Phytotoxin(s) Produced in Culture by the Pierce's Disease Bacterium

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ABSTRACT

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A phytotoxin that caused burning and scorching of leaves of grape, almond, and plum was obtained by washing cell cultures of rickettsialike bacteria that had been isolated from grapevines affected with Pierce's disease (PD), almond affected with almond leaf scorch, and alfalfa affected with alfalfa dwarf. Detached leaves and leaves on dormant and rooted grape cuttings treated with a bacteria-free solution of toxin developed scalding and necrosis on the leaf margins, symptoms that are characteristic of PD. Although no bacteria were present in the solution containing the toxin, they were easily isolated from rooted and nonrooted leafy cuttings similarly treated with cell suspensions of the bacteria. Leaves and shoots of other woody and herbaceous plants bioassayed with the toxin were not visibly affected, except for periwinkle and Eureka lemons, which showed general wilting.

Additional key words: xylem-limited bacteria.

Rickettsialike bacteria (RLB) are prokaryotes that cause or are associated with about 20 plant diseases (6). Recently some of the RLB from plants have been cultured on artificial media. Thus, Koch's postulates can be fulfilled (1) and these RLB characterized. The RLB which incites Pierce's disease (PD) of grapevines (*Vitis vinifera*) also causes almond leaf scorch (ALS) and alfalfa dwarf (AD) (1,4,8,12).

Bacterial cells, tyloses, and gums block xylem vessels of grape and almond infected by the PD bacterium (3,12–14). Mircetich et al (12) observed that only 15% of the xylem vessels contained RLB in almond (*Prunus amygdalus* Batsch.) with severe leaf scorch symptoms. Mollenhauer and Hopkins (13,14) indicated that in cross sections of grape leaves with the marginal burn symptom of Pierce's disease, rarely more than 40% of the vessels contain RLB and, even then, parts of these vessels were not totally occluded. Vascular occlusions do not account for the marginal leaf scorch, especially since wilting is not part of the syndrome. Furthermore, chlorosis followed by marginal scorching of leaves frequently preceded the detection of RLB in ALS (12). The possible involvement of a toxin in the foliar symptoms of ALS and PD was suggested (12). Hopkins (7) suggested that there may be sufficient blockage over the length of a PD-affected grape petiole to produce sufficient water stress to result in leaf marginal necrosis.

We report the isolation of a phytotoxic preparation from RLB grown in pure culture that causes foliar symptoms of PD and ALS. The preparation produced the burning and scalding of the leaves characteristic of the disease in grapes and almond. A preliminary report of this work has been published (10).

MATERIALS AND METHODS

Isolates of Pierce's disease bacteria. Recently isolated cultures of Pierce's disease bacteria from grapevines from Costa Rica (5), Mexico (15), and California, and also from almond and alfalfa in California were used to produce toxin. Bacteria were grown for 10 days at 29 C on PD2 agar medium in 10-cm-diameter petri dishes (2).

Collection of phytotoxin. The phytotoxic preparation was collected by washing the bacterial cells from PD2 agar plates with 25 ml of sterile, glass-distilled, deionized water. This wash contained between 10^7 to 10^8 cells per milliliter. The wash was then centrifuged at 17,000 *g* for 10 min at 4 C, and the supernatant was passed through a 0.2 μm nitrocellulose filter. The filtrate freed of bacterial cells, which will be referred to as crude culture filtrate (CCF), was either used immediately or lyophilized and stored under vacuum at 4 C.

Plant materials. Dormant and green grapevine cuttings were collected from healthy mother vines growing in an isolated foundation vineyard of the Foundation Plant Materials Service, University of California, Davis. The dormant cuttings were stored at 4 C in plastic bags until needed. Green and dormant cuttings were rooted in a vermiculite rooting medium in a mist chamber. Herbaceous plants and rooted cuttings were grown in UC-mix in either glass or fiberglass greenhouses (11). Healthy almond, Santa Rosa plum (*Prunus salicina* Lindl.), and peach seedling (*Prunus persica* L.) leaves and spurs were obtained from the college farm, University of California, Davis.

