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SHORT COMMUNICATION / *COMUNICAÇÃO*

Confirmation of the presence of the *Citrus leprosis virus C* (CiLV-C) in Southern Mexico

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ABSTRACT

Citrus leprosis was detected in sweet oranges in Chiapas, Mexico, in 2005 based on symptoms. The disease was soon after observed in sweet orange orchards at Huimanguillo and Cunduacan, state of Tabasco. Leaf samples of leprosis-affected Valencia or Hamlin sweet oranges were collected and subjected to ultrastructural examination and molecular detection of CiLV-C by RT-PCR. Cytopathic effects typical of CiLV-C infection as presence of short, baciliform particles in the endoplasmic reticulum cisternae and electron dense, vacuolated viroplasma in the cytoplasm, as well as hypertrophy of some spongy parenchyma cells were observed in the tissues of the leaf lesions. RT-PCR using specific primers for CiLV-C produced DNA fragments of the expected size. *Brevipalpus* mites were present in these orchards and identified as *B. phoenicis*, the known vector for CiLV-C. Transmission tests with these mites confirmed their role as leprosis vectors to sweet orange but not to lemon, Volkamerian lemon, C35 citrange and Carrizo citrange. These data confirm the presence of CiLV-C in Tabasco, Mexico and that the citrus leprosis found in Chiapas was caused by CiLV-C.
Key words: *Brevipalpus phoenicis*, Chiapas, citrus leprosis, electron microscopy, molecular detection, Tabasco.

RESUMO

Confirmação da ocorrência da leprose dos citros tipo citoplasmático no sul do México

Em 2005 houve um relato da presença da leprose dos citros no Estado de Chiapas, ao sul do México, baseado em sintomatologia. Subsequentemente a doença foi constatada em pomares de laranjeiras doces no Estado de Tabasco, nas localidades de Huimanguillo e Cunduacan. Amostras de folhas com lesões lepróticas de laranjeiras Valencia ou Hamlin foram coletadas e posteriormente examinadas ao microscópio eletrônico e submetidas a ensaios de detecção molecular por RT-PCR usando primers específicos. Estudos ultraestruturais revelaram alterações citopatológicas típicas causadas pelo CiLV-C (partículas baciliformes curtas no lúmen do retículo endoplasmático e a presença de viroplasmas vacuolados e elétron-densos no citoplasma) e hipertrofia em algumas células do parênquima lacunoso. Ensaios de RT-PCR resultaram na amplificação, na maioria das amostras de folhas dessecadas, de fragmento de DNA de tamanho esperado. Ácaros *Brevipalpus* coletados nos pomares foram identificados como *B. phoenicis*. Ensaios de transmissão com este ácaro confirmaram seu papel como vetor do CiLV-C para laranja doce, mas não houve transmissão para limão, limão Volkameriana, citrange C35 e citrange Carrizo. Todos estes dados considerados em conjunto confirmam que CiLV-C está presente em Tabasco, México e que a leprose dos citros descrita em Chiapas é causada pelo CiLV-C.
Palavras-chave: *Brevipalpus phoenicis*, Chiapas, detecção molecular, leprose dos citros, microscopia eletrônica, Tabasco.

Citrus leprosis was first reported in the early 1900's in Florida, US (Fawcett, 1911). It was afterwards reported in many South American countries and in the last 10 years it has been spreading through Central America (Rodrigues et al., 2003; Bastianel et al., 2010). Sanchez Anguiano (2005) reported the occurrence of leprosis in southern Mexico based on symptoms on sweet oranges. This caused serious concern in the Mexican citrus industry, which is going through a considerable expansion, with important production areas in several Mexican states (Chiapas, Tabasco, Veracruz, Tamaulipas, Nuevo Leon, Chihuahua, Sonora). Mexico has 526,000 ha of citrus plantation of which ca. 70% are sweet orange, with a net yield of 6.7 million tons with a market value of US$ 700 million (SIAP, 2011).

