Association of a Rickettsialike Organism with Pierce's Disease of Grapevines and Alfalfa Dwarf and Heat Therapy of the Disease in Grapevines

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ABSTRACT

Ultrathin sections of grapevines affected with Pierce's disease and alfalfa plants affected with dwarf showed rickettsialike organisms in the lumen and between the vessels of the xylem tissues when examined with an electron microscope. They were not present in sections from healthy grapevines until such vines were inoculated by exposure to infective leafhoppers. The organisms had cell walls, cytoplasmic membranes, and a cytoplasm with electron-dense organelles. They measured ca. 0.4 μ in diam and up to 3.2 μ in length. The cell wall, of several layers, was rippled, much like the cell walls of other rickettsiae known in animal hosts. The organisms could not be cultured on an artificial medium. Grapevines were freed of the causal agent of Pierce's disease by immersion of the entire plant in water at 45 C for 180 min, 50 C for 20 min, or 55 C for 10 min. The vines tolerated immersion at 45 C for 24 hr, 50 C for 2.5 hr, and 55 C for 10 min. Plants freed of the causal agent remained healthy. Such plants were susceptible to the disease when reinoculated by infective leafhopper vectors.

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Additional key words: Hordnia circellata, bacterial disease.

Pierce's disease (PD) is an important problem in the culture of grapevines and alfalfa in California and the southeastern United States, but it apparently has never spread beyond the North American Continent. During early days of expansion of the grape industry in California, Pierce's disease probably was introduced from the southeastern United States (4) to the Santa Ana River Valley in Orange County, where it destroyed thousands of acres of vineyards and forced the industry to move to other parts of the

state. Cyclical epidemics of PD threaten the grape industry in the coastal valleys of northern California and in the Central Valley, where the bulk of the viticultural industry is now located.

Although PD was known in the 1880's and was the subject of scientific investigations intermittently for several decades, the pathogenic agent that causes it remained obscure. Pierce (11) believed that a microorganism must be the causal agent, and he observed bacteria associated with diseased plants. He could not, however, culture the bacteria and produce the disease by inoculation. Weimer (13), investigating alfalfa dwarf, a similar disease, also observed bacteria and bacterialike bodies in diseased plants, but could not isolate and reinoculate them to healthy plants. He showed, however, that dwarf was graft-transmissible, and on this basis he assumed that a virus was the causal agent. Hewitt (3) established that PD was transmissible by grafts and buds in grapevines, and also suggested that it was caused by a virus. Subsequently, Hewitt et al. (5) established that leafhoppers were natural vectors of the disease, and a few years later (6) showed that PD and alfalfa dwarf were incited by the same infectious agent. Further work (7) demonstrated that the PD agent was limited to xylem tissues, which often became plugged with gum and tyloses. Hopkins & Mortensen (8) suggested that the Pierce's disease agent might be a mycoplasma rather than a virus, because tetracycline treatments caused some remission in disease symptoms.

Renewed efforts (Goheen et al., *unpublished data*) to understand and control PD indicated that the causal agent, whatever it might be, was easily inactivated by hot water in vivo. This suggested that the agent was not a true virus, but might well be a mycoplasma or some other microorganism easily inactivated by heat. This led to a critical experiment with hot water treatments for disease inactivation, and to electron-microscopic studies of the xylem tissues of host plants affected with PD. The results of our studies are reported here.

MATERIALS AND METHODS.—PD-affected grapevines of the cultivars Ruby Cabernet and Carignane, showing conspicuous symptoms, were selected in midsummer from commercial vineyards in Napa Valley. Individual canes, as needed, were cut from such vines, placed in polyethylene bags, and transported immediately to Davis. At Davis they were held for periods of 2 to 3 days, with their bases in a container of water, and used as sources of the PD agent.

Hordnia circellata Baker, one of the most efficient vector leafhoppers for PD, was used to move the agent to potted grapevines. We obtained the leafhoppers by sweeping wild vegetation along the banks of streams in Napa Valley. The insects were placed on plants in screened cages, or occasionally in large plastic bags and transported to Davis. They were not checked for natural infection, but were immediately caged on affected canes and allowed to feed for 1 or 2 days. Lots of ca. 25 insects were moved from the diseased canes to small cages on healthy vines of the cultivars Mission or Carignane and allowed to feed for 2 days. They were then either moved to inoculate additional plants or destroyed. The inoculated plants were held in the greenhouse along with noninoculated controls to await appearance of disease symptoms.

