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USING A RIBOSOMAL PROBE FOR THE DETECTION OF THE PHYTOPLASMA ASSOCIATED WITH ´BUNCHY TOP SYMPTOM OF PAPAYA´ (BTS) IN PLANT AND INSECT HOSTS

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ABSTRACT: **Recently, a phytoplasma of the 16SrII, *Candidatus* Phytoplasma aurantifolia group was associated with Bunchy Top disease (BTD) in eastern Cuba. Total DNA from more than 200 plant and insect leaf samples surveyed during 2005 and infected by the 16SrII phytoplasma was indexed by a non-radioactive nucleic acid hybridization assay (nrNAH). Phytoplasma 16S rDNA PCR products of selected samples were purified, labeled with alakaline phosphatase and used as a probe for the detection of such phytoplasma by the system of direct alkaline phosphatase labelling and chemiluminiscent detection (AlkPhos, Amersham LIFE SCIENCE, UK). The nrNAH assay detected the BTD phytoplasma in both papaya plants and *Empoasca papayae* Oman. The probe yielded hybridization signals reacting with reference controls and samples infected with the BTD phytoplasma. The nrNAH is considered a valuable diagnostic tool for the national phytosanitary surveillance, seed certification and breeding programs and a pathway to develop further specific assays.**

*(Key words: phytoplasma; papaya; **Empoasca papayae**; **Candidatus** Phytoplasma aurantifolia; non-radioactive nucleic acid hybridization)*

USO DE UNA SONDA RIBOSOMAL PARA LA DETECCIÓN DE FITOPLASMA ASOCIADO CON LA ENFERMEDAD DEL SÍNTOMA DEL COGOLLO ARREPOLLADO (BTS) EN PLANTAS E INSECTOS HOSPEDANTES

RESUMEN: **Recientemente, el grupo fitoplásmico 16SrII, *Candidatus* Phytoplasma aurantifolia fue asociado con la enfermedad similar al cogollo arrepollado, Bunchy Top disease (BTD) en el Este de Cuba. El ADN total de más de 200 muestras de plantas e insectos se muestreó durante el 2005, y las que resultaron infectadas por el fitoplasma BTD, fueron evaluadas mediante un ensayo de hibridación de ácidos nucleicos no radioactiva (nrNAH). Los productos de PCR correspondientes al ARN ribosomal 16S de muestras seleccionadas se purificaron, marcaron con fosfatasa alcalina y se utilizaron como sonda para la detección de este fitoplasma mediante el sistema de marcaje directo con fosfatasa alcalina y detección quimioluminiscente (AlkPhos, Amersham Life Science, UK). El ensayo nrNAH detectó el fitoplasma BTD tanto en plantas de fruta bomba como en *Empoasca papayae* Oman. La sonda arrojó señales de hibridación al reaccionar con los controles de referencia y las muestras infectadas con el fitoplasma BTD. El ensayo nrNAH se considera una valiosa herramienta diagnóstica para los programas de vigilancia fitosanitaria, certificación de semilla y mejoramiento genético, y una vía para desarrollar futuros ensayos específicos.**

*(Palabras clave: fitoplasma; papaya; **Empoasca papayae**; **Candidatus** Phytoplasma aurantifolia; hibridación de ácidos nucleicos no radioactiva)*

INTRODUCTION

Papaya (*Carica papaya* L.) is widely cultivated in Cuba for the national consumption and export, with a production between 55 and 60000 t per year (5). It is distributed throughout the country representing 19% of the total of fruits produced, being Maradol Roja, the main variety (3).

Papaya is affected by various phytoplasma diseases (16) like mosaic and yellow crinkle associated with 16SrII group (*Candidatus* Phytoplasma aurantifolia) and dieback, with 16SrXII group (Stolbur).

Phytoplasmas present particular problems in the pursuit of Koch's postulates to establish them as the cause of a disease as they cannot be cultured *in vitro* (21). Therefore, molecular methods are the most feasible for their detection, identification and characterization in plant and insect vector hosts (1, 7, 9, 20).

Bunchy Top disease (BTD) was first reported in Cuba (3). Plants affected with the disease have shown a mix of symptoms similar to those caused by dieback, yellow crinkle and mosaic phytoplasmas in Austalia (16, 17), as well as those caused by a rickettsia associated with BTD in Puerto Rico (13), all of them related to losses over 70% (28). Although losses caused by BTD disease have not been yet quantified, the disease is widespread throughout all the provinces of the country (6). No vectors of such diseases have been identified, except the leafhopper *Orosius argentatus* Evans, which has been thought to be involved in epidemic outbreaks of dieback disease (28). However, *Empoasca papayae* Oman has been identified as the leafhopper candidate to vector BTD disease in Cuba (5).

