New Experimental Hosts and Further Information on Citrus Leprosis Virus

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ABSTRACT. *Tetragonia tetragonioides* (Tetragoniaceae), *Atriplex hortensis, A. latifolia, Beta vulgaris* ssp *cicla* and *Chenopodium bonus-henricus* (Chenopodiaceae) were found to be new experimental hosts of citrus leprosis virus (CiLV), extending its experimental host range outside the Rutaceae to 13 species belonging to 5 genera of the Centrospermae. All the herbaceous hosts react to mechanical inoculation only with local infection. Thirty three other species in 25 families, that have not been previously tested, were not susceptible to mechanical inoculation with CiLV. *In vitro* properties of CiLV in *C. quinoa* were further checked. The virus retained infectivity after 45 mo storage in dried-leaf and three days in infected sap at room temperature. Infectious CiLV was recovered in leaves of *C. quinoa*, 24 to 42 h after inoculation, or one to two days before symptom appearance. The typical particles and structures of CiLV have been seen in the thin sections obtained from local lesions of infected *Chenopodium quinoa* leaves. During 1997 and 1998 winter (from July to September) in São Paulo State no new leaf infections were found on sweet orange plants. Symptoms where present on old leaves of Cleopatra mandarin, but the virus content was very low, probably because of decrease of virus multiplication during the winter months.

Knowledge on citrus leprosis virus (CiLV) has advanced during the last five years (5, 6, 10), but there is still much to be done to improve virus purification (5) and to prepare antisera or probes for rapid identification of the virus. CiLV causes only local infection in all known plant hosts and it would be useful to find a systemic host. Here we describe work to expand experimental host range of CiLV with special emphasis on finding a systemic host. Information is also presented on the presence of CiLV particles inside necrotic lesions on *C. quinoa*, replication of the virus in *C. quinoa* leaves before the appearance of the symptoms, and its longevity *in vitro* at room temperature and in infected dried leaves. Ecological observations on leprosis disease in citrus and Cleopatra mandarin were also made.

MATERIALS AND METHODS

The CiLV isolates used were obtained from Cleopatra mandarin and sweet orange and multiplied in *C. quinoa*. The mechanical inoculation tests were done as previously described (10), using the PDET extraction 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). From 0.5 to 1% activated charcoal was also used.

Thirty eight plant species belonging to 25 families not previously tested for susceptibility to CiLV were mechanically inoculated (Table 1 and 2). At least three plants of each species were inoculated and grown in insect-proof glasshouse kept at day temperatures from 24° to 35°C and night temperature not lower than 21°C. Most of the tests were repeated in different seasons. Both positive and negative infections were checked by back inoculation to at least three *C. quinoa* plants with a total of eighteen susceptible leaves.

Longevity *in vitro* at room temperature (23 to 29°C) was determined with crude extracts obtained by crushing one part (w/v) symptomatic *C. quinoa* leaf tissue in three part of PDET buffer containing 1% charcoal. Preservation of infectivity in dried leaf of *C. quinoa* was checked in tissue dried under vacuum and stored at -20°C.

Tests for replication of CiLV in *C. quinoa* leaves prior to the appearance of symptoms were made with

TABLE 1
LIST OF PLANT SPECIES SUSCEPTIBLE OR RESISTANT TO CILV BELONGING TO THE RUTACEAE AND THE CENTROSPERMAE (AMARANTHACEAE, CHENOPODIACEAE, TETRAGONIACEAE)

