Proteins Associated with Citrus Blight

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ABSTRACT

Derrick, K. S., Lee, R. F., Brlansky, R. H., Timmer, L. W., Hewitt, B. G., and Barthe, G. A. 1990. Proteins associated with citrus blight. Plant Dis. 74:168-170.

Proteins in crude extracts from healthy and blight-affected citrus trees were compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Complex patterns of proteins from healthy and diseased trees were observed in extracts prepared by pulling buffers through sections of roots under vacuum. Several proteins present in extracts from diseased trees were either absent or present in much lower concentrations in healthy trees. The additional proteins observed from trees with blight appeared to be diagnostic for the disease. Protein patterns characteristic of trees with blight were observed in assays of 17 blight-symptomatic trees that had been infected by root grafting.

Blight is the most serious disease of citrus in Florida. The disease has been the subject of numerous research efforts, but its cause remains unknown. Attempts to transmit the disease by grafting or reproduce the disease by reconstituting blighted trees from root sprouts and buds from diseased trees have failed (11–13, 18,19). The recent demonstration that blight can be transmitted by root grafting (6,16) has renewed interest in determining the cause of the disease. The Pierce's disease bacterium was found in citrus trees affected with blight (3). Hopkins (2) recently reported that young citrus trees have developed some symptoms of blight after inoculation with the xylem-limited bacterium (XLB) (*Xyllella fastidiosa* Wells et al). The author concluded that the evidence is very strong for the involvement of XLB in the blight syndrome in Florida (2). Hopkins' conclusion contrasts to that of Timmer, Lee, and Brlansky (*unpublished*). After extensive studies, they were unable to consistently isolate XLB from blighted citrus trees, to establish XLB infections in citrus by various inoculation techniques, or to find XLB in the xylem of blighted trees by immunofluoresence or by light or electron microscopy. In addition, the incidence of XLB in sharpshooter vectors was not associated with blight in groves (15).

Since conventional methods for associating a pathogen with blight have been unsuccessful, we have compared protein preparations from healthy and diseased trees using a variety of tissues and extraction procedures. The detection of unique proteins in diseased trees (which could be related to either a pathogen or stress) could lead to methods for detecting presymptomatic trees and possibly to characterization of the cause of the disease. M. G. Bausher (*personal communication*) has recently detected two distinct proteins with molecular masses of about 12.5 kD in diseased trees using ultrafiltration of leaf extracts followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). We report finding several proteins in vacuum extracts of root pieces from diseased trees that either are absent or are present in much lower concentrations in similar preparations from healthy trees.

MATERIALS AND METHODS

Root samples were taken from 20-yr-old Valencia sweet orange (*Citrus sinensis* (L.) Osbeck) trees on rough lemon (*C. jambhiri* Lush.) rootstock. These trees had been inoculated 2 yr earlier by grafting the roots with eight to 10 root pieces per tree or by approach-root grafting to a diseased tree (6,16). Trees adjacent to either inoculated or donor trees served as healthy controls. Definite visible symptoms of blight were observed in many of the inoculated trees about 22 mo after grafting; diagnosis of blight was confirmed by water uptake tests (7) and by increased zinc levels in trunk wood (17). The protein assays reported here were made approximately 2 mo after the onset of symptoms.

Roots (about 1–2 cm in diameter) were collected and assayed the same day or stored overnight at 4 C. The roots were cut into 10-cm lengths and their bark was removed. Vacuum extracts were prepared by pulling 400 μl of TTM (0.05 M Tris containing 2% Triton X-100 and 0.5% 2-mercaptoethanol, pH 7.5) or 400 μl of TACM (0.05 M Tris containing 0.1% ascorbic acid, 0.1% cysteine, and 0.5% 2-mercaptoethanol, pH 8.0) through root 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. Total extracts were prepared by chopping the wood into fine pieces, grinding with liquid nitrogen, and boiling for 5 min in sample buffer. The extracts were assayed by SDS-PAGE on 1.5-mm thick, 12 or 18% acrylamide gels (5). Molecular weights were estimated by comparison to these standard proteins (molecular weights in parentheses): 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 (9).

To minimize possible in vitro proteolysis, vacuum extracts were routinely collected and kept on ice until being mixed with sample buffer and boiled. To determine whether in vitro proteolysis was significantly affecting the SDS-PAGE patterns, some samples were kept at 37 C for 1 hr both with and without added sample buffer before being prepared for electrophoresis.

