

ABSTRACTS

Toward Characterizing Stem Pitting Determinants of *Citrus tristeza virus*

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Identification of sequence motifs responsible for *Citrus tristeza virus* (CTV) pathogenicity requires a workable genetic system. Presently, the only system available is based on a cDNA clone of the T36 genomic RNA whose RNA transcripts are infectious in *Nicotiana benthamiana* protoplasts and virions produced are mechanically transmitted to citrus. Subcloning this cDNA in the binary vector pCAMBIA also allowed production of infectious CTV virions in agroinfiltrated *N. benthamiana* leaves. To identify CTV genes responsible for stem pitting in grapefruit or sweet orange we started preparation of a cDNA based on the sequence of the severe isolate T318A. For this purpose we synthesized cDNA from two natural defective RNAs (12-13 Kb) containing the *cis*-elements required for autonomous replication (ORFs 1a and 1b and variable portions of the 3' terminal region) as a first step to obtain infectious replicons. This objective was achieved only after inserting a plant intron in the 5' proximal region of the CTV genome and subcloning the cDNA in a BAC vector to reduce toxicity and improve stability of the construct in bacterial cultures. After checking infectivity of a pool of 14 clones by agroinfiltration of *N. benthamiana* leaves, replication of individual clones in agroinoculated leaves was monitored by Northern blot and real time quantitative RT-PCR analyses. All clones showed replication and accumulated at different levels in infected cells. Minireplicons with higher accumulation level will be used to prepare full-genome clones by insertion of the lacking central genes.

Characterization of a Severe Isolate of *Citrus tristeza virus* in Commercial Citrus Varieties

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Citrus tristeza virus (CTV) isolate T318A, an aphid transmitted sub-isolate of the severe isolate T318, is composed of a major sequence variant whose nucleotide sequence was determined. To characterize its effects in commercial varieties, 3-4 plants of each Washington navel, Salustiana, Valencia and Berna sweet orange, Nules Clementine, Star Ruby grapefruit and Pink pummelo propagated on Carrizo citrange, were graft-inoculated with T318A and one additional plant left as healthy control. CTV infection was confirmed by ELISA and DTBIA in all inoculated plants, except in Pink pummelo that only yielded positive detection by RT-PCR. CTV accumulation was monitored in three consecutive flushes using an optimized real-time RT-PCR protocol that allows absolute quantification of gRNA molecules in citrus. Highest CTV titers were observed in sweet orange and mandarin varieties, followed by Star Ruby grapefruit, whereas accumulation in Pink pummelo was 3-4 orders of magnitude lower. In most plants, virus titer was higher in the first and third flushes (Autumn and Spring) than in the second (Winter period). Growth of infected Pink pummelo, Nules Clementine, and Berna, Valencia and Salustiana sweet orange plants was similar to that of healthy controls, some Washington navel orange plants were slightly stunted, and Star Ruby grapefruit showed seedling yellows and severe dwarfing. Mild stem pitting was observed in Washington navel, Valencia and Salustiana sweet orange, moderate pitting in Berna, and moderate to intense in Star Ruby grapefruit. Our results suggest that T318A is a potential threat for the citrus industry and that Pink pummelo is partially resistant to this isolate.

Sequence Analysis of the Coat Protein and the RNA-dependent RNA Polymerase Genes of a *Citrus tristeza virus* Isolate from Turkey

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The presence of *Citrus tristeza virus* (CTV) in Turkey has been known for long time and biological and serological properties of a few isolates were determined to some extent. However, molecular characteristics of Turkish isolates have not yet been studied. To initiate molecular characterization of CTV isolates from Turkey, Iğdır isolate, one of the first CTV isolates identified from Jaffa sweet orange grafted on sour orange and maintained in Mexican lime for 25 yr, was selected. dsRNA was isolated from infected bark tissue and the coat protein (CP) and the RNA-dependent RNA polymerase (RdRp) genes were amplified by two-step reverse transcription-polymerase chain reaction (RT-PCR) method using gene specific primers. PCR products of the CP and the RdRp genes were individually cloned into the pGEM-Teasy vector and clones containing the CP and the RdRp genes were sequenced. Sequence of the CP and the RdRp of the Iğdır isolate were compared with CP and RdRp of other CTV isolates from different citrus growing region of the world available in the GenBank or other sources. Sequence comparison revealed that the CP of Iğdır isolates showed 96-97% sequence identity with of the CP from other CTV isolates. Based on CP sequence, the Iğdır isolate was most similar to B53, a stem pitting inducing isolate. Phylogenetic analysis of the CP indicated that Iğdır isolate has closer genetic relationship with severe isolates of CTV. On the other hand, the RdRp of Iğdır isolates showed 85-95% sequence identity with RdRp from other CTV isolates. Based on RdRp sequence Iğdır isolate was most similar with T30 and T3585, two mild isolates of CTV. Phylogenetic analysis of the RdRp suggested that the Iğdır isolate has closer genetic relationships with mild isolates of CTV.

Detection and Identification of *Citrus tristeza virus* Isolates from Different Citrus Growing Regions of Turkey

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Field surveys were conducted in five different citrus growing regions of Turkey in 2005 and 2006. A total of 201 samples were collected from different citrus varieties and tested for the presence of *Citrus tristeza virus* (CTV) by DAS-ELISA and RT-PCR. While DAS-ELISA showed that 41 trees were infected with CTV, 54 trees were found to be infected with CTV by RT-PCR. For further characterization 39 CTV positive samples were also tested by western blot using a polyclonal antibody detecting all CTV isolates and the monoclonal antibody MCA13 specific to the severe isolates of CTV. All 39 isolates tested by western blot gave positive reaction with the polyclonal; however, 32 isolates especially the ones from Satsuma, also gave positive reaction with MCA13. These isolates were also tested by bidirectional/PCR (BD/PCR) allowing differentiation of the MCA 13 positive and negative isolate and detection mixed infection. Results of BD/PCR were generally in agreement with the results of western blot assay with MCA13. A DNA fragment of about 300 bp specific to MCA13 reacting isolates were amplified from most of the isolates but a 400 bp fragment were amplified only from seven isolates in BD/PCR. In addition, both 300 bp and 400 bp DNA fragments were amplified from several isolates indicating mixed infection of MCA13 positive and negative isolates. A total of 28 isolates representing different geographical regions and host species were selected for biological indexing. Although none of these 28 isolates showed any symptoms on sour

orange, grapefruit, or sweet orange, all isolates induced vein clearing symptom on Mexican lime. Additionally, all tested Satsuma isolates and one kumquat isolate produced stem pitting symptoms on Mexican lime.

Two Distinct Evolutionary Pathways for *Citrus tristeza virus*: Recombination Defines Two Gene Modules and Provides for Increased Genetic Diversity in a Narrow Host Range Plant Virus

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Phylogenetic analysis of the full or partial genomic sequences of the *Citrus tristeza virus* (CTV) isolates T36, T68-1 and NS25 showed phylogenetic incongruities between sequences involved in viral RNA replication and those involved in movement and other viral functions. This incongruity was not found for these sequences for isolates T3, T30, T385, VT and T318A. Distance analysis of the replication sequences of T36, T68-1 and NS25 showed these are phylogenetically distinct from each other and from replication sequences of isolates T3, T30, T385, VT and T318A, which formed a cluster of related sequences. Sequences not directly associated with replication were highly similar among all CTV sequences examined. Potential recombination points were identified in the genomes of T36, T68-1 and NS25, indicating that recombination joined disparate replication sequences with highly conserved sequences for movement and other viral functions. The evidence suggests that the CTV genome has evolved two gene modules. Large increases in genetic diversity have occurred in the replication module when distantly related replication sequences were introduced into infected citrus trees and recombination occurred with the more conserved sequences of a CTV population already present in these trees. Sequence conservation in the movement/encapsidation module may be linked to the virus' adaptation and restriction to citrus.

Influence of Climatic Variability on *Citrus tristeza virus* Epidemiology in Two Regions of Cuba

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Climate changes with time affect agroecosystems, so it is important to characterize and determine such climatic variations in order to find out the most favorable periods for insect vectors appearance. We aimed at the characterization of favorable climatic conditions for the development of aphid vector populations and the increase of *Citrus tristeza virus* (CTV) infections in the Cuban citrus regions of Matanzas and Isla de la Juventud by using two complex indexes. Seasonal risk conditions favoring the appearance and rise of aphid populations, as well as the increase in CTV incidence, were identified. *Toxoptera citricida* seasonal variation patterns were generally found in the second semester of the year, while the *Aphis spiraecola* + *Toxoptera aurantii* populations present a clear bimodality (March-April and September-October). There is a higher risk of vector population increase when high values of the IB_{t,1,M} index occur along with negative minimum values of IB_{t,2,M} index. This is a situation of high anomaly and corresponds to low light and warm conditions with high humidity. Finally, the association pattern between climatic conditions and the virus-vector complex, as well as the response periods of aphid populations to

favorable climatic conditions, were identified. With respect to the increase in CTV incidence, correlations were obtained with a delay of up to 4 mo after the appearance of the vector.

Preliminary Evaluation of *Citrus tristeza virus* Isolates from Apulia (Southern Italy)

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During field surveys carried out in spring 2002 and 2003 in Apulia (southern Italy), two *Citrus tristeza virus* (CTV) foci were identified in commercial orchards of the Taranto province. In the first focus (Castellaneta) infected Navelina orange trees were symptomless or stunted, whereas typical decline was observed in the second focus (Massafra). In addition, a tree with quick decline symptoms was found in a grove near Palagianello. Molecular profiles of Apulian CTV isolates were obtained using Hilf and Garnsey's genotype-specific primers. CTV populations of the two foci proved to consist of T30-like genotypes and a mixture of T30 and VT genotypes, respectively, while the isolate from Palagianello was identified as a T36-like severe genotype. Three isolates (CTV-0032, CTV-0036 and CTV-0038), showing varied CTV symptoms, were characterized based on coat protein (CP) gene sequences. The CTV CP gene was amplified by RT-PCR using CP degenerate primers yielding a 672 bp amplicon. The RFLP profile, nucleotide and deduced amino acid sequences were analyzed and compared to each other and also to some other exotic CP gene sequences of CTV isolates available in databases. The results revealed that Apulian isolates CTV-0032 and CTV-0036 have high similarity to Florida T30 and Spanish T385 mild isolates, while CTV-0038 is closely related to the severe Argentinian isolate C269-6. Additional work on different genomic regions of CTV-0038 may contribute to the study of the variability in CTV populations of this area.

