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SHORT COMMUNICATION

Survey of 16SrIX phytoplasmas associated with HLB-symptoms in weeds and leafhoppers at citrus orchards

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Abstract

Citrus plants presenting the typical huanglongbing (HLB) symptoms have been associated to 16SrIX phytoplasma in the States of São Paulo (SP), Minas Gerais (MG), Bahia (BA) and, more recently, Distrito Federal (DF), Brazil. Although in SP and MG states '*Candidatus* Liberibacter asiaticus' is more commonly associated with HLB, single infection by phytoplasmas also occurs. To date, only 16SrIX phytoplasma induces HLB symptoms in BA and DF, a region free of '*Ca*. Liberibacter spp.'. The objective of this work was to survey for alternative hosts and potential vectors of the 16SrIX phytoplasma in citrus orchards from DF using quantitative real-time PCR (qPCR) detection. Fifty-three specimens encompassing fifteen species of weeds were collected from three Tahiti lime (*Citrus latifolia* Tanaka) and Ponkan mandarin (*Citrus reticulata* Blanco) orchards. Only five plants, one *Euphorbia* sp., one *Sida* sp., and three *Bidens pilosa* were positive for the presence of the 16SrIX phytoplasma. Regarding the leafhoppers, 16SrIX phytoplasma was detected in 11/ 38 specimens of *Scaphytopius* sp. and not detected in 32 specimens of *Planicephalus flavicosta.* These results suggest that spontaneous vegetation and the leafhoppers *Scaphytopius* may harbor 16SrIX phytoplasma that can further infect citrus orchards. The capacity of *Scaphytopius* insects to vector the phytoplasma to citrus plant should be better investigated.

Keywords *Bidens pilosa* · *Scaphytopius* sp. · Huanglongbing - associated phytoplasma · '*Candidatus* Phytoplasma phoenicium'

Phytoplasmas of 16SrIX group have been associated to citrus plants with HLB symptoms in the States of São Paulo, Minas Gerais, Bahia and Distrito Federal, Brazil (Teixeira et al. 2008; Silva et al. 2013; Wulff et al. 2015; Sanches et al. 2016). Other phytoplasma groups have also been reported infecting citrus in Brazil (Wulff et al. 2019). The presence of weeds and leafhoppers in citrus orchards has been described as a source for phytoplasmas spread (Marques et al. 2012; Martínez-Bustamante et al. 2018). The leafhopper *Scaphytopius marginelineatus* was pointed out as a potential vector of the 16SrIX phytoplasma from citrus. This species was associated with the weeds *Alternanthera tenella*, *Commelina* sp., *Panicum maximum,* and *Sida rhombifolia* present in citrus orchards (Marques et al. 2012). The citrus orchards infected with 16SrIX phytoplasma group in Brazlândia, DF, presented several weeds and leafhoppers, but their association with phytoplasmas was not studied (Sanches et al. 2016). The objective of this work was to investigate the presence of 16SrIX phytoplasma group in the spontaneous vegetation and leafhoppers occurring in citrus orchards where this HLB-associated phytoplasma group has been found.

The survey was performed in three orchards of Tahiti lime [*Citrus latifolia* (Yu. Tanaka) Tanaka] and Ponkan mandarin (*Citrus reticulata* Blanco) in the region of Brazlândia-DF, where HLB symptoms occur and the presence of the 16SrIX phytoplasma was previously reported by our group (Fig. 1) (Sanches et al. 2016). Citrus plants positive for the presence of 16SrIX phytoplasma group were negative for '*Ca.* Liberibacter asiaticus' and for '*Ca.* Liberibacter americanus', the bacteria associated to HLB in Brazil (Sanches et al. 2016). The weeds surrounding infected citrus plants and presenting typical symptoms induced by phytoplasmas (yellowing, foliar

