

Relation of Pierce's Disease Bacterium to a Wilt-Type Disease in Citrus in the Greenhouse

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Florida Agricultural Experiment Station Journal Series Paper 3273.

I am grateful to Connie Thompson and Richard Roof for technical assistance and to Steve Garnsey for providing citrus seedlings.
Accepted for publication 11 February 1982.

ABSTRACT

Hopkins, D. L. 1982. Relation of Pierce's disease bacterium to a wilt-type disease in citrus in the greenhouse. *Phytopathology* 72:1090-1092.

The Pierce's disease (PD) bacterium produced symptoms in rough lemon (*Citrus jambhiri*) seedlings in the greenhouse similar to those observed in field trees affected by citrus blight. The symptoms, including dieback of branches and leaf drop, appeared 8–12 mo after inoculation. Water-flow rates were reduced in stem sections of diseased rough lemon seedlings. PD bacteria were detected in stem extracts of rough lemon by immunofluorescence. Survival and multiplication of the PD bacterium in

rough lemon petioles were determined by reisolation and dilution plating over a 6-wk period. The PD bacterium also produced a disease in seedlings of the trifoliolate orange (*Poncirus trifoliata*), Carrizo citrange (*P. trifoliata* × *C. sinensis*), and Rangpur lime (*C. limonia*), but not in those of alemow (*C. macrophylla*), Cleopatra mandarin (*C. reticulata*), or sour orange (*C. aurantium*). Symptomatology and varietal susceptibility of this disease were similar to citrus blight.

Citrus blight, also known as young tree decline, has been present in Florida citrus groves since at least the 1870s and may be endemic to Florida (13). It causes extensive losses and currently is the most serious disease affecting Florida citrus. The etiology of this blight has not been determined.

Citrus blight and Pierce's disease (PD) of grapevine, which limits grape culture in Florida, have many characteristics in common (8). Both produce wilt-type symptoms, which suggest that xylem dysfunction is a major characteristic of both diseases. Delayed spring growth flush, wilting, decline of vigor, canopy thinning or leaf drop, and twig dieback also occur. Tetracycline antibiotics suppress symptoms of citrus blight (15) and PD (7). A leafhopper vector of the PD bacterium was used to recover the bacterium from citrus trees with blight and to transmit it to grapevine (9). Rickettsialike bacteria have been detected in rough lemon roots of blighted citrus by electron microscopy and immunofluorescent techniques (2,10). However, the PD bacterium was not cultured directly from blighted citrus.

Rough lemon (*Citrus jambhiri* Lush.) is known from field observations to be the rootstock that is most susceptible to blight; sour orange (*C. aurantium* L.) and sweet orange (*C. sinensis* [L.] Osb.) are less susceptible (16).

The object of this study was to determine whether the PD bacterium is pathogenic to citrus.

MATERIALS AND METHODS

Inoculation of citrus seedlings in the greenhouse. Two isolates of the PD bacterium (CB-1 and CB-9) that had been recovered from blighted citrus (9) and one isolate (PD-1) from grapevine were used to inoculate 1-yr-old rough lemon seedlings. Cloudy suspensions of bacteria ($>10^8$ cells per milliliter) in succinate-citrate-phosphate (SCP) buffer were used in the inoculations. The SCP buffer was trisodium citrate (1.0 g/L) and disodium succinate (1.0 g/L) in 0.015 M phosphate buffer, pH 7.0.

Six seedlings of rough lemon were inoculated with each isolate in December 1977. An L-shaped cut was made halfway through the stem and extended 3 cm longitudinally in the stem. The spur resulting from this cut was submerged in a bacterial suspension in a vial. At least 1 ml of the liquid suspension was taken up by the spur, but the actual number of bacteria entering the plant vascular

system was unknown. In March 1978, bacterial suspensions were vacuum-infiltrated into 10- to 12-cm-long Valencia sweet orange cuttings (1,3). Infiltrated cuttings were cleft-grafted onto five of the inoculated rough lemon seedlings. Thus, there were five inoculated Valencia on rough lemon grafted plants and 13 inoculated rough lemon seedlings. These were all reinoculated in December 1978 by pinpricking the stems through drops of bacterial suspension. As checks, seven rough lemon seedlings and two Valencia on rough lemon grafted trees were pinpricked through SCP buffer. Greenhouse temperatures were 28–33 C in the daytime and 20–25 C at night. Screenhouse temperatures were more variable; summer temperatures ranged from 22 to 35 C and winter temperatures ranged from –4 to 27 C.