Effectiveness of Antibodies Developed to the Recombinant Coat Protein of *Citrus tristeza virus*

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Polyclonal antibodies specific for the recombinant coat protein (rCP) p25 gene of *Citrus tristeza virus* (CTV) isolate MX14 from Mexico were developed in goats and rabbits. The reactivity of rCP antibodies was evaluated using healthy and CTV infected tissue. The combination of rCP antibodies developed in goats used as primary (coating) antibodies, and rCP antibodies developed in rabbits, as intermediate antibodies, reacted efficiently, with optical density (OD₄₀₅) values between 0.400 and 2.000 for CTV infected tissue of CTV isolates from diverse geographic origins. OD₄₀₅ values for healthy tissue were lower than 0.100. The OD values obtained with the combination goat/rabbit rCP antibodies gave consistent results for positive and negative sample discrimination as the commercial CTV detection kit available from Agdia. Consistent results were also obtained using the Central California Tristeza Eradication Agency (CCTEA) antibodies used for large scale CTV detection. CCTEA detection kit uses goat anti-CTV antibodies for trapping developed against purified virus preparations by conventional methods and rCP rabbit anti-CTV antibodies as the intermediate antibody. Results of this work provide evidence

that rCP antibodies can be efficiently used for both capture and detecting antigen in double antibody sandwich indirect ELISA.

Occurrence of Genetic Bottlenecks During *Citrus tristeza virus* Acquisition by *Toxoptera citricida* in Field Conditions

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Diverse reports refer to the existence of strain segregation during *Citrus tristeza virus* (CTV) transmission by *Toxoptera citricida*. However these effects, as well as the occurrence of genetic bottlenecks, have not yet been shown to occur in the field or quantified. In this study we address the involvement of *T. citricida* in strain segregation and genetic bottleneck events by comparing the nucleotide diversity of CTV coat protein (CP) gene variants present in field grown trees with that of variants retrieved from single wingless aphids. Plant material and aphids were collected in orange orchards in the northern part of Portugal. Two trees harboring haplotypes from four and six CP gene phylogenetic groups were analysed. Analysis of Molecular Variance revealed that most of the variation of the virus was found among individual aphids (FSC: 0.766) within each location. Computer simulations of random virus acquisition by single aphids showed that in 54% of the cases, only virions from a single CP gene phylogenetic group was acquired and that there is about a 60% probability that the effective size of the viral population be reduced to less than one tenth of the original value. However, a small number of aphids (e.g six) was enough to acquire the full complement of phylogenetic groups present. These results suggest that in nature one does not expect to find epidemiological changes during nearby transmission by *T. citricida* (e.g. in the same orchard), while, less frequent, long distance transmission might originate a shift in the viral population properties.

A Comparison Between a Coat Protein Gene Targeting System and Dispersed Genome Markers for Strain Discrimination of *Citrus tristeza virus*

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In consequence of the worldwide spread of *Citrus tristeza virus* (CTV), development of quick methods for discrimination of virus variants is essential for selective quarantine and eradication policies, to prevent movement of severe strains into new areas and for meaningful epidemiology. In the recent years we have developed a discrimination system based on the phylogenetic analysis of the major CP gene. More recently Hilf et al., 2005 (Phytopathology 95: 909-917) developed a system based on genetic markers targeting diverse regions of the CTV genome. In this work we took a collection of CTV isolates whose CP gene sequences were known and analysed them using Hilf's system. Only one group was consistently typed with both systems: T30 (Hilf) which corresponded to the CP based group M. The VT (Hilf) group corresponded to isolates from the CP based groups 2, 3b, 4, and 5. The T3 (Hilf) group corresponded to isolates from CP based groups 3a and 4. Some isolates from CP based group 5 could not be assigned to any of Hilf's groups. The T36 (Hilf) group was not detected in our collection which is consistent with the absence of group 1 (CP based) isolates in this collection. A real time PCR system based on molecular beacons and Taqman probes is being developed based on the CP gene sequence.

Identification of Turkish strains of *Citrus tristeza virus* (CTV) by Analysis of Double Stranded RNA Methods

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This paper reports on results obtained in dsRNA analysis of CTV isolates of different citrus areas in Turkey. The nine isolates that used in this study all induced vein clearing symptoms in Mexican lime leaves, but no symptoms of Mexican lime stem pitting or seedling yellows in sour orange occurred. Double stranded RNA analysis enabled us to identify three different patterns among the nine CTV isolates assayed. T-36 isolates used for positive control. All isolates showed a strong band of 0.5×10^6 da corresponding to the seedling yellows strains however, they were not show any reaction in biological indexing. Nine isolates showed band of 13.3×10^6 da, corresponding to the full-length replicative form.

Development of Transgenic Mexican Lime Plants for Resistance to *Citrus tristeza virus* Through Post-transcriptional Gene Silencing

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Two obstacles which may hinder the development of citrus resistant to *Citrus tristeza virus* (CTV) via post-transcriptional gene silencing (PTGS) are the sequence diversity of CTV, and the presence of multiple suppressors of PTGS in the CTV genome. To address the diversity of CTV, we sequenced 114 coat protein (CP) genes of Hawaiian isolates and used this data, along with the CP genes of isolates from around the world present in GenBank, to create a synthetic CTV CP gene segment. This 626 bp untranslatable segment is $\geq 94.6\%$ similar to the CPs of all known strains of CTV. We used *Agrobacterium* to introduce this gene segment into Mexican lime in sense, antisense, and inverted repeat configurations. CTV also possesses at least three PTGS suppressors: p20, p23, and CP, whose expression *in planta* may prevent a resistant phenotype. To target their expression, we linked p20 and p23 gene segments and the entire 3' untranslated region to a segment of the synthetic CTV CP gene and introduced this construct into Mexican lime using *Agrobacterium*. Molecular analyses indicate these plant lines are transgenic, and greenhouse evaluation of their resistance to CTV is currently underway.

First Monitoring and Characterization of *Citrus tristeza virus* and Relative Vectors in Syria

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The main Syrian citrus growing areas of Lattakia and Tartous were surveyed to assess the presence of *Citrus tristeza virus* (CTV) and its relative vectors. Eight nurseries, two budwood source fields and 19 groves of the main

citrus varieties were visually inspected and budwood indexed. A total of 4% of infected sources was assessed by DTBIA in two nurseries, in two budwood source fields and in six groves. Based on the reactions with several MAb, CTV sources were serologically included in four serogroups. Only one Valencia CTV-source induced severe symptoms on Mexican lime (vein clearing, leaf cupping, stunting and stem pitting). *Aphis spiraecola* (50%), followed by *A. gossypii* (27%) were the most common aphids found, whereas there was no evidence of presence of *Toxoptera citricida*. This is the first report of CTV in Syria, but apparently, the virus has not spread by the vectors. Hybridization of amplicons from the coat protein gene (CPG) to a set of strain-specific probes evidenced a complex strain mixture, The SSCP analysis and the subsequent nucleotide analysis of five Syrian CTV isolates enabled their distinction in mild and severe group.

Biological and Molecular Characterization of Two Virulent *Citrus tristeza virus* Isolates Found in Central California

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Vigilant survey and eradication efforts for *Citrus tristeza virus* (CTV) are critical to protect citrus from infection and spread of severe CTV strains. Two notable CTV isolates were detected by the Central California Tristeza Eradication Agency (CCTEA), Tulare, CA in 1994 and 2000. The isolates, identified as Rocky Hill (RH) and Dekopon (Dk) were positive with monoclonal antibody (MAb) MCA13 and produced severe reactions in CCTEA biocharacterization tests. In 2005-2006, new investigations were conducted to compare these isolates with local ones. *Aphis gossypii* transmission (AT) efficiency was 74 and 23% for RH and Dk, respectively. All RH isolates including the AT sub-isolates were negative with the MAb 3E10; whereas one Dk source was positive and the other negative. Most of the Dk AT sub-isolates were negative suggesting that a mixture of strains were present. This was confirmed by SSCP profiles, nucleotide sequence, and symptoms in indicator plants. RH was a strong SY isolate and had a non-standard VT genotype. The Dk sources, in contrast, contained VT-, T3- and T30-like genotypes. Sequences from the different Dk AT sub-isolates indicated that the T3 genotype component was predominantly transmitted by aphids. In general, the Dk AT sub-isolates were more severe compared to the parent isolate suggesting that Dk symptom expression was ameliorated by the mixture. Despite these differences, both RH and Dk isolates were designated as stem pitting strains with distinct genotypes and severe host reactions compared to those of local isolates.

Tissue print-ELISA[®] Complete Kit for Screening of Severe *Citrus tristeza virus* Isolates at Large Scale Testing

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A complete “Tissue print-ELISA[®]” or “Immunoprinting-ELISA[®]” kit including nitrocellulose membranes pre-printed with tissues infected with severe and mild *Citrus tristeza virus* (CTV) isolates and negative controls, has been designed based on the CTV-specific monoclonal antibody MCA13 reaction. The kit has been evaluated in large

scale analyses of 3,115 field adult trees grafted on Troyer and Carrizo citrange in Castellón and Valencia, Spain. Only 7% of those trees reacted positively with MCA13. The kit was also assayed with 154 characterised CTV isolates from IVIA and international collections. A positive reaction with this kit was associated with at least one of the following symptom expression patterns in the greenhouse: vein clearing, vein corking and stem pitting on Mexican lime; seedling yellows; pitting on sweet and/or grapefruit seedlings, and weak shoots or no sprouting of sour orange buds grafted on CTV-infected Pineapple sweet orange. Isolates inducing only mild to moderate symptoms in Mexican lime were considered mild. Isolates causing noticeable symptoms on sweet orange, grapefruit, sour orange or lemon seedlings, or in sour orange grafted in CTV-infected sweet orange plants were considered severe. The reaction of the CTV-specific MCA13 in DASI-ELISA and in Tissue print-ELISA were similar but not identical. The use of immobilized targets in membrane allowed more positive MCA13 detections. The available kit could be a suitable alternative to standard biological indexing, conventional ELISA (using extracts) or molecular methods for detecting the more aggressive isolates in large surveys. A positive reaction must be considered as a strong indication of the presence of severe CTV isolates. Nevertheless, this situation must be confirmed by biological indexing, at least for the first isolates detected.

