Variability of Virulence in Grapevine Among Isolates of the Pierce's Disease Bacterium

D. L. Hopkins

Professor of plant pathology, University of Florida, Agricultural Research and Education Center, Leesburg 32749-0388.
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ABSTRACT

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The average virulence of Pierce's disease (PD) bacterial isolates obtained from various grape species (*Vitis vinifera*, *V. labrusca*, *V. rotundifolia*, *V. munsoniana*, and other *Vitis* spp.) in *V. vinifera* 'Carignane' and *V. rotundifolia* 'Carlos' were similar. There was no indication of any host-specific race for grape species among these grape isolates. PD isolates from American elder (*Sambucus canadensis*), however, were less virulent in Carignane than were isolates from grape, and failed to produce symptoms in Carlos muscadine grape. Symptoms in the more resistant muscadine grapevine appeared to depend more on the level of virulence of the individual PD isolate rather than on its host of origin. PD isolates from all host species varied in virulence from avirulent to highly virulent, even if all the isolates had been obtained from a single plant. Virulence was lost during 18 mo of serial transfers.

Additional key words: rickettsialike bacteria, xylem-limited bacteria.

Gram-negative, xylem-limited bacteria (XLB), often referred to as "rickettsialike" bacteria, cause diseases that result in heavy losses in such economically important crops as grapevine, peach, almond, and plum (2,8,13). These bacteria also cause serious leaf scorch and decline diseases of urban trees (7,16).

Pierce's disease (PD) is the major limiting factor in the production of bunch grapes (*Vitis vinifera* L., *V. labrusca* L., and other *Vitis* spp.) in the southeastern United States. The grape industry there is based primarily on resistant *Vitis* species native to the Gulf Coastal Plain of the United States, especially muscadine grapes (*V. rotundifolia* Michx.) (11). Muscadine grape production is a rapidly expanding industry in the southeastern states. However, some muscadine cultivars are susceptible to PD and a few of them cannot be grown commercially in Florida.

The PD bacterium has a very wide host range (4,15). In Florida, it has been isolated from naturally infected Virginia creeper (*Parthenocissus quinquefolia*), peppervine (*Ampelopsis arborea*), American elder (*Sambucus canadensis*), blackberry (*Rubus procerus*), beautyberry (*Callicarpa americana*), and citrus (*Citrus jambhiri* and *C. sinensis*) (*unpublished*).

Little is known about the range and variability of virulence in a natural population of the PD bacterium. In one study, the virulence of PD isolates obtained from grapevines in Florida was quite variable (9). The occurrence of severe PD in muscadine grapes, which were thought to be resistant, and the isolation of the PD bacterium from a diverse assortment of natural hosts make especially pertinent the study of virulence and the search for bacterial strains specific for muscadine or bunch grapes. An understanding of the variability in virulence of the PD bacterium to grapevine is essential to further work on mechanisms of pathogenicity, epidemiology, and control of PD.

The primary goals of this study were to assess the variability in virulence to grapevine of PD isolates obtained from various hosts and to look for isolates that may be host specific for muscadine or bunch grapes.

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MATERIALS AND METHODS

PD strains were isolated from the petioles of leaves with marginal necrosis from bunch grapes, muscadine grapes, wild grapes (*V. munsoniana* and *V. riparia*), American elder, Virginia creeper, and peppervine. The petioles were surface sterilized in 1% sodium hypochlorite for 3 min and rinsed four times in sterile water. They were aseptically cut into 0.5–1.0 cm sections, which were squeezed with forceps. The sap exuding from each section was blotted onto PD2 medium modified by substituting 2 g of soluble starch per liter for bovine serum albumin fraction five (2,3). PD strains were also cultured from root and stem segments of citrus with blight (10). Vacuum infiltration of the segments (5) 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) was used to extract the PD bacteria. This vacuum extract was streaked onto the PD2 medium. Inoculated PD2 plates were incubated at 28 C for 5–10 days.

Identification of the PD bacterium from petioles was not difficult because there were few contaminants. Contamination was a much more serious problem in the vacuum extracts of citrus roots and stems. Any bacterial colony visible to the unaided eye within 3 days was discarded, but any colony visible after 3 days was streaked onto nutrient agar. If the bacterium did not grow on nutrient agar, it was tested in an indirect immunofluorescence test (6) with antisera to the PD bacterium. Reactive isolates were considered to be the PD bacterium.

Isolates from single cells were obtained by dilution plating and the selection of isolated individual colonies. Pure cultures were tested immediately for virulence or were lyophilized to maintain virulence for later testing.

