# BLIGHT

## Purification of Virus-like Particles from Blight-Affected Citrus Trees

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ABSTRACT. Citrus blight continues to be one of the most economically important diseases of citrus in Florida, where it has been known for over 100 yr. It has also has been reported in Argentina, Brazil, South Africa, Venezuela and Australia. Blight can be transmitted by root grafting and, therefore, appears to be caused by a transmissible organism, still unknown. Using a combination of sucrose density and cesium sulfate gradient centrifugation techniques, rod-shaped virus-like particles were isolated from the roots of blight-affected trees. Polyclonal antisera were prepared and reacted positively in immunogold labeling and ELISA tests. When 33 healthy sweet orange trees on Carrizo rootstock were inoculated with sucrose gradient fractions containing the particles, one-third of the trees (but none of the control trees) became ELISA-positive. Some dieback symptoms similar to those of blight were seen in some of the inoculated trees.

Citrus blight has been known in Florida for over 100 yr (25). The disease reached epidemic proportions after rough lemon rootstock was planted throughout many of the citrus growing areas of Florida. The disease has been reported from many citrus growing areas of the world, namely Brazil (9), Argentina (9), Venezuela (9), South Africa (31) and Australia (10); however, it has not been reported from areas where a Mediterranean type of climate is present (e.g., California, Spain, Italy, or Israel).

The main symptoms of citrus blight are a permanent wilt and decline in the tree canopy with off-color small leaves and twig dieback. Fruit appear normal but may have high acidity and few fruit are set. Water flow in the xylem of affected trees is reduced (20) due to the presence of amorphous plugging materials (7) and this results in a permanent wilt of the trees. The root systems of blight-affected trees are at first unaffected, but as the disease progresses both fibrous and major roots die. The disease has been characterized by high levels of Zn (26, 30) first in the bark and later in the xylem of affected trees, and the presence of blight-associated proteins (4, 14).

Many different theories have been suggested as to the cause of citrus blight, but none have been proven. In 1984 in Florida citrus blight was transmitted using root grafts from tree to tree (29), and later using root pieces of infected trees grafted onto healthy trees (27, 28). Positive root graft transmissions were repeated in South Africa (21) and Brazil (22). These results suggest that the most likely cause is a transmissible agent.

Electron microscopic studies have been done in an attempt to determine the causal agent of citrus blight (6). These studies examined parts of blight-affected trees both above and below ground. Fastidious prokaryotes, fungi, and phytoplasmas agents have not been found specifically associated with diseased trees. No viroid or virus except *Citrus tristeza virus* (CTV) was found. Since blight is graft-transmitted using roots from affected trees, we attempted to purify a virus particle from the roots of affected trees using sucrose and cesium sulfate gradient centrifugation methods. We report here the findings from this work.

### MATERIALS AND METHODS

**Plant materials**. Blight-affected sweet orange trees on Carrizo cit-

range and rough lemon rootstocks were selected in citrus groves in Central Florida. Diagnosis for blight was done using water uptake tests (20), blight protein tests (14), and the presence of amorphous xylem plugs (7, 8). Roots, approximately 1-4 cm in diameter, were cut from affected trees and kept at 4°C. The roots were washed and cleaned. The bark was cut and gently removed from the xylem and kept on ice. The cambial side of the phloem was scraped from the bark piece and then mixed with cold (4°C) extraction buffer (0.1 M sodium phosphate buffer, pH 8.0 containing 0.1 M sodium EDTA, 0.1% sodium diethyldithiocarbamate, 0.1% 2-mercaptoethanol, and 0.5% bentonite). Healthy controls were root samples from sweet orange on Carrizo rootstock trees in the California Clonal Protection Program (D. Gumpf, Univ. of Calif., Riverside) where citrus blight has not been reported, and also from Florida citrus trees of the same scion/rootstock combination that tested negative using the described diagnostic assays.

**Purification of virus-like particles.** Both blight-affected and healthy control tissues were utilized. The tissue was homogenized in extraction buffer and squeezed through cheesecloth; 1% Triton X-100 was added and this mixture was centrifuged at 17,000 g for 10 min. After centrifugation, 1 ml of 30% PEG in 0.6 M sodium chloride was added for every 4 ml of supernatant and stirred overnight at 4EC. The mixture was centrifuged at 17,000 g for 10 min, and the pellets were resuspended in 0.1 M sodium phosphate buffer, pH 8.0, then centrifuged at 17,000 g for 10 min. The supernatant was layered onto a 10-40% sucrose density gradient in 0.1 M sodium phosphate buffer, pH 8.0 and centrifuged at 71,000 g for 4 h. The top 2 ml of the gradient was removed using a syringe and discarded, and the next 2.5 ml was placed into a 3.7 M cesium sulfate solution in 0.1 M sodium phosphate, pH 8.0 and centrifuged for 22 h at 151,000 g. The resultant bands were measured and collected.

