Citrus and Coffee Strains of *Xylella fastidiosa* Induce Pierce's Disease in Grapevine

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ABSTRACT

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Xylella fastidiosa causes citrus variegated chlorosis (CVC) disease in Brazil and Pierce's disease of grapevines in the United States. Both of these diseases cause significant production problems in the respective industries. The recent establishment of the glassy-winged sharpshooter in California has radically increased the threat posed by Pierce's disease to California viticulture. Populations of this insect reach very high levels in citrus groves in California and move from the orchards into the vineyards, where they acquire inoculum and spread Pierce's disease in the vineyards. Here we show that strains of *X. fastidiosa* isolated from diseased citrus and coffee in Brazil can incite symptoms of Pierce's disease after mechanical inoculation into seven commercial *Vitis vinifera* varieties grown in Brazil and California. Thus, any future introduction of the CVC strains of *X. fastidiosa* into the United States would pose a threat to both the sweet orange and grapevine industries. Previous work has clearly shown that the strains of *X. fastidiosa* isolated from Pierce's disease– and CVC-affected plants are the most distantly related of all strains in the diverse taxon *X. fastidiosa*. The ability of citrus strains of *X. fastidiosa* to incite disease in grapevine is therefore surprising and creates an experimental system with which to dissect mechanisms used by *X. fastidiosa* in plant colonization and disease development using the full genome sequence data that has recently become available for both the citrus and grapevine strains of this pathogen.

Pierce's disease (PD) of grapevines (*Vitis vinifera* L.), caused by a strain of the bacterium *Xylella fastidiosa* (39), was first noted in California near Anaheim in 1884, and has been an important disease problem since that time in California. The disease prevents the cultivation of *V. vinifera* and French hybrid grapes in the southeastern United States, where the glassy-winged sharpshooter (GWSS), *Homalodisca coagulata* Say, is one of its insect vectors (4). The introduction (1) and establishment of the GWSS in California has accelerated the spread of the bacterium in vineyards (26,30). The insect has been found in 15 counties in California and is established in 9 of these counties, threatening the $30 billion California grape industry.

Citrus variegated chlorosis (CVC) is a destructive disease of citrus, first described from Macaubal in São Paulo State, Brazil, in 1987 (35), and later shown to be caused by a strain of *X. fastidiosa* (5,14). The disease can be transmitted by contaminated budwood, sharpshooters, and natural root grafts (15). In the year 2000, 35% of the 200 million sweet orange trees in São Paulo State showed CVC symptoms (2), representing a direct loss of more than US$100 million. CVC also has been found in almost all the other citrus production areas in Brazil, as well as in some regions of Argentina (3) and Costa Rica (W. Villa Lobos, *personal communication*).

Coffee leaf scorch (CLS), also known as Requeima do Café, is another economically important disease caused by a strain of *X. fastidiosa*. First described in São Paulo, Brazil, the disease is widespread and has apparently been present in Brazil as an unrecognized problem for a long time (9,20), reducing coffee production in some plantations. Recently, CLS was also discovered in Costa Rica (34). Strains of *X. fastidiosa* isolated from citrus and coffee plants are closely related (32) and share the same sharpshooter insect vectors, and we have shown that the strains of *X. fastidiosa* from citrus could cause CLS disease in coffee plants (20).

