

Citrus leprosis resistance within the citrus group

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Abstract Reported in Brazil since the 1930's, citrus leprosis, caused mainly by citrus leprosis virus C, has been a major concern for the national sweet orange production. In recent years, the disease has spread to several other countries and it is now considered a worldwide threat. The occurrence of the disease has been studied almost exclusively in sweet oranges because other citrus genotypes are of secondary relevance in Brazil and in some other American countries where it occurs. Here we report 12 resistant citrus genotypes among 160 accessions evaluated. After 90 days of the infestation with viruliferous mites, asymptomatic genotypes were observed in sour orange, lemon, grapefruit, mandarins, tangelo, and tangor groups. The results revealed promising genotypes resistant to the disease, which can be incorporated in citrus breeding programs aiming to obtain varietal resistance, and confirmed the susceptibility of many citrus genotypes to CiLV-C. This assay ratify the already reported uneven level of susceptibility within the citrus group.

Keywords Citrus breeding · Viral disease · *Brevipalpus* spp. · CiLV-C · *Cilevirus*

Introduction

Leprosis is one of the most important viral diseases in the Brazilian citrus production, caused by citrus leprosis virus C (CiLV-C) that is transmitted by the mite *Brevipalpus* spp. (Acari: Tenuipalpidae) [4, 22]. Recent taxonomic revision of the *B. phoenicis* group included several new morphological parameters and redefined the group into eight species [5]. So far, only *B. yothersi* and *B. papayensis* have been reported in main citrus production areas in Brazil [29], whereas *B. phoenicis* stricto sensu has been reported only in backyard citrus plants in São Paulo state [21].

Different viruses associated with *Brevipalpus* spp. and causing leprosis-like symptoms (chlorotic or necrotic localized lesions in citrus leaves, stems, and fruits) have been reported in citrus in Brazil [13, 21], Panama [11], Mexico [9, 25], Colombia [26, 27] and Hawaii [17]. However, in most places—and particularly in Brazil—the prevalent and most important leprosis-inducing virus is CiLV-C [14, 20].

This virus presents low genetic variability, with only two different phylogenetic clades, one originally found in Cordeirópolis, city in the São Paulo (SP), Brazil (CRD), detected from Mexico to Argentina, and the infrequent isolate from São José do Rio Preto, SP, Brazil (SJP) with reports only in the central-northern region of São Paulo State, Brazil, naturally infecting sweet orange and *Commelina benghalensis* plants [18, 20]. Under laboratory conditions, *B. yothersi* can transmit both isolates to sweet orange plants with ~ 90% of efficiency [20].

It is known that sweet oranges (*Citrus sinensis* L. Osbeck) are more susceptible to leprosis than mandarins (*C. reticulata* Blanco) [2]. Lemons [*C. limon* (L.) Osbeck], and limes [*C. aurantifolia* (Christmann) Swingle] are

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considered resistant to the virus [12]. However, few studies have assessed the response to leprosis extensively, and most of them were based solely on field observations. In fact, investigating the incidence and severity of leprosis in other citrus genotypes could strategically (i) clarify the importance of these plants in the disease epidemiology, (ii) determine which genotypes are susceptible to the virus and, consequently, expand the available information on the host range within the Rutaceae family, (iii) serve as important source of information for countries that produce other citrus genotypes but are still free of the disease, and (iv) identify possible sources of genetic resistance that can be used in breeding.

There are still many unsolved questions regarding the inheritance of leprosis resistance. Crosses between resistant and susceptible genotypes resulted in hybrids which generated F_1 (first generation progeny) with a Mendelian segregation ratio, and studies with QTLs (Quantitative Trait Loci) indicated that few genes were involved in the resistance [2, 3]. This paper reports the results of a large-scale screening for CiLV-C resistance in several citrus genotypes, indicating the presence of potential candidates for future breeding programs aiming for resistance towards leprosis.