To determine whether any of the blight-specific proteins could be concentrated by centrifugation, vacuum extracts were centrifuged at 13,600 × *g* for 5 min. The resulting supernatant fluid was centrifuged at 174,000 × *g* for 1 hr. Extracts and preparations fractionated by centrifugation were applied to filmed grids, stained with 2% uranyl acetate, and examined with an electron microscope.

Florida Agricultural Experiment Station journal series R00128.

Accepted for publication 19 September 1989 (submitted for electronic processing).

RESULTS

The protein patterns from total extracts of healthy and diseased root wood (where tissue was ground and boiled with sample buffer) were identical (Fig. 1). The patterns were typical of those seen in numerous experiments in which protein patterns of healthy and diseased trees were compared using various tissues, including leaves at various stages of growth and bark from young stems, old stems, and roots. Using vacuum extracts, however, there were obvious differences in the SDS-PAGE pattern of proteins from roots of diseased and healthy trees (Figs. 2–4). Similar results were obtained using TTM (Fig. 2) or TACM (Fig. 3) as the extraction buffer. Because the Triton X-100 in TTM buffer caused excess foaming under vacuum, we used TACM for routine assays. Keeping vacuum extracts with and without added sample buffer at 37 C for 1 hr before final preparation for SDS-PAGE did not appear to change the protein patterns.

In 12% gels, there were seven prominent proteins (Figs. 2 and 3) present in vacuum extracts of diseased roots that are referred to as blight-specific proteins. These proteins were either absent from comparable preparations from healthy roots or present in much lower concentrations. A protein with a molecular mass of about 43 kD was present in higher concentrations in extracts from roots of diseased trees. When vacuum extracts were centrifuged at 13,600 × *g* for 5 min, the 43-kD protein was recovered in the pellet (Fig. 3). The remaining blight-specific proteins were, for the most part, found in the supernatant fluid after centrifuging at 174,000 × *g* for 1 hr.

A 35-kD protein was present in samples from diseased roots. This protein was readily seen in freshly stained gels (both 12 and 18%) where it appeared gold in color compared to most other proteins (which were brown). This protein was not observed in preparations from healthy trees. Three proteins of between 26 and 31 kD were present in high concentrations in samples from diseased trees; there were several proteins in this area of the gels. Proteins of similar sizes were seen in samples from healthy trees but at much lower concentrations. A 23-kD protein—seen as a brown band on the lower edge of a large gold band in freshly stained 12% gels—was present in samples from diseased trees but not in samples from healthy trees. The concentration of the gold band above the 23-kD, blight-specific band was diminished in some samples from diseased trees (Fig. 2, lane 2). Better separation and detection of the 23-kD protein was obtained on 18% gels (Fig. 4). In 12% gels, proteins of about 18 and 15 kD were seen in samples from diseased trees but not in those from healthy trees. The concentrations of these proteins varied considerably, but the 15-kD protein was seen in all samples from diseased trees that had the blight-specific protein pattern. The 15-kD band, which was at the limit of resolution for 12% gels, was separated into proteins of 15, 12, and 10 kD on 18% gels (Fig. 4). A protein, seen as 15 kD on 12% gels and 12 kD on 18% gels, was also observed in some inoculated trees that were not showing symptoms. This suggests that this protein occurs earlier than the other blight-specific proteins and that it might be used to diagnose presymptomatic trees.

Root samples from 17 blight-symptomatic, inoculated trees in the root-graft transmission experiment were assayed on 12% gels. In the initial assay, protein patterns typical of blight-infected roots were observed in 15 of these samples. Two samples gave protein patterns typical of healthy trees, but the blight proteins were observed in a second sample (samples were taken at random) from these two trees. None of the blight-specific proteins were observed in assays of 26 healthy trees, including 18 healthy controls from the root-graft transmission experiment. In addition, extracts from three trees showing symptoms of decline induced by citrus tristeza virus and one tree showing wilt symptoms typical of root rot gave protein patterns characteristic of healthy trees. None of the four trees contained any of the blight-specific proteins.

We have also observed blight-specific proteins in vacuum extracts of stems, but their occurrence and concentration appear to be more erratic than in roots. The 15-kD protein was detected in some stem samples from diseased trees and the gold band associated with the 35-kD protein was also seen at times. A few stem samples had all the blight-specific proteins observed in root samples.