Sour orange, Cleopatra mandarin (*C. reticulata* Blanco), alemow (*C. macrophylla* West.), Carrizo citrange (*Poncirus trifoliata* × *C. sinensis*), Rangpur lime (*C. limonia* Osb.), and trifoliolate orange (*P. trifoliata* [L.] Raf.) seedlings, which as rootstocks vary in field susceptibility to blight, were inoculated with isolate CB-9 ($>10^7$ cells per milliliter) by pinpricking the stem in May 1979. They were reinoculated in July 1979 and October 1979 by the same method. Three seedlings of each rootstock were inoculated with CB-9 and two seedlings of each rootstock with SCP buffer as controls.

Growth of the PD bacterium in rough lemon and grapevine. Isolate CB-9 at 10^8 cells per milliliter of SCP buffer (0.25 OD at 600 nm) was used to inoculate rough lemon and grapevine petioles. One drop (0.02 ml) of the suspension was used to inoculate a single site on the petioles by pinpricking through the drop. Petiole sections, 2-cm-long, centered on the inoculation point, were used to determine bacterial populations at the inoculation site at various times after inoculation. The samples were surface sterilized in 1% sodium hypochlorite, rinsed in sterile water, triturated in SCP buffer with a mortar and pestle, and filtered through cheesecloth. These extracts were centrifuged at 5,000 g for 15 min. The pellet was resuspended in 0.4 ml of SCP buffer. The colony-forming units (CFU) in these suspensions were quantified by dilution plating on modified JD-3 medium (1). Each population represents the mean of three petiole samples. Bacterial colonies were counted 7–8 days after plating.

Detection of PD bacteria in citrus tissue. Stem sections (3–4 cm) of inoculated citrus were surface sterilized, rinsed in sterile water, triturated in SCP buffer with a mortar and pestle, filtered through cheesecloth, and centrifuged at 5,000 g for 15 min. The pellet was resuspended in 0.5 ml of SCP buffer. Portions of the suspension were plated on JD-3 medium and others were used for indirect immunofluorescence (4) or enzyme-linked immunosorbent assay

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(ELISA) (11). ELISA was considered positive for a sample only if the absorbance ($A_{405\text{ nm}}$) value was ≥ 3 times that of the control.

Water-flow rates through rough lemon stem sections. Stem sections, 7 cm long and 3–5 mm in diameter, were subjected to water-flow tests. A vacuum of 600 mm Hg was applied to the sections with a vacuum pump and flask (3). The time (seconds) required to pull 1.0 ml of H_2O from a 10-ml pipet (connected to the section with plastic tubing) into the stem section was measured in seconds.

RESULTS

Inoculations of rough lemons. Seven of 13 inoculated rough lemon seedlings were maintained in the greenhouse throughout the study and six were held in the screenhouse. Five of the seven plants in the greenhouse developed symptoms (first observed in August 1978), which included dieback of young shoots from the tip, curling of younger leaves, leaf drop, and reduced leaf size in new growth. The affected plants continued to produce new shoots that died back, exhibiting symptoms similar to those of copper deficiency, often resulting in a bushy-type growth. The plants were routinely fertilized with a copper-containing citrus fertilizer, and additional applications of tribasic copper sulfate to the soil and directly on the foliage were made without affecting symptom development. All efforts to reisolate the bacteria failed. However, bacteria were detected by immunofluorescence in four of the five diseased rough lemon plants. The other two inoculated plants and seven control plants remained symptomless and immunofluorescence tests were negative.

Of the seven inoculated plants in the greenhouse, two of three plants inoculated with isolate CB-9, two of two with CB-1, and one of two with PD-1 developed symptoms. The two citrus isolates (CB-1 and CB-9) produced moderate to severe symptoms within 8–12 mo after inoculation. The grape isolate (PD-1) produced only slight symptoms 24 mo after inoculation and, even then, bacteria were not detected by immunofluorescence.

Two of six rough lemon seedlings inoculated and maintained in the screenhouse developed very slight dieback symptoms. Immunofluorescence was used to detect the PD bacterium in one of these. After the winter of 1978–1979, the new growth flush on these plants was apparently normal and symptoms did not develop further.

Because xylem dysfunction restricts water movement and is apparently responsible for moisture stress symptoms in citrus blight (5,14), I measured water-flow rates in the diseased rough lemon plants (Table 1). Flow rates were reduced to 9–20% of those in the healthy rough lemon stem sections of comparable size and age. Water-flow rates were reduced the most in the 4.0-mm-diameter sections. There was no overlap of values; all healthy samples of comparable size had more rapid rates than any diseased sample.

