OTHER VIRUSES

Partial Characterization of Citrus Leprosis Virus

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ABSTRACT. Citrus leprosis virus (CiLV) was mechanically transmitted to the following nine plant species which all developed necrotic local lesions: sweet orange, *Chenopodium amaranticolor, C. album, C. capitatum, C. foliosum, C. murale, C. polyspermum, C. quinoa* and *Gomphrena globosa*. Mechanical transmission was improved by growing test plants at temperatures above 25°C. Two isolates, one from sweet orange and the other from Cleopatra mandarin, were indistinguishable and were readily transmitted to *C. quinoa*, but could not be transmitted back to citrus from this host. In crude *C. quinoa* sap in PDET buffer, CiLV had a thermal inactivation point of 55-60°C, a dilution end point of 10^{-3} and a longevity *in vitro* at 4°C of 6 days. Attempts to purify CiLV from field citrus samples were unsuccessful. However, PEG-concentrated preparations contained a 25 kDa protein which was absent in extracts of healthy tissue from the same tree. In thin sections of infected sweet orange and Cleopatra mandarin leaves and young bark, bacilliform virus-like particles were detected in enclaves of the endoplasmic reticulum. Rounded structures were occasionally found in the perinuclear space of sweet orange leaf cells. These results support the view that CiLV may be a naked rhabdovirus.

Citrus leprosis virus (CiLV) causes local chlorotic and necrotic lesions on the leaves, branches and fruit of sweet orange (7). Other citrus can also be affected but less severely. It is the only known citrus virus that does not cause a systemic infection, but the local lesions on sweet orange are sometimes numerous and large and cause severe damage. CiLV is transmitted by mites of the genus *Brevipalpus* (15). Recently the virus was mechanically transmitted from sweet orange leaves, fruit peel and young bark to Caipira sweet orange, *Chenopodium amaranticolor, C. quinoa* and *Gomphrena globosa*, using liquid nitrogen, protective buffers and charcoal (3). Kitajima et al. (10) observed rhabdovirus-like particles measuring 100 to 120 × 40 nm in infected sweet orange leaf tissue, some of which appeared to have budded through the nuclear membrane. Recently, however, examination of ultrathin sections of mechanically inoculated citrus, revealed particles resembling non-enveloped rhabdoviruses measuring 120 to 130 × 50 to 55 nm in membranous vesicles of the endoplasmic reticulum (3).

The history of leprosis investigations has recently been reviewed by Rossetti (15).

The present paper presents results on ecological and epidemiological observations, mechanical transmission, host range, *in vitro* properties, further electron microscopy and attempted purification of CiLV.

MATERIALS AND METHODS

Ecological and epidemiological observations. Citrus and non-citrus plants with leprosis-like symptoms and plants which are hosts of the mite vector were examined and tested. The plants were from Alfenas, Limeira, São Paulo and Brasilia. Samples from leaves of *Camellia japonica* and *Magnolia arbustifolia* showing leprosis-like symptoms and growing near CiLV infected citrus in Limeira were mechanically inoculated onto the

same test plants used for CiLV. Other plant species (see Results) were similarly tested.

Mechanical transmission. CiLV-infected sweet orange (Pera, Pera-Rio, Natal, Bahianinha, Valencia and Valencia trepadeira) leaf samples were collected from Bebedouro, Colina and Limeira in São Paulo state, and Alfenas in Minas Gerais. Infected leaves were also collected from a Cleopatra mandarin tree (Fig. 1) in a nursery at Limeira.

C. quinoa was mainly used as the assay host, and inoculated plants were maintained in a growth chamber at temperatures above and below 25°C to determine the optimum conditions for symptom development.

Seventy-three plant species in 29 families, (see Tables 1 and 2) were inoculated. Both positive and negative infections were checked by back inoculation to *C. quinoa*. Extractions were done by grinding one part of tissue in four parts (w/v) TACM (0.05 M Tris, 0.1% ascorbic acid, 0.1% L-cysteine, 0.5% 2-mercaptoethanol, pH adjusted to 8.0 with HCl) or PDET buffer (0.05 M phosphate buffer pH 7, containing 0.005 M Na-DIECA, 0.001 M Na-EDTA and 0.005 M Na-thioglycollate) in the presence of activated charcoal. Test plants were dusted with carborundum prior to inoculation.

