

Field Detection of Citrus Blight Using Immunological Techniques

M. G. BAUSHER, USDA-ARS, Horticultural Research Laboratory, 2120 Camden Road, Orlando, FL 32803, and M. J. SWEENEY, Department of Molecular Biology and Microbiology, University of Central Florida, Orlando 32816

ABSTRACT

Bausher, M. G., and Sweeney, M. J. 1991. Field detection of citrus blight using immunological techniques. *Plant Dis.* 75:447-450.

Eighty-six sweet orange trees from three Florida grove locations were diagnosed as being healthy or blighted using a western blot system with proteins extracted from citrus leaves. Extracted proteins were separated on 18% SDS-PAGE gels, electroblotted onto polyvinylidene difluoride membrane, and probed with citrus blight antisera obtained from blighted citrus trees. This blot system was as reliable as conventional diagnostic systems and in several cases detected the presence of blight before increased tissue concentrations of zinc, decreased water uptake, and wilt were observed. Results were the same for samples taken during winter dormant periods and samples taken during spring growth. The use of leaf samples for the diagnosis of citrus blight has the advantage of allowing repeated sampling, unlike xylem zinc analysis and water injection techniques.

Blight of citrus is an important problem in a number of places in the world, including Argentina, Brazil, Florida, and South Africa (26,29), and the etiology is not known. The disease results in overall decline of plant vigor, leading to removal of affected trees and enormous economic loss. Blight has been known to exist in Florida for a century, and extensive research on the disease has failed to identify a causal agent. The syndrome begins as a delay or lack of new growth flush in the spring and wilting of existing leaves. The wilting is not corrected by irrigation, and subsequent seasonal growth shows increased leaf water potential, abscission of leaves, and death of terminal growing points (20). The metabolism of the tree is disrupted, and chlorides and sodium accumulate in the leaves (28). Ribulose 1,5-bisphosphate carboxylase and carbonic anhydrase are less active in blight-stressed trees (24).

Recently, the disease was apparently transmitted by root grafting (22). Hopkins (9) reported that immature seedlings (< 2 yr) infected with *Xylella fastidiosa* Wells et al, a xylem-limited, gram-negative bacterium, developed some symptoms of blight. In the field, how-

ever, immature trees (< 5 yr) are not susceptible (6,18). Wutscher and Smith (30) failed to infect reconstituted trees, and symptoms did not develop in trees grafted with buds, shoots, or roots from blighted trees. Recently, Lee et al (12) reported that the disease developed in trees approach-grafted with roots from diseased trees. Derrick et al (7) transmitted the disease to healthy trees by grafts with 9- to 12-cm root sections from diseased trees.

Imposition of stress in plants results in metabolic changes and can alter the types of proteins formed in cells (15,23), and new proteins can be induced in plants by pathogens. Proteins have been found in the leaves of blighted sweet orange (*Citrus sinensis* L.) Osbeck 'Hamlin' and 'Valencia' trees that have not been detected in healthy citrus (1). These proteins could not be found in leaves of citrus infected with fungal or viral pathogens, including tristeza and *Phytophthora parasitica* Dastur, or under water stress (-2.5 mPa). Derrick et al (7) reports the presence of similar proteins in roots of blighted citrus trees.

A biological marker might allow diagnosticians and researchers to differentiate citrus blight from other physiological or pathological disorders and to detect the disease in asymptomatic trees. Here we describe a western blot method for detection of proteins that appear unique to trees with citrus blight.

