Recovery of Rickettsialike Bacteria by Vacuum Infiltration of Peach Tissues Affected with Phony Disease

W. J. French, R. G. Christis, and D. L. Stassi

Associate Professor of Plant Pathology, University of Florida, IFAS, Agricultural Research Center, Monticello, FL 32344; Plant Pathologist III, Agronomy Department, University of Florida, Gainesville, FL 32611; Graduate Student, Genetics Group, Biological Sciences, Florida State University, Tallahassee, FL 32306.

Florida Agricultural Experiment Station Journal Series Paper No. 155.

Accepted for publication 24 February 1977.

ABSTRACT

FRENCH, W. J., R. G. CHRISTIE, and D. L. STASSI. 1977. Recovery of rickettsialike bacteria by vacuum infiltration of peach tissues affected with phony disease. Phytopathology 67: 945-948.

A method is described for collecting bacteria from peach trees (*Prunus persica*) affected with phony disease. A solution of 10^{-1}M KOH was drawn through root sections and the resulting extract, when examined by phase-contrast microscopy, contained up to 7.5×10^7 bacterial cells/ml. Electron microscopy of the extracts indicated that the bacteria were morphologically indistinguishable from the rickettsialike bacteria observed in ultrathin-sectioned material. Extracts from stems usually contained lower concentrations than those from roots. A method for aseptically isolating rickettsialike bacteria was developed, but no rickettsialike bacteria grew on any of the media tested.

Rickettsialike bacteria (RLB) have been associated consistently with phony peach disease (PPD) in Florida (3). Nyland et al. (6) described a similar organism associated with a peach tree [*Prunus persica* (L.) Batsch] affected by PPD in Georgia. The RLB observed by electron microscopy in xylem elements of roots (3, 6) and leaf midveins (3) are rod-shaped bodies about 0.25 to 0.4 μm $\times$ 1.0 - 3.0 μm in size. One of the most distinctive morphological features of the organism is the rippled appearance of the outer cell wall and the unitized strands which appear to radiate from the wall (6). Rod-shaped bacteria, similar in size, shape, and host tissue localization in PPD, were liberated with 10^{-1}M KOH from diseased xylem tissues and were observed by phase-contrast microscopy (2). Initial attempts to examine the bacteria by electron microcopy for comparison with RLB were unsuccessful owing to low yields of bacteria and high concentration of host debris in KOH preparations.

The purpose of this paper is: (i) to relate the rod-shaped bacteria observed by phase-contrast optics (2) to those observed in xylem elements by electron microscopy (3, 6) and (ii) to describe a technique for collecting RLB in sufficient quantities for future use in PPD diagnoses, antiserum preparations, and eventual pure culture of the organism.

MATERIALS AND METHODS

Vacuum extraction for microscopy.—Roots and stems from peach trees affected with PPD and from healthy trees were collected in Madison and Jefferson Counties, Florida. Collections were made in January and February when the trees were in a dormant condition. Frozen sections of roots and stems were prepared for phase-contrast microscopy as described previously (2).

The larger roots and stems from the same trees were cut into 6 - 10 $\times$ 70 mm sections. The bark was removed and one end of a xylem cylinder was fitted to a piece of plastic tubing which was attached to a 7 $\times$ 20 mm glass tube. The glass tubing was inserted through a stopper of a vacuum flask and into a centrifuge tube which was contained within the flask. Plastic tubing was attached to the top of the xylem cylinder to form a well which held about 1 ml of liquid. Water and KOH solutions (10^{-5} to 10^{-1}M) used in the procedure were first filtered through 0.2 μm (average pore size) membrane filters, then drawn through a section of root or stem at a vacuum of 25 - 60 cm Hg as required. The resulting extract was centrifuged at about 500 *g* for 5 min. The supernatant fluid was examined with phase-contrast optics and the cell concentration was determined by counting the number of rods per field at $\times$400. To assure a sufficient concentration of cells for electron microscopic examination (an average of 1 cell per 75 $\times$ 300 μm grid opening), it was estimated that a minimum of 2.6×10^5 cells per ml of extract was required.

A drop of suspension was placed for 1 min on a carbon-stabilized Formvar film on a copper specimen grid, and the excess extract was removed by touching with filter paper. A drop of 1.0% potassium phosphotungstate, pH 6.5, was added for 30 sec, then removed with filter paper. The grid was allowed to dry for 5 min and then it was examined with the electron microscope.

Small pieces of the same rootlets and larger roots from the above material were prepared for thin sectioning by fixing overnight at 4 C in Karnovsky's fixative (4). They were postfixed overnight at 4 C in 2% OsO_4. Tissues were dehydrated in an ethanol-acetone series, then embedded in an epoxy-resin mixture (7). Sections were cut longitudinally through the xylem elements, stained with uranyl acetate and lead citrate, and examined with a Philips EM-200 electron microscope.

