Transmission of *Citrus leprosis virus C* by *Brevipalpus phoenicis* (Geijskes) to Alternative Host Plants Found in Citrus Orchards

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Abstract

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The equivalent of US\$75 million is spent each year in Brazil to control *Brevipalpus phoenicis*, a mite vector of *Citrus leprosis virus C* (CiLV-C). In this study, we investigated the possibility that hedgerows and windbreaks normally found in citrus orchards could host CiLV-C. Mites confined by an adhesive barrier were reared on sweet orange fruit with leprosis symptoms then were transferred to leaves of *Hibiscus rosa-sinensis*, *Malvaviscus arboreus*, *Grevilea robusta*, *Bixa orellana*, and *Citrus sinensis*. Ninety days post infestation, the

descendant mites were transferred to Pera sweet orange plants to verify the transmissibility of the virus back to citrus. Nonviruliferous mites which had no feeding access to diseased tissue were used as controls. Local chlorotic or necrotic spots and ringspots, symptoms of leprosis disease, appeared in most plants tested. Results generated by reverse-transcription polymerase chain reaction with primers specific for CiLV-C and by electron microscope analyses confirmed the susceptibility of these plants to CiLV-C.

Citrus leprosis (CL) is a disease of great importance to the Brazilian citrus industry, particularly affecting sweet orange (*Citrus sinensis* (L.) Osb.) production in São Paulo State, the world's leading producer of sweet oranges. The disease has been reported to be absent in the United States since the 1970s (12) but, in recent years, it has spread into other citrus-producing areas of South and Central America and has reached Mexico (20,30,38).

In the state of São Paulo, Brazil, up to US\$75 million per year is spent on chemicals to control the mite vector (41). Leprosis causes fruit drop and extensive defoliation, which both lead to a reduction in fruit quality and yield. CL is not a systemic disease and the localized symptoms include necrotic lesions on twigs, chlorotic ringspots on leaves, and necrotic ringspots with a central depression on fruits. Because the lesions on the twigs can expand and converge, if an affected plant is left untreated for mites, severe dieback can occur, leading to the death of the tree within a few years (6).

Two types of viruses have been reported to cause leprosis symptoms (24). The nuclear type of *Citrus leprosis virus* (CiLV-N) occurs rarely and is associated with short, rod-like particles (40 by 100 to 110 nm) which are found in the nucleus or in the cytoplasm of infected cells. These particles appear frequently associated with membranes of the nuclear envelope or endoplasmic reticulum. The presence of electron lucent viroplasm in the nucleus is another cytopathological characteristic associated with CiLV-N (24). CiLV-N probably belongs to the proposed *Dichorhabdovirus* genus in the *Rhabdoviridae* family, which has *Orchid fleck virus* (OFV) as the type member (29).

The cytoplasmic type, *Citrus leprosis virus C* (CiLV-C) (24), is characterized by short, membrane-bound bacilliform particles (50

to 60 by 110 to 120 nm) present within the cisternae of the endoplasmic reticulum, and by the presence of electron-dense, vacuolated viroplasms in the cytoplasm (14). The complete genome of CiLV-C has been sequenced (34,39) and, based upon unique molecular and biological characteristics, the virus was accepted as the type-member of the new genus *Cilevirus* (8,33,34).

In Brazil, CiLV-C is transmitted by *Brevipalpus phoenicis* Geijskes (Acari: Tenuipalpidae) and affects virtually all sweet orange varieties (2,5,41). Other citrus species and numerous hybrids are less susceptible to the disease (3) but are hosts of the virus and exhibit typical symptoms (4). *B. phoenicis* is cosmopolitan, polyphagous, and has a high degree of adaptation, colonizing at least 786 reported plant host species (11–13).

