Original Article

A phytoplasma representative of a new subgroup 16SrI-Z associated with Bunchy Top Symptoms (BTS) on papaya in Cuba

Fitoplasma representante de un nuevo subgrupo, 16SrI-Z, asociado con síntomas de Cogollo Arrepollado del Papayo (PBT) en Cuba

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ABSTRACT: Phytoplasmas are associated with two papaya diseases in Cuba, Papaya Bunchy Top (PBT) and Bunchy Top Symptoms (BTS), which decrease fruit production. Assessment of the 16S ribosomal DNA by nested PCR, conventional RFLP, sequencing, and *in silico* RFLP analyses allowed the identification of a new phytoplasma subgroup designated BTSp-16SrI-Z associated with BTS disease in the eastern region of the country. BTSp-16SrI-Z subgroup was detected in 60.42 % of the surveyed plants with crown leaf chlorosis and necrosis of the young leaves. Results confirmed that this phytoplasma strain was consistently associated with the observed BTS symptoms, implying new epidemiological constraints for the disease management.

Key words: papaya, nested PCR, phytoplasma, sequencing, *in silico* RFLP.

RESUMEN: Los fitoplasmas se asocian con dos enfermedades del papayo en Cuba, Cogollo Arrepollado del Papayo (PBT) y Síntomas de Cogollo Arrepollado (BTS), que disminuyen la producción de frutos de papaya. La detección del ADN ribosomal 16S por análisis de PCR anidada, RFLP convencional, secuenciación y RFLP *in silico* permitió la identificación de un nuevo subgrupo de fitoplasmas 16SrI-Z asociado a la enfermedad BTS en la región oriental del país. El fitoplasma BTSp-16SrI-Z se detectó en el 60,42 % de las plantas muestreadas con clorosis en las hojas de la región apical y necrosis en las hojas jóvenes. El resultado confirma que este aislado de fitoplasma está consistentemente asociado con síntomas de BTS, lo cual implica nuevas restricciones para el manejo de la enfermedad.

Palabras clave: papayo, PCR anidada, fitoplasma, secuenciación, RFLP *in silico.*

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INTRODUCTION

Phytoplasmas are wall-less, non-cultivable, phloem-limited prokaryotes that affect more than 1000 plant species worldwide (1). Molecular-based analyses have proven to be more accurate and reliable than biological criteria previously used for phytoplasma identification, characterization and diagnosis (2). Meanwhile, RFLP *in silico* analysis has been proposed for the identification of phytoplasma subgroups (3).

Several phytoplasma diseases have been reported worldwide affecting papaya crops, including dieback, yellow crinkle and mosaic in Australia (4) and Nivun Haamir dieback in Israel (5). Others papaya diseases associated with phytoplasmas are dieback in Ethiopia (6), yellow crinkling in Mexico (7), and an axillary shoot proliferation in India (8).

In Cuba, two diseases associated with wall-less prokaryote have been reported: Papaya Bunchy Top (PBT) and Bunchy Top Symptom (BTS), both causing severe losses in papaya crops. Besides, a rickettsia-related proteobacterium associated with PBT was also detected in papaya with PBT symptoms in 2003 (9). However, the causal agents have not been unequivocally identified.

Initial surveys carried out in the provinces of Havana and Villa Clara detected phytoplasmas of the groups 16SrX (apple proliferation) (9) and 16SrXVII (‘*Candidatus* Phytoplasma caricae’) (10) in association with BTS. Later, BTS was associated with the group 16SrII (‘*Ca.* P. aurantifolia’) in the eastern region of the country (11). A group 16SrI phytoplasma has also been associated with papaya affected with BTS-like symptoms in Cuba (12). Finally, a mixed infection including a rickettsia and two new phytoplasma subgroups has been reported in BTS-affected plants (13), and a rickettsia-related proteobacterium is a generalized pathogen in papaya plants affected by bunchy top disease in Cuba (14).

The present research was aimed to determine the classification of a phytoplasma associated with the chlorosis of crown leaves and the necrosis of young leaves, symptoms observed on papaya plants with BTS in Cuba.

MATERIALS AND METHODS

Leaf samples were taken from forty-eight papaya plants with symptoms of chlorosis on the crown leaves and necrosis on the young leaves and from eight symptomless plants growing in papaya plantations in the eastern (Holguín, Las Tunas and Granma provinces) region of Cuba in 2009.

