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Transmission of "*Candidatus* Phytoplasma pruni"-related strain associated with broccoli stunt by four species of leafhoppers

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Abstract

A disease known as broccoli stunt, associated with "*Candidatus* Phytoplasma pruni"-related strain, has been responsible by significant economic losses in crops grown in the State of São Paulo, Brazil. Previous investigations evidenced some species of leafhoppers observed in broccoli fields as potential vectors of the phytoplasma. In this study, the six species more frequently found in broccoli crops were collected to confirm that evidence. Group of five insects of each species were confined per broccoli seedling for an inoculation access period (IAP) of 48 hr. After the IAP, each group was tested for detection of phytoplasma. Evaluation of plants was performed 60 days after inoculation based on the presence of phytoplasma in their tissues. When transmission was positive, genomic fragments corresponding to 16S rDNA were sequenced both for the infected plants and its respective group of insects. The results revealed that the species *Agallia albidula*, *Agalliana sticticollis*, *Atanus nitidus* and *Balcluta hebe* were able to transmit phytoplasma to broccoli seedlings. Based on the estimates of transmission probability by single insects (*P*), the highest transmission rate was observed for *A. nitidus* (24.2%) and the lowest for *B. hebe* (1.9%). The sequencing of 16S rDNA revealed complete similarity between the sequences of the phytoplasma transmitted to broccoli test plants and the sequences of the phytoplasma found in the field-collected leafhoppers. These findings support the inclusion of those species as vectors of phytoplasmas belonging to 16SrIII group in broccolis, providing additional information to improve management of this important disease of endemic occurrence.

KEYWORDS

insect vectors, phloem bacteria, phytoplasma, wall-less bacteria

1 | INTRODUCTION

Broccoli (*Brassica oleracea* var. *italica* Plenck) is one of the most important vegetable crops in Brazil. The state of São Paulo (SP) is the top producer, with 42,227 ton a cultivated area of 2,584 ha in 2016 (IEA, 2016). In commercial fields situated in Bragança Paulista (SP), a municipality located within the green belt area of São Paulo (SP) city, plants showing a disease known as broccoli stunt were observed. Affected plants exhibit stunting, inflorescence malformation, reddening leaves and vessel necrosis. In previous studies performed with broccoli plants sampled in the green belt area, it was demonstrated the presence of phytoplasma of 16SrIII group ("*Candidatus* Phytoplasma pruni" - Davis et al., 2013) in association with the diseased plants (Eckstein et al., 2013). However, the phytoplasma was not identified to level of subgroup. The disease causes yield losses that range from 3% to 10% (Eckstein et al., 2013). Phytoplasmas belonging to 16SrIII were

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also reported in other species of cruciferous such as cabbage (16SrIII-subgroup not identified) (Mello, 2007) and cauliflower (16SrIII-J) (Rappussi et al., 2012) grew in commercial fields. Investigations performed by Eckstein et al. (2014) revealed the presence of phytoplasmas affiliated with 16SrIII group in leafhopper (Hemiptera: Cicadellidae) species collected in the same fields cultivated with broccoli, such as *Agallia albidula Agalliana sticticollis*, *Atanus nitidus*, *Empoasca* spp. and *Planicephalus flavicosta*, which were considered potential vectors of group 16SrIII phytoplasmas. Although numerous species of plants have been reported as phytoplasma hosts in Brazil, the identity of vector species is unknown in most cases (Eckstein et al., 2014). The lack of vector information has been a limiting factor to understanding epidemiological aspects and management of several diseases associated with this type of pathogen (Rappussi et al., 2012; Eckstein et al., 2014). This study was conducted to identify vector species associated with spread of broccoli stunt, by running transmission assays of phytoplasmas to broccolis with field-collected leafhoppers.