In 2006, typical leprosis symptoms (lesions on the leaves, twigs and fruits, precocious fall of fruits) (Figure 1) were observed in many commercial orchards of Valencia and Hamlin sweet orange (*C. sinensis* [L.]) at Huimanguillo and Cunduacan, state of Tabasco. Incidence of symptomatic trees was not high but they were found in most of visited

grooves with clear indication of the dissemination of the disease. Leaf lesions were essentially similar to that caused by *Citrus leprosis virus C* (CiLV-C), the prevalent and more aggressive form of leprosis than that caused by the nuclear form (CiLV-N). Fruits had depressed brownish lesions, usually surrounded by a chlorotic halo (Figure 1A), and many of them were dropped on the soil. Leaf lesions were usually large with pale green color with concentric brownish ring of gummy tissues (Figure 1C-D). There were many chlorotic and necrotic lesions in the twigs, and some stems exhibited large necrotic lesions (Figure 1B). From the size of the leaf and stem lesions, it seems that the initial infection may have occurred at least two to three years before. To confirm that the causal agent of these symptoms was CiLV-C, symptomatic leaf samples were collected randomly, placed in plastic bags and taken to the laboratory of the Comité Estatal de Sanidad Vegetal, Villahermosa, to be processed. Twigs were sampled to verify the presence of *Brevipalpus* mites, which when present were preserved in ethanol 70%. Part of the collected mites was mounted for light microscopy on Hoyer's mounting medium (Moraes & Fletchmann, 2008) and examined under light microscopy at the Laboratório de Acarologia of the Escola Superior de Agricultura Luiz de Queiroz, Universidade de São Paulo (ESALQ/USP), at Piracicaba, SP, Brazil. The remaining mites were air-dried, mounted either dorsally or ventrally on a double side carbon tape, covered by a thin layer of gold in a Baltec SD050 sputter, and examined in a LEO 435 VP scanning electron microscope at the Núcleo de Apoio à Pesquisa em Microscopia Eletrônica aplicada à Pesquisa Agropecuária (NAP/MEPA) of ESALQ/USP, Piracicaba, SP, Brazil.

Small pieces of lesions from still fresh leaves were cut with a sharp razor blade and immersed in a mixture of

