

## UNVEILING THE AETIOLOGY OF PAPAYA DISEASES IN CUBA, DEMOCRATIC REPUBLIC OF CONGO (DRC) AND ETHIOPIA

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**ABSTRACT:** Papaya is amongst the most common fruits grown in Ethiopia and it is being affected by a decline disorder recently reported as causing yield losses over 30% in the major farms. In North Kivu, DRC, papaya is the major cash crop grown for latex, which is exported to Europe for papaine production. Since 1997, a disease has been gradually spread and seriously affected the papaya crop and latex yields. In Cuba, papaya has become one of the most important export crops, mainly for the Canadian and European markets. Recently, a papaya bunchy top like disease (PBT-like) has been associated with the 16SrII phytoplasma group, *Candidatus Phytoplasma aurantifolia*, in the east of Cuba; however, there is no information about the situation of the disease throughout the country. On the other hand, it is required to know the pathogens associated with papaya diseases in Ethiopia and DRC. During 2005-06, leaf samples, including from healthy plants, were collected from symptom papaya plantations surveyed in the three countries and they were tested for rickettsias, papaya ringspot virus and phytoplasmas, which have been the most common plant pathogens associated with papaya diseases worldwide. Total DNA and RNA were extracted and indexed by PCR assays and sequencing of target genes. A 16SrII phytoplasma was identified in more than 95 % of the samples affected with PBT-like in Cuba and the decline disorder in Ethiopia, while a Moroccan Watermelon Mosaic Virus (MWMV) isolate (*Potyviridae* family) was associated with the papaya disease in DRC.

(Key words: papaya; phytoplasmas; papaya ringspot virus; potyvirus; watermelon mosaic virus; rickettsias; PCR).

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## DESCUBRIENDO LA ETIOLOGÍA DE ENFERMEDADES DE LA FRUTA BOMBA EN CUBA, REPÚBLICA DEMOCRÁTICA DEL CONGO (DRC) Y ETIOPÍA

**RESUMEN:** La fruta bomba se encuentra entre los frutales más comunes que se cultivan en Etiopía. Está siendo afectada por un desorden tipo declinamiento, recientemente informado en las principales parcelas, y causando pérdidas superiores al 30%. En North Kivu, DRC, es el principal cultivo de supervivencia, cultivado para la obtención de látex que se exporta a Europa, para la producción de papaina. Desde 1997, la enfermedad se ha expandido gradualmente, afectando seriamente los niveles de látex. En Cuba, la fruta bomba se ha convertido en uno de los más importantes cultivos de exportación, principalmente para los mercados canadiense y europeo. Recientemente, la enfermedad similar al cogollo arrepollado, papaya bunchy top like-disease (PBT-like) ha sido asociada con el grupo fitoplásmico, 16SrII, *Candidatus Phytoplasma aurantifolia*, en el este de Cuba; sin embargo, no existe información acerca de la situación de la enfermedad a lo largo del país. Por otra parte, se requiere conocer los patógenos asociados con las enfermedades de la fruta bomba en Etiopía y DRC. Durante el 2005-06, se colectaron

**muestras foliares de los tres países, incluyendo plantas sanas, las cuales fueron evaluadas para rickettsias, virus de la mancha anular y fitoplasmas, los cuales han sido los patógenos de plantas comúnmente asociados con enfermedades de la fruta bomba a nivel mundial. El ADN y ARN total fueron extraídos y analizados mediante ensayos de PCR y secuenciación de genes blanco. El fitoplasma 16SrII fue identificado en más del 95% de las muestras afectadas con PBT-like en Cuba y declinamiento en Etiopía. Por otra parte, se asoció un aislado del virus del mosaico del melón de Marruecos, *Moroccan watermelon mosaic virus* (MWMV) (familia *Potyviriidae*) con la enfermedad en fruta bomba en DRC.**

(Palabras clave: fruta bomba; fitoplasmas; virus de la mancha anular; potyvirus; virus del mosaico del melón de Marruecos; rickettsias; PCR)

## INTRODUCTION

Papaya (*Carica papaya* L.) is native to the American tropics but it is now cultivated widely throughout tropical and sub-tropical areas of the world for both local consumption and export of fruit, and to a lesser extent, for production of papain (5). A number of viral diseases has been associated with papaya diseases; among them are the apical or droopy necrosis caused by rhabdovirus, and previously reported in Venezuela and Florida (24), and the ringspot caused by a potyvirus and considered the most destructive for papaya. Among non-viral diseases of papaya, Bunchy Top, PBT, caused by a rickettsia of sub-group 1-a of *Protobacteria*, and first reported in Puerto Rico in 1931 (9), is considered the major limiting factor in the production of papaya in the American tropics. On the other hand, different phytoplasma diseases such as Dieback (PD), Yellow Crinkle (PYC) and Mosaic (PM) have been associated with papaya in Australia causing losses over 70% (13,14,19). Particularly, Dieback has caused losses of 100% in some plantations being the most economically significant disease of papaya in the country (12,19).

