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## First report of *Citrus variegation virus* in Palestine Sweet Lime, as Coffee Shade in Costa Rica

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**ABSTRACT.** Symptoms resembling those of *Citrus variegation virus* (CVV) were observed in old Palestine sweet lime trees (*Citrus limettioides* Tan.) used as coffee shade in the Central Valley in Costa Rica. The symptoms include leaf flecking, mosaic, malformation, and dwarfing. This disease was transmitted by grafting to “Valencia” sweet orange (*C. sinensis* L.), Etrog citron (*C. medica* L.), and sweet lime under greenhouse conditions. Symptoms were severe in Palestine sweet lime all year long, but sweet orange and Etrog citron were asymptomatic under high temperature. Reverse transcription polymerase chain reaction (RT-PCR) was used to confirm the presence of CVV in young leaves from sweet lime trees collected from field, and other *Citrus* spp. grafted and maintained in the greenhouse at University of Costa Rica. Fresh tissue from trees infected with CVV obtained from the USDA (Riverside, California), was used as positive control. Furthermore, the sequence obtained (605bp) was analyzed by Blastn algorithm which showed 97% homology with CVV RNA 3 (GenBank Accession No. AF434912). Additionally, small scale virus purification was carried out and isometric particles (26 to 32 nm) were observed under the electron microscope. To our knowledge, this is the first report of the presence of CVV in Costa Rica infecting Palestine sweet limes. Also, we remark on the potential of Palestine sweet lime as a host plant indicator in biological indexing of CVV.

**Keywords:** CVV, Ilarvirus, biological indexing, infectious variegation, Palestine sweet lime

The use of different citrus species as shading trees in coffee plantations is very common in Costa Rica. Many of these citrus plants were introduced in the country without any quarantine regulation. Since 2005, old sweet lime trees (*Citrus limettioides* Tan.) used as coffee shade in the Central Valley in Costa Rica showing leaf flecking, mosaic (Fig 1A), malformation, and dwarfing were observed. The symptoms resembled those associated to *Citrus variegation virus* (CVV), a member of subgroup 2 of genus *Ilarvirus* (*Bromoviridae*) (2, 22, 23). CVV has been detected in a wide range of citrus species and cultivars across Mediterranean Basin (Spain, Italy, Greece, Albania, Turkey, Israel, Algeria, and Morocco), North and South America [United States of America (Florida, California), Argentina, Uruguay], and other locations (1, 2, 6, 7, 14,18, 24, 25). CVV induces symptoms usually mild on oranges and mandarins but may be severe on citron and lemons, with an associated reduction in yield and fruit malformation. Two strains of the virus have

been described on the basis of the symptoms on citrus trees in the field: infectious-variegation strain, and crinkly leaf strain. The first one causes chlorotic mottle with variable severity on the leaves, and crinkles symptoms may be present. The crinkly leaf strain induces distorted, puffed or puckered leaf segments but without variegation. The infection is spread by grafting and mechanical transmission, however, no vector has been identified yet (1, 2).

To confirm CVV presence in Costa Rica different biological and molecular assays were carried out.

### MATERIAL AND METHODS

Graft transmission using budwood from symptomatic Palestine sweet lime (*C. limettioides*) trees to sweet orange (*Citrus sinensis* L.) var. “Valencia”, Etrog citron (*Citrus medica* L.), and Palestine sweet lime, all grafted on Swingle citrumelo (*C. paradisi* x *Poncirus trifoliata*) at least a year old were done under greenhouse conditions. The plants were observed weekly during 12

mo. During 2008 and 2009, when the experiment was carried out, the average temperature was 20°C in the area where the greenhouse is located (Latitude N09°56'04.53'', Longitude W84°02'41.9'', altitude 1236 m). In Costa Rica, the less warm temperatures are from December to February. The minimum and maximum average temperatures recorded in the greenhouse's area during those months were 16°C and 21°C, respectively. On the other hand, average temperatures from March to November were between 19.3°C and 21.1°C (data from CIGEFI-UCR meteorology station).

Partial virus purification using leaves from graft infected trees were achieved by a modification of a method used in *Sugarcane mild mosaic virus* (ScMMV) (17). Observation of negative stained particles was carried out by transmission electron microscope (H-7100).

