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Nota/Note

Survey for phytoplasmas and "*Candidatus* Liberibacter sp." from HLB-like symptomatic citrus plants in Brazil

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SUMMARY

The huanglongbing (HLB) is the most important citrus disease in the world. In Brazil, phytoplasmas from 16SrIX group were associated with symptomatic-HLB citrus plants without *Candidatus* Liberibacter spp. in São Paulo and Bahia States. This work objectives to investigate the presence of phytoplasmas in HLB-symptomatic citrus plants in other regions of Brazil to monitor the spread of these phytoplasmas and their association with HLB. Leaves of sweet orange, lime and mandarin from commercial citrus orchards in the States of Rio Grande do Sul (RS), Minas Gerais (MG), Distrito Federal (DF), Bahia (BA) and Pará (PA) with HLB-like symptoms were collected and submitted to diagnosis. The PCR or qPCR with specific primers for *Ca.* Liberibacter asiaticus/ americanus and 16SrIX phytoplasma were used. A total of 150 samples were analyzed from 141 orchards inspected. Group IX phytoplasmas were detected in six samples from Brazlândia-DF. *Candidatus* Liberibacter asiaticus was detected in MG in the regions of Bonfim, Campanha and Perdões. There was no detection of both 16Sr group IX and *Ca.* Liberibacter asiaticus in RS, BA and PA, currently HLB-free areas. Moreover, *Ca.* Liberibacter americanus was not detected in the samples. The survey will continue to elucidate the importance of phytoplasmas in HLB-like symptomatic citrus plants.

Index terms: 16SrIX-Phytoplasma, *Ca*. Liberibacter spp., huanglongbing.

Levantamento para fitoplasmas e "*Candidatus* Liberibacter sp." em plantas de citros com sintomas semelhantes a HLB no Brasil

RESUMO

O *huanglongbing* (HLB) é a doença de citros mais importante no mundo. No Brasil, fitoplasmas do grupo 16SrIX foram associados a plantas de citros sintomáticas para HLB, sem presença de *Candidatus* Liberibacter spp. nos Estados de São Paulo e Bahia. Este trabalho teve

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objetivo de investigar a presença de fitoplasmas em plantas de citros com sintomas de HLB em outras regiões do Brasil para monitorar a disseminação destes fitoplasmas e sua associação com HLB. Folhas de laranja doce, lima e tangerina de pomares comerciais de citros nos Estados do Rio Grande do Sul (RS), Minas Gerais (MG), Distrito Federal (DF), Bahia (BA) e Pará (PA) com sintomas semelhantes a HLB foram coletadas e submetidas à diagnose. Foram realizados testes PCR ou qPCR com primers específicos para *Ca.* Liberibacter asiaticus/americanus e fitoplasma 16SrIX. Um total de 150 amostras foram analisadas a partir de 141 pomares inspecionados. Fitoplasmas do grupo IX foram detectados em seis amostras de Brazlândia-DF. *Ca.* Liberibacter asiaticus foi detectado em MG nas regiões de Bonfim, Campanha e Perdões. Não houve detecção de fitoplasmas do grupo 16SrIX e *Ca.* Liberibacter asiaticus no RS, BA e PA, atualmente áreas livres de HLB. Além disso, *Ca.* Liberibacter americanus não foi detectada nas amostras. O levantamento irá continuar para elucidar a importância dos fitoplasmas em plantas de citros com sintomas semelhantes a HLB.

Termos de indexação: Fitoplasma 16SrIX, *Ca.* Liberibacter spp., *huanglongbing.*

INTRODUCTION

It was estimated that in the last decade pests and diseases caused the eradication of 40 million citrus trees in Brazil, especially due to citrus variegated chlorosis, citrus canker, citrus sudden death and more recently huanglongbing (HLB) (Neves et al., 2010). Currently, the most serious concern for citrus growers worldwide is HLB. The disease is present in Brazil since 2004 and it was initially associated with *Candidatus* Liberibacter asiaticus and *Ca.* Liberibacter americanus (Coletta-Filho et al., 2004; Teixeira et al., 2005).

In 2007, phytoplasmas of 16SrIX group were associated to citrus plants with HLB-symptoms. The plants did not present *Ca*. L. asiaticus neither *Ca*. L. americanus and were distributed in 11 municipalities of São Paulo State (Teixeira et al., 2008b). This phytoplasma was also detected from symptomatic plants without the presence of *Ca*. Liberibacter in Bahia State (Silva et al., 2013; Wulff et al., 2015).

In addition, phytoplasmas of groups 16SrIII, VII and IX were associated with citrus plants in São Paulo State. However, in this case the symptoms were different from those exhibited in HLB-infected plants (Barbosa, 2010). In China, phytoplasmas belonging to 16SrI-B and 16SrII-A groups were found in citrus trees with HLB-symptoms, associated or not to *Ca*. L. asiaticus (Chen et al., 2009; Lou et al., 2013). In Mexico, phytoplasmas belonging to 16SrI-B and 16SrI-S groups were also found in association or not with *Ca*. L. asiaticus (Arratia-Castro et al., 2014), while 16SrIX phytoplasmas were found alone in citrus plants that presented HLB-symptoms (Wulff et al., 2015).