Inoculations of Valencia grafted on rough lemon rootstocks. All inoculated Valencia on rough lemon trees (one inoculated with CB-9, two with CB-1, and two with PD-1) and two SCP checks remained healthy throughout the 3 yr of this study. Attempts to reisolate the bacteria or to detect them by immunofluorescence failed.

Multiplication of the PD bacterium in rough lemon and grapevine tissue. Because I could not reisolate the PD bacterium from the stems of the diseased rough lemon seedlings, I attempted to assay the growth of the PD bacterium in petioles, which are easier to grind and to use in dilution plating techniques than are stems. Differences between the growth curve of CB-9 in grape petioles and in citrus petioles are striking (Fig. 1). Bacterial populations in both citrus and grape petioles declined during the first 3–4 days. This was followed by a rapid growth phase in the grape petioles, and populations over 10^6 CFU per centimeter of petiole were attained 14–15 days after inoculation. A rapid growth phase apparently started in the citrus after 7 days, but populations of live bacteria then declined slightly and stabilized. However, populations of 10^2 – 10^4 CFU per centimeter of petiole were maintained in rough lemon throughout the 6-wk study. Isolate

PD-1 maintained similar, but more variable, populations in rough lemon petiole tissue.

Inoculation of citrus rootstock seedlings. Leaf drop and dieback symptoms were first observed on Rangpur lime 8 mo after inoculation and on Carrizo citrange and trifoliate orange 12 mo after inoculation (Table 2). Inoculated sour orange and Cleopatra mandarin remained symptomless through 18 mo. One of the three inoculated alemow seedlings was an off-type (it developed severe symptoms at 18 mo); the other two remained healthy. Bacteria were detected (either by immunofluorescence or ELISA) in all seedlings with symptoms. Isolation attempts from the seedlings were unsuccessful. None of the SCP-inoculated control seedlings developed symptoms.

DISCUSSION

The PD bacterium incited a disease in rough lemon, Carrizo citrange, trifoliate orange, and Rangpur lime seedlings in the

TABLE 1. Water-flow rates through stem sections of rough lemon seedlings inoculated with Pierce's disease bacterium

Plant sample ^a	Water-flow rates ^b		
	3.0 mm	4.0 mm	≥ 5.0 mm
CB-9 (1)	1,741	7,367	...
CB-9 (2)	2,567	1,381	549
CB-1 (3)	7,000	4,565	189
SCP buffer (1)	386	117	...
SCP buffer (2)	642	424	35
SCP buffer (3)	620	664	106
Average, inoculated	3,769	4,438	369
Average, buffer control	549	402	71

^aSamples were stem sections from seedlings (1 to 3) inoculated with isolates CB-9 or CB-1 and three seedlings (1 to 3) inoculated with buffer only as a control.

^bData are given as the times in seconds required to pull 1.0 ml of water into a 7-cm-long stem section of given diameter with 600 mm Hg vacuum.

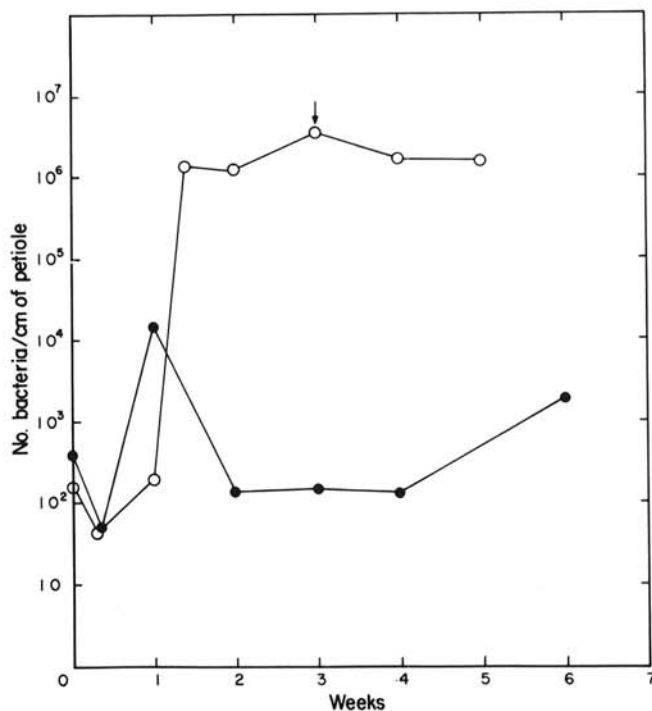


Fig. 1. Growth curves of the Pierce's disease bacterium (isolates CB-9) in plant tissue. Each petiole was inoculated at 0 time with 0.02 ml of a suspension containing 10^8 cells per milliliter. \circ = populations in grape petioles and \bullet = populations in rough lemon petioles. \downarrow = times of first appearance of Pierce's disease symptoms in the grapevine leaves. Each data point represents the mean of three samples.