Control measures based upon strategies of integrated pest management try to reduce the production costs and the environmental impact caused by indiscriminate use of pesticides. Recommendations for CL management in citrus groves include several actions to control the vector, such as alternate administrations of acaricides with different modes of action to avoid the development of resistant populations and application of acaricides that preserve the natural enemies of the mite. Other actions widely used by growers emphasize avoidance of mite dispersion through the use of windbreaks, cleaning of harvest boxes, elimination of fruit fallen to the ground, and pruning of symptomatic twigs in order to reduce the foci of the disease (21,41). The use of hedgerows as barriers to prevent transit of people and animals and to reduce wind that could act to disperse mites is also advised for citrus groves (21). Successful experimental transmission of CiLV-C to herbaceous plants was obtained by mechanical inoculation to *Chenopodium amaranticolor* Coste & Reyn., *C. quinoa* Willd. and *Gomphrena globosa* L. (14), and by mites to *Solanum violaeifolium* Schott. and *Phaseolus vulgaris* L. (22,42). With the exception of dry bean (*P. vulgaris*), which can be used as an intercrop plant in citrus orchards in some countries, these species have little importance to the citrus agroecosystem. Of greater concern relative to the spread of CL is the report of the natural infection of CiLV-C in *Swinglea glutinosa* (Blanco) Merr. plants grown as hedgerows near citrus orchards. Although *S. glutinosa* is a rutaceous relative of *Citrus* spp., this was the first reported non-citrus natural host of the virus (31). After the thorough compilation done by Childers et al. (13) on the host

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*The e-Xtra logo stands for “electronic extra” and indicates that Figure 1 appears in color in the online edition.

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range of *Brevipalpus* spp., other authors have searched for yet more plant species colonized by the mite. Maia and Oliveira (35,36) demonstrated that *B. phoenicis* can colonize some of the plants growing as weeds or windbreaks or in hedgerows near citrus groves and suggested that they could also be hosts of CiLV-C. The objective of this work was to extend these studies to confirm whether or not CiLV-C can infect these plants.

Materials and Methods

***B. phoenicis* populations and CiLV-C inoculum.** Unripe Pera sweet orange fruit with scab lesions were used to propagate *B. phoenicis* populations, because the scab lesions are important oviposition niches for the mites. The fruit were cleaned, dried, and partially submerged in liquid paraffin to prevent desiccation and the area for mite propagation was surrounded by a barrier of the adhesive Tanglefoot (9). The nonviruliferous stock of mites was initially collected in a citrus orchard without leprosis and a laboratory population was initiated from their eggs. These eggs were manually transferred by using a small brush under a stereoscopic microscope to healthy fruit, where the mites were reared. The viruliferous mite population was collected from a citrus grove with a high incidence of leprosis in Jaboticabal, São Paulo State, and the mites were reared on symptomatic fruit. Specimens of both mite populations were mounted on slides with Hoyer's medium and identified using phase-contrast microscopy according to Prichard and Baker (40). Scanning electron microscopy (SEM) analyses were also performed to confirm mite species, as described by Kitajima and Leite (25).

The fruits were placed on plastic trays and kept at $25 \pm 1^\circ\text{C}$, with relative humidity of $60 \pm 5\%$ and a 14-h photoperiod.

Host range. In addition to Pera sweet orange plants, the following plant species were used in this study: *Hibiscus rosa-sinensis* L. (Malvaceae), *Malvaviscus arboreus* Cav. (Malvaceae), *Grevillea robusta* A. Cunn. (Proteaceae), and *Bixa orellana* (Bixaceae). These four species serve as hedgerows in citrus groves and were selected for study based on the work done by Maia and Oliveira (35,36), who suggested that not only the mite but also the virus might colonize them.

Test-plant species were transplanted to 5-liter plastic pots containing equal parts of soil, sand, and manure. Five test plants of each species were infested with viruliferous mites while five of them were infested with nonviruliferous mites. Tanglefoot was applied on each leaf petiole to confine the mites to the selected leaves.

Mite inoculation of CiLV-C. Twenty viruliferous female mites were transferred to a single leaf of each of the test plants, which were maintained in the greenhouse for 90 days. A similar procedure was used for infestation of control plants by the nonviruliferous mites.

Ninety days after colonization, progeny mites were recovered and transferred to Pera sweet orange plants, which were periodically assessed for the appearance of leprosis symptoms.

