Occurrence of Pierce's Disease Bacteria in Plants and Vectors in California

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ABSTRACT

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Wild hosts of the Pierce's disease (PD) bacterium were identified in riparian weeds along the Napa River and near springs on Spring Mountain in Napa County, CA, by enzyme-linked immunosorbent assay (ELISA). The PD bacterium was found in field-collected *Fragaria vesca* var. *californica, Montia linearis, Rubus procerus*, and *Vinca minor*. The bacterium that was cultured in vitro from *R. procerus* and *V. minor* produced typical symptoms of PD following inoculation of healthy grape indicator plants. Bacteria were not isolated in vitro from ELISA positive *F. californica* and *M. linearis* on any media tested. ELISA was used to identify PD bacterium in populations of different homopterans collected in widely scattered locations in California. The PD bacterium was found in wild populations of *Carneocephala fulgida, Draeculacephala minerva, Graphocephala atropunctata, Helochara delta, Pagaronia tredecimpunctata*, and *Philaenus spumarius*. The PD bacterium was cultured in vitro from ELISA positive groups of *C. fulgida, D. minerva, G. atropunctata*, and *P. tredecimpunctata*. The same groups of insects also transmitted PD to healthy *Vitis vinifera* 'Mission' indicator plants. Using ELISA, it was possible to distinguish the PD bacterium in pure culture and in host tissues from other morphologically similar xylem-limited bacteria.

Pierce's disease (PD) was described from grapevines in the Santa Ana River Valley in Southern California in 1892 (15). For many years a virus etiology was suspected because the pathogen could be transmitted from diseased to healthy grapevines by grafting (9), and because several different homopterous insects were shown to be vectors of the disease agent (11). In 1973, workers in California (6) and Florida (12) reported a fastidious bacterium with a rippled cell wall occurring in the xylem elements of diseased vines. When the bacterium was successfully cultured 5 yr later (1), serological techniques were developed to identify the bacterium in grape tissue (14) as well as in tissue from other affected hosts (7,19).

Pierce's disease was originally called "California vine disease" or "Anaheim disease," implying that it originated in the district in southern California where it was first observed and studied. Later it was found in many areas of North America with mild winter climates, including Costa Rica in the American tropics (8,10,18,21). Dwarf in alfalfa (11) and leaf scorch in almonds (2,13) were shown to be caused by the same agent. In addition to these economically important hosts the disease was identified in an array of other plant species (3).

Before the causal bacterium was cultured, identification of PD agent in symptomless hosts required insect transmission tests (3). Colonies of healthy sharpshooter leafhoppers, which were obtained and maintained with much difficulty; a supply of healthy grape and alfalfa indicators, which occupied considerable greenhouse space; and time periods of 2 to 3 mo were needed for symptom development. Freitag (3) established that PD could be transmitted to 75 host plant species of 100 tested, using these experimental methods.

A high titered antiserum specific for PD bacterial antigens (14) was developed against a pure culture of the bacterium. With this antiserum we were able to adapt ELISA and in vitro isolation methods to identify the wild hosts, *Conium maculatum* (poison hemlock), *Cyperus eragrostis* (umbrella sedge), and *Paspalum dilatatum* (dallis grass), infected with the PD bacterium. These weeds were growing in riparian areas in Napa Valley where the disease rapidly killed grapevines (19).

Here, we report additional wild host species in the Napa Valley infected with the PD bacterium. We also report the isolation of PD bacterium from insects and the adaptation of the ELISA method to assay insects for the presence of PD bacterium. The possible use of ELISA to distinguish PD bacterium from other similar xylem-limited bacteria and results of tests from several leafhopper populations and a spittle bug collection for PD bacterium are described.

MATERIALS AND METHODS

Antiserum production and ELISA tests. A PD bacterial isolate (PD-NA-5) from Napa Valley, CA, was grown on sterile RG-7 agar medium (17). Bacteria were maintained aerobically at 28 C and subcultures were made each week onto fresh medium. The PD-NA-5 isolate was used to produce an antiserum with a reciprocal titer of 4,096 by injections into female New Zealand white rabbits (18).

