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Amaranthus caudatus subsp. *mantegazzianus*: A new host of '*Candidatus* Phytoplasma hispanicum' (subgroup 16Sr XIII-A)

Maria Cristina Noelting[1] | Jacson Ferreira[2] | Sarah Rodrigues Galvão[2] | Eduardo José Greizerstein[3] | Maria del Cármen Molina[4] | César Gabriel López[3] | Ivan Paulo Bedendo[2]

[1]College of Agricultural and Forestry Sciences, National University of La Plata, La Plata, Argentina

[2]Plant Pathology and Nematology Department (ESALQ), University of São Paulo, Piracicaba, Brazil

[3]College of Agricultural Sciences, National University of Lomas de Zamora, Lomas Zamora, Argentina

[4]The National Scientific and Technological Research Council, Buenos Aires, Argentina

Correspondence
Ivan Paulo Bedendo, Plant Pathology and Nematology Department (ESALQ) University of São Paulo, CP 09, 13418-900 Piracicaba, SP, Brazil.
Email: ibedendo@usp.br

Abstract

In Argentina, amaranth is a promising crop due to high nutritional quality and ability to grow in a diversity of environments. In areas cultivated with amaranth, were observed plants exhibiting slow growth, deformed leaves, proliferation of shoots and malformed lateral panicles. Field survey revealed up to 96% disease incidence and 92% of the seeds collected from mother plants produced diseased seedlings. A phytoplasma was detected in association with seedlings and adult plants using nested PCR assays. Molecular identification by computer-simulated RFLP and phylogenetic analysis evidenced the occurrence of a '*Candidatus* Phytoplasma hispanicum'-related strain, affiliated with 16SrXIII-A subgroup. The findings implicate amaranth as a new host for this subgroup and as a potential reservoir of the pathogen for other cultivated species. In addition, to the best of our knowledge, this study reports for the first time the presence of 16SrXIII-A phytoplasma in Argentina and in South America. Furthermore, transmission assays pointed that naturally infected seed is an important vehicle of dissemination of the pathogen, threatening the expansion of the crop for new geographical areas.

KEYWORDS
amaranth, fastidious bacteria, mollicutes, phytopathogenic prokaryote, yellows disease

1 | INTRODUCTION

The *Amaranthus* genus is widely distributed around the world, and its diverse cultivated species serve as important sources of nutrients, although numerous species are considered as aggressive weeds in fields cultivated with several commercial crops (Yang, Zhao, Li, & Zhu, 2011). This herbaceous plant presents desirable traits for cultivation, among them good growth in poor soils, tolerance to water restriction and resistance to pathogens and insects (Ochoa-Sánchez, Parra-Cota, Aviña-Padilla, Délano-Frier, & Martínez-Soriano, 2009).

In relation to phytosanitary aspects, besides fungi and virus, phytoplasmas have been reported in association with amaranth in Mexico (Rojas-Martínez, Zavaleta-Mejía, Lee, & Aragón-García, 2009 and Ochoa-Sánchez et al., 2009) and China (Yang et al., 2011). In Mexico, a phytoplasma representative of the 16SrII group, a '*Candidatus* Phytoplasma aurantifolia'-related strain, was identified in association with *Amaranthus hypochondriacus* and *A. cruentus* plants that exhibited unusual colouration and excessive proliferation of stems and shoots (Ochoa-Sánchez et al., 2009). Also in India, a phytoplasma affiliated to the 16SrII group was reported infecting *Amaranthus* sp. plants with yellowing leaves (Rao, Mall, Raj, & Snehi, 2011). In Mexico, a group 16SrIII phytoplasma, '*Ca*. Phytoplasma pruni'-related strain, was associated with *A. hypochondriacus* plants with a complex of symptoms including panicle proliferation, leaf

yellowing, shortening of internodes, and some time stunted shoots (Rojas-Martínez et al., 2009). Interestingly, in China, a phytoplasma belonging to 16SrV, '*Ca*. Phytoplasma ziziphi'-related strain, was molecularly characterized in asymptomatic plants of *A. retroflexus* species (Yang et al., 2011). In Argentina, amaranth is a promising crop due to high nutritional quality of the grains and capacity of the plants to grow in diverse climate and soil conditions. Despite fungal diseases were important (Noelting, 2010), recently a phytoplasma affiliated with 16SrIII group was detected in germinated seeds of amaranth (Noelting, Rojas-Martínez, & Molina, 2015).