**Electron microscopy (EM) of gradient fractions and blight-affected tissues.** Fractions (bands) from the purifications were prepared for electron microscopy by the method of Christie et al. (12). Ten microliters of the sample were put on formvar carbon coated 300 mesh electron microscope grids for 1 min. The sample was removed with the point of a filter paper triangle and the grid was washed with 1.25 ml of a 250 µg/ml bacitracin solution with continuous removal of the solution from the grid surface with filter paper. The samples were washed with distilled water in the same manner and then stained for 10 s with 2% uranyl acetate in a 250 µg/ml bacitracin solution.

Root samples also were fixed for embedding, sectioning and viewing as described previously (7). Particle preparations and sections were treated with an antibody prepared to the isolated particles (see below), and positive labeling was detected by using gold-conjugated goat anti-rabbit antibody as described previously (5).

**Virus-like particle characterization.** SDS polyacrylamide gel electrophoresis (SDS-PAGE) was performed on combined fractions from the cesium sulfate gradients. Twelve percent SDS-PAGE gels were used as described (19). dsRNA analysis was conducted according to the procedure of Dodds and Jarupat (15). Attempts were made to isolate RNA from the purified particles using procedures described previously (2, 23). Total nucleic acids from 0.5 ml fractions from the sucrose gradient fractions 4-9 were prepared as described (2) and run on a 1.2% agarose gel in Tris-borate EDTA buffer (TBE).

**Antibody preparation and testing.** Dialyzed fractions collected from cesium sulfate gradients that

contained virus-like particles were used for preparation of rabbit antisera (Cocalico Inc., Reamstown, PA). The antibodies produced were cross absorbed to remove plant reactions by incubating a 1/100 dilution of the antibody in 23 ml of ELISA coating buffer (0.02 M sodium carbonate, pH 9.6) and 2 ml of a 1/10 dilution of healthy citrus root extract in the same buffer for 30 min at 35°C. Antigen plate trapping ELISA was performed to determine the reactivity (specificity) of the antibodies against the purified particles and not to CTV antigens and healthy plant extracts. Once the antibodies were shown to be specific against the purified particles, IgG was purified from the crude antisera (13) and some of the IgG was conjugated to alkaline phosphatase (1). DAS-ELISA was performed to determine the optimal concentrations of both IgG and conjugate (17). After optimization, aliquots from the purification procedure were removed at various steps and DAS-ELISA was run using the cross-absorbed IgG.

**Association of virus-like particles with blight-affected trees.** Particle purifications and ELISA were used to detect any correlation of particle presence with blight-affected trees. Forty-two trees, both blight-affected and healthy, in various locations in Florida were sampled and assayed.

**Plant inoculations.** Shoot-tip grafted Hamlin sweet orange on Carrizo citrange rootstock were used for inoculation with sucrose gradient fractions 6 and 9. Since blight is a disease of mature trees, the trees were grown in pots and were approximately 2.5 to 3 yr old before inoculation. Trees of this age were flowering and producing some fruit. Inoculations were done by cutting and peeling back a bark flap, dropping inoculum between the bark flap and the xylem, and then replacing the bark and sealing with grafting tape. Healthy controls were inoculated with sucrose solutions. Gradient fractions from healthy appearing field trees were not used since these trees could contain the blight causal agent. The plants were maintained in a growth room at 26°C and were fertilized and pest treated as necessary. They were monitored for presence of the virus-like particle antigen with ELISA and for symptom development.