Thermal inactivation point (TIP) and longevity *in vitro* (LIV) at 4°C were determined with crude infected sap obtained by crushing one part symptomatic *C. quinoa* leaf tissue in four parts PDET (w/v) containing 0.05% charcoal at 4°C. PDET was used to dilute the sap for dilution end point (DEP).

Purification. Methods used to purify plant rhabdoviruses or other unstable viruses were attempted: (i) a protocol modified from Doi et al. (6) for orchid fleck virus extraction and that of Hsu and Black (8) for the concentration of potato yellow dwarf virus; (ii) the nucleocapsid purification protocol for tomato spotted wilt virus of de Avila et al. (4), modified for soursop yellow blotch virus nucleocapsid purification (Martin, de Avila and Kitajima, unpublished data); (iii) the protocol of Derrick et al. (5) for citrus ringspot virus; and (iv) the minipurification protocol described by Lane (12). The tissue used was symptomatic Pera sweet orange and Cleopatra mandarin leaf tissue, selecting the less necrotic areas. Citrus leaves without symptoms were used as controls. Infectivity of extracts was tested by inoculation to *C. quinoa*. The details of method (i) were as follows. Local lesions from citrus leaves collected in the field were pulverized in liquid nitrogen, and then homogenized with 0.1 M phosphate buffer (pH 7 containing 0.1 M DIECA, 0.1% L-ascorbic acid, 0.5% Na sulfite and 5% Triton X-100) at a rate of 1:4 (w/v). The homogenate was filtered through cheesecloth and centrifuged for 10 min at 12,000 *g*. For concentration, 10% PEG 6,000 and 0.1% NaCl were added to the supernatant, stirred for 2 hr at 4°C and then centrifuged for 5 min at 8,000 *g*. The resultant pellet was resuspended in 0.1 M phosphate buffer (pH 7) and centrifuged for 5 min at 8,000 *g*. The supernatant was then centrifuged at 20,000 *g* for 1 hr and the pellets were resuspended in 1 ml 0.1 M phosphate buffer. For controls, extracts from leaves without symptoms from the same tree were similarly extracted.

PAGE and Western blots. SDS-PAGE was done with partially purified preparations and crude preparations from infected and healthy citrus field leaves. Fifty µl of each sample were mixed with an equal volume of 2.5 mM Tris-HCl pH 6.8, 8% SDS, 4% 2-mercaptoethanol and bromophenol blue as marker, and heated at 100°C for 5 min. Aliquots of 10 µl were run in a discontinuous system (11). Gels were silver stained for protein bands.

Gels were blotted to nitrocellulose membranes using Trans-Blot SD (Bio Rad) according to manufacturer's instructions. The membranes were incubated in TBS (50 mM Tris, pH 7.5, 0.15 M NaCl, 0.05% Tween 20) containing 5% dry milk powder, washed in TBS and incubated overnight in TBS containing various rhabdovirus antisera, diluted 1/20,000. Antisera included barley yellow striate mosaic virus, lettuce necrotic yellows virus, maize mosaic virus (type isolate and Brazilian isolate), potato yellow dwarf virus, Sonchus yellow net virus, Annona muricata rhabdovirus and sowthistle yellow vein virus.

The membranes were washed and incubated for 6 hr in TBS containing 0.5% bovine serum albumin with anti-rabbit IgG-alkaline phosphatase diluted 1/15,000. For visualization of the reaction, membranes were incubated in 100 mM Tris-HCl pH 9.5; 100 mM NaCl; 5 mM $MgCl_2$, containing 330 µg/ml 5-bromo 4-chloro 3-indolyl phosphate and 165 µg/ml nitro blue tetrazolium.

Electron microscopy. Ultra thin sections were prepared as previously described (2). Samples were taken from naturally infected leaves, fruit peel and young bark lesions of Pera sweet orange, leaves of Cleopatra mandarin, and from experimentally infected leaves of *C. amaranticolor*. Healthy leaf tissues from the two citrus hosts were used as controls.

