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Research Article

Acerola shoot proliferation induced by a phytoplasma enclosed in the subgroup 16SrIII-F

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ABSTRACT: Acerola bushes were observed showing symptoms of shoot proliferation, generalized stunting, yellowing and decline. Since these symptoms are typically induced by phytoplasmas, this survey was carried out with the aim of detecting, identifying and classifying the supposed phytoplasma present in symptomatic bushes. Total DNA was extracted from symptomatic and asymptomatic samples and used in nested PCR conducted by the primer pairs R16mF2/mR1 followed by R16F2n/R2. Amplifications of expected genomic fragments of 1.2 kb revealed the presence of phytoplasma in 73 % of the symptomatic samples. Molecular analyses, using computer-simulated RFLP patterns, similarity coefficient calculation and phylogenetic analysis allowed for classifying the bacterium as a '*Candidatus* Phytoplasma pruni' – related strain (subgroup 16SrIII-F). The phytoplasma induced the same symptoms in healthy acerola plants inoculated by grafting and showed molecular identity with the strain identified in naturally infected bushes. Although various strains belonging to distinct subgroups within the 16SrIII group have been previously identified in Brazil, this is the first report of the presence of a representative of the 16SrIII-F subgroup in the Brazilian agroecosystem. Considering that phytoplasmas can be systemically distributed throughout the plant and acerola plants are vegetatively propagated, it is recommended that propagation material be obtained from mother plants free of the pathogen.

Keywords: *Malpighia emarginata*, bacteria, phytopathogenic mollicutes, yellows disease, wall-less prokaryote

Introduction

Acerola (*Malpighia emarginata* DC) is a bush belonging to the Malpighiaceae family, a native species of South and Central America (Leffa et al., 2014; Silva et al., 2019). The fruit presents medicinal properties containing high antioxidant activity, substantial amounts of ascorbic acid, and mineral compounds (Chang et al., 2018). In Brazil, acerola has been commercially exploited from the early 1980s as a result of high commercial demand, making the country the world's largest producer and exporter (De Rosso and Mercadante, 2005). The United States of America, Japan and several European countries have a considerable demand for products based on acerola, mainly due to its high vitamin C content (De Rosso and Mercadante, 2005).

Phytoplasmas are wall-less prokaryotes, phloem inhabitants, spread and transmitted by insect vectors. These obligate intracellular bacteria are associated with numerous diseases found in several hundred plant species, inducing, in the main, symptoms of shoot proliferation, phyllody, yellowing and generalized stunting (Bertaccini et al., 2014). Molecular identification was carried out on the basis of analyses of conserved genes, mainly nucleotide sequences of the gene 16S rRNA (Wei et al., 2008). In addition, since phytoplasmas can be transmitted through grafting (Lee et al., 2000), biological assays were performed in order to verify their role and transmissibility in the disease.

Materials and Methods

The present study deals with acerola plants that exhibited symptoms possibly induced by phytoplasmas characterized by shoot proliferation, generalized stunting, yellowing and decline. The disease reduces the normal growth and the quantity and quality of fruit, as well as delaying production.

Samples of leaves and shoots from 11 symptomatic and two asymptomatic bushes were collected and the total DNA was extracted following a prescribed protocol (Doyle and Doyle, 1990).

Phytoplasma detection was performed by nested PCR primed by the pairs R16mF2/mR1 followed by R16F2n/R2, as described in the literature (Gundersen and Lee, 1996). DNA from periwinkle plants knowingly infected by 16SrIII phytoplasma (GenBank GU193977 – Flôres et al., 2013) were used as the positive control and healthy acerola plants represented the negative control.

The DNA products amplified by nested PCR were cloned into *Escherichia coli* DH5α. Sequences corresponding to the 16S rRNA gene of phytoplasmas from four samples were sequenced (three clones for each sample) using the SP6/T7 primers (Malembic-Maher et al., 2008). The sequences were aligned, compared against themselves and phytoplasma sequences found in GenBank employing the Bioedit DNA analysis programs. A majority consensus sequence was selected as reference for the phytoplasma identified in acerola plants and sequences of phytoplasmas belonging to distinct subgroups within the 16SrIII group, deposited in the GenBank database, were submitted to computer-simulated analysis with a set of 17 restriction enzymes, as described by Wei et al. (2008). Based on the colletive restriction profiles a similarity coefficient (F) was calculated for each pair of phytoplasmas considering the strain representative of the acerola phytoplasma and phytoplasmas enclosed