Quantitative Detection of *Citrus tristeza virus* by Direct Tissue-print and Squash Real-time RT-PCR Procedures

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A TaqMan real-time RT-PCR was developed to detect and quantify *Citrus tristeza virus* (CTV) in routine screening of plant materials and individual aphids immobilized on membranes. This direct method does not require plant extract preparation or nucleic acid purification. Samples were immobilized on nitrocellulose, nylon or paper membranes by the tissue-print procedure or by direct squash of aphid species. Pieces of membranes harbouring the samples were inserted into 96 well trays or microtiter plates. One hundred μ l glycine buffer (0.1M glycine, 0.05M NaCl, 1mM EDTA) were added, incubated at 95°C for 10 minutes, vortexed and placed on ice. Five μ l of this extract were directly used as template for large scale analysis by real-time RT-PCR. The reliability of tissue-print real-time RT-PCR (using 10 overlapping imprints from five shoots or 10 leaf pedicels/tree in the same piece of membrane) was similar to conventional real-time RT-PCR (using purified total RNA from the same printed material). When the method was compared with the serological tissue print-ELISA assay, an overall good correlation was observed between the two techniques, although real-time amplification was more consistent. Successful amplification was achieved from imprints already developed by tissue print-ELISA. The number of CTV targets detected in different plant tissues collected around the canopy of adult trees ranged from 1.4×10^4 to 4.0×10^6 . The lowest average Ct value was obtained from fruit peduncles. In single squashed *Aphis gossypii* the number of CTV copies ranged from 3.5×10^3 to 1.3×10^5 . An additional advantage of this user-friendly method for accurate quantification is that print or squash real-time RT-PCR probably only detect genomic RNA from virions, encapsidated large dRNA and some stable dsRNAs, whereas the remaining RNA population is probably degraded by RNases in the porous membranes.

Present Situation of *Toxoptera citricida* and *Citrus tristeza virus* in Northern Spain

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Aphid species caught in four Moericke yellow traps located along the coast of Asturias (northern Spain) in 2002-2004 were identified in December 2004. *Toxoptera citricida* was unexpectedly found in all traps from the 3 years. The species was previously detected, also accidentally, in mainland Portugal and in Spain near the Portuguese border in December 2003 and in 2004. *T. citricida* and *Citrus tristeza virus* (CTV) were surveyed in citrus trees in northern Spain from Portugal to France. *T. citricida* was found in citrus trees from the Portuguese border to eastern Cantabria, just in the limit with the Basque Country. CTV was found only in three sweet orange on Carrizo citrange trees out of 914 analyzed by tissue print-ELISA. Extremely low populations of adults survive in winter in flower buds or in the insertion of the peduncle with the fruit in isolated backyard lemon trees. *T. citricida* populations increase in May. The first winged aphids are caught from mid May to the end of June and the last by the end of November. In July 2007, *T. citricida* was found infesting a single *Berberis* sp. plant. Its role in the life cycle of this aphid species is under evaluation. No sexual phase has been detected. Parasitoids and predators of *T. citricida* in northern Spain are relatively abundant and are the same species described in the Mediterranean areas for other aphid species. The origin of the pest remains unknown. Risk evaluation predicts spread to the Mediterranean citrus growing areas via Portugal if containment is unsuccessful.

Replication and Synergism of Components and Symptoms of *Citrus tristeza virus* Capão Bonito Complex in Mexican Lime Plants

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Two components of *Citrus tristeza virus* (CTV) Capão Bonito isolate, I-1 and I-2, inoculated singly and in groups by grafting inoculation into Mexican lime plants were evaluated by symptom expression, reaction with monoclonal antibody 30.G, 39.8 and 3DF1+3CA5, immunolabeling with polyclonal antiserum 1006 and real-time TaqMan RT-PCR (RTqPCR) assay for the p25, p27 and p23 genes. The goal was to verify interactions with synergistic effects between the two components. Plants inoculated with both components (I-1+I-2) showed symptoms typical of the isolate and had higher virus with 30.G 02, 39.08 and 3DF1+3CA5 MAbs. Immunolabeling detected viral particles in large quantity in plants inoculated with I-1 or I-2 singly. In real-time RT-qPCR all the genes evaluated were more strongly expressed in plants infected with the components (I-1+I-2). Thus, the low replication observed in plants infected with only one of the two components (I-1 or I-2), suggest that the interaction between these components makes the virus replication more efficient, increasing the virus titer in the plant. This contributes to the expression of symptoms in plants infected with the two virus components. These results are the first on a study of synergism among CTV components.

Differential Expression of *Citrus tristeza virus* Genes in Tolerant and Resistant Hosts

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Citrus tristeza virus (CTV) is the causal agent of tristeza, one of the most important citrus diseases worldwide. Its p23 gene has a probable regulatory role in the viral cycle and / or pathogenesis, and p25 and p27 are the CTV coat proteins. The differential expression of these genes of a severe (CTV-CB) and a mild and protective (CTV-IAC) CTV isolates, were evaluated by Real time PCR (qPCR) at different days after inoculation in a highly susceptible variety, Pera sweet orange (*Citrus sinensis* L. Osb.) and a tolerant variety Ponkan mandarin (*Citrus reticulata* Blanco). The qPCR allowed detection and quantification of CTV genes from the two isolates in tolerant and susceptible hosts, indicating that these genes are differentially expressed. The main difference observed was concerned with the host susceptibility and tolerance, being the expression of the CTV genes higher in tissues of the susceptible host than in tissues of the tolerant one. The technique has potential to be used in fast and safe CTV diagnosis, combining high sensibility, specificity, speed and easiness to run the test. This approach can be used as a tool for early selection of susceptible and tolerant hosts, based on the CTV RNA concentration in the tissues.

Purification and Secondary Structure Characterization of the *Citrus tristeza virus* Coat Protein

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The coat protein (CP) is one of the major protein components of the virion, which is primarily required for genome protection. The CP may also be involved in translocation into the host cell and for cell-to-cell movement, due to a probable interaction with host cells via specific receptors to start an infection. The capsid of *Citrus tristeza virus* (CTV) is made up of two types of CPs with 25 and 27 KDa. Therefore, the CTV coat protein from a Capão Bonito complex (São Paulo, Brazil) was produced in *Escherichia coli* BL21 (DE3) and purified by nickel affinity chromatography. The recombinant protein has a predictive molecular mass of 25 950 Da whose identity was confirmed by ELISA and Western blot analyses. The purified CP protein was analyzed for secondary structure through circular dichroism (CD) spectroscopy and analysis showed that CP comprises 44% α -helix, 12% β -sheet and 44% random coil content. In addition, the region between amino acid residues 25 and 100 of CTV CP is predicted to be an antigenic epitope region on the basis of its hydropathicity plot and comparison with the CP sequences of CTV. Our results contribute to structural characterization of CP and may help to understand its biological and physiological functions.

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Epitope Mapping of *Citrus tristeza virus* Capsid Proteins Recognized by Monoclonal Antibodies

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Tristeza causes one of the most economically important viral diseases of citrus industry of the world. It is caused by *Citrus tristeza virus* (CTV) which is transmitted by aphids in a semi-persistent manner and also by grafting during citrus propagation. We characterized the epitopes of the bacterially expressed CTV coat protein (p25) of the Brazilian CTV strain "Capão Bonito". The four monoclonal antibodies which had been previously produced using the *E. coli* expressed recombinant proteins, CB-22 and CB-104, were analyzed by several immunological methods: ELISA, Dot blot, Western blotting, direct tissue blotting and immunoelectron microscopy. The MAb reactivities were primarily screened by the recombinant proteins which contain the sequences of the severe and mild isolates of the Brazilian CTV virus. After determination of the hydrophobicity regions by the ProtScale program (ExpAsy Proteomics Server), we designed six pairs of primers to CB-104 and eight pairs of primers to CB-22 proteins which peptide fragments were expressed and their reactivity were tested in indirect ELISA and Western blot. The MAbs 30 and 37 demonstrated reactivity in ELISA with the fragments **NLHIDPTLI** and **TQQNAALNRDLF** at the protein amino acid position 32-40 and 50-61 from CB-22 and CB-104 proteins. Furthermore, the MAb 39 recognized the epitope **TDVVFNSKGIGN** at the position 120-131 from CB-104. So, these antibodies are positive in ELISA and Western blot we can conclude that these epitopes are linear. Finally, these monoclonal antibodies have been used in CTV crop field diagnostic and can differentiate the mild and severe strains of CTV.

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Serological and Molecular Variability in a Collection of Mediterranean *Citrus tristeza virus* Isolates

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Mediterranean *Citrus tristeza virus* (CTV) isolates from a collection of the CIHEAM/Mediterranean Agronomic Institute in Italy, which had been previously biologically indexed on Mexican lime, were serologically and molecularly characterized. A selective panel of MAbs of different origin, MCA13 included, highlighted the presence of eight serogroups. A broad spectrum reaction was obtained with 30% of the CTV tested sources, whereas 65% reacted positively with MCA13. Hybridization of amplicons from the coat protein gene (CPG) to a set of strain-specific probes showed high frequency of samples harboring a mixture of variants. It also demonstrated a great diversity in their strain composition ranging from mild to severe. A high degree of homology with worldwide isolates by nucleotide sequences of CPG was demonstrated and different clusters were made. A preliminary assessment by SSCP assays showed a great conformational difference between the same isolates. Molecular assays

put into evidence frequent reaction with quick decline-specific probes and few positive results with sweet orange stem pitting type. Until now, the latter was only limited to the Balkan area but not reported in the rest of the Mediterranean area.

Detection of *Citrus tristeza virus* and Citrus Viroids Associated with Citrus in Oman

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Different varieties of citrus mainly grapefruit, sweet lime and oranges are cultivated in northern and southern part of Oman and it is the most important fruit crop after date palm. Recently disease symptoms such as leaf yellowing, decline, stem pitting has been observed in citrus orchards reminiscent to citrus viruses and viroids. Samples from 2,700 symptomatic citrus trees grown in various regions of Oman were collected for serological and molecular analysis. About 11% samples tested positive for *Citrus tristeza virus* (CTV) by DAS-ELISA using alkaline phosphatase labeled monoclonal antibodies according to EPPO protocol. The presence of CTV in ELISA positive samples was reconfirmed by immuno capture reverse transcriptase polymerase chain reaction (IC-RT-PCR) using CTV specific primers. *Citrus exocortis viroid* (CEVd) and citrus cachexia viroid (CCVd) were detected by reverse transcriptase polymerase chain reaction (RT-PCR) using specific primers. 33% citrus samples were found associated with CEVd and 23% with CCVd by RT-PCR test. The prevalence of citrus exocortis and citrus cachexia viroids was found to be higher than CTV in symptomatic citrus samples.