Pieces of leaves with leprosis lesions and healthy leaf tissue of both citrus species and leaf lesions of *C. amaranticolor, C. quinoa, C. polyspermum* and *Gomphrena globosa* were ground in 0.1 M phosphate buffer pH 7, containing 0.3% sucrose and 1% glutaraldehyde, and from this extract, preparations for electron microscopy were made as previously described (3). Sweet orange leaf pieces, fruit peel, young bark lesions and leaf lesions of *C. amaranticolor* were also pulverized in liquid nitrogen and the powder collected in drops of TACM or PDET buffers and prepared for electron microscopy. Preparations were examined in a Philips EM 300 or an EM 210 electron microscope.

RESULTS

Ecological and epidemiological observations. Severe symptoms were found on Cleopatra mandarin, a previously unrecorded host. The symptoms occurred mainly on leaves as large chlorotic areas (Fig. 1), often coalescent, sometimes causing the entire leaf to be chlorotic. Necrotic symptoms were rare, twigs were only occasionally symptomatic, but no fruit symptoms were observed. Nearby sweet orange trees had characteristic leprosis symptoms, but a nearby pummelo tree and a rutaceous plant, *Balfourodendron riedelianum,* had no disease symptoms. Although *Camellia japonica, Magnolia arbustifolia, Palicourea rigida, Pera glabrata* and *Aspidosperma macrocarpum* had leprosis-like symptoms, no transmission from them to herbaceous hosts of CiLV could be accomplished.

Mechanical transmission and host range. Susceptible test plants did not exhibit symptoms at temperatures below 22°C. Best results were obtained in growth chambers at 30 to 32°C (day for 12 hr) and 24-25°C (night). Table 1 shows the plant families that had species which developed local lesions, namely one species of citrus (Rutaceae), seven species of *Chenopodium* (Chenopodiaceae) and *Gomphrena globosa* (Amaranthaceae). All were successfully back inoculated to *C. quinoa*. Table 2 shows the families that were tested but contained no host species of CiLV; the species tested were also negative when back-inoculated. *C. quinoa* was the most reliable indicator host, but back inoculation to citrus was not successful. Back inoculation from sweet orange to

Fig. 1. Field symptoms of CiLV on Cleopatra mandarin.

sweet orange, however, was successful.

The TIP for CiLV was 55-60°C, LIV was 6 days at 4°C and DEP was 10^{-3}. Infected leaves frozen at -80°C retained infectivity for approximately 4 mo., and for about 1 mo. held at -15°C. Dried leaves retained infectivity as did preparations in TACM buffer containing Triton X100.

Purification and protein analysis. None of the preparations obtained in attempts at purification were infectious, and no particles were detected in them by electron microscopy. With purification methods (i) and (ii) some protein was detected, especially with method (i). In SDS-PAGE, a protein of approximately 25 kDa was detected in a partially purified preparation of CiLV infected sweet orange and Cleopatra mandarin, and not in corresponding healthy preparations (Fig. 12). No reaction was obtained in Western blots with any of the antisera tested.

Electron microscopy. In ultrathin sections of infected Cleopatra mandarin leaves (Fig. 7) and Pera sweet orange bark (Fig. 8), bacilliform particles similar to those of non-enveloped rhabdoviruses were observed in enclaves of the endoplasmic reticulum of mesophyll and vascular parenchyma. The particles measured 120 to 130 × 50 to 55 nm, although some 300 nm-long particles were seen. Rounded particles, 55 nm in diameter, were common in the ER, and occasionally in the perinuclear spaces in sweet orange (Fig. 9). In sub-epidermal cells of young bark of sweet orange, viroplasms (Fig. 10) and vesicles associated with tonoplasts (Fig. 11) were seen. In Cleopatra mandarin, the former were rare and the latter absent. No particles were seen in lesions of any of the four herbaceous hosts examined, and no particles nor intracellular alterations were seen in healthy tissues.