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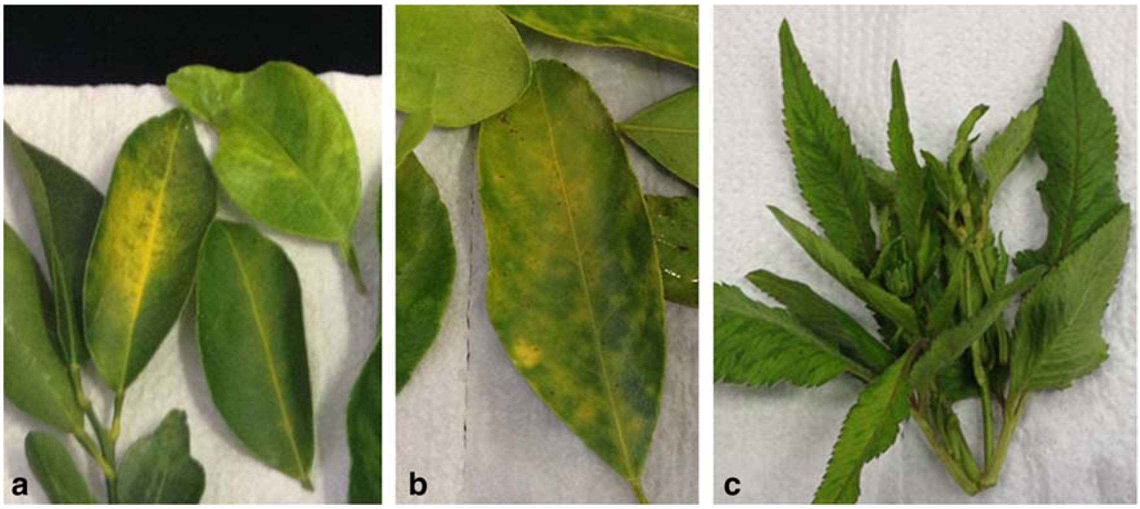

Fig. 1 Symptoms observed in leaves of Tahiti lime plants (A) Ponkan mandarin plants (B) and *Bidens pilosa* (C) infected with 16SrIX phytoplasma group in orchards of Distrito Federal, Brazil

distortion, stunting, virescence, witches' broom, and phyllody, among others) were collected and total DNA was extracted from leaves using a CTAB method, according to Teixeira et al. (2008). The potential vectors were collected using yellow stick traps placed on citrus plants or with yellow pan traps placed below the citrus trees. The insects were conserved in 70% ethanol for taxonomic identification in morphospecies. Members of Deltocephalinae subfamily, which some have been reported as phytoplasmas vectors, had DNA extracted with DNeasy blood and tissue kit (Qiagen). Molecular detection of phytoplasmas from weeds and insects DNA was performed using qPCR with primers FITf, FITr, and the probe

Table 1 Weeds and insects samples with positive results for the presence of 16SrIX phytoplasma in orchards of Distrito Federal

Samples	Collecting date	Symptom	*Cycle threshold (Ct)* value
Weeds			
Bidens pilosa	Nov/2016	Mottling	34.9
Euphorbia sp.	Nov/2016	Stunting, foliar distortion, virescence	33.4
Bidens pilosa	Nov/2017	Foliar distortion, stunting, mottling	33.4
Bidens pilosa	Nov/2017	Virescence, witches' broom, chlorosis	34.9
Sida sp.	Nov/2017	Stunting, yellowing	33.4
Positive control	N.A. *	N.A.	30.3
Negative control	N.A.	N.A.	N.D.**
Insects			
Scaphytopius sp.	April/2016	N.A.	32.2
Scaphytopius sp.	Jan/2016	N.A.	33.1
Scaphytopius sp.	Mar/2016	N.A.	33.2
Scaphytopius sp.	April/2016	N.A.	33.7
Scaphytopius sp.	Jun/2016	N.A.	33.7
Scaphytopius sp.	May/2016	N.A.	34.3
Scaphytopius sp.	Jun/2016	N.A.	34.9
Scaphytopius sp.	May/2016	N.A.	34.9
Scaphytopius sp.	Jul/2016	N.A.	33.5
Scaphytopius sp.	Mar/2016	N.A.	34.6
Scaphytopius sp.	Jul/2016	N.A.	34.8
Positive control	N.A.	N.A.	29.9
Negative control	N.A.	N.A.	N.D.

*N.A. – Not applied

**N.D. – Non-detected

FITp, specific for the 16SrIX phytoplasma (Wulff et al. 2015). The positive control was obtained from a citrus plant previously tested as positive for 16SrIX phytoplasma group and confirmed by amplicon sequencing. The negative controls were obtained from a citrus plant previously tested as negative for phytoplasma and '*Ca*. Liberibacter sp.' and kept in a quarantine greenhouse. Also, ultrapure water was used as an additional negative template control. The qPCR analysis was performed twice for each sample.