Vacuum collection and media preparation for pure culture.—Roots from peach trees affected with PPD were collected in Madison County, Florida during July, August, and September 1976. Roots were exposed so as to avoid introducing contaminants through the bark. Soil surrounding the roots was brushed away from the area to be excised. Each root was rinsed with sterile, distilled water, and surface-sterilized in situ with 70% EtOH. A section of root, approximately 6-10 × 150 mm was excised with flame-sterilized shears. To determine the titer of RLB in the sample, a 50-mm section was cut from one end and infiltrated with a 10^{-1}M KOH solution as described above. The resulting suspension was examined immediately with a phase-contrast microscope. The exposed ends of the remaining section of root were flame-sterilized. The root then was rinsed in 70% EtOH and flamed. These samples were transported to the laboratory in sterile jars stored on ice.

To obtain RLB for attempts at pure culture, aseptically collected roots were vacuum-extracted as previously described, except that all equipment was sterilized by autoclaving and that standard-saline-citrate (SSC), 0.15 M NaCl, water and liquid media were used in place of KOH for collection.

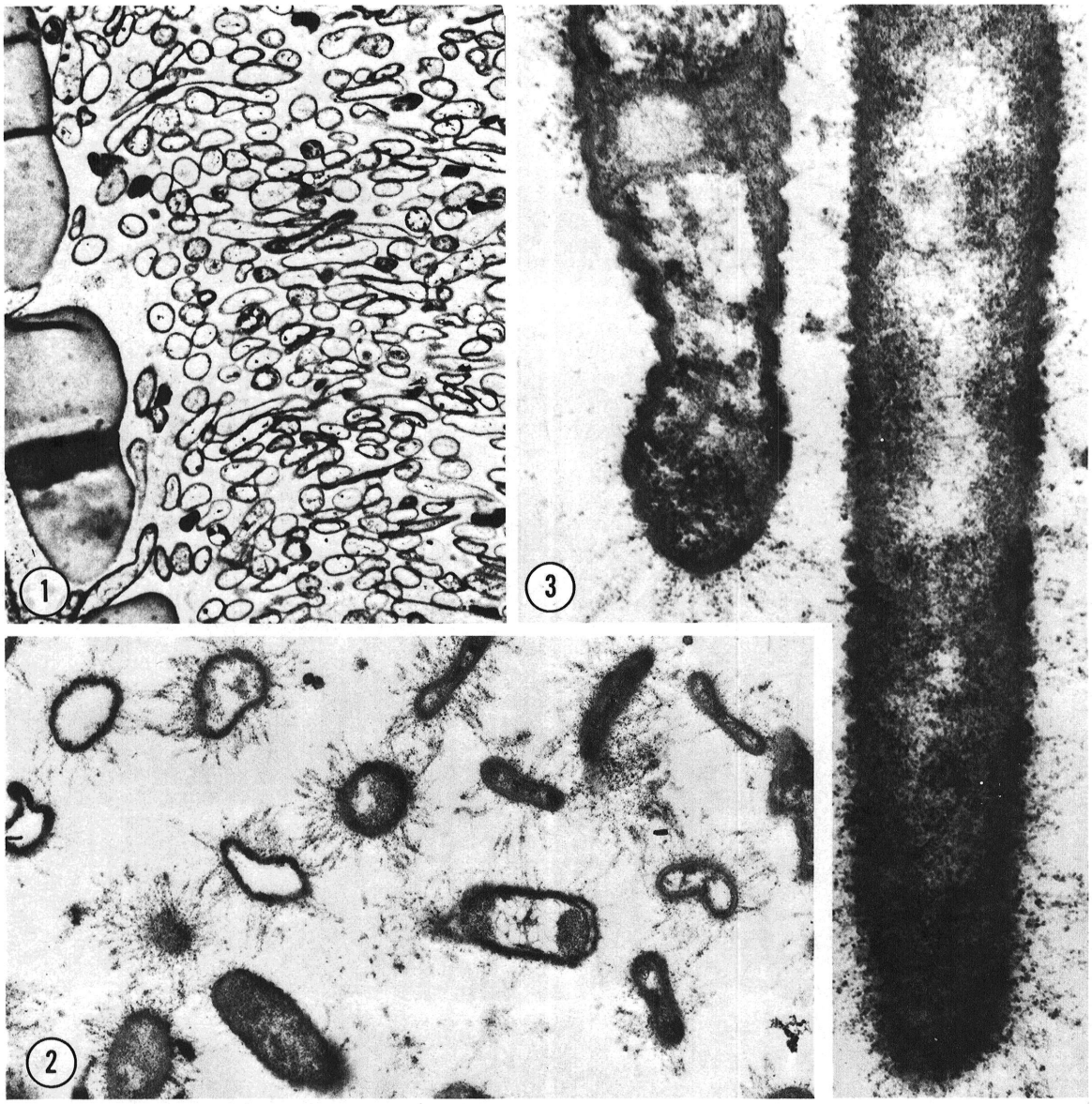

Fig. 1-3. Micrographs of thin sections of peach roots from trees with phony peach disease. **1)** Longitudinal section of a root shows a mass of rickettsial cells in the vessel lumen (× 7,600). **2)** Electron-dense strands radiate from the cell walls (× 23,700). **3)** The cell walls are characteristically ridged (× 69,000).

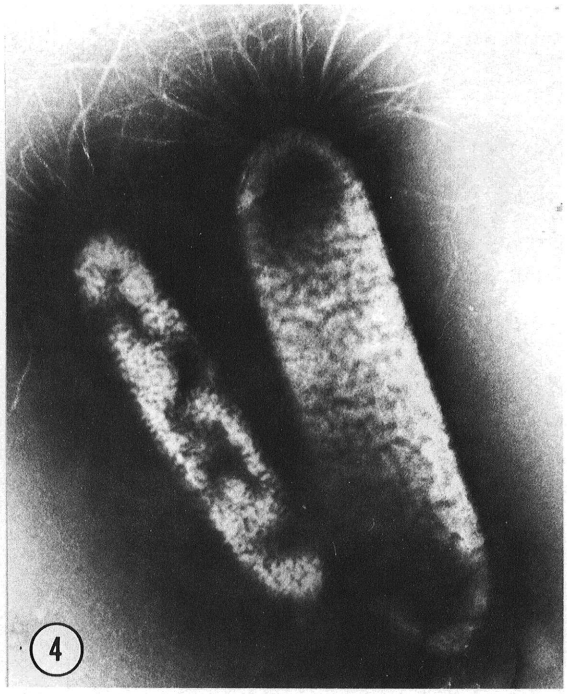

Fig. 4. Micrograph of bacteria extracted from roots of peach trees infected with phony disease. The preparation was negatively stained with potassium phosphotungstate. Note variation in patterns of wall rippling and locations of filaments associated with cell walls. The filaments usually are polar but may also be found over the entire surface of the cell wall. (× 30,000).