DISCUSSION

Cleopatra mandarin is a new natural host for CiLV, but no others were found during this study. However, several *Chenopodium* spp. and *Gomphrena globosa* did develop local lesions, and *C. quinoa* proved to be

TABLE 1
RESULTS OF INOCULATING PLANT SPECIES BELONGING TO THE AMARANTHACEAE, CHENOPODIACEAE AND RUTACEAE WITH CITRUS LEPROSIS VIRUS

Plant species	Inoculation results[x]	Local lesion type[y] (days to appear)	Back inoculation to *C. quinoa*[x]
Amaranthaceae:			
Alternanthera brasiliana	-		-
Alternanthera tenella	-		-
Amaranthus spinosum	-		nd
Amaranthus viridis	-		nd
Celosia cristata	-		-
Gomphrena globosa	+	rb (7)	+
Chenopodiaceae:			
Chenopodium album	■	n (3)	+
Chenopodium amaranticolor	+	n (3)	+
Chenopodium ambrosioides	-		-
Chenopodium capitatum	■	n (5)	+
Chenopodium foliosum	■	n (5)	+
Chenopodium murale	+	v.small n (6)	+
Chenopodium polyspermum	■	rb, ch (15)	+
Chenopodium quinoa	+	n (3)	+
Chenopodium schraderanum	-		nd
Spinacia oleracea	-		nd
Rutaceae:			
Caipira sweet orange	*	bs, ch (17)	+
Madame Vinous sweet orange	-		-
Tarocco sweet orange	-		-
Lime	-		-
Sour orange	-		-
Rough lemon	-		-
Lemon	-		-
Cleopatra mandarin	-		nd
mandarin	-		-
Orlando tangelo	-		-
Dweet tangor	-		-
Citrus bigaradia	-		-
Esenbeckia grandiflora	-		-
Esenbeckia leiocarpa	-		-
Murraya exotica	-		-
Ruta graveolens	-		-
Skimmia reevesiana 'Rubella'	-		-

[x]+positive results obtained with inocula from citrus and/or *C. quinoa*.
*positive results obtained with inocula from sweet orange.
■positive results obtained with inocula from *C. quinoa* and sometimes also from the other herbaceaous hosts.
-negative results: no symptoms observed.
[y]bs = brown spot; ch = chlorotic halo; n = necrotic; rb = reddish brown
[x]nd = not done

an ideal assay plant, developing consistently clear lesions in 3 days. It was, therefore, used for the TIV, LIV and DEP determinations, and to assay for infectivity. The *in vitro* properties of CiLV are in line with those of the great majority of the rhabdoviruses, namely TIP of 50-60°C, LIV of 1-7 days at 4°C and DEP of 10^{-3} to 10^{-5}.

