Serological Tests for Citrus Blight*

K. S. Derrick, G. A. Barthe, B. G. Hewitt, and R. F. Lee

ABSTRACT. The symptoms of trees with blight are similar to those of a number of other declines of citrus. The finding of distinctive proteins in leaves and roots of infected trees has led to the development of serological tests that are useful in distinguishing trees with blight from those declining from other disorders. Two blight-associated proteins (35 and 12 kd) were purified by preparative electrofocusing and SDS-PAGE. Polyclonal antisera were produced to both proteins, and a monoclonal antibody was produced to the 12-kd protein. Both proteins were readily detected in crude extracts from blighted trees by immunospot and western blot assays. In several experiments, trees with symptoms of blight that were positive by water uptake tests and zinc wood analyses were also positive in the serological tests. Some bearing trees were found to contain the two proteins up to one year before blight symptoms developed. The 12-kd protein was detected in young trees three months after root-graft inoculations.

Citrus blight is a serious disease in many areas of the world, and is frequently a problem when citrus is grown in hot, humid areas. Blight has been reported in Cuba, Belize, Colombia, South Africa, Australia, and Florida (15). Based on symptomatology and diagnostic assays, several diseases in South America, including citrus declinio in Brazil, declinamiento in Argentina, marchitamiento repentino in Uruguay and sudden decline in Venezuela are probably identical to blight (4, 12, 15).

Trees on sour orange are resistant to blight, but many of the common rootstocks used as alternatives to sour orange are very susceptible. Blight has become a major disease of citrus in many areas when citrus industries have been reestablished on blight susceptible rootstocks following large scale losses of trees on sour orange due to citrus tristeza virus (CTV).

The cause of blight is not known, but induction of all the symptoms of blight in several replicated root-graft transmission experiments indicate a pathogen is involved (9,11,13,14,17). The symptoms of the disease, which occur only on fruit-bearing trees, are similar to those of a number of other declines of citrus. Several diagnostic tests have been developed for identifying trees with blight, including zinc analysis (1,18), water uptake (10) and, more recently, assays for blight-associated proteins (2,3,5,6,7,8)).

Some groves remain free of blight for many years suggesting that if the cause and means of spread were known, strategies could be developed for controlling the disease. Since trees less than four years of age do not show visible symptoms of blight, there is an obvious need to be able to identify infected trees before they show symptoms. A number of proteins have been found to occur in blighted trees that are absent or present in much lower concentrations in healthy or trees declining with other disorders. These proteins have properties similar to pathogenesis related proteins that have been observed in other host/pathogen combinations. We report the development of rapid serological methods for detecting two proteins that appear to be associated with blight and may be indicators of presymptomatic infections.

MATERIALS AND METHODS

Isolation of blight-associated proteins and production of antisera. Xylem fluid from healthy and blighted trees was collected as previously described (6). The extracts were precipitated with three volumes of acetone and held at -20 C for 1 hr. The precipitates were collected by centrifugation at 10,000 g for 15 min and resuspended in 50 ml of water plus 2 ml of pH 3-10 isoelectric focusing ampholyte. The

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mixture was subjected to preparative isoelectric focusing using a Bio-Rad Rotofor, (Bio-Rad Laboratories, Richmond, California) following the manufacturers instructions. The resulting fractions were assayed by SDS-PAGE as previously described (6). Fractions containing blight-associated proteins were precipitated with acetone as described above, resuspended in water, mixed with an equal volume of 250 mM Tris-HC1, 8% sodium dodecyl sulfate (SDS), 4% 2-mercaptoethanol, pH 6.8 (TB) and heated at 100 C for 5 min. The individual preparations were subjected to preparative SDS-PAGE (1.5 mm thick, 12% gels). The gels were stained with 0.3 M $ZnCl_2$. Bands of interest were cut out, frozen in liquid nitrogen, pulverized and lyophilized. The preparations were sent to Cocalico Biologicals, (Reamstown, Pennsylvania) for antisera production. Using a similar antigen preparation of the 12-kd blight-associated protein, a monoclonal antibody was produced by the University of Florida Interdisciplinary Center for Biotechnology Research.

