Proteins and Filamentous Structures Associated with Citrus Blight

K. S. Derrick, R. F. Lee, R. H. Brlansky, L. W. Timmer, B. G. Hewitt, and G. A. Barthe

ABSTRACT. Extracts from healthy and blighted trees were prepared by vacuum extracting sections of roots and stems. Analysis of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed several proteins in extracts from diseased trees that were absent or present in much lower concentrations in extracts from healthy trees. When the extracts were centrifuged and the resulting pellets examined by electron microscopy, filamentous structures were observed. The nature and function of these structures are unknown, but they were present in much higher concentrations in preparations from blighted than healthy trees.
Index words. SDS-PAGE electrophoresis, electron microscopy.

Blight is a serious disease of citrus in Florida. The disease has been the subject of numerous research efforts, but its cause remains unknown. Attempts to transmit blight by grafting or to reproduce the disease by reconstituting blighted trees from root sprouts and buds from diseased trees have failed (6,7,8,11,12). These failures led to the conclusion that blight is a nonparasitic disorder, but recent demonstrations that blight can be transmitted by root grafting (4,9,10) suggest that disease is caused by a systemic (viruslike) pathogen.

We recently reported finding several proteins in vacuum extracts of root and stem pieces from diseased trees that are absent or are present in much lower concentrations in similar preparations from healthy trees (2). These proteins are referred to as blight proteins, but at this time it is not known if they are pathogen or pathogenesis related. We report here the potential for using assays for blight proteins for early detection of blight in presymptomatic trees and the presence of unusual filamentous structures in blighted trees.

MATERIAL AND METHODS

Root and stem samples were collected from blighted and healthy trees. Samples were also taken from healthy-appearing (presymptomatic) trees that had been root-graft inoculated. Roots and stems, about one to two cm in diameter, were collected and assayed the same day or stored overnight at 4 C. The samples were cut into 10-cm lengths, and the bark was removed. Vacuum extracts were prepared by drawing up to 4 ml of 0.05 M Tris, pH 8.0 containing 0.1% ascorbic acid, 0.1% cysteine, and 0.5% 2-mercaptoethanol (TACM) through the root or stem pieces using a vacuum pump. The extracts were mixed with an equal volume of double-strength SDS-PAGE sample buffer and heated at 100 C for 5 min. The extracts were assayed by SDS-PAGE on 1.5 mm 12% acrylamide gels (3). Molecular weights were estimated by comparison to standard proteins (molecular weights in parenthesis): phosphorylase B (94,000), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,000), and lysozyme (14,300). The gels were stained with silver nitrate (5).

A crude-vacuum extract from blighted roots was used to produce an antiserum in rabbits by COCALICO Biologicals, Inc. P.O. Box 265, Reamstown, PA. For western blot analysis, SDS-PAGE gels were electroblotted onto nitrocellulose using The Semi-Dry Electroblotter (JKA-BIOTECH, Denmark) following in-

Florida Agricultural Experiment Station Journal Series No. R-00631.

structions provided by the manufacturer. The membranes were blocked with crude-extracts from healthy roots before application of the primary antibody. Immunostaining of nitrocellulose membranes was done using the ProtoBlot Western Blot AP System following instructions supplied by the manufacturer (PROMEGA, 2800 S. Fish Hatchery Road, Madison, WI).

For electron microscopy, vacuum extracts were centrifuged at 20,000 X g for 5 min. The resulting pellets were suspended in 0.1 ml of water and applied to filmed electron microscope grids. Following staining with 2% uranyl acetate in water, the grids were examined with an electron microscope. Serologically specific electron microscopy (SSEM) was done as previously described (1).

RESULTS AND DISCUSSION

As previously reported (2), blight proteins were detected in root and stem samples from infected but not from healthy trees (Fig. 1). On occasion, a given sample from a blighted tree had a protein profile characteristic of a healthy tree. But, invariably a second assay of a different stem or root from the same tree was positive for blight proteins. Blight proteins were also readily detected in presymptomatic trees 2 yr after they had been root-graft inoculated. These mature, bearing trees subsequently developed blight symptoms several months after being identified as positive for blight proteins. In addition, blight proteins have been detected in graft-inoculated young trees, indicating they may be infected. If these trees are indeed infected it could be several years before they develop symptoms. Additional research and observations are obviously required, but at this point it appears that the blight proteins may be an early indictor of infection.

An antiserum made to a crude-vacuum extract from blighted roots readily detected the 35 kilodalton (Kd) blight protein in western blot assays (Fig. 1). In subsequent experiments, it was found that the 35 Kd protein has an affinity for immunoglobulins and was detected in western blots using "normal serum." However, the sensitivity of the assay using normal serum was much less than with the antiserum to the blight extract. Preliminary experiments have indicated that the 35 Kd protein has an isoelectric point above 10, which may explain its affinity for immunoglobulins.