Evaluation of virulence of PD isolates. PD isolates were grown at 28 C on modified PD2 medium. From cultures 4–6 days old, bacterial suspensions were prepared in SCP buffer. Inocula were adjusted to $A_{600\ nm} = 0.25$ (10^7–10^8 colony-forming units [cfu] per milliliter) with a Bausch & Lomb Spectronic 2000 spectrophotometer (9).

Rooted cuttings from five Carignane bunch grape and three Carlos and three Higgins muscadine source plants were used in the virulence studies. Rooted cuttings from three rough lemon citrus plants and seedlings of American elder were also used. Rooted cuttings and seedlings had a minimum of six nodes prior to inoculation.

Inoculations were made with a pin-pricking technique (9). A drop (0.02 ml) of inoculum was placed on the first and third internode from the base of the rooted cutting or seedling. A dissecting needle was used to pierce the grapevine stem three to five times through the drop, resulting in the inoculum being pulled into the plant. This means that $\sim 4 \times 10^5$ to 4×10^6 cfu were inoculated into each plant (9). Inoculated plants were kept in the greenhouse at 28–33 C in the daytime and 20–25 C at night.

Disease incidence, based on symptoms, was recorded every 2 wk. Culturing the PD bacterium from petioles, as described above, was used to confirm visual diagnoses. Inoculated plants were observed for 4 mo after inoculation. After 4 mo, severity ratings of PD symptoms were made with an arbitrary 0–5 scale: 0 = no symptoms, 1 = leaf marginal necrosis (MN) in the basal leaf, 2 = MN in one-third or fewer of the leaves, 3 = MN in one-third to one-half of the leaves, 4 = MN in one-half to three-quarters of the leaves and with dead growing points, and 5 = MN in all of the leaves or a dead plant.

Effect of weekly serial transfers and passage through a grapevine host on virulence of PD isolates. In January 1981, ten isolates of PD were assayed, as described in the preceding section, for virulence to Carignane and the isolates were lyophilized. These same 10 isolates were also transferred weekly to modified PD2 media for 18 mo. Virulence of the isolates that had been transferred weekly for 18 mo was then compared with that of the same 10 lyophilized isolates.

To study the effect of passing isolates through Carignane grapevine on virulence, leaf petioles were inoculated by pin-pricking the petiole through a drop of inoculum (10^7–10^8 cfu per milliliter) as described above. After 30 or more days, the isolates were recultured from the inoculated petiole. Passage through Carignane was repeated six times with each isolate. Virulence of each PD isolate was compared with its virulence prior to passage through Carignane.

Serology. Antisera to PD isolates were prepared in New Zealand White rabbits by intravenous injections of washed bacterial suspensions of 10^8–10^9 cells per milliliter in phosphate buffered saline. Injections of 0.2, 0.5, 1.0, and 2.0 ml were given on days 1, 6, 11, and 18, respectively. Three days after the final injection, the rabbits were bled at the marginal ear vein. The whole blood was placed in a 37 C water bath for 1 hr and centrifuged at 1,000 *g* for 10 min. The serum was recentrifuged at 1,500 *g* for 15 min to eliminate red blood cells, and the clear antiserum was collected and frozen. The agglutination titer of the antisera was greater than 1:1,280. Preimmunization sera were used as normal sera.

Ouchterlony double diffusion tests were performed in 0.75% Noble agar, 0.05 M (pH 8.0) tris-buffered physiological saline, and 0.02% sodium azide. Wells were 7 mm in diameter with 5 mm between wells. Antigens were prepared by grinding 2 ml of bacterial suspension (10^8–10^9 cells per milliliter) with 1 cc of glass beads (0.17–0.18 mm in diameter) for 2 min at top speed on a Vortex-Genie mixer (Scientific Industries, Inc.). The diluted antiserum (1:2) was placed in the center well and the antigens in the outer wells. Plates were incubated at 24 C in a moist chamber for 2–3 days before readings were made.

For enzyme-linked immunosorbent assay (ELISA), gamma globulin was purified from the PD antisera and conjugated with alkaline phosphatase as described by Clark and Adams (1). The ELISA was done as previously described (14), with slight modification. Flat-bottom microtiter plates were coated with gamma globulin by incubating them at 37 C for 2–4 hr. The bacterial samples were incubated overnight at 6 C. The enzyme-labeled gamma globulin was incubated at 37 C for 4–6 hr. The enzyme substrate (*p*-nitrophenyl phosphate) at a concentration of 1 mg/ml was allowed to react for 30 min, and the reaction was stopped by adding 30 μl of 5N NaOH per well. Plates were kept on ice until readings were made at $A_{405\ nm}$ with a Bausch & Lomb Spectronic 20 spectrophotometer. PD bacteria concentrations of 10^5 cells per milliliter were used in the test samples.