Occurrence, Distribution and Characterization of *Citrus tristeza virus* and Relative Vectors in Apulia the Region of South-East Italy

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Tristeza outbreaks first appeared in the Apulian region of Italy in 2002 along the Ionian coast, and originated by infected propagating material supplied by extra-regional nurseries. In the last few years about 80,000 trees were tested by DTBIA and showed different rates of *Citrus tristeza virus* (CTV) infections in commercial citrus groves of Clementine and sweet oranges of different ages. A few infected plants were detected in the nurseries located in the CTV foci. By aphid vector monitoring, 2,252 apterae aphid adults were identified in commercial orchards. *Aphis spiraecola* (47,5 %) and *A. gossypii* (38,25%) were the most abundant compared to *Toxoptera aurantii* and *Myzus persicae*. Virus transmissibility using *A. gossypii* and selected CTV isolates was successfully carried out under protected conditions. A total of 22 Apulian CTV sources were biologically, serologically and molecularly characterized. All tested sources showed clear cut tristeza symptoms on Mexican lime. A selective panel of Mabs highlighted the presence of six distinct serological reaction patterns, two of which reacted with MCA13. Hybridization of amplicons from the coat protein gene (CPG) to a set of strain-specific probes indicated some

isolates as “pure” and the remaining ones as a complex strain mixture. These results were confirmed by SSCP analysis on 11 isolates. According to the previous analysis, four CTV isolates were chosen for partial genome sequencing. The phylogenetic tree clustered the selected Apulian isolates close to CP sequence of the mild isolate T30 (Florida).

Influence of the *Brevipalpus phoenicis* Endosymbiont *Cardinium* sp. in the Transmission of Citrus leprosis virus

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Citrus leprosis is a viral disease of significant economic and environmental impact in Brazil and some other countries in the Americas. CiLV, its causal agent, is transmitted by *Brevipalpus phoenicis* (Acari: Tenuipalpidae), a polyphagous mite that reproduces through thelytokous parthenogenesis. The presence of *Cardinium* symbiont is associated to the feminization in these mites and reproduction alterations in several other arthropod hosts. In some plant virus-vector relationships, endosymbiont bacteria play important roles in the arthropod capacity to transmit plant viruses. However, it was not known whether or not the *Cardinium* has any influence on CiLV transmission by the mite vector. Our research program, which also addresses the prevalence and variability of the *Brevipalpus* endosymbiont, investigated this vector-symbiont-virus interaction. Three populations of *B. phoenicis* were used (1) mites (males) free of *Cardinium* (originated from a tetracycline treatment of the female parental), (2) females treated with antibiotic, and (3) control group (females without the antibiotic treatment). These populations were maintained in sources of inoculum of leprosis for 72h, and then used for the infestation of sweet orange var. Pera plants. The symptoms started to appear 25 days after the infestation, and all of the plants from all treatments presented typical leprosis lesions. These preliminary results suggest that both acquisition and inoculation of CiLV by *B. phoenicis* are not *Cardinium*-dependent and, hence, the endosymbiont does not seem to play a role in the citrus leprosis pathosystem.

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Initial Responses of Sweet Orange to Citrus leprosis virus Detected by ESTs

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Citrus leprosis virus, cytoplasmic type (CiLV-C), transmitted by the tenuipalpid mite *Brevipalpus phoenicis*, causes an economic important disease in Brazil and other countries in South and Central America. ESTs sequencing and analyses can bring important information on the response of different genotypes to a pathogen. The main objective of this work was to identify genes in *Citrus sinensis* cv. Pera plants that were differentially expressed in the beginning of CiLV-C infection, up to 48 hours after viruliferous mite inoculation. In order to accomplish that,

cDNA libraries were constructed from healthy and CiLV-inoculated sweet orange leaves and yielded 8,188 and 4,852 valid reads, respectively. Two hundred and fifty four genes were found to differ significantly in terms of expression, with 193 of them being induced and 61 repressed by the pathogen, and distributed within 19 categories classified according to the MIPS FunCat. A sub-set of such categories was chosen to be studied in details due to their involvement in stress responses. They included, in addition to genes involved in cell rescue, defense and virulence, those involved in cellular communication/signal transduction mechanisms, metabolism, and energy. Here we discuss the possible roles of some of these genes in the initial steps of the disease.

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Evidence Suggesting That *Brevipalpus phoenicis*-Citrus leprosis virus Interaction May Not be of Circulative-Propagative Type

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The false spider mite, *Brevipalpus phoenicis*, is the only vector of Citrus leprosis virus (CiLV) in Brazil. This virus, which induces the appearance of only local lesions in its plant hosts and does not invade them systemically, is currently considered the most important viral agent affecting citrus in the country due to the severe economic losses it causes. With the complete sequencing of the CiLV genome, we are now focusing on studies that address functional genomics of host-virus pathosystem as well as the virus-vector-endosymbiont interactions. It is widely accepted in the literature that the CiLV-*Brevipalpus* interaction is of the circulative propagative type. However, the difficulty in finding particles and the apparent absence of viroplasm in viruliferous mites, noted by transmission electron microscopy analyses, has raised doubts concerning the replication of the virus within its vector. Real time quantitative PCR (RT-qPCR) allows the reliable and specific quantification of small amounts of a target-sequence, and was used to monitor whether or not CiLV appears to replicate in the *Brevipalpus* mite. Four treatments, with three biological replicates, were tested: a) mites constantly feeding in the source of CiLV inoculum; b) viruliferous mites in inert medium; c) viruliferous mites reared onto non-citrus plant (*Clerodendron* sp.); d) viruliferous mites reared onto the resistant Rangpur lime fruits. Periodic evaluations were performed for up to 21 days. For each treatment, in each assessment, a bulk of ten mites was collected and analyzed for the relative quantification of the virus. The results indicate that the *Brevipalpus* mite loses viral particles when reared onto a non-CiLV host or inert medium, suggesting that the virus may circulate, but likely not propagate, in its vector.

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Haplotype Characterization and Genetic Variability of Two Genes of CiLV-C Through SSCP in Brazilian Citrus Orchards

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With the molecular characterization of Citrus leprosis virus-cytoplasmic (CiLV-C), it was possible to develop molecular tools for viral detection and variability studies. Previously, we used SSCP to determine CiLV-C diversity among isolates from several Brazilian states. Here we present data on the variability of CiLV-C from citrus orchards within São Paulo State, responsible for more than 80% of the sweet orange production in Brazil. Approximately seventy symptomatic samples of 14 sweet orange varieties from 36 municipalities within the main citrus producing regions of the state were assessed. Asymptomatic sweet orange plants were used as negative controls. Samples were submitted to RT-PCR with two primer pairs that amplify regions within the CiLV-C replication-associated and the movement protein genes. Amplicons were used for SSCP (single strand conformation polymorphism) analyses in 8% polyacrylamide gels. POPGENE and PHYLIP software were used to estimate statistic patterns of genetic diversity, and genetic distance and phenogram, respectively. Data analyses of molecular variance were performed with AMOVA statistics by WINAMOVA software. Different polymorphic patterns were confirmed by sequencing. Seventy to 80% of the samples exhibited the same SSCP pattern, constituting the prevalent haplotype. The results revealed allelic frequencies equally distributed among the isolates for the two genes in all geographic regions, with few exceptions. The West region showed the lowest rate of genetic diversity and the highest distances were observed between the isolates from the North and the West regions. Despite low, genetic diversity was detected regardless of the region or the variety assessed.

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Response of Mandarin Cultivars and Hybrids to Citrus Leprosis

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The global importance of citrus leprosis has significantly increased in the last years, with the northbound dissemination of the virus to new areas in South and Central America. Sweet oranges (*Citrus sinensis*) are considered the most susceptible species to Citrus leprosis virus (CiLV) and, even though there are differences in susceptibility amongst them, none of the commercial varieties are resistant to the disease. It has been accepted that mandarins and some of their hybrids exhibit higher levels of resistance. However, few studies have been carried out to date with the objective to compare resistance amongst those genotypes. In this work we evaluated, through symptom analysis, the response to leprosis of 25 different genotypes of mandarins and hybrids from a ten-year-old orchard with a long time history of the disease. We observed higher resistance level under natural conditions among *C. reticulata* varieties and some of their hybrids, such as the Murcott tangor (*C. reticulata* x *C. sinensis*), which did not present any leprosis symptoms. *C. deliciosa* and *C. clementina* accessions and hybrids, such as the Lee tangelo [(*C. clementina* x (*C. reticulata* x *C. paradisi*)], exhibited the highest level of susceptibility to the disease under natural conditions. However, even in those genotypes, most of the symptoms were observed in leaves and not in fruits, as often observed in oranges.

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Studies on the Possible Causes of Spread of *Citrus psorosis virus*

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Research and observations in Argentina and Texas suggested a natural spread of the psorosis disease of citrus. Recent studies also suggested the possibility of transmission of the disease from tree to tree by an *Olpidium*-like fungus. In order to study the natural spread of the disease we initiated a complete tree by tree survey by counting trees showing symptoms. This was begun in October, 2006 in Tucumán, Argentina in an orchard of nucellar Westin sweet orange trees. The severities of symptoms were rated as: 0 (no symptoms); 1 (some bark peeling and/or gumming), 2 (psorosis like scaling) and 3 (classic psorosis bark scaling). Seventeen trees were rated as 3 and budwood of four of these were indexed to Pineapple sweet orange indicator seedlings. Psorosis was confirmed in all four of these trees. The results of the survey on 5,910 trees, showed 2,263 trees with symptoms rated as 1 to 3 (38, 29%). This survey will be continued for four more years. In this same orchard, we planted Pineapple sweet orange seedlings around the canopies of seven orange trees showing different severities of psorosis symptoms. In addition, soil was taken from the surrounding canopies of these same trees, placed in containers and planted with Pineapple sweet orange seedlings. Negative control seedlings were planted in peat moss. These are now being held under greenhouse conditions and after one year, we will search for the presence of any *Olpidium*-like fungus in the roots of the sweet orange seedlings growing in the greenhouse and also from seedlings growing in the orchard soils.