MATERIALS AND METHODS

Extraction of proteins from plant material. Samples (30-gm) of young, fully expanded leaves were harvested from sweet orange trees located in three areas of Florida: Fort Pierce during

December and February, St. Cloud during December and April, and Davenport during July and August. The trees were either cv. Valencia or cv. Hamlin grafted to rough lemon (*C. limon* L.) N.L. Burm.) or sour orange (*C. aurantium* L.) rootstock. Although the trees varied in age from 15 to 30 yr, most were 30 yr old. The harvested samples were washed in 1% (v/v) sodium hypochlorite (20% household bleach) for 5 min, rinsed in tap water, washed in deionized water, and blotted dry before extraction. Wearing powder-free vinyl gloves, we removed tissues from the midribs of the leaves. The tissue was immediately frozen and crushed in liquid nitrogen with a mortar and pestle. The ground leaves were placed in an extraction buffer (50 mM Tris, 1 mM CaCl₂, 1% [w/v] insoluble polyvinylpyrrolidone [PVP], 0.15 M NaCl, pH 8.4, 4 C), 10 ml/g fresh weight of tissue. The tissue slurry was stirred at 4 C for 10 min and filtered through Miracloth. The liquid phase was centrifuged for at least 10 min at 20,000 g and the pellet discarded. Ammonium sulfate was added to 85% (w/v). This mixture was stirred for 18 hr at 4 C. The resulting precipitate was sedimented by centrifugation for 20 min at 20,000 g. The pellet was dissolved in ×20 volume of water, and the solution was dialyzed against 10 changes of water using a 6,000- to 8,000-*M_r* cutoff tubing (Spectrapor). The extract was freeze-dried and stored in sealed glass containers under vacuum at -20 C. Another method of extraction used involved protease inhibitors and acetone precipitation (25).

Electrophoresis. The SDS-PAGE (18% acrylamide) system of discontinuous electrophoresis of Lammeli (10) was used with a Mini-Gel apparatus (BioRad, Richmond, CA). The sample buffer consisted of 4% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 20% (v/v) glycerol, and 0.01% (w/v) bromophenol blue in 62.5 mM Tris, pH 6.8. Electrophoretic conditions were constant 200 V for 1 hr (4 C). The SDS-PAGE gels were stained with either Coomassie Blue R-250 or Coomassie Silver (BioRad) (19). Reagents for electrophoresis and low molecular weight standards were from BioRad.

Antiserum preparation. Proteins less than 30,000 *M_r* were removed from the total soluble leaf extracts by ultrafiltration (30,000-*M_r* cutoff filters) and

Mention of a trademark, warranty, proprietary product, or vendor does not constitute a guarantee by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

Accepted for publication 2 October 1990.

This article is in the public domain and not copyrightable. It may be freely reprinted with customary crediting of the source. The American Phytopathological Society, 1991.

electrophoresed. The bands just below the lysozyme standard (14,600 M_r) were electroeluted, and the SDS was removed (1). The antigen (200 μg) was dissolved in 1 ml of sterile PBS adjuvant solution (16) containing monophospholipid A (250 $\mu\text{g}/\text{ml}$) (14), trehalose dimycolate (250 $\mu\text{g}/\text{ml}$), cell wall skeleton of mycobacteria (250 $\mu\text{g}/\text{ml}$), squalene (20 μl), and Tween 80 (0.2%), v/v. The solution was injected intramuscularly into 4-month-old male rabbits at a 14-day interval (16). Blood was collected from the peripheral ear vein 10 days after the second injection (14). The clotted sera were spun at 1,500 g to remove the red blood cells, and the serum was stored at -20°C . IgG was removed from the serum by the use of agarose-bound recombinant protein A (Beckman, Fuller-

ton, CA) (2,11) as per the manufacturer's protocol.

Protein blotting. Proteins were transferred onto a polyvinylidene difluoride (PVDF) (Immobilon P, Millipore) transfer membrane by a semidry system (Nova blot) (21). Blot conditions for the citrus blight antisera probing were 0.8 mA/cm^2 for 25 min with a buffer system consisting of 39 mM glycine, 48 mM Tris, 0.0375% (w/v) SDS, and 20% methanol. Slot blots stained with Coomassie Blue R-250 were used to determine the amount of protein delivered to each electrophoresis lane (5). Stained protein content was measured with a Shimadzu CS9000 (Kyoto, Japan) scanning densitometer at 595 nm. Controls were derived from bovine serum albumin (Sigma Type V). After probing with citrus blight anti-

sera was completed, blots were visualized by use of alkaline phosphatase conjugated goat antirabbit secondary antibody (Bio-Rad).

