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Research Article

Sunn hemp, a major source-plant of the phytoplasma associated with huanglongbing symptoms of sweet orange in São Paulo State, Brazil.

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Abstract

In São Paulo State (SPS), sweet orange (*Citrus sinensis*) trees with huanglongbing (HLB) symptoms are infected with *Candidatus* (*Ca.*) *Liberibacter* (*L.*) *asiaticus* (*Las*) or *Ca. L. americanus* (*Lam*). However, in 2007, 3 years after HLB was first reported in SPS, some trees with characteristic HLB symptoms were found free of liberibacters, but infected with a phytoplasma of 16Sr group IX. This phytoplasma was further characterized by PCR amplification of ribosomal protein genes *rpsC-rplV-rpsS* and amplicon sequencing. A qPCR test to detect the phytoplasma in plants and insects was also developed on the basis of the ribosomal protein genes. The phytoplasma was transmitted from citrus-to-citrus by grafting. The 16Sr group IX phytoplasma associated with HLB symptoms in sweet orange in SPS and characterized by the above techniques was named “HLB-phytoplasma”. Although the HLB-phytoplasma is widely distributed in many municipalities of central, northern, and northwestern SPS, the number of HLB-phytoplasma-infected trees in each municipality is very small. Experiments have been undertaken to identify the origin of the HLB-phytoplasma and the source of inoculum on which a putative insect vector could become infected with the HLB-phytoplasma. In SPS, sunn hemp (*Crotalaria juncea* L.) is a major, widely distributed cover crop. A 16Sr group IX phytoplasma was detected in sunn hemp plants with witches' broom and virescence symptoms, and was shown to have 16Sr DNA sequences and ribosomal protein gene sequences with 100% identity to the corresponding sequences of the sweet orange HLB-phytoplasma. Transmission electron microscopy revealed the presence of phytoplasma cells in the phloem sieve tubes of infected *C. juncea* stalks. These results were taken as evidence that the sunn hemp phytoplasma and the sweet orange HLB-phytoplasma were identical. *Scaphytopius marginelineatus*, a leafhopper frequently found in sweet orange orchards, was shown to acquire the HLB-phytoplasma efficiently from affected sunn hemp plants, but acquisition from, and transmission rates to, sweet orange were very low. On the whole, these data suggest that (i) sunn hemp is a major source of inoculum of the HLB-phytoplasma, (ii) *S. marginelineatus* becomes infected on sunn hemp and transmits the phytoplasma to sweet orange, and (iii) transmission from sweet orange to sweet orange occurs only rarely, if at all. 16Sr group IX phytoplasmas, very closely related to the SPS HLB-phytoplasma, have also been detected in citrus in Minas Gerais and Bahia states (Brazil) and Mexico.

Keywords: Brazil, *Citrus sinensis*, *Crotalaria juncea* L., huanglongbing, phytoplasma, *Scaphytopius marginelineatus*

Introduction

Before 2004, only 2 species of *Candidatus* (*Ca.*) *Liberibacter* (*L.*) were known to be associated with citrus huanglongbing (HLB): *Ca. L. africanus* (*Laf*) in Africa and *Ca. L. asiaticus* (*Las*) in Asia (Bové 2006). Liberibacters are endogenous, uncultured, sieve tube-restricted alpha-proteobacteria (Gram-negative bacteria) (Jagoueix et al. 1994, 1997). In 2004, HLB was reported for the first time from America and, more precisely, from

central São Paulo State (SPS), Brazil. Two *Ca. L.* species were identified and could be detected by specific PCR techniques (Teixeira, Ayres, et al. 2005; Teixeira, Saillard, et al. 2005). Most trees were infected with a new liberibacter species, *Ca. L. americanus* (*Lam*), and only very few trees carried *Las*, the known Asian species. However, over the years, the proportion of *Las*-infected trees has increased greatly, while *Lam*-infected trees have become difficult to find (Lopes et al. 2009). Since 2005, when Florida, USA, reported for the first time the

presence of HLB in North America, many countries in North, Central, and South America have become affected with HLB, with Las being the most widespread disease-associated liberibacter.

Two psyllid vectors are responsible for the spread of HLB. *Trioza erytreae*, the African citrus psyllid, transmits Laf in Africa, and *Diaphorina citri*, the Asian citrus psyllid, is the vector of Las in Asia and America. In Brazil, *D. citri* was reported for the first time in 1942 and transmits both Las and Lam (Yamamoto et al. 2006).

Characteristic HLB symptoms on citrus trees are (i) leaf blotchy mottle and (ii) lopsided fruit with color inversion, necrotic seeds, and brownish stained vascular bundles at the peduncular end of the columella (Bové 2006). In February 2007, sweet orange (*Citrus sinensis* L. Osbeck) trees with these symptoms were encountered in Barretos and Colômbia, 2 municipalities (MUs) of northern SPS, a region previously free of HLB. Unexpectedly, these trees gave negative PCR tests for all three citrus liberibacter species, Las, Lam, and Laf, but they could be shown to be infected with a phytoplasma having 99% 16SrDNA sequence identity with the pigeon pea witches'-broom phytoplasma of 16Sr group IX (Teixeira et al. 2008). In agreement with this result, phytoplasma cells could be detected by transmission electron microscopy in sieve tubes from midribs of symptomatic leaves. While liberibacters are walled, Gram-negative bacteria, phytoplasmas are wall-less bacteria. Liberibacters and phytoplasmas have in common that they are endogenous, non-cultured, and sieve tube-restricted. Both are transmitted by insects feeding on sieve tube sap: psyllids in the case of liberibacters and, in the case of phytoplasmas, mainly leafhoppers, but also psyllids.

The first sweet orange trees showing HLB symptoms and found to be infected with the 16Sr group IX phytoplasma came initially (February 2007) from 2 MUs in northern SPS. By September 2007, with additional surveys, the number of such MUs rose to 16 (Teixeira et al. 2008) and by December 2008, 65 MUs in northern, central, and southern SPS were affected (Wulff et al. 2009). These figures suggested that the source plant on which the putative phytoplasma insect-vector became infected was relatively well distributed throughout SPS (Teixeira et al. 2008; Wulff et al. 2009). This plant could have been a weed or a crop plant. Legumes such as *Cajanus* sp. and *Crotalaria* spp. are used as cover crops in the sugar cane and citrus industries; they have a wide distribution in SPS. Indeed, *Crotalaria juncea* L. (sunn hemp) plants growing in between rows of sweet orange trees in citrus orchards were found to be infected with the 16Sr group IX phytoplasma (Wulff et al. 2009). Thus, sunn hemp could have been a source of inoculum on which a putative insect vector became infected with the 16Sr group IX phytoplasma. Marques et al. (2012) have identified such a vector: the leafhopper *Scaphytopius marginelineatus*, dominant and frequent in citrus plots, and found to be infected with the 16Sr group IX phytoplasma.

Even though the 16Sr group IX phytoplasma is widely distributed throughout SPS, the number of trees affected by the phytoplasma in citrus farms is small, the disease incidence ranging from 0.1% to 1.76% affected trees. Also, most of the affected trees are distributed randomly and, in 80% of cases, the minimum distances between affected trees are higher than 100 m (Teixeira et al. 2008). These results suggest that primary infection of citrus trees with the 16Sr group IX phytoplasma by the putative leafhopper vector, possibly *S. marginelineatus*, is a rare event, and that secondary infections from citrus-to-citrus by *D. citri*, the Asian HLB psyllid vector, do not occur.

Since 2007, when sweet orange trees infected with the 16Sr group IX phytoplasma were identified for the first time in SPS (Teixeira et al. 2008), additional citrus trees infected with this phytoplasma have been detected in 2008 in Minas Gerais State (Wulff, unpublished) and in 2012 in Bahia State (Silva et al. 2013). Citrus DNA samples received in 2011 from Mexico were analyzed at Fundecitrus in Araraquara, Brazil, and found to be infected with the phytoplasma of 16Sr group IX (Wulff, unpublished). In China, Chen et al. (2009) have also reported a phytoplasma associated with HLB symptoms in citrus, but the phytoplasma belonged to 16Sr group I, not IX. The 16Sr group I phytoplasma has also been reported from Mexico (Arratia-Castro et al. 2014). Recently, the occurrence of a phytoplasma of 16Sr group II, sub-group A, was reported as being associated with HLB-like, blotchy mottle symptoms in grapefruit (*Citrus paradisi* Macfad.) in China (Lou et al. 2013). The phytoplasma responsible for witches'-broom disease of lime (Zreik et al. 1995) in small-fruited, acid lime (*Citrus aurantifolia* (Cristm.) Swingle) belongs also to 16Sr group II, but sub-group B, and could not be transmitted to grapefruit by graft-inoculation (Bové et al. 1996).