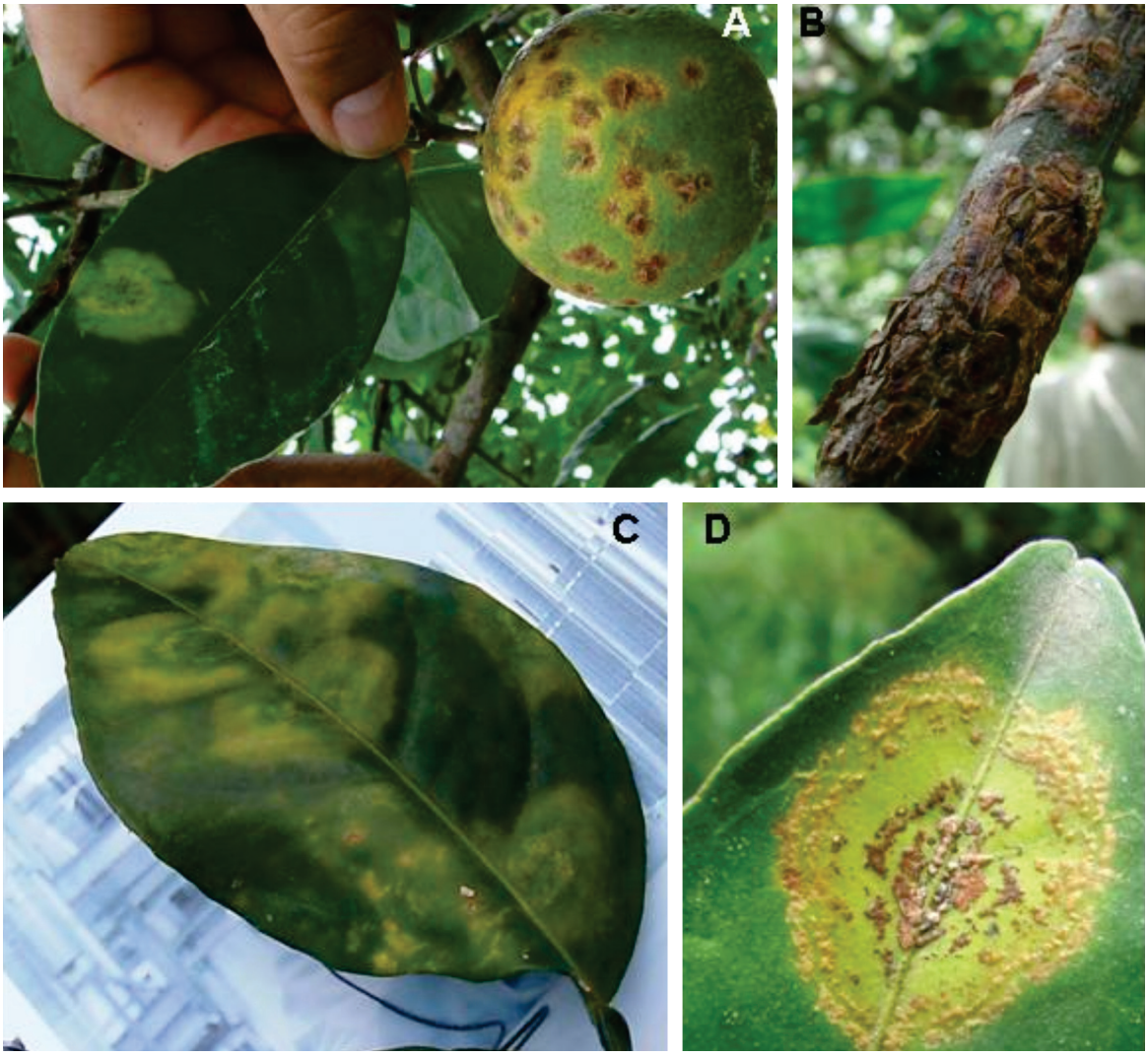

FIGURE 1 - Leprosis symptoms found in sweet oranges in the state of Tabasco. **A.** Leaf and fruit lesions of Valencia sweet orange with leprosis (Cunduacan); **B.** Necrotic lesions on a stem of Valencia sweet orange (Huimanguillo); **C.D.** Different aspects of lesions on the leaves of Valencia sweet orange (Huimanguillo).

2.5% glutaraldehyde and 2% paraformaldehyde in 0.05M, pH 7.2 cacodylate buffer for fixation. These samples were used for transmission electron microscopy (TEM). Several leaves with lesions were air dried for RT-PCR analysis to detect CiLV-C by molecular techniques.

Fixed leaf samples were further processed (Maunsbach & Afzelius, 1999) at the NAP/MEPA, for TEM. Tissues were washed in cacodylate buffer, post fixed in 1% OsO_4, dehydrated in acetone and embedded in low viscosity Spurr's epoxy resin. Blocks were trimmed and sectioned in a Leica UC6 ultramicrotome equipped with a diamond knife. Sections were collected on a copper grid, stained with 3% uranyl acetate and Reynold's lead citrate and examined in a Zeiss EM900 transmission electron microscopy.

Dried leaves were processed for RT-PCR according to the protocol and specific primers for CiLV-C as described by Locali et al. (2003) at the Centro APTA Citros Sylvio Moreira (CCSM), Cordeirópolis, SP, Brazil and Instituto de Fitosanidad- Colegio de Postgraduados, Texcoco, México.

Half of the 12 samples of leprotic leaf lesions collected from Huimanguillo and Cunduacan examined by TEM revealed the presence of short, bacilliform particles within cisternae of the endoplasmic reticulum and/or electron dense viroplasm in the cytoplasm of either palisade and/or spongy parenchyma cells (Figure 2 A and B), a characteristic cytopathic effect of the infection by CiLV-C (Rodrigues et al., 2003; Kitajima et al., 2003). Also, hypertrophied cells were commonly seen in the spongy parenchyma as previously observed in leaf tissues infected by CiLV-C (Marques et al., 2007).

RT-PCR assays of dried leaf samples, using specific primers for CiLV-C (Locali et al., 2003) promoted the amplification of DNA fragments of expected size, in most of the analyzed samples (data not shown), whose sequence (GenBank accession HQ292778) exhibited similarity with that of CiLV-C (GenBank accession YP_654542). Further RT-PCR assays carried out at the Instituto de Fitosanidad, Colegio de Postgraduados in additional samples from Tabasco and Chiapas, resulted in positive results.

Light microscope examination indicated that the mite species is *Brevipalpus phoenicis* Geijskes, based on the number (5) of the laterodorsal setae and the number of the solenidia (2) at the end of the tarsus of the leg II (Welbourn et al., 2003). Similar findings were made when these mites were examined under the SEM (Figure 2 C-D).