Blight determinations. We used two methods of diagnosis on healthy and blighted plants: 1) zinc analysis (6,17,27) and water uptake (13) and 2) the western blot system.

RESULTS

Western blots of extracts from blighted trees contained multiple protein bands (Fig. 1, lanes 5-7), whereas a single band or no positive reactions were observed for healthy trees (Fig. 1, lanes 1-4 and 8). Blight-associated proteins occurred below the molecular weight standard of 14,600 (lysozyme) (Fig. 1, lane 9). Thus, the M_r of the blight proteins was about 13,000. The molecular weight standards used were 2 μg of protein per lane, whereas the unknown tree samples were 3.2 μg . The multiple banding pattern is suggestive of several compounds as antigens in blight (Fig. 2, lanes 4-7).

Extracts from diseased trees of both Valencia and Hamlin reacted to the antiblight IgG fraction of the sera (Fig. 3). Leaf proteins from Hamlin were precipitated by ammonium sulfate (Fig. 3, lanes 1 and 2) and leaf proteins from Valencia, by acetone containing protease inhibitors (Fig. 3, lanes 3 and 4). The banding patterns with both extracts were identical. Leaves from blight trees (Fig. 3, lanes 1 and 3) had multiple bands, and those from healthy trees (Fig. 3, lanes 2 and 4) had single bands.

Extracts of leaf samples from a typical double-banding pattern for the blight leaves and a single-banding pattern (or no band) for the healthy leaves were found in samples from all three grove locations. A sample from a blight-positive tree was electrophoresed, blotted, and

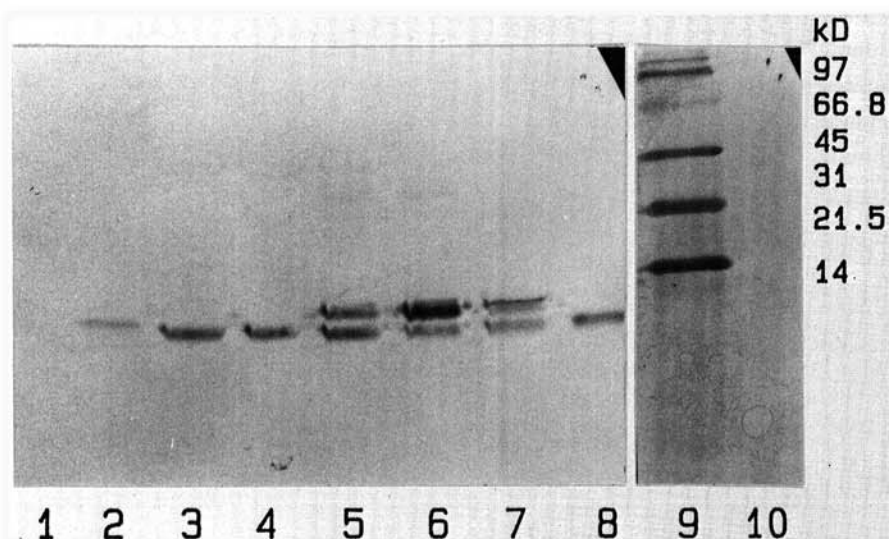


Fig. 1. Western blot of 18% polyacrylamide gel with leaf tissue of (lanes 1-4 and 8) healthy and (lanes 5-7) blighted Valencia orange trees; lane 9 is standards stained with Coomassie Blue R-250, and lane 10 is blank.