In the work presented here, available PCR techniques have been used and new ones have been developed for improved detection and characterization of phytoplasmas in citrus, sunn hemp, and leafhoppers. Also, in view of the possible role of (i) sunn hemp as a source of inoculum of the 16Sr group IX phytoplasma, and (ii) *S. marginelineatus* as the vector, it was shown that *S. marginelineatus* can acquire the 16Sr group IX phytoplasma when feeding on *C. juncea* plants infected with this phytoplasma. We have further studied (i) the distribution of the 16Sr group IX phytoplasma in citrus and sunn hemp in SPS, (ii) the occurrence, in sunn hemp, of phytoplasmas other than the 16Sr group IX phytoplasma, and (iii) the possible acquisition of the 16Sr group IX phytoplasma by the Asian citrus psyllids for secondary infection of citrus. The abbreviation "HLB-phytoplasma" will be used for the 16Sr group IX phytoplasma associated with citrus HLB symptoms.

Materials and methods

Plant material

For easier viewing, Table 1 lists phytoplasmas from selected plants (#1 to #13) and techniques used for their

detection, including further characterization as based on PCR amplification of 16SrDNA and ribosomal protein genes (RPGs).

Sweet orange. To identify MUs in SPS where the HLB-phytoplasma was present in symptomatic sweet orange leaf samples, data were retrieved from the Fundecitrus Diagnostic Laboratory, in charge of analyzing the samples received from citrus farmers throughout SPS, and sometimes neighboring Minas Gerais State as well. The HLB-phytoplasma was detected using the protocol of Teixeira et al. (2008) by 16SrDNA PCR amplification with primers D7f2/D7r2. By this means, 2 young, symptomatic sweet orange field trees from SPS were detected, one from Potirendaba MU (tree #0, not included in Table 1) and one from Parisi MU (tree #1); they were transferred in 2008 from the orchards to 100 L containers inside the Fundecitrus greenhouse in Araraquara, SPS.

The phytoplasmas from these 2 sweet orange trees were confirmed as the HLB-phytoplasma by nested PCR amplification of 16Sr DNA with primers P1/P7 followed by primers fU5/rU3, and sequencing of the nested amplicon (Table 1, PCR technique “a1”). Indeed, the sequences from tree #1 and tree #0 were identical and had 100% sequence identity with the similar sequence of the HLB-phytoplasma of Teixeira et al. (2008). The tree from Parisi MU is the sweet orange tree #1 in Tables 1, 3, and 4, and has been used as the reference tree for further characterization of the HLB-phytoplasma. The tree #0 from Potirendaba MU was used for acquisition and transmission assays with insects.

Sweet orange trees #4 from Bahia State, Brazil, and #5 and #6 from San Luis Potosi, Mexico, were collected because they showed blotchy mottle symptoms and were sent to Fundecitrus as leaf (#4) or DNA samples (#5 and #6). These 3 trees were found to be infected with 16Sr group IX phytoplasmas by PCR amplification of RPGs with primers specific for 16Sr group IX phytoplasmas (Table 1, PCR techniques M&M “c” and “d”).

Sunn hemp. Sunn hemp plants grown between citrus rows or in fields close to citrus orchards (Fig. 1A) were sampled for the detection of phytoplasmas. A systematic assessment of phytoplasma infection in sunn hemp was carried out in 10 areas located in 9 MUs of SPS between May 2010 and February 2011: Angatuba, Agudos, Bocaina, Catanduva, Ibirá, Itápolis, Lençóis Paulista, Promissão, and Votuporanga (Fig. 2); Catanduva MU had 2 areas. For each of these 10 areas, 5 sunn hemp plots measuring 100 m² (20 m x 5 m) each were surveyed for symptoms typical of phytoplasma diseases: shoot proliferation, upper stalk-end shaped as a spiral, witches'-broom (WB) (Fig. 1C, D), WB with virescence (Fig. 1E.a), small leaves, leaf yellowing, leaf mosaic, and malformed leaves. Ninety-eight plants with such symptoms were collected and analyzed by nested PCR amplification of 16Sr DNA with universal phytoplasma primers P1/P7 followed by phytoplasma-specific nested primers fU5/rU3 to identify among the 98 symptomatic plants, those 48 which were infected with a phytoplasma.

The 16Sr group phytoplasmas within the 48 phytoplasmas of these plants were determined from the sequence of their nested fU5/rU3 amplicon (Table 1, PCR technique M&M “a1”). In Tables 1, 3, and 4, sunn hemp plants #3, 10, 11, 12, and 13, having, respectively, phytoplasmas of 16Sr groups IX, I, III, VII, and XV, were selected from within the 48 phytoplasma-infected plants (see legend of Table 2). The 16Sr groups of the phytoplasmas from these 5 plants were also determined from the sequence of their P1/P7 amplicon (Table 1, PCR technique M&M “a2”).

For detection of phytoplasmas in the above 98 symptomatic sunn hemp plants (including plants #3 and 10 to 13), a second technique, in addition to PCR amplification of 16Sr DNA, has been used: PCR amplification of RPGs, with (Table 1, PCR techniques M&M “b”) or without amplicon sequencing (Table 1, PCR techniques M&M “c” and “d”).

Symptomatic sunn hemp plants were occasionally collected from farms located in the MUs of Botucatu, Fernandópolis, Olímpia, Potirendaba, and Tanabi (Fig. 2); these plants were only analyzed for 16Sr group IX phytoplasmas by PCR technique M&M “c” (Table 1). Four sunn hemp plants with witches'-broom symptoms and infected with the HLB-phytoplasma, as well as 1 plant without symptoms (Fig. 1B), were selected from Potirendaba and transferred to 12 L containers inside the Fundecitrus greenhouse to carry out acquisition and transmission experiments and provide samples for electron microscopy (EM). One of the 4 sunn hemp plants from Potirendaba is plant #2 in Tables 1, 3, and 4. Two of these 4 HLB-phytoplasma infected plants from Potirendaba (including plant #2) were used for EM.

Additional plants from various origins. Plant DNA samples also came from Costa Rica (*Gliricidia sepium* #7 and #8) and Italy (*Picris echioides* #9). These samples were found to be infected with 16Sr group IX phytoplasmas by PCR amplification of RPGs with primers rp(IX)F2 and rp(IX)R2, specific for 16Sr group IX phytoplasmas (Table 1, PCR technique M&M “c”). The phytoplasma in *P. echioides* from Italy was transmitted to *Catharanthus roseus* and kept in the phytoplasma collection at INRA Bordeaux, France. The DNA sample used came from the Bordeaux *C. roseus*.

Leafhoppers and psyllids

The Asian citrus psyllid, *D. citri*, was collected on *Murraya paniculata*, and reared and multiplied in cages on liberibacter-free *M. paniculata* seedlings for several generations. Adults were collected with a mouth aspirator and used for transmission experiments. Individuals of the leafhopper *S. marginelineatus* were collected with a net in citrus orchards, and reared and multiplied on caged *Sida rhombifolia* seedlings for several generations (Marques 2011). Adults were collected with a mouth aspirator. *S. rhombifolia* is a preferred host of *S. marginelineatus*, but is not a host of the HLB-phytoplasma.

Table 1

Phytoplasmas from selected plants and PCR techniques used for their detection and further characterization as based on PCR amplification of 16SrDNA and on the ribosomal protein genes (RPGs) *rpsC-rpIV-rpsS*.

Plants ^a	Origin	Symptom	16Sr group	PCR Techniques ^b				
				Amplification of 16Sr DNA		Amplification of RPGs		
				M&M "a1"	M&M "a2"	M&M "b"	M&M "c"	M&M "d"
1. Sweet orange	Parisi, SPS, Brazil	Blotchy mottle	IX	yes, sequencing ^c	nd	rp(IX)R2 / rpL2F3, sequencing	Yes (+)	Yes (+)
2. Sunn hemp	Potirendaba, SPS, Brazil	Witches'-broom	IX	idem	nd	idem	Yes (+)	Yes (+)
3. Sunn hemp ^c	Itápolis, SPS, Brazil	Witches'-broom	IX	idem	yes, sequencing	idem	Yes (+)	Yes (+)
4. Sweet orange	Bom Jesus da Lapa, Bahia, Brazil	Blotchy mottle	IX	nd	nd	idem	Yes (+)	Yes (+)
5. Sweet orange	San Vicente, San Luis Potosi, Mexico	Blotchy mottle	IX	nd	nd	idem	Yes (+)	Yes (+)
6. Sweet orange	Tamuin, San Luis Potosi, Mexico	Blotchy mottle	IX	nd	nd	idem	Yes (+)	Yes (+)
7. <i>G. sepium</i>	Limonal, Costa Rica	Little leaf	IX	nd	nd	idem	Yes (+)	nd
8. <i>G. sepium</i>	Vuelta de Jorco, Costa Rica	Little leaf	IX	nd	nd	idem	Yes (+)	nd
9. <i>P. echioides</i>	INRA Collection	Witches'-broom	IX	nd	nd	idem	Yes (+)	nd
10. Sunn hemp	Catanduva, SPS, Brazil	Leaf yellowing	I	yes, sequencing	yes, sequencing	rp(D)RIA and rpL2F3, sequencing	Yes (-)	Yes (-)
11. Sunn hemp	Ibirá, SPS, Brazil	Shoot proliferation	III	idem	idem	idem	Yes (-)	Yes (-)
12. Sunn hemp	Agudos, SPS, Brazil	Shoot proliferation	VII	idem	idem	idem	Yes (-)	Yes (-)
13. Sunn hemp	Ibirá, SPS, Brazil	Shoot proliferation	XV	idem	idem	rp(D)RIA and rpF1C, sequencing	Yes (-)	Yes (-)

^a – See M&M, plant material and legend of Table 2.