An Immunocapture RT-PCR Procedure Using *Apple stem grooving virus* Antibodies Facilitates Molecular Genetic Characterization of Citrus tatter leaf virus from the Original Meyer Lemon Host

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A magnetic bead-based immunocapture system using polyclonal antiserum against *Apple stem grooving virus* (ASGV) successfully facilitated polymerase chain reaction (PCR) amplification of sequences from three Citrus tatter leaf virus (CTLV) isolates originally isolated from the citrus host Meyer lemon. Primers designed from a pairwise alignment of genomic sequences of CTLV isolates from lily and from kumquat amplified two non-overlapping genomic regions of 625 and 1165 bp (~28% of the CTLV genome) which were cloned and sequenced. Despite being propagated separately in the glasshouse for more than 40 yr, the CTLV sequences from separate Meyer lemon sources were identical, but had only ~80% nucleotide identity with the homologous regions of CTLV genomes of isolates from lily and from kumquat. Neighbor-joining phylogenetic analysis indicated the CTLV isolates from Meyer lemon were distinct from but more closely related to CTLV from kumquat than from lily, and these CTLV sequences showed equivalent genetic distances from two ASGV isolates.

Viroids and Rootstocks Effects on Field Performance of Tahiti Lime in Brazil

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Some viroids reduce citrus tree growth on different rootstocks and may be used for tree size control in high density plantings that can provide higher productivity than conventional ones. To study the effects of citrus viroids inoculation on vegetative growth, yield and fruit quality of Tahiti lime [*Citrus latifolia* (Yu.Tanaka) Tanaka] grafted on six rootstocks (main treatments): FCAV and Rubidoux trifoliolate oranges [*Poncirus trifoliata* (L.) Raf.], FCAV and Limeira Rangpur limes [*Citrus limonia* Osbeck] Carrizo citrange [*C. sinensis* (L.) Osbeck x *Poncirus trifoliata* (L.) Raf.] and Sunki mandarin [*Citrus sunki* Hort. ex Tanaka] and inoculated with three viroids isolates (secondary treatments): [*Citrus Exocortis Viroid* (CEVd) + *Hop stunt viroid* (HSVd - CVd-II, a non cachexia variant) + *Citrus III viroid* (CVd-III)], [*Hop stunt viroid* (HSVd - CVd-II, a non cachexia variant) + *Citrus III viroid* (CVd-III)] and control [no inoculation], an experiment was set up in February 2001, in Bebedouro, São Paulo State, Brazil. The experimental design was a split plot design in randomized blocks with six main treatments and three secondary ones with three plants per plot. Inoculation was made at field 10 months after planting by bud grafting. The trifoliolate oranges rootstocks induced less vigor. Both isolates reduced tree size. Trees not inoculated yielded better (total of four harvests) than inoculated ones but the productivity (kg/m³) was the same. Fruit quality was affected by viroids inoculation but without commercial importance. There is not agricultural or economical advantage in the use of citrus viroids for Tahiti lime plantings.

Effect of Viroids on Resistance to *Phytophthora* Infection of Citrus

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Citrus viroid-induced resistance to *Phytophthora* infection in citrus was measured by the number of *Phytophthora* sporangia in Rio Red grapefruit bait tissue, infected with citrus viroids compared with non-inoculated control. Different viroid isolates containing mixtures of viroids (CEVd, HSVd, CVd III, and CVd IV) were designated by plant numbers and sources. Source 13E was associated with the lowest number of sporangia in bark, leaves, and roots used as baits, while CEVd E9 a known severe CEVd isolate, significantly reduced the number of sporangia in leaves and bark. Sources 1A, 2E, 3E, 4D, and 6E showed significantly reduced number of sporangia on bark, leaves, and roots compared to healthy and 44A; however their effect was not as pronounced as that of E9 and 13E. Sources 12E and 44A did not suppress sporangia production. RT-PCR analysis revealed that all source plants had mixed infections with several viroids, while 12E and 44A contained no viroids. In addition to confirming the earlier reports on the suppression of *Phytophthora* infection in general, our study showed a significantly reduced *Phytophthora* sporangia development due to a number of viroids in mixed infection, but there did not appear to be any effect related to viroid species. Extraction of phenolic acids with 80% ethanol was more efficient compared to 100% methanol and acetonitrile-water mixture. High Performance Liquid Chromatography revealed no notable detection of salicylic acid in healthy and viroid infected plants, but there was a small peak corresponding to salicylic acid in *Phytophthora*-infected and both viroid- and *Phytophthora*-infected plants. Flavone was detected in all the source plants with a slight increase in *Phytophthora*-infected and both viroid- and *Phytophthora*-infected plants. A peak corresponding to quercetin dehydrate was detected in *Phytophthora*-infected plants.

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Twelve Rootstocks Effects on the Intensity of Citrus Variegated Chlorosis in ‘Folha Murcha’ Sweet Orange in Bebedouro, SP, Brazil

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In a study conducted in a Folha Murcha sweet orange grove, located at Bebedouro, in the State of São Paulo, Brazil, twelve rootstocks have been evaluated on their influence on yield and CVC intensity. The experiment orchard was planted in 2001, at 7 x 4 m spacing, according to a randomized block design, with five replications and two trees per plot. The 12 rootstocks (treatments) were as follows: Carrizo citrange; the hybrids Rangpur lime x Swingle citrumelo and Changsha x English Small; the Sunki and Sun Shu Sha Kat mandarins; Limeira and FCAV Rangpur limes; Swingle citrumelo; Orlando tangelo and the trifoliates Rubidoux, FCAV and Flying Dragon. No supplementary irrigation or chemical control of vectors was applied. In 2006 and 2007 the CVC severity was evaluated by visual observation of the symptoms in the trees' canopy, using a 1-4 severity scale. Yield was evaluated by weighing all the fruits. Yield data collected in 2006 were plotted against the CVC scales. Although no significant differences were found among the treatments, an evident increase of CVC's severity was observed for all the rootstocks in time. Nevertheless, Flying Dragon, Changsha x English Small and FCAV trifoliolate showed the lowest increment on CVC's severity between both years. Highest yields were recorded on trees showing mild CVC symptoms, while a clear reduction on yield was observed on trees with stronger CVC symptoms. The evaluation of CVC intensity must continue and further research is necessary to clarify the rootstock effect on symptoms appearance and on yield.

Navelina Isa 315 Sweet Orange: A Citrus Variegated Chlorosis Resistant Cultivar

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Citrus variegated chlorosis (CVC), caused by *Xylella fastidiosa* subsp. *pauca*, is a serious disease in Brazil. The utilization of resistant cultivars is compulsory to allow a long-term coexistence with the disease. The Navelina ISA 315 cultivar - a clone recovered by *in vitro* culture of undeveloped ovules, was introduced from Italy for CVC resistance studies and showed to be carrier of cachexia during indexing procedures. It was established in two field blocks in 2000 (block 1) and 2001 (block 2) with a total of three and eight trees. One and four plants were inoculated by approach grafting of CVC infected nursery trees, respectively. Inoculation was performed in the field nine to eleven months after planting. Nursery trees of the cultivar were inoculated with bacterial suspension in 2006 and 2007. Twenty sweet orange trees showing severe symptoms of CVC were topworked with Navelina ISA 315 with a total of 248 buds grafted in 2007. Isolation of the bacteria infecting the field and topworked trees was performed. From 2000 through 2007, the CVC the presence of typical symptoms of the disease in the trees was evaluated visually twice a year. PCR tests using specific primers for *X. fastidiosa* were conducted for all trees in the two blocks and for the nursery and the topworked ones. None of the trees challenged showed symptoms despite the positive PCR results and bacteria recovering from the topworked trees. Studies on the possible influence of cachexia on CVC symptoms expression are in progress.

***Xylella fastidiosa* Multiplication in Pera Sweet Orange x Murcott Tangor Citrus Hybrids**

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Twenty hybrids of Pera sweet orange x Murcott tangor were infected with *Xylella fastidiosa* and the spectrum of tolerance was evaluated within of under greenhouse conditions. Efficiency of inoculation, multiplication of bacteria within the plants, and symptoms expression were the parameters analyzed. The rate of infection ranged from 40% to 100% (average of 70%) for all genotypes analyzed. *X. fastidiosa* populations ranged from log 0.59 to log 2.13 cell mg⁻¹ of tissue for the resistant hybrids. These values were different ($P = 0.05$) from those obtained for the tolerant or susceptible hybrids (from log 3.02 to log 4.06 cell mg⁻¹). With the exception of resistant hybrids, *X. fastidiosa* was recovered from all the other hybrids (from log 2.31 to 5.03 CFU mg⁻¹ of tissue). The first foliar symptoms appeared after 90 days post-inoculation but the time varied according to the genotype. The high broad-based heritability index for resistance obtained (0.96) at 210 days from *X. fastidiosa* inoculations, using bacterial quantification by real-time PCR, could indicated that the influence of the number of bacteria was due to plant genes response rather than environment variations.

Behavior of Six Sweet Orange Varieties Under High Inoculum Pressure of Citrus Variegated Chlorosis

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The use of resistant varieties is known as the most efficient measure of control to the citrus variegated chlorosis (CVC), caused by *Xylella fastidiosa* subsp. *pauca*, a serious disease to the Brazilian citriculture. The aim of the current work was to evaluate the disease evolution and severity on six sweet oranges varieties, selected by showing fewer leaf symptoms in the greenhouse. The experimental design was a randomized block, arranged in split-plots in time. Each block was composed by a rootstock and each experimental unit by eighteen trees. The trees were evaluated by visual assessments performed once a year using a 4-note scale. For each evaluation all plants were inspected. With the number of trees inspected and their grades the disease index was calculated for the two years. Results were submitted to analysis of variance using the Fischer's test and the means compared by the Tukey test ($P < 0.05$). The Folha Murcha sweet orange showed the minor disease index while São Miguel and Vaccaro Blood were the varieties that showed more leaf symptoms and the highest disease index.

Behavior of Five Valencia Sweet Orange Selections Under High Inoculum Pressure of Citrus Variegated Chlorosis

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Citrus Variegated Chlorosis (CVC), caused by the *Xylella fastidiosa* subsp. *pauca* is one of the most important diseases that affect the Brazilian citrus industry. Sweet oranges are the most susceptible to the bacteria. The aim of the current work was to evaluate the disease evolution on five selections of the Valencia and Natal and D.João sweet orange cultivars grafted on Swingle citrumelo and Sunki mandarin rootstocks in field conditions of high inoculum pressure, in Bebedouro, SP, Brazil. The experimental layout was a complete randomized block design, with four replicates and seven treatments with two trees per plot. The trees were evaluated by visual assessments performed once a year using a 4-rank scale. With the number of trees inspected and their grades the disease index was calculated for the two years. Results were submitted to analysis of variance using the Fischer's test and the means compared by the Tukey test ($P < 0.05$). The Valencia Late Burjasot IVIA 35-2 on Swingle citrumelo showed both the highest number of trees showing leaf symptoms and disease index. The Valencia variety, had not presented leaves symptoms. For trees on Sunki mandarin rootstock, the Valencia variety showed slightly more leaf symptoms and disease index compared to the other cultivars.