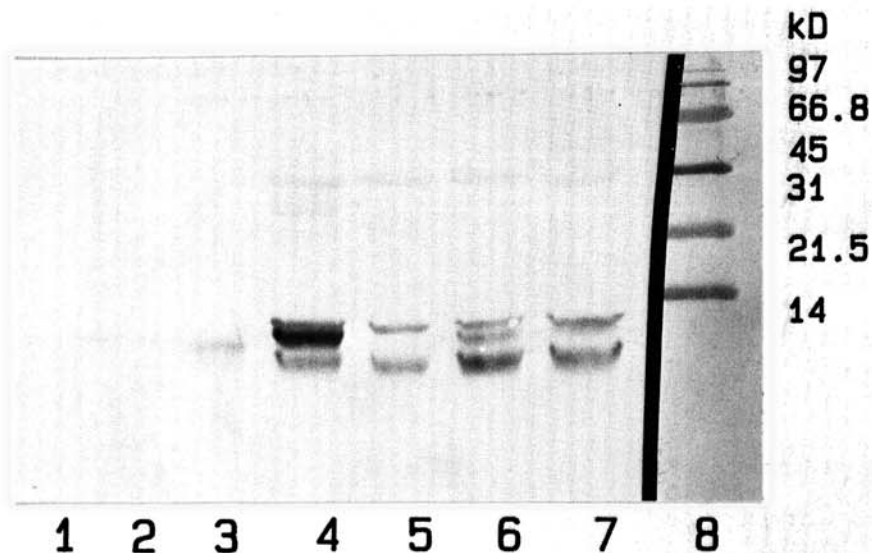


Fig. 2. Blot patterns for leaf extracts from (lane 1) Valencia orange tree protein sources and Bio-Rad low molecular weight standards, (lanes 2 and 3) healthy trees, and (lanes 4-7) blighted trees probed with antiblight IgG. Lane 8 is Bio-Rad low molecular weight standards stained with Coomassie Blue R-250.

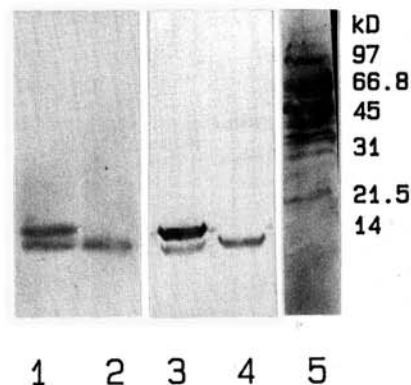


Fig. 3. Western blot of extracts from diseased and healthy (lanes 1 and 2) Hamlin and (lanes 3 and 4) Valencia orange trees. Further extracts were precipitated from Hamlin with ammonium sulfate and from Valencia with acetone from buffer extracts containing protease inhibitors. Lane 5 is leaf extract transferred to a PVDF membrane without further treatment and stained with Coomassie Blue R-250.

cut from the original PVDF membrane and stained with Coomassie Blue R-250 (Fig. 3, lane 5). Clearly, a number of protein bands from leaf tissue extractions were visible in the Coomassie-stained blot that did not appear after antiblight probing, indicating specificity to the antibody.

The antisera reacted with the blight-associated proteins from the three grove locations tested. A third band was observed in the St. Cloud sample (*data not shown*), suggesting that the upper band has more than one component.

For the 10 trees from Fort Pierce, the blight antiserum was positive for all trees diagnosed as having blight by method 1 and negative for all trees diagnosed as apparently healthy (Table 1). Of the 19 trees sampled from St. Cloud, 10 were diagnosed as blighted by method 1 and 15 by method 2. None of the trees diagnosed as blighted by method 1 were rated blight-free in the antisera test. Subsequently, each of the trees diagnosed as blight-free by method 1 and blighted by method 2 developed the elevated zinc and water stress symptoms characteristic of citrus blight.

Two groups of trees were tested at Davenport (K. S. Derrick and L. W. Timmer, *personal communication*). In the first group (37 trees), 24 trees were diagnosed with blight by method 1 and 25 by method 2. The tree considered diseased by method 2 and healthy by method 1 was later found to be in the early stages of blight (K. S. Derrick, *personal communication*). In the second group (20 trees), 11 trees were diagnosed as blighted by method 1 and 13 by method 2. Again, none of the trees diagnosed as blighted by method 1 were diagnosed as blight-free by method 2. No cross-reaction occurred with preimmune rabbit sera or the alkaline phosphatase secondary antibody with the western blotted proteins from leaf sources.