Materials and methods

Genotypes

In total, 160 accessions from the citrus germplasm collection of the Centro de Citricultura Sylvio Moreira/Instituto Agronômico de Campinas (CCSM/IAC, Cordeirópolis, SP, Brazil) were evaluated for leprosis resistance/susceptibility, including 85 common mandarins (*Citrus reticulata*), 23 willowleafs (*C. deliciosa*), 16 tangors (*C. reticulata* × *C. sinensis*), six satsumas (*C. unshiu*), four clementines (*C. clementina*), three sour oranges (*C. aurantium*), one lemon (*C. limon*), three pummelos (*C. grandis*), three grapefruits (*C. paradisi*), six tangelos (*C. reticulata* × *C. paradisi*), four rootstock mandarins (*C. sunki* and *C. reshni*), two kumquats (*Fortunella* spp.) and two limequats (lime vs kumquat hybrids). Two sweet orange (*C. sinensis*) accessions were used as controls of inoculation since all accessions of this species are considered susceptible to leprosis [19].

Mites and virus inoculation

Citrus seedlings were maintained in small pots with an appropriate substrate on greenhouse conditions, in the CCSM/IAC, throughout the experiment. *B. yothersi*, viruliferous for CiLV-C isolate São José do Rio Preto (SJP),

were used to inoculate 6-month-old seedlings (about 20 cm). These mites were reared onto symptomatic sweet orange fruits and confirmed to be viruliferous by RT-PCR, following the protocol reported by Kubo et al. [15]. Five viruliferous mites were transferred to each seedling, with three to five repetitions (seedlings) per genotype, as described by Rodrigues et al. [24].

Disease evaluation

The incidence of leprosis symptoms was monitored weekly and evaluated at 90 days after mites infestation. The infection was assessed through the identification of the typical symptoms of the disease: localized chlorotic or necrotic spots on the leaves and stems. Disease incidence was obtained by the number of symptomatic plants/infested plants.

Severity was evaluated through the average of the three to five plants of each genotype using a descriptive scale developed by Rodrigues [19], where 0 = no lesions; 1 = few lesions in any organ, restricted to a section of the plant; 2 = lesions in more than one organ and/or distributed in more than one section of the plant; 3 = abundant lesions in all organs and evenly distributed in the plant; 4 = abundant lesions (all over the plant) and leaf or fruit drop; and 5 = the same as 4, with the addition of dieback.

RT-PCR assays

Symptomatic and non-symptomatic leaves from one representative of each group (mandarin and hybrids, sweet orange, sour orange, and lemon) were analyzed by RT-PCR using primers that specifically amplify part of the putative coat protein (p29) gene of CiLV-C [16]. The assays were performed using three to five replicates of each genotype, according to methodology described by Locali et al. [16].

Results and discussion

Of the 160 accessions evaluated, 148 (92.50%) exhibited leprosis symptoms in leaves and/or stems, regardless of severity. As expected, most citrus genotypes were susceptible to CiLV-C.

Incidence and severity of leprosis disease in mandarins and hybrids

Not only the sweet orange accessions were highly susceptible to leprosis, but also several mandarin genotypes of different species (*C. deliciosa*, *C. reticulata* and *C. clementina*) were symptomatic for the disease. These data corroborate those obtained by Bastianel et al. [3], who

Table 1 Analysis of the leprosis symptoms in mandarin and hybrids infested with mites viruliferous for CiLV-C