RESULTS

Virulence of PD isolates obtained from various hosts. Results of preliminary tests demonstrated that the 0–5 disease severity rating was a simpler and more consistent method of evaluating virulence of PD isolates than were either the time interval from inoculation to first symptoms or the minimum bacterial concentration that produced symptoms. The time-interval-to-symptoms method was too variable; it seemed to depend on size, age, and vigor of the indicator host plant and on environmental condition. The minimum-bacterial-concentration-that-produced-symptoms method was laborious and required excessive greenhouse space for the inoculated indicator plants.

There were no differences in average virulence to Carignane of PD isolates obtained from different species of grape (Table 1). However, isolates from American elder produced much milder PD symptoms in Carignane than did the grapevine isolates. In general, PD isolates were less virulent to Carlos than to Carignane. In Carlos, PD isolates from muscadine were slightly less virulent than were those from bunch grape. Isolates from American elder failed to produce PD symptoms in Carlos.

Two PD isolates from Virginia creeper and three from peppervine had mean disease ratings in Carignane of 3.0 and in Carlos of 0.8, which were very similar to the isolates from grapevine. Virulence of three isolates from citrus was high (rating

TABLE 1. Virulence of Pierce's disease bacteria obtained from various host plants

Source of isolates[a]	Isolates (no.)	Mean disease rating[b] Carignane	Carlos
Bunch grapes (*Vitis vinifera*, *V. labrusca*, and *Vitis* spp.)	11	3.2 ± 0.5	1.9 ± 0.3
Muscadine grapes (*V. rotundifolia*)	11	3.7 ± 0.5	0.7 ± 0.3
Wild grapes (*V. munsoniana* and *V. riparia*)	11	2.9 ± 0.7	1.3 ± 0.4
American elder (*Sambucus canadensis*)	11	1.4 ± 0.3	0

[a] Isolates obtained in the summer of 1982.

[b] Four months after inoculation, disease severity was rated on a scale of 0 to 5: 0 = no symptoms, 1 = leaf marginal necrosis (MN) in the basal leaf, 2 = MN in one-third or fewer of the leaves, 3 = MN in one-third to one-half of the leaves, 4 = MN in one-half to three-quarters of the leaves and a dead growing point, and 5 = MN in all of the leaves or a dead plant. Three Carignane and three Carlos grape plants were inoculated with each isolate. The mean disease ratings of the isolates by source are given with the standard deviations.

TABLE 2. Distribution of virulence ratings of Pierce's disease bacteria obtained from single plants and from different plants of the same species

Source of isolates	No. of isolates with a given disease rating in grape cultivar Carignane:[a] 0	1	2	3	4	5	Mean disease rating
Bunch grapes in the vineyard (18 grapevines)	1	1	2	6	2	6	3.4 ± 0.4
Single grapevine in the vineyard (20 isolates)	1	2	4	6	4	3	3.0 ± 0.3
Single inoculated grapevine in the greenhouse (16 isolates)	2	1	2	4	4	3	3.0 ± 0.4
American elder plants in the field (12 plants)	1	5	4	1	1	0	1.7 ± 0.3
Single American elder plant in the field (18 isolates)	1	1	7	7	2	0	2.4 ± 0.2

[a] Four months after inoculation, disease severity was rated on a scale of 0 to 5: 0 = no symptoms, 1 = leaf marginal necrosis (MN) in the basal leaf, 2 = MN in one-third or fewer of the leaves, 3 = MN in one-third to one-half of the leaves, 4 = MN in one-half to three-quarters of the leaves and a dead growing point, and 5 = MN in all of the leaves or a dead plant. Each isolate was used to inoculate three Carignane plants. The mean disease ratings of the isolates by source are given with the standard deviations.

of 4.7) in Carignane and was similar to bunch grape isolates in Carlos (rating of 2.0).

Isolates obtained from the different species of grape and from citrus, Virginia creeper, peppervine, and American elder infected American elder seedlings and could be reisolated more than 2 yr after inoculation. However, symptoms were mild and isolates could not be compared for virulence, as was done in grapevine. Symptoms in inoculated American elder consisted of yellowing of older leaves with leaf drop, dieback in winter, and stunting of new growth. In rough lemon citrus, only a few of the more virulent isolates produced any symptoms, and those symptoms were primarily stunting with a little dieback. Virulence ratings, therefore, were not taken from the citrus. Producing symptoms in rough lemon were isolates from bunch grape, wild grapes, Virginia creeper, peppervine, and citrus.