Thus taking into account the occurrence of phytoplasma in amaranth crops in Argentina and the limited knowledge of the disease, the present study was carried out to (a) estimate the disease incidence in a crop installed in natural conditions; (b) evaluate the possible transmission of the pathogen through the seeds; and (c) characterize molecularly the phytoplasma associated with diseased plants.

2 | MATERIAL AND METHODS

The disease incidence was estimated through an assay installed in experimental field using five seed lots belonging to *Amaranthus caudatus* ssp. m*antegazzianus* (L.) Prior to sowing, the seeds were disinfested by immersion in a 2% sodium hypochlorite solution for 5 min, followed by two rinses with sterile distilled water and subsequently dried between sterile filter papers. The sowing in the field was carried out manually in four-row plots of 4 m long, separated from each other at 0.5 m, with three replications. The incidence was evaluated at 64 and 129 days after the emergence by visual inspection of the plants. The percentage of incidence of symptomatic plants was calculated using the following formula: Incidence (%) = N ° Total of symptomatic plants × 100/Total plants.

Seed transmission test was performed in trays of 72-pot cells using sterile substrate employing 80 seeds from two symptomatic plants positive for phytoplasma and 200 seeds sampled from positive asymptomatic mother plants of the five seed lots. Before sowing, seeds were prepared as described above. Subsequently, the plants were transplanted into 120 cc capacity telgopor containers. The incubation was conducted in a growth chamber at 25°C and 12-hr photoperiod, during 120 days. During the test, the substrate was kept moistened by the capillarity technique, which consists of adding water to the trays containing the spildings. Transmission of the pathogen was determined based on the percentage of incidence of symptomatic seedlings, which was calculated based on the formula mentioned above. Molecular analysis for phytoplasma detection was carried out from symptomatic plants.

Detection of phytoplasma was conducted using young shoots of two symptomatic plants (samples Arg1 and Arg2) and one asymptomatic plant (sample Arg5) sampled from experimental field, and two plants (samples Arg3 and Arg4) that manifested symptoms under controlled conditions (growth chamber). A Promega kit (The Wizard® Genomic DNA Purification) was employed for extraction, and the total DNA was used in nested PCR assays primed by the primers P1/P7 (Deng & Hiruki, 1991; Schneider, Seemuller, Smart, & Kirkpatrick, 1995) followed by R16F2n/16R2 (Gundersen & Lee, 1996). Each phytoplasma found in each amaranth sample was considered as a strain.

Molecular characterization was performed with amaranth phytoplasma found in the samples Arg1, Arg3 and Arg5, which were selected after sequencing of the 16S rRNA gene. DNA sequences of 1.2 kb were cloned using pGEM Easy Vector System I (Promega), transformed in *Escherichia coli* DH5α strain, and the resulting plasmids were sequenced. Three clones were sequenced for each strain, and one sequence was selected for each strain due to sequence identity among the clones. DNA fragments were aligned and compared among themselves and with sequences of phytoplasmas belonging to distinct groups of phytoplasmas deposited in GenBank, using software DNA programs (Bioedit, Blast and Multiple Sequence Alignment – CLUSTALW). The three-sequence representatives of each strain were identical to each other, and one of them was selected to represent the phytoplasma found in amaranth. A phylogenetic tree was constructed with the sequence representative of amaranth phytoplasma and sequences of the 16S rRNA gene of different phytoplasmas belonging to the subgroups classified within the 16SrXIII group, employing MEGA program with the neighbour-joining method. Bootstrapping was performed 1,000 times, and *Acholesplasma laidlawii* served as outgroup. A computer-simulated RFLP analysis of the 16S rRNA gene was used, employing the sequence of the amaranth phytoplasma of approximately 1.2 kb. The trimmed and aligned sequence was exported to the pDRAW32 program (ACACLONE Software) for computer-simulated restriction digestion and virtual gel plotting (Wei, Davis, Lee, & Zhao, 2007). The digestion in silico was processed with 17 restriction enzymes, and a virtual 3% agarose gel electrophoresis image was plotted and captured as a PDF file (Wei et al., 2007). The virtual RFLP profiles generated by the strain representative of the phytoplasma found in amaranth plants were compared with those reported for various phytoplasmas belonging to different subgroups within the 16SrXIII group. A similarity coefficient (*F*) was calculated for each pair of phytoplasma based on restriction patterns generated by computer-simulated RFLP analysis (Wei et al., 2007).