Common name/code	I	S	Common name/code	I	S
<i>Mandarin and hybrids</i>					
Tangerina IAC 574	0/5	0.0	Artanigne IAC 554	3/5	1.4
Dieberger IAC 456	0/5	0.0	Mandarina IAC 209	5/5	1.4
Mel IAC 205	0/5	0.0	Monselise IAC 433	5/5	1.4
Shikai IAC 551	0/5	0.0	Muscia IAC 228	5/5	1.4
Yousself Efendi IAC 501	0/5	0.0	Pectinifera Shekwasha IAC 526	5/5	1.4
Imperatriz IAC 565	1/3	0.3	Szinkon × Tizon IAC 568	4/5	1.4
Fremont IAC 543	1/3	0.3	W.S. IAC 446	4/5	1.4
Harris IAC 516	1/3	0.3	Cravo tardia IAC 436	5/5	1.6
Heen Naran IAC 559	1/3	0.3	Dancy IAC 207	5/5	1.6
Szibat 16 × Tizon IAC 524	1/3	0.3	Guidote IAC 452	5/5	1.6
Vermelha IAC 506	1/3	0.3	Jaraguá do Sul IAC 500	4/5	1.6
Dr Silvio IAC 572	2/5	0.4	Szinkon × Batangas IAC 569	5/5	1.6
Fortuna IAC 223	2/5	0.4	Wilking IAC 215	5/5	1.6
Mauritius IAC 563	2/5	0.4	Israel IAC 514	2/3	1.7
Kishiu IAC 550	3/5	0.6	Szibat IAC 558	2/3	1.7
Mand. × Clementina IAC 230	2/5	0.6	Vermelha IAC 510	2/3	1.7
Swatow IAC 171	3/5	0.6	Kaula IAC 496	4/5	1.8
Szinkon × Ladu IAC 548	3/5	0.6	Kinnow IAC 218	4/5	1.8
Tancan IAC 502	3/5	0.6	Large Local IAC 513	5/5	1.8
Tresca × Dancy IAC 211	2/5	0.6	Parson IAC 227	5/5	1.8
Cravo IAC 182	2/3	0.7	Shekwasha × Tizon IAC 542	5/5	1.8
Portuguesa IAC 186	1/3	0.7	Cravo Guidoti IAC 434	5/5	2.0
Cape Nartjee IAC 522	1/3	0.7	Ladu IAC 539	2/3	2.0
Emperor IAC 597	1/3	0.7	Rino IAC 517	5/5	2.0
Campiona IAC 191	2/5	0.8	Romana IAC 432	5/5	2.0
Nicaragua IAC 549	2/5	0.8	Sunwuikon IAC 562	5/5	2.0
Parker IAC 254	4/5	0.8	Tancan IAC 444	5/5	2.0
Surino IAC 538	4/5	0.8	Sunwikon IAC 576	3/3	2.0
Tangerina IAC 197	4/5	1.0	Branca IAC 184	5/5	2.2
Natsu Mikan IAC 187	4/5	1.0	Ladu × Batangas IAC 534	5/5	2.2
Ponkan IAC 172	4/5	1.0	Sul da Africa IAC 529	5/5	2.2
Vermelha IAC 508	3/3	1.0	Shekwasha × Calamondin IAC 573	5/5	2.2
Wilking IAC 566	1/3	1.0	Sylhat IAC 512	5/5	2.2
Cravo × Pomelo IAC 435	4/5	1.2	Oneco IAC 532	3/3	2.3
Jaraguá do Sul IAC 499	5/5	1.2	Shekwasha 149-007 IAC 420	5/5	2.6
Montevideo IAC 438	5/5	1.2	Carpe Nartjee IAC 521	5/5	2.8
Weshart IAC 210	5/5	1.2	Fairchild IAC 533	5/5	2.8
King Orange IAC 212	1/3	1.3	De Wildt IAC 545	3/3	3.0
Osceola IAC 503	2/3	1.3	Small 627 IAC 561	5/5	3.2
Thomas IAC 519	3/3	1.3	Kinnow IAC 176	3/3	3.3
Hansen IAC 596	1/3	1.3	Batangas IAC 504	3/3	3.3
Warnuco IAC 547	3/3	1.3	Depressa IAC 525	3/3	3.3
África do Sul IAC 557	5/5	1.4			
<i>Willowleaf mandarins</i>					
Late IAC 585	1/3	0.3	Ipanema IAC 208	4/5	1.4
Precoce IAC 581	1/3	0.3	Mogi das Cruzes IAC 606	5/5	1.4
117477 IAC 540	2/5	0.4	Rio IAC 584	4/5	1.4
Avana IAC 594	4/5	0.8	Corsega IAC 586	3/5	1.6
114412 IAC 577	4/5	1.0	Paiva IAC 588	5/5	2.0