PD isolates from grapevine varied in virulence to Carignane from avirulent to highly virulent, regardless of the source species. For example, isolates from 18 different bunch grape plants produced severity ratings ranging from 0–5 in Carignane (Table 2). A similar range of virulence was observed in 20 isolates obtained from a single grapevine. A Carignane plant in the greenhouse, with a disease rating of 5 after inoculation with a single PD isolate, yielded 16 isolates with a range of virulence comparable with that of the isolates from the 18 vineyard bunch grape plants and that of the 20 isolates from a single plant. The mean virulence ratings of the 18 isolates, 20 isolates, and 16 isolates were 3.4, 3.0, and 3.1, respectively. The isolates from American elder plants also had a wide range of virulence to Carignane (0–4), but none of the American elder isolates produced a severity rating of 5.

There does not appear to be any host specificity for grape species among the PD isolates from grape (Table 1). The isolates from bunch grapes were even more virulent in cultivar Carlos of *V. rotundifolia* than were isolates originally isolated from muscadine grapes. Rather than any host specificity, the ability to produce symptoms in the more resistant muscadine grapevine compared with bunch grape was dependent on the level of virulence of the individual isolate (Table 3). Approximately 90% of the isolates that had a virulence rating of 4 or 5 in Carignane produced symptoms in Carlos (ratings ranging 1–4), but 47% of those with a rating of 2 or 3 in Carignane and 15% of those with a rating of 0 or 1 produced symptoms in Carlos. Only a few of the highly virulent isolates (rating of 4 or 5 in Carignane) produced symptoms in the highly resistant cultivar Higgins muscadine grapevine.

Effect of weekly serial transfer and six passages through a grapevine host on virulence of PD isolates. After 18 mo of weekly serial transfer, 9 of the 10 isolates tested were less virulent (Table 4). Only one isolate, which was weakly virulent in the beginning (rating of 1) remained the same. Seven isolates had a disease rating of 4 or 5 in Carignane in the beginning, but only one isolate had a rating of 4 after 18 mo. Freeze-drying of the PD isolates was somewhat effective in maintaining virulence; however, four isolates were slightly less virulent after freeze-drying.

Since avirulent PD isolates survive and multiply in grape tissue (12), an attempt was made to restore virulence to seven avirulent or weakly virulent isolates (disease ratings of 0, 1, or 2) by inoculating Carignane grapevines and reisolating 4 wk, or more, later. This was done six times with each isolate. None of these isolates increased in virulence after six passages through the grapevine host.

Comparisons of the cultural characteristics and serological relationships of PD isolates from various hosts. There were no differences in colony type or growth rates related to the host of origin for the PD isolates. Regardless of the host, primary isolations from a single host plant yielded a range of colonies that varied in growth rate and in colony type from domed colonies with entire margins to flat colonies with irregular margins. After 2 or 3 mo in culture, however, all colonies were domed and had similar growth rates.

The PD isolates from various hosts were also very similar in gel double diffusion and ELISA serological tests. In the gel diffusion, there was considerable variability in the intensity of bands, but there were no bands unique to any isolate or group of isolates. Variability in intensity of bands was as great among isolates from a single host as among those from different hosts. In the ELISA tests, the mean $A_{405\ nm}$ values for the bunch grape, muscadine grape, wild grape, and American elder isolates were nearly identical (Table 5). The large standard deviation also indicates variability among isolates from a single host, as did the gel diffusion tests.

DISCUSSION

The identification of severe PD symptoms in muscadine grapevines (previously thought to be resistant) indicated that a race of the PD bacterium that is host specific to *V. rotundifolia* may

TABLE 3. Comparison of the virulence of Pierce's disease bacteria to plants of grape cultivars Carignane and Carlos

	No. of isolates with a given disease rating in grape cultivar Carlos:[a]					
Disease rating in Carignane[a]	0	1	2	3	4	5
4 or 5 (19 isolates)	2	7	5	4	1	0
2 or 3 (17 isolates)	9	5	3	0	0	0
0 or 1 (13 isolates)	11	2	0	0	0	0

[a] Four months after inoculation, disease severity was rated on a scale of 0 to 5: 0 = no symptoms, 1 = leaf marginal necrosis (MN) in the basal leaf, 2 = MN in one-third or fewer of the leaves, 3 = MN in one-third to one-half of the leaves, 4 = MN in one-half to three-quarters of the leaves and a dead growing point, and 5 = MN in all of the leaves or a dead plant. Each isolate was used to inoculate three Carlos plants.