Sample preparation. Five to 10 mature leaves were collected at random from each tree. The leaves were washed with tap water and passed while wet through a leaf roller to prepare extracts for assay. The resulting extract was collected in a microfuge tube and incubated at overnight at 37 C. The samples were microcentrifuged and the resulting supernatants were used for analysis. Xylem fluid from roots and stems was collected as described previously (6).

Immunospot and western blot analysis. For immunospot analysis, 3-µl samples were spotted onto dry nitrocellulose membranes. The membranes were air dried. For 12-kd protein assays, the proteins were denatured by boiling or autoclaving the membranes in water for 10 min. For western blot analysis, samples were mixed with an equal volume of TB and boiled for 5 min. Two µl samples were subjected to SDS-PAGE and following electrophoresis the gels were blotted to nitrocellulose membranes using a Janssen semi-dry blotter (Janssen Life Sciences Products, Piscataway, New Jersey), as described by the manufacturer. The membranes for immunospot or western blot analysis were processed using the Promega ProtoBlot Western Blot AP System (Promega Corporation, Madison, Wisconsin). Monoclonal ascites to the 12-kd protein was used at a dilution of 1:1,000,000 without added extract of healthy plants. To use the polyclonal antiserum to the 12-kd protein for immunospot analysis, the antiserum was diluted (1:16,000) with an extract of healthy leaves prepared by grinding 2.5 g of leaf tissue in a tissumizer with 10 ml of 10 mM Tris-HCl, 150 mM NaCl, pH 8.0 (TBS) and 0.2 g each of sodium sulfite and polyvinyl pyrrolidone 40. The extract was filtered through two layers of cheesecloth and centrifuged at 10,000 x g for 10 min. The supernatant was collected and Tween 20 was added to 0.05% before use.

RESULTS AND DISCUSSION

Antisera were produced to 12 and 35-kd blight-associated proteins that had been purified from xylem fluid from infected roots. These proteins were readily detected in crude extracts from blighted trees by immunospot and western blot analysis (Fig 1-3). The 12-kd protein was in leaves, roots and stems of trees with blight, but not in comparable preparations from healthy trees. The 35-kd protein occurred in approximate equal concentrations in leaves of both infected and healthy trees; however, it was not present in xylem fluid of stems and roots of healthy trees but was readily detected in these fluids from trees with blight (Fig. 1,2).

The serological tests for citrus blight proteins were useful in distinguishing trees with blight from those declining with other disorders. Extracts from trees with CTV, Phytophthora root rot, or citrus variegated chlorosis were readily distinguished from trees with blight (Fig. 1). Other