Plant species[z]	Natural hosts (mite transm.)	Mechanical inocul. results[x]	Local lesion types[y]	Days to appear
Rutaceae				
Citrus aurantifolia	+	—		
C. aurantium	+	—		
C. bigaradia		—		
*C. histrex**		—		
C. jambhiri	+	—		
C. latifolia	+			
C. limon	+	—		
C. medica	+			
C.paradisi	+			
C. reshni	+	—		
C. reticulata	+	—		
C. reticulata × *C. paradisi*		—		
C. reticulata × *C. sinensis*	+	—		
C. sinensis	+	(+)	bs, ch	17
C. sinensis × *Poncirus trifoliata*	+			
Esenbeckia grandiflora		—		
E. leiocarpa		—		
Murraya exotica		—		
Ruta graveolens		—		
Skimmia reevesiana		—		
Amaranthaceae				
Alternanthera brasiliana		—		
Al. tenella		—		
*Amaranthus bouchonii**		—		
*Am. paniculatus**		—		
*Am. retroflexus**		—		
Am. spinosum		—		
*Am. tricolor**		—		
Am. viridis		—		
*Celosia argentea**		—		
C. cristata		—		
Gomphrena globosa		+	rb	5
Chenopodiaceae				
*Atriplex hortensis**		+	cb	5
*Atriplex latifolia**		+	sn	4
*Beta vulgaris ssp cicla**		+	dg	6
Chenopodium album		+	n	3
C. amaranticolor		+	sn	3
C. ambrosioides		—		
*C. bonus-henrici**		+	er, n	6
*C. botrys**		—		
C. capitatum		+	n	5
C. foliosum		+	n	5
C. murale		+	sn	5

[z]The species marked with * are first reported in this paper. The others are previously reported (6, 10).
[y]bs = brown spot; cb = clear brown; ch = chlorotic halo; dg = dark green on leaves becoming chlorotic; er = erratic reaction; n = necrotic; rb = reddish brown; sn = small or very small necrotic; ws = water-soaked.
[x]+ = positive results; (+) = positive results obtained only with inocula from sweet orange; — = negative results.

TABLE 1 (CONTINUED)
LIST OF PLANT SPECIES SUSCEPTIBLE OR RESISTANT TO CILV BELONGING TO THE RUTACEAE AND THE CENTROSPERMAE (AMARANTHACEAE, CHENOPODIACEAE, TETRAGONIACEAE)

Plant species[z]	Natural hosts (mite transm.)	Mechanical inocul. results[x]	Local lesion types[y]	Days to appear
C. polyspermum		+	rb, ch	6
C. quinoa		+	ws, n	3
C. schraderianum		—		
Spinacia oleracea		—		
Tetragoniaceae				
*Tetragonia tetragonioides**		+	ws, n	4

[z]The species marked with * are first reported in this paper. The others are previously reported (6, 10).
[y]bs = brown spot; cb = clear brown; ch = chlorotic halo; dg = dark green on leaves becoming chlorotic; er = erratic reaction; n = necrotic; rb = reddish brown; sn = small or very small necrotic; ws = water-soaked.
[x]+ = positive results; (+) = positive results obtained only with inocula from sweet orange; — = negative results.

extracts prepared from at least three leaves harvested from three *C. quinoa* plants 18 to 54 h after inoculation. Inoculations were made to six leaves on each of three *C. quinoa* plants at intervals of 3 h. To check on possible infectivity due to CiLV particles present on the surface of inoculated leaves, plants immune to CiLV (*Ocimum basilicum*, *Nicotiana benthamiana*, *N. glutinosa*, or *N. tabacum*) were inoculated and then tested in parallel to *C. quinoa*.

Thin sections of *C. quinoa* local lesions caused by CiLV were obtained after aldehyde-osmium fixation and epoxy embedding (11), and viewed in a Philips CM 10 microscope. The lesions of water-soaked consistency were embedded few days after their appearance.

Epidemiological observations were made in a nurseries at Limeira during July through September 1997, the coldest months in São Paulo State. They were repeated during October and early November 1998, a period still moderately cold and rainy.

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

Experimental host range. Of all the 38 plant species tested, only *Tetragonia tetragonioides* (= *T. expansa*) (Tetragoniaceae), *Atriplex hortensis*, *A. latifolia*, *Beta vulgaris* ssp *cicla* and *Chenopodium bonus-henricus (Chenopodiaceae)* were found to be susceptible (Fig. 1). All species found susceptible to mechanical inoculation in this and previous investigations (6, 10) belong to Centrospermae. In Table 1, the susceptible and non susceptible Centrospermae are listed, indicating the type of symptoms and the days to appear. No virus could be detected in all the species that gave no symptoms when mechanically inoculated with CiLV. The list of the plant species not belonging to Amaranthaceae, Chenopodiaceae, Tetragoniaceae and Rutaceae, resistant to mechanical inoculation with CiLV is given in Table 2.