ELISA tests were performed in flat bottom micro-ELISA test plates (Dynatech Laboratories, Alexandria, VA 22314) as described previously (14,17). The plates were read at $A_{405\ nm}$ 30 min after adding the substrate and without adding NaOH in a Multiskan ELISA reader (Flow Laboratories, Inglewood, CA 90302).

Sampling of plants and insects. Leaves and young stems from wild plant species growing in the vicinity of PD-affected grapes along the Napa River and near springs on Spring Mountain in Napa County were collected, transported on ice to the laboratory, and tested in ELISA. Several plants of each species were sampled from June 1979 through May 1981. Healthy grape, periwinkle, and tomato tissues, as well as phosphate-buffered saline (0.1 M PBS, pH 7.4) were included as controls in each ELISA test. In vitro isolations from ELISA-positive plants were made on RG-7, PD-2 (2), BC-YE (22) or BC-ZE (20) media according to methods described elsewhere (19,20).

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In vitro isolations of PD bacterium from *Corneocephala fulgida* Nott., *Draeculacephala minerva* Ball, *Graphocephala atropunctata* Signoret, and *Pagaronia tredecimpunctata* Ball from Napa Valley that tested positively in ELISA were processed as follows: insect heads were removed, groups of two to five heads were surface sterilized (18), rinsed in sterile distilled water, and ground in 5–10 ml of RG-7 liquid medium or phosphate buffer saline (0.1 M PBS, pH 7.0). Serial twofold dilutions were made in RG-7 liquid medium or PBS and 1–2 ml of it was inoculated onto RG-7 or PD-2 medium.

Populations of sharpshooters including *C. fulgida, D. minerva, D. crassicornis* Van Duzee, *G. atropunctata, Helochara delta* Oman, *P. tredecimpunctata*, and the spittlebug, *P. leucophalins* L., were collected at different times of the year from riparian vegetation in several counties of California, especially in areas in or near to Napa County. They were transported live to the laboratory in plastic bags on ice, and were kept on dry ice for 5 min before testing in ELISA.

Preparation of plant and insect tissues for ELISA. Plant samples were prepared as described before (17) with some modifications. One gram of tissue consisting of leaves and stems was ground in 15 ml of extraction buffer (phosphate buffer saline, pH 7.4, + 2% polyvinyl pyrrolidone + 0.05% Tween-20) for 10–20 sec in a Polytron (Brinkman Instruments, Weekburg, NY 11590); the extract was filtered through four layers of cheesecloth and centrifuged at 23,000 *g* for 15 min. The pellet was collected, resuspended in 1 ml of the extraction buffer and sonicated for 10–15 sec at 52.5 w/cm^2 with a Bronwill®-III sonicator (Bronwill Scientific Co., Rochester, NY 14603). The sonicate was then tested by ELISA.

Insects were processed in an identical fashion in lots consisting of four to 10 individuals from one insect population. Samples of sharpshooter leafhoppers that were known either to carry PD bacterium or to be free of it were previously obtained from Alexander H. Purcell, Berkeley, CA, or from PD-affected grapes and tested to determine if ELISA could detect the bacterium from insect tissues. Four insects from an infective population were required for a positive ELISA test based on the studies where 100 known infective *G. atropunctata* were tested in groups consisting of 1, 2, 4, 8, and 16.

Specificity of PD bacterial antiserum in ELISA. Antiserum prepared against the PD bacterium was tested for its specificity against plum leaf scald (PLS) (20) affected plum petioles. *D. minerva* and *G. atropunctata* infected with the PD bacterium or healthy based on transmission studies with seedlings of *V. vinifera* 'Pinot Noir' were tested in ELISA using PD and PLS bacterial antisera along with miner's lettuce and wild strawberry collected from Napa Valley, CA. Bacteria were harvested in sterile water from BC-ZE agar medium and were prepared for ELISA as previously described (17). Plant and insect samples were prepared as described herein.