^b – See M&M, PCR techniques. "a1": PCR amplification with primers P1/P7 followed by nested PCR with primers fU5/rU3 and sequencing the nested 16Sr DNA amplicon; "a2": PCR amplification with primers P1/P7 and sequencing the 16Sr DNA amplicon; "b": PCR amplification with 16Sr group-specific primers and RPG amplicon sequencing; "c": PCR amplification of RPGs with 16Sr group IX specific primers rp(IX)F2 and rp(IX)R2; "d": qPCR as described in this work (primers FITf/FITr probe FITp). Yes: the technique was applied to sample; nd: the technique was not applied to sample. (+): positive PCR reaction, (-): negative PCR reaction.

^c – "sequencing" indicates that the respective amplicons were sequenced and their sequences analyzed and submitted to GenBank.

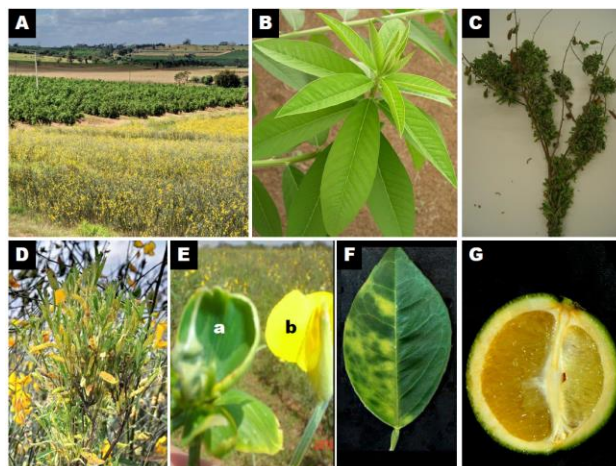


Fig. 1. (A) Sunn hemp field in foreground and sweet orange orchard in middle ground, Ibirá, SPS. (B) Symptomless sunn hemp. (C, D, and E) Sunn hemp infected with HLB-phytoplasma showing witches'-broom symptoms (C and D), virescence (E a: virescent petal; b: normal petal). (F) HLB-phytoplasma induced blotchy mottle on sweet orange leaf and (G) HLB-phytoplasma induced lopsided sweet orange fruit with black seed and brownish vascular bundles at peduncular end of fruit axes.

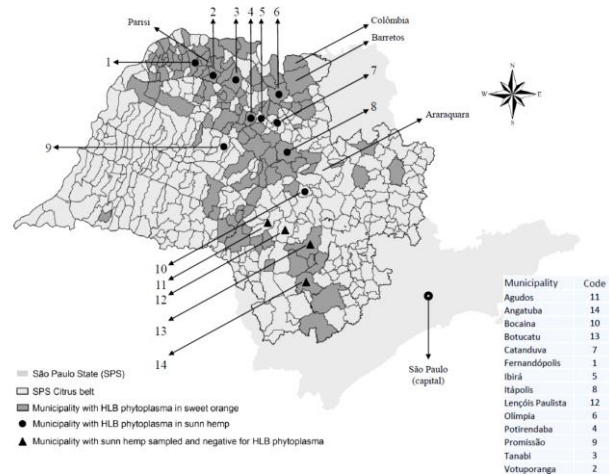


Fig. 2. Municipalities in São Paulo State (SPS) where HLB-phytoplasma was detected in sweet orange (dark gray areas) or in sunn hemp (black circles ●). Black triangles (▲) are municipalities with sunn hemp sampled and negative for HLB phytoplasma.

PCR techniques for detection and characterization of phytoplasmas

DNA extraction for PCR was according to the CTAB protocol of Murray and Thompson (1980) with slight modifications by Teixeira et al. (2008). For DNA extraction, a sunn hemp stalk sample of 0.5 g was collected from each plant at a position close to the most severe symptom. DNA from petioles and midribs of sweet

orange leaves was obtained with the same protocol. DNA from psyllids and leafhoppers was also extracted according to the above CTAB protocol. A plastic rod was used to grind up insects within a plastic tube in the presence of CTAB extraction buffer. DNA quality and concentration were assessed with a NanoDrop instrument (Thermo Scientific, Waltham, MA).

a) Detection of phytoplasmas by PCR amplification of 16SrDNA with universal primers for ribosomal RNA genes and sequencing of the amplicons for 16Sr group identification.

a1) Nested PCR amplification – PCR was carried out with universal phytoplasma primers P1/P7 (Deng and Hiruki 1991; Schneider et al. 1995) followed by a nested amplification with primers fU5 and rU3 (Seemüller et al. 1994; Teixeira et al. 2008), leading to an amplicon of approximately 850 bp. The presence of a fU5/rU3 nested amplification product upon agarose gel electrophoresis was taken as evidence that the sample was phytoplasma-positive; in the absence of a PCR product, the sample was considered as negative. For identification of the 16Sr group to which the phytoplasma belonged, the sequence of the nested fU5/rU3 amplicon was used (Table 1, PCR technique M&M “a1”).

a2) Conventional PCR amplification – PCR was carried out with universal phytoplasma primers P1 and P7 giving an amplicon close to 1.8 kb. The sequence of the P1/P7 amplicon was also used for 16Sr group identification (Table 1, PCR technique M&M “a2”). PCR was carried out with Taq DNA polymerase (Invitrogen) and amplification products were cleaned with the PCR cleanup kit (Promega Corporation, Madison, WI).

Sequence analyses of PCR amplicons from P1/P7 (PCR technique “a2”) and nested PCR amplicons from P1/P7 followed by fU5/rU3 (PCR technique M&M “a1”) were performed after sequence determination with big dye terminator and contig analysis with CodonCodeAligner v. 4.1.1 (CodonCode Corporation, Centerville, MA). The sequence of the P1/P7 amplicon and the sequence of the nested fU5/rU3 amplicon were used for identification of the 16Sr group phytoplasmas with, respectively, iPhyClassifier (Zhao et al. 2009) and Blastn (Altschul et al. 1997).

b) Characterization of 16Sr phytoplasma groups by PCR amplification of RPGs *rpsC-rplV-rpsS* with specific primers and amplicon sequencing. In above section “a”, the various 16Sr phytoplasma groups (I, III, VII, IX, and XV) from the 48 sunn hemp plants (M&M, Sunn hemp) were identified on the basis of their 16SrDNA sequences (Table 1, PCR techniques M&M “a1” and “a2”). This group identification was confirmed on the basis of the *rpsC-rplV-rpsS* RPG sequence using the PCR primers designed by Martini et al. (2007) (Table 1, PCR technique M&M “b”). For phytoplasmas of 16Sr group I, III, and VII, the primers used were rp(I)RIA and rpL2F3; for 16Sr group IX, they were rp(IX)R2 and rpL2F3; and for 16Sr group XV, rp(I)RIA and rpF1C were used. In the case of 16Sr group IX phytoplasmas, the DNA samples analyzed came not only from sunn hemp but also from (i) sweet orange from Brazil (São Paulo and Bahia States) and Mexico, (ii) *G. sepium* from Costa Rica, and (iii) *P. echioïdes* from Italy (Table 1, samples 4 to 9). *G. sepium* had been previously reported as a host of 16Sr group IX phytoplasmas (Kenyon et al., 1998).