3 | RESULTS

In field conditions, the early symptoms of disease were observed 3 days after emergence, characterized as chlorotic cotyledons rolled inwards found in seedlings of amaranth grown in the experimental plots. The symptoms were progressively accentuated during the growth of the plants. Plants of lower height, axillary regrowths of small size, and shortening internodes were observed 64 days after emergence (Figure 1a). At the stage of onset of panicle formation, 106 days after emergence, symptomatic plants exhibited small lateral panicles, atrophied shoots with leaves curved inwards, and leaves with necrotic margins (Figure 1b). In some cases, affected

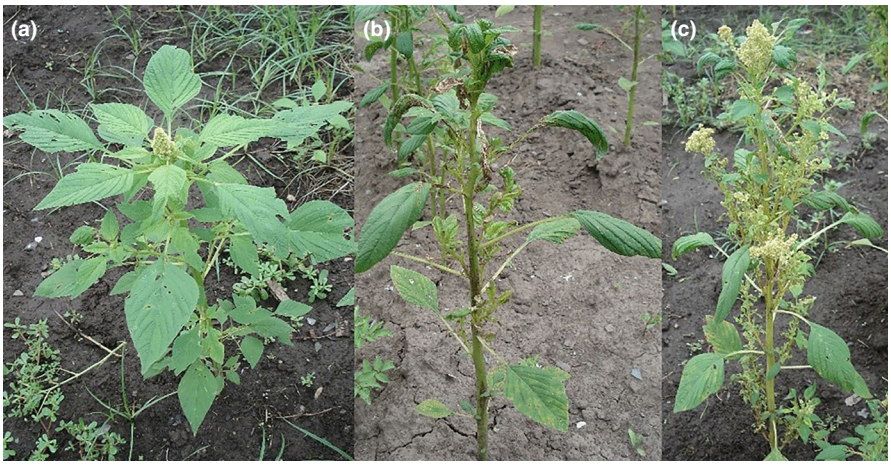

FIGURE 1 Amaranth plants exhibiting some symptoms associated with phytoplasma: (a) asymptomatic plant; (b) symptomatic plant 64 days after emergence; (c) symptomatic plant 109 days after emergence

plants displayed yellowing of the foliage, phloem necrosis and poor root development. The disease incidence ranged from 49% to 96% for the evaluations performed to the 64 and 106 days after emergence of the plants, respectively. The association between phytoplasma and diseased plants was demonstrated by the amplification of approximately 1.2 kb DNA fragment using nested PCR primed by the P1/P7-16F2n/R2 primer pairs.

The pathogen transmission test by seeds revealed a germination rate of 100%. Immediately after emergence, were verified 257 symptomatic seedlings derived from both symptomatic and asymptomatic mother plants, corresponding to almost 92% of the total of emerging seedlings. These seedling displayed symptoms of chlorosis and curling of the cotyledons similar those observed in the field assay. Evaluation made at the 109 days after emergence allowed to observe typical symptoms of phytoplasma characterized by malformations in young leaves and buds, excessive proliferation of axillary buds, and narrow and curved inward leaves. Phytoplasma was detected in symptomatic plants sampled at random, through amplification of DNA fragments of 1.2 kb generated by nested PCR, confirming the presence of this pathogen in tissues from diseased plants.