TABLE 1. ELISA detection of Pierce's disease bacterium in tissue samples of weed hosts and other plants collected from areas adjacent to streams and springs in Napa County, CA

Plant species	Common name	Individuals assayed (no.)	Plants infected with Pierce's disease bacterium[a] (no.)
Aesculus californica	California buckeye	68	0
Agropyron sp.	wheat grass	24	0
Artemisia absinthium	mugwart	14	0
Cynodon dactylon	Bermuda grass	45	0
Daucus carota	wild carrot	21	0
Elymus sp.	wild rye	11	0
Eschscholzia californica	California poppy	32	0
Fragaria californica	wild strawberry	48	3
Fritillaria sp.	fritillary	20	0
Hedera helix	english ivy	30	0
Hordeum nodosum	wild barley	14	0
Lactuca serriola	pickly lettuce	16	0
Montia linearis	miner's lettuce	62	3
Nasturtium officinale	water cress	33	0
Nerium oleander	oleander	14	0
Plantago lanceolata	plantain	26	0
Polygonum ramosissimum	knotweed	18	0
Prunus armeniaca	apricot	17	0
Quercus dumosa	scrub oak	30	0
Rhus diversiloba	poison oak	3	0
Rosa californica	wild rose	16	0
Rubus procerus	Himalaya blackberry	92	13
Salix bebbiana	willow	41	0
Sambucus caerulea	elderberry	72	0
Sorghum halepense	Johnson grass	40	0
Sorghum vulgare var. *sudanense*	sudan grass	23	0
Vitis californica	wild grape	50	0
Vinca minor	periwinkle	96	19

[a] Plants were considered infected when $A_{405_{nm}}$ values in ELISA tests were at least twice those of control phosphate-buffered saline and known healthy plant tissue.

RESULTS

Wild plant populations tested by ELISA showed additional infected individuals of *C. maculatum, C. eragrostis*, and *P. dilatatum*, in addition to those previously reported (18). In addition, we found naturally infected individuals of *Fragaria californica* (wild strawberry), *Montia linearis* (miner's lettuce), *Rubus procerus* Himalaya (blackberry), and *Vinca minor* (periwinkle) (Table 1). Plants were considered infected when $A_{405\ nm}$ values in ELISA were at least twice those of known healthy plant tissue.

Not all plants within the same area were infected according to the ELISA results. Both healthy and infected individuals were found within a sampling site. The $A_{405\ nm}$ values for bacteria-infected samples were significantly greater than those of PBS and healthy samples. The minimum/maximum $A_{405\ nm}$ values for healthy and infected *R. procerus* were 0.024/0.031 and 0.94/0.208, respectively. Similarly, the minimum/maximum $A_{405\ nm}$ values for healthy and infected *V. minor* were 0.026/0.040 and 0.091/0.205, respectively. Monthly sampling and testing by ELISA of blackberry, periwinkle, and grape indicated that infected plants of blackberry and grape could be detected in summer using ELISA. On the other hand, infected periwinkle could be detected in winter (Table 2). Blue-

TABLE 2. Detection of Pierce's disease bacterium by ELISA in grape and selected perennial wild plants throughout the year at two locations in California

Plant[a]	Location[b]	Month[c] J	F	M	A	M	J	J	A	S	O	N	D
Rubus procerus													
blackberry	1	–	–	–	–	+	+	–	+	+	–	–	–
	2	–	–	–	–	–	+	+	+	+	–	–	–
Vinca minor													
periwinkle	1	+	+	+	–	+	–	–	–	–	–	+	+
	2												
Vitis vinifera													
grape	1	–	–	–	–	+	+	–	+	+	+	–	–
	2	–	–	–	–	+	+	+	+	+	–	–	–

[a] Four plants were selected at each location and the same plants were sampled each month. Petioles and stems from each plant were used as test materials. Grape cultivars, Cabernet Sauvignon (location 1), and Chardonnay (location 2) were used.
[b] Location 1 was in Napa, next to Napa River and location 2 was on Spring Mountain.
[c] Collections were made from January to December 1980.

green sharpshooters collected from these hosts were also positive in ELISA when the plant tissue was positive and vice versa. Infected grape stems and petioles could be detected by ELISA during April through November (Table 2). However, the bacterium could be isolated from roots at all times. ELISA tests with monthly collections of blackberry and periwinkle tissue showed that the bacterial concentration in these hosts may not be the same throughout the year like in grape (Table 3).