PCR products were amplified with Phusion proofreading DNA polymerase (Thermo Scientific),

cleaned, and cloned directly in plasmid pBS or, after adenylation, in pGEMT-Easy. Plasmids were extracted with commercial kits (Promega Corporation) and 3 plasmids from each cloned PCR product were sequenced by the Sanger method with primer m13, primer T7 promoter, and group specific primers (Martini et al. 2007). Sequence analysis was as described above (see M&M “a”).

c) Detection of 16Sr group IX phytoplasmas in sunn hemp and sweet orange by PCR amplification of RPGs with group IX specific primers. The DNA samples were analyzed for the presence of 16Sr group IX phytoplasmas by the group IX specific PCR primer set rp(IX)F2 and rp(IX)R2 for amplification of RPGs *rpsC-rplV* (Martini et al. 2007) (Table 1, PCR technique M&M “c”).

d) TaqMan qPCR for detection and quantification of 16Sr group IX phytoplasmas. Primers and the probe for qPCR detection of the HLB-phytoplasma were designed with Primer Express v. 3.0 (Applied Biosystems, Foster City, CA), using as template the partial ribosomal protein locus *rpsC-rplV-rpsS*. Primers FITf (AACGAAGGTTTACGTTTAAACAAGAC) and FITr (GAGGTGCGACTACTATTGTAATATG) (this study) amplify a 100 bp fragment, and the probe FITp (FANCGATCTGCTCTCCCTTTTGCCCTAG-MGB) (this study) was used with these primers. Primer concentration was assayed in a matrix containing 150, 250, 350, or 500 µmoles of primer and 0.20 µmoles of probe. Annealing temperatures of 59, 60, 61, 62, 63, and 64 °C were assayed. Regular detection and quantification of the HLB-phytoplasma was carried out under the following conditions: 0.35 µmoles of each primer, 0.20 µmoles for FITp probe, 1x Path ID Master Mix (product 4388643 Applied Biosystems), 3 µL of DNA template in a final volume of 12 µL. Amplification conditions were 10 min at 95 °C followed by 40 cycles, each cycle for 15 s at 94 °C and 60 s at 62 °C. Specificity of qPCR for the detection of the HLB-phytoplasma was assessed with sunn hemp samples known from previous analyses to be infected with one of the following 16Sr group phytoplasmas: I, III, VII, IX, and XV.

Transmission electron microscopy detection of the HLB-phytoplasma in sunn hemp

Small stalk pieces of sunn hemp plant #2 (Table 1) and a second plant, both displaying symptoms of witches'-broom were collected in Potirendaba MU (see M&M, sunn hemp), fixed for 3 h in a mixture of 2.5% glutaraldehyde and 2% paraformaldehyde in 0.05 M, pH 7.2 cacodylate buffer, post-fixed in 1% osmium tetroxide in the same buffer, dehydrated in acetone and embedded in the Spurr low viscosity epoxy resin (Kitajima and Nome 1999). Longitudinal and transversal sections of the vascular region were obtained with a Leica EM U6 microtome equipped with a Diatome diamond knife, and the sections were stained with 3% uranyl acetate and Reynold's lead citrate before being examined in a Zeiss EM 900 or Jeol JEM 1011 transmission electron

microscope and the images recorded digitally. A symptomless sunn hemp plant testing negative for phytoplasmas by nested PCR with primers P1/P7 followed by fU5/rU3 was used as a phytoplasma-free control.

*Assay for acquisition of the HLB-phytoplasma from sunn hemp by *S. marginelineatus* leafhoppers*

Four sunn hemp plants with witches'-broom symptoms were collected in Potirendaba MU, transferred to containers, and kept in the Fundecitrus greenhouse. These plants were positive for the HLB-phytoplasma as determined by PCR amplification of RPGs with 16Sr group IX specific PCR primers rp(IX)F2 and rp(IX)R2 from Martini et al. (2007) (Table 1, PCR technique M&M "c"). *S. marginelineatus* leafhoppers were confined within a net cage on sunn hemp plants for a 13-day-acquisition period (Marques 2011). Groups of 4 or 5 surviving leafhoppers were collected and processed for DNA extraction and qPCR as described in this work. The same procedure was employed for symptomless, phytoplasma-negative sunn hemp plants.

Results

Distribution of the HLB-phytoplasma in sweet orange in SPS as of April 2014

Symptomatic sweet orange trees infected with the HLB-phytoplasma were found initially in February 2007 in 2 MUs of northern SPS, Barretos and Colômbia (Teixeira et al. 2008). By December 2008, 65 MUs were affected (Wulff et al. 2009) and as of April 2014, the number had increased to 108 MUs in northern, central, and southern SPS (Fig. 2). There were also 5 affected MUs in neighboring Minas Gerais State (MGS). These data are from the Fundecitrus Diagnostic Laboratory where symptomatic citrus leaf samples from SPS and sometimes MGS are regularly analyzed for citrus liberibacters (Las and Lam) and the HLB-phytoplasma. A total of 68,855 samples have been analyzed for HLB detection from August 2007 to April 2014. Of all samples analyzed, the number of citrus samples positive for phytoplasmas or liberibacters were 1.3% and 62.5%, respectively.

The reference tree from Parisi MU is the sweet orange tree #1 in Tables 1, 3, and 4, and the accession number of its nested amplicon sequence (PCR technique "a1") is KM877314, and this sample was used for further characterization of the HLB-phytoplasma. The reference tree from Potirendaba MU was used for insect-acquisition and transmission assays, as well as for showing that the HLB-phytoplasma was transmissible from sweet orange to sweet orange by graft-inoculation. Indeed, budwood sticks from the Potirendaba reference sweet orange tree were graft-inoculated onto five 1-year-old sweet orange plants on Rangpur lime and kept in the greenhouse. Fourteen months after graft-inoculation, 1 of the 5 test plants was found to be PCR-positive with primers D7f2/D7r2, which were developed for detection of the

HLB-phytoplasma (Teixeira et al. 2008). The sequence of the amplification product was 100% identical to that of the HLB-phytoplasma in the donor sweet orange tree, indicating that the HLB-phytoplasma had been transmitted by graft inoculation. Blotchy mottle leaf symptoms developed 26 months after graft-inoculation.

The 16Sr group IX HLB-phytoplasma in sweet orange and sunn hemp; phytoplasmas of 16Sr groups I, III, VII, and XV in sunn hemp

The discovery in sweet orange trees of a phytoplasma associated with HLB symptoms, which were previously known to be induced only by liberibacters, came as a surprise and the question of the phytoplasma's origin, i.e., its initial host plant, was immediately raised. Since the HLB-phytoplasma was found to be closely related to the pigeon pea witches'-broom phytoplasma of 16Sr group IX (Teixeira et al. 2008), pigeon pea (*C. cajan* (L.) Huth.) was an obvious plant to investigate, but its use as a cover crop in SPS was rare. On the contrary, sunn hemp (*C. juncea*) is a tropical legume, widely used in northwestern SPS in particular, as cover crop between citrus rows and during renewal of sugar cane fields. The first symptomatic sunn hemp sample testing PCR-positive for the HLB-phytoplasma was collected in May 2008 and came from the MU of Fernandópolis, precisely in northwestern SPS (Fig. 2). The phytoplasma in this sunn hemp plant was analyzed by nested PCR amplification of 16Sr DNA with primers P1/P7 followed by primers fU5/rU3. The nested sunn hemp amplicon was sequenced (Accession number: KM879341) and found to have 100% sequence identity with (i) the corresponding sequence of the HLB-phytoplasma from the reference sweet orange tree from Parisi MU (sweet orange tree #1 in Tables 1, 3 and 4; KM877314) and (ii) the HLB-phytoplasma sequences (HQ423159/EU266074) from Teixeira et al. (2008). These results were assumed to show that the phytoplasma from sunn hemp was identical to the sweet orange HLB-phytoplasma (Wulff et al. 2009).

Regular sampling for additional sunn hemp plants infected with the HLB-phytoplasma started thereafter as described in M&M, plant material section. Table 2 lists the phytoplasmas detected in sunn hemp plants using symptoms likely to be shown by phytoplasma-affected plants. Nine MUs were surveyed in detail. A total of 98 symptomatic sunn hemp plants were collected in these 9 MUs (Fig. 2, MUs 2, 5, 7, 8, 9, 10, 11, 12, and 14). Samples from the symptomatic plants were first analyzed to determine whether they were infected with a phytoplasma or not. Therefore, nested PCR amplification of 16Sr DNA with universal phytoplasma primers P1/P7, followed by nested phytoplasma primers fU5/rU3, was used. Table 2 shows that 48 of 98 plants were infected with a phytoplasma. The symptoms most frequently associated with phytoplasma infection were "witches'-broom + virescence" (13 plants PCR-positive/13 plants tested), "witches'-broom" (10/12) and "shoot proliferation" (14/32). Next, the 48 16Sr DNA amplicons from the above fU5/rU3 nested PCR were sequenced and

the sequence was used to identify the 16Sr group of the phytoplasma. For instance, of the 32 sunn hemp plants with shoot proliferation symptoms, 14 were infected with a phytoplasma (Table 2). Of these 14 plants, 1 was infected with a phytoplasma of 16Sr group I, 2 plants carried a 16Sr group III phytoplasma, 1 plant harbored a 16Sr group VII phytoplasma, 7 plants contained a 16Sr group IX phytoplasma, and 3 plants had a 16Sr group XV phytoplasma (Table 2). Of the 48 phytoplasma-infected plants, those infected with a 16Sr group IX phytoplasma were the most numerous: 31; for all these 31 phytoplasmas, the PCR amplicons obtained with nested primers fU5/rU3 had 100% sequence identity with the amplicon sequence from the HLB-phytoplasma of reference sweet orange tree #1 (Table 1, 3, and 4) and also with the sequence of the HLB-phytoplasma deposited in GenBank (HQ423159). Symptoms of sunn hemp plants infected with the HLB-phytoplasma were essentially “witches’-broom + virescence”, “witches’-brooms”, and “shoot proliferation”. In addition to the 31 plants harboring the HLB-phytoplasma, 5 plants were infected with a phytoplasma of 16Sr group I and, similarly, 5, 4, and 3 plants, respectively, were infected with a phytoplasma of 16Sr group III, VII, and XV (Table 2; Bianco et al. 2014). As indicated in the legend of Table 2, one phytoplasma-infected plant from each 16Sr group was selected for PCR amplification of 16Sr DNA with primers P1/P7 and sequencing of the amplicon. The size of the P1/P7 amplicons varied from 1,807 bp for group XV (Table 3, plant #13) to 1,830 bp for group I (Table 3, plant #10).

