Preliminary Purification and Double Stranded RNA Analysis of Citrus Leprosis Virus

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ABSTRACT. Partial purification of citrus leprosis virus (CiLV) from local lesions of Cleopatra mandarin was achieved using an extraction buffer of low molarity, viral precipitation with PEG-NaCl, and ultra-centrifugation in a 10 to 50% linear cesium sulfate gradient. Most of the virions disintegrated during the processing, but a limited number of preserved particles, about 45 to 50 × 80 to 120 nm were seen under the electron microscope. Four main bands of dsRNAs were extracted from CiLV local lesions of *Chenopodium quinoa* leaves infected with the Cleopatra or sweet orange isolates, and separated by PAGE. Similar dsRNAs were also extracted from local lesions on field infected leaves of sweet orange and analyzed the same way. Values of 6 to 8 MDa were estimated. In all cases, the dsRNA bands were absent in tissue extracts from leaves not infected with CiLV.

Citrus leprosis virus (CiLV), as far as we know, causes only local lesions but no systemic infection on susceptible plants, and is unstable in plant sap preparations. As a consequence, the virus is difficult to purify (8), and that has hampered investigations on its properties and on the production of antisera, that would be extremely useful for diagnosis and comparison with other similar viruses.

Evidence has been obtained that CiLV can be mechanically transmitted from sweet orange and Cleopatra mandarin to herbaceous hosts and, with some difficulty, from sweet orange to Caipira sweet orange (2, 7, 8). Bacilliform virus particles have been seen in thin sections of naturally and experimentally infected sweet orange plants (2, 8), and in experimentally infected *Chenopodium quinoa* leaves (7).

New investigations on purification and on dsRNAs extracted from CiLV-infected plants are reported in this paper.

MATERIALS AND METHODS

The isolate of CiLV used for these investigations was from naturally infected Cleopatra mandarin in Limeira, São Paulo State (Brazil). About 20 g of local lesion tissue from Cleopatra mandarin leaves were pulverized with liquid nitrogen, homogenized in presence of extraction buffer (0.05 M phosphate buffer

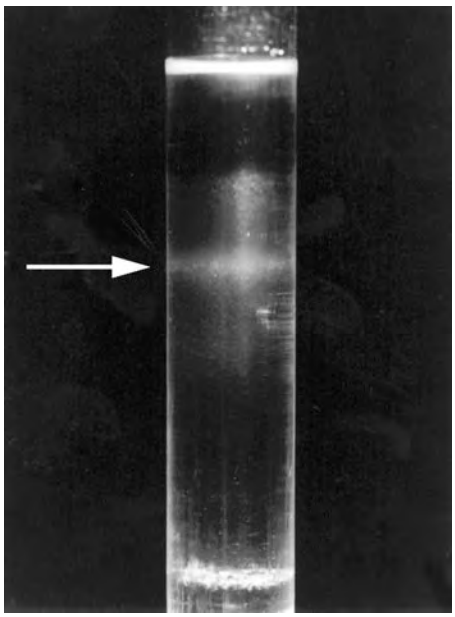

Fig. 1. Light-scattering band (arrow) formed after ultra-centrifugation in a cesium sulfate linear gradient of PEG-precipitated extract from local lesions of CiLV-infected Cleopatra mandarin leaves.

pH 7.0 containing 0.01M Na DIECA, 0.1% ascorbic acid and 0.02 M Na sulfite). The extract was filtered through cheesecloth and centrifuged at low speed. CiLV was precipitated once or twice with 6% PEG-0.5% NaCl. The resulting pellet was resuspended in the same buffer, and centrifuged at 10,000 g for 20 min. The supernatant with the virus suspension was loaded on a 10 to 50% cesium sulfate linear gradient and centrifuged at 150,000 g for 4 h in a Sorvall TH 641 swing in bucket rotor. The light-scattering bands observed were withdrawn, and the virus was recovered by dilution, then centrifugated at 196,000 g for 2 h.

A similar preliminary procedure was used with a CiLV isolate from Cleopatra mandarin multiplied in *C. quinoa*, up to the PEG precipitation step.