Total RNA from sweet lime symptomatic leaves, virus-free citrus tissue as negative control, and fresh tissue with CVV infection as positive control (kindly provided by Dr. R. F. Lee, USDA-ARS, Riverside, CA) were obtained using the "RNeasy Plant mini kit" (Qiagen, Hilden, Germany). cDNA synthesis with CVVR primer (21) was achieved using "RevertAid H Minus kit" (Fermentas, St. Leon-Rot, Germany) following manufacturer's recommendations. PCR was performed using the primers CVVR (tca ttc ttc aac aac caa gaa att rct tgg) and CVVF (gaa gtc tcc tcc tcc act ttt acg t), and PCR conditions according to Roy et al. (21). PCR products were purified and sequenced at Macrogen Inc. (Korea). A BLASTn homology search in GenBank, <http://blast.ncbi.nlm.gov/Blast.cgi>, was carried out using the sequences obtained. All sequences were aligned using the BioEdit Sequence Alignment Editor software (12) and then manually adjusted. A multiple alignment was generated with the coat protein gene of isolates from Spain (CL-903-15 and AF434920), Florida, (USA) (AF434917), Corsica (France) (AF434912) and Campania (Italy) (EU650678). A

dendrogram was constructed by Neighbor joining test (bootstrap=500) using MEGA 5.0. Distances were calculated with Kimura's model (K2P), from the alignment of the RNA3 gene sequences of the CVV obtained in this work, compared with many of those available at the Nucleotide Database of GenBank, <http://www.ncbi.nlm.nih.gov/nucleotide>.

## RESULTS AND DISCUSSION

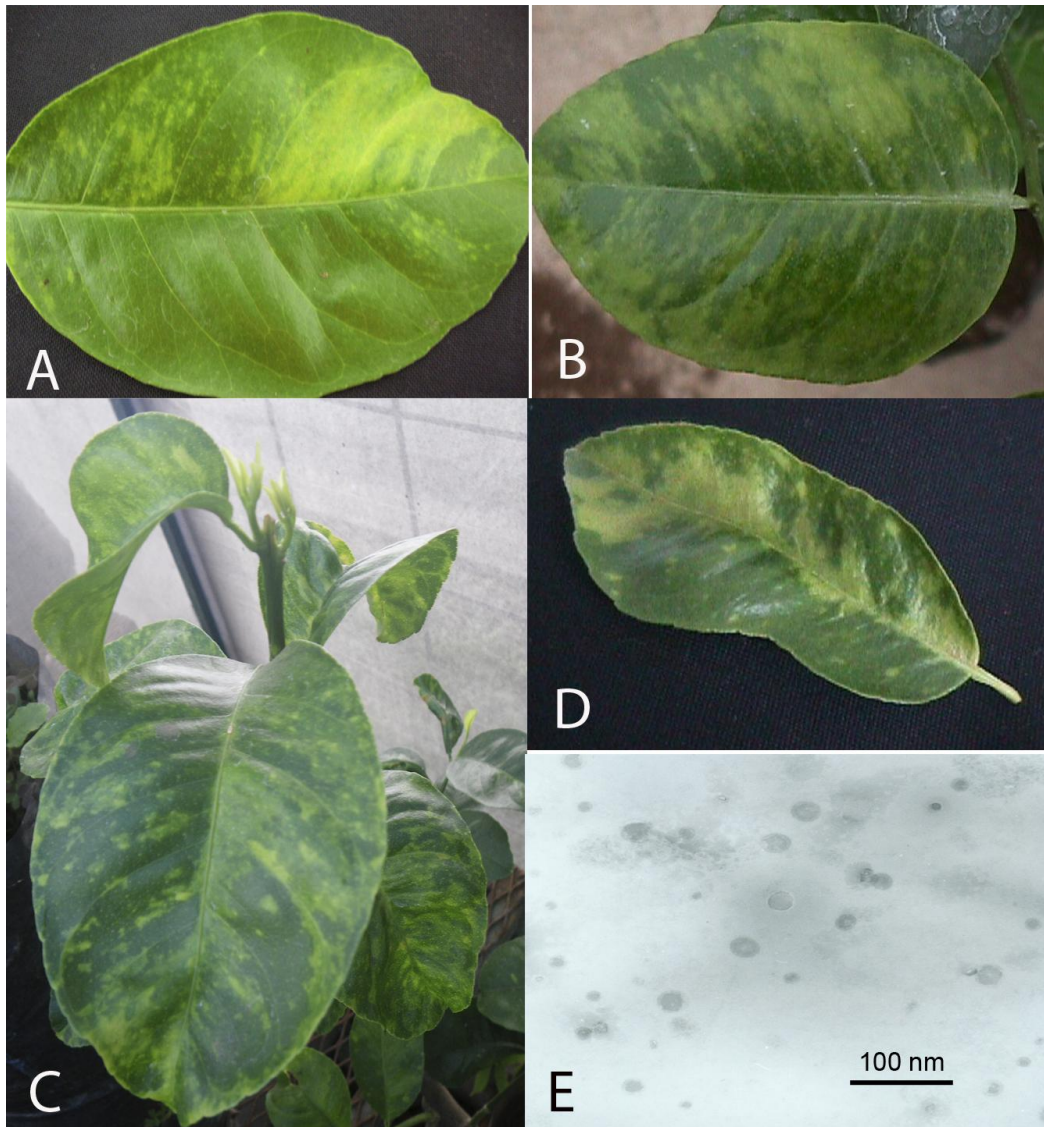
The symptoms (Fig. 1A) detected in Palestine sweet lime trees in coffee plantations in Costa Rica resembled those reported to be associated with CVV (1, 2, 9). Additionally, the symptoms were noticed in few young leaves. The disease was graft transmitted successfully to sweet orange, Etrog citron, and sweet lime (Fig. 1 B, C, D). The leaves of host grafted trees showed symptoms four to six month after graft inoculation.