Nucleic Acid Hybridization assays (NAH) have been widely used to differentiate phytoplasma species (4), being considered one of the most accepted methods for the diagnosis of phytoplasma diseases. There is a high tendency to use non radioactive nucleic acid hybridization methods (nrNAH) due to their research biosafety and technological advantages over PCR assays like minimizing contamination and speeding up the sample analysis (4, 33).

Recently, an nrNAH assay has been developed in Cuba for the generic diagnosis of phytoplasma diseases (4) by using a phytoplasma 16S ribosomal DNA (16S rDNA) probe. Regarding the potential menace of BTD for the Cuban papaya agriculture, it is required to evaluate the feasibility of this system for the field-scale diagnosis of the BTD phytoplasma in both plant and insect material.

In this paper, leaf and insect samples surveyed during 2005 and carrying the BTD phytoplasma were used to obtain a probe for the optimization of a non-radioactive nucleic acid hybridization for the detection of the phytoplasma associated with BTD in Cuba.

MATERIALS AND METHODS

Plant, insect material, and reference controls. Total DNA from 230 papaya plants with (177) and without (53) BTD symptoms and 67 adult species of *E. papayae* leafhoppers, previously collected from papaya plantations of Guantánamo, Santiago de Cuba, Holguín, Camagüey and Granma, and identified as infected by the 16SrII phytoplasma (6) was evaluated by nrNAH. Phytoplasma DNA from Faba Bean phyllody (FBP, group 16SrII, *Candidatus* Phytoplasma aurantifolia), Sweet potato little leaf (SPLL, group 16SrII, *Candidatus* Phytoplasma aurantifolia) and Green Valley X (GVX, 16SrIII group, *Candidatus* Phytoplasma pruni) were used as reference controls.

Development of the ribosomal phytoplasma probe. Two samples from each plant species and *E. papayae* were selected and evaluated by more than five repetitions of a nested PCR (nPCR) assay with generic phytoplasma primers P1 (14) and P7 (29) that amplify the conserved region of the 16S r DNA using a programmable thermocycler (MJ Research) and following PCR conditions previously described (5).

PCR products were purified from a 1% agarose gel according to the manufacturer's specifications (QIAquick Gel Purification kit (QIAgen, UK). Concentration of the purified DNA was quantified by a nanospectrophotometer (NanoDrop, UK) at 260 nm. Five mL of the PCR product (20 ng/mL) were llabelled as described by the kit of direct labelling of DNA probes with alkaline phosphatase and chemioluminiscent detection with CPD-Star (AlkPhos, Amersham Life Science, UK).

Nucleic acid hybridization analysis. Ten microliters of **s**amples and control DNAs were denatured at 100°C, during 5 minutes with denaturing solution (Sodium hydroxide, 0.5M NaOH and Sodium chloride, 1.5M NaCl) and neutralized with neutralizing solution (1M Tris-HCl, pH 8.0, and 0.5M NaCl) (4). DNAs were blotted into a nylon membrane (Hybond NX+, Amersham), previously treated with 2% Sodium Dodecyl Sulfate (SDS) during 10 minutes.

DNAs were fixed to the membrane at 80°C, during 2 hours (4). Hybridization was performed at 40°C. Both low and high stringency washes were performed at 40°C and room temperature, respectively, for 15

minutes each and using wash solutions previously described (4).

The hybridization signal was detected by using the chemioluminiscent detection of the hybridization signal with CPD-Star, according to the manufacturer's instructions (AlkPhos, Amersham LIFE SCIENCE).

RESULTS AND DISCUSSION

Very well defined and strong hybridization signals were yielded by 172/177 symptom papaya samples, 37/53 asymptomatic papaya samples, and 63/67 *E. papayae* samples (Figure 1).

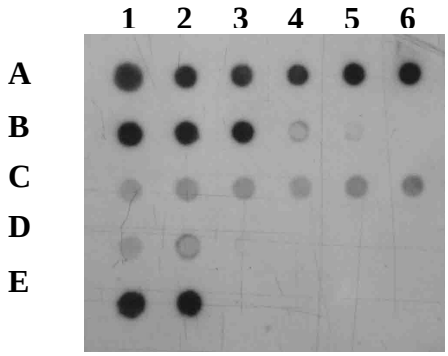

FIGURA 1. Hybridization signals obtained with the ribosomal phytoplasma probe. A1-A6: Papaya samples infected by the BTD phytoplasma (A1-A4: symptom papayas, A5, A6: asymptomatic papayas). B1-B3: FBP, SPLL and GVX reference controls. B4-D3: *E. papayae* samples. D4: Healthy papaya. D5: Healthy *E. papayae*. D6: TE1x Control. E1-E2: Probe controls./ *Señales de hibridación con la sonda ribosomal. A1-A6: Muestras de frutabomba infectadas con el fitoplasma BTD. B1-B3: Controles de referencia FBP, SPLL y GVX. B4-D3: Muestras de* ***E. papayae*** *infectadas con el fitoplasma BTD (A1-A4: Frutabombas sintomáticas, A5, A6: Frutabombas asintomáticas). D4: Frutabomba sana. D5:* ***E. papayae*** *sano. D6: Control TE1x. E1-E2: Controles de sonda.*