Virus detection in plant tissues was carried out by transmission electron microscopy (TEM) and by reverse transcription followed by the polymerase chain reaction (RT-PCR). The analyses were performed on symptomatic tissues when available or on tissues from the delimited area of mite feeding for the asymptomatic plants.

TEM analyses. For the cytological studies, small pieces of tissue from the lesions (1 by 3 mm) were submerged in a modified Karnovsky solution (2.5% glutaraldehyde and 2% paraformaldehyde in 0.05 M cacodilate buffer, pH 7.2, and 0.001 N M CaCl_2) for 1 to 2 h, washed with 0.05 M cacodilate buffer, post fixed with 1 % OsO_4 in 0.05 M cacodilate buffer for 1 h, and en bloc stained for 12 h with a 0.5% aqueous solution of uranyl acetate. Fixed tissues were dehydrated in acetone and embedded in the low-viscosity Spurr's epoxy resin. Thin sections were obtained from these blocks with a Leica UCT ultramicrotome equipped with diamond knife. Sections were stained with 3% aqueous uranyl acetate and Reynold's lead citrate (27), and examined with a Zeiss EM 900 TEM.

RT-PCR detection of CiLV-C. Leaves (0.1 g) from each test plant were ground to powder in liquid nitrogen and used for total RNA extraction using a cetyltrimethylammonium bromide method (18,32). The cDNA synthesis was performed according to standard protocol, using 4 μl of 5 $\times$ buffer, 2 μl of 2 mM dithiothreitol, 1 μl

Fig. 1. Leprosis symptoms on leaves of different hosts infested by mites viruliferous for *Citrus leprosis virus C*. **A**, *Bixa orellana*; **B**, *Citrus sinensis* Pera; **C**, *Hibiscus rosa-sinensis*; **D**, *Grevillea robusta*; and **E**, *Malvaviscus arboreus*.

of reverse transcriptase (M-MLV-RT), 1 µl of 2.5 mM dNTP, 1 µl of random primers, 0.5 µl of RNase inhibitor (all Life Technologies reagents), 3 µl of total RNA, and 7.5 µl of diethylpyrocarbonate-treated H₂O, and incubated at 37°C for 90 min.

PCR was performed with 10 pmol of MP-F and MP-R oligonucleotides that amplify a fragment of 344 bp within the tentative movement protein (*mp*) gene of CiLV-C, as described by Locali et al. (32), in 1× buffer with 2.5 mM MgCl₂, 10 mM dNTP mix, 1 U of *Taq* DNA Polymerase (all Life Technologies reagents), and 3 µl of cDNA, adjusted up to a final volume of 25 µl with MilliQ purified H₂O. The reaction was performed using a PTC 100 (MJ Research Waltham, MA) thermocycler programmed for 1 cycle at 94°C for 3 min followed by 35 cycles at 94°C for 30 s, 56°C for 30 s, and 72°C for 40 s. Aliquots of 8 µl were analyzed in 1% agarose gels, prepared in 0.04 M Tris-acetate and 0.001 M EDTA, containing ethidium bromide at 0.5 mg/ml.

Sequencing and analysis of amplicons. Purified amplicons were cloned and transformed into *Escherichia coli* DH5α according to standard procedures. Recombinant clones were sequenced with an ABI Prism 3700 DNA sequencer (Applied Biosystems, Foster City, CA).

The nucleotide consensus sequence for each amplicon was analyzed by the program Phred-Phrap-Consed and compared with sequences available at the GenBank database (www.blast.ncbi.nlm.nih.gov), using the BlastX tool to confirm their identity.

Results

Confirmation of *Brevipalpus* spp. Mite species were confirmed as *Brevipalpus phoenicis* using phase contrast microscopy and SEM (data not shown).

Symptom development. The symptoms of leprosis in the test plants were diverse and included necrotic lesions in *M. arboreus*, chlorotic ringspots and necrotic spots in *H. rosa-sinensis*, and pin-point necrotic lesions in *Bixa orellana* and *G. robusta* (Fig. 1).

