

# Transmission of viruses associated with cytoplasmic and nuclear leprosis symptoms by *Brevipalpus yothersi* and *B. californicus*

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**Abstract** Two groups of viruses cause the citrus disease complex known as leprosis: the cytoplasmic type, of the genera *Cilevirus* and *Higrevirus*, and the nuclear type, of the genus *Dichorhavirus*. It has been shown that the cilevirus *Citrus leprosis virus C* (CiLV-C) is transmitted by *Brevipalpus yothersi*. Within the genus *Dichorhavirus*, CiLV-N is a recently described and distinct species present in Brazil and transmitted by *B. phoenicis* sensu stricto, whereas the species found in Mexico and Colombia are strains of *Orchid fleck virus* (OFV-citrus), suspected to be vectored by *B. californicus*. A study was conducted to determine whether *B. californicus* and *B. yothersi* can acquire and inoculate Mexican isolates of CiLV-C and OFV-citrus to sweet and acid citrus species (sweet orange, mandarin, grapefruit, sour orange, Persian lime and Mexican lime) in experiments set up in areas in Mexico with prevalence of the respective viruses. *Brevipalpus californicus* acquired OFV-citrus and transmitted it to all the citrus cultivars included in the experiment, while *B. yothersi* acquired CiLV-C and transmitted it only to sweet citrus cultivars (sweet orange, mandarin, and grapefruit). Both mite species were able to become established and reproduce in the experimental plants for nearly a year. This study represents

the first experimental evidence of OFV-citrus transmission by *B. californicus*, as well as evidence that *B. yothersi* cannot transmit this virus.

**Keywords** *Brevipalpus*-transmitted diseases · *Cilevirus* · *Citrus* · *Dichorhavirus* · Mexico

## Introduction

Leprosis is a group of diseases characterized by the appearance of local lesions on the leaves, stems and fruits of different citrus species, cultivars and hybrids (genus *Citrus*, family Rutaceae). Losses caused by leprosis to crops include reduced yield, increased production costs and death of twigs (Rodrigues et al. 2003).

The first reports of citrus leprosis date back to the mid-nineteenth century in Florida, USA. There, a disease of sweet orange (*Citrus x sinensis* Osbeck) that caused severe symptoms on twigs and fruits was called “scaly bark” or “nail head rust” (Fawcett 1911). In Paraguay a disease appeared that attacked sweet orange and caused similar symptoms to those observed in Florida (Spegazzini 1920); this disease was later found in Argentina and Brazil (Bitancourt 1955) and was named “lepra explosiva” or “variola”. Knorr and DuCharme (1951) compared the symptoms of lepra explosiva in South America (Argentina, Brazil, and Paraguay) to those of scaly bark in Florida, concluded that they were synonymic and generalized the name of citrus leprosis to refer to this disease.

Pioneer works on the etiology of citrus leprosis were controversial and inconclusive. Its proposed causal agents were fungi (Fawcett 1911), mites (Vergani 1945) or viruses (Frezzi 1940; Knorr 1968). Although with some difficulty, leprosis was mechanically transmitted, giving support to its viral etiology (Colariccio et al. 1995).

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Kitajima et al. (1972) observed samples of sweet orange and grapefruit (*Citrus x paradisi* Macfad) with leprosis symptoms under the transmission electron microscope (TEM) and found viral particles and translucent inclusions in the cell nuclei. Also using TEM, Kitajima et al. (1974) and Colariccio et al. (1995) observed viral particles and dense, vacuolated inclusions in the cell cytoplasm of sweet orange samples with leprosis symptoms. This led Kitajima et al. (2003) and Rodrigues et al. (2003) to postulate that there are two different types of leprosis, the nuclear and the cytoplasmic types (provisionally named CiLV-N and CiLV-C, respectively, by these authors).

A method to detect CiLV-C by reverse-transcription polymerase chain reaction (RT-PCR) was developed by Locali et al. (2003). Later, the complete genome of this virus was sequenced and it was classified as the type member of the genus *Cilevirus* (Locali-Fabris et al. 2006). In sweet orange plants with leprosis-like lesions collected in Colombia, cytopathological symptoms characteristic of leprosis of the cytoplasmic type were observed by TEM but those samples failed to react to enzyme-linked immunosorbent assay (ELISA) and RT-PCR-based methods to identify CiLV-C. A new virus was extracted from these samples, and its genome was characterized; it was called *Citrus leprosis virus C2* (CiLV-C2) and placed in the genus *Cilevirus* (Roy et al. 2013a).