2 | MATERIAL AND METHODS

Insects were collected with a sweep net from broccoli fields in Bragança Paulista (SP) (22°57′07″S; 46°32′32″W) showing high incidence of broccoli stunt, when the plants presented inflorescence. In the laboratory, collected specimens of the leafhoppers *A. albidula*, *A. sticticollis*, *A. nitidus*, *Balcluta hebe*, *Empoasca sp.* and *Scaphytopius fulginosus* were separated based on their external morphology. Voucher specimens were preserved, and the species identification was carried out by leafhopper taxonomist (Keti Zanol, Univ. Federal do Paraná, Curitiba, Brazil).

Healthy broccoli seedlings (test plants) were grown in pots kept in a screened greenhouse to avoid contact with insects. At 3 weeks after seeding, the test plants were exposed to the field-collected leafhoppers for an inoculation access period (IAP) of 48 hr. Four test plants were inoculated for each leafhopper species by confining groups of five insects per plant, except in the case of *B. hebe*, for which 15 leafhoppers were used per plant. Four test plants non-exposed to leafhoppers were kept as negative control of the transmission experiment.

After the IAP, each group of insects was separately collected and submitted to PCR assays for detection of phytoplasmas. The test plants were evaluated 60 days later for phytoplasma infection based on PCR assays. Total DNA from plants was extracted using the Dneasy Plant Mini Kit (Qiagen) following the manufacturer's instructions. Leafhopper DNA was extracted as described by Marzachi, Veratti, and Bosco (1998). Nested PCRs were performed with universal primers P1/Tint (Deng & Hiruki, 1991; Smart et al., 1996) and group-specific primers R16 (III)F2/R16(III)R1 according to Lee, Gundersen-Rindal, Hammond, and Davis (1994). Positive and negative controls were represented by DNA from chayote infected with witches' broom phytoplasma (16SrIII-J) and broccoli test plants non-exposed to insects, respectively.

When transmission was positive, products of 0.8 Kb generated by nested PCR with specific primers R16(III)F2/R16(III)R1 were sequenced both for the infected plant and its respective group of leafhoppers. Sequenced fragments of 16S rRNA gene from phytoplasma found in plants and batches of insects were compared. Both fragments were also compared with DNA sequence of the chayote witche's broom phytoplasma, a representative of the 16SrIII-J subgroup. Subsequently, diverse products generated by P1/Tint were also submitted to nested PCR assays, now using 16F2n/R2 for re-amplification (Gundersen & Lee, 1996), to classify the phytoplasmas from broccoli and insects within subgroups belonging to 16SrIII group. The amplified products were cloned in *Escherichia coli* DH5alpha strain using pGEM easy Vector System (Promega). All sequences were sequenced by specialized enterprise. The identity of phytoplasmas was determined using an online phytoplasma classification tool, *i*PhyClassifier based on 16S rRNA gene F2n/R2 sequences (Zhao et al., 2009).

TABLE 1 Transmission of group 16SrIII phytoplasmas by leafhoppers collected in fields affected by broccoli stunt in Bragança Paulista, SP, Brazil

		Transmission to broccoli plants	
Leafhopper species	Proportion of PCR-positive insect samples	Proportion of infected test plants	P^c
Agallia albidula	3/4[a]	2/4[b]	0.129
Agalliana sticticollis	3/4	2/4	0.129
Atanus nitidus	4/4	3/4	0.242
Balcluta hebe	2/4	1/4	0.019
Empoasca sp.	1/4	0/4	0
Scaphytopius fulginosus	1/4	0/4	0

[a]Number of samples of five insects that were PCR-positive for group 16SSrIII phytoplasmas over the total number of samples tested.
[b]Number of broccoli test plants that were PCR-positive for group 16SrIII phytoplasmas over the total number of test plants. Each test plant was inoculated by a group of five insects, except in the case of *B. hebe* for which 15 insects were used per test plant.
[c]Transmission probability by single insects (P) was calculated as described by Swallow (1985), with the formula $P = 1 - (1 - p)^{1/k}$, where p is the proportion of infected test plants and k is the number of insects used for inoculation of each test plant.