Table 1 continued

Common name/code	I	S	Common name/code	I	S
Rio IAC 593	4/5	1.0	Tardia IAC 582	4/5	2.0
Nobilis IAC 199	4/5	1.0	Paulista IAC 567	5/5	2.2
Pernambucana IAC 578	5/5	1.0	Tardia IAC 591	5/5	2.4
Pará IAC 193	3/5	1.2	Revero IAC 195	5/5	2.8
10630 IAC 579	1/3	1.3	Willow IAC 186	5/5	2.8
Paraguaia IAC 600	1/3	1.3	Céu IAC 587	5/5	3.7
Comum IAC 583	5/5	1.4			
<i>Satsuma mandarin</i>					
Satsuma Wase AC 167	1/5	0.2	Satsuma IAC 429	4/5	0.8
Satsuma Anã IAC 428	2/5	0.4	Satsuma Owari IAC 219	5/5	1.8
Satsuma IAC 216	3/5	0.6	Satsuma IAC 527	5/5	2.4
<i>Clementine mandarin</i>					
Clementina IAC 174	4/5	0.7	Clementina IAC 431	5/5	1.4
Clementina Montreal IAC 231	4/5	0.8	Clementina IAC 175	2/3	2.0
<i>Tangelo</i>					
Minneola IAC 224	0/5	0.0	Seminole IAC 235	1/3	1.0
Minneola IAC 239	3/5	0.4	Webber IAC 232	2/3	1.3
São Jacinto IAC 238	2/3	1.0	Orlando IAC 225	5/5	2.0
<i>Tangor</i>					
Temple IAC 200	0/5	0.0	Mimosa IAC 253	5/5	1.4
Temple IAC 247	0/5	0.0	Reticulata IAC 246	5/5	1.4
Mimosa IAC 215	1/3	0.3	Maracujá IAC 251	5/5	2.0
Sabará IAC 241	1/3	0.7	Índia IAC 249	5/5	2.6
Baia × Mexeriqueira IAC 605	4/5	0.8	Baía × Cravo IAC 218	2/3	2.7
Bunca IAC 250	4/5	0.8	Rei IAC 255	2/3	2.7
Santa Maria Madalena IAC 603	4/5	1.0	Pera × Cravo IAC 217	3/3	2.7
Sangue de Boi IAC 242	2/3	1.3	Moreira IAC 602	5/5	3.4

Incidence (I) is the number of the symptomatic plants/total plants. Severity (S) is the average values of notes assigned to each plant, according to the descriptive scale developed by Rodrigues [19]

reported that, although often considered resistant, some mandarins are susceptible to CiLV-C. In this study, all of the 23 willowleaf mandarins (*C. deliciosa*), six satsumas (*C. unshiu*) and four clementines (*C. clementina*) tested exhibited conspicuous leprosis symptoms after mite inoculation, some cases with high susceptibility (Table 1). Additionally, only five out of the 85 common mandarins (*C. reticulata*) tested, IAC 574, Dieberger IAC 456, Mel IAC 205, Shikai IAC 551 and Youself Efendi IAC 501, remained asymptomatic throughout the experiment (Table 1). Other common mandarin genotypes exhibited variable severity scores when evaluated with the descriptive scale above described, ranging from 0.3 (few symptoms) to 3.3 (highly symptomatic) (Table 1). Altogether, this does not only point out to heterogeneity in the response of different mandarin groups to leprosis but also indicates that mandarins are overall less resistant than originally suggested. Interestingly enough, within the mandarins,

symptoms in the field tend to be less evident and concentrated in leaves rather than in fruits or stems, as often occur with sweet oranges [3]. This characteristic may lead to a decreased time of available source of inoculum in the field, less attraction of the lesions to the vector (since *Brevipalpus* mites reach higher population densities on fruits and stems than on leaves) and less damage to the plant. Consequently, this could explain at least part of the reduced losses associated with leprosis observed in mandarin orchards.