in subgroups of the 16SrIII group. Additionally, the majority consensus sequence representative of the phytoplasma present in acerola plants was also submitted to the interactive online tool *i*PhyClassifier (Zhao et al., 2009). A phylogenetic tree was constructed with the sequence of the acerola phytoplasma and sequences from representatives of the diverse ribosomal subgroups belonging to the 16SrIII group, using the MEGA 6.0 program (Tamura et al., 2013). The nucleotide sequence of *Acholeplasma palmae* was used as the outer root of the tree. The phylogenetic tree was built using the 'Neighbor-joining' method and 'bootstraping' processed 1,000 times.

A transmission test was carried out with 15 acerola rootstocks cultivated in pots, 18 months old, generated from branches of a mother plant, and previously submitted to nested PCR in order to verify the absence of phytoplasmas. The scions approximately 2 cm long were collected from symptomatic trees proven to be harbouring the phytoplasma and grafted onto the potted plants. Samples were collected from grafted symptomatic plants maintained in a greenhouse with screens to avoid insects, and the total DNA was used in nested PCR. The detected phytoplasma was molecularly identified by sequencing the 16S rRNA gene and computer-simulated analysis.

Further assays were conducted in order to verify the presence of virus in the acerola samples. The RNA extracted with a Purelink viral RNA/DNA kit was used in RT-PCR (Reverse Transcription-Polymerase Chain Reaction) for detecting cucumber mosaic virus (CMV) and the virus belonging to the *Potyvirus* and *Begomovirus* genera. The RT-PCR reactions for CMV were performed according to a protocol previously described by Rizos et al. (1992). The assays for detecting Potyvirus were carried out with primers CIFor/CIRev (Ha et al., 2008), while the RT-PCR reactions for Begomovirus were primed by the PAL/PAR pair (Rojas et al., 1993).

Results and Discussion

The association of phytoplasma with affected plants was demonstrated by the amplification of expected genomic fragments of approximately 1.2 kb from the nested PCR. These were detected in 73 % of the analyzed samples (Figure 1). PCR assays yielded products from positive control, but there was no amplification from the negative control. The RT-PCR generated no product, thereby indicating the absence of virus in the analyzed samples.

The sequence selected to represent the phytoplasma identified in the acerola bushes was denominated ASP-Br01 (Acerola Shoot Proliferation-Brazil 01) and deposited in the GenBank database under accession number MT153591. This sequence shared 99.6 % identity with sequences of 16S rRNA of '*Candidatus* Phytoplasma pruni'-related strains. The analysis by the interactive tool online *i*PhyClassifier indicated that the acerola phytoplasma belongs to the 16SrIII-F subgroup (Figure 2). The identity between ASP-Br01 phytoplasma and the phytoplasma of the 16SrIII-F subgroup (Genbank accession number AF510724) was confirmed since the similarity coefficient (F) value was 1.0. Phylogenetic analysis was consistent with the computer-simulated RFLP analysis because the branching pattern of the phylogenetic tree showed that the acerola phytoplasma emerges from the same branch from the representative of the 16SrIII-F subgroup (Figure 3).

The phytoplasma was transmitted in 70 % of the grafted acerola plants. The typical and conspicuous symptoms of generalized stunting, yellowing and shoot proliferation were observed 80-90 days after grafting (Figure 4). The presence of phytoplasma was consistently detected in 100 % of the symptomatic plants and the disease was identified as acerola shoot proliferation (ASP). Molecular identification confirmed that the phytoplasma detected was a member of the 16SrIII-F subgroup. Samples from grafted plants also showed negative results for the presence of vírus in their tissues.