None of the infected wild plant species in the study, other than *C. maculatum* and *P. dilatatum*, showed symptoms either in the field or in the greenhouse when collected from the field and transported and grown in the greenhouse. Three plants each of *F. californica* and *M. linearis* and five plants each of *R. procerus* and *V. minor* were observed for symptoms for 1 yr. Infected plants of *C. maculatum* in the field showed marginal burning of leaves and infected plants of *P. dilatatum* were stunted. Leaf scorching was produced in healthy *C. maculatum* grown from seed in the greenhouse by inoculating plants with a pure culture of the PD bacterium by the needle-injection method (18).

Ten each of healthy plants of *C. eragrostis, R. procerus*, and *V. minor* were inoculated with a pure culture of PD-NA-5 by needle injection (18) and held in the greenhouse. None of these plants showed symptoms after 16 wk. However, after 1 yr six of inoculated *R. procerus* showed leaf scorching which started from the tip of the leaf and progressed downward. The scorching was similar to that of PD bacterium inoculated into grape. All of the inoculated plants of *V. minor* showed chlorosis of leaves 1 yr after inoculation. Uninoculated controls remained healthy. None of the inoculated plants were dead 18 mo after inoculation. During the same period, none of the inoculated *C. eragrostis* showed any symptoms. Bacterial colonies were isolated from these plants, as well as from the symptom-expressing plants of *C. maculatum* on RG-7 or PD-2 medium after 10 days of aerobic incubation at 27 C. No bacterial colonies were isolated from uninoculated plants of the same species. The colonies were circular, with entire margins, white, smooth, and convex, all of which are characteristic of PD bacterium. The isolates were serologically indistinguishable from the PD bacterial isolate, PD-NA-5, in immunodiffusion or ELISA tests. This was the PD bacterial isolate used to inoculate the plants. The same type of bacterium was also isolated from 12 ELISA-positive samples of blackberry and periwinkle collected from Napa Valley. The bacteria, when inoculated in the greenhouse to indicator cultivar Mission grapes, produced typical PD. Bacteria were not isolated on any of the media we tested from ELISA-positive *M.*

TABLE 3. ELISA detection of Pierce's disease bacteria in the xylem-feeding vectors collected from foliage of weed host plants growing adjacent to permanent water sources in several California counties

Site no.[a]	Place of collection	Insect species	Individuals in test population (no.)	Composite lots (no.)	Lots positive for Pierce's disease bacteria in ELISA tests[b] (no.)	(%)
1.	Tehama County					
	Red Bluff	*Draeculacephala minerva*	10	2	0	0
2.	Butte County					
	Chico	*D. minerva*	25	5	2	40
3.	Mendocino County					
	Ukiah	*D. minerva*	13	3	0	0
4.	Sonoma County					
	Alexander Valley	*Graphocephala atropunctata*	11	2	0	0
		D. minerva	15	3	0	0
	Jenner	*Philanenus leucophalius*	20	4	1	25
	Santa Rosa	*D. minerva*	12	3	1	33
	Sonoma	*G. atropunctata*	19	4	0	0
		D. minerva	12	3	0	0
5.	Napa County					
	Napa River & tributaries	*G. atropunctata*	1020	170	49	29
		D. minerva	664	121	40	33
		Pagaronia tredecimpunctata	141	28	4	14
6.	Solano County					
	Vallejo	*D. minerva*	9	2	1	50
	Wooden Valley	*G. atropunctata*	12	3	1	33
		D. minerva	12	3	2	66
		P. tredecimpunctata	16	4	1	25
7.	Contra Costa County					
	Martinez	*D. minerva*	57	14	2	14
8.	San Mateo County					
	Half Moon Bay	*D. minerva*	20	4	1	25
9.	Santa Cruz County					
	Santa Cruz	*D. minerva*	12	3	0	0
	Watsonville	*D. minerva*	11	2	0	0
10.	Monterey County					
	Castroville	*D. minerva*	10	2	0	0
	Monterey	*D. minerva*	15	3	0	0
	Moss Landing	*D. minerva*	30	6	0	0
	Salinas	*D. minerva*	12	3	0	0
11.	San Luis Obispo County					
	Morro Bay	*D. minerva*	10	2	0	0
12.	San Joaquin County					
	Manteca	*Carneocephala fulgida*	12	3	2	66
		D. minerva	4	1	0	0
13.	Tulare County					
	Sequoia National Forest	*Helochara delta*	29	5	1	20
		D. crassicornis	21	4	0	0