DNA of phytoplasma reference controls belonging to the group 16SrI (American aster yellows, AAY) were obtained from the collection at Rothamsted. In addition, DNA of pepper little leaf (PLL, subgroup 16SrI-S), tomato little leaf (ToLL, subgroup 16SrI-T) and potato purple top (PPTJAL8, subgroup 16SrI-U, and PPT-SON18, subgroup 16SrI-V) were used for the direct comparison of the RFLP patterns.

DNA was extracted from 300 mg of leaf tissue from 48 symptomatic papaya plants by the method of Doyle & Doyle (15). A nested PCR assay was performed using puReTaq Ready-To-Go PCR beads (Amersham Biosciences) with phytoplasma 16S rDNA primers R16mF1/R16mR1 for the first round and R16F2n/R16R2 (16) for the nested reaction. A programmable thermocycler (MJ Research) was used. PCR conditions were those previously described (13).

PCR products were digested with the restriction endonucleases *Alu*I, *Bfa*I, *Bst*UI, *Hha*I, *Hinf*I, *Hpa*II, *Mse*I, *Sau*3AI, *Rsa*I and *Taq*I (New England Biolabs), according to the manufacturer's instructions. Digestion products were electrophoresed on 3% agarose gels and visualized under UV light after staining with ethidium bromide in a transilluminator. RFLP patterns were compared with previously

published patterns of phytoplasma in papaya (13).

Phytoplasma R16F2n/R16R2 amplicon with diverse RFLP patterns, PCR-amplified from plants with BTS from each region of Cuba, were purified on spin columns (QIAquick gel extraction kit, Qiagen). PCR products were inserted in the pGEM-T-Easy vector (Promega) and cloned in *Escherichia coli* DH5α. The inserts were sequenced in both directions with M13 primers by the Sequencing Service of the School of Life Sciences, University of Dundee, UK (http://www.dnaseq.co.uk).

Virtual RFLP was performed for the 16S rDNA sequences (1246 bp) of the papaya phytoplasma strains by using virtual gel analysis in the iPhyClassifier program. Each 16S rDNA sequence was digested *in silico* with *Alu*I, *Bam*HI, *Bfa*I, *Bst*UI, *Dra*I, *Eco*RI, *Hae*III, *Hha*I, *Hinf*I, *Hpa*I, *Hpa*II, *Kpn*I, *Sau*3AI, *Mse*I, *Rsa*I, *Ssp*I, and *Taq*I restriction endonucleases (3).

The 16S rDNA sequences obtained were aligned using the MUSCLE algorithm implemented in MEGA v. 6.0 (17). A phylogenetic tree was constructed using the maximum likelihood (ML) method with the general time reversible nucleotide substitution model with gamma-distributed rate variation in invariant sites (G+I). Bootstrapping (3,000 replications) was performed to estimate the stability and support for the branches.

RESULTS

The detection of phytoplasma DNA in 85.42% of the 48 papaya plants displaying symptoms (Figure 1) by nPCR/*Hae*III supported the presence of phytoplasma in the diseased papaya plants.

In vitro RFLP analysis of the R16F2n/R16R2 amplicon obtained from all the nPCR/*Hae*III positive plants and the phytoplasma controls belonging to 16SrI subgroups (Figure 2), showed that *Bst*UI profiles of the BTSp-16SrI-Z strain (twenty-nine samples) displayed a polymorphic pattern different from that displayed by the several subgroups used as controls (PPT-16SrI-S, PPT-16SrI-T, PPT-16SrI-U and PPT-16SrI-V), including 16SrI-B and 16SrI-X subgroups associated with BTS disease in a previous study (13).

When the same *Bst*UI endonuclease was used, the BTSp-16SrI-Z strain also showed a pattern different from that of the rest of the 16SrI subgroups reported by Lee et al. (18).