## RESULTS

**Particle purification and electron microscopy of the gradient fractions**. There were no visible bands produced in the sucrose gradients; however, 0.5 ml fractions were taken and filamentous virus-like particles were found in fraction 6 and fractions 8-9 from the meniscus. In the cesium sulfate gradients, bands were observed. From the top of the tube one band was found at 9-11 mm, one to two bands at 14-17 mm, and one band at 24-26 mm (Fig. 1). The virus-like particles were only found in the bands 14-17 mm from the top. The particles measured from 315 nm to 556 nm in length and were all about 10 nm in width (Figs. 2 and 3). Pieces of CTV virions were often found in some

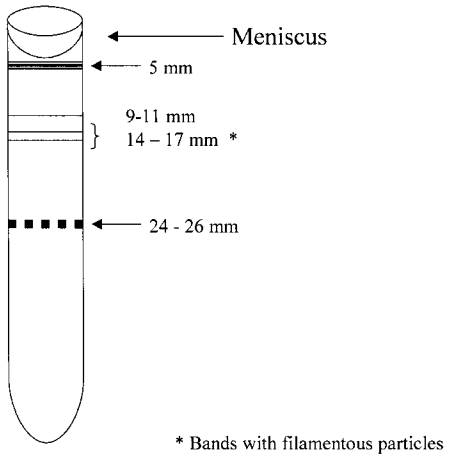


**Fig. 1. Diagram of the location of the light scattering bands obtained in the cesium sulfate gradient from blight affected root material.**

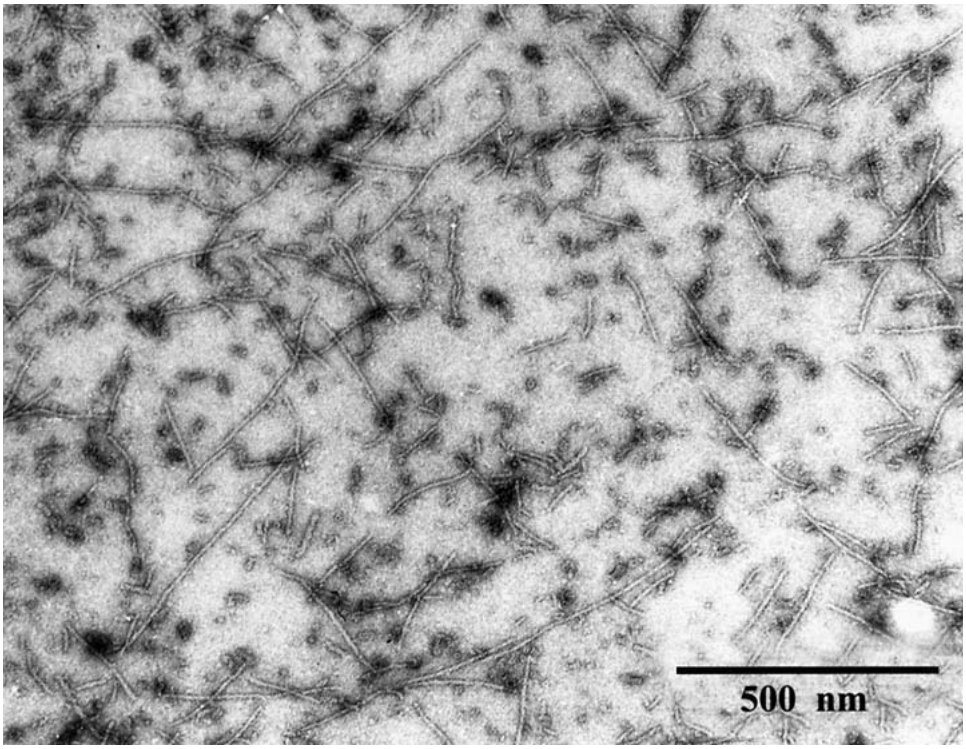


**Fig. 2. Electron micrograph of filamentous particles from cesium sulfate gradients of blight material.**

preparations. No particles were found in equivalent areas of the gradients from three healthy tree root samples from the California Clonal Protection Program.

**Detection in field trees.** In Florida, 50 blighted trees and 10 healthy trees were analyzed. Particles were found in 39 (78%) of the blighted trees and five (50%) of the healthy trees. Three of the five healthy trees that were found to contain the particle have now been diagnosed as blight positive trees. The particles were consistently purified from trees during spring and fall when the temperatures were cooler. Purifications were done on two trees with blight in Australia (10) and both were found to have similar particles.

**Characterization of virus-like particles.** In SDS-PAGE, protein bands were found in the PEG pellet preparations and in the cesium gradient fractions. One band of approximately 23 kD was found consistently and often a second band of 29 kD (Fig. 4). In assays for dsRNA, bands approximately 0.4 to 0.6 kb in size were found in some of the preparations (Fig. 5). ssRNA was not detected in the isolated particles.