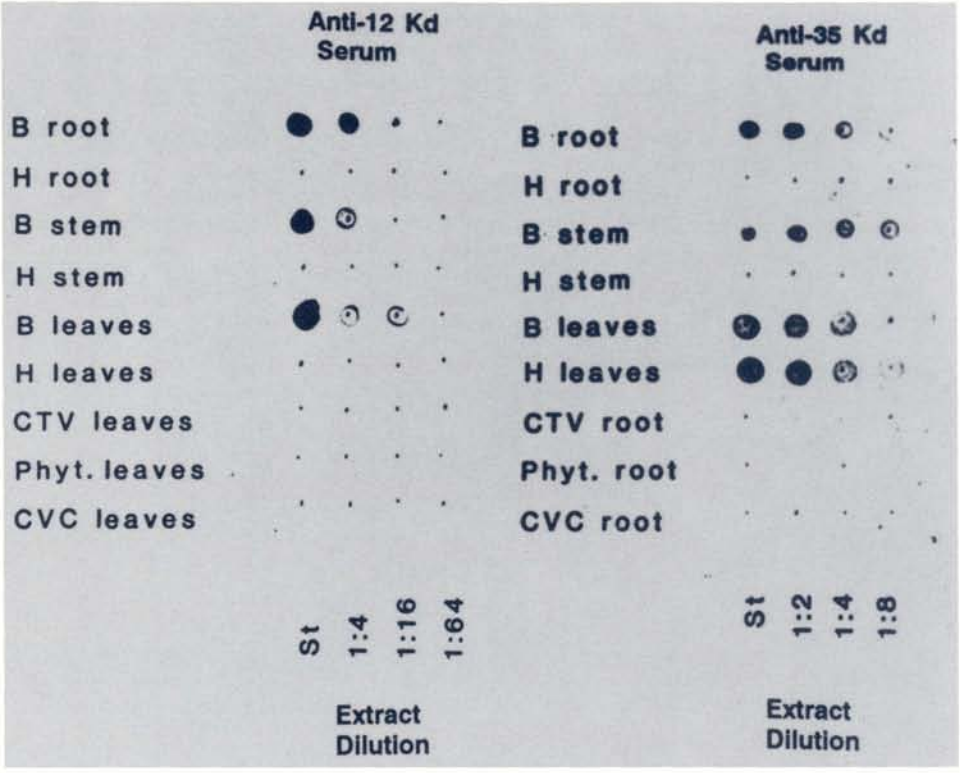
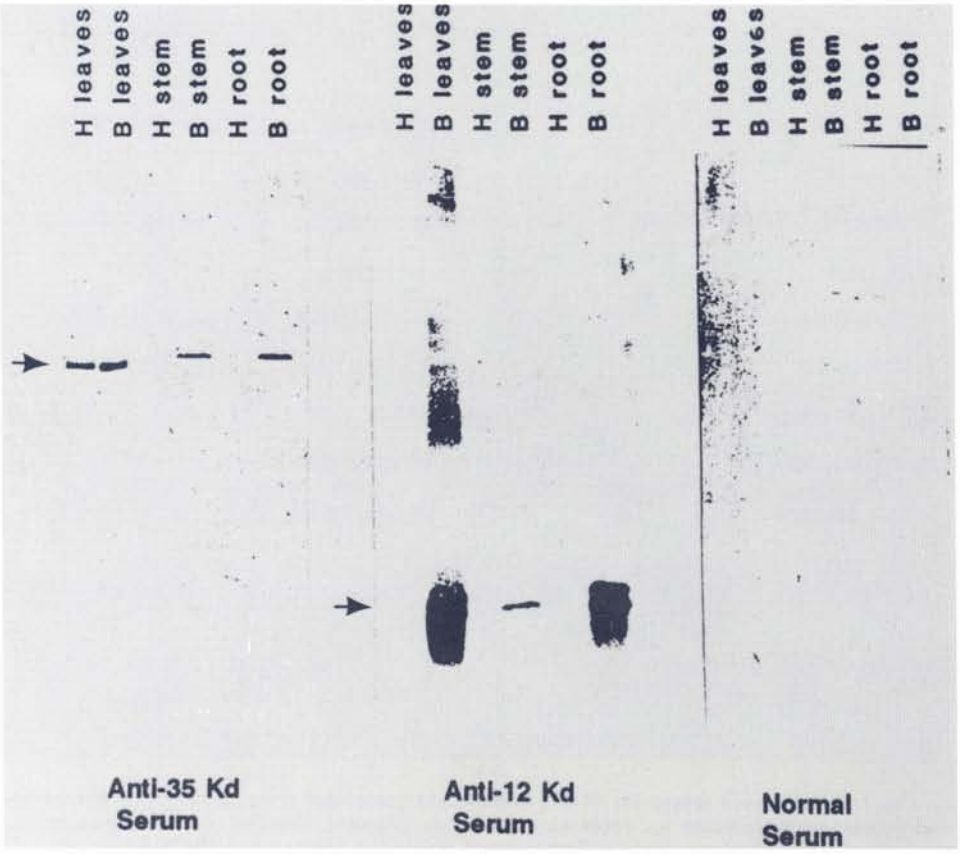