Prior to the description of CVC disease, several strains of *X. fastidiosa* were isolated from sweet orange trees by Hopkins in Florida (19). Research has shown that these strains were probably PD strains resident in citrus, since PD was transmitted from these trees to grapevines using insect vectors (19) and PCR-based genomic characterization of these strains placed them within the PD group, but not the CVC group, of *X. fastidiosa* (28,32). This indicates that the PD strains of *X. fastidiosa* could survive in, colonize, and be transmitted from citrus plants. Recent epidemiological data showed that proximity to citrus increased the incidence and severity of PD in grapevines in the Temecula Valley of California (25). This relationship occurs because in California the GWSS preferentially feeds and reproduces to high levels on citrus. Taken together, this suggests that commercial citrus plantations could serve as a reservoir of inoculum for PD strains of *X. fastidiosa.* However, despite numerous attempts, *X. fastidiosa* has not been isolated or detected in citrus that was experimentally inoculated with California grape strains of *X. fastidiosa* or from citrus trees growing adjacent to vineyards experiencing epidemic PD losses in Southern California (B. Hill, M. Blua, B. Kirkpatrick, and A.H. Purcell, *unpublished data*). It should also be pointed out that the PD strains of *X. fastidiosa* present in the United States apparently are not capable of inciting CVC, because citrus has been grown commercially in California and Florida for more than a century in the presence of PD-affected grapevines, and CVC has never been reported in the United States. However, it is not known whether the CVC strains of *X. fastidiosa* from South America, which clearly do colonize, multiply, and induce disease in citrus in South America, are capable of multiplying within and causing disease in grapevine. The objectives of this study were to determine if the citrus and coffee strains of *X. fastidiosa* could induce PD symptoms in grapevines after mechanical inoculation. If the CVC strains could incite PD, the introduction of these strains into California would pose a tremendous threat to both the sweet orange and wine grape industries, given the high populations of GWSS found in many areas of California.

MATERIALS AND METHODS

Plant materials and management. Grape (*V. vinifera*) seedlings were used in the experiments carried out at Fundecitrus, Araraquara, SP, Brazil. Seeds of grape varieties 'Pinot Noir' and 'Cabernet Sauvi-

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gnon' were kindly provided by Alexander Purcell, University of California, Berkeley. Seeds of grape varieties 'Beni Taka', 'Italia', 'Niagara', and 'Rubi' (all *V. vinifera*) were obtained from table fruit purchased at a market in Araraquara, SP, Brazil. The seeds were soaked in water with aeration for 24 h, and then stored at 4°C for 30 days. The seeds were then sown in vermiculite and germinated after 2 weeks. Seedlings were transplanted 1 week later to pots (2 liters in volume) containing PlantMax (Eucatex, São Paulo, Brazil) based substrate consisting of the coffee formulation of PlantMax and composted bovine manure (50/50), supplemented with 300 g of Osmocote fertilizer (14:14:14) per 100 liters of substrate. Plants were watered daily, and Ridomil was applied as necessary as a soil drench in Brazil.

Rooted cuttings of grape variety 'Chardonnay' were used for the experiments carried out in ARS-USDA, Beltsville, MD. These plants were provided by Bruce Kirkpatrick, from the Foundation Plant Materials Service at the University of California, Davis. At both locations, all plants were maintained in screen-protected greenhouses throughout the experiments to preclude unintended transmission of the pathogen by insect vectors.

***X. fastidiosa* strains.** In the experiment carried out at Fundecitrus, *X. fastidiosa* strains 9a5c (21,37) and 2105 (20) from citrus and 3124 from coffee (20) were used to inoculate grapevines. These strains had previously been triply cloned and shown to be pathogenic in citrus or coffee plants. Bacteria were grown in liquid PW medium (8), at 30°C for 7 days prior to inoculation. All medium components were "tissue-culture tested" grade (Sigma Chemical Co., St. Louis, MO). In the experiment carried out at Beltsville, three strains of *X. fastidiosa* were used. One was a PD strain (Temecula 042801) kindly provided by Bruce Kirkpatrick, University of California, Davis; the second strain was CVC strain 9a5c (21,37), and the third was a fresh citrus isolate designated L001. This strain was isolated in April and triply cloned in May and June 2001 from CVC-affected 'Pera' sweet orange twigs collected from the Bebedouro Citrus Experiment Station, Bebedouro, SP, Brazil. Bacteria were grown in liquid PW medium at 30°C for 7 days prior to inoculation.