[a] Pierce's disease (PD) on grapes has been previously found at sites 2, 5, 6, 7, and 12. No PD on grapes has been previously reported from the other sites.
[b] Insects were considered to be infected when $A_{405\ nm}$ values were at least twice those of phosphate-buffered saline or known healthy insect samples.

linearis (miner's lettuce) and *F. californica* (wild strawberry) or from ELISA-negative blackberry in 10 attempts representing five plants.

Pierce's disease bacterium was also detected by the ELISA tests in insect populations collected in several different counties (Table 3). Some of the insect collections were made from areas where PD in grape or alfalfa dwarf has not been observed and from areas where no grapevines have ever been grown.

Infected *D. minerva* were detected near Chico in the northern Sacramento River Valley, an area in which Pierce and others (5) found diseased vines at about the turn of the century. Infected *H. delta* were found in a mountain meadow at an elevation of 2,200 m in the Sequoia National Forest near the top of the Sierra Nevada Mountains east of Fresno, confirming a report made by Freitag and Frazier (4). Infected spittle bugs and leafhoppers were found at Jenner and Half Moon Bay, respectively, localities on the Pacific sea coast.

Infected leafhopper populations ranging from 25 to 64% were found along streams that drain into the bays comprising the flooded lower reaches of the San Joaquin and Sacramento rivers. *D. minerva* was the most common sharpshooter encountered in the samplings. Populations of this species from the counties bordering the Sacramento river delta region carried the bacterium but collections of the insect along the central California coast from Santa Cruz to Morro Bay were free of it.

In the Napa Valley, populations of *G. atropunctata* collected in early May 1979, were carrying PD bacteria. Infected populations of this insect were detected by ELISA tests throughout the growing season through April 1981. This was confirmed by transmission studies using cultivar Mission grape seedlings. The $A_{405\ nm}$ values varied from one group of insects to the other. However, the $A_{405\ nm}$ values for infected leafhoppers were significantly greater than those of healthy ones and the PBS control. The minimum/maximum $A_{405\ nm}$ values for healthy and infected *G. atropunctata* were 0.027/0.041 and 0.080/0.204, respectively. The minimum/maximum $A_{405\ nm}$ values for *D. minerva* were 0.011/0.046 and 0.103/0.179, respectively.

Colonies that showed characteristics of PD bacterium in culture were observed in 8 of 10 attempts on RG-7 or PD-2 medium when isolations were made from infected *C. fulgida, D. minerva, G. atropunctata*, and *P. tredecimpunctata*. These colonies were separated from other bacterial colonies that also developed occasionally and a pure culture was obtained. The bacterial isolates from these colonies were serologically indistinguishable from PD bacterial isolates from diseased grape tissue. *Carneocephala fulgida, D. minerva*, and *G. atropunctata* from the same collections were also inoculated to cultivar Mission grapes which developed typical PD symptoms in 3–4 mo. Pierce's disease bacteria were reisolated in vitro from these plants.

Populations of *G. atropunctata* were frequently collected from blackberries and *Vitis rupestris* 'St. George' rootstocks in the Napa Valley. On several occasions we obtained positive ELISA readings in both the leafhopper population and the blackberry plant but we did not detect the PD bacterium in plants of St. George from which we collected leafhoppers that produced positive ELISA readings. In vitro isolations from stems and petioles of cultivar St. George were also negative.