CiLV was easily recovered from *T. tetragonioides*, *A. hortensis*, *A. latifolia*, *Beta vulgaris* and *C. bonus-henricus*, but with the inocula from these species and the other susceptible herbaceous hosts, we were unable to infect sweet orange, or Cleopatra mandarin leaves even

TABLE 2
LIST OF PLANT SPECIES NOT FOUND INFECTED AFTER MECHANICAL INOCULATION WITH CiLV[z]

Angiospermae, Dicotyledonae
- Piperales:
  - Peperomiaceae: *Peperomia* sp.
- Urticales:
  - Moraceae: *Ficus bengalensis*; *F. carica*, *Ficus* sp.
- Polygonales:
  - Polygonaceae: *Fagopyrum esculentum**
- Centrospermae (Amaranthaceae, Chenopodiaceae and Tetragoniaceae are listed in Table 1):
  - Aizoaceae: *Aptenia cordifolia**; *Dorotheanthus bellidiformis**
  - Nyctaginaceae: *Mirabilis jalapa*; Photolaccaceae: *Rivina humilis**
  - Portulacaceae: *Portulaca oleracea**; *Talinum paniculatum*
- Ranales:
  - Lauraceae: *Laurus nobilis*
- Rhoeadales:
  - Cruciferae: *Brassica oleracea acephala*
- Rosales:
  - Leguminosae: *Glycine max**; *Phaseolus vulgaris* 'Saxa'; *Vigna unguiculata* 'Black'; *Wisteria sinensis*
- Geraniales (Rutaceae are listed in Table 1):
  - Geraniaceae: *Pelargonium zonale*
  - Meliaceae: *Melia azedarach*
- Sapindales:
  - Anacardiaceae: *Schinus molle**
  - Balsaminaceae: *Impatiens walleriana*
- Rhamnales:
  - Vitidaceae: *Vitis vitifera*
- Malvales:
  - Malvaceae: *Hibiscus rosa-sinensis*, *Gossypium hirsutum*
  - Sterculiaceae: *Brachychiton populneus*
- Parietales:
  - Begoniaceae: *Begonia* sp.
  - Passifloraceae: *Passiflora bignonioides**
- Myrtiflorae:
  - Myrtaceae: *Feijoa sellowiana**
  - Punicacea: *Punica granatum**
- Umbelliflorae:
  - Cornaceae: *Aucuba japonica**
  - Umbelliferae: *Apium graveolens*, *Petroselinum sativum**
- Primulales
  - Myrsinaceae: *Ardisia crenata**
- Contortae:
  - Oleaceae: *Ligustrum lucidum*, *Ligustrum ovalifolium*, *Ligustrum vulgare**
  - Gentianaceae: *Eustoma grandiflorum*
  - Apocynaceae: *Catharanthus roseus*
- Tubiflorae:
  - Bignoniaceae: *Macfadyena unguis-cati**
  - Convolvulaceae: *Ipomea purpurea*
  - Labiatae: *Ocimum basilicum, Coleus blumei**, *Salvia splendens**
  - Pedaliaceae: *Sesamum indicum*
  - Solanaceae: *Capsicum annuum, Datura metal., D. stramonium, Lycopersicon esculentum, Nicotiana benthamiana, N. clevelandii, N. glutinosa, N. tabacum* 'White Burley', *Petunia hybrida, Physalis pubescens, Solanum capsicastrum**
  - Scrophulariaceae: *Antirrhinum majus**
  - Verbenaceae: *Verbena* × *hybrida**
- Rubiales:
  - Rubiaceae: *Coffea arabica* 'Mundo Novo', 'Unknown'

[z]Plant species are arranged under their taxonomic position according to Willis (16). The species marked with * are first reported in this paper. The others are previously reported (6, 10).