Plant inoculations. The inoculations in both experiments were done using 7-day-old *X. fastidiosa* cultures in liquid PW medium (10^8 to 10^9 CFU/ml). Each plant was inoculated at 20 points on the stem using a 5-ml syringe with a 20G needle to simultaneously wound and deliver a droplet of inoculum (20). At Fundecitrus, each of the three strains of *X. fastidiosa* was injected into eight plants of each of the six grape cultivars (Table 1) on 15 March 2001. Eight control plants were mock-inoculated with liquid PW medium only. The plants in the experiments carried out at Beltsville were inoculated on 28 June 2001, using five plants per strain of *X. fastidiosa* and another five plants inoculated with liquid PW medium as control. In the experiments carried out at Fundecitrus, the plants were 45 days old when inoculated and had four to six leaves. At Beltsville, the plants were cut 10 cm above the soil, and one new shoot was trained for each plant. The shoot was inoculated when it had four to six leaves and was allowed to grow after inoculation to as long as 2 m. At both locations, plants were observed regularly for symptoms of PD (38).

Detection of *X. fastidiosa*. All test plants were assayed for *X. fastidiosa* by using double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) and polymerase chain reaction (PCR). DAS-ELISA was performed according to Chang et al. (5), with a polyclonal antibody raised against strain 9a5c (Sanofi Diagnostic Pasteur, Marnes la Coquette, France). One gram of leaf petiole and midribs was ground in a Polytron blender in 2 ml of phosphate-buffered saline (PBS). The suspension was filtered through cheesecloth, and an aliquot of 150 µl of the final filtrate was used for each well of the ELISA plate. For positive controls, cell suspensions of strains 9a5c, 2105, and 3124 were adjusted to a Klett-Summerson colorimeter reading of 50 (10^8 to 10^9 CFU/ml). Aliquots of 150 µl of the suspension were used per well of the ELISA plate. Colorimetric detection was done using alkaline phosphatase labeled antibody, and the plates were read at 405 nm after 30 min of incubation.

PCR assays were performed on xylem extracts obtained from petioles by using a syringe to displace the xylem contents with sterile deionized water (MilliQ, Bedford, MA) (10). The xylem extracts were diluted 1,000-fold in deionized water. Diluted extracts (200 µl) were transferred to PCR tubes and centrifuged at 12,000 × *g* for 15 min. The supernatant was removed, and the pellet was dried at 65°C for 15 min and then suspended in 20 µl of deionized water. PCR reagents and primers specific for *X. fastidiosa,* 272-1-int and 272-2-int (28), were added directly into the extracts and used for PCR amplification in a final reaction volume of 40 µl. The amplification program began with an incubation at 94°C for 4 min to lyse the bacteria, followed by 35 cycles of 94°C for 1 min, 64°C for 1 min, and 72°C for 1 min, followed by a final extension cycle of 10 min at 72°C. Following electrophoresis through agarose gels, PCR products were visualized by staining with ethidium bromide.

Table 1. Evaluation of grape plants inoculated with *Xylella fastidiosa* from citrus and coffee[w]

Grape variety	*X. fastidiosa* strain	Number inoculated	ELISA[x] positive (%)	PCR[y] positive (%)	Symptomatic plants (%)
Beni Taka	9a5c	8	37.5	62.5	62.5
	2105	8	25.0	50.0	50.0
	3124	8	37.5	50.0	62.5
Subtotal/mean	3	24	33.3	54.2	58.3
Italia	9a5c	8	25.0	37.5	37.5
	2105	8	12.5	12.5	0
	3124	8	37.5	50.0	37.5
Subtotal/mean	3	24	25.0	33.3	25.0
Niagara	9a5c	8	62.5	75.0	87.5
	2105	8	25.0	37.5	50.0
	3124	8	50.0	62.5	62.5
Subtotal/mean	3	24	45.8	58.3	66.7
Rubi	9a5c	8	37.5	50.0	62.5
	2105	8	25.0	25.0	37.5
	3124	8	37.5	62.5	75.0
Subtotal/mean	3	24	33.3	45.8	58.3
Pinot Noir	9a5c	8	37.5	62.5	75.0
	2105	8	25.0	50.0	50.0
	3124	8	50.0	62.5	75.0
Subtotal/mean	3	24	37.5	58.3	66.7
Cabernet Sauvignon[z]	9a5c	16	31.3	56.3	68.8
	2105	16	25.0	37.5	50.0
	3124	16	37.5	62.5	81.3
Subtotal/mean	3	48	31.3	52.1	66.7
All cultivars	9a5c	56	37.5	57.1	64.3
	2105	56	23.2	35.7	41.1
	3124	56	41.1	58.9	67.9
Total	3	168	33.9	50.6	57.7