Roy et al. (2015b) analyzed samples of different citrus species from Querétaro State, Mexico, with symptoms of leprosis; they all resulted negative to CiLV-C and CiLV-C2 but presented translucent inclusions in the cell nuclei, as well as viral particles in the nuclei and cytoplasm, like those described by Kitajima et al. (1972). With samples from different localities in Querétaro state, Mexico, Roy et al. (2013b, 2013c, 2015b) determined the complete genome of a nuclear type leprosis virus and named it CiLV-N. Independently, Cruz-Jaramillo et al. (2014) determined the complete genome of a closely related virus isolated from sour orange (*Citrus aurantium* L.) collected in Jalisco, Mexico; it was designated as Citrus necrotic spot virus or CNSV. Meanwhile, Hartung et al. (2015) partially characterized the genome of a virus present in orange peels with leprosis symptoms collected in Florida in the 1950s. It was closely related to the virus called CiLV-N by Roy et al. (2013b, 2015a, 2015b) and was designated as CiLV-N0.

The acronym CiLV-N was applied to a virus causing the nuclear type of leprosis that was found in Querétaro, Mexico (Roy et al. 2013b, 2015a, 2015b). However, its close relatedness with OFV was recognized, leading Dietzgen et al. (2014) to propose that it is a strain of OFV instead of a distinct virus. In addition, Ramos-González et al. (2017) characterized a distinct virus causing nuclear leprosis from citrus samples collected in the state of São Paulo, Brazil. The samples examined by Kitajima et al. (1972), later shown to correspond to nuclear

leprosis, were collected in the same area. By priority, Ramos-González et al. (2017) proposed that this virus should be named *Citrus leprosis N* (CiLV-N), whereas the virus causing nuclear leprosis in Mexico, as a citrus-infecting strain of OFV, should be named OFV-citrus. The above nomenclature is followed in this paper. Since there are several strains of OFV-citrus, they could be distinguished by numbers; for instance, the strain characterized by Hartung et al. (2015) could be OFV-citrus0.

Among citrus genotypes, sweet orange is the main host of most viruses causing leprosis, both cytoplasmic and nuclear types (Bastianel et al. 2010). In contrast, OFV-citrus in Mexico has a considerably wider host range that includes several species of both sweet and acid citrus (Roy et al. 2015b). CiLV-C has expanded its distribution northward in the last decades, reaching southeast Mexico in 2005 (Izquierdo-Castillo et al. 2011). Recently, citrus plants with symptoms of leprosis have been found in many sites in Mexico, even very close to the border with the USA, although information is not available about which type of leprosis is present at each site (SAGARPA 2017).

Mites of the family Tenuipalpidae have consistently been observed associated with leprosis symptoms, so their possible role as causative agents of these symptoms or vectors of a possible pathogen has been considered. Frezzi (1940) identified *Tenuipalpus pseudocuneatus* Blanchard as a vector of a possible virus that causes leprosis. Meanwhile, Musumeci and Rossetti (1963) associated *Brevipalpus phoenicis* (Geijskes) to leprosis, and Knorr (1968) reproduced symptoms of this disease when inoculating *Brevipalpus californicus* (Banks). Recent works have confirmed the role of *Brevipalpus* mites as vectors of both CiLV-C and CiLV-C2; in every case, the identity of the virus strain was confirmed by RT-PCR (Nunes et al. 2012a, 2012b; Roy et al. 2013a).

The specific identity of the involved *Brevipalpus* mites has been subject to discussion. *Tenuipalpus pseudocuneatus* was later qualified as a synonym of *Brevipalpus obovatus* Donnadieu by Pritchard and Baker (1951), while Beard et al. (2015) determined that *B. phoenicis* sensu lato is really a group made up of eight species. Important research data on the species involved in transmitting leprosis cite *B. phoenicis* as a vector (Boaretto and Chiavegato 1994; Colariccio et al. 1995; Rodrigues et al. 1997; Roy et al. 2013a), so the specimens used for transmission must be reviewed, if they are still available, to corroborate which species it truly is within the *phoenicis* group. Moreover, mixed populations of *B. californicus* and *B. phoenicis* have been documented (Knorr 1968; Salinas-Vargas et al. 2013) as well as *B. phoenicis* with *B. obovatus* (Cáceres et al. 2013). Thus, a clear relationship between a specific type of virus and its vector is not always available.