The cultivation of papaya has increased considerably in Ethiopia in the last six decades (21); however, a decline disorder has been observed in the major papaya farms, including the Melkassa area of east Shewa, where significant yield losses have been associated with it. More than 300,000 families in North Kivu, DRC, depend on papaya industry, where papaya is grown for latex that is exported to Europe for papaine production. Since 1997, a disease has been gradually spread and seriously affected the papaya crop and latex yields throughout North Kivu. As field production has declined, this has been offset by expansion into new forest areas. This is having a serious environmental impact on the fragile ecosystems of the equatorial highlands.

Recently in eastern Cuba, a 16SrII phytoplasma, *Candidatus* Phytoplasma aurantifolia, has been associated with a papaya bunchy top like-disease

(PBT-like) (5), and *Empoasca papayae* Oman identified as the potential vector. Although losses caused by PBT-like have not been quantified yet, the disease is widespread throughout the country.

The present paper pursues to identify the plant pathogens associated with papaya diseases in Ethiopia, DRC, as well as in the western and central regions of Cuba, and to contribute elements to the knowledge on epidemiology and the management improvement of such diseases.

## MATERIALS AND METHODS

**Symptoms:** Symptoms exhibited by those diseased papaya plants in all papaya plantations visited in Ethiopia, DRC and Cuba were evaluated and described.

**Plant material and reference controls.** Leaf samples were collected from 12 symptomatic papaya plants in farms of the Melkassa area of east Shewa, Ethiopia, during September 2005. Apparently healthy (symptomless) weeds growing in the field like *Galinsoga parviflora* Cav., *Parthenium hysterophorus* L., and *Ricinus communis* L. were also surveyed. During September 2006, 47 leaf samples from papaya plants showing symptoms and additional healthy plants were collected from surveys carried out in North Kivu. A national 2005-06 survey of PBT-like was carried out in Western (Pinar del Río and Havana) and Central (Matanzas, Cienfuegos and Villa Clara) Cuba. Leaf samples from 532 papaya plants with (416) and without (116) symptoms and 66 assorted weed species with and without symptoms of yellowing, little leaf, phyllody, virescence and witch's broom were collected from papaya plantations. Weeds were identified as *Anoda acerifolia* Cav. (Malvaceae); *Echinochloa colona* (L.) Link (Poaceae); *Euphorbia heterophylla* L. (Euphorbiaceae); *Macroptilium lathyroides* (L.) Urb. (Fabaceae); *Malvastrum coromandelianum* (L.) (Malvaceae); *Malacra alceifolia* Jack. (Malvaceae);

*Momordica charantia* L. (Cucurbitaceae); *Parthenium hysterophorus* L. (Asteraceae); *Rynchosia minima* (L.) DC. (Fabaceae). Positive reference controls corresponded to the peanut witches' broom phytoplasma of the 16SrII group, PWB, and the papaya ringspot virus (PRSV), both from the Rothamsted Research collection; as well as, the PBT rickettsia, gently provided by Dr Brunner from the University of Puerto Rico.

**Polymerase chain reaction (PCR) and sequence analysis.** Total DNA was extracted from plant material (11) and analyzed for papaya bunchy top rickettsia through a simple PCR with the primers PBTF1/PBTR1 that amplify the rickettsial flavoprotein subunit of the succinate dehydrogenase gene (*sdhA*) according to Davis *et al.* (9). In addition, DNA was indexed in a nested PCR with phytoplasma generic primers P1/P7 (10) and R16F2n/R16R2 (20) that amplify the conserved 16S ribosomal DNA region. RNA was extracted with a RNA purification kit (QIAGEN, UK), and used as template in a RT-PCR with a generic potyvirus primer Uni3 and oligo dT (SIGMA-Aldrich, UK), which amplify the partial NIb gene, the entire coat protein and 3' untranslated region. PCR products of randomly selected samples were purified on spin columns (QIAquick gel extraction kit; QIAGEN, UK) and directly sequenced using the Sequencing Service, School of Life Sciences, University of Dundee, UK (<http://www.dnaseq.co.uk>), with Applied Biosystems Big-Dye version 3.1 chemistry on an automated capillary DNA sequencer model 3730. The 16S rDNA sequence from survey samples was compared with others in Genbank by BLAST protocol (1) for a final identification.