[w] All inoculated plants evaluated for the presence or absence of disease symptoms and tested for the presence of *X. fastidiosa* by both enzyme-linked immunosorbent assay (ELISA) and polymerase chain reaction (PCR).

[x] Test was scored as positive for *X. fastidiosa* if the absorbance at 405 nm was three times that of the uninoculated control plants (5).

[y] Test was positive if a 600-bp product was produced by primer pair 272-1-int and 272-2-int (28).

[z] Combined data of eight plants from each of two seed sources.

Disease incidence and severity. At Fundecitrus, grapevines were scored for the presence or absence of typical symptoms of PD, and the data were recorded as percentage of plants showing symptoms. At Beltsville, a disease severity index (DSI) was calculated for 'Chardonnay' as follows, using data recorded at 10-day intervals following inoculation. The first 20 leaves of each plant were evaluated for the presence or absence of symptoms. The DSI was calculated based on the degree of symptom severity as measured by leaf scorch symptom: 0 = healthy leaf, 1 = leaf with less than 5.0% scorched area, 2 = leaf with 5.1 to 10.0% scorched area, 3 = leaf with 10.1 to 25.0% scorched area, 4 = leaf with 25.1 to 50.0% scorched area, 5 = leaf with more than 50% scorched area. The leaf scorch severity ratings were summed for each plant (20 leaves per plant) and divided by the number of leaves rated times the maximum possible rating (20 × 5 = 100) to give the final DSI, with a range from 0 to 1.

Analysis of variance was performed on the means of the disease severity index, and when significant, this was followed by a means comparison test (Tukey's) using a confidence level of $P = 0.05$.

RESULTS

Development of Pierce's disease. At Fundecitrus, the 'Niagara' seedlings began to show typical PD leaf scorch symptoms 2 months postinoculation. The seedlings of the other five cultivars (Table 1) started to develop symptoms 3 months postinoculation. Strains 9a5c (Fig. 1) and 2105 from citrus and 3124 from coffee caused typical symptoms of PD in 'Niagara' seedlings, which included the presence of "green islands" on the stems caused by irregular lignification of the bark. With the exception of the seedlings of grape 'Italia' inoculated with CVC strain 2105, which did not show typical PD symptoms, the disease incidence (symptomatic/inoculated) ranged from 0.375 to 0.875 among seedlings of the six grape cultivars (Table 1).

At Beltsville, 'Chardonnay' plants developed symptoms 1 month postinoculation. Strains 9a5c (Fig. 2A) and L001 from citrus produced symptoms in 'Chardonnay' that were the same as those produced by the PD strain Temecula 042801 (Fig. 2B). However, the mean DSI was higher for the PD strain than for the CVC strains at 50 and 60 days postinoculation (Table 2). All 'Chardonnay' plants inoculated with PD or CVC strains produced typical PD symptoms in the experiment at Beltsville. No symptoms were observed on control plants mock-inoculated with pure PW medium at either Fundecitrus or Beltsville.