Concerning molecular characterization of the amaranth phytoplasma, the analyses of the nucleotide sequences of the 16S rDNA showed perfect identity each of others, considering all the nine sequenced clones, allowing select one of them as representative of the amaranth phytoplasma. The sequences of the clones representatives of the samples Ag1, Ag3 and Ag5 were denominated ASP-Ar1, ASP-Ar3 and ASP-Ar5, respectively, where the ASP means Amaranth Shoot Proliferation. These three sequences were deposited in GenBank database, under accession number MK440609, MK440610 and MK440611, respectively. The sequence ASP-Ar1 was selected to represent the phytoplasma detected in amaranth plants. The sequence of the ASP-Ar1 phytoplasma shared 98.8% similarity with sequence of the reference phytoplasma of the group 16Sr XIII (Mexican periwinkle virescence phytoplasma) a 'Ca. Phytoplasma hispanicum'-related strain (Davis, Zhao, & Dally, 2016). Based on computer-simulated virtual RFLP patterns, a value of similarity coefficient (F) equal to 0.98 was obtained, when the ASP-Ar1 phytoplasma identified in amaranth was contrasted with the representative of the subgroup 16SrXIII-A (Table 1). However, this strain showed values of (F) lower than 0.97 when compared with members of the subgroups into the 16SrXIII group. According to the current scheme adopted for phytoplasma classification, (*F*) values higher 0.97 allow to aggregate strains in a same subgroup (Wei et al., 2007). The phylogenetic tree generated with DNA sequences from phytoplasmas belonging to the main groups reported in Brazil, representatives of distinct subgroups within 16SrXIII group, and the amaranth phytoplasma evidenced that the latter is closely related to others members of 16SrXIII group (Figure 2). Specifically, the phylogenetic analysis pointed that ASP-Ar1 phytoplasma is tightly clustered with the reference phytoplasma of the 16SrXIII-A subgroup.

4 | DISCUSSION

The increase of diseased plants observed between the first (64 days) and second (129 days) evaluations could be attributed to late manifestation of symptoms by infected plants or even late transmission by possible insect vectors, as discussed by Camarena-Gutiérrez and De La Torre-Almaraz (2008). The symptoms present in affected amaranth plants are recognized as typically induced by phytoplasmas (Lee, Davis, & Gundersen-Rindal, 2000). Moreover, some symptoms identified in this study were coincident with those described in Mexico in amaranth plants infected by distinct phytoplasmas, such as excessive proliferation of shoots, leaf yellowing, shortening of internodes, and stunted shoots (Rojas-Martínez et al., 2009 and Ochoa-Sánchez et al., 2009). The consistent detection of phytoplasma by amplification of 1.2 kb DNA fragments confirmed the initial presumption of the association of the symptoms exhibited by the affected plants with this type of pathogen. These results are coincident with those reported from Mexico, which revealed firmly the association of phytoplasmas with diseased amaranth plants (Ochoa-Sánchez et al., 2009; Rojas-Martínez et al., 2009). Interestingly, our results also showed asymptomatic plants harbouring phytoplasma; this fact is not rare for diseases associated with phytoplasmas and can be attributed to diverse factors, among them absence of symptoms due to late infection in a species of short growing cycle

TABLE 1 Similarity coefficient (*F*) values obtained from amaranth shoot proliferation phytoplasma (ASP-Ar1) and phytoplasmas affiliated with subgroups belonging to the 16SrXIII group

Subgroups	16SrXIII-A	16SrXIII-B	16SrXIII-C	16SrXIII-D	16SrXIII-E	16SrXIII-F	16SrXIII-G	16SrXIII-H	16SrXIII-I	16SrXIII-J	ASP-Ar1
16SrXIII-A (AF248960)	1										
16SrXIII-B (U96616)	0.95	1									
16SrXIII-C (AF495882)	0.86	0.82	1								
16SrXIII-D (FJ914647)	0.92	0.89	0.82	1							
16SrXIII-E (JQ792171)	0.92	0.87	0.79	0.70	1						
16SrXIII-F (KJ921641)	0.89	0.80	0.74	0.83	0.81	1					
16SrXIII-G (DQ444264)	0.91	0.90	0.95	0.87	0.83	0.80	1				
16SrXIII-H (JX626329)	0.95	0.94	0.91	0.91	0.91	0.84	0.92	1			
16SrXIII-I (KT444663)	0.87	0.92	0.84	0.87	0.85	0.78	0.86	0.88	1		
16SrXIII-J (EU719108)	0.83	0.76	0.74	0.75	0.77	0.92	0.72	0.76	0.70	1	
ASP-Ar1 (MK440609)	0.98	0.95	0.88	0.89	0.95	0.89	0.89	0.96	0.85	0.79	1