Samples taken at various steps during the purification process were examined by electron microscopy after negative staining with 2% uranyl acetate. Other aliquots were tested for infectivity by inoculation on *C. quinoa*.

The dsRNA analysis was done either from extracts of local lesions from Pera sweet orange leaves, collected in Bebedouro, Brazil or from local lesions from *C. quinoa* leaves. The *C. quinoa* plants had been inoculated with CiLV from either the Cleopatra or sweet orange isolate. CiLV dsRNA was extracted by routine methods (4), involving disruption of the local necrotic lesions in liquid nitrogen. From 10 to 20 g of local lesion tissue was used. Molecular weight estimates of the CiLV-associated dsRNA bands were based on the values of the tobacco mosaic

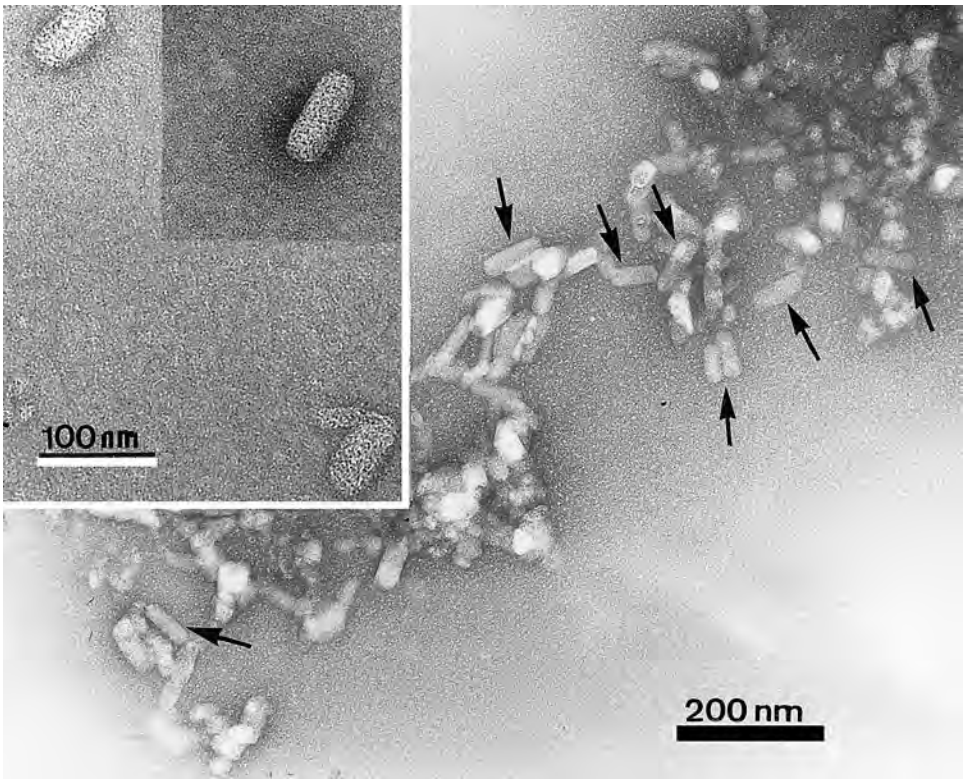

Fig. 2. Electron micrograph of negatively stained CiLV particles (arrows) in partially purified preparation from Cleopatra mandarin. Insert: magnified particles showing outer surface structure.

tobamovirus replicative dsRNAs (9) used as a marker in the first experiments (from sweet orange), and on the values of the dsRNA genome segments of maize rough dwarf fijivirus (1) in the second one (from *C. quinoa* and Cleopatra mandarin).

Most of the investigations with CiLV in Cleopatra mandarin and sweet oranges were done in São Paulo, while the ones with CiLV in *C. quinoa* were done in Torino, Italy. This is because the winter temperature of São Paulo was not suitable for CiLV multiplication in *C. quinoa* (7), and in Torino the virus in *Citrus* spp was not available.