A second technique was used to analyze the 98 symptomatic plants in Table 2: PCR amplification of RPGs (*rpsC-rplV*) with primer pair rp(IX)F2 and rp(IX)R2, specific for 16Sr group IX phytoplasmas (M&M, PCR technique: “c”) Martini et al. (2007). The very same 31 plants, found to be infected with the HLB-phytoplasma by nested PCR amplification of 16Sr DNA and amplicon sequencing (Table 2), were again found to be infected with the HLB phytoplasmas, this time by PCR amplification of RPGs, confirming the previous identification. Specificity of the technique for 16Sr group IX phytoplasmas is shown on Fig. 3C, where PCR amplification is positive for the 16Sr group IX phytoplasmas (lanes 6 to 9), but negative for group I, III, VII, and XV phytoplasmas (respectively lanes 2, 3, 4, and 5).

In addition, symptomatic sunn hemp plants were occasionally collected from farms located in the MUs of Botucatu, Fernandópolis, Olímpia, Potirendaba, and Tanabi (Fig. 2); these plants were analyzed for the presence of 16Sr group IX phytoplasmas only by PCR amplification of RPGs with the above primers rp(IX)F2 and rp(IX)R2 (M&M, PCR technique “c”). Plants from the last 4 MUs, but not from Botucatu MU, were positive for HLB phytoplasmas. Two of 4 symptomatic sunn hemp plants from Potirendaba MU (Fig. 2), testing positive for the HLB-phytoplasma, were kept in the Fundecitrus greenhouse and examined by electron microscopy to confirm the presence of the phytoplasmas in the sieve tubes.

Table 2
Phytoplasma infections of symptomatic sunn hemp plants in SPS, as based on PCR amplification of 16Sr DNA.

Sunn hemp symptoms ^a	Number of symptomatic plants	Phytoplasma-positive plants/ Symptomatic plants tested ^b	16Sr groups of infecting phytoplasma ^c					I + III + VII + IX + XV
			I	III	VII	IX	XV	
Shoot proliferation	32	14/32	1/14	2/14 ^e	1/14 ^f	7/14	3/14 ^h	14/14
Stalk end turned into a spiral	23	2/23	0/2	0/2	0/2	2/2	0/2	2/2
Witches’-broom & virescence	13	13/13	0/13	0/13	0/13	13/13	0/13	13/13
Witches’-broom	12	10/12	1/10	0/10	1/10	8/10 ^g	0/10	10/10
Little leaf	7	3/7	0/3	3/3	0/3	0/3	0/3	3/3
Leaf yellowing	5	3/5	3/3 ^d	0/3	0/3	0/3	0/3	3/3
Leaf mosaic	4	3/4	0/4	0/4	2/3	1/3	0/4	3/3
Malformed leaves	2	0/2	0	0	0	0	0	0/0
Total	98	48/98	5/48	5/48	4/48	31/48	3/48	48/48

^a – Sunn hemp plants with various phytoplasma symptoms were collected in 10 areas within 9 municipalities, each area having 5 100 m² (20 m x 5 m) plots.
^b – Phytoplasma detection was by nested PCR with universal phytoplasmas primers P1/P7 followed by nested primers fU5/rU3 (M&M, PCR technique “a1” without amplicon sequencing).
^c – Number of plants (with a given symptom) positive for a phytoplasma of a given 16Sr group, as determined from the sequence of the nested fU5/rU3 PCR amplicon (technique “a1” with amplicon sequencing).
^d – One of the 3 infected plants is plant 10 of Tables 1 and 3.
^e – One of the 2 infected plants is plant 11 of Tables 1 and 3.
^f – The infected plant is plant 12 of Tables 1 and 3.
^g – One of the 8 infected plants is plant 3 of Tables 1, 3, and 4.
^h – One of the 3 infected plants is plant 13 of Tables 1 and 3.

Table 3

Plants used for further characterization of phytoplasmas as based on the sequences of 16Sr DNA and ribosomal proteins genes (RPGs) *rpsC-rplV-rpsS*.

Plant ^a	16Sr group	Size of 16SrDNA sequence of P1/P7 amplicon (bp) ^b	GenBank accession*	Size of <i>rpsC-rplV-rpsS</i> sequence (bp) ^c	GenBank accession*
1. Sweet orange	IX	nd	nd	1,316	KJ806622
2. Sunn hemp	IX	nd	nd	1,316	KJ806623
3. Sunn hemp	IX	1,817	KF941131	1,316	KJ806621
4. Sweet orange	IX	nd	nd	1,316	KJ806624
5. Sweet orange	IX	nd	nd	1,316	KJ806625
6. Sweet orange	IX	nd	nd	1,316	KJ806626
7. <i>G. sepium</i>	IX	nd	nd	1,316	KJ806627
8. <i>G. sepium</i>	IX	nd	nd	1,316	KJ806628
9. <i>P. echioides</i>	IX	nd	nd	1,316	KJ806629
10. Sunn hemp	I	1,830	KF878383	1,531	KJ806619
11. Sunn hemp	III	1,813	KF941133	1,541	KJ806617
12. Sunn hemp	VII	1,821	KF941132	1,573	KJ806620
13. Sunn hemp	XV	1,807	KF878382	1,287	KJ806618

^a – Plants: as in Table 1.

^b – PCR technique M&M “a2” in Table 1. nd: not determined.

^c – PCR technique M&M “b” in Table 1.

* – Accession numbers deposited in GenBank from sequences obtained in this work.

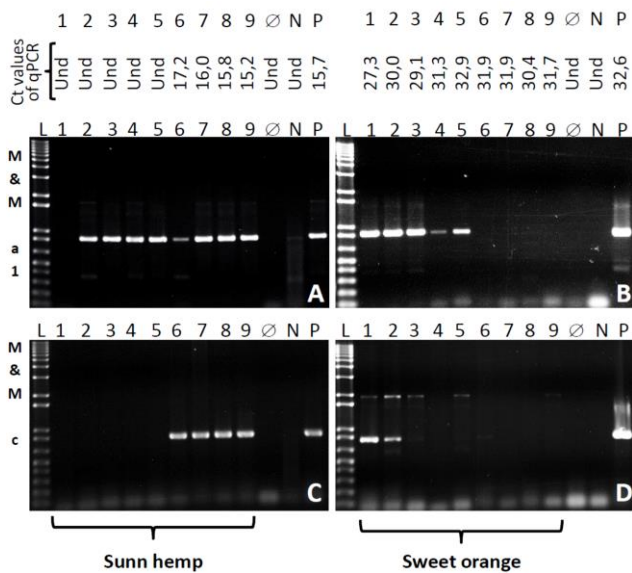


Fig. 3. Phytoplasma detection by PCR in sunn hemp (A and C) and sweet orange (B and D) plants. (A and B) PCR amplification of 16Sr DNA with primers P1/P7 followed by nested primers fU5/rU3. (C and D) PCR amplification of ribosomal protein genes with primers rp(IX)F2 and rp(IX)R2, specific for 16Sr group IX phytoplasmas. Ct values of qPCR (average value of three replicates): at top of figure. For qPCR (left), (A and C), 1: asymptomatic sunn hemp; 2, 3, 4, 5, and 6 to 9: sunn hemp plants infected with a phytoplasma of, respectively, 16Sr group I, III, VII, XV, and IX; Ø: no template control; N: healthy sunn hemp control; P: 16Sr group IX phytoplasma positive control. For qPCR (right), (B and D), 1 to 9: sweet orange plants infected with HLB-phytoplasma (16Sr group IX phytoplasma); Ø: no template control; N: healthy sweet orange control; P: 16Sr group IX phytoplasma positive control. L: Ladder 1 kb plus Invitrogen; Und: undetected.

One of the 4 sunn hemp plants is plant # 2 of Table 1, 3, and 4. A high number of wall-less structures (Fig. 4A and 4B), indistinguishable from phytoplasma cells, and clearly different of the walled, Gram-negative liberibacter cells (Fig. 4C), were found in cross sections of most sieve tubes. The structures were essentially circular with diameters from 200 to 1000 nm. No Liberibacter-like bacterial cells could be seen on electron micrographs from phytoplasma-positive samples. Phytoplasma-like bodies were not found in the sieve tubes from negative-control sunn hemp plants.

