

# Citrus Vein Enation Virus, a Probable Luteovirus

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**ABSTRACT.** Citrus vein enation virus is persistently transmitted by aphids. Isometric virus-like particles have been observed in the phloem of infected rough lemon seedlings and in the hindgut and accessory salivary glands of viruliferous aphids. Small numbers of 28-nm isometric particles were purified from excised enations and in one case the presence of a 33 kd protein was observed. Double-stranded RNA analysis of infected bark showed four bands ranging from  $3.6 \times 10^6$  to  $0.5 \times 10^6$  daltons. Luteovirus is the only plant virus group with all the above characteristics, and it is suggested that CVEV be provisionally classified as a member of this group.

Citrus vein enation/woody gall (CVEV) has been known for well over thirty years (5, 17). CVEV can be transmitted by aphids (8, 12), however only little information was known about the etiology of the disease.

Electron microscope work in this laboratory in 1986 showed the presence of virus-like particles in the phloem of CVEV-infected rough lemon (14). Subsequently, we demonstrated that the concentrations of these particles were highest in the enations, and that similar particles could be found in the hindgut and in the accessory salivary glands of viruliferous aphids (*Toxoptera citricidus* Kirk.) (10). We also showed that CVEV is a persistently transmitted virus, with a latent period of 2-3 days (11). These properties are common characteristics of the luteovirus group.

## MATERIALS AND METHODS

Citrus tristeza virus (CTV)-free rough lemon seedlings inoculated with CVEV using aphids (*T. citricidus*) were used as source plants for ds-RNA extraction and virus purification.

DsRNA extraction was done as described for CTV by Lee (9). Samples of 8-12 g young bark tissue were extracted and two extracts were pooled so that the dsRNA from 16-24 g was layered in a gel lane. Similar extracts from healthy rough lemon bark and avocado leaves (*Persea americana* cv. Fuerte) for dsRNA

standards were prepared at the same time. The samples were separated on 5% acrylamide gels run at 100v for 6 hr. Gels were stained with ethidium bromide, and photographed over a UV transilluminator with long exposure (up to 20 min) to detect faint bands. Extractions were carried out at various seasons of the year.

Two different approaches were used for virus purification. Assuming CVEV is in low concentrations, purification from 1 kg of young leaves was attempted, following the method of Takanami and Kubo (16) for tobacco necrotic dwarf virus and potato leafroll virus (PLRV). Because driselase was expensive, this method was tried initially without the enzyme, and then with a 1.125% pectinase Rohament P5/0.375% cellulase TC (Serva Feinbiochemica) mixture, which was considerably cheaper and was as effective as driselase for PLRV purification (13).

In a second approach, extractions were made from 1-2 g excised enations, since higher concentrations of particles had been observed there (10). The method, termed the clarified virus concentrate (CVC) procedure was developed by S. R. Christie, D. E. Purcifull, W. E. Crawford, and N. A. Ahmed (unpublished information) to purify viruses from small amounts of tissue. It was tried without enzymes, and with both driselase (Sigma Chemical Co.) and the pectinase/cellulase mixture.

In all extractions, the tissue was ground in liquid nitrogen and then macerated in 0.1M phosphate buffer,

pH 7.5. Enzyme treatment was done at this stage with 2% driselase for 2 hr at 28 C, or pectinase/cellulase for 16 hr at 28 C (18). The extracts were then expressed through cheesecloth, clarified with an equal volume of chloroform:butanol (1:1) and then spun at 10,000g for 10 min. Polyethylene glycol was then added to a final concentration of 6%, and after it had dissolved the mixture was centrifuged at 10,000g for 20 min. For the CVC procedure, all centrifugations were carried out in a microfuge. The pellets were resuspended in a small volume of 0.01M phosphate buffer and clarified by low speed centrifugation. Drops of supernatant were placed on formvar-coated grids, negative stained with 1% potassium

phosphotungstate, pH 7.5, and viewed in a JEOL 100CX electron microscope.

When virus-like particles were observed, the extract was analysed for proteins by SDS-PAGE, using an extract of healthy tissue as a control and standard molecular weight markers (Sigma Chemical Co.).

## RESULTS

No dsRNA bands from CVEV-infected tissue were observed when the gels were examined on the transilluminator. However when the gels were photographed with 20-min exposure faint bands were observed with molecular weights of 3.6, 2.6, 1.9 and  $0.5 \times 10^6$  daltons (Fig. 1). These bands were only detected in samples collected in the autumn. Attempts to prove their double-stranded nature were unsuccessful due to their low concentrations.

The only virus-like particles observed were in extracts of excised enations. Low numbers of 28-nm isometric particles were observed irrespective of whether enzymes were used (Fig. 2). SDS-PAGE analysis of one such extract showed the presence of a 33 kda protein which was absent in the extract of healthy tissue (Fig.3).

## DISCUSSION

Phloem-limited viruses are difficult to purify and characterise, especially from perennial plants because of their low virus titres. Dale *et al.* (3) have identified the agent of banana bunchy top (BBT) disease as a luteovirus on the basis of its persistent transmission by aphids, and the dsRNA pattern. Dale (2) reported the presence of low numbers of isometric particles in extracts.