The development of NAH signals by 97.1% of symptomatic samples suggests the robustness of the nrNAH assay as previously described (4) and corroborates the association of BTD with phytoplasmas extending the information of previous reports (3, 5). The development of NAH signals indicates that 40°C is the optimal hybridization temperature for the detection of the BTD phytoplasma in both papaya plant and *E. papayae*. Reference controls yielded similar NAH signals extending the use of this ribosomal probe for the detection of phytoplasmas of 16SrIII group, which is the most related phylogenetically to 16SrII phytoplasma group (5) where BTD phytoplasma belongs to. This points the need to identify highly variable genomic regions to achieve the specific detection of the BTD phytoplasma.

The 16SrII group has representatives in South-East Asia, South Pacific, Africa, Arabian Peninsula, Europe (Italy), Australia, and America (12) in many different plant species including citrus, peanut, potato, sweet potato, alfalfa, cacti, apple, faba bean, soybean, weeds (15, 23, 25, 26, 27, 34), and also includes two important diseases of papaya: papaya yellow crinkle (PYC) and papaya mosaic (PM) (16). However, as far as we know, it is the first record of 16SrII phytoplasma group in papaya in America and the Caribbean.

Phytoplasmas seem to have a limited distribution correlated to the geographic region (24). The 16SrII group along with members of 16SrXI, *Candidatus* Phytoplasma oryzae do appear to be restricted to the South-East Asia region (18, 19, 20, 22, 24, 30).

The phytoplasma associated with an original plant host can become dispersed and re-distributed throughout a wide area by the exchange of germplasm in the form of seeds and plants, which along with the potential of exotic insects to vector local phytoplasma pathogens can cause changes in the balance between disease and epidemic (24). The presence of phytoplasmas in coconut embryos (11), *in vitro* grown seedlings of alfalfa with witches' broom (23), symptomatic seedlings of wild carnation (31), as well as tomato seedlings and lime plantlets (8), leads to hypothesise that the BTD phytoplasma could have originally been imported through seed exchange, a route which is currently under investigation.

Latent infections of phytoplasma infections habitually occur for perennial and long cycle crops (2, 32). Phytoplasmas multiply and move throughout the plant during a period of incubation, where they can be detected in symptomless plants (10). Symptom expression may occur when the infected plant is under unfavourable growth conditions or other stress factors (32).

The study of BTD in the eastern Cuba yielded a 69.8% of BTD latent infection. Therefore, asymptomatic papayas are an escape route for the 16SrII phytoplasma, and may play an important epidemiological role in spreading the disease in the field. It is an important element for the future design of practices for BTD management. Further studies will be required to understand the genetic mechanisms of the BTD phytoplasma in symptomless papaya and the environmental factors involved. The fact that the nrNAH assay detects phytoplasma DNA at early stages

makes this assay feasible to introduce in the seed certification and breeding programs throughout the country.

O. argentatus has been identified as a putative candidate for transmission trials in Australia (28), although the identification of leafhopper vectors of papaya phytoplasmas is still under investigations. The BTD phytoplasma was detected in 94% of *E. papayae* collected from BTD affected papaya fields of eastern Cuba. These findings point to *E. papayae* as the potential vector of BTD disease in Cuba remaining as a target for future transmission studies. It also means a high constraint for the national papaya production as the apparently healthy papayas might be latent phytoplasma reservoirs from where vectors can acquire and spread it. Therefore, it is urgent to consider the control of *E. papayae* populations as part of the management of BTD (21, 24).

As far as we know, this is the firs report of using a ribosomal phytoplasma probe through an nrNAH assay for the detection of a phytoplasma associated with a papaya disease in America and the Caribbean. The ribosomal probe allows detecting the phytoplasma associated with BTD in both papaya plants and *E. papayae*, providing a valuable tool to support diagnosis purposes for the national quarantine, phytosanitary surveillance, seed certification and breeding programs. In addition, it is a start point to improve the nrNAH assay for the specific detection of such phytoplasma using highly variable genomic regions. On the other hand, the identification of alternative BTD hosts and *E. papayae* as the potential vector is a crucial element for the improvement of the BTD management in Cuba.

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