A ‘*Candidatus phytoplasma asteris*’ (l6Srl group) phytoplasma associated with delayed maturity in soybean plants in Brazil

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Abstract Phytoplasmas were detected using nested PCR with extracts of soybean plants which exhibited delayed maturity. PCR assays, phylogenetic and virtual RFLP analysis identified the phytoplasma as a member of the l6Srl-B subgroup.

Keywords Aster yellows · Etiology · Mollicutes · Wall-less bacteria

Brazil is the second largest producer of soybean in the world (WAP 2017). There, this crop is affected by an important disease where stems of diseased plants remain green, and retain leaves at the stage in the cropping cycle when plants normally reach maturity, die off and are harvested. In addition, many pods abort, resulting in few pods per plant, and the number of grains per pod is also reduced (Meyer and Hirose 2010; Meyer 2015). High incidences of the disease cause crop yield loss of 40–60%, reduced grain quality, and difficulty in harvesting (Meyer and Hirose 2010). Initially, the problem was attributed to biotic agents such as mites and plant parasitic nematodes, or abiotic factors such as nutritional imbalance (Meyer and Hirose 2010; Meyer 2015). Elsewhere in the Americas, in Colombia (Granada 1979a), Mexico (Fletcher et al. 1984), USA (Derrik and Newsom 1984; Lee et al. 2002, 2011; Mollov

et al. 2014), Costa Rica (Villalobos et al. 2009), and Cuba (Acosta et al. 2015), similar symptoms have been associated with phytoplasmas. To investigate the possible occurrence of phytoplasma in diseased soybean plants in Brazil, eighteen symptomatic and six asymptomatic samples were collected from two commercial fields in the municipality of Sorriso (state of Mato Grosso), from a region that is one of the most important areas of soybean cultivation in that nation.

DNA was extracted from leaves and petioles using a Qiagen DNeasy Plant Mini Kit. Phytoplasma detection was conducted by nested PCR with universal primers Rl6mF2/Rl6mRl and Rl6F2n/16R2 (Gundersen and Lee 1996). Nested PCRs, primed by Rl6mF2/Rl6mRl followed by l6Srl group-specific primers R16(l)Fl/Rl (Lee et al. 1994), were employed for molecular identification. The nested PCR with universal primers generated DNA fragments of 1.2 kb for all symptomatic samples, but not for asymptomatic plants. Further nested PCR, primed by l6Srl group-specific primers, yielded a product of 1.1 kb from all symptomatic samples, indicating the occurrence of a phytoplasma of the l6Srl group.

DNA products size 1.2 kb from four phytoplasma isolates taken from different samples, were cloned using the pGEM-T Easy Vector System I (Promega), transformed into the *Escherichia coli* DH5 α strain and sequenced. Since no polymorphism was detected, a majority consensus sequence was selected for each isolate. The four sequences were indistinguishable and a consensus sequence of 1246 bp, designated Soy-Br1, was chosen to represent the soybean phytoplasma, and deposited in GenBank (JQ065052). This nucleotide sequence shared 99% similarity with sequences of l6Srl phytoplasmas. It was then aligned and compared with

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Fig. 1 Phylogenetic tree based on sequences of 16S rDNA from the phytoplasma found in association with soybean plants (Soy-Br1) and representatives affiliated with several subgroups of the 16SrI group. *Acholeplasma palmae* was used as outgroup. The numbers on the branches represent bootstrap values for 1000 replicates

sequences of phytoplasmas affiliated with distinct subgroups within the 16SrI group, employing DNA analysis programs (Phred phrap, Bioedit, and Multiple Sequence Alignment CLUSTALW). A phylogenetic tree was constructed, using file MEGA-5 program, with the neighbor joining method (Fig. 1). Moreover, phylogenetic analysis showed that the soybean phytoplasma was closely related to the representative of subgroup 16SrI-B, since they emerged from the same branch of

the phylogenetic tree (Fig. 1). Virtual RFLP analyses were simulated using the sequence representative of the phytoplasmas found in soybean and sequences of representatives of subgroups of the group 16SrI (Table 1). The trimmed and aligned sequences were exported for restriction analysis using the pDRAW32 program developed by AcaClone Software, for computer-simulated restriction digestion and virtual gel plotting (Wei et al. 2008). Similarity coefficients

Table 1 Similarity coefficients (F) derived from the sequence of the phytoplasma identified in soybean plants and reference phytoplasmas belonging to various subgroups within the 16SrI group