TABLE 4. Loss of virulence of Pierce's disease bacteria to cultivar Carignane grape plants during 18 mo of weekly serial transfer

	Disease rating[a]		
Isolate	Jan 1981	June 1982	June 1982 (FD)
CB80-4	5	1	3
PD80-1	5	0	3
PD80-2	5	2	5
PD80-5	4	2	3
PD80-8	2	1	1
PD80-12	5	1	5
PD80-23	1	1	1
PD79-8	1	0	1
PD-15	5	0	5
PD-1	5	4	5
Mean (10 isolates)	3.8	1.2	3.2

[a] Four months after inoculation, disease severity was rated on a scale of 0 to 5: 0 = no symptoms, 1 = leaf marginal necrosis (MN) in the basal leaf, 2 = MN in one-third or fewer of the leaves, 3 = MN in one-third to one-half of the leaves, 4 = MN in one-half to three-quarters of the leaves and a dead growing point, and 5 = MN in all of the leaves or a dead plant. Each isolate was used to inoculate three cultivar Carignane grape plants. Inoculations were made in January 1981 to test the virulence of the isolates at the beginning, in June 1982 to test their virulence after 18 mo of serial transfer, and in June, 1982 to test their virulence after 18 mo of storage as a freeze-dryed (FD) suspension.

TABLE 5. Comparison of Pierce's disease bacteria from various host plants by enzyme-linked immunosorbent assay (ELISA)

Source of isolates	Isolates (no.)	Mean absorbance ($A_{405\ nm}$)[a]
Bunch grapes (*Vitis vinifera*, *V. labrusca*, and *Vitis* spp.)	11	0.83 ± 0.13
Muscadine grapes (*V. rotundifolia*)	11	0.86 ± 0.25
Wild grapes (*V. munsoniana* and *V. riparia*)	7	0.88 ± 0.29
American elder (*Sambucus canadensis*)	5	0.88 ± 0.23

[a] The mean absorbance for the isolates by source are given with the standard deviation. The $A_{405\ nm}$ value for buffer alone was ≤ 0.02.

exist. In this study, there was, however, no indication of host specificity for grape species among the PD isolates from grape. Instead, the ability of PD isolates to induce symptoms in the more resistant muscadine grapevine depended on the level of virulence of the individual isolate, regardless of its host of origin.

Isolates from American elder differed from other isolates in that none of them produced a disease rating of 5 in Carignane and none of them induced symptoms in the Carlos muscadine grape. This could mean that the isolates from American elder are a different race or strain of the PD bacterium that may be host specific for *V. vinifera* and non-pathogenic in *V. rotundifolia*, or even a different XLB that also happens to cause mild symptoms in *V. vinifera*. Another possible explanation is that only weakly virulent PD isolates occur in American elder or can be cultured from it. The relatively few isolates obtained from Virginia creeper and peppervine, which are members of the family Vitaceae, were similar to the grape isolates in virulence to Carignane and Carlos.

If the criterion for determining whether an XLB is the PD bacterium is its ability to produce symptoms in a susceptible grapevine, then the American elder bacterium, by this criterion, is the PD bacterium. The PD bacterium has a very wide host range (4) and it is also possible that more than one XLB could produce symptoms in grapevine.

The variation in virulence among PD isolates from grapevine species could have resulted from natural variation in the bacterial populations in the host or from a loss of virulence in culturing. Isolates obtained from a single grapevine that had been inoculated with a pure culture of a highly virulent isolate in the greenhouse had a similar range of virulence as isolates obtained from different grapevines in the vineyard. This indicates that most of the variability in virulence results from a loss of virulence in culturing. Long-term culturing of the PD bacterium by weekly serial transfer resulted in further loss of virulence. This lost virulence was not regained when the bacterium was reinoculated into the grapevine host and allowed to multiply for long periods of time.

If we assume that other XLB may also lose virulence in culturing and single-cell purification, tests of pathogenicity of an unknown XLB should either be made with a large number of isolates or with a mixture of colonies from the primary isolation without purifying the isolates from single cells. Otherwise, there could be the risk of using avirulent isolates of the XLB.

Virulence of PD isolates used in a research program must be tested frequently. Storage of lyophilized stock cultures is one way to maintain virulent isolates. Also, storage in silica gel (17) is a convenient way of preserving virulent working stocks of the XLB. The bacteria are easily recovered when needed and virulence has been constant for at least 1 yr.

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