ELISA, PCR, and isolation of the pathogen. At Fundecitrus, 2 months postinoculation, most of the symptomatic plants gave positive ELISA reactions. However, *X. fastidiosa* was not detected by ELISA from more than one-third of the symptomatic plants of all six grape cultivars inoculated with different *X. fastidiosa* strains (Table 1). PCR tests for *X. fastidiosa* were negative from all parts of the plants of all six varieties unless the extracts were first washed in 1,000 volumes of water, indicating that grapevine extracts contained strong inhibitors of the PCR. Sodium ascorbate and polyvinylpyrrolidone (22) failed to prevent the inhibition in our experimental conditions. However, *X. fastidiosa* could be detected by PCR with the primer pair 272-1-int and 272-2-int (28) in extracts washed in 1,000 volumes of water from all of the ELISA-positive plants and some ELISA-negative ones. *X. fastidiosa* strains were easily isolated from the symptomatic plants during the period from 1 to 2 months postinoculation. Before or after this period, there were relatively more contaminants that interfered with the isolations. All of the inoculated strains were reisolated from the inoculated plants, and they did not differ morphologically (light microscope), serologically (ELISA), or by growth in PW medium compared with the original strain used as inoculum. The reisolated and original strains of *X. fastidiosa* produced a band of 600 bp after PCR amplification with the specific primer pair 272-1-int and 272-2-int. No control plants gave positive ELISA or PCR reactions, and *X. fastidiosa* was never isolated from any control plants.

Susceptibility of grape cultivars to *X. fastidiosa*. Among the six grape cultivars inoculated in experiments at Fundecitrus, 'Niagara', 'Pinot Noir', and 'Cabernet Sauvignon' were the most susceptible to *X. fastidiosa* (Table 1). 'Niagara' had the highest rate of positive ELISA reactions among the three cultivars. 'Italia' was the least susceptible variety, and 'Beni Taka' was moderately susceptible to *X. fastidiosa*. At Beltsville, 100% of 'Chardonnay' plants inoculated either with PD strain Temecula 042801 or CVC strains from citrus plants developed typical symptoms of PD, consistent with the fact that 'Chardonnay' is very susceptible to PD (38). However, it is difficult to directly compare the susceptibility of 'Chardonnay' with that of the six grape cultivars used in the experiments in Brazil, because the greenhouse conditions at the two locations, al-

Fig. 1. Symptoms of Pierce's disease in 'Niagara' grapevine caused by the citrus strain 9a5c (37) of *Xylella fastidiosa*. The inoculated plant is on the left and the control plant is on the right.

though similar during the summer seasons when these experiments were conducted, were not exactly the same. PD strains of *X. fastidiosa* could not be included in the experiment in Brazil for phytosanitary reasons.

Relative pathogenicity *X. fastidiosa* strains from citrus and coffee. There were no consistent differences in the response of grapevines to strain 9a5c from citrus and strain 3124 from coffee. Both produced typical symptoms of Pierce's disease (Table 1), and the two strains induced the same disease symptoms after mechanical inoculation of grape cultivars 'Benitaka', 'Italia', and 'Pinot Noir'. When inoculated on 'Niagara', strain 9a5c induced more diseased plants than did strain 3124; however, strain 3124 induced more diseased plants than strain 9a5c when inoculated on grape 'Rubi' and 'Cabernet Sauvignon'. The other CVC *X. fastidiosa,* strain 2105 from citrus, induced significantly fewer ELISA-positive, PCR-positive, and symptomatic plants, in all six inoculated grape cultivars, than did the other CVC and CLS strains. No typical symptoms of PD were observed from the inoculated plants of grape 'Italia' with the strain 2105. Taken together, this suggests that there may be variable interactions between these *X. fastidiosa* strains and specific *V. vinifera* varieties.