The investigations carried out in Torino were conducted under quarantine precautions with authorization from the Quarantine Service of the "Ministero per le Politiche Agricole".

RESULTS

Purification. After the cesium sulfate step, an opalescent band was formed 38 to 42 mm from the meniscus (Fig. 1). No bands were present in the control carried out with healthy tissues of Cleopatra mandarin. Samples taken before the cesium sulfate step and in the opalescent band contained short, irregularly bacilliform particles, similar to those found in tissues of infected leaves. These particles measured around 45 to 50 × 80 to 120 nm, and occasionally were thinner and longer. They tended to flatten, and were apparently unpenetrated by stain (Fig. 2). No infection on *C. quinoa* leaves was obtained with aliquot of the same samples taken during purification steps.

In preliminary purification trials with infected *C. quinoa* leaves, which were used fresh or following storage at -80°C, no virus particles were seen in the electron microscope, but typical local lesions were produced in *C. quinoa*-inoculated with samples taken after PEG precipitation. No infection was detected in samples purified from Cleopatra mandarin leaves.

DsRNA analysis. Four major dsRNA bands not observed in healthy controls were consistently found in extracts infected with CiLV-*C. quinoa* leaves (Fig. 3). From naturally-infected Pera sweet orange leaves (Fig. 4), the major bands were apparently there, but the first was

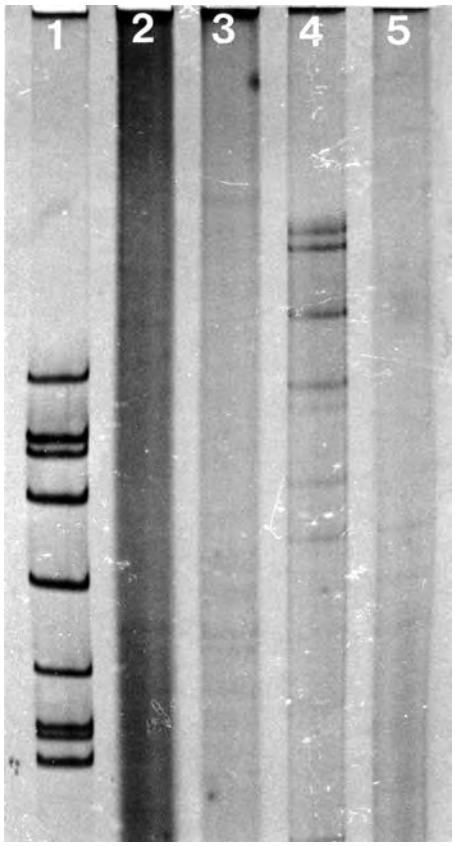

Fig. 3. DsRNA bands observed following polyacrylamide gel electrophoresis of extracts from CiLV-infected and healthy *Chenopodium quinoa* and Cleopatra mandarin leaves: lane 1, ds RNA components of maize rough dwarf fijivirus. The estimated molecular weight of MRDV genomic RNAs are, in order of decreasing mobilities, 2.57, 2.22, 2.22, 2.14, 1.93, 1.60, 1.25, 1.09, 1.04, and 1.00 × 10^6; lane 2, extract of healthy Cleopatra; lane 3, extract of CiLV-infected Cleopatra; lane 4, extract of CiLV-infected *C. quinoa*; and lane 5, extract of healthy *C. quinoa*.