Results of the *in silico* RFLP analysis confirmed the previous results obtained by the

FIGURE 1. Papaya plants, cv. Maradol Roja, showing symptoms associated with BTSp: (A) chlorosis of crown leaves; (B) necrosis of the young leaves./ *Plantas de papayo cv. Maradol Roja, mostrando síntomas asociados a fitoplasmas causante de BTS: (A) chlorosis de hojas de corona; (B) necrosis de las hojas jóvenes.*

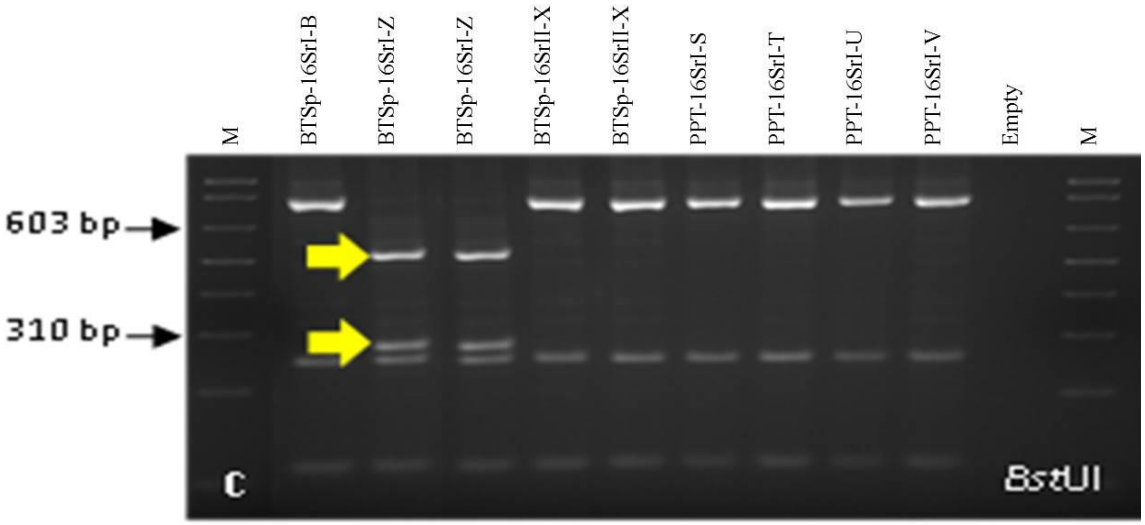

FIGURE 2. RFLP analysis using *Bst*UI endonuclease for digestion of the 1246 bp 16S rDNA fragment from the BTSp-16SrI strain detected in papaya plants and the positive controls BTSp-16SrI-B, BTSp-16SrI-X, PPT-16SrI-S, PPT-16SrI-T, PPT-16SrI-U, and PPT-16SrI-V. M, Size marker (1 Kb plus DNA Ladder, Invitrogen)./ *Análisis de RFLP empleando la endonucleasa BstUI para la digestión de un fragmento de 1246 pb del ADNr 16S de los aislados BTSp-16SrI detectados en plantas de papayo y los controles positivos BTSp-16SrI-B, BTSp-16SrI-X, PPT-16SrI-S, PPT-16SrI-T, PPT-16SrI-U, y PPT-16SrI-V. M, Marcador (1 Kb plus DNA Ladder, Invitrogen).*

conventional RFLP with a restriction endonuclease. To the novel phytoplasma strain BTSp-16SrI-Z, polymorphism was observed with *Bst*UI (Figure 3), which demonstrated the genetic divergence of this strain within the group 16SrI and established a new subgroup associated with BTS affected papaya plants.

The phylogenetic tree strongly supports the results obtained by the conventional RFLP analysis, the new strain, identified as BTSp-16SrI-Z (JF781311), clustered with the strain identified as PYC from papaya in Mexico (FJ602434) in a phylogenetic branch different from the strains BTSp-16SrI-X (JF781308) and BTSp-16SrI-B (JF781307), all in the group 16SrI (Figure 4). However, they clustered in different branches from phytoplasmas associated with PYC and papaya mosaic (PM) in Australia (Y10096 and Y10097).

DISCUSSION

Results from the present study confirmed the previously reported association of the 16SrI phytoplasma group with BTS in Cuba (12,13). In addition, for the first time it is reported a new 16SrI phytoplasma subgroup identified in papaya plants exhibiting symptoms of chlorosis of the crown leaves and necrosis of the young leaves, named BTSp-16Sr-Z.