**Antibody detection assays.** Two antisera, designated 149 and 150, were produced. In Western blots of preparations that contained filamentous particles, the antibodies detected two bands approximately 23 and 29 kD in size (Fig. 6). Particles from the cesium sulfate gradient fractiosn reacted with immunogold labeled antibody (Fig. 7).

ELISA was done on each step of the purification: 1) after initial clarification; 2) after resuspension of the PEG pellet; 3) on each fraction from the sucrose gradient; and 4) on the bands obtained from the cesium sulfate gradient. Positive ELISA results

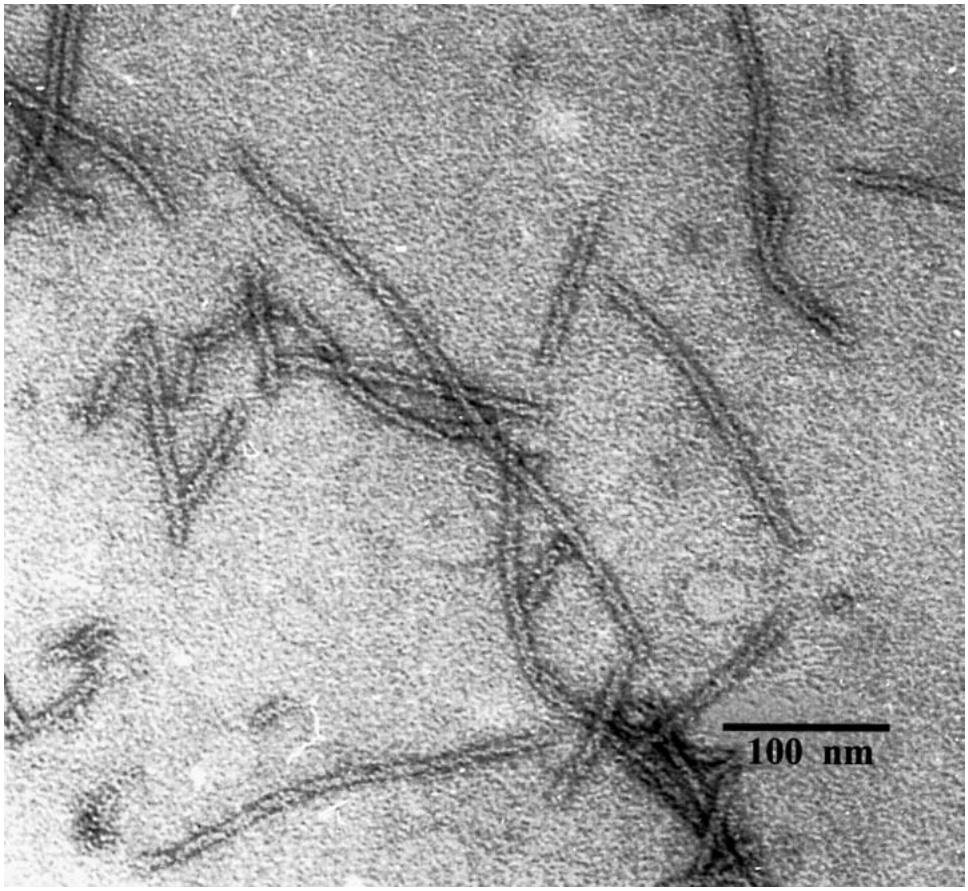


**Fig. 3. High magnification of the filamentous particles isolated in cesium sulfate gradients.**

were obtained with a sample from the initial purification step, with a sample of the resuspended pellet and on fractions 6 and 9 from the sucrose gradients. Positive ELISA results were also obtained on the cesium sulfate fractions in the 14 to 17 mm bands which corresponded to where the particles were seen.

**Pathogenicity tests.** Thirty-three trees were inoculated with either fraction 6 or fraction 9 from the sucrose gradients or a combination of both fractions that contained the suspected virus. Of these trees 11 (33%) were found to be ELISA positive after 2 yr. None of the 10 trees inoculated with sucrose were ELISA positive. Many of the trees, inoculated with the positive particle preparations, showed some type of twig dieback symptom with leaf drop. In order to maintain the trees in the growth room, and thus free from insects, they had to be trimmed. Often after trimming, the trees exhibited some dieback symptoms. In some cases the entire scion died and the rootstock usually sprouted.