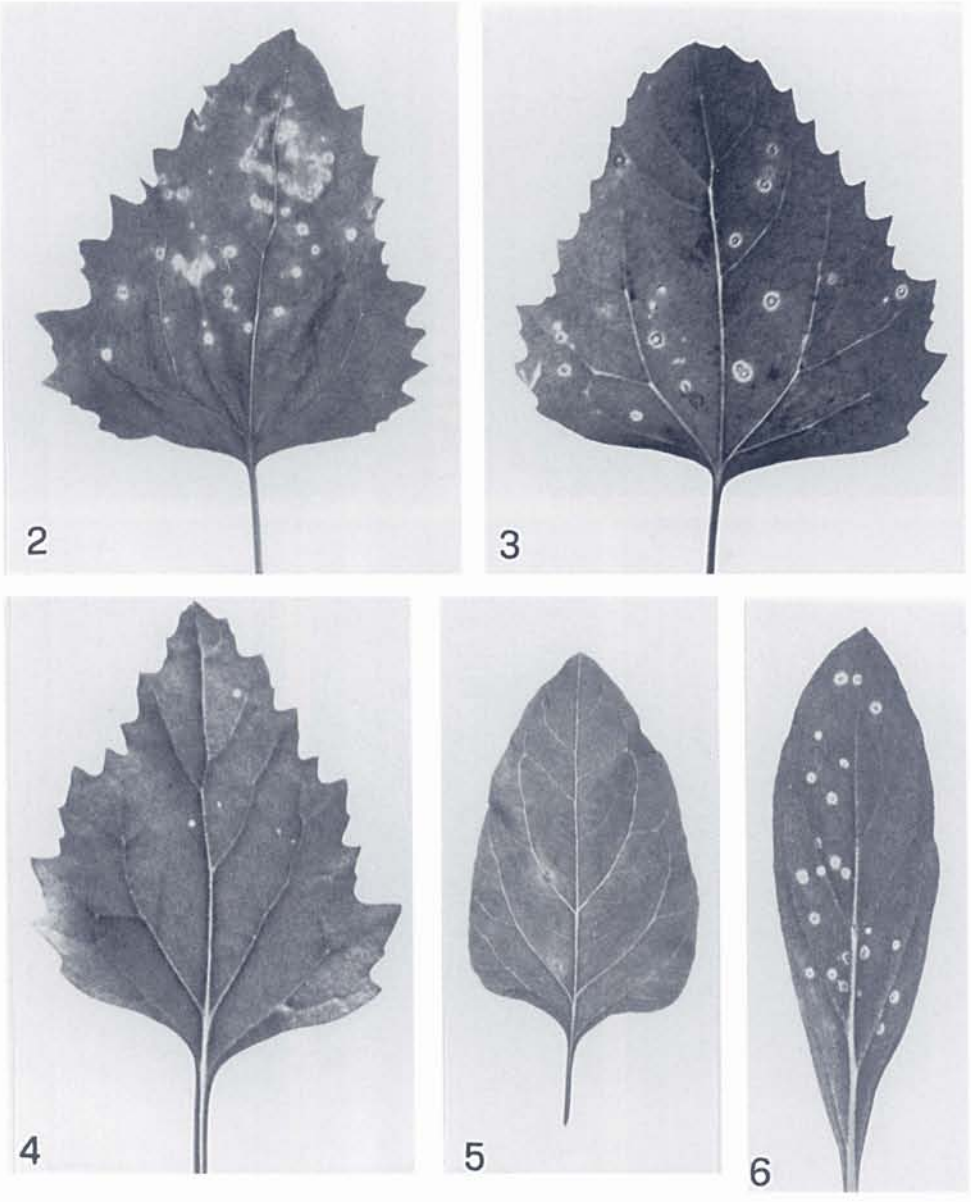
The failure to detect any virus particles in extracts may be due to

TABLE 2
NON-HOSTS OF CITRUS LEPROSIS VIRUS

Family & species	Family & species
Apocynaceae	Nyctaginaceae
Catharanthus roseus	*Mirabilis jalapa*
Balsaminaceae	Oleaceae
Impatiens walleriana	*Ligustrum lucidum*
Begoniaceae	*L. ovalifolium*
Begonia sp.	Palmae
Campanulaceae	*Trachycarpus fortunei*
Platycodon sp.	Pedaliaceae
Convolvulaceae	*Sesamum indicum*
Ipomea purpurea	Peperomiaceae
Cruciferae	*Peperomia* sp.
Brassica olearacea acephala	Portulacaceae
Cucubitaceae	*Talinum paniculatum*
Cucumis sativus	Rubiaceae
Cucurbita pepo	*Coffea arabica* cvs 'Mundo Novo' and unknown
Gentianaceae	Solanaceae
Eustoma grandiflorum	*Capsicum anuum*
Geraniaceae	*Datura metel*
Pelargonium zonale	*D. stramonuim*
Labiatae	*Lycopersicon esculentum*
Ocimum basilicum	*Nicotiana benthamiana*
Lauraceae	*N. clevelandii*
Laurus nobilis	*N. glutinosa*
Leguminoceae	*N. tabacum* 'White Burley'
Phaseolus vulgaris 'Saxa'	*Petunia hybrida*
Vigna unguiculata 'Black'	*Physalis pubescens*
Wisteria sinensis	Sterculiaceae
Malvaceae	*Brachychiton populneus*
Hibiscus rosa-sinensis	Umbelliferae
Gossypium hirsutum	*Apium graveolens*
Meliaceae	Vitidaceae
Melia azedarach	*Vitis vinifera*
Moraceae	
Ficus bengalensis	
F. carica	
Ficus sp.	