Currently *Ca.* Liberibacter spp. are regulated pest in Brazil restricted to Paraná, São Paulo and Minas Gerais States. The importance of phytoplasmas for the general context of the HLB is still unknown. However, the presence of HLB-symptoms associated to phytoplasmas is enough to cause commercial concerns in other States considered *Ca.* Liberibacter–free areas. This work aimed to investigate the presence of phytoplasmas in HLB-like symptomatic citrus plants in other regions of Brazil to monitor the spread of these phytoplasmas and its association with *Ca.* Liberibacter spp.

Commercial citrus orchards located in different regions of Brazil (Figure 1) were inspected for the presence of typical HLB symptoms such as blotch mottling in the leaves and lopsided fruits with abnormal maturation. Symptomatic citrus trees were found in States of MG, DF, BA and PA and the leaves and fruits were collected for analysis. Symptomatic trees were not found in 110 citrus orchards inspected in RS State from regions of Vale do Caí, Western, Northwestern and Alto Uruguai. São Paulo and Paraná States were not included in this survey due to the presence of *Ca*. L. asiaticus and *Ca*. L. americanus for a long time, what reduces the chance of phytoplasmas detection (Teixeira et al., 2008b; Wulff et al., 2015).

Total DNA was extracted from leaves and/or fruits columella of sweet orange, lime and mandarin with CTAB method, according to Teixeira et al. (2008b). The detection of *Ca*. L. americanus and asiaticus was performed with duplex PCR according to Teixeira et al. (2008b) with primers GB1/GB3 (Teixeira et al., 2005) and A2/J5 (Hocquellet et al., 1999) or with quantitative real-time PCR (qPCR) according to Teixeira et al. (2008a). The detection of 16SrIX phytoplasma was performed using qPCR with primers FITf and FITr and the probe FITp (Wulff et al., 2015).

The *Ca*. Liberibacter asiaticus was detected only in samples of mandarin Ponkan from MG State (Table 1). The plants presented lopsided fruits and abnormal maturation, with most of the leaves being smaller and showing asymmetrical chlorosis. Typical blotchy mottle was

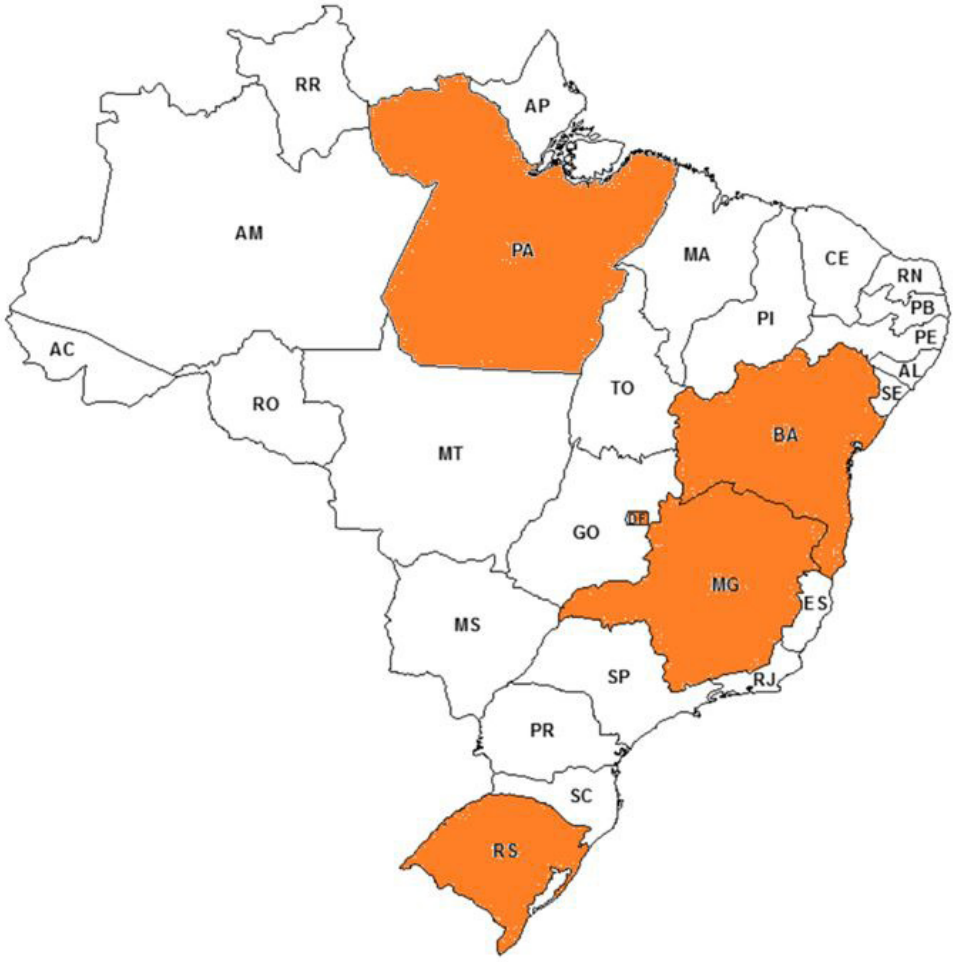

Figure 1. Map of Brazil showing the States selected for inspection and sampling of HLB-like symptomatic leaves and/or fruits in citrus commercial areas.