The conventional RFLP allowed identification and differentiation of a new 16SrI subgroup associated with BTS-affected papaya plants in Cuba. The novel strain BTSp-16SrI-Z was detected in 60.42% of the samples displaying chlorosis of the crown leaves and necrosis of the young leaf symptoms. This strain is different of BTSp-16SrI-X identified by Acosta et al. (13) in the same region. Similarly, this approach has been used to distinguish among phytoplasma subgroups associated with papaya diseases such as the 16SrI-C subgroup in Mexico (7) and a 16SrII group in India (8,19).

In silico RFLP with sequences of the strain BTSp-16SrI-Z present in BTS-affected papaya plants confirmed the presence a new phytoplasma subgroup within the 16SrI group. This technique has been used in the

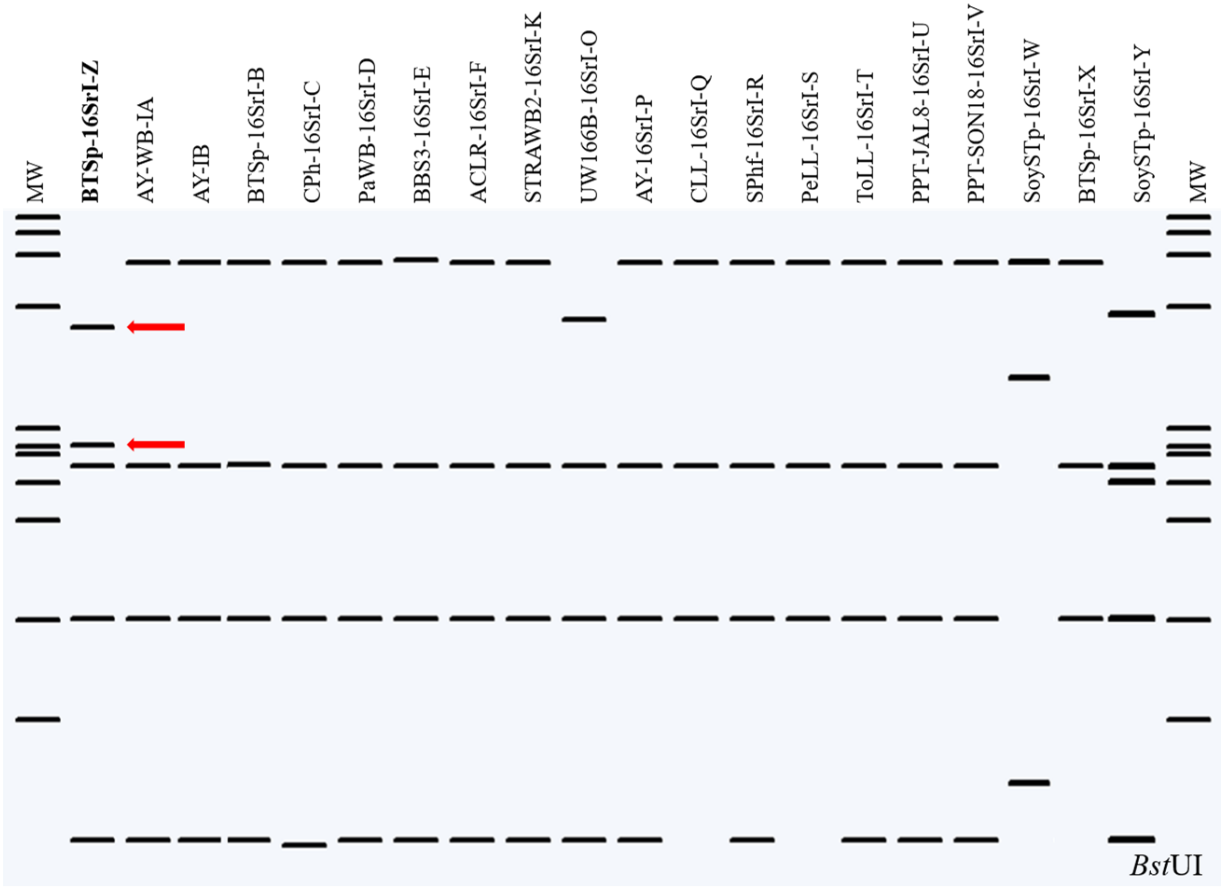

FIGURE 3. RFLP virtual patterns with *Bst*UI derivate from *in silico* digestion of a 16S rDNA fragment amplified with primers R16F2n/R16R2 from the strain BTSp-16SrI-Z detected in papaya plants (in bold) and previously classified as belonging to the group 16SrI in papaya and other crops. MW, molecular weight marker (øX174 DNA digested with *Hae*III); fragment sizes (bp) from top to bottom: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72./ *Patrones de RFLP virtuales con BstUI derivados de digestión in silico de fragmentos de ADNr 16S amplificado con iniciadores R16F2n/R16R2 del aislado BTSp -16SrI-Z detectado en plantas de papayo (en negrita) y los previamente clasificados como pertenecientes al grupo 16SrI en papayo y otros cultivos. MW, marcador de peso molecular (øX174 DNA digerido con HaeIII); tamaños de los fragmentos (pb) de arriba hacia abajo: 1353, 1078, 872, 603, 310, 281, 271, 234, 194, 118, 72.*

identification of new phytoplasma subgroups within groups (3,20-22).

Phylogenetic analysis strongly supported the results obtained by both conventional and *in silico* RFLP, showing that the strain BTSp-16SrI-Z clustered within the same branch with the strains BTSp-16SrI-B and BTSp-16SrI-X. However, they clustered in different branches from the phytoplasmas associated with PYC and papaya mosaic (PM) in Australia, which were classified as subgroup 16SrII-D. In this sense, Padovan and Gibb (23) indicated that yellow crinkle and dieback diseases were caused by two different groups of phytoplasma strains, and that the main factors increasing phytoplasma diversity in papaya were the hemipteran vector abundance and the presence of weed hosts growing in the same agroecosystem. However, in our case, ecological niches in unmanaged areas of the eastern region may favour strain diversity. Similarly, Acosta et al. (13) found that