TABLE 2 (CONTINUED)
LIST OF PLANT SPECIES NOT FOUND INFECTED AFTER MECHANICAL INOCULATION WITH CiLV[z]

Cucurbitales:	
	Cucurbitaceae: *Cucumis sativus*, *Cucurbita pepo*, *Citrullus lanatus**
Campanulatae:	
	Campanulacae: *Platycodon* sp.
	Compositae: *Cichorium intybus**, *Lactuca sativa**, *Zinnia* sp.
Angiospermae. Monocotyledones	
Pincipes:	
	Palmae: *Trachycarpus fortunei*
Microspermae:	
	Orchidaceae: *Dendrobium nobile**, *Paphiopedilum* sp.*
Pteridophyta	
Filicopsida, Filicida, Pteridales:	
	Adiantaceae: *Adiantum capillus-veneris**

[z]Plant species are arranged under their taxonomic position according to Willis (16). The species marked with * are first reported in this paper. The others are previously reported (6, 10).

after a partial purification of the sap extracted from CiLV-infected *C. quinoa* leaves.

Comparative inoculations of herbaceous hosts with four isolates of CiLV obtained from Cleopatra mandarin and 'Pera' sweet orange conducted during different periods of the year showed no differences in symptomatology, host range, or *in vitro* properties, even though two of the isolates were mechanically passed 19 and 28 times during the span of about two years.

Longevity *in vitro* at room temperature and recovery of infectivity from *C. quinoa* fresh and dried leaves. *In vitro* longevity at room temperature was 3 days. No infectivity was found in assays made after 4, 5 and 6 days.

Tests for replication of CiLV in *C. quinoa* leaves before symptom appearance are summarized in Table 3. Only occasional lesions were observed in assays made 24, 27 and 30 h after inoculation. Titer remained low at 33, 36 and 39 h after inoculation, but increased in plants tested 42 and 45 h after inoculation. Titer approached normal level at 48 and 54 h after inoculation. No lesions were obtained from extracts of inoculated *O. basilicum*, *N. benthamiana*, *N. glutinosa,* and *N. tabacum* plants, used as control against the possibility that infections was associated with the original inocula.

It was also found that CiLV infectivity was maintained after 45 mo in dried leaves. While a good number of local lesions on *C. quinoa* were obtained in tests done after 22 mo storage, only a few lesions were obtained after 45 mo storage, a duration probably near to the limit.

Electron microscopy. In ultrathin sections of the local lesions from CiLV-infected *C. quinoa* leaves, most of the cells were greatly damaged and few ultrastructural details could be seen. In some parenchyma cells, CiLV elliptical particles, about 45 nm in diameter, were present in the lumen of the endoplasmic reticulum (Fig. 2). While numerous particles were present in some cells (Fig. 2B), in others only few particles were contained in enclaves of the endoplasmic reticulum (Fig. 2A). Additionally, viroplasm structures were present. Both particles and viroplasms were similar to those found in CiLV-infected citrus leaves (6, 10).

Epidemiology. During the winter months of 1997 we noted the scarcity and often absence of leprosis symptoms on the leaves of susceptible sweet orange plants. Only