In the group of mandarins and their hybrids, Murcott is one of the most widely cultivated and consumed in Brazil. It is also known as highly resistant to leprosis [2, 4, 23] and, hence, it has been used in our breeding program as the parental line for leprosis resistance. Similarly to what has been reported to Murcott, two tangor accessions (Temple IAC 200 and Temple IAC 247) exhibited resistance to leprosis in our trial (Table 1). Other tangors,

Table 2 Analysis of the leprosis symptoms in citrus accessions infested with mites viruliferous for CiLV-C

Common name/code	I	S	Common name/code	I	S
<i>Sweet orange</i>					
Pêra Ovo IAC 181	5/5	1.6	Natal IAC 188	5/5	2.0
<i>Sour orange</i>					
Azeda IAC 245	0/5	0.0	Azeda Vitoria IAC 609	2/5	0.4
Azeda Spanish Italian IAC 246	0/5	0.0			
<i>Lemon</i>					
Meyer IAC 627	0/5	0.0			
<i>Pummelo</i>					
Hawaiian IAC 339	2/5	0.4	151427 IAC 340	4/5	1.6
Chinesa IAC 241	4/5	0.8			
<i>Grapefruit</i>					
Royal IAC 314	0/5	0.0	Marsh Seedless IAC 321	4/5	1.2
Rubi IAC 315	5/5	1.0			
<i>Rootstock mandarins</i>					
Sunki IAC 200	4/5	0.8	Suen-Kat IAC 202	5/5	1.4
Suen-Kat IAC 201	5/5	1.4	Cleópatra IAC 199	5/5	1.6
<i>Fortunella and hybrids</i>					
Kumquat Meiwa IAC 424	4/5	1.0	Limequat Eustis IAC 425	5/5	1.0
Kumquat Nipon IAC 422	5/5	1.0	Limequat Lakeland IAC 426	5/5	1.0

Incidence (I) is the number of the symptomatic plants/total plants. Severity (S) is the average values of notes assigned to each plant, according to the descriptive scale developed by Rodrigues [19]

however, displayed susceptible reactions, with severity scores ranging from 0.3 to as high as 3.4 (Table 1). This difference in susceptibility to leprosis is most likely related to the response of their parental lines. While there is no report of sweet orange genotypes resistant to CiLV-C [4], there are genotypes within the group of the mandarins with high levels of resistance, as previously reported [3] and observed in this work (Table 1).

Leprosis disease in other citrus groups

Among the 42 other citrus genotypes tested, only lemon Meyer IAC 627 (*C. limon*), grapefruit Royal IAC 314 (*C. paradisi*), Minneola IAC 224 tangelo, two sour orange and two Temple tangor (mandarin × sweet orange hybrid) accessions remained asymptomatic 90 days after inoculation (Table 2).

There are no confirmed reports of leprosis infecting lemons in the field, although Bitancourt [6] observed leprosis-like symptoms many decades ago in lemon trees. Since then, neither field observations nor experimental trials have suggested that *C. limon* might be a host of CiLV-C. Melzer et al. [17] identified leprosis-like symptoms in *C. volkameriana* in Hawaii caused by *Hibiscus green spot virus 2* (HGSV), the type member of the genus *Higrevirus* that share some characteristics with the cileviruses such as CiLV-C. Roy et al. [28] reported that

lemons, in addition to other six species of *Citrus* spp., are natural hosts of orchid fleck virus (OFV) strain citrus, previously known as citrus leprosis virus nuclear type [1, 10] causing leprosis-like symptoms in Mexico. Similarly, to our knowledge, there are no confirmed reports of CiLV-C infecting grapefruits, although such species is a reported host of OFV-citrus in Mexico [28] and of a different virus reported in Brazil more than 40 years ago causing leprosis-like symptoms by Kitajima et al. [13]. Such virus is likely citrus leprosis virus N, recently sequenced and characterized by Ramos-González et al. [21].