The correct identification of the mites capable of transmitting a certain type of leprosis is especially important since

these mites seem to be specific in vectoring a given virus. Knorr (1968) reproduced symptoms of leprosis when inoculating plants with *B. californicus* and *B. obovatus*, but failed when inoculating *B. phoenicis*. Roy et al. (2015a) observed in Mexico that the distribution of *B. yothersi* widely coincides with that of CiLV-C in warm lowlands, while the distribution of *B. californicus* widely coincides with that of OFV-citrus in temperate highlands. CiLV-C and CiLV-C2 are vectored by *B. yothersi* (Ramos-González et al. 2016; Roy et al. 2013a), while CiLV-N is vectored by *B. phoenicis sensu stricto* (Geisjkes) (Ramos-González et al. 2017). The distribution areas of the mentioned *Brevipalpus* species are partially or totally disjunct (Roy et al. 2015a; Salinas-Vargas et al. 2013) and the distribution of the different types of leprosis viruses seem to be those of their respective vectors (Ramos-González et al. 2017). These facts have important implications in the management of the group of diseases known as leprosis. Because of this, the objective of the present work was to define whether *B. californicus* and *B. yothersi* have the capability to acquire and inoculate CiLV-C and/or OFV-citrus.

## Material and methods

### Acquisition of the biological material

Mites of two species of the genus *Brevipalpus* were collected in areas free of CiLV-C and OFV-citrus: Texcoco, state of Mexico, Mexico, at 19°27'46.42"N and 98°54'14.75"W, with an altitude of 2246 m, and Atoyac de Alvarez, Guerrero, Mexico, at 17°12'01.51"N and 100°25'56.39"W, with an altitude of 45 m. Colonies were founded on sour oranges with the collected mites, using the method described by Franco et al. (2007) with slight modifications. In short: each fruit was partially submerged in molten paraffin leaving the area where the mite would be placed uncovered. On the paraffin-free area, a mixture of plaster, flour, and sand (1:1:1 by weight) was placed and humidified to form a thick paste which, once dried, would mimic scale-like lesions to facilitate the establishment of the mites. A single female was placed on each fruit. The oranges were replaced when they appeared wilted, approximately every 3 weeks.

Once the colonies of mites from the mentioned locations were established, individuals were mounted in slides with Hoyer liquid (Krantz and Walter 2009), or processed for observation under the scanning electron microscope following the method by Bozzola and Russell (1992) with slight modifications. A Carl Zeiss Axiostar Plus phase contrast microscope and a Zeiss ΣIGMA VP scanning electron microscope were used. The identity of the species was confirmed based on the coincidence of redescrptions in revision works (Baker and Tuttle 1987; Beard et al. 2015; Pritchard and Baker 1951; Welbourn et al. 2003).

To confirm that these mites were virus-free, total RNA was extracted from groups of at least 10 specimens using the CTAB technique (Locali et al. 2003), and analyzed through RT-PCR using the RNeasy Mini and OneStep RT-PCR kits (Qiagen), following the manufacturer's instructions. To detect CiLV-C, the MPF and MPR primers were used (Locali et al. 2003), while for OFV-citrus, the CiLV-N-NPF and CiLV-N-NPR primers were used (Roy et al. 2014). The Nad5f and Nad5mr primers were included in the reactions to amplify 180 bp segments of plant DNA as controls to minimize the risk of false negatives (Menzel et al. 2002).

### Acquisition of CiLV-C and OFV-citrus by *B. californicus* and *B. yothersi*

*B. californicus* and *B. yothersi* specimens from the virus-free colonies were used. Groups were selected including 10 larvae, nymphs, or adults of each species, with three replicates of each stage. They were confined on sweet orange leaves with symptoms of leprosis, cytoplasmic type, from Huimanguillo, Tabasco, Mexico, for periods of 24, 48, and 72 h, in order for them to acquire CiLV-C. Separately, and using the same method, the test was repeated with sour orange leaves with leprosis symptoms, nuclear type, from Toluimán, Querétaro, Mexico.

Once the time periods transpired, total RNA was extracted from each group of 10 mites, and the presence of CiLV-C or OFV-citrus was determined through RT-PCR as previously described. The same technique was applied to 250 mg of leaves used to feed the mites. The positive samples were processed and sent to Macrogen (South Korea) to obtain their genomic sequences, which were edited with the BioEdit software to obtain consensus. They were compared against sequences deposited in GenBank with the Blast software.