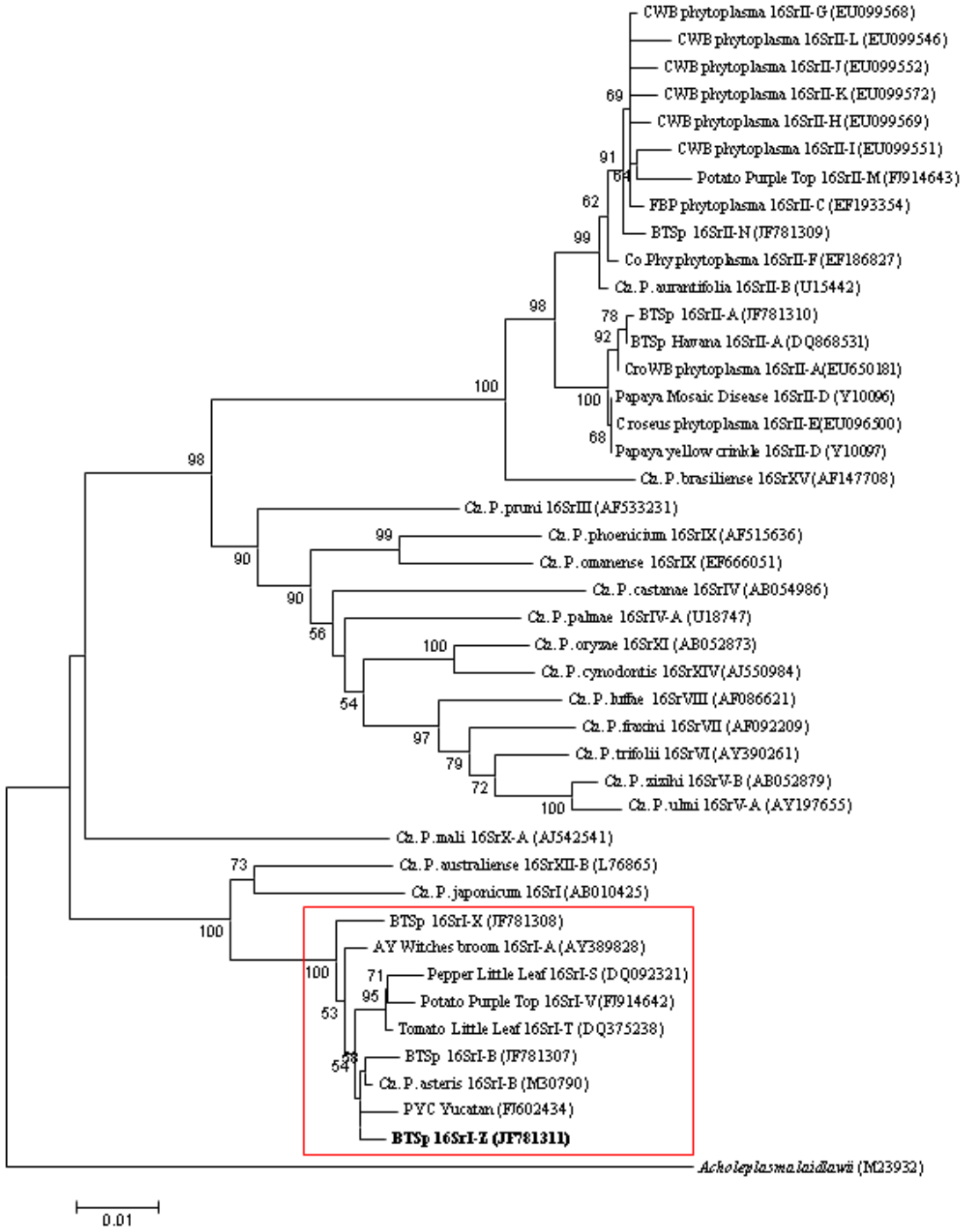

FIGURE 4. Phylogenetic tree based on the sequence of a 1246 bp fragment of the 16S rDNA from the BTSp-16SrI-Z subgroup detected in BTS in papaya plants (in bold), and including sequences of *Ca.* phytoplasma species belonging to 15 groups. *Acholeplasma laidlawii* was used as the out-group. Bootstrap values (3,000 replications). / *Árbol filogenético basado sobre las secuencias de un fragmento de 1246 pb del ADNr 16S del subgrupo BTSp-16SrI-Z detectado en plantas de papayo afectadas por BTS (en negrita), incluyendo secuencias de especies Ca. phytoplasma perteneciente a 15 grupos. Acholeplasma laidlawii fue empleado como grupo base. Valores de bootstrap (3,000 replicaciones).*

ecological niches in weedy areas favoured strains diversity of the phytoplasma group 16SrII in papaya. In the weedy ecosystems, papayas are apparently propagated through true seed, suggesting that, assuming the lack of seed transmission, insect vector(s) may carry diverse phytoplasma strains to or from neighbouring plant species, including weeds.

In summary, the papaya-infecting phytoplasmas from eastern Cuba exhibited a remarkable genetic diversity. The extensive biodiversity of 16SrI strains suggests an ongoing evolution of phytoplasmas in adaptation to their geo- and bioecological niches. The strain diversity observed in the present study could be explained by differences in vector species. In either case, co-existence of diverse phytoplasma strains in the same geographical location and in the same host species may favour phytoplasma-phytoplasma, phytoplasma-vector, and phytoplasma-plant interactions, providing increased opportunities for genetic recombinations and the emergence of new phytoplasma plant diseases.

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REFERENCES

1. Bertaccini A, Duduk B. Phytoplasmas and phytoplasma diseases: a review of recent research. Phytopathologia Mediterranea. 2009; 48:355-78.