One hundred and thirty-five diseased plants were chosen for treatment with hot water from a group inoculated in September 1969. These consisted of 66 Mission and 69 Carignane vines in 1-gal containers. All inoculated plants of both cultivars showed excellent symptoms of PD by the end of November, at which time they were moved to a bench in the lath house for holding over winter. In March, the diseased dormant plants were divided into 18 lots, each of which contained seven plants made up of three plants of one and four plants of the other variety. The remaining six Carignane plants and the three Mission plants were designated as diseased controls. Each lot of seven plants was removed from the pots, the soil was shaken from the roots, and the plants were immersed in a hot water bath. Treatments were immersion for 30, 60, 180, 480, 1,440, or 2,880 min at 45 C; 5, 10, 20, 30, 60, or 150 min at 50 C; and 2, 5, 10, 20, 40, or 100 min at 55 C. Immediately after immersion, the plants were repotted in pasteurized soil and held in a warm greenhouse. Control plants were removed from their pots, freed of soil, dipped in water held at 25 C, then repotted.

Electron-microscopic examinations were made on thin sections of xylem tissues from the veins of leaves that were beginning to show symptoms. Such leaves were obtained from diseased Ruby Cabernet and Carignane from the field, and from insect-inoculated Mission and Carignane vines in the greenhouse. Xylem tissues from healthy Mission and Carignane vines were also examined by electron microscopy. Diseased tissues were obtained from a single alfalfa plant affected with alfalfa dwarf which was supplied by the late B. R. Houston of the Plant Pathology Department, Davis.

Discs of leaf tissue, 1-2 mm thick, were cut from the midribs and petioles of leaves showing typical symptoms of PD, and from leaves and young shoots of the alfalfa plant expressing symptoms of dwarf. Sections of similar leaf tissues were taken from healthy grape plants as checks. The tissues were held in 2.5% glutaraldehyde for 2 hr, then washed in 0.1 M sodium phosphate buffer at pH 7.0. They were postfixed in OsO_4 for 4 hr or longer. After dehydration in an acetone series, tissues were embedded in Spurr's medium (12). Ultrathin sections were cut both across and parallel to the xylem elements with a diamond knife and mounted on copper grids. The thin sections were stained with uranyl acetate and lead citrate and examined with the electron microscope.

Similar tissues were taken from leaves showing symptoms of PD for attempted culturing. The fragments came both from leaves of diseased vines in the field and from leaves of vines experimentally inoculated by insect vectors. Fragments were surface-sterilized in a sodium hypochlorite solution (5%), plated, and incubated on 523 agar (9), a rich

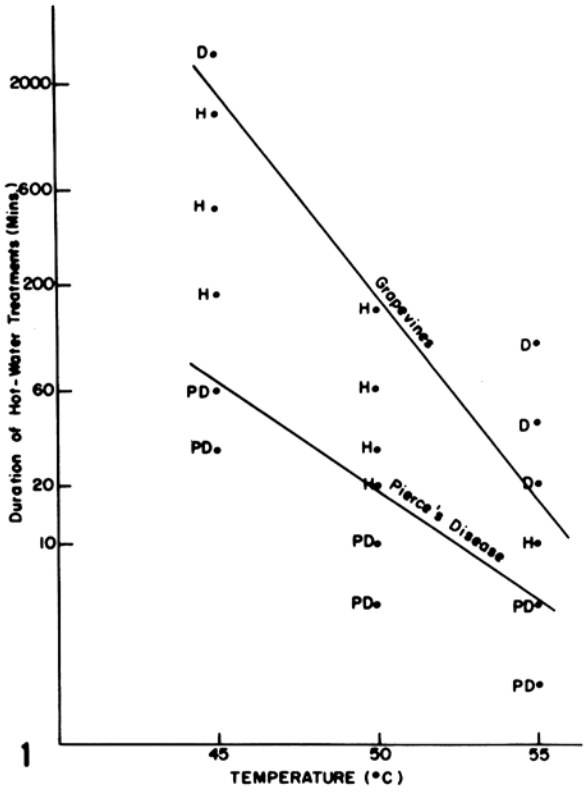


Fig. 1. 1) Thermal death time curves of grapevines and the agent of Pierce's disease of grapevines as determined by immersion of whole plants in hot water. PD = vines that survived but were still diseased; H = vines that survived and were freed of PD; D = vines that were killed by immersion in hot water.

medium used for culturing plant-parasitic bacteria.

