Ultrastructure of Citrus Chlorotic Dwarf-Infected Leaves and Bark

D. S. Howd, J. S. Hartung, and R. H. Brlansky

ABSTRACT. Citrus chlorotic dwarf (CCD) is a virus-like disease discovered in Turkey in the mid 1980s. Infected plant material was sent to the USDA, ARS exotic citrus quarantine facility in Beltsville, MD and was grafted into *Citrus macrophylla* seedlings. Symptoms of CCD include various chlorotic patterns and leaf crinkling or other types of distortion. Leaves are small and often have a notch near the apex. Leaves showing symptoms of chlorosis, leaf crinkling and distortion, and bark from the symptomatic shoots were collected and prepared for ultrathin sectioning and transmission electron microscopy. In sections of tissue, high numbers of ribosomes and endoplasmic reticulum (ER), especially rough ER, were observed in both leaf and bark tissues. Chloroplasts showed partial to complete breakdown of the grana in all tissues and very little starch was observed. Cellular membranes and cell walls remained intact and most cell contents were recognizable. In bark sections, there were numerous membrane-bound vesicles and some phloem cells were found to contain an unknown filamentous material, which appeared virus-like.

In the mid-1990s, a new disease of citrus was discovered in Turkey a few years after the introduction of the bayberry whitefly (*Parabemesia myricae* (Kuwana)) (8, 9, 10). Symptoms included various chlorotic patterns, leaf crinkling, and other types of leaf distortion. All cultivars of citrus are susceptible. The disease has been transmitted in the greenhouse by the bayberry whitefly and by grafting. Although symptomatology and transmission results suggest that the causal agent of this disease may be a virus, attempts to purify a virus particle have been unsuccessful. Infected leaf and young bark were examined using transmission electron microscopy in an attempt to identify possible virus particles and to examine the effects of the disease at the cellular level.

Fully expanded leaves and bark from CCD-infected *Citrus macrophylla* (Webster), Beltsville isolate B332, maintained in the exotic citrus pathogen collection at the USDA, ARS quarantine facility in Beltsville, MD, as well as fully expanded leaves and bark from healthy *C. macrophylla*, were fixed with 3% glutaraldehyde in 0.066 M phosphate buffer pH 6.8, post-fixed with 2% osmium tetroxide in the same buffer, dehydrated in a 25-100% acetone series and embedded in Spurr's resin. Tissue was sectioned on a Reichert Ultracut E microtome and stained with uranyl acetate and Reynolds lead citrate. Sections were examined using a Philips 201 transmission electron microscope.

Healthy *C. macrophylla* leaf (Fig. 1A) and bark tissue (Fig. 1B) contained numerous chloroplasts with well developed grana, large starch granules and osmiophilic globules. Organelles were arranged around a central vacuole. Ribosomes were few in number and there was little endoplasmic reticulum (ER).

CCD-infected *C. macrophylla* leaf (Fig. 1 C, E) and bark tissue (Fig. 1 D, F) showed changes in the chloroplasts, which contained no starch and very few small osmiophilic globules. Some chloroplasts within a cell had normal grana, while others showed loss of structure and some breakdown of their contents although their membranes remained intact. Large numbers of ribosomes were present and the ER, especially the rough ER, was more highly developed than in the healthy controls. There was more P-protein found in infected cells than in healthy cells. Some cells in CCD-infected bark tissue, but not leaf tissue, had membrane-bound vesicles