### Citrus leprosis transmission by *Brevipalpus californicus* and *B. yothersi* to sweet and sour citrus

Due to the disjunct distribution of CiLV-C and OFV-citrus in Mexico and the quarantine established by the Mexican government (COFEMER 2006), this study had to be done in two locations: one at Colegio de Postgraduados, campus Tabasco, in the municipality of Cárdenas, Tabasco State, which is an area with prevalence of cytoplasmic leprosis (CiLV-C). It is located at 17°59'10.45"N and 93°35'2.70"W, and an altitude of 22 m. The second location was Toluimán, Querétaro, which has a prevalence of nuclear leprosis (OFV-citrus), located at 20°53'35.8"N and 99°54'59.9"W, with an altitude of 1607 m. At each site there was an enclosed nursery, and in each an experiment was established where citrus plants were separately inoculated with *B. californicus* or *B. yothersi*.

At the location with a prevalence of CiLV-C (Cárdenas, Tabasco), the experiment was performed with potted plants

of Dancy mandarin (*C. reticulata*), Fairchild tangerine (*C. clementina* Hort. exTanaka  $\times$  *Citrus*  $\times$  *tangelo* Ingram and Moore), Satsuma mandarin (*C. unshiu* Marcow), Rio Red grapefruit (*C. paradisi*), Valencia orange (*C. sinensis*), Campbell orange (*C. sinensis*), Marrs orange (*C. sinensis*), sour orange (*C. aurantium*) and Persian lime (*C. latifolia*). At the location with a prevalence of OFV-citrus (Tolimán, Querétaro), the experiment was done with potted Valencia orange, Navel orange, sour orange, Reina mandarin (*C. reticulata*), Dancy mandarin, Monica mandarin (*C. reticulata*), Rio Red grapefruit, Persian lime, Mexican lime (*Citrus aurantifolia* [Christm.] Swingle) and sweet lime (*C. limetta*). All plants used in the experiments were 7 to 8 months old with a height between 100 and 150 cm. These plants came from a certified nursery, and were thus likely to be leprosis-free; however, this was confirmed through RT-PCR using the techniques above mentioned.

At each experimental site, a completely randomized design was used with four replicates per treatment. All plants received a spray of elemental sulfur (250 mL/100 L water) 1 month prior to the experiment. To facilitate the establishment of the mites on the plants, a paste was made with plaster, sand, wheat flour and water as mentioned before, which was applied with a small brush on the leaves where the mites were going to be transferred, until forming a 3 cm diameter circle. The plants of each species or variety were infested with mites from the following background: (1) mites collected from sweet orange leaves with leprosis symptoms and presence of CiLV-C (Cárdenas, Tabasco) or OFV-citrus (Tolimán, Querétaro), confirmed through RT-CPR; (2) mites from the virus-free colonies which were previously confined for 72 h on leaves with leprosis lesions (cytoplasmic in Cárdenas, Tabasco; nuclear in Tolimán, Querétaro) for feeding and acquisition; (3) mites from virus-free colonies; (4) control plants, without inoculation. Each plant received 10 mite specimens (larvae or nymphs). The plants were separated by at least 1 m from each other, and both sites had walls to limit wind movement. In Cárdenas, Tabasco, a single infestation with *B. yothersi* was done (July, 2014), while another group of plants was infested twice with *B. californicus* (March and June, 2015). In Tolimán, Querétaro, a group of plants was infested three times with *B. yothersi* (January, April, and June, 2014), while another was infested twice with *B. californicus* (March and June, 2015).

The plants were kept in their respective nurseries to register the appearance of leprosis symptoms. A photographic record of each plant was taken to describe the progress of the disease symptoms. Finally, samples of leaves with or without leprosis symptoms were taken to confirm the presence of mites and to detect OFV-citrus through RT-PCR, with the primers and techniques above mentioned. The positive samples were sent to Macrogen (South Korea) to be sequenced, edited with BioEdit and compared against sequences in GenBank.

## Results

### Identification of the collected mites

The mites collected in Atoyac de Alvarez were identified as *B. yothersi*, while those collected in Texcoco were identified as *B. californicus* species group B. Figure 1 shows the diagnostic characteristics of each species; they are the ornamentation pattern in the propodosoma, opisthosoma and microplates, as well as the shape of the spermatheca (Beard et al. 2012). With these mites, the colonies were founded and later used in the CiLV-C and OFV-citrus acquisition and inoculation tests.