Multidrug Resistance in *Xylella fastidiosa* Biofilm

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Xylella fastidiosa (*Xf*) is the causal agent of citrus variegated chlorosis that affects sweet orange production in Brazil. The pathogenicity results from the occlusion of xylem vessels by biofilm formation. Cells within biofilms differ from planktonic cells, showing higher resistance to biocides, antibiotics and host defense responses. We evaluated the sensitivity of biofilm and planktonic *Xf* cells to three different antibiotics. A colorimetric assay based on the reduction of a tetrazolium salt (XTT) was used to measure biofilm cell viability in high antibiotic concentration. We evaluated alterations in the biofilm after antibiotics addition through Scanning Electron Microscopy (SEM). Quantitative RT-PCR was utilized to monitor the expression of genes encoding two different types of efflux pumps, belonging to the RND family and ABC transporters, in different antibiotics concentrations. Our results show that biofilms are less sensitive to all the tested antibiotics compared to planktonic cells. The level of expression of the genes was different according to the antibiotic and concentrations tested. The analyses of gene expression demonstrated that resistance in *Xf* biofilm might involve different molecular mechanisms of resistance. In high concentrations of antibiotic, the cells still showed metabolism response, small biofilm (SEM) and total RNA was obtained. These results suggest that the cells could be in a resistant physiological state or a bacterial programmed cell death is taking place. These mechanisms are important for a bacterium that is constantly under stress conditions in the host, which suggest that the control could be more difficult when biofilm is formed.

Diagnosis of *Xylella fastidiosa* of Citrus Variegated Chlorosis by Immunomolecular Techniques

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Xylella fastidiosa pv. *pauca* is the causal agent of Citrus Variegated Chlorosis (CVC) one of the most destructive disease of citrus in Brazil. The most efficient method of diagnosis of *X. fastidiosa* has been performed by PCR, which required many stages including DNA extraction from plant tissues or through petiole perfusion. These methods require considerable time and several steps which become the diagnosis expensive and increase the risk of contamination. Therefore, to reduce time and costs this work describes two advanced PCR-based diagnosis methods. The first was Immuno Capture associated with Polymerase Chain Reaction (IC-PCR). The *X. fastidiosa* cells were eluted from citrus tissues, immunocaptured by a polyclonal antiserum following the bacterial DNA extraction and subsequent pathogen detection by PCR. The second one was Immuno-PCR. The antigen was detected by a *X. fastidiosa* biotinylated antiserum that binds to a biotinylated-reporter DNA, that was amplified by PCR. The antiserum titer in ELISA was 1:10,000. The IC-PCR products were an amplified of 500 pb using CVC-1 and 272-int primers and Immuno-PCR amplifies a fragment of 1,100pb using a specific primers to *uspA1* gene. The sensitivity was higher in Immuno-PCR (1×10^2 bacteria) followed by IC-PCR (1×10^3 bacteria) and ELISA (1×10^6 bacteria). Thus, the IC-PCR was efficient to capture and detect *X. fastidiosa* being characterized as a quick and cheap assay, but requires an extraction process that maintains the integrity of bacteria wall. In the other hand, Immuno-PCR was more reliable and sensitive, although was more expensive and requires considerably time to be executed. Financial support: FAPESP, CNPq and CAPES

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Surveys for Citrus Huanglongbing and its Asian Citrus Psyllid Vector in Texas

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The Asian citrus psyllid, *Diaphorina citri*, one of the vectors of citrus huanglongbing (HLB) or greening, was first recorded in Texas in the Lower Rio Grande Valley (LRGV) in 2001. After the discovery of HLB in Florida in 2005, a USDA-APHIS-PPQ funded survey was conducted in 2006 to detect the presence of HLB and determine the distribution of the psyllid in all areas of Texas where citrus is grown, both commercially and as dooryard trees. Psyllids were found throughout south Texas, with a northern limit from Del Rio in the west, through San Antonio to the coastal area north of Corpus Christi. They were also found on dooryard citrus trees in Houston and surrounding areas. Psyllids were present on citrus trees in 33 of the 87 counties where citrus was located. No typical HLB symptoms were observed anywhere, but 309 leaf samples with various yellowing patterns were sent to the USDA-AMS laboratory in Gastonia NC for HLB analysis by PCR, with no positives being detected thus far. The surveys resumed in 2007, with particular attention being given to the commercial citrus of the LRGV, and the residential properties in the cities of Corpus Christi and Houston.

Distribution and Quantification of *Candidatus Liberibacter americanus* in Various Leaves from a Huanglongbing-affected Westin Sweet Orange Tree in São Paulo State, Brazil

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Huanglongbing-affected trees have frequently symptomless shoots and branches together with symptomatic ones. Symptomless leaves may occur next to leaves with severe blotchy mottle. Various growth flushes do not show similar leaf symptoms. In addition to mottle, leaves may show zinc deficiency symptoms. Are liberibacters present in both of these symptomatic and asymptomatic leaves? The distribution and quantification of *Candidatus Liberibacter americanus* has been studied by conventional 16S rDNA PCR, nested 16S rDNA PCR, and quantitative SYBR Green real time (RTi) PCR in 822 leaves samples collected on a four-year-old, HLB-affected Westin sweet orange tree in the Araraquara region of São Paulo state. The sensitivity of RTi-PCR was such that a positive reaction was still obtained with 10 liberibacters per gram of leaf midrib tissue (1/g). Nested PCR was almost as sensitive, while conventional PCR was 1000 times less sensitive. One of the five major branches of the tree was symptomless, and none of its 217 leaf samples gave a positive reaction with any of the three PCR techniques, suggesting that the branch was not yet infected or had a liberibacter titer too low to be detected by even the most sensitive PCR technique. All 111 leaf samples with blotchy mottle symptoms were PCR positive even with the least sensitive PCR, conventional 16S rDNA PCR. These leaves had the highest liberibacter titers, averaging 10⁷ 1/g, and even their lowest titer (4.6x10⁵ 1/g) was still 10 times higher than the limit of detection of conventional PCR. This explains why leaves with blotchy mottle are the most reliable tissues for HLB diagnosis. 61 of 77 leaf samples with zinc deficiency symptoms, and 81 of 634 samples without symptoms, gave also positive reactions with conventional PCR. Nested PCR detected in addition 4 samples with zinc deficiency, and 30 samples without symptoms; these samples had lower liberibacter titers (10³ to 10⁵ 1/g). RTi-PCR gave positive reactions with only relatively few additional samples, which had even lower liberibacter titers (10² to 10³).

Differential Responses to Temperature of Citrus Plants Affected by *Candidatus Liberibacter americanus* and *Ca. Liberibacter asiaticus*

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In Brazil, HLB is caused by *Ca. Liberibacter americanus* (*Lam*) and *Ca. Liberibacter asiaticus* (*Las*). Currently HLB is present in 1, 2 and 124 municipalities of Minas Gerais, Paraná and São Paulo (SP) states, respectively. In SP, affected municipalities are distributed mainly within the central and southern regions. The extreme northern and western regions appear to remain free of HLB. The number of annual total hours above 32C in the north and west areas is about five times higher than that of central and south, which could be a factor affecting HLB distribution. Aiming to determine if there is any influence of temperature on infection progress and HLB symptom development, three growth chamber experiments were conducted involving naturally inoculated 1.5-yr-old Hamlin field plants and graft-inoculated greenhouse plants of Hamlin, Natal, Pera and Valencia, all grafted on Rangpur lime. Healthy plants served as controls. All branches were cut at or close to the trunk and the plants were transferred to chambers with diurnal temperature regimens of 22C/8h, 24C/16h and 27C/8h, 32C/16h. Sixty days later all *Las* affected plants (50/50) showed mottled leaves and tested positive by PCR, regardless of temperature

regimen and cultivar, contrary to *Lam* affected plants, which showed mottled leaves and tested positive by PCR only on those maintained at 22-24C (25/25). Mottling of leaves on *Lam*-affected plants was more intense. All plants were then transferred to a HLB favorable greenhouse environment. Two years later, all field plants from the 27-32C chamber that were previously affected *Lam*, remained asymptomatic and tested negative by PCR. Further growth chamber experiments involving more realistic field diurnal temperature regimens are underway to determine temperature limits for *Las* and *Lam*. In addition, ongoing field surveys should yield more detailed information on geographical distribution of both liberibacters in São Paulo state.

Graft Transmission Efficiencies of *Candidatus Liberibacter americanus* and *Ca. Liberibacter asiaticus* to Citrus Plants

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In Brazil, HLB is caused by *Ca. Liberibacter asiaticus* (*Las*) and *Ca. Liberibacter americanus* (*Lam*). Since the first report of the disease in 2004, transmission experiments have been conducted aiming to assess the reaction of commercial sweet oranges to pathogen infection and to determine levels of aggressiveness between the two liberibacters. Budsticks obtained from symptomatic branches of field trees were grafted at the trunk of 1-yr-old potted Hamlin, Natal, Pera, and Valencia plants in the greenhouse. One year after inoculation, higher transmission rates were observed for *Las* in both experiments, as determined by PCR analysis of total DNA extracted from leaf midribs. Conversely, mottled leaves associated with stronger zinc deficiencies appeared on plants infected by *Lam*. *Las* transmitted to 73.3, 73.3, 70.0, and 66.6%, and *Lam* to 10.0, 16.6, 16.6, and 23.3% of Hamlin, Natal, Pera, and Valencia, respectively, confirming the high susceptibility of these cultivars to the disease. In the second experiment, 'Natal' plants were inoculated with *Las* or *Lam* alone, or simultaneously with both liberibacters. *Las* was detected in 53.9% and *Lam* in 40.3%, respectively, in plants inoculated with *Las* or *Lam*. In co-inoculated plants, 12.9% contained just *Lam*, 40.3% just *Las*, and 19.3% both liberibacters. The higher transmission rates observed for *Las* may be the result of a higher titer of this pathogen in affected trees.