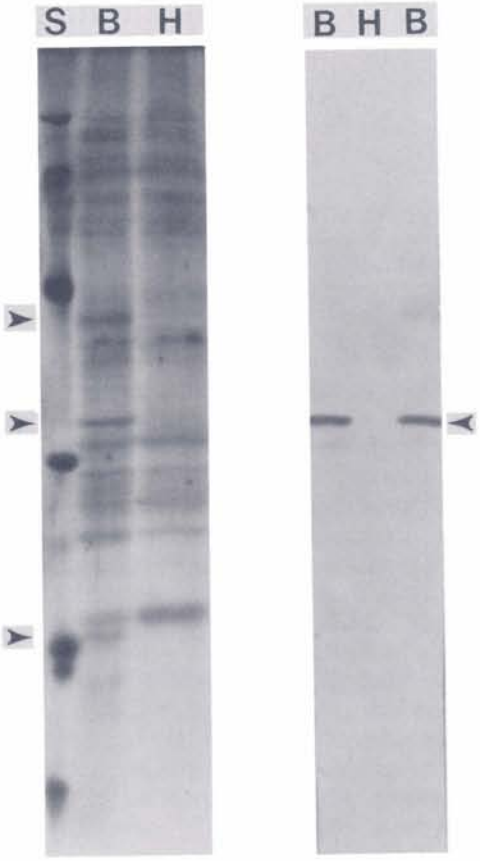

Fig. 1. The figure on the left shows SDS-PAGE of proteins in extracts from roots of blighted (lane B) and healthy (lane H) trees. The lanes marked (S) contain proteins used as molecular weight markers. The arrowheads mark 43, 35, and 23 Kd proteins that appear to be related to blight. The figure on the right is a western blot showing the 35 Kd protein immunostained using antiserum to an extract from blight roots.

In attempts to associate a pathogen with citrus blight, we routinely

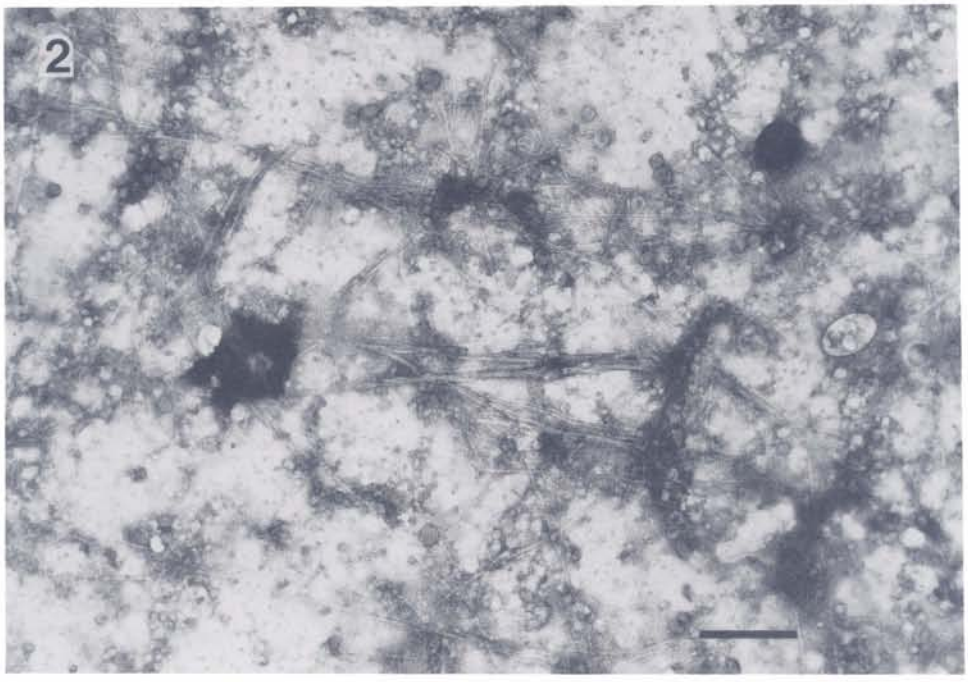

Fig. 2. Electronmicrograph of filamentous structures found in a vacuum extract from roots of a blighted tree. The scale bar represents 500 nm.