The constant detection of phytoplasma confirmed the initial presumption of its presence in acerola bushes. The detection level over 70 % observed for symptomatic plants reinforced the concept that the symptoms were related to the presence of phytoplasma, since the

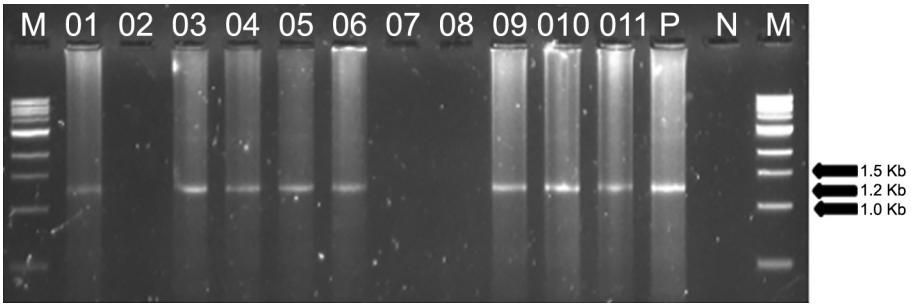

Figure 1 – Amplification of genomic fragments of 1.2 kb from 16S rRNA of the phytoplasma found in 8 out of 11 samples of the symptomatic acerola plants, using nested PCR primed by the pair R16mF2/mR1 followed by R16F2n/R2. Column: M- 1 kb Lader molecular marker; Columns: 01-011 samples of acerola; Column: P = Positive control (GenBank GU193977); Column: N = Negative control.

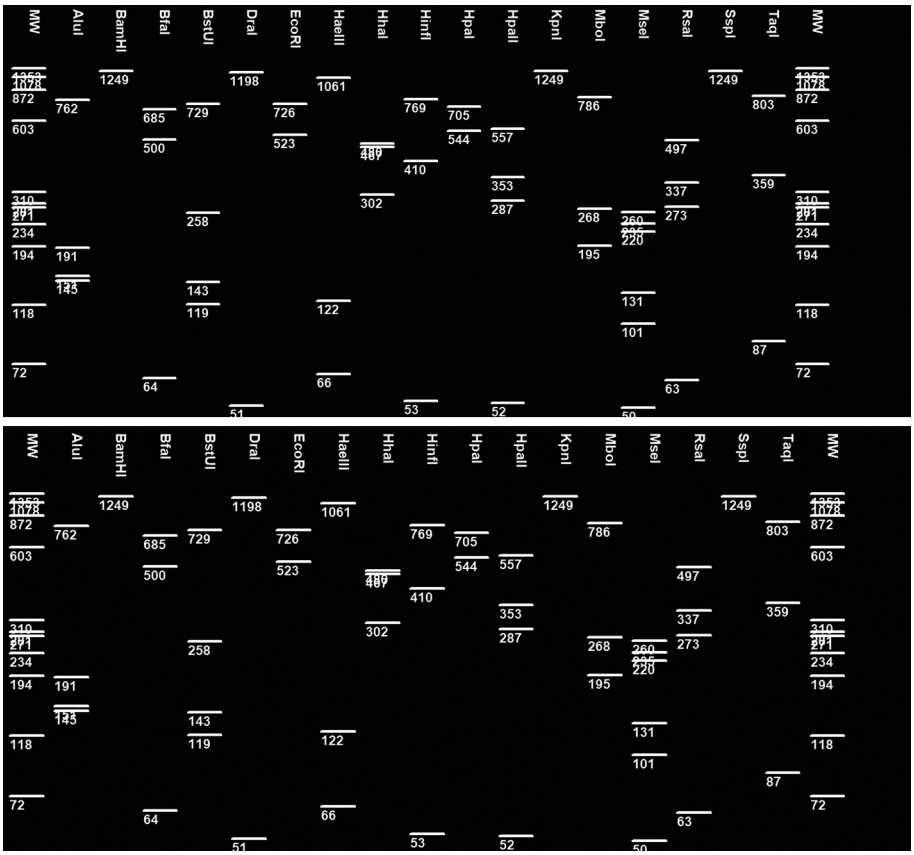

Figure 2 – Virtual RFLP patterns from in silico digestions of 16S rRNA gene R16F2n/R16R2 fragments from acerola phytoplasma (GenBank MT15359- Above) and from reference phytoplasma of the 16SrIII-F subgroup (GenBank AF510724- Below). Molecular Weight: ϕX174RFHaeIII digest.