Symptoms were strong in sweet lime all year long, regardless of temperature conditions (Fig. 1D). On the other hand, sweet orange and Etrog were asymptomatic at high temperature (over 35°C), and symptoms were conspicuous only in leaves developed during cool conditions (15°C-20°C). Similar results have been mentioned by Davino et al. (5).

Therefore, Palestine sweet lime may be a good indicator plant to be used in biological indexing where serological and molecular techniques are not available; alternatively, this indicator plant may represent an useful tool to increase titer of CVV from citrus plants to be tested using serological and molecular assays. Different authors pointed out that detection of CVV by serological techniques is limited by the low titer and the irregular distribution of virus in infected plants and, by the narrow period of testing application during the year because detection being reliable only in young tissues from spring to early summer in subtropical and temperate regions (5, 16).

Viral isometric particles (26 to 32 nm) were seen under the electron microscope (H7100) from negative stained small scale

virus purification (Fig. 1 E). The shape and size of particles resembled those reported to CVV (10).



**Fig. 1.** (A) Leaf from old Palestine sweet lime trees (*Citrus limettioides* Tan.) showing flecking and mosaic resembling CVV symptoms detected in the Central Valley in Costa Rica. Leaf mosaic developed after transmission by grafting budwood from symptomatic trees to (B) sweet orange var. “Valencia”, (C) Etrog citron, and (D) Palestine sweet lime under greenhouse conditions at Universidad de Costa Rica (San Jose, Costa Rica). (E) Viral isometric particles seen under the electron microscope from negative stained small scale virus purification from Palestine sweet lime trees showing leaf flecking and mosaic.