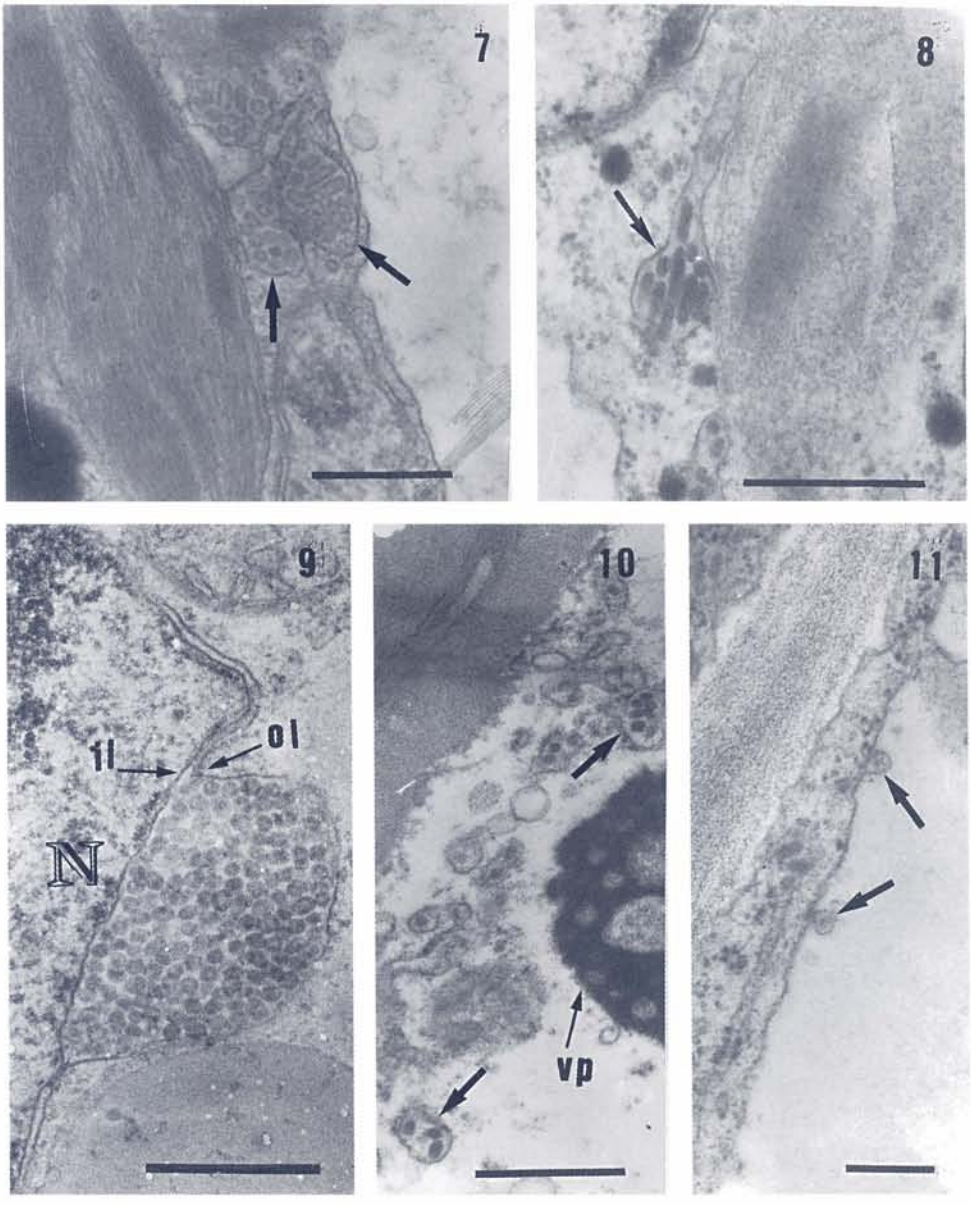
instability of the particle, although its infectivity was maintained for 6 days. Attempts at purification are continuing using a variety of stabilizing techniques. The presence of a 25 kDa protein in symptomatic tissue and its absence in healthy tissue suggests it may be associated with CiLV. If it were from a systemic virus such as tristeza it would also have been present in the controls. The M proteins of other rhabdoviruses have a similar size, that of wheat striate mosaic virus being also 25 kDa (16).