CiLV-C detection by TEM. Groups of short, bacilliform particles within endoplasmic reticulum cisternae and electron dense and vacuolated viroplasm, typical of CiLV-C infected cells (14,26), were consistently found in the parenchymal cells of leaf lesions on *H. rosa-sinensis*, *M. arboreus* and *Citrus sinensis* plants that were colonized by viruliferous mites (Table 1; Fig. 2). However, neither viral particles nor viroplasms were observed in cells of *G. robusta* or *B. orellana* tissues.

Table 1. Detection of *Citrus leprosis virus C* in some plant species experimentally infected by viruliferous mites using different techniques

Plant species	Total/positives ^a		
	SL	RT-PCR	TEM
<i>Bixa orellana</i>	5/3	3/3	3/0
<i>Citrus sinensis</i> Pera	5/5	5/5	5/3
<i>Grevillea robusta</i>	5/3	3/3	3/0
<i>Hibiscus rosa-sinensis</i>	5/3	3/2	3/1
<i>Malvaviscus arboreus</i>	5/3	3/2	3/2

^a SL = symptoms in Pera sweet orange plants after transfer of mites from the alternative hosts, RT-PCR = reverse-transcription polymerase chain reaction, and TEM = transmission electron microscopy.

Fig. 2. Transmission electron micrographs of thin sections of leaves of experimentally infected plants of **A**, *Citrus sinensis* and **B**, *Malvaviscus arboreus* exhibiting particles of *Citrus leprosis virus C* within cisternae of the endoplasmic reticulum in leaf parenchymal cells, and **C**, *Hibiscus rosa-sinensis* showing electron-dense viroplasm.

RT-PCR detection and amplicons sequencing. CiLV-C was detected when RT-PCR assays amplified the expected 344-bp fragment from symptomatic tissues of *H. rosa-sinensis*, *M. arboreus*, *G. robusta*, *B. orellana*, and *C. sinensis* Pera infested with viruliferous mites. Control samples, infested with nonviruliferous mites, did not yield any bands (Table 1).

The consensus sequences amplified from *H. rosa-sinensis*, *M. arboreus*, *B. orellana*, *G. robusta*, and *C. sinensis* Pera showed high identity with *mp* sequences of CiLV-C available in the GenBank (Table 2), confirming that these species are hosts of the virus.

Discussion

The data obtained in this study confirm the early evidence (35,36) that CiLV-C is able to infect non-citrus plants that often occur near citrus orchards. Prior to this work, the virus had been transmitted with relative difficulty to only a few herbaceous hosts by mechanical inoculation (14). In more recent studies, Rodrigues et al. (41) and Groot et al. (22) demonstrated that viruliferous mites are able to transmit CiLV-C to *Solanum violaeifolium* and *P. vulgaris*, respectively, although it was not possible to transmit the virus back from *S. violaeifolium* to citrus. In Colombia, *Swinglea glutinosa* trees, a relative of citrus in the Rutaceae family that is commonly used as a hedgerow in orange groves, were found naturally infected by CiLV-C (31). Maia and Oliveira (35) claimed that CiLV-C could be transmitted to citrus from alternative hosts using the mite vector but the authors did not confirm the presence of the virus in those plant species.

This work demonstrates that CiLV-C may have a broader host range than previously thought, including plants in several families as diverse as the Bixaceae, Malvaceae (two genera), and Proteaceae. The fact that most of these alternative hosts are often associated with citrus orchards and also host the mite vector suggests that they may serve as reservoirs of the virus under field conditions.