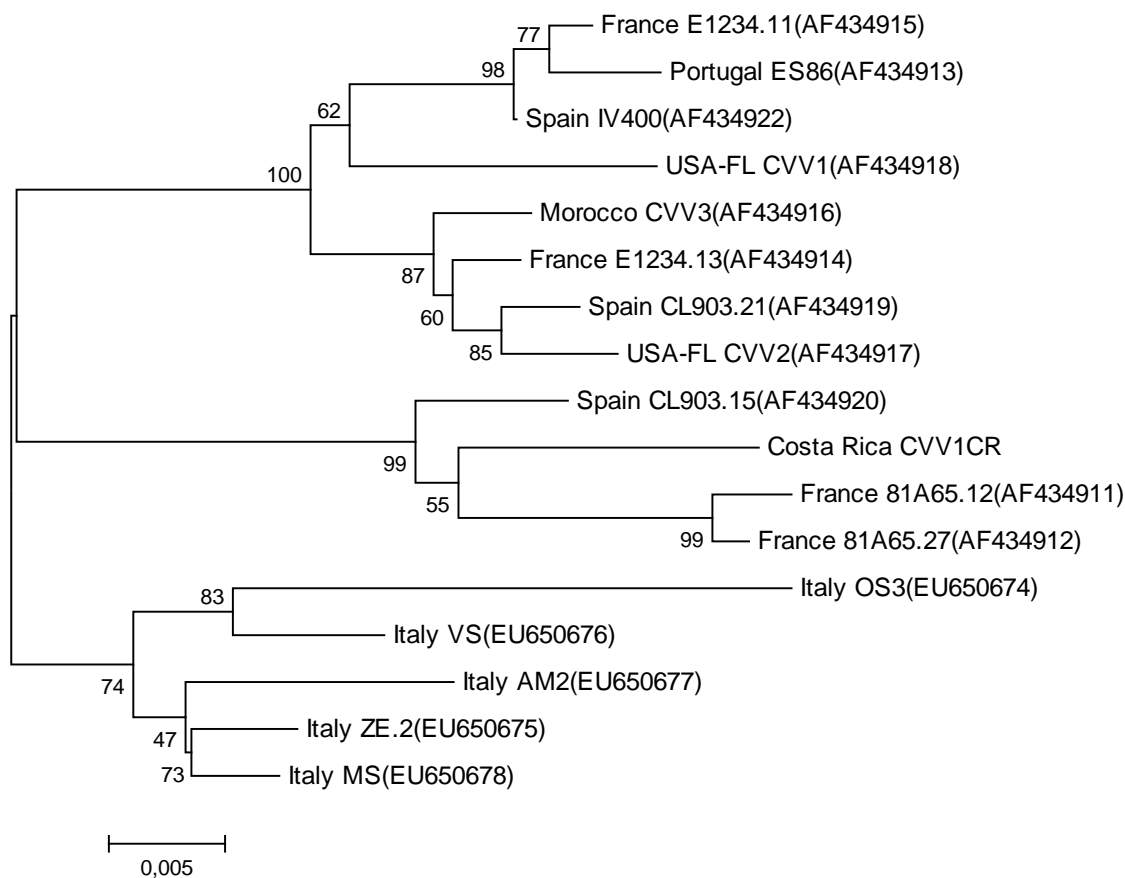




The CVV infection was confirmed by RT-PCR, amplicons of approximately 700 bp were obtained from all samples of symptomatic trees and positive control (data not shown). The sequence obtained (605 bp) using the CVVF and CVVR primers (21) was analyzed by BLASTn algorithm, showing 97% homology with the sequence of the coat protein gene (CVV RNA 3) of an isolate from Corsica (AF434912).

Alignment some GenBank CVV coat

protein sequences and on Costa Rican's strain (Gen Bank Accession No. JN899604) showed three transitions involving amino acid changes (Arg<sup>AGG</sup> → Lys<sup>AAG</sup> at position 103, Ile<sup>ATA</sup> → Val<sup>GTA</sup> at position 116, and Thr<sup>ACC</sup> → Ala<sup>GCC</sup> at position 181) (Fig. 2). More studies are needed to understand those substitutions in terms of selection (i.e. host range). A dendrogram showed a closer relationship between CVV1CR and Spanish and French strains (Fig 3).



**Fig. 3. Dendrogram showing relationships between CVV isolates from Italy, France, Morocco, Portugal, Spain, USA (Florida) and Costa Rica (CVV1CR) (NJ & bootstrap 500, distances calculated by Kimura's method with 2 parameters).**

According to Bennani et al. (2), the CVV does not seem to be naturally transmissible by pollen, but is highly transmissible by mechanical means between citrus species and herbaceous hosts.

It has been speculated that ilarviruses occur naturally in native species and on rare

occasions move to crops in which they subsequently cause disease problems as the result of asexual propagation and/or spread through pollen (18, 23). This idea has been supported by association of *Parietaria mottle ilarvirus* (PMoV), first reported in the weed species *Parietaria officinalis* (4),

and later, with diseases in tomatoes in Italy, France, and Greece (8, 19, 20) and bell pepper (*Capsicum annuum*) reported in Spain (13).

Lovisolò (18) suggested that CVV and *Citrus leaf rugose virus* (CLRv) were a result of a single transfer of a virus to citrus with differences in host range and symptom expression resulting of differential selection within citrus over time. A very close serological relationship was found between CVV and CLRv (10, 11), so both have been included as members of Iilarvirus group 2 (15). However, according to Scott et al. (23) CVV is more related to other ilarviruses, as *Elm mottle virus* (EMoV), *Asparagus virus 2* (AV-2), and *Spinach latent virus* (SPLV) than to CLRv at molecular level, suggesting that mutations and deletions could explain the differences between them. On the other hand, molecular studies carried out by Boulila (3) included both citrus viruses in a new tentative subgroup III encompassing CLRv, *Tulare apple mosaic virus* (TAMV), SPLV, EMoV, and CVV.

The origin of this virus in Costa Rica is difficult to track because many plants were

introduced without any quarantine regulation; and more data will be necessary to clarify this issue. A survey in Costa Rica should be done as soon as possible to know if CVV is present in other places in the country or in alternative host plants. However, it is necessary to establish measures to prevent the dispersion of CVV, detection at early stages of infection and use virus-free trees in propagation.

Benani et al. (2) emphasized the Garnsey et al. (9) recommendation, although this ilarvirus has not been a major citrus production problem; repeated discovery of the virus in unexpected and unrelated sites indicates that continued surveillance is necessary, especially in certification programs.

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