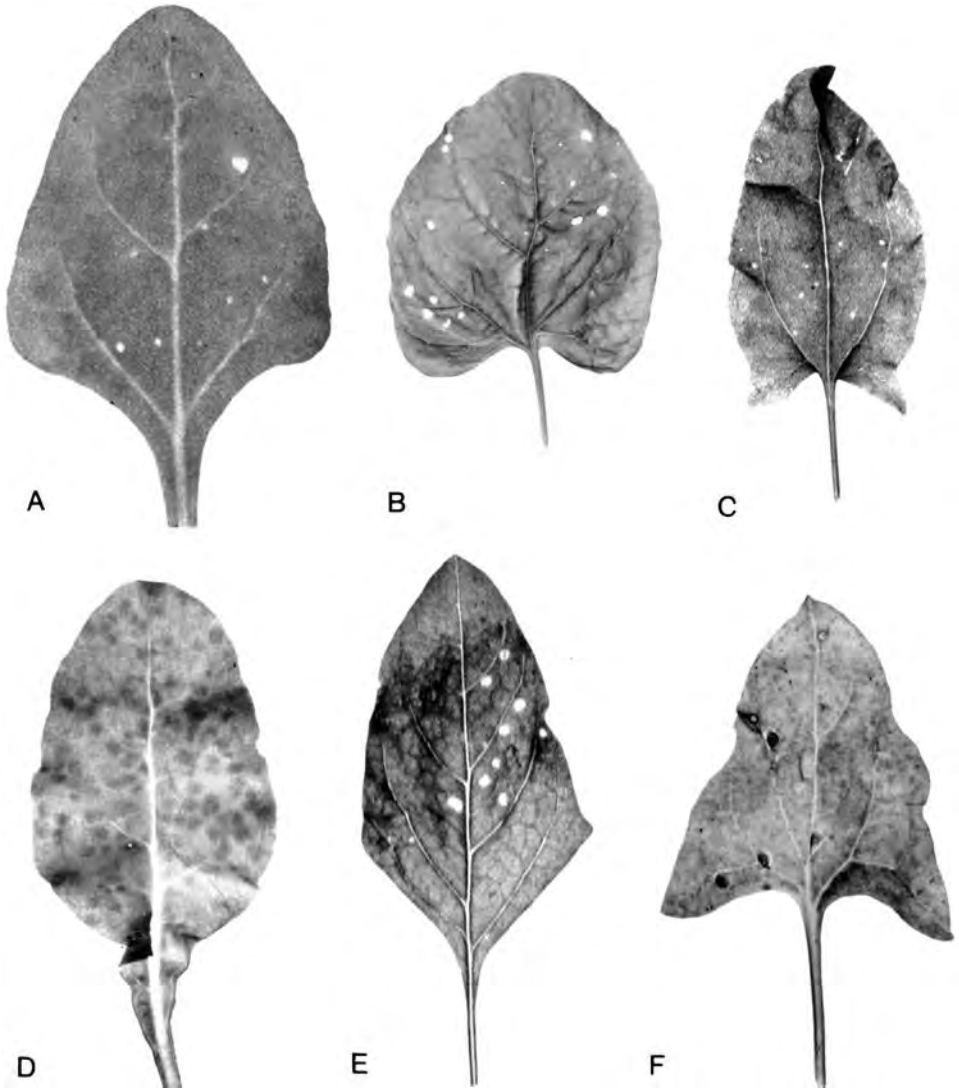

Fig. 1. Local lesions on test plants mechanically inoculated with CiLV. a) *Tetragonia tetragonioides*, 7 d after inoculation. b) *Atriplex hortensis*, 20 d after inoculation. c) *Atriplex latifolia*, 5 d after inoculation. d) *Beta vulgaris* ssp *cicla*, 12 d after inoculation. e) *Chenopodium polyspermum*, 10 d after inoculation. f) *C. capitatum*, 7 d after inoculation

Cleopatra mandarin leaves were found with symptoms, probably because the symptoms are less severe in sweet orange and were present in leaves of the internal lower part of the canopy. Similar behavior was also observed during winter months of 1998 in Cleopatra and sweet orange. No leprosis symptoms were observed on leaves of Bahia sweet orange in an abandoned plot, while severe cankers were present on mature stems and branches and a few small cankers were seen on young green stems.

The absence of leaves with symptoms in sweet orange could be connected with drop of leaves with symptoms promoted also by drought and/or cold weather and decrease of virus multiplication and new infections during winter months.