Disease severity on 'Chardonnay' grapevine. There was no significant difference in disease severity among the different *X. fastidiosa* strains 40 days postinoculation in 'Chardonnay' (Table 2). Fifty and sixty days postinoculation, disease severity was significantly higher for strain Temecula 042801 than for strains 9a5c or L001. These results demonstrate that Temecula 042801 from grapevine was more pathogenic on grapevine than CVC strains 9a5c and L001. There was no significant difference in the disease severity index of strains 9a5c and L001, indicating that there was no significant difference between the pathogenicity of the citrus strains 9a5c and L001 when inoculated into 'Chardonnay' grapevine.

DISCUSSION

X. fastidiosa has an exceptionally broad host range, with strains of the bacterium causing important diseases on a very wide range of shade and fruit trees (17,29). In some cases, individual strains are able to infect and incite disease in different hosts (20,23,40), but in other cases cross infectivity experiments have given negative results (36). The bacterium is also able to infect and colonize a wide range of alternate hosts without causing disease (11,18,33). The recent introduction and establishment of the glassy-winged sharpshooter into California has dramatically increased the threat that PD poses to the California grape and wine industries because it is a much more abundant vector for the pathogen than are the other leafhopper species native to California. California also has a significant sweet orange industry that is threatened by the potential introduction of strains of *X. fastidiosa* that cause CVC disease in Brazil.

Our results show that strains of *X. fastidiosa* that cause both CVC disease and CLS disease in South America also are capable of infecting, colonizing, and producing symptoms of PD in commercial grape cultivars (*V. vinifera*). While PD strain Temecula 042801 killed one of the five 'Chardonnay' plants inoculated, neither of the citrus strains caused symptoms that progressed until the plants died. However, this experiment was abruptly terminated 88 days after inoculation, when a tornado destroyed the greenhouse. Therefore, the longer-term consequences of infection of 'Chardonnay' by the citrus strains could not be determined in this study. However, it is clear that an inadvertent introduction of CVC into the United States would directly threaten the grape as well as the citrus industry.

Analyses of genomic (16,27,32) and plasmid (6,16) DNA, as well as sequence comparisons of the 16SrDNA region from cultured *X. fastidiosa* (7,16), have consistently shown the existence of distinct groups of strains associated with host and geographic regions of origin. The relationships among citrus, coffee, and grapevine strains were estimated using rep-PCR–based and random amplified polymorphic DNA (RAPD)-PCR–based DNA markers. The citrus and coffee strains were only distantly related to the grapevine strains, having a coefficient of similarity of only 0.4 (32). The citrus and coffee strains also induce symptoms in their hosts of origin that are very different from those associated with Pierce's disease of grapevine (5,14,20,38). In spite of this distant genetic and pathologic relationship to PD strains,