In these and other experiments where we looked for a pathogen associated with blight, extracts and fractionated preparations were routinely examined with an electron microscope for the possible presence of bacteria, viruslike particles, or any unusual structures that may be specific to preparations from infected tissue. In all cases, the results were negative.

DISCUSSION

The failure to transmit citrus blight by budding and ready transmission by ap-

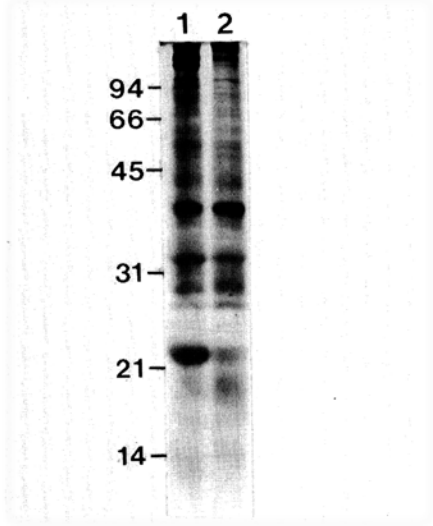

Fig. 1. SDS-PAGE (12% polyacrylamide) of a total protein extract of wood from the roots of a healthy (lane 1) and a diseased (lane 2) tree. Molecular weights (× 10^{-3}) of standard proteins are shown on left.

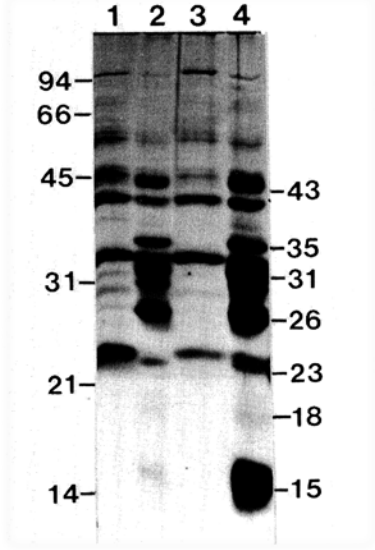

Fig. 2. SDS-PAGE (12%polyacrylamide) of vacuum extracts from four trees prepared using TTM extraction buffer. Lanes 1 (100 μl) and 3 (100 μl) are from healthy roots; lanes 2 (50 μl) and 4 (50μl) are from roots of diseased trees. Molecular weights (× 10^{-3}) of standard proteins are shown on left and blight-specific proteins are shown on right.

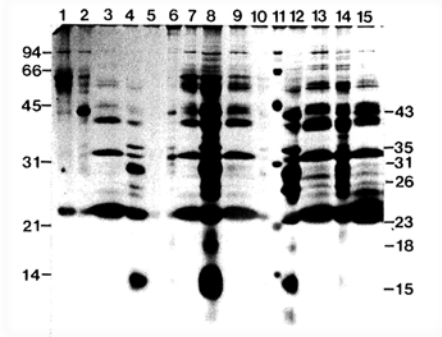

Fig. 3. SDS-PAGE (12% polyacrylamide) of vacuum extracts prepared using TACM extraction buffer. Lanes 1 (100 μl, H[ealthy]) and 2 (50 μl, B[lighted]) are 13,600 × *g* pellets from vacuum extracts of roots. Lanes 3 (50 μl, H) and 4 (25 μl, B) are 174,000 × *g* supernatant fluids from vacuum extracts of roots. Lanes 5 (200 μl, H) and 6 (200 μl, B) are 174,000 × *g* pellets from vacuum extracts of roots. Lanes 7 (50 μl, H) and 8 (25 μl, B) are assays of original extracts used to prepare samples shown in lanes 1–6. Lanes 9 (50 μl, H) and 10 (5 μl, B) duplicate lanes 7 and 8, respectively. Lane 11 is molecular weight markers. Lanes 12 (25 μl, B), 13 (50 μl, H), 14 (25 μl, B), and 15 (50 μl, H) are assays of roots from individual trees. Molecular weights (× 10^{-3}) of standard proteins are shown on left and blight-specific proteins are shown on right.