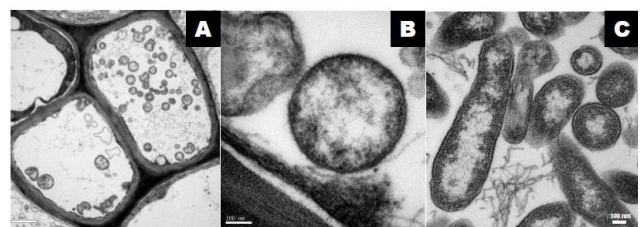


Fig. 4. Electron micrograph of phytoplasma cells in sieve tube of sunn hemp plant, PCR positive for HLB-phytoplasma. (A) phytoplasma cells in sieve tube. (B) wall-less phytoplasma cells surrounded by a single membrane (cytoplasmic membrane). (C) *Ca. Liberibacter* cells in periwinkle sieve tubes; the dark outer layer represents the Gram-negative cell wall.

Comparison of the HLB-phytoplasmas from sweet orange, sunn hemp, and other plants in SPS and other regions

In Table 3, the plants from SPS (sweet orange tree #1, sunn hemp plants #2, #3, and #10 to #13) were known to be infected with a phytoplasma of a given 16Sr group on the basis of PCR amplification of 16Sr DNA and amplicon sequencing (Table 1, PCR techniques M&M “a1” and “a2”). A second technique was also used to

detect and characterize the above phytoplasmas: PCR amplification of RPGs *rpsC-rpIV-rpsS* with specific primers from Martini et al. (2007) and sequencing of the amplicon (Table 1 PCR technique M&M “b”). For instance, the primer pair specific for 16Sr group IX phytoplasmas is rp(IX)R2 and rpL2F3 (Table 1) with which a 1,316 bp amplicon was obtained (Table 3). The sizes of the amplicon for 16Sr groups I, III, VII, and XV were 1,531 bp, 1,541 bp, 1,573 bp, and 1,287 bp, respectively (Table 3).

The sequence of a PCR amplification product of 1,316 bp covering the RPGs was obtained from the reference sweet orange tree with blotchy mottle leaves from Parisi MU (Table 3, sweet orange tree #1), and a sunn hemp plant with witches'-brooms (Table 3, plant #2), from Potirendaba MU. Since the amplification products of the RPGs from the sweet orange phytoplasma and the sunn hemp phytoplasma had not only the same size (1,316 bp) but also the same sequence, it was assumed that the 2 phytoplasmas were identical and that the HLB-phytoplasma from citrus was present in sunn hemp. The RPG amplicon sequence from a second sunn hemp plant (Table 3, plant #3) in a third MU, Itápolis, was identical to that of plants #1 and #2.

As shown in Table 3, all the RPG amplicons of the 16Sr group IX phytoplasmas from SPS (plants #1 to #3) or from Bahia (Brazil), Mexico, Costa Rica, or Italy (plants #4 to #9), and present in 4 different plant species (sweet orange, sunn hemp, *G. sepium*, and *P. echinoides*) have the same size (1,316 bp). In addition, for the phytoplasmas of plants #1 to #3, the RPG amplicon has not only the same size, but also the same sequence and this sequence will be referred to as the “SPS-type-sequence” of the HLB-phytoplasma.

Table 4 shows that the RPG amplicons of the phytoplasmas from plants #4 to #8 had 99.77% to 99.32% sequence identity with the SPS-type-sequence, but not 100% identity, due to 3, 6, or 9 single nucleotide polymorphisms (SNPs) when compared to the SPS-type-sequence of samples #1, #2, or #3, respectively; the total number of SNPs amounted to 14 for plants 1 to 8.

Similarly, the 2 Costa Rica phytoplasmas (Table 4, plants 7 and 8) also shared the same RPG sequence (Costa Rica-RPG-sequence), but was different from the SPS-type-sequence as well as from the Mexico-RPG-sequence because of 9 SNPs. Interestingly, the Bahia-RPG-sequence was different from the SPS-type-sequence by only three SNPs (Table 4, plant #4, underlined G, T, and C) and none of these nucleotide changes induced amino acid substitutions. On the contrary, with the Mexico- and Costa Rica-RPG-sequences, there are 4 SNP induced amino acid substitutions (Table 4, underlined N, N, V, and S).

qPCR: Detection and quantification of 16Sr group IX HLB-phytoplasma

The sequences from the RPGs *rpsC-rpIV-rpsS* (Tables 3 and 4) led to the development of a qPCR protocol capable of specifically detecting all the 16Sr group IX phytoplasmas in sweet orange and sunn hemp studied in this work, whether from SPS, Bahia, or Mexico. Indeed, all 98 symptomatic sunn hemp samples of Table 2 were assayed with the qPCR protocol and only the 31 plants infected with the HLB-phytoplasma tested positive. Sunn hemp samples infected with phytoplasmas of 16Sr groups I, III, VII, or XV were negative in the qPCR test (Table 1, PCR technique M&M “d”). In addition, the number of DNA target copies of the HLB-phytoplasma was found to be 70 per ng of DNA in sweet orange whereas in sunn hemp, it reached as many as 77,000 copies, i.e., a titer more than 1,000 times higher in sunn hemp than in sweet orange. This explains why the HLB-phytoplasma in sunn hemp is easily detected. However, with sweet orange, in which the phytoplasma titer is much lower, high sensitivity of the phytoplasma detection technique is important.

In Fig. 3, phytoplasma detection in sunn hemp (Fig. 3, A and C) and sweet orange (Fig. 3, B and D) was by three techniques (Table 1); first, technique M&M “d”: qPCR (Ct values at the top of the figure); second, technique M&M “a1”: PCR amplification of 16Sr DNA with universal primers P1/P7 followed by nested PCR with primers rU5/fU3 (Fig. 3, A and B); and third, technique M&M “c”: PCR amplification of RPGs with primers rp(IX)F2/rp(IX)R2, specific for 16Sr group IX phytoplasmas (Fig. 3, C and D). As technique M&M “a1” uses 2 pairs of universal phytoplasma primers, it is able to detect sunn hemp phytoplasmas of 16Sr group IX (Fig 3A, lanes 6 to 9) as well as of groups I, III, VII, and XV (Fig 3A, lanes 2 to 5). Techniques “d” and “c” are specific for the 16Sr group IX phytoplasma and do not detect the phytoplasmas of the other groups (Fig 3C and qPCR). For sweet orange trees from SPS, in which only the 16Sr group IX HLB-phytoplasma occurs, qPCR was the most sensitive technique as it detected the phytoplasma in the samples from all 9 plants tested, whereas techniques M&M “a1” and “c” detected the phytoplasma in only 5 and 2 plants, respectively (Fig. 3, B and D). As a matter of fact, this qPCR was shown to be more sensitive in the detection of the HLB-phytoplasma than the protocols of Teixeira et al. (2008) that were used previously. qPCR is used since August 2012 at the Fundecitrus Diagnostic Service.

Transmission electron microscopy of HLB-phytoplasma infected tissues

Single walled, pleomorphic bodies were consistently found in the lumen of sieve tubes from HLB-phytoplasma-infected sunn hemp (sample #1 and from Potirendaba MU, which were absent in symptomless, control plants (Fig. 4, A and B). They differ remarkably from liberibacter, which characteristically have an outer cell wall as those of Gram-negative bacterium (Fig. 4 C).

Table 4

Single nucleotide polymorphisms (SNPs) in the nucleotide sequence of the ribosomal protein genes (RPGs) *rpsC-rplV-rpsS* among 16Sr group IX phytoplasmas^a.