2. Lee IM, Hammond RW, Davis RE, Gundersen-Rindal DE. Phytoplasma: Phytopathogenic mollicutes. Annual Review of Microbiology. 2000; 54: 221-255.
3. Zhao Y, Wei W, Lee I-M, Shao J, Suo X, Davis RE. The iPhyClassifier, an interactive online tool for phytoplasma classification and taxonomic assignment. Methods in Molecular Biology. 2013; 938: 329-338.
4. White DT, Blackall LL, Scott PT, Walsh KB. Phylogenetic positions of phytoplasmas associated with dieback, yellow crinkle and mosaic diseases of papaya and their proposed inclusion in '*Candidatus* Phytoplasma australiense' and a new taxon, '*Candidatus* Phytoplasma australasia'. Int. J. Syst. Evol. Microbiol. 1998; 48: 941-51.
5. Gera A, Mawassi M, Zeidan A, Spiegel S, Bar-Joseph M. An isolate of '*Candidatus* Phytoplasma australiense' group associated with Nivun Haamir dieback disease of papaya in Israel. Journal Plant Pathology. 2005; 54:560.
6. Bekele B, Hodgetts J, Tomlinson J, Boonham N, Nikolic P, Swarbrick P, et al. Use of a real-time LAMP isothermal assay for detecting 16SrII and XII phytoplasmas in fruit and weeds of the Ethiopian Rift Valley. Plant Pathology. 2011; 60:345-355.
7. Rojas RI, Zavaleta-Mejía E, Rivas-Valencia P. Presencia de fitoplasmas en papayo (*Carica papaya*) en México. Revista Chapingo Serie Horticultura. 2011; 17: 47-50.
8. Verma R, Mungekar D, Gaikwad P, Tomer SPS, Datar VV. 2012. First report of a phytoplasma associated with an axillary shoot proliferation disease in papaya in India. New Disease Reports. 2012; 25: 18.
9. Arocha Y, Horta D, Peralta E, Jones P. First report on molecular detection of phytoplasmas in papaya in Cuba. Plant Disease. 2003; 87:1148.
10. Arocha Y, Horta D, Roque A, Peralta E. Molecular detection of phytoplasmas in papaya in Cuba. Rev. Protección Veg. 2005; 20: 20-26.
11. Arocha Y, Piñol B, Acosta K, Almeida R, Devonshire J, Van de Meene A, et al. Detection of phytoplasma and potyvirus pathogens in papaya (*Carica papaya* L.) affected with 'Bunchy Top Symptom'