***Murraya paniculata* as an Alternate Host of *Candidatus Liberibacter americanus* and *Ca. Liberibacter asiaticus* in Brazil**

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In Brazil, *Murraya paniculata*, a very common ornamental tree in urban areas, was found to naturally host *Ca. Liberibacter americanus* (*Lam*) and *Ca. Liberibacter asiaticus* (*Las*) (Lopes et al., Summa Phytopathologica 2005, 31:48-49, and Fitopatologia Brasileira 2006, 31:303). During a sampling survey conducted in the last 3 yr in the areas most affected by citrus HLB, a total of 443 adult *M. paniculata* trees were sampled. The incidence of affected *Murraya* trees was estimated to be 31.5, 24.1, 22.2, 16.6, 11.6, 11.1, 7.3, and 0% for Motuca, Bueno de Andrada, Silvânia, Américo Brasiliense, Araraquara, Santa Lúcia, Matão, and Rincão, respectively. Of 443 trees, only two were infected by *Las* (0.5%) and 55 (12.4%) by *Lam*. The *Las* infected trees showed yellow leaves only, and most of the trees infected by *Lam* showed shoot dieback, suggesting older infections. Spatial distribution of affected trees varied considerably with higher incidences in districts closer to citrus farms affected by the HLB. The potential of *Murraya* as source of liberibacter to infect citrus was investigated. *Murraya* and citrus trees affected by *Lam* or *Las*, with symptoms in one sector of the canopy, were divided in quadrants (*Murraya*) or main shoots (citrus), and each quadrant or shoot divided in two parts. In most cases, only symptomatic tissues tested positive by

PCR, an indication that pathogen distribution in affected Murraya and citrus follows the same pattern. In graft transmission experiments involving 20 to 30 inoculated plants per treatment (host/inoculum source combination), *Las* was transmitted from Murraya to Murraya in one of eight plants and from citrus to Murraya in one of two plants, and *Lam* was transmitted from Murraya to Murraya in five of 10 plants. The Murraya budstick used as inoculum died in most citrus grafted plants due, probably, to tissue incompatibility.

Survey for “*Candidatus*” *Liberibacter* species on Citrus in South Africa

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“*Candidatus*” *Liberibacter asiaticus* has recently spread to previously Huanglongbing-free Sao Paulo State, Brazil and Florida, USA. Along with this, a new “*Ca.*” *Liberibacter* species, namely “*Ca.*” *L. americanus* was detected in Brazil. This prompted the question whether “*Ca.*” *L. africanus* and “*Ca.*” *L. africanus* ssp. *capensis*, previously found in South Africa, remain the only *Liberibacter* species present here. Leaf samples were collected from 279 citrus trees in 57 groves distributed throughout the citrus production areas of South Africa. Samples displayed Greening/Huanglongbing or related symptoms. DNA extraction was performed on the samples using a standard CTAB extraction procedure. A multiplex PCR combining the previously published rplA2/rplJ5 and GB1/GB3 primer sets was optimized to simultaneously detect the three *Liberibacter* species. This was used to test all DNA extracts. None of the samples yielded amplicons of 1027bp size, indicative of “*Ca.*” *L. americanus* infection. Amplicons ranging from about 660bp to 710bp were obtained for 197 samples. As amplicon concentrations varied considerably, resolution of the expected band size of “*Ca.*” *L. africanus* (669bp) and “*Ca.*” *L. asiaticus* (703bp), was not reliable. Amplicons from 112 sources were subjected to direct sequencing to identify the *Liberibacter* species. Amplicons from these sources yielded sequences with identities ranging between 97.6 and 100% from the cognate “*Ca.*” *L. africanus* Nelspruit sequence in Genbank (U09675). No instances of “*Ca.*” *L. africanus* ssp. *capensis* were found in citrus.

The *rplKAJL-rpoBC* Operon of the *Liberibacter*s: Further Proof that *Candidatus Liberibacter americanus* is a Distinct Species

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The *rplKAJL-rpoBC* operon or β operon is a classic bacterial gene cluster, which codes for proteins K, A, J, and L of the large ribosomal subunit, as well as proteins B (β subunit), and C (β' subunit) of RNA polymerase. In the early 1990s, the operon was obtained as a 2.6 kbp DNA fragment (In-2.6) by random cloning of DNA from periwinkle plants infected with the Poona (India) strain of the huanglongbing agent, later named *Candidatus (Ca.) Liberibacter (L.) asiaticus*. DNA from periwinkle plants infected with the Nelspruit strain (South Africa) of *Ca. L. africanus* was amplified with a primer pair designed from In-2.6, and yielded, after cloning and sequencing, a 1.7 kbp DNA fragment (AS-1.7) of the β operon of *Ca. L. africanus*. In-2.6 and AS-1.7 have been used as specific hybridization probes for the detection of the Asian and African liberibacter strains, respectively. They have served to design PCR primers A2 and J5 for liberibacter detection by amplification of ribosomal protein genes. Finally, by

comparing the sequences of In-2.6 and AS-1.7, the Asian and the African liberibacters were confirmed as two distinct species. The β operon of the American liberibacter, as well as the three upstream genes (*tufB*, *secE*, *nusG*) have now also been obtained by the technique of chromosome walking, and extend over 4673bp, comprising the following genes: *tufB*, *secE*, *nusG*, *rplK*, *rplA*, *rplJ*, *rplL*, and *rpoB*. The sequence of the β operon was also determined for a Brazilian strain of *Ca. L. asiaticus*, from *nusG* to *rpoB* (3025bp), and was found to share 99% identity with the corresponding β operon sequences of an Indian and a Japanese strain. Finally, the β operon sequence of *Ca. L. africanus* was extended from 1673bp (*rplA* to *rpoB*) to 3013bp (*nusG* to *rpoB*), making it possible to compare the β operon sequences of the African, Asian and American liberibacters over a length of ~3000bp, from *nusG* to *rpoB*. While *Ca. L. africanus* and *Ca. L. asiaticus* shared 81.2% sequence identity, the percentage for *Ca. L. americanus* and *Ca. L. africanus* was only 72.2%, and for *Ca. L. americanus* and *Ca. L. asiaticus*, only 71.4%. The ~3000bp *nusG-rpoB* sequence was also used to construct a phylogeny tree, and this tree was found to be identical to the known 16SrDNA-based tree. These results confirm earlier findings that *Ca. L. americanus* is a distinct liberibacter species, more distantly related to *Ca. L. africanus* and *Ca. L. asiaticus*, than is *Ca. L. africanus* to *Ca. L. asiaticus*.

Current Status of Citrus Huanglongbing (HLB) in São Paulo State, Brazil, Based on Molecular and Visual Diagnosis

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HLB was first seen in Brazil in March of 2004, in Araraquara county, which is located in the central area of São Paulo State. Based on ribosomal DNA differences, two species of the causal agent (*Candidatus Liberibacter* sp.) were considered: the already known *Ca. L.* and the newly named *Ca. L. americanus* variants. Regular PCR techniques were developed for both species and confirmation in other counties were soon observed. By the end of 2004 the disease had already alarmed most of the industry, and the common sense was to try to mitigate HLB as soon as possible by mandatory removal of affected trees. In March of 2005 legal support was created and a 'task force' was assembled using three organizations: Fundecitrus (www.fundecitrus.com.br), the Agricultural Defense System, (www.cda.sp.gov.br) and the 'Centro APTA Citros Sylvio Moreira' (www.centrodecitricultura.br). The challenge was obviously enormous because the initial surveyed area had around 80 million trees. Detailed characterization of symptoms, development of quantitative real time PCR and studies assessing the distribution of the bacterium in affected trees were essential to allow a massive visual diagnostic for HLB in São Paulo. Up to July 31st of 2007, 401,334 trees were officially diagnosed positive for HLB in São Paulo, and the total number of tree losses reached around 2 million trees, including volunteered removal done by farmers. The disease is now present in 132 counties in São Paulo, in one county in Minas Gerais and in another county in Paraná State.

Initial Attempts to Obtain Huanglongbing Resistant or Tolerant Sweet Orange by Embryo Rescue from Healthy Chimeras of Diseased Fruit

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Huanglongbing remains a threat in the cooler citrus production areas of South Africa, despite restrictions on the movement of citrus material from infected areas, as well as cultural control measures, such as the use of systemic insecticides for vector control, planting of healthy certified trees and the eradication of infected material. The ultimate control strategy would be the use of resistant plant material. Attempts to obtain resistance with conventional breeding were unsuccessful. A new approach is to utilize embryo rescue of seed from healthy chimera sections of diseased fruit. The embryos were obtained from wide, asymptomatic sections of symptomatic fruit and cultured on Murashige and Tucker medium. Once the clones have developed to approximately 1 cm, they were micro-grafted to virus-free rootstocks. After further development to 10–15 mature buds, they were multiplied on virus-free rootstocks by budding. When the buds started to grow, they were challenged with “*Candidatus*” *Liberibacter africanus* by means of the insect vector *Trioza erytreae*. After a 7-day challenge on a young shoot, the psylla were collected from the plants and tested by PCR and dot blot hybridization, using the primer pair A2/J5, or probes from the amplicon, to determine if the insects used for challenges were infectious. Three months after the *Liberibacter* challenge, the same primer pair was used to evaluate the plants by PCR for the presence of the bacterium. Two clones remained negative after challenge, but the ultimate evaluation will be in the field where they are exposed to repeated natural infection by the insect vector.

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A Phytoplasma Closely Related to the Pigeon Pea Witches'-Broom Phytoplasma is Associated with Citrus Huanglongbing Symptoms in São Paulo State, Brazil

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In February 2007, sweet orange trees with characteristic symptoms of huanglongbing (HLB) were encountered in a region of São Paulo state (SPs) hitherto free of HLB. These trees tested negative for the three liberibacter species associated with HLB. A polymerase chain reaction (PCR) product from symptomatic fruit columella DNA amplifications with universal primers fd1/rP1 was cloned and sequenced. The corresponding agent was found to have highest 16S rDNA sequence identity (99%) with the pigeon pea witches'-broom phytoplasma of group 16Sr IX. Sequences of PCR products obtained with phytoplasma 16S rDNA primer pairs fU5/rU3, fU5/P7 confirm these results. With two primers D7f2/D7r2 designed based on the 16S rDNA sequence of the cloned DNA fragment, positive amplifications were obtained from more than one hundred samples including symptomatic fruits and blotchy mottle leaves. Samples positive for phytoplasmas were negative for liberibacters, except for four samples, which were positive for both the phytoplasma and ‘*Candidatus* *Liberibacter asiaticus*’. The phytoplasma was detected by electron microscopy in the sieve tubes of midribs from symptomatic leaves. These results show that a phytoplasma of group IX is associated with citrus HLB symptoms in northern, central, and southern SPs. This phytoplasma has very probably been transmitted to citrus from an external source of inoculum, but the putative insect vector is not yet known.

