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Identification of potential leafhoppers vectors of phytoplasmas (16SrIII group) associated with broccoli stunt disease in Brazil

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Abstract Recently, disease in broccoli plants (*Brassica oleracea* var. *italica*) was associated with three distinct phytoplasmas in Brazil. The disease named broccoli stunt (BS) has caused significant economic losses in São Paulo State. Group 16SrIII phytoplasmas is the most common group that have been associated with BS. The BS disease is still poorly understood, and the lack of information about the vectors further impairs its management. In this study, leafhoppers belonging to 18 different species were collected from weeds thriving near broccoli fields that were affected by group 16SrIII phytoplasmas. Specific primers revealed the presence of group 16SrIII phytoplasmas in five leafhoppers: *Empoasca* spp., *Agallia albidula* Uhler, *Agalliana sticticollis* (Stål), *Planicephalus flavicosta* (Stål), and *Atanus nitidus* (Linnavuori). The identity of the phytoplasmas was confirmed through DNA sequencing analysis. The leafhoppers were infected by phytoplasmas of the 16SrIII group (proposed "*Candidatus* Phytoplasma pruni" species) and are phylogenetically related to the broccoli stunt phytoplasma (BSP) strains detected in the study area and, thus, are considered potential vectors of group 16SrIII phytoplasmas to broccoli plants.

Keywords Insect vector · Phytoplasma · Broccoli · Leafhopper

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Introduction

Leafhoppers (Hemiptera: Cicadellidae) are exclusively phytophagous insects and are major vectors of phytoplasmas (Weintraub and Beanland, 2006). Phytoplasmas are plant pathogenic prokaryotes associated with approximately 1000 plant diseases throughout the world (Hogenhout et al., 2008). These have been classified into groups based on restriction fragment length polymorphism (RFLP) patterns from the 16S rRNA gene or in putative "*Candidatus* Phytoplasma" species based on the sequence similarity of the same region (Lee et al., 1998; IRPCM, 2004). In Brazil, phytoplasmas (mainly of the 16SrI and III groups) have been associated with more than 30 plant species. Many of these pathogens cause economically significant crop diseases. Broccoli (*Brassica oleracea* var. *italica*) is one of the most important vegetable cultures in Brazil, whose trading volume in the São Paulo General Warehousing and

Centres Company (CEAGESP) is approximately 14,5 t per year (FNP Consultoria e Comercio, 2013). Recently, a phytoplasma associated with the disease named broccoli stunt (BS) has caused losses in around 10 % of broccoli crops in São Paulo State, Brazil. Group 16SrIII phytoplasmas are more commonly found in diseased broccoli plants, whereas group 16SrI and XIII have also been detected (Eckstein et al., 2013).

Despite the large number of known host plants for phytoplasmas, only one leafhopper species has been recorded as a vector in Brazil, namely *Dalbulus maidis* (DeLong and Wolcott), which transmits maize bushy stunt phytoplasma (Nault, 1980). The lack of information about vectors of phytoplasmas has been a limiting factor to the understanding and control of plant diseases associated with these pathogens. This is also the case for BS disease, which is still poorly understood and the phytoplasma vectors remain unknown. To identify possible vectors of phytoplasmas belonging to the 16SrIII group associated with the BS disease, we surveyed leafhoppers thriving on weed vegetation that was located near a broccoli field and tested these for the presence of group 16SrIII phytoplasmas.

Material and methods

During July to December 2009, leafhoppers were collected from weeds thriving near a broccoli agricultural area located at the municipality of Bragança Paulista, in the state of São Paulo, Brazil. The broccoli plants were infected by the phytoplasmas (BSP) of the group 16SrIII. The weeds at the surveyed area were mainly of the species Sida sp., Nicandra physalodes, Leonurus sibiricus, Palicourea marcgravii, Emilia sonchifolia, Sonchus oleraceus, Erigeron sp., Bidens pilosa, Crotalaria sp., and Ageratum conyzoides. The collected leafhoppers were separated according to species based on their external morphology. A part of the specimen was used for taxonomic identification and the remaining was stored in 70 % ethanol and subjected to molecular tests for detection and identification of phytoplasmas.