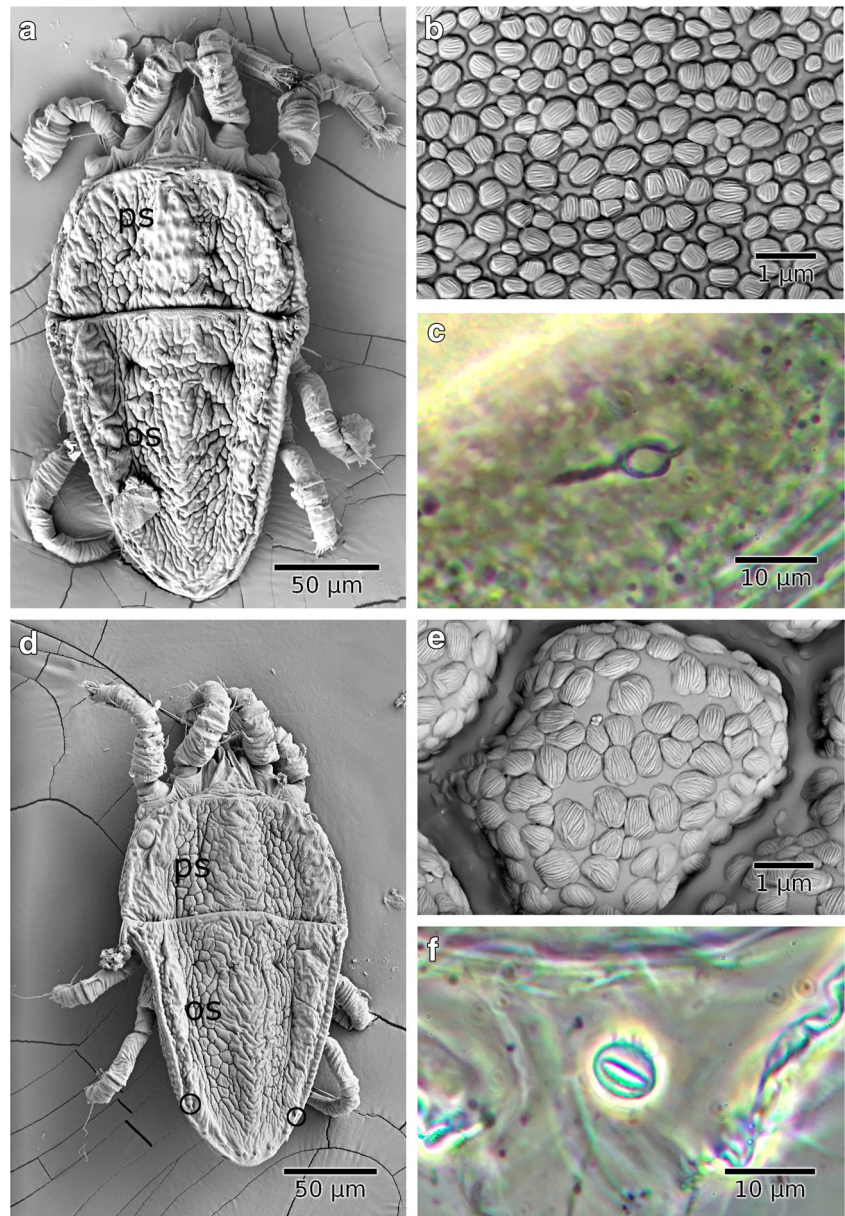
### CiLV-C and OFV-citrus acquisition by *B. yothersi* and *B. californicus*

The presence of CiLV-C was detected in *B. yothersi* larvae and nymphs after feeding on symptomatic leaves for 24 and 48 h (one of three in both cases), while two out of three adult mite groups were CiLV-C positive when they had been fed with symptomatic tissues for 24 and 48 h. All the cases where *B. yothersi* fed from OFV-citrus infected leaves were negative for the presence of this virus. In the case of *B. californicus*, no presence of CiLV-C was detected in any developmental stage or feeding time with symptomatic tissues (Table 1). In the OFV-citrus acquisition test, only in *B. californicus* was the virus detected in nymphs and adults (one of three) that had fed on symptomatic tissues for 48 or 72 h (Table 2). All the amplified segments were sequenced and compared against their respective cognates in GenBank (Table 3).

### CiLV-C transmission by *B. californicus* and *B. yothersi* to sweet and acid citrus cultivars

When trying the infestation with *B. yothersi* for the transmission of CiLV-C on nine citrus cultivars, symptoms characteristic of cytoplasmic leprosis appeared in Valencia orange, Campbell orange, Marrs orange, Satsuma mandarin and Dancy mandarin infested with mites from treatment 1 (mites collected from plants with leprosis symptoms) and from treatment 2 (mites from the virus-free colony forced to feed on symptomatic leaves). No leprosis symptoms were observed in the plants infested with mites from treatment 3 (mites from the virus-free colony), or the control plants with no mite infestation. Through RT-PCR the presence of CiLV-C was confirmed in the plants that developed leprosis symptoms, while all the asymptomatic samples came out negative. After a year of observations, none of the citrus cultivars inoculated with *B. californicus* developed leprosis symptoms nor was the presence of CiLV-C detected.