RESULTS.—By mid-May 1970, plants subjected to hot water treatments and repotted in late March had become re-established. All plants that were held for 180 min or longer at 45 C, 20 min or longer at 50 C, or 10 min or longer at 55 C were free of PD at this time. All plants that were held for 1,440 min or less at 45 C, 150 min or less at 50 C, or 10 min or less at 55 C survived hot water exposure. Thermal death curves for the agent of PD and for grapevines were constructed from these data (Fig. 1).

The diseased control plants and the plants treated in hot water, but not cured, all died during the summer of 1970 and were discarded. The healthy plants remained healthy. Cured plants from treatments near the thermal death time of the PD agent as well as a few survivors from longer treatments were held a 2nd year. No PD developed in these plants in 1971.

One healthy Mission plant from the treatment at 180 min at 45 C was saved, and several plants were propagated from it during the summer of 1971. In September, one of these daughter plants was exposed to *H. circellata* that had been held on a plant that had PD. Another daughter plant was not inoculated. The healthy vine remained healthy 2 years after treatment; the insect-inoculated one showed distinct symptoms of PD by May 1972 (Fig. 2, 3). Thus, the agent did not become latent after hot water treatment but was completely eliminated from the affected plant by the treatment. When the infective agent was reintroduced by infective leafhoppers, the cured plants again showed symptoms of PD.

We consistently found a rickettsialike organism in the xylem of infected leaves of both grapevines and alfalfa as illustrated in the electron micrographs (Fig. 4-7). It occurred in grape leaves from diseased plants in the field as well as in leaves of plants that were inoculated in the greenhouse by insect vectors, but not in leaves of noninoculated control plants. The organism occurred both inside and between xylem cells (Fig. 6, 7). It did not occur in the phloem or parenchyma tissues. An organism of similar appearance was found in the xylem tissues of the alfalfa plant affected with alfalfa dwarf (Fig. 5).

The organism (Fig. 4) has an apparent cell wall of two or more layers and a definitive cytoplasmic membrane. The cells appear to be highly organized