DISCUSSION

Proteins found in leaves from blighted citrus trees are antigenic and diagnostic for a change in metabolism of the leaves. Proteins may arise *de novo* from the host plant as a result of an unknown stress or they may be produced by a pathogenic agent. The production of these proteins appears to be for the onset specific to blight, because even without the presence of visible water stress in the leaves, the proteins are present (*unpublished*). These proteins may hold a link to a pathogen as the blight protein source; they could be either host- or pathogen-encoded.

The use of PVDF membranes and alkaline phosphatase-conjugated antibodies greatly increased the sensitivity of this method over the use of nitrocellulose. Apparently, the PVDF (8) membranes are hydrophobic longer during the transfer and blocking procedures than the nitrocellulose (8). The use of alkaline

Table 1. Diagnosis of citrus blight at three locations in Florida

Location	No. of trees	Method 1 ^a		Method 2 ^b	
		Blighted	Healthy	Blighted	Healthy
Fort Pierce	10	5	5	5	5
St. Cloud	19	10	9	15	4
Davenport					
Site 1	37	24	13	25	12
Site 2	20	11	9	13	7

^aZinc analysis by atomic absorption (7) and water injection by syringe technique (13).

^bWestern blot using blight leaf protein antibody.

phosphatase provided greater sensitivity than would peroxidase-conjugated secondary antibodies, because of the increased color reaction and color stability of the phosphatase. According to the manufacturer, alkaline phosphatase-conjugated materials have a 10-fold increase in sensitivity over peroxidase (3,4).

Also, no cross-reaction was found with extracts from citrus or grapes that had been inoculated with *X. fastidiosa* to the blight leaf protein antisera. The proteins appear resistant to protease activity, since extracting with or without a protease inhibitor cocktail and long dialysis times do not affect the results. The western blot system for the diagnosis of citrus blight is highly specific and uses easily acquired leaf tissue in small samples (28). It also holds more promise as an earlier diagnostic procedure than either zinc or water injections. Additionally, trees of all ages may be sampled as long as leaf tissue is available, which is a distinct advantage to currently employed diagnostic methods. Further work is being pursued to determine properties of the proteins, since they were always found in leaves with the symptoms of blight. Work is under way to confirm the structure, period of onset of these blight-associated proteins, and their presence in other citrus anatomy and to produce monoclonal antibodies to these proteins.

ACKNOWLEDGMENTS

We thank Dorothy Payne for diligent technical assistance and Heinz Wutscher, Ken Derrick, L. W. Timmer, and Robert Pelosi for access to field locations.

LITERATURE CITED

- Bausher, M. G. 1991. Electrophoretic and immunological evidence of unique proteins in leaves of citrus trees: Application to citrus blight detection. *Electrophoresis* 11:830-834.
- Bigbee, W. L., Vanderlaan, M., Fong, S. S. N., and Jensen, R. H. 1983. Monoclonal antibodies specific for the M- and N-forms of human glycoporphin A. *Mol. Immunol.* 20:153.
- Bio-Rad Laboratories. 1987. Western blotting detection systems: How to choose. Bull. 1310. Bio-Rad, Richmond, VA.
- Blake, M. S., Johnston, K. H., Russell-Jones, G. L., and Gotschlich, E. C. 1984. A rapid, sensitive method for detection of alkaline phosphatase-conjugated antibody on western blots. *Anal. Biochem.* 136:175-179.
- Bradford, M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248.