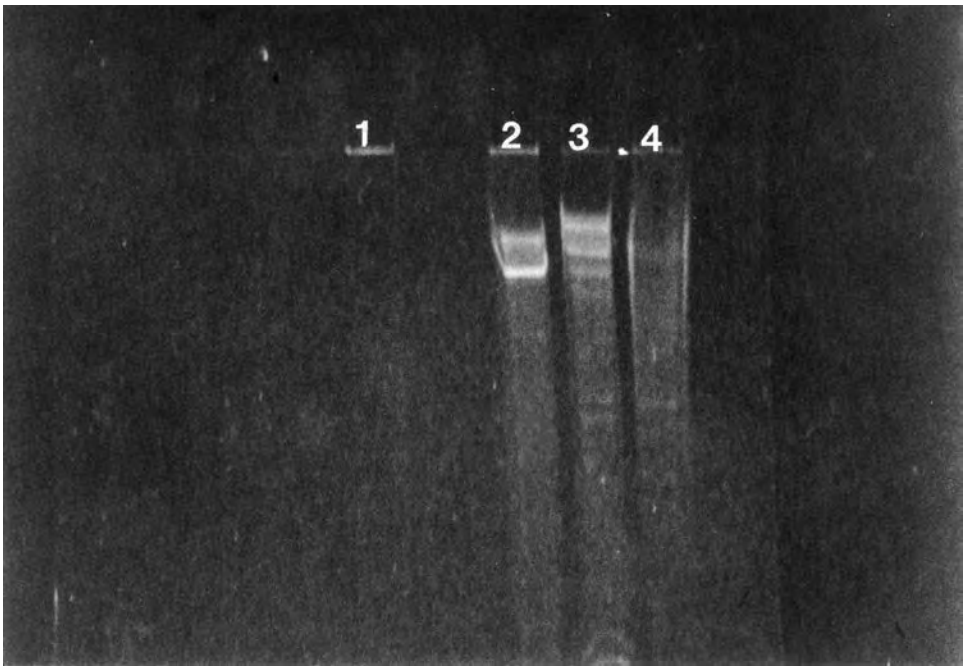

Fig. 4. DsRNA bands observed following polyacrylamide gel electrophoresis of extracts from CiLV field infected and healthy Pera sweet orange leaves: lane 1, extract from healthy tobacco; lane 2, tobacco mosaic tobamovirus replicative form. The estimated molecular weight of TMV replicative RNAs are, in order of decreasing mobilities, 4.00 and 2.25 × 106; lane 3, extract from CiLV-infected Pera sweet orange; and lane 4, extract from healthy tissues of the same Pera sweet orange.

probably a doublet formed by the first two bands as seen from *C. quinoa*. No bands were seen in the extract from Cleopatra leaves collected at Limeira during winter months of 1997. The standards used did not allow accurate determination of the sizes of the CiLV dsRNAs, but values of 6 to 8 MDa were estimated.

DISCUSSION

Investigations on CiLV-infected *C. quinoa* dsRNA were done with virus multiplied under controlled conditions, whereas trials with Pera sweet orange were on field infected leaves. The dsRNAs found in symptomatic sweet orange tissues are likely to be those of CiLV because they were not present in symptomless tissues coming from the same plants and they were similar to those found in experimentally infected *C. quinoa*. The dsRNAs bands of citrus tristeza virus, which could have been present in Pera sweet orange plants, have, however, different mobilities than those found (3).

Data on dsRNA of Rhabdo and Rhabdolike viruses are not available, as far as we know. The values estimated for CiLV dsRNAs suggest that the corresponding ssRNA could have molecular weight similar to those of the Rhabdoviruses, which range from 4.0 to 4.6 MDa.

Partially purified preparations contained short, bacilliform particles, similar to those observed in thin sections. Such particles were not detected in comparable healthy tissues which suggests that they represent CiLV virions.

CiLV particles were apparently unpenetrated by the negative stain, which made it impossible to visualize their inner structure. This might

be connected with the purification method, and should be further investigated.

The lack of infectivity of partially purified preparations from field-infected Cleopatra mandarin leaves is probably due to the very low concentration of CiLV, and the poor multiplication of CiLV in *C. quinoa* during winter. Partially purified preparations from *C. quinoa* leaves were infective because these experiments were done under optimal conditions for CiLV multiplication (7).

CiLV particles are probably less stable than those of orchid fleck virus (OFV), at least when in plant sap extracts. The purification method previously used for CiLV (8) which mainly followed a protocol modified from Doi et al. (5) for OFV was unsuitable for CiLV, probably because of the high molarity of the extraction buffer. From recent investigations it became more clear that CiLV and OFV are quite different viruses (6, 7).

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