Leafhopper DNA was extracted according to Marzachi et al. (1998). The samples were composed of individual insects or batches of insects, arranged according to size (Table 1). Phytoplasma detection, was performed using nested polymerase chain reaction (PCR) assays with phytoplasma universal primers P1/Tint (Deng and Hiruki, 1991; Smart et al., 1996), followed by R16F2n/R16R2 (F2n/R2) (Gundersen and Lee, 1996), as described by Lee et al. (1998). Positive samples for F2n/R2 assays were subjected to other PCR tests for the detection of group 16SrIII phytoplasmas using group-specific primers R16 (III) F2/R16 (III) R1, then used in nested PCR assays for P1/Tint amplicons (Lee et al., 1994). Negative and positive controls of the PCR reactions included water and DNA from periwinkle plants infected with group 16SrIII phytoplasmas, respectively.

Table 1 Detection of phytoplasmas and specific group 16SrIII phytoplasmas in leafhoppers species collected near a broccoli field affected by group 16SrIII phytoplasmas at Bragança Paulista, SP, Brazil

Leafhopper species	N° individuals collected	Number of positive/tested samples[a]	Group 16SrIII
Subfamily *Megophthalminae*			
Agallia albidula (Uhler, 1895)	218	2/52 (3–5) [b]	Positive
Agalliopsis sp.	1	–	
Agallia cezia (Dutra, 1967)	2	0/1 (1)	
Agalliana sticticollis (Stål, 1859)	30	1/6 (3)	Positive
Subfamily *Deltocephalinae*			
Atanus nitidus (Linnavuori, 1955)	67	12/27 (1–3)	Positive
Atanus sp.(1)	1	–	
Atanus sp.(2)	1	–	
Chlorotettix minimus (Baker, 1898)	5	0/1 (1)	
Exitianus obscurinervis (Stål, 1859)	1	–	
Balclutha hebe (Kirkaldy, 1906)	264	2/56 (5)	Negative
Dalbulus maidis (DeLong and Wolcott, 1923)	3	–	
Planicephalus flavicosta (Stål, 1862)	30	1/15 (1–3)	Positive
Unerus colonus (Uhler, 1895)	11	0/4 (2)	
Scaphytopius fuliginosus (Osborn, 1923)	17	0/4 (2)	
Osbornellus infuscatus (Linnavuori, 1955)	2	–	
Subfamily *Typhlocybinae*			
Protalebrella brasiliensis (Baker, 1899)	25	0/4 (5)	
Parallaxis donaldsoni (Baker, 1903)	9	0/2 (3)	
Empoasca spp.	726	2/58 (10–15)	Positive
Total	1423	21/234	

[a] Number of leafhoppers samples in which phytoplasmas were detected by nested-PCR using universal primers F2n/R2 over the total tested

[b] The variable number of individuals per sample is indicated within parenthesis. The number of individuals per sample was determined according the leafhopper size

–: not tested

Nested PCR products were analysed in agarose gel (1 %), stained with Sybr® safe (Invitrogen, Carlsbad, CA, USA) and visualised on a UV transilluminator. The PCR products from a partial 16S rRNA gene were obtained using the universal primer pair F2n/R2. One positive sample from each leafhopper species that generated positive results for F2n/R2 and group specific primer pairs was submitted for direct nucleotide sequencing. The nucleotide sequences were deposited to GenBank as Accession Numbers JQ742079 to JQ742084. Sequence analysis and alignments were performed using the Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1997). A phylogenetic tree was constructed using MEGA 4.0 (Tamura et al., 2007). Alignments and phylogenetic trees were submitted to TREEBASE under ID 14423.

Results

A total of 1,423 leafhoppers specimens of the family Cicadellidae, representing three subfamilies (18 taxa and 14 genera), were collected. The majority of the leafhoppers (61.1 %) belonged to Deltocephalinae, whereas 22.2 % and