**Fig. 1 a–c** Diagnostic characters of *B. yothersi*. **a** Scanning electron (SEM) micrograph of dorsal overview showing central propodosomal area with areolae (ps) and central opisthosomal area with V-shaped folds (os). **b** SEM micrograph of microplates. **c** Phase contrast micrograph of spermatheca with an apical stipe. **d–f** Diagnostic characters of *B. californicus*. **d** SEM micrograph of dorsal propodosoma showing irregular reticulations (ps), central opisthosomal area with V-shaped folds (os) and setae f2 (encircled). **e** SEM micrograph of microplates. **f** Phase contrast micrograph of a highly sclerotized oval spermatheca



Out of all the treatments where mites were inoculated, at least some specimens were recovered, which were mounted on slides to verify their identity. In every case, the species identified was the same that had been inoculated. For the time period between mite inoculations and

their recovery from the experimental plants, the recovered mites were considered descendants of the original mites inoculated. Their presence confirmed that both *B. californicus* and *B. yothersi* had established on the experimental plants.

**Table 1** CiLV-C acquisition by *Brevipalpus californicus* and *B. yothersi* larvae, nymphs, and adults fed on Valencia orange leaves with cytoplasmic leprosis symptoms

Developmental stage	CiLV-C acquisition by <i>B. californicus</i>			CiLV-C acquisition by <i>B. yothersi</i>		
	24 h	48 h	72 h	24 h	48 h	72 h
Larvae	0/3 <sup>a</sup>	0/3	0/3	1/3	1/3	0/3
Nymphs	0/3	0/3	0/3	1/3	1/3	0/3
Adults	0/3	0/3	0/3	2/3	0/3	2/3

<sup>a</sup> Positive (pooled 10 mite specimens)/total cases

**Table 2** OFV-citrus acquisition by *Brevipalpus californicus* and *B. yothersi* larvae, nymphs, and adults fed on sour orange leaves with nuclear leprosis symptoms

Developmental stage	OFV-citrus acquisition by <i>B. californicus</i>			OFV-citrus acquisition by <i>B. yothersi</i>		
	24 h	48 h	72 h	24 h	48 h	72 h
Larvae	0/3 <sup>a</sup>	0/3	0/3	0/3	0/3	0/3
Nymphs	0/3	1/3	1/3	0/3	0/3	0/3
Adults	0/3	1/3	1/3	0/3	0/3	0/3

<sup>a</sup> Positive (pooled 10 mite specimens)/total cases

### Description of cytoplasmic leprosis symptoms

In Valencia orange, the symptoms appeared as round chlorotic spots with a light green center surrounded by a yellow, or lighter green, chlorotic halo in an approximate proportion of 50% for each chlorotic halo, and sometimes two or more symptoms together, forming irregular shapes. In some cases, small brown spots could be seen on the division between both halos, appearing to form a brown ring. In Marrs orange, the spots were oval shaped, not so rounded as in Valencia orange, with a light green halo in the center, sometimes triangular, surrounded by a yellowish chlorotic halo. This halo was larger than the central one. When the spot was small, the central halo could not be distinguished and it had a uniform yellow coloring. In Campbell orange, the spots were rounded and light yellow, with no differently colored center, as in the case of Valencia orange and Marrs orange. The edges of the spot were light green with undefined limits. In Dancy mandarin, the spots were rounded to oval shaped with a light green chlorotic center surrounded by a ring of dead tissue, and a third, external, yellow, chlorotic halo. In Satsuma mandarin, the spots were rounded and light green, with occasionally a green center. This cultivar had the highest number of spots, as well as the smallest spots. In twigs, the spots were very similar to those on the leaves, with rounded shape and a brown to necrotic center 3 months after their appearance, surrounded by a light green to yellow chlorotic halo. Figure 2a and b illustrate these symptoms.

### OFV-citrus transmission by *Brevipalpus californicus* and *B. yothersi* to sweet and acid citrus cultivars

When 10 citrus cultivars were infested with *B. yothersi*, a year passed with no apparent characteristic nuclear leprosis symptoms. All the plant tissue samples (asymptomatic) were negative for OFV-citrus through RT-PCR. When the plants were infested with *B. californicus* to transmit OFV-citrus to all the 10 different citrus cultivars, 4 months after the second infestation characteristic nuclear leprosis symptoms appeared in Valencia orange, sour orange, Reina mandarin, Dancy mandarin, Rio Red grapefruit, Persian lime, Mexican lime, and sweet lime infested with mites from treatments 1 and 2. The samples of tissues with symptoms of nuclear leprosis were positive for OFV-citrus through RT-PCR (Table 4). Similarly to the experiment conducted in Tabasco, the recovered mites were the same species as those inoculated.