The following media were prepared in liquid form, or with the addition of 15g/liter agar (hard agar), 7g/liter agar (soft agar) or 200g/liter gelatin. Incubation of RLB on these media was at 22 C and 30 C for up to 2 mo. Nutrient broth (Difco), 30 g/liter brain-heart infusion (Difco) with or without Vogel's Medium N(8), trypticase-soy medium (Difco) and D_2 medium (5) with 10g/liter sucrose were the nutrient-rich bacteriological media used in an attempt to culture RLB.

Nutrient media were supplemented with peach xylem sap, peach root extract, leafhopper homogenate and soil extract in an attempt to mimic the natural environment of the organism. Peach xylem sap was collected from healthy Junegold trees by employing the vacuum apparatus described above. Peach root extract was prepared by boiling approximately 20 g of minced root in 100 ml of distilled water for 5 min. To make leafhopper homogenate, 30 frozen leafhoppers [*Homalodisca coagulata* (Say)] were homogenized in 3 ml of distilled water with a Virtis "45" homogenizer. The homogenate was centrifuged at 1,000 *g* for 10 min. The resulting supernatant fluid was collected and filter-sterilized. To prepare soil extract, 50 g of Norfolk soil (1), collected from around the roots of phony peach trees, was soaked in 100 ml of distilled water for 2 hr. The suspension then was centrifuged at low speed. The supernatant fluid was collected and filter-sterilized. Combinations of media used were as follows: nutrient broth with soil extract base; nutrient broth with Difco yeast extract (5g/liter), with or without dextrose (5g/liter), with 5% (v/v) homogenized leafhopper or in peach root extract base; nutrient broth, yeast extract, dextrose, nicotinamide adenine dinucleotide (NAD - 10 μg/ml) with homogenized leafhopper or root extract base; SSC and dextrose, with homogenized leafhopper or root extract base; peach root sap with or without NAD; coconut (*Cocos nucifera* L.) phloem sap (supplied by R. E. McCoy, University of Florida), with or without NAD; and nutrient agar with 25% coconut sap.

RESULTS

Microscopy.—Rod-shaped bacteria (0.24 - 0.66 μm × 1.60 - 3.0 μm) were released from frozen sections of phony disease roots treated with 10^{-1}M KOH (2). The rods were obtained from all roots from which subsamples were used for vacuum extraction and electron microscopy. No rods were obtained from roots from trees without PPD symptoms.