(Rapussi et al., 2012). Occurrence of phytoplasma in tissue of symptomless amaranth plants was also reported in China in plants collected at random from different parts of an orchard (Yang et al., 2011). In another case, an aster yellows phytoplasma was found in a large proportion of asymptomatic canola plants (Olivier, Galka, & Sèguin-Swartz, 2010).

Phytoplasma transmissibility by seed has been investigated since the beginning of 2000s (Bertaccini & Duduk, 2009). One of the first indications was found in alfalfa plants infected by phytoplasmas that showed evidences of phytoplasma transmission by seeds. In another cases, seeds produced by disease plants of lime and tomato, which were allowed to germinate, displayed the presence of phytoplasmas in several growth stages. Amaranth seeds as vehicle of transmission of phytoplasma demonstrated in our assays reinforce these previous reports. Specifically, our results are in perfect agreement with study performed in Mexico, in which the occurrence of phytoplasma was detected in germinated amaranth seeds sampled from symptomatic plants naturally infected (Rojas-Martínez et al., 2009). Although transmission of phytoplasma by seeds is still controversial, the mentioned results point in positive sense to transmission. In agreement with these findings, aster yellows phytoplasma was detected in up to 80% of the seedlings derived from misshapen seeds and 60% of seedlings from normal-looking seeds produced by symptomatic canola plants (Olivier et al., 2010). In addition, seeds from symptomatic or asymptomatic plants germinated up to 90% and canola plants derived from infected seedlings exhibited malformations such as shrivelled leaves and general growth delay. Diverse phytoplasmas were also identified in seedlings from oil seeds and tomato emerging of seeds sampled of symptomatic plants, reinforcing the transmissibility of these pathogens via seeds (Calari et al., 2011). Seeds as phytoplasma vehicle were also reported in studies with *Sesamum indicum*, *Brassica napus*, *Solanum lycopersicum* and *Zea mays*, which revealed that seeds derived from mother plants infected by different ribosomal groups of phytoplasmas generated seedlings, whose the presence of phytoplasmas was detected in different stages of growth (Satta, 2017). Transmission through seeds was also confirmed by the detection of phytoplasma in commercial seedlings of alfalfa (Khan, Botti, Paltrinieri, Al-Subhi, & Bertaccini, 2002). In addition, in alfalfa, a severe effect was observed on seed production, which frequently caused the death of plants and reduced the vigour in those that survived (Pilkington et al., 2003). Thus take into consideration it is very feasible that the increase of phytoplasma diseases in several crops worldwide it would be due to its transmission by seed in some of them, which has been ignored for several decades.

The collective virtual RFLP patterns and similarity coefficient calculation provided support for delineation of the phytoplasma associated with amaranth shoot proliferation as belonging to the 16SrXIII-A subgroup since the values of sequence similarity and similarity coefficient were in perfect concordance. In addition, due to the value of $F = 0.98$, the ASP-Ar1 phytoplasma can be considered a variant 16SrXIII-A** (Wei, Lee, Davis, Xiaobing, & Zhao, 2008), which make perfect sense taking into account that the detection of this subgroup jumped from Mexico to Argentina and the geographic