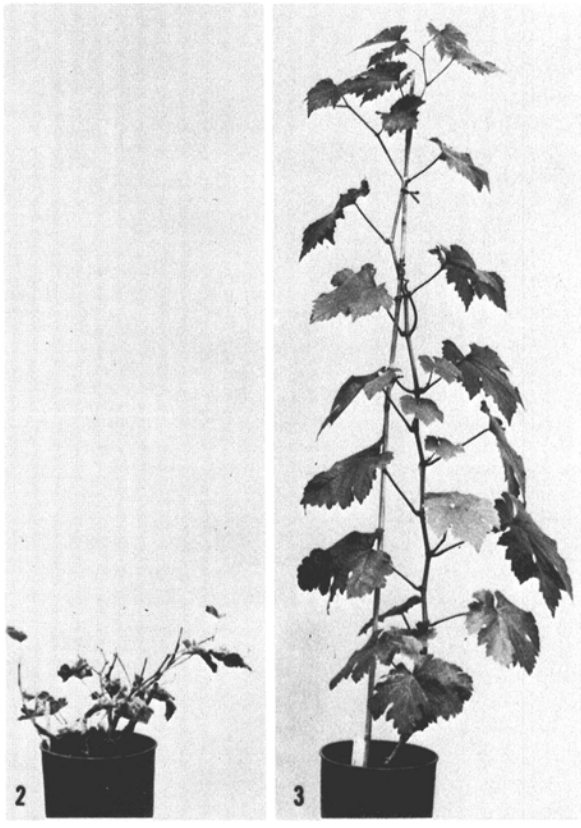
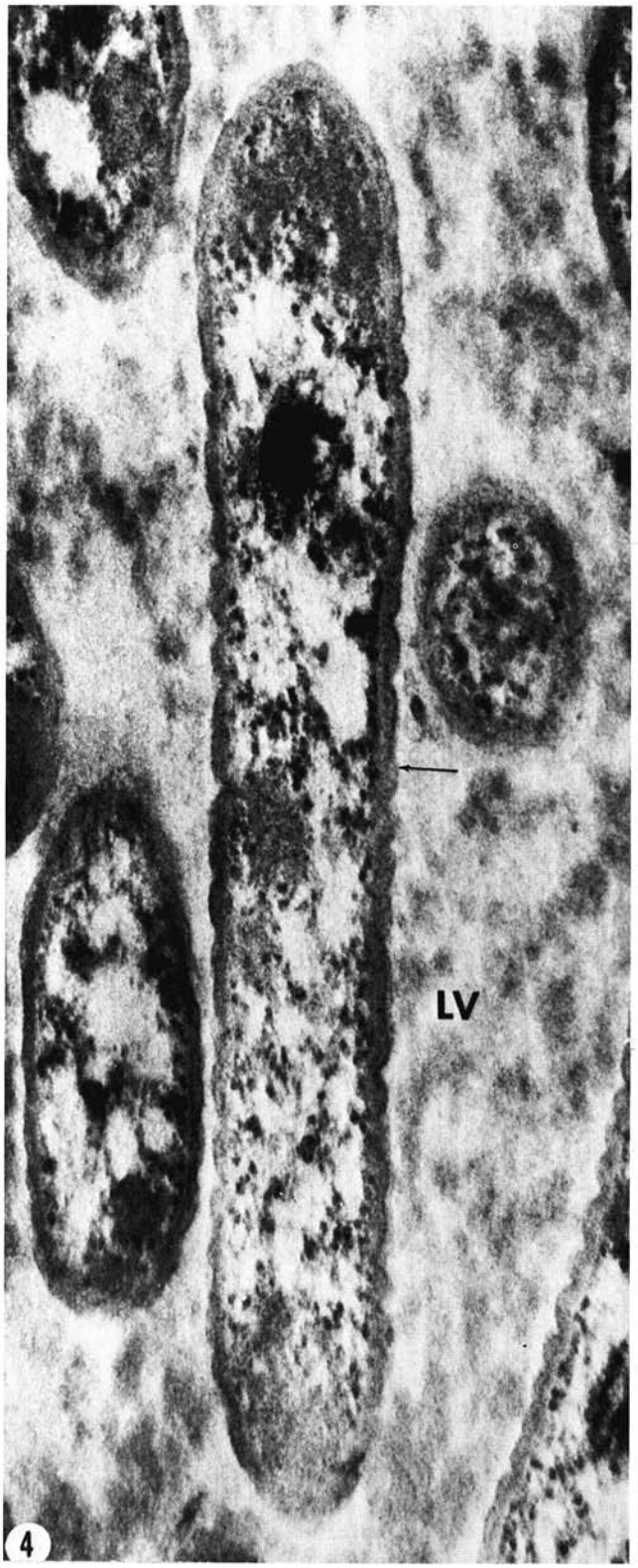