## DISCUSSION

Citrus blight continues to be a problem for citrus in Florida and in other citrus growing areas of the world. It is estimated, in Florida, that over 900,000 trees annually are removed because of citrus blight. Since the disease can be transmitted by root grafting it is most likely

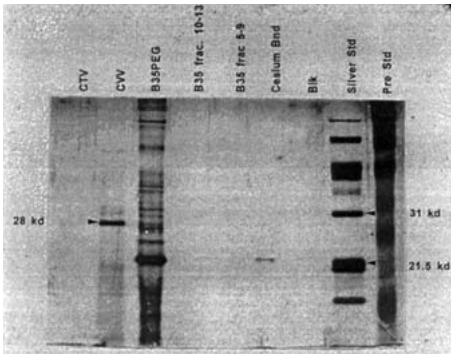


**Fig. 4. SDS-PAGE gel with a 23 kD band from the cesium sulfate fractions. An equivalent size protein was found in the PEG pellet.**

caused by a graft transmissible agent. Since no agents such as bacteria, fungi or fastidious prokaryotes, have been found, a virus is a likely suspect. As we show in this paper we have attempted to purify a virus from blight-affected field trees and in Florida and Australia. The purification of particles from tree roots was attempted because no virus or fastidious prokaryotes have been detected from the scions of affected trees and because the disease has been shown to be transmitted by root grafting.

Filamentous virus-like particles were recovered from cesium sulfate gradient fractions from roots of blight-affected trees, although the particles were in low concentrations; dsRNA's were found in some preparations. Unsuccessful attempts to isolate a nucleic acid tend to lessen the chances that the particles are viral. The characterization of a nucleic acid, cloning and sequencing are needed.

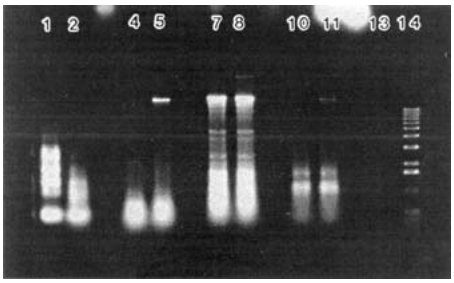


**Fig. 5. dsRNA analysis of preparations from blight-affected trees. The lanes are as follows: Lanes 1 and 2 RNA ladder (0.24, 1.35, 2.37, 4.40, 7.46, and 9.4 kb), lanes 4 and 5 healthy control tissue, lanes 7 and 8 CTV infected tissue, lanes 10 and 11 blight affected tissues, 13 and and lane 14 DNA ladder. Lanes 1, 4, 7, 10 and 13 were treated with DNase. Lanes 2, 5, 8, 10, and 14 were treated with RNase. Lanes 3, 6, 9 and 12 are blank.**

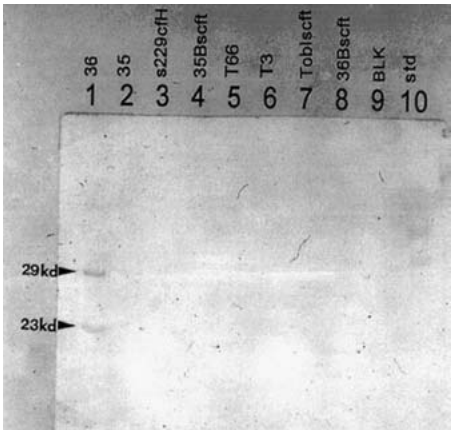


**Fig. 6. Western blot analysis of citrus blight preparation from cesium sulfate gradient fraction that contained filamentous rod-shaped particles. Bands are present at 23 kd and 29 kd from the cesium sulfate gradient fractions 6 and 9 (Lane 1) but absent from (Lane 2) healthy preparations (Lane 3), a preparation from blight shoots (Lane 4), preparations from CTV isolates T66 and T3 (Lanes 5 and 6), healthy tobacco (Lanes 7 and 8). Lane 9 was blank and lane 10 contained the protein standards.**

The particles were in low concentration in the roots. Purification was attempted from the scion of affected trees (data not shown) and some cases similar particles were recovered. The particles also were found in some healthy field trees that did not test positive for blight using the recommended diagnostic tests. These healthy trees could be infected with the blight causal agent, but may be symptomless.