Plants ^b	IR				rpsC			IR			rplV			rpsS				SNPs	% identity
	107 ^c	259	274	337	402	433	757	901	989	1013	1137	1163	1251	1277					
1. Sweet orange, SPS, Brazil	A	C	T	G	G	T	A	T	T	A	T	G	G	T	0	100			
2. Sunn hemp, SPS, Brazil	A	C	T	G	G	T	A	T	T	A	T	G	G	T	0	100			
3. Sunn hemp, SPS, Brazil	A	C	T	G	G	T	A	T	T	A	T	G	G	T	0	100			
4. Sweet orange, Bahia, Brazil	<u>G</u>	C	T	G	<u>T</u>	<u>C</u>	A	T	T	A	T	G	G	T	3	99.77			
5. Sweet orange, Mexico	A	C	<u>C</u>	G	G	T	<u>C</u>	<u>A</u>	<u>C</u>	A	<u>G</u>	G	<u>T</u>	T	6	99.54			
6. Sweet orange, Mexico	A	C	<u>C</u>	G	G	T	<u>C</u>	<u>A</u>	<u>C</u>	A	<u>G</u>	G	<u>T</u>	T	6	99.54			
7. <i>G. sepium</i> , Costa Rica	A	<u>T</u>	T	<u>A</u>	G	<u>C</u>	<u>C</u>	T	T	<u>T</u>	<u>G</u>	<u>A</u>	<u>T</u>	<u>C</u>	9	99.32			
8. <i>G. sepium</i> , Costa Rica	A	<u>T</u>	T	<u>A</u>	G	<u>C</u>	<u>C</u>	T	T	<u>T</u>	<u>G</u>	<u>A</u>	<u>T</u>	<u>C</u>	9	99.32			
9. <i>P. echiooides</i> (INRA Collection)	A	C	C	G	G	T	C	A	T	A	G	A	T	T	61	95.36			
SPS type sequence	A	C	T	G	G	T	A	T	T	A	T	G	G	T	-	-			
SPS consensus amino acid residues ^d	-	I	G	S	-	I	K	I	I	G	L	L	A	I	-	-			
	-	I	G	S	-	I	<u>N</u>	<u>N</u>	I	G	<u>V</u>	L	<u>S</u>	I					

^a– The RPG sequence was obtained by PCR technique “b”, Table 1.

^b– Plants: as in Table 1.

^c– Numbers positioned above the sequence alignments indicate the nucleotide position in each gene region (gene names are positioned at the corresponding sequence number from the first SNP). Bases that are different from the SPS type sequence are underlined. For *P. echiooides* only SNPs common to the other 16Sr group IX are shown, with exclusive SNPs not shown. IR: intergenic region.

^d– Amino acid residues that are different from the consensus residues due to nucleotide changes are underlined. SNPs in intergenic regions are indicated as “–” in the corresponding amino acid sequence. I = Isoleucine; G = Glycine; S = Serine; K = Lysine; N = Asparagine; L = Leucine; V = Valine; A = Alanine.

Acquisition of the HLB-phytoplasma by the leafhopper S. marginelineatus on infected sunn hemp plants and transmission assays of the HLB-phytoplasma from the infected leafhoppers to sweet orange

Marques et al. (2012) have shown by PCR amplification and amplicon sequencing that DNA samples from field-collected *S. marginelineatus* leafhoppers in SPS were positive for the HLB-phytoplasma. In the work reported here, the leafhopper was found to survive quite well on sunn hemp plants, as it stayed alive up to 20 days under greenhouse conditions. Therefore, acquisition of the HLB-phytoplasma by *S. marginelineatus* from infected sunn hemp plants was attempted. Sunn hemp plants with witches’-broom symptoms from Potirendaba MU and PCR-positive for the HLB-phytoplasma, as determined by group specific primers rp(IX)F2 and rp(IX)R2 (Martini et al. 2007), were used as donor plants (plant #2 in Table 1). Symptomless sunn hemp plants testing PCR-negative for the HLB-phytoplasma were used as negative “donor” plants. In assay I, 200 insects reared on *S. rhombifolia* seedlings were transferred to 2 symptomatic sunn hemp donor plants. After 13 days of acquisition feeding, 153 insects survived and were allowed to stay again on *S. rhombifolia* for 7 days to possibly favor multiplication of the phytoplasma in the vector; then 6 batches of 20 to 33 insects were caged each on a 9-month-old sweet orange plant grafted on Rangpur lime. After 5 days of infection feeding, the insects were recovered for detection of the HLB-phytoplasma by qPCR. The HLB-phytoplasma was detected in 30 of 30 insect samples (5 insects per sample). Similarly, in a second assay, 42 of 50 insects survived and

were caged on 2 individual sweet orange test plants; finally, 4 of 11 insect samples (4 insects per sample) were qPCR-positive for the HLB-phytoplasma. In the control experiments with symptomless, phytoplasma-negative sunn hemp plants, all insect samples were qPCR negative at the end of the assays.

This experiment shows that *S. marginelineatus* is capable of acquiring the HLB-phytoplasma when feeding on HLB-phytoplasma-infected sunn hemp plants. However, transmission of the HLB-phytoplasma to 8 sweet orange plants (6 from assay I and 2 from assay II) with batches of ~20 insects per plant was negative.

Transmission assays of the HLB-phytoplasma from sweet orange to sweet orange by D. citri psyllids

Transmission of the HLB-phytoplasma from citrus-to-citrus by the Asian citrus psyllid was studied in 4 independent assays, each initiated in 1 of the 4 seasons of the year, winter (assay 1), spring (assay 2), summer (assay 3), and fall (assay 4). The reference sweet orange tree from Potirendaba MU was maintained in the Fundecitrus greenhouse and used as a source of inoculum. Seventy adult psyllids were caged on a symptomatic branch for 2 weeks of acquisition feeding; they were then caged, for an inoculation period of 2 weeks on 2 healthy citrus test plants (9-month-old Pera sweet orange grafted on Rangpur lime). Each test plant received half of the insects that remained alive. Assay 2 had only one test plant due to the low number of insects recovered alive. At the end of the inoculation period, insects were collected for DNA extraction and PCR with primers D7f2/D7r2 for detection

of the HLB-phytoplasma (Teixeira et al., 2008). The test plants were maintained in a screen house for visual evaluation of symptoms and sample collection for PCR analysis. Symptom evaluations and sample collections took place for each assay every 4 months from 13 months post inoculation to 38 months.

None of the psyllids caged for a 2-week-acquisition period on the symptomatic, HLB-phytoplasma-infected sweet orange tree gave a positive PCR amplification and none of the sweet orange test plants showed symptoms or gave positive PCR tests for the HLB-phytoplasma. Thus, no transmission of the HLB-phytoplasma from sweet orange to sweet orange by *D. citri* psyllids was obtained. The test plants were discarded after 3 years.

Transmission assays of the HLB-phytoplasma from sweet orange to sweet orange by the leafhopper S. marginelineatus

Three hundred and twenty leafhoppers were confined on symptomatic branch #1 of the donor sweet orange tree (reference sweet orange tree from Potirendaba MU, M&M, Plant Material); after an acquisition period of 48 h, leafhoppers, which were still alive, were caged for 18 days on *S. rhombifolia* plants, to possibly favor multiplication of the phytoplasmas in the leafhoppers; 154 leafhoppers still alive were then transferred to Pera sweet orange test plant #1 (6-month-old Pera sweet orange grafted on Rangpur lime) for an inoculation period of 48 h, after which 133 leafhoppers were still alive and used for qPCR detection of the HLB-phytoplasma. Similarly, in a second assay, 315 leafhoppers were caged on branch #1 of the above donor sweet orange tree, of which 83 were eventually recovered alive after 48 h on sweet orange test plant #2 and used for HLB-phytoplasma detection by qPCR. The same procedure was repeated with a second branch of the donor tree and ended up with sweet orange test plants #3 and #4, from which, 8 and 76 leafhoppers, respectively, were recovered for qPCR detection of the HLB-phytoplasma. The 4 sweet orange test plants, #1 to #4, were maintained in a screen house for evaluation of symptoms and PCR analyses with primers D7f2/D7r2 for detection of the HLB-phytoplasma (Teixeira et al. 2008). The first evaluation was done 8 months after inoculation and then 4 more times with 4-month intervals, up to 24 months post inoculation.

All the leafhoppers recovered after the inoculation periods and all 4 sweet orange plants tested negative for the HLB-phytoplasma, indicating that the leafhoppers did not acquire the HLB-phytoplasma from the infected sweet orange donor plant and thus, no transmission of the HLB-phytoplasma from sweet orange to sweet orange occurred.

Discussion

The HLB-phytoplasma in sweet orange and sunn hemp in SPS

The HLB-phytoplasma, a 16Sr group IX organism, was characterized on the basis of its 16Sr DNA sequence (Accession number: HQ423159) (Teixeira et al. 2008).

Since 2008, additional sweet orange trees infected with a group IX phytoplasma have been identified in SPS. The reference sweet orange tree from Parisi MU (Tables 1, 3, and 4, plant #1) is such a tree. The phytoplasma in these trees and the initial HLB-phytoplasma (Teixeira et al. 2008) were found to share the same 16Sr DNA sequence: above sequence HQ423159 and sequence KM877314 from plant #1 have 100% sequence identity. This sequence is that of the nested amplicon obtained from PCR amplification with primers P1/P7 followed by primers fU5/rU3 (Table 1, PCR technique “a1”). A further characteristic gene sequence of the HLB-phytoplasma has been obtained from the above referenced sweet orange tree #1 from Parisi MU: the RPGs *rpsC-rpIV-rpsS* (Table 3, sweet orange #1, Accession number: KJ806622). The RPG sequence comprises 1,316 bp.