Electron microscopy of sections of rootlets from peach affected with PPD revealed numerous bacteria in the lumen of xylem elements. (Fig. 1). The organisms were similar in size and morphology to those reported previously (3, 6). Many of the RLB had strands radiating from the periphery of their cell walls (Fig. 2, 3) as described by Nyland et al. (6). No bacterial cells were seen in root or stem sections from trees which appeared healthy.

Extracts obtained by drawing sterile distilled water through diseased roots and stems contained few bacteria (10^3 - 10^5 cells/ml); however, resuspended pellets formed at 6,000 *g* contained enough cells for electron microscope examination (i.e., a minimum of 2.6×10^5 cells/ml). As the concentration of KOH was increased from 10^{-5} to 10^{-1}M the concentration of RLB in the extract increased from 10^3 - 10^5 cells/ml to 6.5×10^6 cells/ml. When 0.5 ml of 10^{-1}M KOH was drawn through a root xylem cylinder, the resulting extract contained up to 7.5×10^7 cells/ml. Although bacteria in excess of 10^7 cells/ml were obtained in KOH extracts from some stem sections, the mean concentration from 52 stems was 9.5×10^5 cells/ml and the mean concentration from 26 root samples was 8.3×10^6 cells/ml. Bacteria occurred in all of the roots and in 78% of the stems that were sampled.

Grids prepared from H_2O or KOH extracts all contained bacteria similar in appearance to the RLB found in the sections of tissues infected with PPD (Fig. 4). The RLB in the extracts were very distinctive due to the rippled cell wall, and the presence of many strands associated with the cell wall (Fig. 4). KOH at 10^{-1} M did not cause deterioration of the cell wall or strands.

Extraction for attempted pure culture.—To obtain material for pure culture of RLB, 30 roots of 15 trees affected with PPD were extracted aseptically with SSC, H_2O, 0.15 M NaCl, or liquid medium. Bacterial concentrations ranging from 1.8×10^5 to 1.8×10^6 cells/ml were obtained routinely. When viewed under the phase-contrast microscope, none of the extracts was seen to contain organisms which appeared obviously different from RLB. Bacteria in nine of the extracts were incubated on the media described above, but no RLB grew on these media.

DISCUSSION

The rod-shaped bacteria observed by phase-contrast optics in xylem extracts from peach affected by PPD appear to be the same as the RLB observed in tissues by electron microscopy.

The vacuum extraction technique with KOH solution, SSC, or water is an effective method for collecting RLB in numbers suitable for further study. The technique is also a useful diagnostic method for PPD. Although the KOH treatment of frozen sections is a fast and reliable method for diagnosing PPD, repeated sampling to avoid missing "pockets" of RLB in the xylem tissue is required. Vacuum extraction is fast, and is more reliable than the frozen-section method because of the larger volume of tissue sampled in each extraction. The relative purity of the root fluids suggests that this vacuum extraction method may be useful for serological studies of the RLB.

We are as yet unable to grow RLB on an artificial medium. Further efforts will be made using chick embryo and plant tissue culture techniques.

The constant association of this organism with PPD as evidence in thin sections and in frozen and vacuum extracts and the observed similarity of the bacteria obtained by these methods strengthens the hypothesis that PPD is caused by a rickettsialike bacterium rather than a virus.

LITERATURE CITED

1. BRYON, O. C. 1960. Soils of Florida and their crop adaptation. Fla. Dep. Agric. Bull. 42. 48 p.
2. FRENCH, W. J. 1974. A method for observing rickettsialike bacteria associated with phony peach disease. Phytopathology 64:260-261.
3. HOPKINS, D. L., H. H. MOLLENHAUER, and W. J. FRENCH. 1973. Occurrence of a rickettsia-like bacterium in the xylem of peach trees with phony disease. Phytopathology 63:1422-1423.
4. KARNOVSKY, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolarity for use in electron microscopy. J. Cell Biol. 27:137A-138A.
5. KADO, C. I., and M. G. HESKETT. 1970. Selective media for isolation of Agrobacterium, Corynebacterium, Erwinia, Pseudomonas, and Xanthomonas. Phytopathology 60:969-976.
6. NYLAND, G., A. C. GOHEEN, S. K. LOWE, and H. C. KIRKPATRICK. 1973. The ultrastructure of a rickettsialike organism from a peach tree affected with phony disease. Phytopathology 63:1275-1278.
7. SPURR, A. R. 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct. Res. 26:31-43.
8. VOGEL, H. J. 1956. A convenient growth medium for Neurospora. Microbiol. Genet. Bull. 13:42.