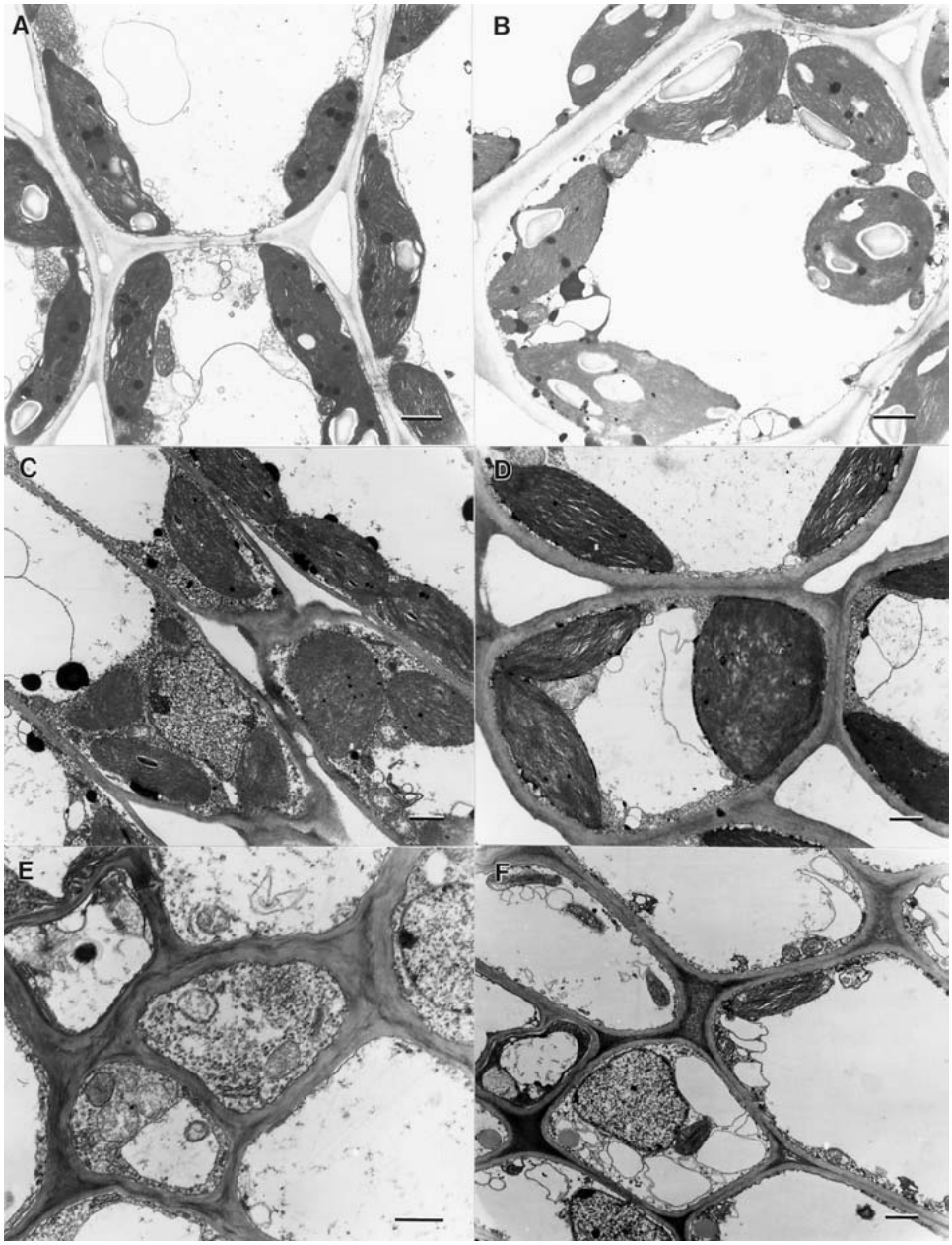

Fig. 1. Transmission electron micrographs of *Citrus macrophylla* tissue, either healthy (A, B) or citrus chlorotic dwarf-infected (C-F). Leaf palisade parenchyma (A), bark parenchyma (B), leaf palisade (C), bark parenchyma (D), spongy parenchyma (E), and phloem parenchyma (F). Bar = 1 µm.

not found in healthy bark. The vesicles appeared to originate from the tonoplast. Healthy and CCD-infected tissue were similar in mitochondrial numbers and structure. Some phloem cells from infected tissue contained masses of dark staining filamentous virus-like particles (Fig. 2), round in cross section (Fig. 3) that were not seen in healthy phloem.

When this work began, it was assumed that the causal agent was

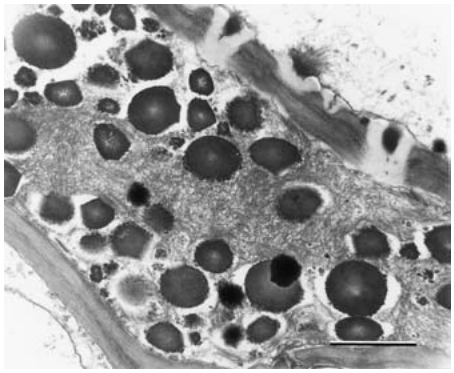

Fig. 2. Phloem parenchyma cells in citrus chlorotic dwarf-infected *Citrus macrophylla* containing filamentous virus-like particles. Bar = 1 µm.

