	NT
	3	+	19	NT	NT
	4	+	21	NT	NT
PD-1	1	−	13	0.91	NT
	2	+	19	0.87	174
PD-15	1	−	20	1.21	NT
None	1	−	9	1.21	86
	2	−	9	1.10	NT

[y] Data represent time in seconds required to pull 1.0 ml of water into a 7-cm-long stem section with 60 mm Hg vacuum.

[z] Samples for determination of water-soluble phenolics and zinc were taken from the main trunk of the tree. Relative heights of the absorption peaks at 277 nm were used to compare amounts of water-soluble phenolics. NT = not tested.

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 Lobinske is gratefully acknowledged.

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