## RESULTS AND DISCUSSION

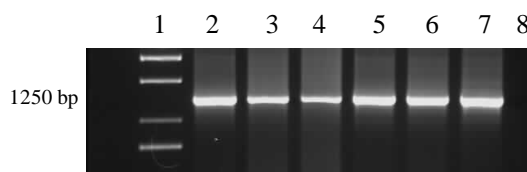
The first symptom observed in papaya affected with the decline disorder in Ethiopia was a bright yellowing of the upper young leaves; later symptoms of mosaic, crinkling and leaf tip necrosis developed followed by drying of the upper leaves that progressed to the whole plant death.

In North Kivu, DRC, plants are affected at any age and all the local papaya varieties are susceptible to the disease. Symptoms observed in the leaves range from yellow mosaic patterns to deformation and shoestrings as the disease progresses. Oily streaks in petioles and variations in shape and size of the ring spots on the fruits are also seen. Within the trunk, pink discoloration and tumor-like growths appear that become brown and necrotic as the disease progresses. In the last stage, plants stand as a pole with a tuft of small leaves at the apex and the internal trunk becomes fibrous.

Symptoms of PBT-like in Cuba have been previously described (4). Those observed in PBT-like affected papaya in eastern Cuba were characterized by leaf stunting, crinkling and yellowing, short internodes, bunched appearance of the inner crown leaves, rigid petioles extending more horizontally from the main stem than normal, lost or decrease of latex fluid, and reduction of fruit size. In some cases, phyllody and virescence were observed.

None of the papaya plants evaluated yielded PCR bands when indexed by using the PBT rickettsial primers suggesting that rickettsias were not involved in the development of either the decline disorder in Ethiopia, PBT-like in Cuba or the papaya disease in DRC.

Amplicons of the expected size 1250 bp corresponding to phytoplasma DNA were obtained for 12/12 symptomatic papaya plants from Melkassa area of east Shewa, Ethiopia. For Cuban samples, PCR amplifications were yielded by 398/416 symptomatic papaya, 71/116 asymptomatic and 46/66 of the weed species evaluated (Fig. 1).



**FIGURA 1.** Nested PCR from phytoplasma-infected papaya. Lane 1: 1Kb Molecular weight marker. Lanes 2-3: Samples from Melkassa, east Shewa, Ethiopia. Lanes 4-6: Samples from western, central and eastern regions of Cuba, respectively. Lane 7: PWB. Lane 8: Healthy papaya. / *PCR anidada a partir de fruta bomba infectada por fitoplasmas. Pocillo 1: Marcador molecular 1Kb. Pocillos 2-3: Muestras de Melkassa, Shewa este, Etiopía. Pocillos 4-6: Muestras de occidente, centro y oriente de Cuba, respectivamente. Pocillo 7: PWB. Pocillo 8: Fruta bomba sana.*

The sequencing of the 16S ribosomal DNA of randomly selected symptomatic papayas from Ethiopia (Genbank accession No. DQ285659) and Cuba (Genbank accession No. DQ286948) along with their comparison with the rest of 16S rDNA sequences deposited in Genbank, identified a member of the 16SrII, *Candidatus* *Phytoplasma aurantifolia* group in the samples analyzed. The Ethiopian isolate showed the highest similarity (98%) with that of papaya yellow crinkle in Australia (Genbank accession No. Y10097, a member of the 16SrII group), while the Cuban isolate showed the highest sequence homology (99%) with that of *Polygalla phyllody* phytoplasma (Genbank accession No. AY787140), also a member of the 16SrII group.

The 16SrII group has been detected in South-East Asia, South Pacific, Africa, Arabian Peninsula, Europe, Australia and America (8), and includes papaya yellow crinkle (PYC) and papaya mosaic (PM), two important diseases of the Australian papaya industry; however, as far as we know, it is the first record of 16SrII phytoplasma group in papaya in Ethiopia, as well as in the Caribbean.