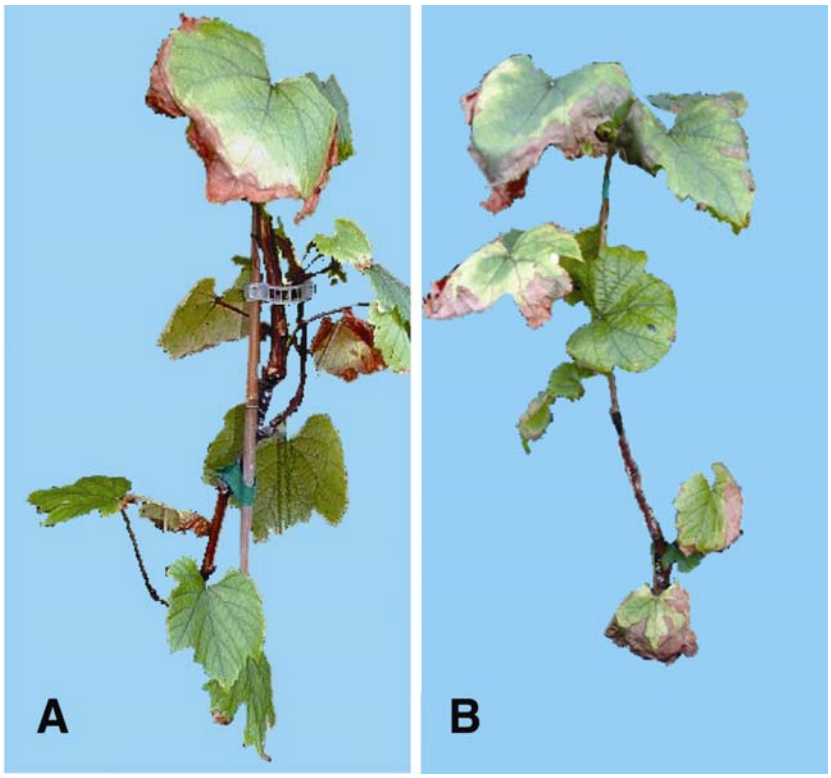

Fig. 2. Symptoms of Pierce's disease in 'Chardonnay' grapevine. **A,** *Xylella fastidiosa* citrus strain 9a5c (37). **B,** *X. fastidiosa* grape strain Temecula 042801.

Table 2. Pierce's disease severity in 'Chardonnay' grapevines inoculated with different strains of *Xylella fastidiosa*

	Mean disease severity index after inoculation[y,z]		
Strains[x]	**40 Days**	**50 Days**	**60 Days**
PD 042801	0.370 B	0.696 A,a	0.798 A,a
CVC9a5c	0.340	0.380 b	0.380 b
CVCL001	0.292	0.326 b	0.372 b

[x] Pierce's disease strain Temecula 042801, and citrus strains CVC9a5c and CVCL001.
[y] See text for explanation of how the disease severity index was calculated. The range of values is 0 to 1.
[z] When the mean disease severity index values differed over time for a particular strain, differences are denoted by uppercase letters. When the mean disease severity differed among strains for a given date, differences are denoted by lowercase letters. Means followed by the same letter are not significantly different ($P = 0.05$)

our work establishes that citrus and coffee strains can incite PD symptoms in grapevine. It is interesting that PD was reported in Costa Rica in 1979 (12), and more recently *X. fastidiosa* has been reported to be causing both CVC (W. Villa Lobos, *personal communication*) and CLS (34) in Costa Rica. Are both PD and CVC/CLS strains of *X. fastidiosa* present in Costa Rica, or is the PD reported in Costa Rica caused by infection of grapevine by CVC/CLS strains? We note that although *X. fastidiosa* causes epidemic diseases of both citrus (14) and coffee (9) in Brazil, PD of grapevine has not been reported there. Some hosts of *X. fastidiosa* may show moderate symptoms when grown in the greenhouse, but none when grown in the field (A. H. Purcell, *unpublished observations*). Thus, it may be possible that Brazilian grapevines are infected with CVC strains of *X. fastidiosa* without showing clear disease symptoms.

The basis of both host selection and disease induction by *X. fastidiosa* are not understood. However, full genome sequence data reveal that *X. fastidiosa* does not possess the *HRP*/*avr* gene system described in other bacterial pathogens of plants and animals (37). We note that although the citrus and coffee strains of *X. fastidiosa* produced PD symptoms after inoculation into 'Chardonnay' grapevine, disease severity was significantly less for these strains than what were observed for the "homologous"' strain Temecula 042801 (Table 2). We have also shown that strains of *X. fastidiosa* isolated from diseased coffee and citrus in Brazil are able to induce symptoms of PD in a set of six commercial grape (*V. vinifera*) cultivars (Table 1) and identified a strain of *X. fastidiosa*, 2105 (citrus), that overall induced less severe symptoms than did the other strains tested from citrus and coffee. Strain 2105 also induced fewer symptomatic plants in each of six tested grape varieties than did the strains from citrus and coffee (Table 1). These observations could form the basis for experimental dissection of the mechanisms that underlie virulence in the citrus, as well as grapevine, strains of *X. fastidiosa*, using the recently available genomic sequence information (37) and systems for genetic analysis of this pathogen (13,24,31) that are being developed.

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