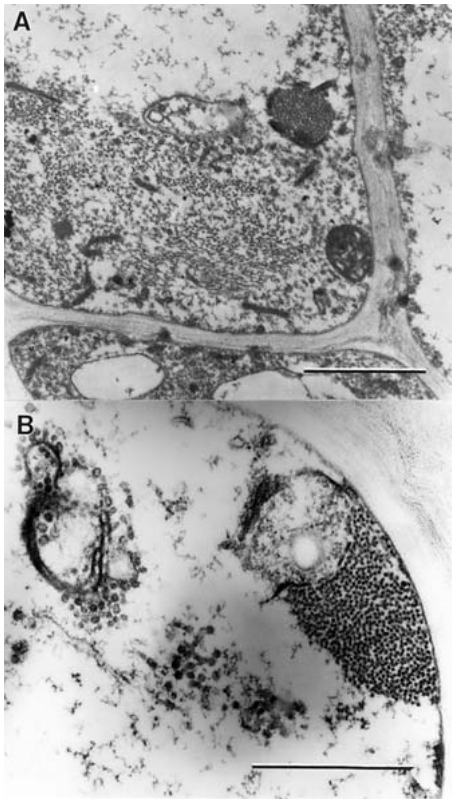

Fig. 3. Phloem parenchyma cells in citrus chlorotic dwarf-infected *Citrus macrophylla* containing filamentous virus-like particles in cross section. Bar = 1 µm.

most probably an isometric virus (S. M. Garnsey, pers. comm.), but to date, none have been seen in sections of CCD-infected tissue. However, masses of filamentous particles with virus-like appearance were seen in both leaf and bark phloem tissue. These particles appeared as fibrous masses such as often described for many viruses (11).

Both diseased and healthy plant tissues often contain material referred to as P-protein. Esau (5) showed P-protein in ultrathin sections as individual filaments or as bodies referred to as slime bodies. P-protein filaments may be confused with filamentous virus particles due to their size and aggregation in the phloem. Esau (5) pointed out the association and the difference between P-protein and virus particles in cells infected with *Beet yellows virus* (BYV) and *Tobacco mosaic virus* (TMV). Often in cross section, P-protein tubules have an unstained core, whereas of BYV and TMV particles are generally thinner in diameter and have cores that stain. Esau showed the difference between P-protein and BYV (her figure 79, page 158) where the former is distinctly wider in diameter than the BYV particles and appears more tubular in shape. BYV virions have been found to be 13 nm in diameter while P-protein reported by Esau (5) as 23 nm in diameter. Other structures described by Esau as similar to plant viruses are the X-tubules associated with TMV infection. Esau stated that it is often difficult to distinguish between P-protein and X-tubules when seen in different cells. Esau reported the size of X-tubules as 28 nm in diameter.

However in some electron micrographs, P-protein and virions may be difficult to distinguish, as P-protein is found in different forms. In four species of legumes Wergin et al. (15) studied the structure and development of P-protein in the phloem parenchyma and companion cells. Tubular P-protein was the most commonly encountered component in phloem sieve elements, but was also present in the phloem paren-

chyma cells. The P-protein components found by Wergin et al. (15) were granular, fibrillar, and crystalline structures in parenchyma cells of all the species studied and in the companion cells of *Melilotus alba*.

Various types of viral inclusion bodies may occur in virus infected plants (3, 4, 11). Infected phloem and phloem parenchyma cells have been found to contain crystalline and paracrystalline virus inclusion bodies that in some instances may resemble crystalline or fibrillar P-protein.

Typically, virus-infected plant tissues show an increase in starch (1, 6, 7, 12, 14) and osmiophilic globules (6, 13, 14). In CCD-infected tissue we found a marked decrease in starch and only a few small osmiophilic globules. Starch content usually decreases as necrosis progresses in viral local lesions (1), but there were no signs of necrosis in the CCD-infected tissues.

According to Lesemann (11), no one organelle is believed to be specifically involved in the cytopathology of infection by any filamentous virus; however, the most conspicuous alteration is often the proliferation of ER, which may be significant in viral synthesis. Increases in the amount of ER, both rough and smooth, and increased populations of ribosomes, have been reported for infections involving widely different viruses (1, 2, 6, 7, 11, 12, 13, 14), and usually indicate the presence of abnormally active metabolism. In our study, we found higher numbers of ER as well as ribosomes.

The changes we report suggest infection by a virus, possibly a filamentous virus. Purification of a virus from CCD-infected materials and proof of pathogenicity will determine if the filamentous structures seen here are the causal agent of CCD. This will enable further studies on the effect of this presumed virus on various citrus tissues.

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