Bioassay for phytotoxin activity. Leaves from various grape cultivars were excised under water with a razor blade previously rinsed in acetone. The water was sterilized by filtration through a 0.2-μm nitrocellulose filter. After approximately 1 hr in water, the leaves, with an adhering drop of water on the petiole bases, were transferred to 5-ml glass serum vials (No. 223738, Wheaton Glass Co., Millville, NJ 08332) containing CCF. Two leaves were placed in each vial. The vials were placed under a Gro-Lux fluorescent light at 25 C. Detached leaves and young shoots from plants other than grape were tested similarly for sensitivity to CCF. Autoclaved CCF and CCF from uninouclated PD2 agar plates were used as controls.

Dormant grape cuttings were freshly pruned on both ends, the basal end was placed in CCF, and the vacuum line with 50 cm Hg vacuum was attached to the top end. Rooted cuttings were similarly vacuum infiltrated except the root tips were clipped off. One to 3 ml of CCF was drawn into each cutting or rooted cutting.

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Column chromatography. Lyophilized CCF was resuspended in 1/10 of its original volume and 1-ml aliquots were chromatographed on a Sephadex G-50 column (1.5 × 40 cm) equilibrated with 0.05 M potassium phosphate buffer, pH 7.0, or distilled, deionized water, either of which had been sterilized by filtration through a 0.2-μm nitrocellulose filter. The flow rate was 28 ml/hr, 10-ml fractions were collected and bioassayed for phytotoxin activity by using detached grape leaves.

RESULTS

Treatment of detached leaves, cuttings, or rooted cuttings from grape with CCF produced the foliar symptoms characteristic of Pierce's disease (Fig. 1). Detached grape leaves bioassayed in CCF developed burning and scalding beginning at the leaf margin and progressing in concentric zones toward the leaf base (Fig. 1). Plant parts placed in autoclaved CCF and CCF from uninoculated PD2 plates did not develop these symptoms. Differential reactions of detached leaves to CCF were observed among susceptible and tolerant grape cultivars (Table 1). Leaves from PD-susceptible cultivars Pinot Noir, Mission, and St. George, developed symptoms in 6–12 hr, whereas tolerant cultivars (Dog Ridge and Salt Creek), *V. rotundifolia,* and *V. munsoniana* did not show the effects for 48–72 hr. The CCF from isolates of PD bacteria from Costa Rica, Mexico, almond, and alfalfa (*Medicago sativa* L.) produced results similar to those reported in Table 1 when bioassayed with detached grape leaves. Rooted grape cuttings of Pinot Noir, Chardonnay, Chenin blanc, and St. George, vacuum infiltrated with CCF, produced the foliar symptoms of PD within 24 hr, whereas rooted cuttings of Dog Ridge and Salt Creek did not develop leaf symptoms until 72 hr after treatment. When dormant grape cuttings were treated with CCF then planted in a rooting bed, the newly emerging leaves that appeared after 1–2 wk displayed the foliar symptoms of PD.

Lyophilized CCF resuspended at 2 mg dry weight per milliliter of sterile water (about one-tenth the original volume) severely affected dormant and rooted grape cuttings. Most of the cuttings died within 48 hr. However, dormant and rooted grape cuttings vacuum infiltrated with 10^8 cells per milliliter of the PD bacterium that had been washed three times in 0.02 M potassium phosphate buffer (pH 7.0) containing 0.85% sodium chloride, took 5–8 wk to develop PD symptoms. The PD bacterium could be readily isolated and cultured from PD bacterium-infiltrated cuttings, but not from the CCF-infiltrated cuttings. No symptoms developed on detached leaves or on dormant or rooted cuttings infiltrated with either autoclaved CCF or washes from uninoculated plates. The CCF was checked for presence of bacteria by centrifuging at 17,000 *g* for 10 min, decanting the supernatant, resuspending the pellet in 50 μl of sterile water, and observing under a phase-contrast microscope. Further tests for PD bacteria were made by placing the resuspended CCF on a protein-coated microscope slide and staining with fluorescent antisera specific for PD bacteria (9). Fresh and resuspended CCF were streaked on PD2 plates to detect any PD bacteria remaining in the CCF after sterilizing filtration. No rickettsialike bacteria were detected in CCF by these procedures.