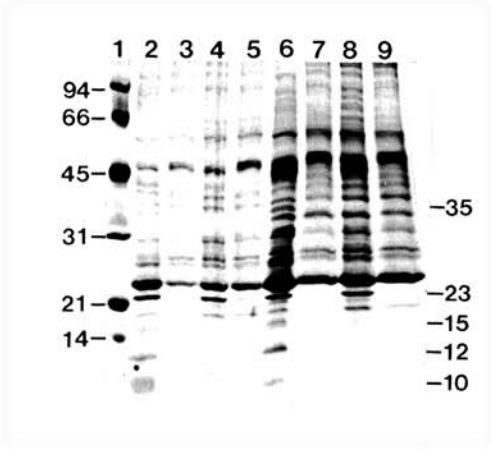

Fig. 4. SDS-PAGE (18% polyacrylamide) of vacuum extracts prepared using TACM extraction buffer. Lane 1 is molecular weight markers. Lanes 2 (25 μl, B[lighted]), 3 (50 μl, H[ealthy]), 4 (25 μl, B), and 5 (50 μl, H) are assays of roots from individual trees. Lanes 6–9 are 2× sample volume duplicates of samples shown in lanes 2–5, respectively. Molecular weights ($\times 10^{-3}$) of standard proteins are shown on left and blight-specific proteins are shown on right.

proach-root grafting may indicate something about the cause of the disease. With regard to transmission, blight is similar to phony peach, which could not be transmitted by budding but was readily transmitted by root grafting (4). It is now known that phony peach is caused by a systemic bacterium that is concentrated in the xylem and is thus located in the wood of roots and stems. The small amount of wood associated with a bud apparently does not contain sufficient bacteria for consistent bud transmission. A systemic bacterium has been suggested as the cause of citrus blight (1–3), and a low number of bacteria have been observed in preparations of both diseased and healthy citrus (1). In an earlier study (10), examination of samples from trees with blight by transmission electron microscopy failed to implicate a mycoplasmalike agent in the cause of the disease. The injection of oxytetracycline into diseased trees was reported to lower the zinc levels in trunk wood but did not cause remission of disease symptoms (8,14). If blight is caused by a systemic bacterium or mycoplasmalike agent, the concentration of the pathogen in infected tissue appears to be extremely low; bacteria or mycoplasmalike agents have not been observed in repeated examinations of tissue from blighted trees by light microscopy or by transmission and scanning electron microscopy (Brlansky, *unpublished*).

In our search for a blight-specific protein, we compared numerous samples of leaves and bark from diseased and healthy trees. In these experiments, extracts and preparations fractionated by differential and sucrose-density gradient centrifugation were analyzed by SDS-PAGE. No proteins that appeared to be blight-specific were found. This failure and the possible analogy of blight to phony peach, which suggests that the blight pathogen (whatever its nature) is concentrated in the wood, led to the experiments reported here.

It is conceivable that at least some of the proteins in vacuum extracts that appear to be associated with citrus blight are produced by the pathogen that causes the disease. The 35-kD, 23-kD, 15-kD, 12-kD, and 10-kD proteins appear to be unique to blight in that comparably sized proteins were not observed in samples from healthy trees. A protein of 43 kD and three proteins between 31 and 26 kD appear in healthy tissue but appear in higher concentrations in infected tissue. This suggests that they may be host proteins that are synthesized at higher rates in diseased trees. The 12-kD protein that we observed in blight samples may be similar to the proteins recently found in partially purified preparations from leaves (M. G. Bausher, *personal communication*).

The failure to detect blight-specific proteins in some stem samples and in root samples from two of 17 trees with symptoms supports the view that the blight pathogen is unevenly distributed in infected trees. Trees remained healthy after being reconstituted using root sprouts forced from infected trees and using buds from infected trees (18,19). This was taken as proof that the cause of blight was not transmitted by grafting. A possible explanation is that the forced root sprouts were from sections of roots that did not contain the pathogen; these sections would tend to produce root sprouts much earlier than infected roots. Some roots from an infected tree have very poor feeder root growth and low rates of water transport. These roots produce root sprouts more slowly than more normal-appearing roots from the same tree that have higher rates of water transport (Lee, Brlansky, Timmer, and Graham, *unpublished*).

The ability to detect several blight-specific proteins, whether they are related to pathogenesis or produced by the pathogen that causes the disease, could lead to characterization of this elusive pathogen. Our failure to detect a pathogen by electron microscopy in preparations that contain the blight-specific proteins and the observation that all of the proteins but one do not sediment when centrifuged suggest the proteins are not attached to a pathogen in vacuum extracts. Procedures based on partial amino acid sequence analysis or serological detection could be used to isolate the blight-specific protein genes and ultimately determine whether they are indeed related to the pathogen.

ACKNOWLEDGMENT

We thank M. G. Bausher for allowing us to read his manuscript before publication.

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