Subgrupo	A	B	C	D	E	F	K	L	M	O	P	Q	R	S	W	Soy-Br1
16SrI-A	1															
16SrI-B	0,92	1														
16SrI-C	0,91	0,93	1													
16SrI-D	0,93	0,97	0,90	1												
16SrI-E	0,91	0,93	0,92	0,90	1											
16SrI-F	0,86	0,86	0,89	0,85	0,87	1										
16SrI-K	0,90	0,92	0,91	0,89	0,91	0,92	1									
16SrI-L	0,89	0,97	0,90	0,94	0,90	0,85	0,89	1								
16SrI-M	0,88	0,96	0,93	0,95	0,89	0,84	0,88	0,93	1							
16SrI-O	0,87	0,87	0,80	0,84	0,84	0,75	0,81	0,84	0,83	1						
16SrI-P	0,92	0,94	0,93	0,91	0,93	0,94	1,00	0,91	0,90	0,81	1					
16SrI-Q	0,84	0,92	0,89	0,89	0,89	0,84	0,84	0,89	0,92	0,81	0,86	1				
16SrI-R	0,91	0,93	1,00	0,90	0,92	0,87	0,91	0,90	0,93	0,80	0,93	0,89	1			
16SrI-S	0,90	0,92	0,93	0,89	0,91	0,88	0,92	0,89	0,88	0,81	0,94	0,84	0,93	1		
16SrI-W	0,86	0,92	0,86	0,92	0,85	0,82	0,88	0,90	0,92	0,85	0,91	0,86	0,87	0,86		
Soy-Br1	0,92	1	0,93	0,97	0,93	0,88	0,92	0,97	0,95	0,87	0,94	0,92	0,93	0,92	0,92	1

(F) were calculated for each pair of phytoplasmas from distinct subgroups within the 16SrI group and the soybean phytoplasma, using the method of Wei et al. (2008). Restriction patterns generated by the soybean phytoplasma were identical to those produced by a reference phytoplasma of subgroup 16SrI-B. Therefore, the similarity coefficient (F) calculated between the soybean phytoplasma and the representative of subgroup 16SrI-B was equal to 1.0 (Table 1). Analysis by iPhyClassifier program (Zhao et al. 2009) suggested that the virtual RFLP pattern derived from the tested 16S rDNA F2n/R2fragment is identical (similarity coefficient 1.00) to the reference pattern of 16Sr group I, subgroup B.

Evidence of associations between phytoplasmas and soybean plants, with similar symptoms to those observed in Brazil, have been reported since the 1960s (Kitajima and Costa 1979). Thus, this disease was first found in Colombia, where the presence of phytoplasmas was demonstrated by leafhopper transmission and remission of symptoms using tetracycline (Granada 1979a, b). Later, in Mexico, association of phytoplasmas with plants showing green colouration and lengthening of the vegetative cycle was shown by electronic microscopy and leafhopper transmission (Fletcher et al. 1984). A disease characterised by delayed maturity was also reported in the United States of America (USA) and associated phytoplasmas were detected by microscopic observation (Derrick and Newsom 1984). Development of molecular tools enabled further characterisation. Phytoplasmas of the 16SrVI group and subgroup 16SrI-B were identified from soybean plants grown in the USA, which had green stem and leaves at the end of the crop cycle (Lee et al. 1998). Later, also in the USA, phytoplasmas of subgroups 16SrI-A and 16SrI-O were identified in plants with symptoms collectively called green-stem syndrome (Lee et al. 2002). In Costa Rica, a phytoplasma of the 16SrXXXI group was found in association with plants that remained green at harvest time (Villalobos et al. 2009; Lee et al. 2011). More recently, in the USA, a 16SrI-B phytoplasma was found associated with soybeans displaying bud proliferation and stunting (Mollov et al. 2014). Finally, in Cuba, stunting and aborted seed pods were associated with a phytoplasma of the 16SrI-B subgroup (Acosta et al. 2015).

The findings of the present study are thus in agreement with previous reports from other countries and provide further evidence that a phytoplasma of the 16SrI-B group is associated with diseased soybean plants in

Brazilian crops. In Brazil, phytoplasmas affiliated with the 16SrI-B subgroup have been also found in association with important diseases represented mainly by maize bushy stunt (Bedendo et al. 2000) and sugarcane yellow leaf (Silva et al. 2009). Thus, our findings show that soybean is an additional host species for a phytoplasma of the subgroup 16SrI-B in Brazil.

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