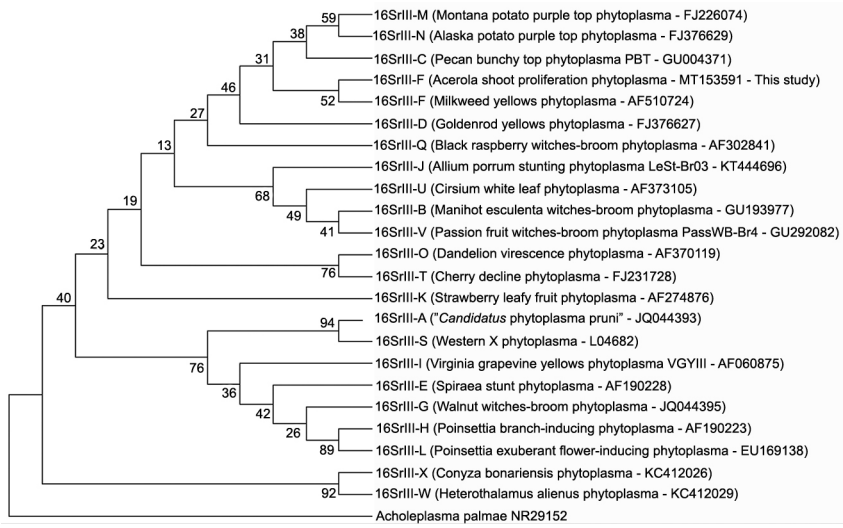

Figure 3 – Phylogenetic tree constructed with the sequence of the phytoplasma identified in the acerola plants (GenBank MT153591) and phytoplasmas belonging to subgroups of the 16SrIII group.

virus was not detected in the samples. In addition, the preliminary suspicion that the disease was associated with the presence of an infectious agent was corroborated by a grafting experiment, which provided evidence where the phytoplasma occurring in the symptomatic bushes was transmitted by grafting to healthy plants. Moreover,

Figure 4 – Acerola healthy branch (left) and symptomatic branch produced by plant grafted with scions from naturally diseased plant (right).

the sequence of the 16S rRNA gene confirmed that the same microorganism was present in naturally infected and grafted plants.

Studies on phytoplasmas have been carried out on numerous pathosystems in Brazil, indicating the occurrence of a broad range of hosts and pathogens. Surveys have revealed that the diverse representatives of the 16SrIII group are those most frequently found in cultivated and uncultivate species, such as *Brassica oleracea* (Rappussi et al., 2012; Eckstein et al., 2013; Amaral-Mello et al., 2011); *Manihot esculenta* (Flôres et al., 2013; Souza et al., 2014); *Solanum melongena*; *Passiflora edulis*, *Cucurbita pepo*, *Sechium edule*, *Solanum licopersicum*, *Melia azedarach*, *Catharanthus roseus and Euphorbia pulcherrima* (Amaral-Mello et al., 2011); *Leonurus sibiricus* (Flôres and Bedendo, 2013); *Celosia* sp. (Eckstein et al., 2012); *Bougainvillea spectabilis* (Silva et al., 2014); and *Brachiaria decumbens* (Fugita et al., 2017). Interestingly, the subgroup 16SrIII-F had not been reported in Brazilian territory. However, members of this subgroup were described inducing phyllody, stunting, yellowing, and abnormal growth in *Heracleum sosnowskyi* specifically in Lithuania (Valiunas et al., 2007), in *H. sosnowskyi*, *Dictamnus albus*, *Vaccinium corymbosum*, and *V. myrtillus* in various European countries, and in *Asclepias syriaca* in North America (Valiunas et al., 2004).

The findings of this study indicated that the disease is associated with a phytoplasma, acerola being a new host for a strain belonging to the 16SrIII group, and the first occurrence of a 16SrIII-F phytoplasma in Brazil. In addition, to the best of our knowledge, this is the first report of an acerola disease associated with the presence of a phytoplasma on a worldwide scale. As a result of demonstrating the association between acerola shoot proliferation and phytoplasma, two control measures are proposed: i) seedlings used for the initial establishment of commercial orchards which are produced by cuttings, are propagative material, represented by branches, that should be obtained from mother plants free of the pathogen and ii) although insect vector transmission is unknown, it is suggested that seedlings should be produced in protected nurseries, so as to avoid the entry of possible vectors.

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Authors' Contributions

Conceptualization: Ferreira, J.; Pereira, T.B.C.; Bedendo, I.P. **Data acquisition:** Pereira, T.B.C.; Ferreira, F. **Data analysis:** Ferreira, J.; Almeida, C.A.; Pereira, T.B.C.; Favara, G. **Design of methodology:** Ferreira, J.; Almeida, C.A.; Bedendo, I.P. **Software development:** Ferreira, J.; Bedendo, I.P. **Writing and editing:** Ferreira, J.; Bedendo, I.P.

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