Table 1. Citrus orchards inspected and analysis performed in Minas Gerais (MG), Bahia (BA), Distrito Federal (DF) and Pará (PA) States, Brazil

Region	Orchards inspected/ symptomatic samples	Phytoplasma* 16SrIX	Ca. Liberibacter asiaticus*	Ca. Liberibacter americanus*
Perdões-MG	7/20	0	8	0
Campanha-MG	5/13	0	13	0
Bonfim-MG	1/2	0	2	0
Bom Jesus da Lapa- BA	1/ 5	0	0	0
Núcleo Rural Tabatinga-DF	3/ 25	0	0	0
Núcleo Rural Taquara-DF	3/ 31	0	0	0
Brazlândia-DF	3/42	6	0	0
São Domingos do Araguaia-PA	1/1	0	0	0
Marabá-PA	4/8	0	0	0
Castanhal-PA	3/3	0	0	0

*Number of positive samples.

not observed in Ponkan leaves, although leaves presented symptoms typically related to HLB (Figure 2). The cycle threshold (*Ct*) of qPCR reactions for *Ca*. Liberibacter asiaticus from samples of MG ranged from 18 to 35 (Limit of detection is *Ct* 35). *Ca*. Liberibacter americanus was not detected in analyzed samples.

The phytoplasma 16SrIX was detected only at Brazlândia region in DF State. The leaves did not present typical HLB symptoms, although the plants presented nutritional deficiencies and symptoms caused by other pests and/or fungal and bacterial diseases. These samples were collected in small growers' areas that did not adopt the best agricultural practices. The phytoplasma was detected in leaves of sweet orange, lime and mandarin with qPCR *Ct* values close to 35, at the limit of detection for a positive sample, demonstrating low concentration in plant tissues. The results were confirmed by agarose gel electrophoresis of the qPCR product. There was no detection of phytoplasma and *Ca.* Liberibacter spp. together in the samples.

Natural weeds as observed closer to the analyzed plants (Figure 3) could be potential natural reservoir of phytoplasmas. The presence of weeds or another crop inside or adjacent to infected citrus orchards is described by other authors (Bové et al., 2008; Barbosa, 2010; Marques et al., 2012) and reveals a potential source for the phytoplasmas spread. Indeed, Marques et al. (2012) described the leafhopper *Scaphytopius marginelineatus* as positive for the HLB-associated group IX phytoplasmas. The 16SrIII phytoplasmas were found in *Bidens pilosa*, *Leonurus sibiricus* and *Solanum americanum* and the 16SrVII phytoplasmas in *Erigeron bonariensis* and *Euphorbia heterophylla*. These weeds were sampled in commercial citrus orchards in São Paulo State that presented citrus plants infected by these phytoplasmas (Barbosa, 2010). In addition, *Crotalaria juncea* plants were found infected by the 16SrIX phytoplasma in São Paulo State among other phytoplasmas, with DNA sequence identical to the ones found in sweet orange (Wulff et al., 2015; Bianco et al., 2014). The purpose of these plants was to improve the soil between citrus rows, and they presented typical witches' broom symptoms (Bové et al., 2008).

Due to the spatial distribution of the disease in citrus orchards, apparently the transmission occurs from vectors present in adjacent crops (Teixeira et al., 2008b). We observed several leafhoppers and other potential insect vectors in citrus orchards of Brazlândia. The leafhopper *Scaphytopius marginelineatus* was pointed out as potential vector of the 16SrIX phytoplasma. This species was associated to the weeds *Alternanthera tenella*, *Commelina* sp., *Panicum maximum* e *Sida rhombifolia* present in citrus orchards (Marques et al., 2012). In addition, transmission tests showed that *S. marginelineatus* acquires the phytoplasma from *C. juncea* more efficiently than from citrus plants (Toloy et al., 2011). Moreover, Barbosa (2010) found the leafhopper *Agalia albidula* infected by 16SrIII phytoplasma in weeds inside citrus orchards.

In conclusion, the phytoplasma presented low incidence in citrus orchards inspected so far. The survey will continue to elucidate the importance of phytoplasmas in HLB-symptomatic citrus plants. Studies for identification of weeds and leafhoppers associated to infected citrus plants are been carried out to verify their association with phytoplasmas in Distrito Federal State.

Figure 2. Irregular maturation and lopsided fruits in Ponkan fruits in Minas Gerais State, Brazil.

Figure 3. General aspect of the weeds-infested citrus orchard sampled in Brazlândia, DF, Brazil.

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