Fifty-three weeds specimens were collected inside the citrus orchards embracing the following species: *Amaranthus* sp. (1), *Ambrosia* sp. (1), *Bidens pilosa* (14), *Commelina benghalensis* (2), *Conyza* sp. (4), *Cyperus* sp. (1), *Euphorbia* sp. (15), *Lepidium virnicum* (1), *Leonotis nepetifolia* (1), *Momordica charantia* (1), *Proxelis difusa* (1), *Ricinus communis* (1) *Sida* sp. (4), *Sonchus oleraceus* (1) and *Trifolium repens* (1). The taxonomic identification of four specimens was not possible due to the sample condition. Three *Bidens pilosa* plants (Fig. 1C) gave positive results for the presence of 16SrIX phytoplasmas, whereas one *Euphorbia* sp. and one *Sida* sp. also presented positive results (Table 1). These plants were collected in the same orchard, near different citrus trees. From the insects collected in the traps, 74 specimens belonged to the Deltocephalinae subfamily. Among them, 32 specimens were identified as *Planicephalus flavicosta*, four as *Agallia albidula* and 38 as *Scaphytopiu*s sp. according to taxonomic keys and descriptions provided by Nielson (1968), Kramer (1971) and Zahniser and Dietrich (2013). The identification was confirmed by taxonomists specialized in Homoptera. Eleven *Scaphytopiu*s sp. presented positive results for 16SrIX phytoplasmas (Table 1), while all *Planicephalus flavicosta* and *Agallia albidula* specimens tested were negative. Marques et al. (2012) reported that *Scaphytopiu*s *marginelineatus* is frequently found on *Sida rhombifolia* and here we found both, insect (*Scaphytopiu*s sp.) and plant (*Sida* sp.), harboring 16SrIX phytoplasma.

The low incidence of 16SrIX phytoplasma in citrus plants from orchards of the DF region has raised questions about the role of weeds or other plants as reservoirs of this phytoplasma (Sanches et al. 2016). In São Paulo State, the spatial distribution of the disease in citrus orchards pointed out that the phytoplasma transmission occurs from vectors present in adjacent plants (Teixeira et al. 2008). Further research reported that *Crotalaria juncea* presented inside or adjacent to citrus orchards were the most common host for this phytoplasma (Wulff et al. 2015). Also, transmission tests showed that *S. marginelineatus* acquires the phytoplasma from *C. juncea* more efficiently than from citrus plants (Toloy et al. 2011).

In DF, the weed *Bidens pilosa* may be an important host of 16SrIX phytoplasma since 21% of the plants collected during the surveys were infected. In Mexico, the species *B. odorata* was reported as a probable alternative host of the 16SrIX phytoplasma, as well as the green manure crop *Cajanus cajan* (Martínez-Bustamante et al. 2018). *Catharanthus roseus* is also a host for 16SrIX phytoplasma in Brazil (Barbosa et al. 2012). The low titer found in the weeds in our case is about 10 times less than reported in sweet oranges and in both cases, much lower than the titer reached in sunn hemp (Wulff et al. 2015). The *Euphorbia* sp. and *Sida* sp. plants appear to be incidental hosts of 16SrIX phytoplasma group due to the low incidence observed. The leafhopper *Scaphytopiu*s sp. may has a potential role in the spread of the 16SrIX phytoplasma group in DF, since it was detected in about 30% of collected specimens. It was not possible to determine if the specimens collected in DF are *S. marginelineatus*. However, comparisons with previously identified *S. marginelineatus* specimens suggest that *Scaphytopiu*s individuals collected here are a different species.

The findings demonstrated that leafhoppers of *Scaphytopius* genus might have a potential role in the transmission cycle of 16SrIX phytoplasma group between spontaneous adjacent vegetation and citrus. Isolation of pathogen from an insect and experimental transmission in the laboratory are not conclusive to assess the vector's ability in the environment, as Mitchell (2004) pointed out. Therefore, further investigation of *Scaphytopius*' capacity in vectoring 16SrIX phytoplasma with an appropriate experimental design is required.

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Author contribution MLS, OMM, NAW and MMS planed and designed experimental work. MLS, OMM, CMI, ASG and MMS collected insect and weed samples. MLS and SLBS conducted identification and molecular analysis of insects. OMM, GCG and MMS conducted molecular analysis of weeds. MLS, OMM, NAW and MMS wrote the manuscript.

Compliance with ethical standards

Conflict of interest On behalf of all authors, the corresponding author states that there is no conflict of interest.

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