Fig. 2-3. 2) Mission grapevine with symptoms of Pierce's disease following an inoculation with *Hordnia circellata* and **3)** the noninoculated check. Both plants were propagated by cuttings from a plant that had been cured of PD by hot water treatment.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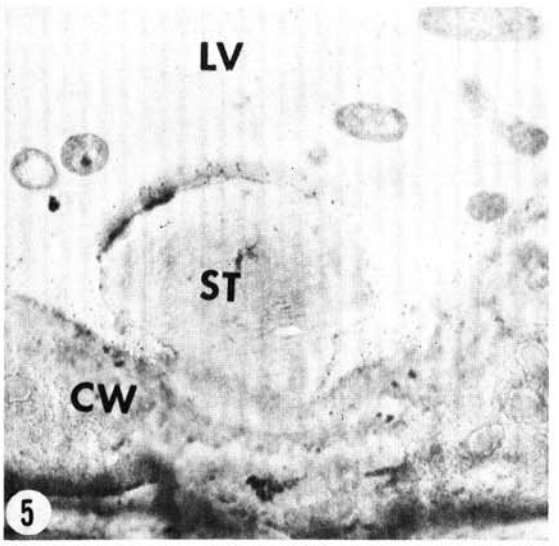
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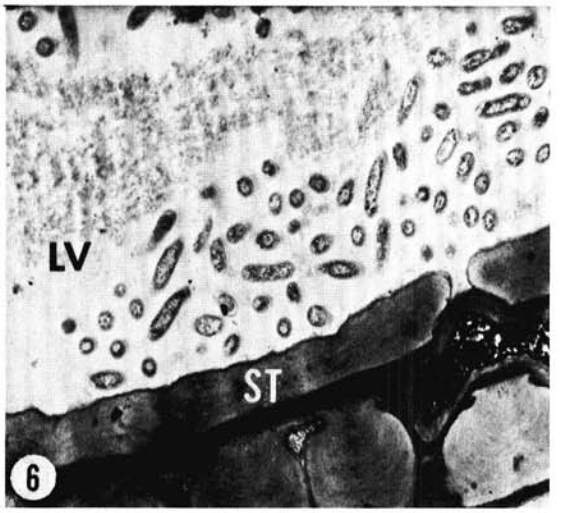
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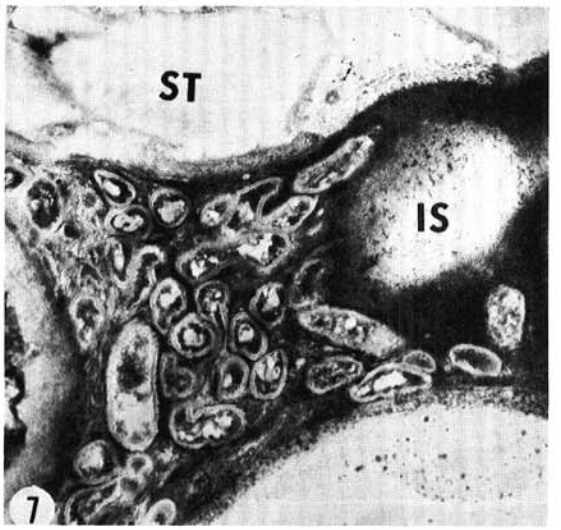
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Fig. 4-7. 4) A rickettsialike organism in the lumen of a vessel of grapevine affected by Pierce's disease. Note the rippled cell wall and the electron-dense area suggesting nucleic acid concentration. The diameter of the organism is 0.4 μ, and its greatest length is about 3.2 μ (× 62,500). **5)** Organisms in lumen (LV) of a xylem cell of alfalfa leaf from a plant affected with alfalfa dwarf. Longitudinal, cross, and oblique sections show the rippled cell wall. CW = cell wall, ST = secondary thickening, LV = lumen of vessel (× 12,500). **6)** Longitudinal section through the xylem of a leaf of grapevine infected with PD showing abundant cells of the etiologic agent in cross and longitudinal section. LV = lumen of vessel, ST = secondary thickening of a vessel wall (× 5,000). **7)** Concentration of cells of the organism in an intercellular space (IS) of the xylem of a grapevine leaf that showed symptoms of PD. ST = secondary thickening of a vessel wall (× 12,500).

with ribosomes and electron-dense areas suggesting nucleic acid. The cell wall is rippled. The organism is about 0.4 μ in diam and up to 3.2 μ in length. This closely approximates the size and morphology of known rickettsiae parasites of animals and insects. The rippled cell wall is a characteristic associated with rickettsiae (1) as seen in electron micrographs.

Attempts to culture an organism from PD-infected tissues were limited in number. However, in the few cultures tried, no organisms were recovered on the medium used.

DISCUSSION.—The occurrence of rickettsialike organisms in the xylem elements of PD-affected grapevines and alfalfa plants and the apparent transmissibility of them by one of the known vectors of PD strongly suggest that the organisms are causally associated with PD. The heat lability and size of the organism in grapevines approximate those of rickettsial pathogens of animals. Rickettsiae are frequently associated with insects and other arthropods (1) and recently they were found in stunted dodder plants in France (2).

Rickettsiae, associated with arthropods and higher animals, are mostly obligate parasites or culturable only with considerable difficulty (10). Failure with culturing and small size distinguish the organism found in grapevines and alfalfa from other plant-pathogenic bacteria. Previous unsuccessful efforts (11, 13), and ours, to culture an organism, along with the evidence presented by Hewitt et al. (6) on the restriction of the infectious agent to the xylem, suggest that the PD agent is a fastidious bacterium confined to its vectors and to the xylem tissues in its host plant.

The lability of the PD agent in hot water is of considerable practical importance. Immersion of propagating wood in hot water for 3 hr at 45 C will eliminate any threat that Pierce's disease might be moved to new areas by diseased wood.

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