The dsRNA pattern and sizes found in CVEV-infected citrus are similar to those of known members of the luteovirus group. A genomic dsRNA of  $3.6 \times 10^6$  has been reported for beet western yellows virus (BWYV) strains STFL and ST9 (4) and for some strains of barley yellow

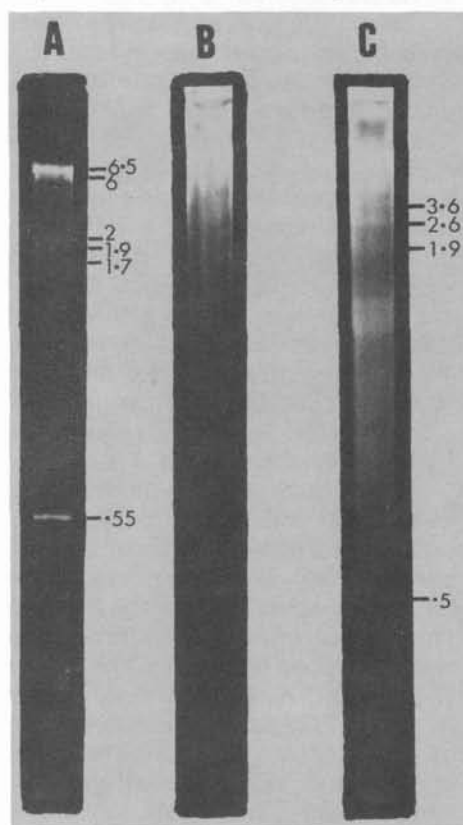


Fig. 1. A 5% polyacrylamide gel stained with ethidium bromide showing dsRNA extracts of: A) Fuerte avocado leaves (as molecular weight standards), B) healthy rough lemon bark, and C) citrus vein enation-infected bark. Molecular weights are shown in kdaltons.

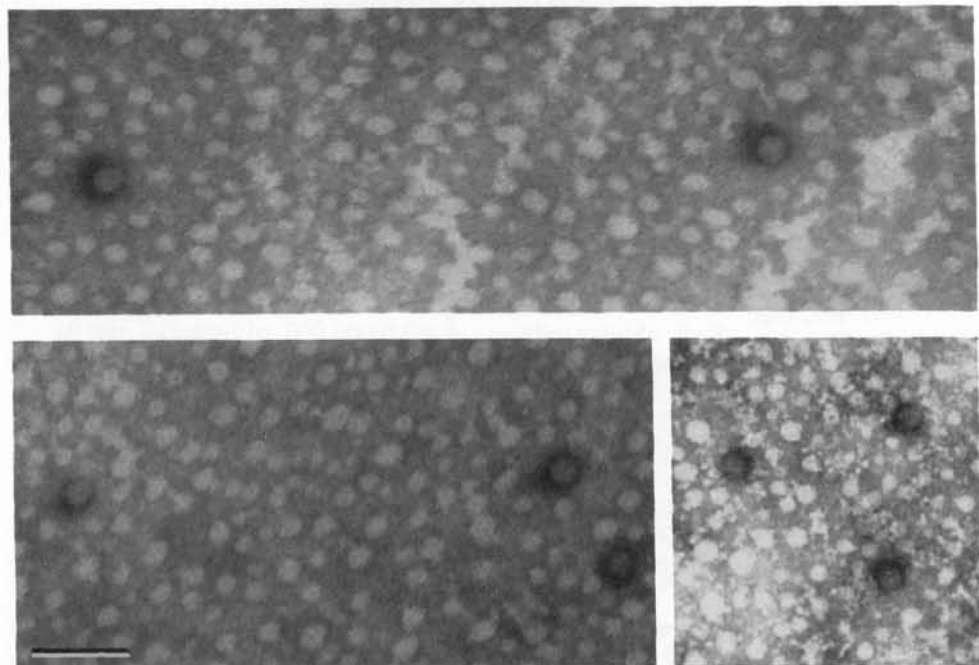


Fig. 2. Isometric particles isolated from excised enations of CVEV-infected rough lemon leaves. Bar scale = 100nm.

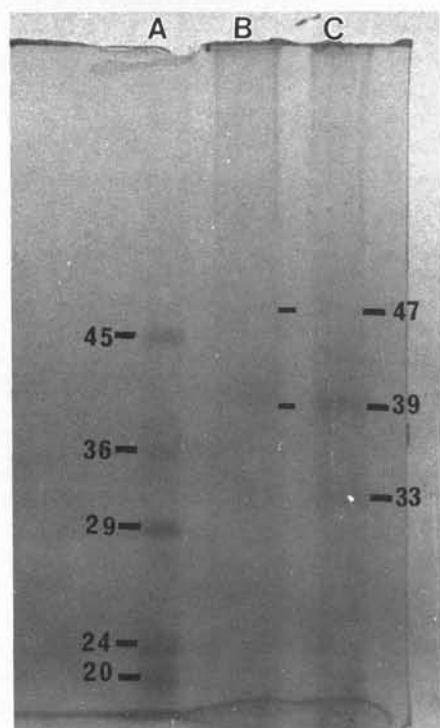


Fig. 3. SDS-PAGE analysis of proteins in healthy (lane B) and CVEV-infected (lane C) citrus leaf extracts. Molecular weight markers are in lane A. Molecular weights given in kdaltons.

dwarf (BYDV) (6). BWYV-STFL possesses only two dsRNA bands and BYDV strains MAV, PAV and SGV have five. However, BWYV-ST9 has four of sizes 3.6, 2.2, 1.4 and 0.46 x 10<sup>6</sup> daltons, similar to the pattern found in CVEV. BBTV also has four dsRNA species associated with it, however the sizes range from 4.4 to 0.48 x 10<sup>6</sup> daltons.

The difficulty in isolating sufficient BBTV (2) for analysis is similar to the problems encountered in this study. There was an indication that the molecular weight of the capsid protein may be 33 kda which is larger than the common 26 kd of most luteoviruses (5, 15, 19). However pea leafroll virus has been reported to possess a 35-kd capsid protein (1).

The cumulative data on CVEV, from its aphid transmission, the presence of 28 nm particles in the phloem and in viruliferous aphids, to the presence four dsRNA bands and the isolation of small isometric particles, indicate that CVEV can be provisionally classified as a member of the luteovirus group.

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