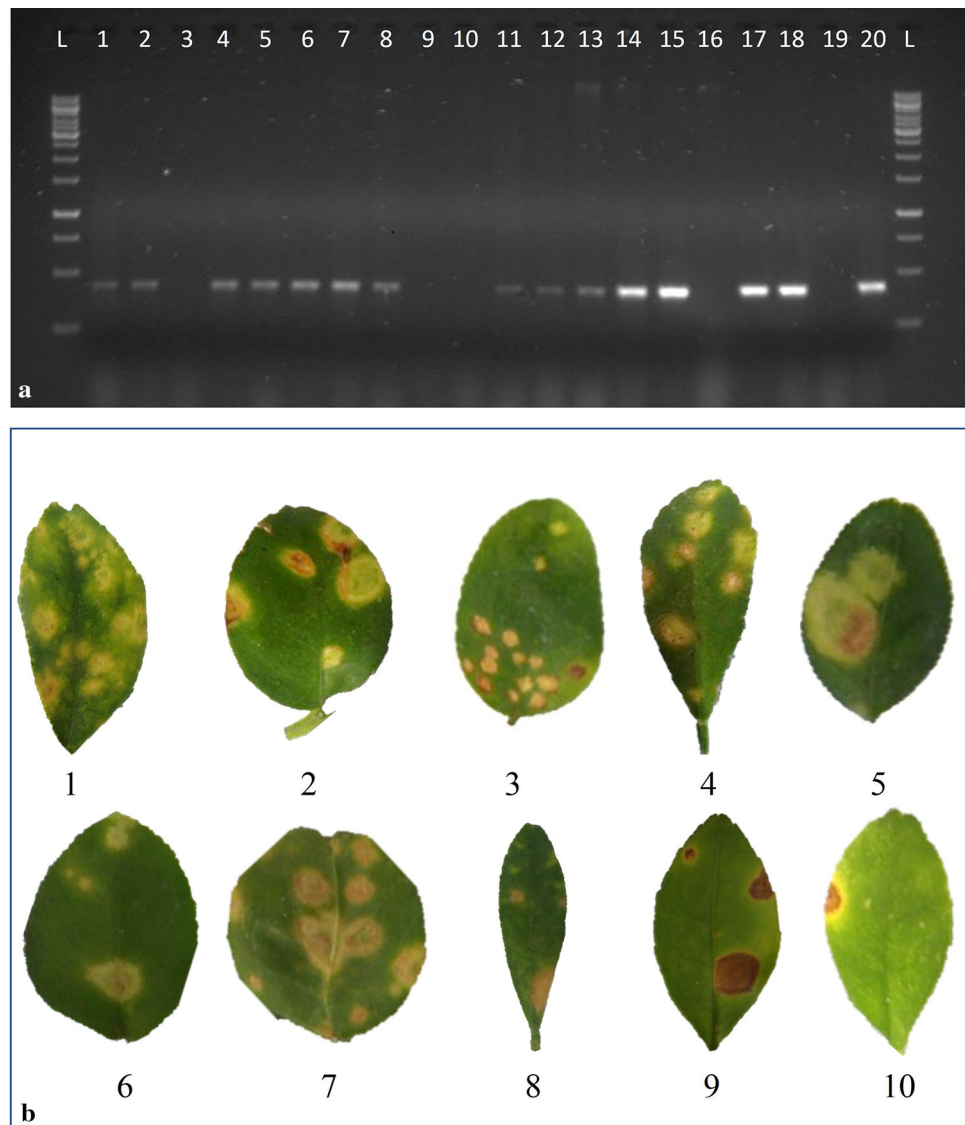
Two accessions of sour orange (*C. aurantium*), Azeda IAC 245 and Spanish Italian IAC 246, remained asymptomatic throughout the experiment. However, within this citrus group, there are previous reports of susceptibility to leprosis [6, 7], indicating variability amongst genotypes, as observed for the tangors and the mandarins.