The inability to work with this disease in young greenhouse grown trees makes purification attempts difficult. Other viruses, such as CTV, are normally present in field

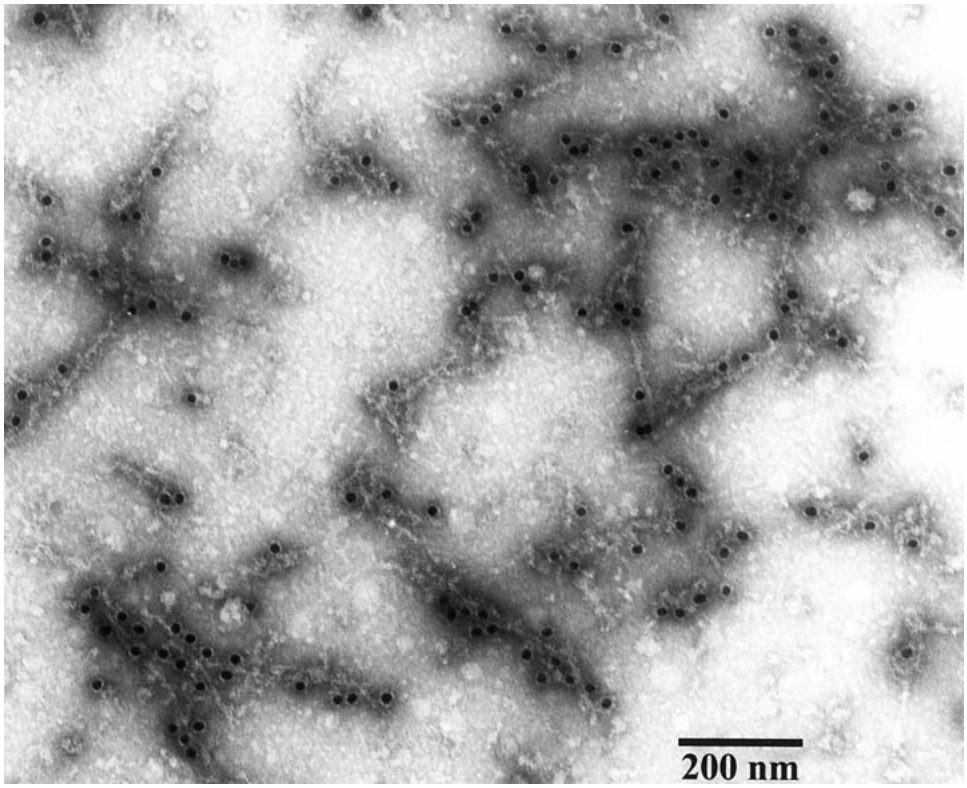


**Fig. 7. Particles from the cesium sulfate gradient fractions immunogold labeled using polyclonal antibody BFD 151.**

trees and must be identified in purification attempts. No bioassay or index host exists for the blight causal agent and the only the decline symptoms and the xylem plugging are definitive symptoms. Blight specific proteins also are used for diagnosis. However, they have not been used successfully in a presymptomatic diagnosis.

Pathogenicity tests (Koch's postulates) are difficult to run since the disease is only found in mature field trees and the inoculation of healthy trees in areas where the disease occurs is difficult since the determination of what constitutes a healthy tree is questionable. Because of this, pathogenicity tests were done using trees propagated from shoot grafted plant materials. Recently, trees on Swingle citrumelo rootstock have been observed to decline from blight at 4-5 yr of age. This may be a good rootstock to use for pathogenicity experiments.

The filamentous particles found in this study are similar to those described previously (3, 11, 18, 24). Particles 6-9 nm in diameter and of variable lengths were found in both CTV- infected and healthy plants. All of the plant materials used was from greenhouse grown plants infected with CTV via grafting. Bar Joseph and Lobenstein (3) and Kitajima et al. (18) commented that the particles that they found had no helical structure or internal detail. Kitajima et al. (18) commented that the particle could be a normal host component or a latent virus which could mislead or be confused with other citrus viruses. Esau (16) showed in sectioned materials the association and difference between P-protein and virus particles in cells infected with *Tobacco mosaic virus* (TMV) and

*Beet yellows virus* (BYV). P-protein often appears as tubules and in cross section the lumen of the particles does not stain. Granules have been found associated with P-protein, but its nature is unknown. Esau stated that it is difficult to distinguish between P-protein and X-tubules when seen in different cells. Esau reported the size of P-protein to be 23 nm in width while X-tubules were 28 nm. The filamentous particles that we report are 10 nm in width.

More work is needed on this filamentous particle and its relationship to citrus, citrus blight and to other citrus diseases. Work must in any case continue on citrus blight to identify the causal agent since specific diagnostics and control methods will only be successful if this is accomplished. As research continues in various aspects of plant and animal diseases we should be open to the discovery of new pathogens and new structures that are found in plant tissues.

## ACKNOWLEDGMENT

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