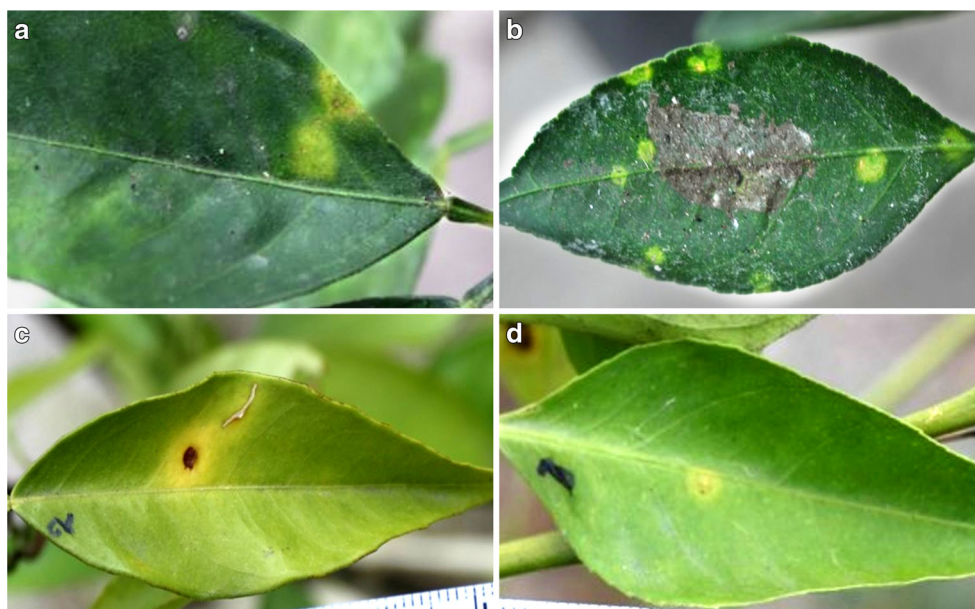
### Description of nuclear leprosis symptoms

In Valencia orange, the lesions were round chlorotic spots with a brown to black necrotic center in the shape of a crust, surrounded by a yellow to light green chlorotic halo, with a larger necrotic center. In Navel orange, the spots were rounded with a brown round halo center which formed dead crusty tissue surrounded by a yellow chlorotic halo in similar proportions. In Reina and Dancy mandarin the spots were rounded with a brown to black necrotic center surrounded by a light

**Table 3** Percent identity of the obtained sequences with sequences of CiLV-C and OFV-citrus available in GenBank

Development stage and virus acquisition time	Virus to be compared with	Identity	GenBank access with the highest identity
Larvae 24 h	CiLV-C	98%	JQ944801
Nymphs 24 h	CiLV-C	98%	JQ944802
Adults 24 h	CiLV-C	98%	JQ944802
Larvae 48 h	CiLV-C	96%	JQ944802
Nymphs 48 h	CiLV-C	98%	JQ944802
Nymphs 48 h	OFV-C	99%	KF209275
Adults 48 h	OFV-C	99%	KF209275
Adults 72 h	OFV-C	98%	KF209275

**Fig. 2** **a, b** Characteristic cytoplasmic leprosis symptoms in two citrus species 30 days after infestation with CiLV-C viruliferous specimens of *B. yothersi*. **a** Valencia orange. **b** Satsuma mandarin. **c, d** Characteristic nuclear leprosis symptoms in two citrus species 60 days after being infested with OFV-citrus viruliferous *B. californicus* specimens. **c** Valencia orange. **d** Sweet lime



green chlorotic halo with undefined edges. As a slight difference, in Dancy mandarin the halo was yellowish. In Rio Red grapefruit and sweet lime the spots were very similar, round with a small brown crusty center surrounded by a yellow chlorotic halo. In sour orange the spots were irregular, similar to those of Dancy mandarin. Figure 2c and d illustrate these symptoms.

## Discussion

Consistently, this research proved that *B. californicus* was capable of acquiring and inoculating OFV-citrus, but there

was no evidence that it acquired or inoculated CiLV-*C. Brevipalpus yothersi* showed the opposite situation. OFV-citrus is a strain of OFV, which originally infects orchids (Childers and Rodrigues 2011), and thus the proof of its transmission by *B. californicus* from and to citrus is an original contribution of the present study.