Molecular diagnostic

The results of the RT-PCR assays confirmed those obtained by phenotypic evaluation which amplify specifically part of the putative coat protein (p29) gene of CiLV-C on the symptomatic leaves from accessions representatives of each group (mandarin and hybrids, sweet orange, sour orange, and lemon) (Fig. 1).

Fig. 1 a Detection of CiLV-C SJP in different citrus accessions by RT-PCR products obtained from *p29* gene. Lane L: molecular weight marker GeneRuler™ 1 kb DNA Ladder; 1: Fairchild IAC 533; 2: Carpe Nartjee IAC 521; 3: Dieberger IAC 456; 4: Sul da Africa IAC 529; 5: De Wildt IAC 545; and 6: Vermelha IAC 508 mandarins; 7: Shekwashe × Tizon IAC 542 mandarin hybrid; 8: Satsuma Owari IAC 219; 9: Azeda IAC 245 sour orange; 10: Meyer IAC 627 lemon; 11: Tardia IAC 591; 12: Paiva IAC 588; and 13: Late IAC 585 willowleaf mandarins; 13: Rubi IAC 315 grapefruit; 14: Clementina IAC 431; 15: Kumquat Nipon IAC 422; 16: Royal IAC 314 grapefruit; 17: Cleópatra IAC 199 mandarin; 18: Pêra Ovo IAC 181 sweet orange; 19: negative control (RT-PCR); 20: positive control (RT-PCR). 1% agarose gel electrophoresis TAE 1X.

b Citrus leprosis symptoms on leaves of 1: Pêra Ovo IAC 181 sweet orange 2: Fairchild IAC 533; 3: Carpe Nartjee IAC 521; 4: Tardia IAC 591; 5: Satsuma Owari IAC 219; 6: Clementina IAC 431; 7: Shekwashe × Tizon IAC 542 mandarin hybrid; 8: Cleópatra IAC 199 mandarin; 9: Rubi IAC 315 grapefruit; 10: Kumquat Nipon IAC 422



The implication of the leprosis occurrence in different citrus groups

In this work, susceptible genotypes were observed in most citrus groups. For satsumas, pummelos (*C. maxima*), kumquat (*Fortunella* spp.) and its hybrid, to the best of our knowledge, this is the first report of the disease caused by CiLV-C. These results support the potential importance of the leprosis disease to areas where citrus genotypes, other than sweet oranges, are cultivated. Sunki and Cleopatra mandarins (*C. sunki* and *C. resnyi*) were also susceptible to leprosis. However, the disease impact on these species should not be comparable to that caused in other citrus species, due to their use as rootstocks in commercial orchards.

Mites were able to not only feed on but also colonize all plants tested in the experiment. The abundant mite

colonization in all genotypes strongly suggests that the asymptomatic plants were indeed resistant to CiLV-C. In this context, Bastianel et al. [2] and Freitas-Astúa et al. [12] observed no correlation between the number of mites and leprosis lesions in citrus and their hybrids. While there was a broad range of response to the disease, no variation in the susceptibility to mite colonization was observed amongst different genotypes (data not shown). However, those are indirect observations and specific studies on mite colonization and biological parameters of the vector should be addressed for an unequivocal comparison between genotype preferences and correlation with CiLV-C transmission and symptom development. Regardless of the disease, our results confirm that *B. yothersi* is well adapted to the citrus group [5]. Most of the citrus genotypes tested here are not listed as hosts for the mite in the compilation organized by

Childers et al. [8] or Beard et al. [5], which suggests that its host range can be significantly broader than reported.

This study identified promising genotypes, which can be incorporated in citrus breeding programs aiming to obtain varietal resistance. Genetic breeding is an approach to reduce the excessive use of pesticides to control the vector and is extremely desired. On the other hand, the identification of genotypes susceptible to leprosis in other citrus groups and genera suggests that the disease can become a concern once it reaches originally indene areas, even where genotypes other than sweet oranges are cultivated.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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