Phytoplasmas habitually inhabit the phloem sieve tubes of non-symptom plants (6, 15, 17, 22). They have been detected in many hosts not exhibiting symptoms of a disease such as alders, apricots, almonds and sugarcane (2, 23). From our study, 61.2% of symptomless papaya samples resulted infected by the 16SrII phytoplasma, what indicates that most of the papaya plants not exhibiting symptoms in the field may be PBT-like latent reservoirs and may play a significant role in the spread of the disease.

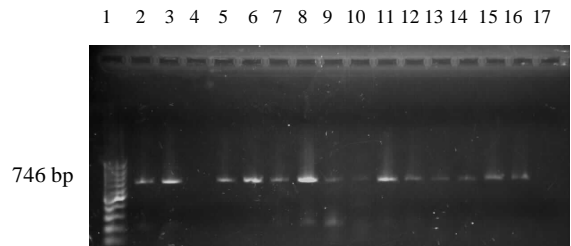
Forty six of the weed species collected from PBT-like affected papaya plantations were infected by the 16SrII phytoplasma. All weeds identified in Ethiopia yielded phytoplasma DNA amplification by nPCR, thus, *G. parviflora*, *P. hysterophorus* and *R. communis* were considered as new alternative 16SrII phytoplasma hosts, even not showing symptoms of any particular phytoplasma disease. Weeds identified in Cuba carrying the 16SrII phytoplasma were *A. acerifolia* displaying leaf yellowing, virescence and phyllody, *E. heterophyllia* showing typical witch's broom and little leaf symptoms, and *M. coromandelianum* and *R. minima*, these latter exhibiting no obvious symptoms.

Many species of weeds that do not exhibit symptoms have been identified as alternative phytoplasma reservoirs. These include *Whiteochloa cymbiformis* in northern Australia (7, 23); *Sorghum stipoideum* (Ewart & Jean White) as a symptomless reservoir of the sugarcane grassy shoot phytoplasma (7), *Clematis vitalba* L. as a reservoir for flavescence dorée and other phytoplasmas in Italy (3), asymptomatic *Tridax procumbens* L., *Cajanus marmoratus* Benth and *Tribulopsis* sp., plants found in die-back affected papaya fields in northern Australia (19), and *M. lathyroides* and *Sorghum halepense* L., collected from fields of sugarcane affected by Yellow Leaf Syndrome (YLS) in Havana, Cuba (4).

Therefore, it is suggested that the native weed population is an important reservoir of inoculum (16) and that its control could help reduce the disease incidence in papaya in both Cuba and Ethiopia.

From papaya samples of North Kivu, DRC, 43/47 exhibited RT-PCR amplification of expected size, 746 bp corresponding to a potyvirus (Fig. 2). The sequencing

of the purified RT-PCR products from representatives of the DRC papaya samples yielded a 739 bp sequence corresponding to part of the NIb gene, the entire coat protein and the 3'untranslated region (Genbank accession No. EF063361). BLAST analysis of such sequence showed an 89% identity with that of *Moroccan watermelon mosaic virus* (MWMV) (AF305545).



**FIGURA 2.** RT-PCR of papaya from DRC infected with a potyvirus, MWMV. Lane 1: 100bp Molecular weight marker. Lanes 2, 3, 5-7, 9-16: Symptomatic papaya samples. Lane 8: PRSV. Lanes 4, 17: Healthy papayas. / *RT-PCR de fruta bomba de DRC infectada con un potyvirus, MWMV. Pocillo 1: Marcador molecular 100 pb. Pocillos 2, 3, 5-7, 9-16: Muestras sintomáticas de fruta bomba. Pocillo 8: PRSV. Pocillos 4, 17: Fruta bombas sanas.*

*Moroccan watermelon mosaic virus* was first reported as a distinct member of the *Potyvirus* genus (*Potyviridae* family) in Morocco in 1972 (18). As far as we know, this is the first record of MWMV both in papaya and in the DRC. Further studies are required to fully characterize the virus and understand its association with this papaya disease.

These studies have allowed identifying the plant pathogens associated with emergent papaya diseases in Cuba, Ethiopia and DRC. Besides, the results achieved contribute with significant information about alternative hosts, including those latent inoculum sources, which may play a determinant role in spreading the disease in the field. Therefore, they constitute important elements to be considered in designing strategies for management, as well as for the further studies required for a complete knowledge of such diseases.

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