TABLE 3
RECOVERY OF INFECTIVITY IN *CHENOPODIUM QUINOA* LEAVES AT DIFFERENT TIME INTERVALS AFTER INOCULATION[z]

Time after inoculation (h)	Evaluation of infectivity
18	0
21	0
24	(+)
27	(+)
30	(+)
33	+
36	+
39	+
42	++
45	++
48	+++
51	+++
54	+++
68: symptoms appearance	+++

[z]0 = no local lesions; (+) = not more than one lesion per plant; + = not more than two lesions per plant; ++ = more than five lesions per plant; +++ = more than 20 lesions per plant.

Mechanical inoculation of *C. quinoa* with sap from infected leaves of Cleopatra mandarin and with macerates from dried CiLV-infected *C. quinoa* during the winter of 1997 in the unheated glasshouses at the Instituto Biológico (São Paulo) did not produced lesions. This failure was probably due to low temperatures that restrict CiLV multiplication. The temperature in the glasshouses often went below 24°C, and even to 14° at night. The same tests repeated in a heated glasshouse in Torino with day temperatures from 24° to 32°C and night temperatures from 21° to 24°C also showed that leaves with symptoms taken during winter contained very little CiLV, as judged by the small number of local lesions produced in *C. quinoa* and the failure to recover dsRNAs, as described by Colariccio et al. (5). Macerates of a similar sample of dried CiLV infected *C. quinoa* leaves were infectious, when tested in a heated glasshouse.

DISCUSSION

Results obtained here confirmed a prior report (10) that CiLV is a thermophile virus that multiplies only if the day temperature is above 24°C and the night temperature is above 21°C.

The 12 known natural hosts of CiLV are all in the genus *Citrus*. Until now, 112 different plant species in 44 families have been tested for susceptibility and the 13 herbaceous species susceptible to mechanical inoculation, all belong to the Chenopodiaceae, Amaranthaceae and Tetragoniaceae, of the order Centrospermae. The Centrospermae are phylogenetically distinct from the other Angiosperms (7), and the red pigments of the Centrospermae are betalains, whereas the red or blue pigments of other higher plants are based on anthocyanins (12).

Within the Chenopodiaceae, the species containing aromatic substances such as *Chenopodium ambrosioides*, *C. schraderianum* and *C. botrys* were immune, while all the other *Chenopodium* species tested were susceptible to CiLV, although to different degrees.

The most consistent test plant for CiLV investigations was *C. quinoa*. *C. amaranticolor*, which in early experiments (6, 10) reacted similarly to *C. quinoa*, in these investigation reacted with only a