In SPS, 31 sunn hemp plants were found to be infected with a 16Sr group IX phytoplasma and for all these phytoplasmas, the sequence of the nested fU5/rU3 amplicon was the same and identical to that of the HLB-phytoplasma in the reference sweet orange tree #1. Also, the phytoplasmas from randomly selected sunn hemp plants #2 and #3 (Table 1) shared with the HLB-phytoplasma of sweet orange tree #1, the following sequence with 100% identity: the 1,316-bp amplicon sequence of the RPGs *rpsC-rpIV-rpsS* (Table 3). In addition, only the 31 sunn hemp 16Sr group IX phytoplasmas and the HLB-phytoplasma from reference tree #1, gave positive PCR amplifications with the qPCR technique developed in this work (Table 1, PCR technique “d” and Fig. 3). Based on these results, there can be little doubt that the 16Sr group IX sunn hemp phytoplasma is identical to the HLB-phytoplasma in sweet orange.

S. marginelineatus, leafhopper vector of the HLB-phytoplasma

In SPS, all sunn hemp plants infected with a 16Sr group IX phytoplasma were, in fact, carrying the HLB-phytoplasma, showing that, at least in central, northern, and northwestern SPS, sunn hemp plants infected with the HLB-phytoplasma are widely distributed within the citrus-growing MUs (Fig. 2). These sunn hemp plants could well be the source of inoculum on which a putative insect vector becomes first infected with the HLB-phytoplasma and transmits it later to sweet orange. If so, the insect vector should be HLB-phytoplasma-positive. Indeed, Marques et al. (2012) have identified such a vector: the leafhopper *S. marginelineatus*. Also, as reported here, this leafhopper was able to acquire the HLB-phytoplasma while feeding on HLB-phytoplasma-infected sunn hemp plants. As the titer of the HLB-phytoplasma in sunn hemp is high, as shown in this work, acquisition of the HLB-phytoplasma from sunn hemp plants by the leafhoppers should be easy and efficient. Indeed, 34 of 41 batches of 5 insects became qPCR positive after 13 days of acquisition feeding on infected sunn hemp plants. Finally, the putative insect vector must be able to feed on, and inject, the HLB-phytoplasma in sweet orange. Marques et al. (2012) have shown that *S.*

marginelineatus occurs frequently and is dominant in citrus orchards, even though it is not a citrus leafhopper. In addition, the leafhopper did transmit the HLB-phytoplasma to sweet orange seedlings, even though at a low rate, as only 2 of 81 sweet orange test plants became infected (Marques 2011). In the work reported here, transmission of the HLB-phytoplasma by HLB-phytoplasma-infected *S. marginelineatus* leafhoppers to 8 sweet orange test plants could not be demonstrated (0 plants infected of 8 tested), probably because the number of sweet orange test plants, 8, was too low in comparison with the 81 plants of Marques (2011). However, in these Marques' experiments, the low infection rate of sweet orange with the HLB-phytoplasma from *S. marginelineatus* is in agreement with the epidemiology of the HLB-phytoplasma in nature: a very low percentage of HLB-phytoplasma-affected sweet orange trees in the farms (< 0.1%), random distribution of the symptomatic trees, no aggregation of symptomatic trees, and minimum distance between symptomatic trees: ~100 m (Teixeira et al. 2008). For all these reasons, *S. marginelineatus* is most probably one of the insect vectors acquiring the HLB-phytoplasma at high rate while feeding on infected sunn hemp plants but transmitting it at low rate and only occasionally to sweet orange trees. Marques (2011) has obtained HLB-phytoplasma-infected *S. marginelineatus* leafhoppers by acquisition feeding on HLB-phytoplasma-infected sweet orange. However, most probably in nature, infected sunn hemp plants, demonstrated to have a high titer of HLB-phytoplasmas and on which the leafhoppers become easily infected, as shown in this work, seem to be at least one of the major source plants on which *S. marginelineatus* leafhoppers acquire the HLB-phytoplasma in many SPS MUs.

Transmission assays of the HLB-phytoplasma from sweet orange to sweet orange

With *S. marginelineatus*. The very low percentage of sweet orange trees affected by the HLB-phytoplasma in citrus orchards does not suggest active transmission of the phytoplasma from sweet orange to sweet orange. Acquisition of the HLB-phytoplasma from sweet orange by *S. marginelineatus* leafhoppers has been reported (Marques 2011), but only 4 of 258 insects were positive by PCR amplification with nested primers D7f2/D7r2 of Teixeira et al. (2008). Transmission of the HLB-phytoplasma to sweet orange was also rare, since only 2 of 81 test plants became infected, as seen above (Marques 2011). Experiments to demonstrate citrus-to-citrus transmission by *S. marginelineatus* were also undertaken in this work, but gave only negative results. In particular, none of the 300 leafhoppers recovered after 2 days of acquisition feeding on symptomatic, HLB-phytoplasma-infected sweet orange plants became infected with the phytoplasma. This negative result might be due to several reasons: (i) the titer of the HLB-phytoplasma in sweet orange is low, 1,000 times lower than in sunn hemp; (ii) sweet orange is not the most preferred host of *S. marginelineatus*, even though some New World

Scaphytopius spp. are able to transmit the agent of citrus stubborn disease, *Spiroplasma citri*, from plant-to-plant (Calavan and Bové 1989); and (iii) a 2-day acquisition access period might have been too short.

With *D. citri*. In SPS, the HLB-associated liberibacters, Lam and Las, are efficiently transmitted from sweet orange to sweet orange by *D. citri*, the Asian citrus psyllid, because this insect is essentially monophagous and feeds exclusively on citrus and a few other rutaceous plants, such as *M. paniculata*. Therefore, transmission of the HLB-phytoplasma by *D. citri* has been examined in this work. Psyllids caged on HLB-phytoplasma-infected sweet orange trees for 2 weeks of acquisition feeding failed to become infected with the HLB-phytoplasma and, of course, no transmission to sweet orange plants was observed.

In summary, even though experimentally *S. marginelineatus*, but not *D. citri*, was shown to transmit the HLB-phytoplasma from sweet orange to sweet orange, even though at a very low rate, it seems likely that, in nature, transmission of the HLB-phytoplasma from sweet orange to sweet orange occurs only rarely, if at all.

The HLB-phytoplasma in SPS and other regions of the world

The HLB-phytoplasmas from sweet orange, sunn hemp, and other plants in SPS and additional regions, have been identified and characterized on the basis of, in particular, PCR amplification of RPGs *rpsC-rpIV-rpsS* with primers rp(IX)R2/rpL2F3, specific for 16Sr group IX phytoplasmas and sequencing the RPG amplicon (Table 1, PCR technique "b"). The RPG amplicons of all these plants have the same size, 1,316 bp (Table 3). Furthermore, in SPS, the RPG amplicons from all sweet orange and sunn hemp plants studied have the same sequence: the SPS-type-sequence (for instance: Table 4, plants 1, 2, and 3). The HLB-phytoplasma from the sweet orange trees in Bahia (Table 4, plant 4) has an RPG amplicon sequence very close to the SPS-type-sequence (99.77% identity), differing only by 3 SNPs and none of these leads to an amino acid change. In the case of the sweet orange trees from Mexico (Table 4, plants 5 and 6), the difference with the SPS-type-sequence involves 6 SNPs (99.54% identity). For the Bahia and Mexico phytoplasmas, one knows that they are HLB-phytoplasmas, in spite of 3 or 6 SNPs respectively, because HLB symptoms were seen in sweet orange trees infected with these phytoplasmas in the absence of liberibacters. In the case of the *G. sepium* plants from Costa Rica (Table 4, plants 7 and 8), the phytoplasma has not been identified in sweet orange and, with 9 SNPs in the RPG amplicon sequence (99.32% identity), it cannot be ascertained that one deals with an HLB-phytoplasma. The *P. echiooides* phytoplasma (Table 4, plant 9), with 61 SNPs and only 95.36% identity with the SPS-type-sequence, is probably not an HLB-phytoplasma. The HLB-phytoplasmas from SPS, Bahia, and Mexico, and perhaps the *G. sepium* phytoplasma from Costa Rica,

might represent different haplotypes, as defined by Nelson et al. (2011).

Phytoplasmas of 16Sr groups I, III, VII, and XV in sunn hemp in SPS

Sunn hemp plants infected with phytoplasmas of 16Sr groups I, III, VII, and XV have also been identified in SPS, but much less frequently than the HLB-phytoplasma: ~5 infected plants of 48 for each group compared to 31 of 48 plants for the group IX HLB-phytoplasma (Table 2). The lower number of sunn hemp plants infected with phytoplasmas of these additional groups might explain why such phytoplasmas have not been found in sweet orange. It remains to be seen if they will eventually show up in citrus in SPS, especially since phytoplasmas of 16Sr groups other than group IX have been found associated with HLB symptoms in citrus: group I phytoplasmas in China (Chen et al. 2009), as well as in Mexico (Arratia-Castro et al. 2014), and phytoplasmas of 16Sr group II, subgroup A, in China also (Lou et al. 2013).

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