Positive results in ELISA were obtained only with PD bacterial antiserum, but not with PLS bacterial antiserum, when four groups (four insects in each group) of field-collected *D. minerva* or *G. atropunctata* were tested by ELISA using PD and PLS bacterial antiserum (Table 4). Two of the field-collected miner's lettuce plants, which were held in the greenhouse, and wild strawberry plants, which were previously found to be infected, reacted positively in ELISA with the PD bacterial antiserum, but not with the PLS bacterial antiserum (Table 4).

TABLE 4. ELISA tests from field-collected sharpshooters, *Montia linearis* (miner's lettuce) and *Fragaria californica* (wild strawberry) with antisera to Pierce's disease (PD) and plum leaf scald (PLS) bacteria

Insect/plant material	No. tested	No. positive	$A_{405\ nm}$ PDB	$A_{405\ nm}$ PLSB
Green sharpshooter (*Draculacephala minerva*)	12	4	0.102[a] (+)	0.025 (−)
Blue-green sharpshooter (*Grapohocephala atropunctata*)	18	9	0.150 (+)	0.036 (−)
Miner's lettuce	12	2	0.104 (+)	0.020 (−)
Wild strawberry	18	2	0.092 (+)	0.028 (−)
PLS-affected plum	4	4	0.113 (+)	0.672 (+)
Healthy plum	4	0	0.030 (−)	0.034 (−)
Phosphate-buffered saline (PBS)			0.028	0.031

[a] + = positive reaction, − = negative reaction. Reactions were considered positive only when the average A_{405nm} values were at least two to three times higher than the values obtained with PBS.

DISCUSSION

The ELISA test can be used as a rapid method for detecting the PD bacterium in wild host species and in populations of insects that become infective by feeding on such hosts. The value of the test is confirmed by experiments showing that the PD bacterium can be isolated from most of the plants or insects that produce positive ELISA reactions. However, the test is not useful in detecting low concentrations of bacteria (14). Because we previously demonstrated that the antiserum prepared against the PD bacterium was highly specific in ELISA with no reaction with other bacteria (14), ELISA may be used to monitor insect populations for the presence of PD bacteria in California. The PD bacterial antiserum reacts with other xylem-limited bacteria, but with less intensity (20). Hence, in vitro isolation should be carried out along with ELISA studies.

Our study has been mainly concerned with the identification of wild host plants of the PD bacterium and potential PD vector populations. During a 3-yr period, we identified some of the important host plant species of PD along the Napa River and on Spring Mountain in the Napa Valley. Blackberry was shown to be one of the most common breeding hosts for *G. atropunctata* (16). By collecting blackberry and periwinkle samples throughout the year and testing them by ELISA, we have demonstrated that the bacterium can not be detected in the aerial parts of a plant throughout the year. This was also observed with grape plants during spring when the young grape leaf is free of bacteria. The wild plants that possess the bacteria play an important role in serving as reservoirs from which the insects can acquire the bacteria and inoculate grape plants (4). Thus, the weeds and wild plants along the permanent water sources in Napa Valley might play an important role in the epidemiology of PD. Control of weed hosts might help in the control of PD. However, the best control of PD should be the simultaneous control of weed hosts and insect vectors and the use of tolerant cultivars. Plants and insects positive in ELISA can be considered as hosts of the PD bacterium. However, plants or insects giving negative results can neither be said to be hosts or nonhosts of the bacterium because ELISA can detect the bacterium only when the concentration is above 10^5 cells per milliliter (14).

Identification of the PD bacterium in several areas far removed from grape cultivation and its absence in other areas where grapes are grown, suggests that the PD bacterium may probably be a native of the riparian vegetation along many of the waterways of the San Francisco Bay area. In the Napa Valley, PD spreads from vegetation only as far as the insect vectors migrate into the vineyards from the hosts from which they acquire the PD bacterium while feeding (4). In the Napa Valley, this is about 100 m or less from a source of permanent water (17). Grape plantings in Napa are usually over 100 m from permanent water sources and are thus free from the threat of disease.

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