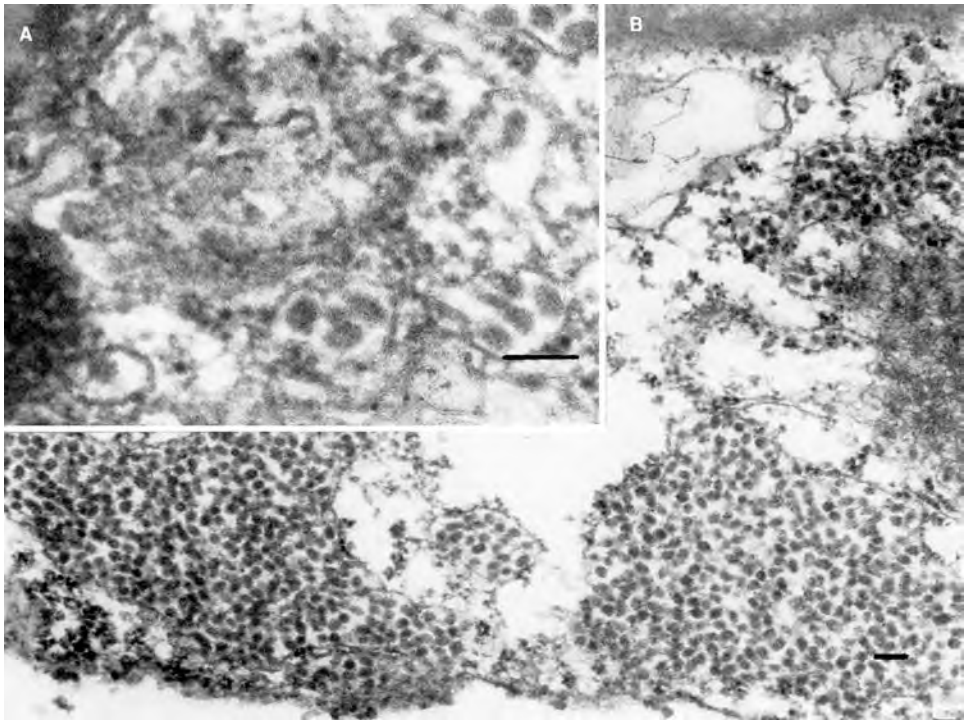

Fig. 2. Portion of the mesophyll of CiLV-infected cells of *Chenopodium quinoa* leaves. Bar = 100 nm. a) Virus particles in enclaves of the endoplasmic reticulum and on the left side a part of a viroplasm structure. b) Cell full of virus particles.

few pin-point local lesions. We think that the stronger reactions reported earlier were because the line of *C. amaranticolor* used was a hybrid of *C. amaranticolor* and *C. quinoa*.

Although CiLV can be easily transmitted mechanically among the susceptible species of the Centrospermae, it has not been possible to transmit the virus back to *Citrus* spp, even after partial purification. Similar behavior has been reported (2) for coffee ringspot virus (CoRSV), which could not be back transmitted from *C. quinoa* to coffee. CiLV can be transmitted mechanically from citrus to citrus (6) but at low percentage. Possibly, the evolution of CiLV and CoRSV allows them to be efficiently transmitted by *Brevipalpus* mites to their natural hosts, but not by mechanical transmission. This aspect and the non-systemic infection of plant hosts should be further investigated.

In spite of its apparent particle instability, CiLV had a rather good infectious rate, and the particle instability may be connected with the loss of an ancestral envelope and/or the fragility of some virus protein. CiLV maintained its infectivity for 45 mo in dried leaves and infectivity is present within inoculated leaves 24 to 42 h before the appearance of symptoms, indicating a rapid replication cycle.

CiLV belong to a small cluster of similar, non-enveloped rhabdo-like viruses transmitted by *Brevipalpus* spp., which probably include the citrus leprosis-type virus of orchids (8), Ligustrum ringspot, LRSV (13), and green spot of passion fruit, GSPFV (9). In addition to their different natural hosts, these viruses seem to have other differences. CiLV does not infect *Ligustrum lucidum*, *L. ovalifolium*, *L. vulgare*, *Dendrobium nobile*, *Paphiopedilum* sp and

Passiflora bignonioides, species which are or could be hosts of LRSV, GSPFV, or the leprosis-type virus of orchids.

CoRSV is another virus transmitted by *Brevipalpus phoenicis* and sap transmissible to *C. quinoa* and *C. amaranticolor*. It also shares with CiLV non-transmissibility by sap from herbaceous hosts to coffee (2). However, CoRSV is an enveloped rhabdovirus (3) and causes systemic infection in *C. amaranticolor* (1). The hypothesis that these viruses are primarily arthropod viruses (10) in different stages of evolution in plants, seems increasingly convincing.

Our epidemiological observations suggest that infected leaves of Cleopatra and stem and branch cankers on sweet orange could be important sources of infection during spring, at least in the Limeira area.

Before our investigations CiLV could be diagnosed practically only through the symptoms observed in citrus. This sometimes gave rise to confusion with other diseases, especially citrus canker (15), and some types of psorosis. Transmission of the disease by the mites can confirm diagnosis but this is rather difficult to do routinely, and takes at least 17 days. Graft transmission from stem local cankers or lesions gives low percentages of positive results and requires long incubations periods (4). By using herbaceous test plants, especially *C. quinoa*, it is possible to detect the presence of CiLV in as little as three days under appropriate conditions. The other herbaceous hosts could be useful to differentiate CiLV from other viruses, mainly the citrus ones (14).

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