Leaves of the following plants were tested for sensitivity to CCF obtained from recently isolated California PD bacterial strains from grape, almond, and alfalfa, but none was affected: tomato, *Lycopersicon esculentum* (L.) Karst. ex Farv.; potato, *Solanum tuberosum* L.; jimsonweed, *Datura stramonium* L.; bell pepper, *Capsicum annuum* L.; cucumber, *Cucumis sativus* L.; tobacco, *Nicotiana tabacum* L.; turnip, *Brassica rapa* L.; olive, *Olea europaea* L.; peach, *Prunus persica* L.; pear, *Pyrus communis* L.; cabbage, *Brassica oleracea* L.; cauliflower, *B. oleracea* L. var. *capitata*; broccoli, *B. oleracea* L.; aster, *Callistephus chinensis* (L.) Nees; and daisy, *Chrysanthemum frutescens* L. Leaves from periwinkle (*Vinca minor* L.) and Eureka lemon (*Citrus limon* (L.) Burm. f.) showed only slight wilting, but did not display burning or scorching symptoms when their petioles were immersed in CCF. Detached leaves, spurs, and young shoots of almond and plum were sensitive to CCF. Burning and necrosis of the leaf margins typical of ALS and plum leaf scald were produced in almond and plum, respectively. There was no apparent difference in the symptoms that developed on treated bioassay plants after treatment with CCF obtained from several different strains of PD, ALS, and AD bacteria.

When chromatographed in a column of Sephadex G-50, toxic activity eluted after the void volume as two distinct fractions, fraction 1 was collected in tubes 13 and 14 and fraction 2 in tubes 18, 19, and 20 (Fig. 2). In the detached grape leaf bioassay, fraction 1 usually produced a wilting symptom, often without necrosis. Fraction 2, which had a much lower molecular weight than fraction 1, produced the more typical burning and necrosis along the leaf margin, but did not cause wilting. When fraction 2 was concentrated (1 mg/ml) and a leaf from a sensitive grape cultivar was used for bioassay, the margin of the leaf became desiccated and died without first turning brown or red. At lower concentrations (100 μg/ml), more typical foliar symptoms of PD were produced.

Bacteria grown on PD2 medium with soluble starch (1 g/500 ml) or activated charcoal (1 g/500 ml) instead of bovine serum albumin

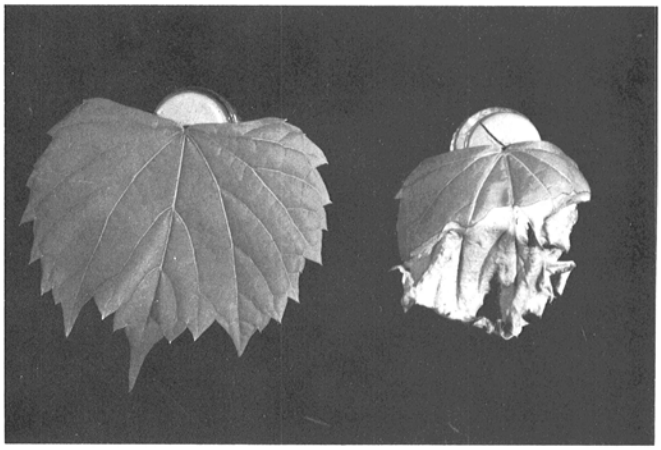

Fig. 1. Effect of crude culture filtrate (CCF) on detached leaves of St. George grape. The leaf on the right, incubated for 12 hr in CCF from a Napa Valley isolate of Pierce's disease bacterium showed the marginal leaf scald symptom characteristic of Pierce's disease of grapevines. The leaf on the left was incubated in autoclaved CCF.

TABLE 1. Time required by different grape cultivars for development of foliar symptoms of Pierce's disease after treatment with CCF[a]

Cultivars	Field susceptibility rating	Hours post-treatment	
		Leaves[b]	Cuttings[c]
Pinot Noir	1[d]	6–12	<24
Mission	1	6–12	ND[e]
St. George	1	6–12	<24
Chardonnay	2	12–18	<24
Chenin blanc	2	12–18	<24
White Riesling	2	12–18	ND
Salt Creek	3	48–60	72
Dog Ridge	3	48–60	72
Vitis munsoniana	4	60–72	ND
Vitis rotundifolia	4	60–72	ND

[a] Treatment was with crude culture filtrate (CCF) collected from a recent California isolate of the PD bacterium. Autoclaved CCF and CCF obtained from uninoculated PD2 plates were used as controls.

[b] Leaf petioles with leaves attached were immersed in CCF; four leaves were used for each cultivar. The results are a summary of three different CCF preparations. The numbers are the time in hr noted for the first and the last leaves to show PD foliar symptoms. Readings were taken at 6-hr intervals.

[c] Twelve rooted cuttings without soil were treated by vacuum infiltration. Observations were recorded at 24-hr intervals and the times indicated were the time when all cuttings showed PD foliar symptoms.