- Cohen, M., and Wutscher, H. K. 1974. Diagnosis of trees with citrus blight (YTD). *Proc. Int. Soc. Citric.* 3:884-886.
- Derrick, K. S., Lee, R. F., Brlansky, R. H., Timmer, L. W., Hewitt, G. G., and Barthe, G. A. 1990. Proteins associated with citrus blight. *Plant Dis.* 74:168-170.
- Gultekin, H., and Heerman, K. H. 1988. The use of polyvinylidenedifluoride membranes as a general blotting matrix. *Anal. Biochem.* 172:320-329.
- Hopkins, D. L. 1988. Production of diagnostic symptoms of blight in citrus inoculated with *Xylella fastidiosa*. *Plant Dis.* 72:432-435.
- Lammeli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.
- Langone, J. J. 1982. Applications of immobilized protein A in immunochemical techniques. *J. Immunol. Methods* 55:277-296.
- Lee, R. F., Brlansky, R. H., Timmer, L. W., Tucker, D. P. H., Graham, J. H., and Derrick, K. S. 1988. Graft transmission of citrus blight. (*Abstr.*) *Phytopathology* 78:1572.
- Lee, R. F., Marais, L. J., Timmer, L. W., and Graham, J. H. 1984. Syringe injection of water into the trunk: A rapid diagnostic test for citrus blight. *Plant Dis.* 68:511-513.
- Nerenberg, S. T., Zedler, P., Prasad, R., Biskup, N. S., and Pedersen, L. 1978. Hematological response of rabbits to chronic, repetitive, severe bleedings for the production of antisera. *J. Immunol. Methods* 24:19-24.
- Okashi, Y., and Matsuoka, M. 1985. Synthesis of stress proteins in tobacco leaves. *Plant Cell Physiol.* 26:473-480.
- Ribi, E., Cantrell, J. L., Takayama, K., Qureshi, N., Peterson, J., and Ribi, H. O. 1984. Lipid A and immunotherapy. *Rev. Infect. Dis.* 6:567-572.
- Smith, P. F. 1974. Zinc accumulation in the wood of citrus trees affected with blight. *Proc. Fla. State. Hortic. Soc.* 87:91-95.
- Smith, P. F., and Reitz, H. K. 1977. A review of the nature and history of citrus blight in Florida. *Proc. Int. Soc. Citric.* 3:881-884.
- Switzer, R. C., III, Merrill, C. R., and Shifin, S. 1979. A highly sensitive silverstain for detecting proteins and peptides in polyacrylamide gels. *Anal. Biochem.* 98:231-237.
- Syvrtsen, J. P., Bausher, M. G., and Albrigo, L. G. 1980. Water relations and related leaf characteristics of healthy and blight affected citrus trees. *J. Am. Soc. Hortic. Sci.* 105:431-434.
- Towbin, H., Staehelin, T., and Gordon, G. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci.* 76:4350-4354.
- Tucker, D. P. H., Lee, R. F., Timmer, L. W., Albrigo, L. G., and Brlansky, R. H. 1984. Experimental transmission of citrus blight. *Plant Dis.* 68:979-980.
- Van Loon, L. C. 1985. Pathogenesis-related proteins. 1985. *Plant Mol. Biol.* 4:111-116.
- Vu, J. C., Bausher, M. G., and Yelenosky, G. 1987. Activity of ribulose-1,5-bisphosphate carboxylase in the leaf tissues of blight-affected 'Valencia' orange trees (*Citrus sinensis* (L.) Osbeck). *Environ. Exp. Bot.* 27:45-51.
- Witt, W., Bausher, M. G., and Yelenosky, G. 1988. Immunoblotting to detect a soluble glycoprotein from trifoliolate orange in a citrus hybrid.

- HortScience 23:768-770.
26. Wutscher, H. K., Campiglia, H. G., Hardesty, C., and Salibe, A. 1977. Similarities between marchitamiento repentino disease in Uruguay and Argentina and blight of citrus in Florida. Proc. Fla. State Hortic. Soc. 90:81-84.
 27. Wutscher, H. K., Cohen, M., and Young, R. H. 1977. Zinc and water-soluble phenolic levels in the wood for diagnosis of citrus blight. Plant Dis. Rep. 61:572-576.
 28. Wutscher, H. K., and Hardesty, C. 1979. Concentrations of 14 elements in tissues of blight-affected and healthy 'Valencia' orange trees. J. Am. Soc. Hortic. Sci. 104:9-11.
 29. Wutscher, H. K., Schwarz, R. E., Campiglia, H. G., Moreira, C. S., and Rossetti, V. 1980. Blight-like citrus tree declines in South America and South Africa. HortScience 15:588-590.
 30. Wutscher, H. K., and Smith, P. F. 1988. Failure to propagate citrus blight in reconstituted trees. Proc. Fla. State Hortic. Soc. 101:62-63.