Fig. 1. Immunospot assays for 12 and 35-kd blight associated proteins. Extracts from leaves and vacuum extracts stems and roots were diluted as indicated. Positive reactions are seen with extracts of blighted leaves, roots and stems, and of healthy leaves when the 35-kd antiserum is used. Extracts of leaves and roots from trees declining with citrus tristeza virus (CTV), Phytophthora root rot (Phyt.) and citrus variegated chlorosis (CVC) did not react.

trees declining with various disorders that have been assayed and found not to contain blight-associated proteins include citrus psorosis/ringspot, murcott collapse, citrus nematode, exocortis, drought stress, and salt stress.

The initial screening of the polyclonal antiserum to the 12-kd protein was done by western blot analysis. The antiserum was produced to SDS-denatured 12-kd protein, and after finding that it did not react with the native protein in immunospot or ELISA assays, we developed an immunospot procedure where the samples were boiled in TB before being spotted to nitrocellulose membranes. This procedure was subsequently modified by spotting native proteins followed by boiling or autoclaving the membranes to denature the proteins. Although the inject antigen used to produce the polyclonal antiserum to the 12-kd protein was purified, it reacted with extracts from healthy plants. Thus, it was necessary to add an extract from healthy leaves to the antibody solution for immunospot assays using this serum. The monoclonal antibody to the 12-kd protein did not react with extracts from healthy leaves and thus can be used without adding healthy extract to the primary antibody solution. The 12-kd protein that we detect by immunospot and western blot assays is most probably the same protein detected by western blot assays using an antiserum produced to a protein isolated from leaves (2).

The 12-kd protein immunospot test for blight has proven very reliable in identifying trees with visible symptoms of blight. Only in a few cases has a known blighted tree failed to give a



Fg. 2. Western blot assays for 12 and 35-kd blight-associated proteins. Arrows indicate 12 and 35-kd proteins.

positive reaction. Whether due to sampling errors or the possible selection of leaves from a sector of the tree that did not contain the protein, a second sampling of the tree, if it indeed did have blight, was usually positive. Trees with visible symptoms of blight usually gave very strong reactions in the immunospot test and confirming western blot assays were rarely needed.

Fig. 3. Immunospot assays for 12-kd blight associated protein of root-graft inoculated two-year-old trees. Every other tree in each row was inoculated. After seven months, six of six trees inoculated with roots from a symptomatic tree were positive. After three months, two of three trees inoculated with roots from a symptomatic tree were positive; none of the three trees inoculated with roots from a predecline (protein positive) tree were positive.

A most important application of any assay procedure for citrus blight is in the detection of presymptomatic infections. The blight-associated proteins are known to occur in trees before visible symptoms develop (6). The 12-kd protein is expressed very early in root-graft-inoculated trees (Fig. 3). In an experiment where two-year-old sweet orange trees on rough lemon were inoculated by root grafting, the 12-kd protein was detected in two of three inoculated trees after 3 months and, in a separate experiment, six of six inoculated trees were positive after seven months. All the trees in the block were blight-protein negative at the beginning of the experiment. The trees on each side of the inoculated trees used as uninoculated controls have remained blight-protein negative. The ability to inoculate and identify young presymptomatic trees by 12-kd assay should be useful in future research on citrus blight.

The concentration of the 12-kd protein in leaves of presymptomatic trees is quite variable and some leaves of a given tree contain the protein whereas others do not. This may correlate with observations that the visible symptoms of a tree with blight are frequently sectored. We attempt to minimize this effect by pooling leaves from different branches of the tree for each sample.

There is an obvious need to identify the pathogen that causes blight and the search will certainly continue. The graft transmission experiments indicate the pathogen is probably restricted to the roots. Due to the ease of sampling and assaying for the 12-kd protein in leaves this test may continue to be used even after methods are developed for detecting the pathogen in the roots. Obviously, the ability to confirm results from leaf protein assays by actual pathogen detection in the roots would be desirable.

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