H. rosa-sinensis and *M. arboreus* are malvaceous plants utilized as hedgerows in several countries and might serve as large-scale sources of CiLV-C. The virus caused only chlorotic spots in *H. rosa-sinensis*, whereas *M. arboreus* reacted with necrotic and green spots in senescent leaves. There are reports of other *Brevipalpus* spp.-transmitted viruses naturally infecting these ornamental malvaceous plants (28,36) but the primers used in this study do not detect these other viruses (J. Freitas-Astúa and E. W. Kitajima, unpublished data). *G. robusta*, of Australian origin, was introduced into several tropical countries to be used as windbreaks in orchards due to its desirable characteristics of being evergreen, growing quickly with an erect form, and having low intraroot competition

Table 2. Comparison among the consensus sequences of the region within the *Citrus leprosis virus C* movement protein gene from different hosts

Host plant	GenBank accession number	Identity with GenBank available sequences
<i>Hibiscus rosa-sinensis</i>	JQ944799	99% (DQ388513.1)
<i>Citrus sinensis</i> Pera	JQ944800	98% (EU257507.1)
<i>Malvaviscus arboreus</i>	JQ944801	99% (DQ272491.1)

(15). *B. orellana* is another plant species largely employed as a windbreak in Brazil. Both species exhibit minor pinpoint symptoms or remain asymptomatic when infected with CiLV-C, suggesting that other hosts not assessed in this study might also be asymptomatic.

The incidence (Table 1) and diversity of symptoms (Fig. 1) can be interpreted as different reactions to the virus, suggesting that these species exhibit various levels of susceptibility, which are also observed within citrus species and hybrids and even other rutaceous plants (4,7,16,17). Moreover, the mites behave differently on different hosts (10,17), with potential impact on viral transmission, because CiLV-C depends solely on the vector for its dissemination in the field (9,40).

The three techniques used to detect the virus in alternative host plants—transmission, RT-PCR, and TEM—have been widely used for the diagnosis of viral diseases, particularly leprosis. Transfer of the mites to citrus plants 90 days after test-plant infestation assures that these individuals are the descendants of the original ones, because the mite life cycle is approximately 25 days, with maximum longevity of 44 days for single mites on different host plants at 25°C and 65 to 70% relative humidity (10,11). Moreover, because there is no transovarial transmission of CiLV-C in *Brevipalpus phoenicis* (4,7,23), these mite descendants had to acquire the virus in the alternative host plants in order to become viruliferous and transmit the virus to the citrus plants. Hence, this was a strong indication that the test plants were, indeed, hosts of CiLV-C.

The RT-PCR results confirmed the presence of CiLV-C in such hosts, indicating a broader host range than initially thought (Table 2). All the amplicons which were cloned and sequenced were similar to the CiLV-C *mp* sequences available in the GenBank, confirming the identity of the virus. The TEM analysis detected CiLV-C in cells of leaf lesions of *H. rosa-sinensis*, *M. arboreus* (Fig. 2), and *C. sinensis* Pera. Despite intense scrutiny, viral particles or cytopathic alterations in *G. robusta* and *Bixa orellana* could not be found by TEM. RT-PCR has been more sensitive than TEM in detecting CiLV-C (31), because RT-PCR uses a larger amount of infected tissue while TEM explores only a very small volume of the lesion. Furthermore, it has been demonstrated that, in new lesions, most of the cells contain virus and viroplasm but, in mature lesions, they are found only in a few cells (19). It is likely that the incidence of CiLV-C infected cells in *G. robusta* and *B. orellana* was very low, because the leaves showed no symptoms. This may have contributed to the lack of CiLV-C detection by TEM.

The identification of these symptoms in the reported alternative hosts may help identify disease foci in citrus groves, allowing surveys near citrus production areas. This is the first work to show that windbreaks and hedgerows plants often found in proximity to citrus orchards can host not only the *Brevipalpus phoenicis* vector but also the leprosis causal agent, CiLV-C. Although the mites only move over short distances on infested plants (1), the presence of alternative host plants may represent a significant risk for disease dispersion, and fences should be considered in strategies of virus control and management. However, whether or not these hosts play any role in the epidemiology of leprosis under natural conditions still needs to be addressed. Another point to be raised is that, thus far, CiLV-C and the *Brevipalpus* spp.-transmitted viruses, with the exception of OFV, seem to be confined to the American continent. The ornamentals *H. rosa-sinensis* and *M. arboreus* may serve as carriers of CiLV-C-viruliferous *B. phoenicis* to virus-free regions, including other continents. Hence, extra care should be taken to avoid dissemination of these hosts to new areas.

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