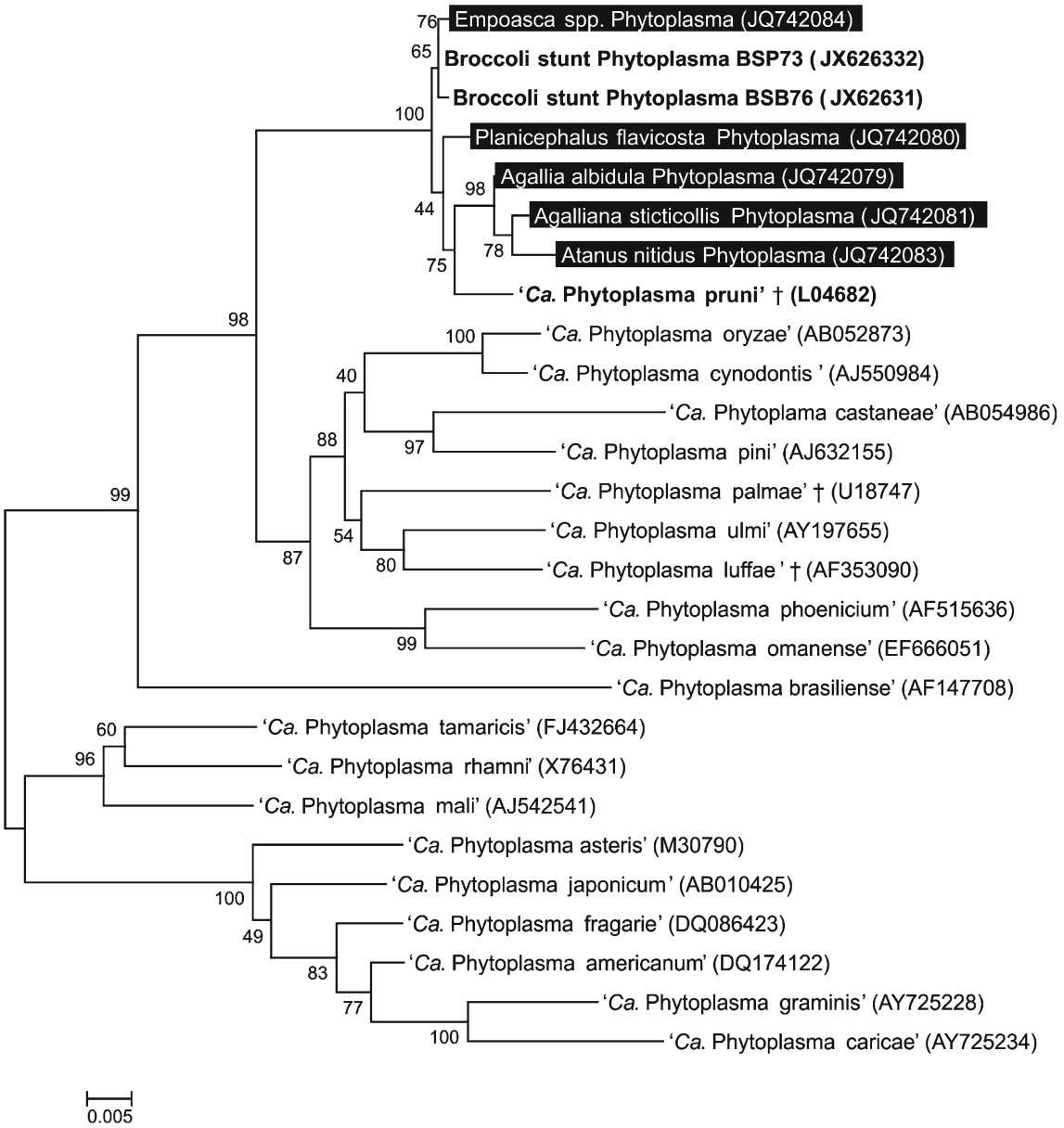

Fig 1 Phylogenetic tree, constructed by the neighbor-joining method, with 16S rRNA gene sequences showing relationship between phytoplasmas strains ofleafhoppers –this communication; boldface - and reference phytoplasmasfrom GenBank. Accession Nos. for sequences are shown in parenthesis alongside the names of the phytoplasmas. † this is an incidental citation and does not constitute prior citation according to Rule 28b of the Bacteriological Code

16.6 % belonged to Megophthalminae and Typhlocybinae, respectively (Table 1). The most abundant leafhopper genus was Empoasca spp. (Typhlocybinae), representing more than 50 % of the total number of specimens

Phytoplasmas were detected in six of the 12 leafhopper species subjected to nested PCR analysis with universal primers: Balclutha hebe (Kirkaldy), Empoasca spp., Agallia albidula Uhler, *Planicephalus flavicosta* (Stål), *Agalliana sticticollis* (Stål), and *Atanus nitidus* (Linnavuori) (Table 1). When primers specific to the 16SrIII phytoplasma were used, phytoplasmas were detected in five species, excluding *Balclutha hebe*.