Because different numbers of insects were used per test plant in transmission assays depending on the leafhopper species, we estimated the transmission rate by single individuals (*P*) with the formula $P = 1–(1–p)^{1/k}$, where *p* is the proportion of infected test plants and *k* is the number of insects used for inoculation of each test plant (Swallow, 1985).

3 | RESULTS

Phytoplasmas were detected in groups of five field-collected specimens of all leafhopper species tested, although the frequency of detection varied among the species (Table 1). The species *A. albidula*, *A. sticticollis*, *A. nitidus* and *B. hebe* were able to transmit phytoplasma to broccoli test plants. Based on the estimates of transmission probability by single insects (*P*), the highest transmission rate was observed for *A. nitidus* (24.2%) and the lowest for *B. hebe* (1.9%) (Table 1). The presence of phytoplasmas in leafhopper and broccoli samples was revealed by amplification of genomic fragments of 0.8 Kb, which evidenced the occurrence of representatives of the 16SrIII group. DNA fragments of 0.8 Kb were consistently amplified from positive control, while none of the plants non-exposed to leafhoppers (negative control) were PCR-positive.

Analysis of nucleotide sequences of 16S rDNA demonstrated 99% similarity between the sequences of the phytoplasma transmitted to broccoli test plants and the sequences of the phytoplasma found in the field-collected leafhoppers. In addition, both sequences share 99% similarity in relation to the sequence of the strain representative of the 16SrIII group used as control. Two sequences (GenBank: MG988412 and MG988413) were selected as representatives of the phytoplasma detected in broccoli test plants, and one sequence (GenBank MG988414) was used as representative of the phytoplasma found in leafhoppers. These sequences were submitted to the classification system *i*PhyClassifier, and all the tree phytoplasmas were identified as belonging to the 16SrIII-X subgroup.

4 | DISCUSSION

Our findings reinforced those previously reported by Eckstein et al. (2014), in which phytoplasmas affiliated with group 16SrIII were detected in insects of the species *A. albidula*, *A. sticticollis*, *A. nitidus* and *Empoasca* sp. In addition, our results revealed that *A. albidula*, *A. sticticollis* and *A. nitidus* transmitted phytoplasmas to broccoli test plants, demonstrating that these species are not only harbouring group 16SrIII phytoplasmas in the field but they are also vectors of these pathogens. Therefore, the previous suspicion considered that these leafhoppers species could transmit phytoplasmas (Eckstein et al., 2014) was confirmed in the present investigation.

Nested PCRs with group-specific primers for detection of phytoplasmas were used in our study because previous results (Eckstein et al., 2014) revealed consistently the prevalence of 16SrIII strains in broccoli plants and insects collected in the sampled fields. Furthermore, the detection with specific primers was more efficient compared with diverse universal primers. Although the broccoli plants did not exhibit symptoms during the evaluation period, transmission was clearly demonstrated by the PCR assays. Analysis processed by the system *i*PhyClassifier revealed a perfect identity among the 16S rRNA sequences of the phytoplasmas providing sufficient support to demonstrate that the strain found in field-collected leafhoppers was transmitted to broccoli test plants. Our findings support the inclusion of the leafhopper species *A. albidula*, *A. sticticollis*, *A. nitidus* and *B. hebe* as vectors of phytoplasmas affiliated with 16SrIII group in broccolis, providing additional information to the understanding of broccoli stunt epidemiology as well as to improve management strategies for this important disease of endemic occurrence in broccoli crops. It is also a relevant contribution to ecological studies of phytoplasmas in Brazilian agroecosystems, where the corn leafhopper *Dalbulus maidis* was until now the only species confirmed as a vector. Because some of these new vectors species (particularly *A. albidula* and *B. hebe*) are highly abundant on weeds present on the spontaneous vegetation of some crops affected by phytoplasmas (Marques, Teixeira, Yamamoto, & Lopes, 2012; Oliveira et al., 2013), they may be involved in the epidemiology of other diseases associated with these pathogens.

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