Mite transmission assays were carried out with adult *B. phoenicis* from a colony maintained on sweet orange fruits. They were transferred onto sweet orange leaves with typical leprosis lesions, collected from leprosis-infected orchard in Huimanguillo and left feeding for 24 h. Then they were transferred onto five healthy seedlings of Valencia sweet orange, lemon (*C. limon* [L.] Burm. f.), Carrizo citrange (*Poncirus trifoliate* x *C. sinensis*), C35 citrange and Volkamerian lemon (*C. volkameriana* Tan. and Pasq.), 12 mites on a single leaf per plant, under greenhouse conditions. Chlorotic spots appeared on infested leaves of sweet orange 5-6 weeks after infestation in all infested sweet orange plants, but not in four control plants, without infestation. RT-PCR of the tissues of the leaf lesions confirmed the presence of CiLV-C. Leprosis symptoms did not appear on lemon, Carrizo

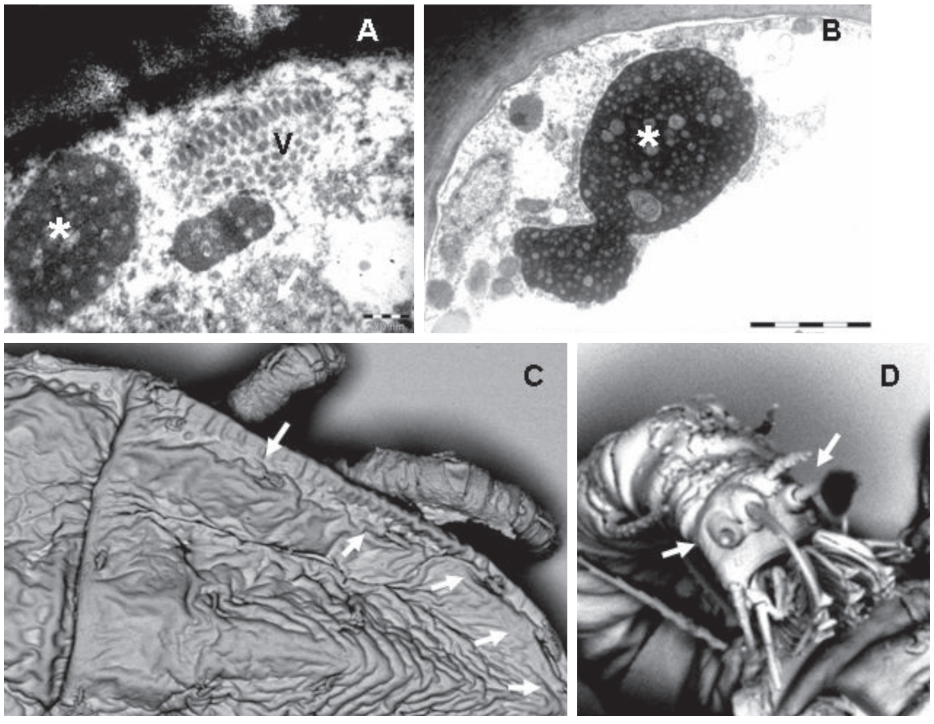

FIGURE 2 - Transmission electron micrographs of thin sections of leaf lesions of Valencia sweet orange caused by CiLV-C. **A.** Part of spongy parenchyma cell showing a viroplasm (*) and a group of short, bacilliform particles (V) within a cisternae of the endoplasmic reticulum. Sample from Huimanguillo; **B.** Similar to A, showing a large viroplasm (*). Sample from Cunduacan; **C.D.** Scanning electron micrographs, using backscattered detector, of *Brevipalpus phoenicis* collected at sweet orange orchard at Huimanguillo, Tabasco. Identification was based on the presence of five latero-dorsal setae (arrows) in the opistosoma (**C**) and two solenidia (arrows) at the end of the tarsus of the leg II (**D**).

citrange, C35 citrange and Volkamerian lemon which are considered highly resistant or immune to CiLV-C (Bastianel et al., 2010).

The results of the different analyses lead to the conclusion that CiLV-C is present in the symptomatic leaf samples collected at Huimanguillo and Cunduacan, state of Tabasco, and RT-PCR assays confirmed that the disease observed in sweet orange orchards at Chiapas (Sanchez Anguiano, 2005) is caused by CiLV-C.

It is not known exactly how CiLV-C was introduced into Mexico, despite quarantine measurements. One speculation is that CiLV-C may have been brought accidentally into Southern Mexico by the flux of immigrants from Guatemala bringing in contaminated orange fruits, discarded during the journey.

Mexican authorities are taking measures to avoid the spread of the disease to other citrus growing regions as well as to reduce or eradicate the main focus in affected areas. Because of the destructive nature of CiLV-C and the high cost of control of the vector, *B. phoenicis*, there is also a serious concern in the Caribbean area about the risk of dispersal of this disease also to other countries, including southern USA, a major citrus producer, and where the disease has not been registered after the 1960's (Childers et al., 2003).

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