The round particles seen in some tissues may or may not be virus particles; they may be artifacts formed from unstable viruses. Recently virus-like particles measuring 30 to 40 × 110 to 130 nm were seen in cells of the mite vector, *B. phoenicis,* and were thought to be badnaviruses (14). We believe that on the basis of data given, it is difficult to determine if this virus is a badnavirus, and further investigations are needed. However, in the 6th Virus Taxonomy Report, CiLV is listed as a non-enveloped virus, possible



Figs. 2 to 6. Local lesions on test plants mechanically inoculated with CiLV:
Fig. 2. *Chenopodium quinoa*
Fig. 3. *C. amaranticolor*
Fig. 4. *C. murale*
Fig. 5. *C. polyspermum*
Fig. 6. *Gomphrena globosa*.

belonging to the plant rhabdoviruses (13). Data presented in this paper, and published elsewhere (3, 7, 10) supports this statement, rather than classifying it as a badnavirus. CiLV particles are wider than badnaviruses, no viroplasms and vesicles associated with tonoplasts have been reported for this group, and badnaviruses occur in the cytoplasm but not in enclaves of the ER (13). Particles with a similar morphology to CiLV



Figs. 7 to 11. Electron micrographs of CiLV-infected cells. Bar = 500 nm.

Fig. 7. Cleopatra mandarin mesophyll cell with particles in enclaves of the endoplasmic reticulum (arrows).

Fig. 8. Sub-epidermal cell from young bark lesion of Pera sweet orange with particles in the endoplasmic reticulum (arrow).

Fig. 9. Pera sweet orange mesophyll cell with particles in the perinuclear space; il = inner lamella, ol = outer lamella.

Figs. 10 and 11. Sub-epidermal cell from a young bark lesion of Pera sweet orange. Fig 10, virolpasm-like body (vp) and particles in enclaves of endoplasmic reticulum (arrows); Fig. 11 tonoplast-associated vesicles (arrows).

have been observed by EM in association with Dendrodium leaf streak virus, Phalaenopsis chlorotic spot virus, orchid fleck virus and Mimosa bacilliform virus. Coffee ringspot has the same mite vector as CiLV and

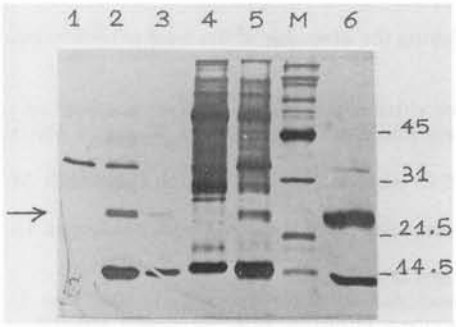


Fig. 12. SDS-PAGE protein pattern of partially purified and crude CiLV preparations from citrus leaves. Lane 1 - partially purified preparation from healthy leaves; lanes 2, 3 and 6 - partially purified preparations from infected leaves; lane 4 - crude preparation from healthy leaves; lane 5 - crude preparation from CiLV-infected leaves; lane M molecular weight markers (kDa). Arrow indicates protein band of 25 kDa in infected samples.

some morphological similarities, but it is an enveloped rhabdovirus (1, 9). Investigations on the relatedness of these viruses are needed.

It is important to confirm the presence of CiLV in the mite vectors. The non-systemic nature of the plant disease implied that the virus may be primarily an arthropod virus. The mite has a wide host range, and it is possible that CiLV may be introduced into other important plant species, but its virtual disappearance in the USA (7) suggests that, at present, it has no alternate hosts in nature. We were unable to demonstrate the presence of CiLV in any of the tested non-citrus species.

ACKNOWLEDGMENTS

We wish to thank the following at the Istituto di Fitovirologia applicata del CNR, Torino, Italy: V. Masenga for assistance with the electron microscopy, and G. Boccardo, M. Conti, V. Lisa, E. Luisoni, P. Roggero and R. G. Milne for critical reading of the manuscript. The financial assistance of the Brazilian CNPq and FAPESP and the Italian CNR is also acknowledged.

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