(BTS) in eastern Cuba. Crop Protection. 2009; 28: 640-646.
12. Acosta K, Zamora L, Fernández A, Arocha Y, Martínez Y, Santos ME, et al. First report of '*Candidatus* Phytoplasma asteris' (group 16SrI) affecting papaya in Cuba. New Disease Report. 2011; 24:29.
13. Acosta K, Zamora L, Piñol B, Fernández A, Chávez A, Flores G, et al. Identification and molecular characterization of phytoplasmas and rickettsia pathogens associated with 'Bunchy Top Symptom' (BTS) and 'Papaya Bunchy Top' (PBT) of papaya in Cuba. Crop Protection. 2013; 45: 49-56.
14. Luis-Pantoja M, Ramos-González PL, Naranjo M, Hernández-Rodríguez L, Rodríguez J, Pérez-López E. *Rickettsia*-related bacteria associated with papaya plants showing bunchy top disease in Cuba. Journal of general Plant pathology. 2015; 81 (2): 166–168.
15. Doyle J, Doyle J. Isolation of plant DNA from fresh tissue. Focus. 1990; 12:13-15.
16. Gundersen D, Lee IM. Ultrasensitive detection of phytoplasmas by nested-PCR assay using two universal primer pairs. Phytopathologia Mediterranea. 1996; 35:144-151.
17. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution. 2013; 30: 2725-2729.
18. Lee I-M, Gundersen-Rindal DE, Davis RE, Bartoszyk IM. Revised classification scheme of phytoplasma based on RFLP analysis of 16S rRNA and ribosomal protein gene sequences. International Journal of Systematic Bacteriology. 1998; 48: 1153-1169.
19. Verma R, Gaikwad P, Mungekar D, Tripathi1 S, Datar V, Singh J. First report of mixed infection of *Papaya ringspot virus* and phytoplasma in papaya in India. Journal of Plant Pathology. 2014; 96 (2): 431-439.
20. Mauricio-Castillo JA, Salas-Muñoz S, Velásquez-Valle R, Ambríz-Granados S, Reveles-Torres LR. '*Candidatus* Phytoplasma trifolii' (16SrVI) en chile mirasol (*Capsicum annuun* L.) cultivado en Zacatecas, México. Revista Fitotecnia Mexicana. 2015; 38 (4): 389- 396.
21. Sharma A, Sharma S, Kang S. First report of a new "*Candidatus* Phytoplasma australasia''-related strain in *Capsicum annuum* in India. Journal of Plant Pathology. 2015; 97 (3): 541-551.
22. Valiunas D, Jomantiene R, Ivanauskas A, Urbonaite I, Sneideris D, Davis RE. Molecular Identification of Phytoplasmas Infecting Diseased Pine Trees in the UNESCO-Protected Curonian Spit of Lithuania. Forests. 2015; 6: 2469-2483.
23. Padovan A, Gibb K. Epidemiology of phytoplasma diseases in papaya in Northern Australia. Journal of Phytopathology. 2001; 149: 649-58.