The partial 16S rDNA sequence analyses using BLAST showed that the phytoplasma detected in *Empoasca* spp. (GenBank Accession No. JQ742084), *Planicephalus flavicosta* (GenBank Accession No. JQ742080), *Atanus nitidus* (GenBank Accession No. JQ742083), *Agallia albidula* (GenBank Accession No. JQ742079), and *Agalliana sticticollis* (GenBank Accession No. JQ742081) shared 98–99 % homology with the sequences of the phytoplasmas strains associated with the BS disease detected in the broccoli fields of Bragança Paulista in 2009 (GenBank Accession Nos. JX626332 and JX626331) (Eckstein et al., 2013), as well as with Canadian peach X phytoplasma (L33733), the reference strain of the 16SrIII group, and type member of the proposed putative species "*Ca*. Phytoplasma pruni" (IRPCM Phytoplasma/Spiroplasma Working Team-Phytoplasma Taxonomy Group 2004).

Phylogenetic analysis confirmed the results obtained using group-specific primers and sequence comparisons (Fig. 1) and showed that group 16SrIII phytoplasmas from *Empoasca* spp., *Agallia albidula*, *Atanus nitidus*, *Planicephalus flavicosta*, and *Agalliana sticticollis* could be classified into a single clade, together with the BS phytoplasma strains previously associated with the diseased broccoli plants in the same study field (Eckstein et al., 2013).

Discussion

This study observed a wide diversity of leafhopper species, most of them belonging to the Deltocephaline subfamily, although the most abundant leafhopper genus was *Empoasca* spp. of the Typhlocybinae subfamily. Deltocephalinae is a very important subfamily of phytoplasma vectors, encompassing 75 % of the known vectors. However, Typhlocybinae and Megophthalminae are also important because these comprise species that are considered as phytoplasma vectors (Weintraub and Beanland, 2006).

The results of the study indicated that various species of leafhoppers might be considered potential vectors of group 16SrIII phytoplasmas associated with BS disease. Although some of these species have been previously reported as vectors or potential vectors of phytoplasmas, our findings showed, for the first time, the association of phytoplasmas with other new species. In the *Empoasca* genus, two species were described as phytoplasma vectors, represented by *E. papaya* Oman, vector of a 16SrII group phytoplasma associated with bunchy top disease in papaya plants in Cuba (Pérez et al., 2010), and *E. decipiens* Paoli, identified as a vector of the 16SrI group phytoplasmas to *Chrysanthemum carinatum* plants (Galetto et al., 2011). Leafhoppers of the *Agalliana* genus were recently associated with phytoplasmas. Group 16SrIII phytoplasmas were detected by PCR assays in the species *Agalliana sticticollis* in the present study and in *Agalliana ensigera* Oman collected in an Argentinian garlic field affected by garlic decline disease that was associated with phytoplasmas (Catalani, 2011). In the *Agallia* genus, *Agallia albidula* was recently considered as a potential vector of group 16SrIII phytoplasmas to citrus plants in Brazil (Barbosa JC, personal communication).

To our knowledge, this is the first report that describes the detection of phytoplasmas in Deltocephalinae leafhoppers of the genus *Atanus*. It is worthy to note that almost 50 % of *Atanus nitidus* batches tested were positive for phytoplasmas, which is a rather high frequency rate, even for known phytoplasma vectors, which strongly suggests an interaction between this leafhopper species and the phytoplasma found in its body tissues. Thus, *Atanus nitidus* may be an important vector in broccoli fields, if it is able to transmit group 16SrIII phytoplasmas. *Planicephalus flavicosta* was already reported as a vector of phytoplasmas in periwinkle plants (Dabek, 1982), but no additional information about this Deltocephalinae species in association with phytoplasmas has been published since then.

In this work, a total of five leafhoppers species were considered as a potential vector of group 16SrIII phytoplasmas present in broccoli. Our findings contribute for additional information pertaining to this research topic in Brazil, particularly on phytoplasmas of the 16SrIII group, which have been frequently detected in association with several plant diseases such as cauliflower stunt (Rapussi et al., 2012), as well as other plant hosts (Montano et al., 2011). Furthermore, for the first time, this study generated evidence for the association of phytoplasmas with leafhoppers belonging to the species *Planicephalus flavicosta*, *Empoasca* sp., *Agalliana sticticollis*, *Atanus nitidus*, and *Agalia albidula* in Brazil. However, it is also well known that some insects can acquire phytoplasmas but are unable to transmit them (Vega et al., 1993). Thus, a next reasonable step involving transmission trials must be developed to determine the ability of these species to transmit phytoplasmas to broccoli, as well as other vegetables.

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