[d] Scale 1–4 (with 4 being most tolerant) based on susceptibility to Pierce's disease in the field.

[e] ND = not determined.

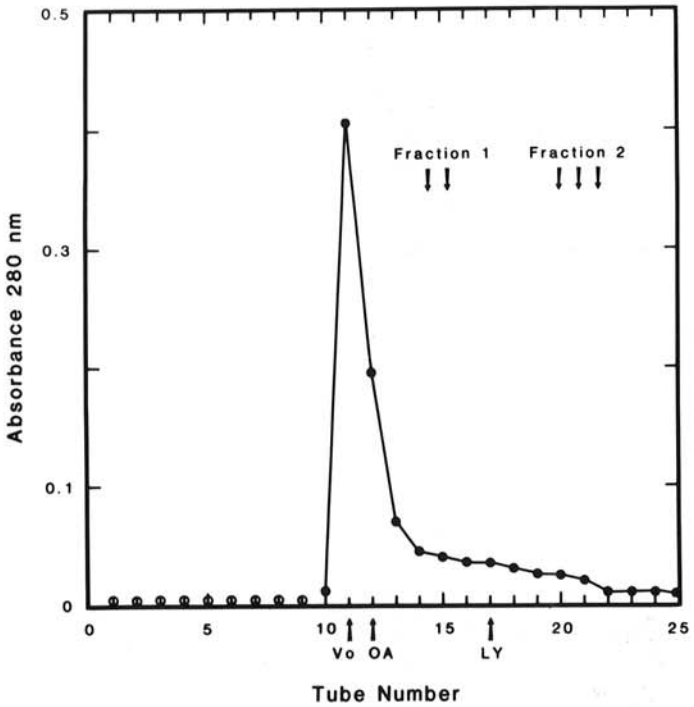

Fig. 2. Fractionation of phytotoxic activity and the absorbance at 280 nm from crude culture filtrate of Pierce's disease bacterium. Fractionation was on a Sephadex G-50 column (1.5 × 40 cm) equilibrated with 0.05 M potassium phosphate buffer, pH 7.0, with a flow rate of 28 ml/hr. Ten-minute fractions were collected, absorbance at 280 nm was determined, and fractions were used for bioassay on detached grape leaves. Phytotoxic activity eluted in tubes 13 and 14 and in tubes 18, 19, and 20 as fraction 1 and fraction 2, respectively. The void volume (V_0), determined by blue dextran, and elution of ovalbumin (OA) (mol wt 4.4×10^4) and lysozyme (LY) (mol wt 1.3×10^4), as determined on previous runs, are indicated by arrows (↓).

still produced a phytotoxic CCF.

CCF preparations lost phytotoxicity after repeated freezing and thawing, but were not adversely affected by lyophilization. Crude culture filtrates incubated at room temperature for 2–4 days lost phytotoxicity. Treatment of CCF preparations with Proteinase K (100 μg/ml for 2 hr at 25 C) did not destroy their phytotoxic properties.

DISCUSSION

The phytotoxin(s) in CCF from all isolates of PD bacterium produced the leaf margin burning and scalding that is characteristic of PD (Fig. 1), ALS, and plum leaf scald. The CCF collected from several recent isolates of PD, ALS, and AD similarly affected all the indicator plants tested. There was no discernable difference in the phytotoxicity of CCF collected from PD strains isolated from the United States, Mexico, or Costa Rica.

The separation of toxic activity of CCF after chromatography through a Sephadex G-50 column indicates that more than one toxin is produced. The toxic activity that elutes as fraction 1 usually produces wilting without necrosis. This may be due to direct physical vascular plugging by high-molecular-weight compounds, the stimulation of occlusion formation in xylem vessels, or a combination of both. Fraction 2 did not produce wilting, but did produce the burning and necrosis of leaf margins typical of the foliar symptoms of PD.

The foliar symptoms characteristic of PD develop very quickly in rooted cuttings of susceptible grape cultivars after treatment with CCF. However, PD bacteria washed in PBS and infiltrated into rooted cuttings do not produce foliar symptoms of PD for 5–8 wk. This suggests the phytotoxic activity produced by PD bacterium may be important in the symptom development of PD. The quick response of susceptible cultivars to the CCF preparations from PD bacteria may be useful for a rapid screening of grape, almond, and plum cultivars for tolerance and/or resistance to this bacterium.

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