TABLE 2. Inoculation of citrus seedlings used as rootstocks with an isolate of the Pierce's disease bacterium

Seedling	Symptoms ^a		
	8 mo	12 mo	18 mo
Carrizo citrange	0/3	3/3 DB, LD	3/3 DB, LD
Trifoliolate	0/3	2/3 LD	3/3 DB, LD
Rangpur lime	2/3 LD	2/3 LD	2/3 DB, LD
<i>C. macrophylla</i>	0/3	0/3	1/3 DB, LD
Cleopatra mandarin	0/3	0/3	0/3
Sour orange	0/3	0/3	0/3

^aThe numerator is the number of rootstock seedlings with symptoms and the denominator is the number inoculated. The symptoms were: DB = dieback of shoots and LD = leaf drop. Two controls of each rootstock remained symptomless.

greenhouse. Although the PD bacterium has a wide host range (6) and has been recovered from blighted citrus (9), this is the first study that shows symptoms produced on citrus by the PD bacterium. The dieback-type symptoms were similar to those of citrus blight in the field. Xylem dysfunction is an internal symptom of blight that also occurred in the inoculated seedlings.

Two isolates from citrus, CB-1 and CB-9, and an isolate from grapevine, PD-1, were pathogenic on rough lemon seedlings. However, isolates from citrus produced earlier and more severe symptoms than did the grape isolate. Further study with more isolates is needed to determine whether host specificity or isolate virulence differences are responsible for this symptom difference. The production of relatively mild symptoms in only two of the six rough lemon seedlings in the screenhouse is puzzling, but may merely reflect the temperature sensitivity of the bacterium (12).

The inability to reisolate the bacterium from the diseased rough lemon seedlings, even though the PD bacterium can be detected in extracts by immunofluorescence, is unexplainable. Possibly, the extraction procedure does not remove viable bacteria from the stems or the bacteria are killed by the procedure. Since stem inoculation points were difficult to assay, growth data on the bacteria in rough lemon had to be based on petiole tissue. The PD bacterium did not multiply to a level that would be readily detectable with a light microscope, as in grapevine petioles. However, bacterial multiplication did occur in the rough lemon, since populations 1 to 6 wk after inoculation were higher than those measured 3 days after inoculation. These populations were mean values and the bacterium was reisolated only from approximately 66% of the inoculated petioles. With such low bacterial populations (10^2 – 10^4 bacteria per centimeter of petiole), the reduced water-flow in rough lemon may not be caused simply by xylem vessel blockage with bacterial aggregates.

Susceptibility to blight in the field varies with the rootstock. Field observations indicated that the rootstocks used in our tests rank for susceptibility between rough lemon (most susceptible), trifoliolate orange, Rangpur lime, Carrizo citrange, alemow, Cleopatra mandarin, and sour orange (least susceptible) (16). In the greenhouse, dieback symptoms were produced in rough lemon, trifoliolate orange, Rangpur lime, and Carrizo citrange but not in sour orange, Cleopatra mandarin, and alemow. Susceptibility of the rootstock seedlings to disease caused by the PD bacterium in greenhouse trials paralleled that of rootstocks to blight in the field. If this similarity holds with more rootstocks and in repeated tests,

inoculation of seedlings could provide a much needed rapid method for screening rootstocks for blight resistance. Currently evaluations must be made in the field on large populations of trees and require a minimum of 10 yr.

Results of this study have shown that the PD bacterium can induce many of the blight symptoms in citrus. This and other studies (2,8,9,10,15) implicate the PD bacterium as a causal agent of citrus blight in Florida. However, symptoms have not developed in the trees of Valencia grafted on rough lemon rootstocks that we inoculated in the greenhouse. To prove conclusively the causal relationship of PD bacterium to blight, typical symptoms will have to be produced on mature field-grown trees of sweet orange grafted on rough lemon rootstocks. These experiments are in progress.

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