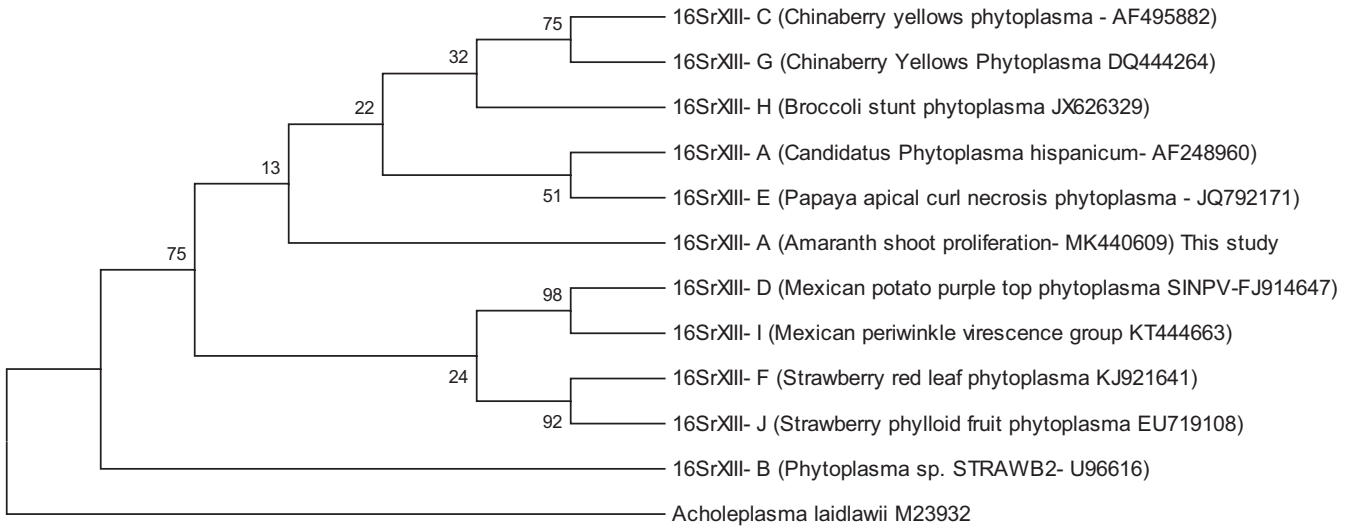

FIGURE 2 Phylogenetic tree constructed based on the sequence of 16S rDNA from amaranth shoot proliferation phytoplasma (ASP-Ar1) and representative strains of subgroups classified within 16SrXIII group

and ecological conditions of this new area are different. However, it is worth noting that, although the three clones analysed showed identical sequences, there is a small chance that a second gene was not sampled at all because closely related 16SrXIII strains have been demonstrated to have heterogeneous 16S rRNA-encoding loci (Pérez-López & Dumonceaux, 2016). On the other hand, the pattern of branching of the phylogenetic tree is consistent with the findings based on RFLP patterns and sequence analysis, indicating ASP-Ar1 phytoplasma related to the representative of the 16SrXIII-A subgroup.

The findings of the present study revealed the occurrence of one more distinct phytoplasma inducing symptoms coincident with those previously described in amaranth caused by different phytoplasmas affiliated with the group 16SrII (Ochoa-Sánchez et al., 2009) and group 6SrIII (Rojas-Martinez et al., 2009). Concerning the phytoplasma classification based on 16S rDNA, few subgroups are recognized within 16SrXIII group and few species of plants are known as harbouring representatives of this group (Melo et al., 2018; Pérez-López & Dumonceaux, 2016; Pérez-López, Luna-Rodríguez, Olivier, & Dumonceaux, 2016). Interestingly, the geographical distribution of members of the 16SrXIII group is apparently limited to American continent with phytoplasmas reported in Argentina, Bolivia, Brazil, Mexico, Paraguay and the United States (Melo et al., 2018). The identification of a 16SrXIII-A phytoplasma revealed amaranth as a new host for this subgroup, contributing for extend the knowledge about the diversity of plant hosts. In addition, we report the first occurrence of 16SrXIII-A phytoplasma in Argentina and consequently in South America, because this strain was previously identified only in Mexico (Davis et al., 2016).

In relation to epidemiological aspect, the incidence level verified in field assay showed the high potential damage of the disease for the amaranth crop. Moreover, asymptomatic plants harbouring the phytoplasma may act as inoculum source in case of occurrence of a possible vector. Finally, transmissibility of the pathogen via seeds is a favourable factor for its dissemination in new areas compromising the expansion of the crop. These aspects reinforce the importance of the pathogen and reveal serious implication to control disease.

ORCID

Ivan Paulo Bedendo https://orcid.org/0000-0003-4481-7533

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