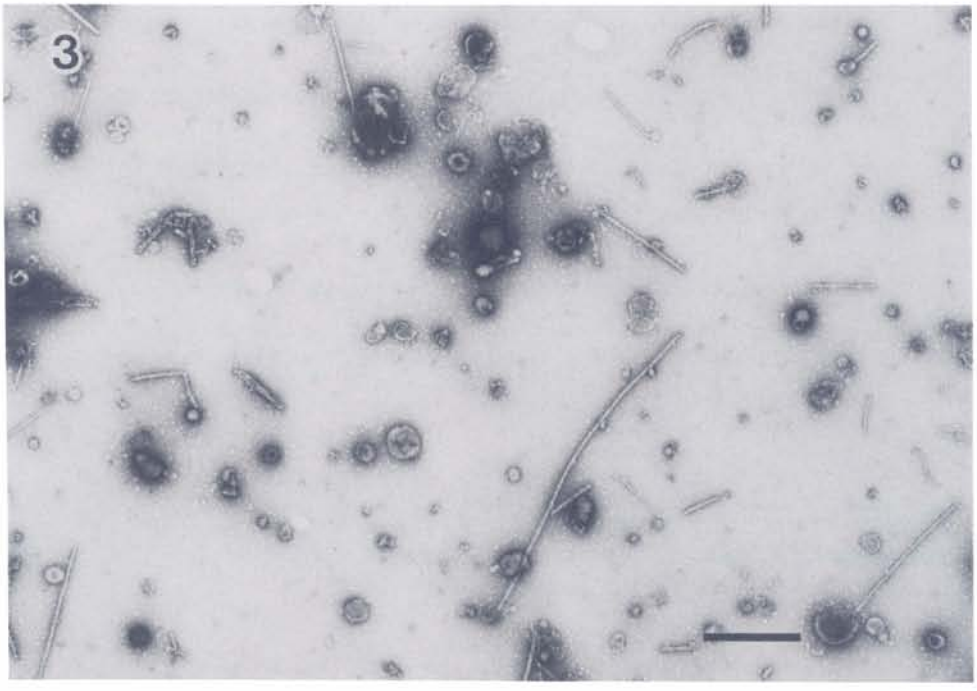

Fig. 3. Electronmicrograph of a SSEM assay of a preparation from roots of a blighted tree. The scale bar represents 500 nm.

examine crude extracts and the various fractionated preparations produced therefrom with an electron microscope. In all cases, we have failed to detect bacteria or particles characteristic of known plant viruses. Numerous filamentous structures were observed in electron microscopic examinations of preparations concentrated by centrifuging vacuum extracts of roots from blighted trees (Fig. 2). Only an occasional filament was observed in preparations from healthy roots. Filaments were readily detected in samples from roots of blighted trees, but not from roots of healthy trees, by SSEM using the antiserum to a crude-extract from blighted roots (Fig. 3). The relatively short filaments seen in SSEM assays may be broken pieces of the longer filaments seen in "dip" preparations. The nature and function of the filamentous structures are not known, but they appear to be diagnostic for blight.

LITERATURE CITED

1. Derrick, K. S., and R. H. Brlansky
1976. Assay for viruses and mycoplasmas using serologically specific electron microscopy. Phytopathology 66: 815-820.
2. Derrick, K. S., R. F. Lee, R. H. Brlansky, L. W. Timmer, B. G. Hewitt, and G. A. Barthe
1990. Proteins associated with citrus blight. Plant Dis. 74: 168-170.
3. Laemmli, U. K.
1970. Cleavage of structural proteins during assembly of the head of bacteriophage T4. Nature 270: 680-685.
4. Lee, R. F., R. H. Brlansky, L. W. Timmer, D. P. H. Tucker, J. H. Graham, and K. S. Derrick
1988. Graft transmission of citrus blight. Phytopathology 78: 1572 (abstr.).
5. Morrissey, J. H.
1981. Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. Anal. Biochem. 117: 307-310.
6. Rhoads, A. S.
1936. Blight, a non-parasitic disease of citrus trees. Florida Agr. Expt. Stn. Bull. 296. 64 pp.
7. Smith, P. F.
1974. History of citrus blight in Florida. Citrus Ind. 55 (9): 13,14,16,18,19; (10): 9,10,13,14; (11): 12,13.
8. Smith, P. F. and H. J. Reitz
1977. A review of the nature and history of citrus blight in Florida. Proc. Int. Soc. Citriculture 3: 881-884.
9. Timmer, L. W., R. H. Brlansky, K. S. Derrick, and R. F. Lee
1989. Transmission of citrus blight by root graft inoculation, pages 000-000. *In* Proc. 11th Conf. IOCV. Riverside.
10. Tucker, D. P. H., R. F. Lee, L. W. Timmer, L. G. Albrigo, and R. H. Brlansky.
1984. Experimental transmission of citrus blight. Plant Dis. 68: 979-980.
11. Wutscher, H. K. and P. F. Smith
1988. Failure to propagate citrus blight in reconstituted trees. Proc. Florida State Hort. Soc. 101: 62-63.
12. Wutscher, H. K., C. O. Youtsey, P. F. Smith, and M. Cohen
1983. Negative results in citrus blight transmission tests. Proc. Florida State Hort. Soc. 96: 48-50.