In a pioneer work, Knorr (1968) observed the reproduction of leprosis symptoms when inoculating citrus plants with possibly viruliferous *B. californicus* specimens, but he did not observe this development of leprosis symptoms when the inoculation was done with *B. phoenicis* specimens. In the mentioned work, there were no microscopic or molecular evidence. However, Hartung et al. (2015) determined that the virus responsible for the leprosis

**Table 4** OFV-citrus transmission test in citrus species and cultivars by *Brevipalpus californicus*

Cultivar	Treatments <sup>a</sup>				Identity with OFV-citrus	GenBank access with highest identity
	1	2	3	4		
Valencia orange	4/4 <sup>b</sup>	0/4	0/4	0/4	97%	KF209275
Navel orange	0/4	1/4	0/4	0/4	97%	KJ195893
Sour orange	1/4	0/4	0/4	0/4	97%	KJ195893
Reina mandarin	3/4	1/4	0/4	0/4	96%	KF209275
Dancy mandarin	1/4	0/4	0/4	0/4	97%	KF209275
Monica mandarin	0/4	0/4	0/4	0/4		
Rio Red grapefruit	2/4	2/4	0/4	0/4	97%	KF209275
Persian lime	0/4	0/4	0/4	0/4		
Mexican lime	0/4	0/4	0/4	0/4		
Sweet lime	2/4	2/4	0/4	0/4	97%	KF209275

<sup>a</sup> Treatments: 1, viruliferous field mites with OFV-citrus; 2, mites fed on OFV-citrus infected leaves; 3, virus-free mites; 4, control (no mite infestation)

<sup>b</sup> RT-PCR results using CiLV-N-NPF and CiLV-N-NPR primers (Roy et al. 2014)

prevalent in Florida in the first half of the twentieth century was of the nuclear type, which they designated as CiLV-N0 (now proposed as OFV-citrus0). The results of both works agree with the results of the present study.

Contrastingly, Roy et al. (2013a) observed a mixed infection of CiLV-C2 and OFV-citrus with *B. phoenicis* and sweet orange in Colombia, while Roy et al. (2015a) observed a mixed infection of CiLV-C and OFV-citrus in sweet orange plants in Tabasco, Mexico, where *B. yothersi* is especially predominant. This suggests that at least two types of virus can be transmitted by a single mite species; in this case, *B. yothersi*. This could also be true in the case of Colombia, where the species was named as *B. phoenicis*, although it could actually be *B. yothersi* according to the revision by Beard et al. (2015).

The amount of viral RNA ingested by a mite is necessarily very small, so its detection through RT-PCR requires at least 10 specimens, which applies to both CiLV-C and OFV-citrus (Kubo et al. 2011; Roy et al. 2015a). Repeated cases of mixed populations of *B. yothersi* with *B. californicus* or *B. obovatus* have been documented (Knorr et al. 1968; Cáceres et al. 2013; Salinas-Vargas et al. 2013), hence there is the possibility that the samples of 10 or more mites used for viral RNA extraction might actually be composed by more than one mite species, each carrying a different virus. In the present research, there was strict control of the identity of the mites used for the experiment, since they came from colonies formed from a single individual (acquisition test and treatment 2 of the transmission test). Other mites were collected from the field, but all the recovered mites were the same species as the inoculated, thus we postulate that the mites subjected to RT-PCR were not a mixture of species.

*Brevipalpus californicus* was apparently not able to acquire or inoculate CiLV-C, while *B. yothersi* did not seem able to acquire or inoculate OFV-citrus. However, this assumption is not fully conclusive, as a mite might simply be less efficient to acquire a certain virus or the virus concentration might be so low as to make it undetectable through the method used, as commented by Choudhary et al. (2015). Nevertheless, the results of the present work are consistent enough to postulate that *B. californicus* is capable of transmitting OFV-citrus to citrus cultivars, while *B. yothersi* transmits CiLV-C. This postulate is supported by all the field data obtained in Mexico, where nuclear leprosis is mainly distributed at high altitudes with a temperate climate and where the predominant species is *B. californicus*, whereas cytoplasmic leprosis is prevalent at lower altitudes with a warm weather, where the predominant species is *B. yothersi* (Roy et al. 2015a). *Brevipalpus californicus* is known as the vector of OFV (Kondo et al. 2003). Thus, the fact that the Mexican virus causing nuclear leprosis has this mite as the vector strengthens the proposition that it is a strain of OFV. In contrast, Brazilian CiLV-N is a distinct *Dichorhavirus* and has a distinct *Brevipalpus* species as vector (Ramos-González et al. 2017).

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