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Quantitative Detection of *Spiroplasma citri* by Real Time PCR

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There is a need to develop an accurate and rapid method to detect *Spiroplasma citri*, the causal agent of citrus stubborn disease for use in epidemiology studies. Quantitative real-time PCR was developed for detection of *S. citri*. Two sets of primers based on sequences from the P58 putative adhesin multigene of *S. citri* resulted in amplicons of estimated sizes of 86 (P 58 1f/2r) and 119 bp.(P 58 3f/4r) The DNA binding fluorophore SYBR Green I was used for the real-time assay. The assay detected *S. citri* from cells in culture and in DNA extracts from columella and leaf petioles from stubborn-infected trees from the greenhouse and field. The assay was estimated to be sensitive to a level of less than 10⁻⁶ ng of *S. citri* DNA using the broad spectrum primer P58-3f/4. Primer P58 1f/2r, however, reacted with a smaller population of *S. citri* strains. The assay successfully detected *S. citri* in two field plots in Kern County and identified two genetic populations in the field as well as from stubborn strains collected in the 1970's and maintained continuously *in planta*. Real time PCR should now provide a reliable new tool to assess stubborn disease incidence. Stubborn is a phloem limited prokaryote like Huanglongbing (HLB). The two diseases have similar symptomatology, however, HLB does not occur in California. Proactive surveys are underway in California for HLB. Therefore, the *S. citri* test can be used with the HLB test to sort out infections.

Transmission of Citrus Sudden Death Associated Symptoms, a Summary of Dates of Field and Greenhouse Assays

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Citrus Sudden Death (CSD) was first seen in 1999 in citrus plants from the Northwestern of São Paulo and Western of Minas Gerais, both Brazilian States. It is a graft transmissible disease that so affects mainly sweet orange grafted on both Rangpur lime and on Volkameriana lemon. The first symptoms associated to this disease are a yellowing stain of the inside part of the rootstock bark and a general pale-green aspect of canopy followed by the death of the tree. An overview of results obtained from a broad transmission experiment conducted in a high infected CSD farm located in Barretos county (northwestern of Sao Paulo State), on the field and under greenhouse conditions is shown here. In 2001, the sweet orange (Pera, Valencia, and Natal) and Ponkan mandarin canopies grafted on Rangpur lime, Volkameriana lemon, Cleopatra mandarin, Swingle citrumelo and *Poncirus trifoliata* rootstocks were inoculated by budwood from CSD disease plants (A) and from health nursery plants (B). A third treatment was composed by non-inoculated plants (C). In the field the first CSD symptoms started at 18 mo in sweet orange and Ponkan grafted on Rangpur lime. After 6 yr 28% of sweet orange plants grafted on Rangpur lime, 22% grafted on Volkameriana lemon, and 100% of Ponkan grafted on Rangpur lime showed CSD symptoms. Plants from treatments B and C also showed CSD symptoms, an undirected evidence of vectoring of causal agent. Valencia on rootstocks of trifoliata, Swingle and Cleopatra which were inarched with Rangpur lime had shown a reduced growth when compared with non-inarched ones. Rangpur lime grafted on canopy of Valencia on Cleopatra shown CSD symptoms too, reinforcing the Rangpur lime susceptibility. No symptoms were found in any plants growth under greenhouse conditions.

Cloning, Expression and Polyclonal Antiserum Production of Recombinant Capsid Protein of Citrus Sudden Death

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Citrus Sudden Death (CSD) is a new disease that has killed more than 1 million orange trees in Brazil. The causes and mechanisms of the disease are yet unknown, however, all infected plants are always associated with *Citrus tristeza virus* (CTV). The DNA of the CTV protein p23 and p25 was obtained from CSD trees by polymerase chain reaction (PCR) using random primers. These genes had been successfully cloned in the pGEM-T cloning vector and subcloned in the pSV282 expression vector to produce a heterolog protein fused to 6xHis-tag and maltose binding protein. The recombinant proteins were expressed in a soluble fraction in *E. coli* BL21 (DE3) cells using lactose as inductor and purified through affinity chromatography using an amylose resin. The p23 sequence had 209 amino acids (23,629.7 Da) and theoretical pI of 8.41, while the p25 had 223 aa (24,884.1 Da) and theoretical pI of 6.83. The p25 sequence differed by eight amino acids from the most common Brazilian strains, such as Pêra IAC and the 'Capão Bonito' complex, while p23 showed only two different amino acids when compared with the same strains. The specific polyclonal antibodies for these proteins have been developed in our laboratory and started to be used in a wide range of serological methods for CSD detection as ELISA and Western blot analysis. Therefore, in the near future it will be used for field screening of CSD.

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Sanitary Characterization of "Quebra-Galho" Acid Lime Tahiti and Selection of Candidate Mother Trees

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In order to sanitarily characterize the clone "Quebra-Galho" of the Tahiti acid lime and select candidate mother trees, 80 trees were evaluated concerning tristeza, exocortis and psorosis symptoms and biologically indexed for all the viruses and by RT-PCR for viroids. The nutritional status, tree size, production and fruit quality were evaluated as well. All the biological tests were positive for tristeza, but negative for cachexia. For exocortis, 82.5% of them were positive whereas for psorosis, 11.2% of the tests were positive although field trees did not present symptoms. Regarding tristeza, the reaction in Galego lime was classified as weak (58.8%), fair (40.0%) and strong (1.2%), without stem pitting in field trees branches. *Hop stunt viroid* (HSVd), *Citrus dwarfing viroid* (CVd-III) and the *Citrus exocortis viroid* (CEVd) were found, respectively in 31.3%, 82.5% and 100.0% of the plants. All trees were infected with tristeza and CEVd, which was found isolated or in combination with other viroids. Differences observed in the expression of tristeza and exocortis symptoms in field trees can be attributed to both interferences

between the viroids and the use of bud donor's trees infected by less aggressive strains for propagation. None of the trees, including candidate mother trees, selected in function of yield and fruit quality, showed constant association among their nutritional status, size, fruit production and quality and the type of contamination by viroids.

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Health Status Testing in the Auscitrus Budwood and Seed Scheme

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The Auscitrus budwood and seed scheme supplies high health status propagation material to the Australian citrus industry. The scheme operates in partnership with the New South Wales Department of Primary Industries (NSW DPI). More than 140 varieties of virus-free mother trees are maintained in insect proof screenhouses in a non-citrus growing area at the Elizabeth Macarthur Agricultural Institute (EMAI), Menangle, Sydney. A separate repository houses over 70 varieties that have been pre-immunized with a mild strain of *Citrus tristeza virus* (CTV). Budwood multiplication trees are propagated directly from mother trees in the EMAI repositories. There are over 1,900 budwood multiplication trees and 700 seed supply trees located at Dareton in the Sunraysia citrus growing area. The past 5 yr have seen average sales of over 700,000 buds per annum supplied to the Australian citrus industry by Auscitrus. Pathogen testing for the scheme is conducted in accredited nursery and laboratory facilities located at EMAI. Repository and budwood multiplication trees are tested every 3 yr for viroids by graft inoculation onto Etrog citron indicator plants. Further analysis by sequential PAGE and RT-PCR is conducted when required. Trees are tested every 6 yr for *Citrus psorosis virus* (CPsV) via biological indexing on Symons sweet orange. At the conclusion of CPsV testing, stems are peeled to observe signs of orange stem pitting strains of CTV. Rootstock seed supply trees are tested regularly for CPsV using direct tissue blot immunoassay (DTBIA). Grapefruit budwood multiplication trees in the scheme are pre-immunized with a mild strain of CTV. These trees are tested annually for the presence of the mild viral strain using West Indian lime indicator plants and RFLP analysis of the p23 gene.

Detection of Virus and Virus-like Disease on Citrus in the Turkish Republic of Northern Cyprus

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A survey program was conducted to determine the virus and virus-like disease in the citrus growing area in Morpho, Famagusta and Kyrenia in the Turkish Republic of Northern Cyprus. The Jaffa, valencia, Washington navel orange, Marsh seedless grapefruit, and Clementine mandarin species that are extensively produced in TRNC were included the survey program. In old plantation *Citrus psorosis virus* (CPsV), *Citrus tristeza virus* (CTV), Citrus stubborn disease, Cristacortis, Citrus vein enation, impietratura, Satsuma dwarf, gummy bark disease, *Citrus exocortis viroid* (CEVd), Citrus cachexia viroid (CCaVd) and Rurple were seen in Cyprus orchards. However in new plantations these citrus diseases were not seen. CPsV and Stubborn disease are very common in citrus orchards. In Morpho and Famagusta a range of 0.34% of orange trees infected with CTV and stem pitting strain was found in Cyprus.

Survey of Citrus Virus and Viroid Diseases in Hunan Province, China

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A survey has been undertaken in three different areas of Hunan province. Samples from six trees of a navel orange variety and three of a clementine variety introduced from abroad were collected near Changsha and in the County of Xin Ning. To identify specific viroids, RT-PCR analysis was carried out with specific primers for *Citrus exocortis viroid* (CEVd), *Hop stunt viroid* (HSVd) and *Citrus viroid III* (CVD-III), as well as for the cachexia variants of HSVd. All the samples were infected by HSVd, eight with CVD-III and six with CEVd. The cachexia variants of HSVd were detected in four out of nine samples. The mixed infections were as follows: one sample had CEVd and HSVd, eight had HSVd and CVD-III, and five all the three viroids. A survey has been carried out also in the area of Xupu in the south-western of the Province. Field observation revealed characteristic bumps of trunk and branches, associated to the introduced 'Albania' foreign blood orange variety, as well as some cases of root rot and decline. Typical scaly bark was discovered in some trees associated to reduction of canopy and fruiting. No symptoms of citrus tristeza or psorosis as well as huanglongbing diseases were observed. Samples from 13 trees were processed for the detection of CTV by monoclonal antisera, whereas for HSVd, CEVd and CVD-III by RT-PCR. All the tested trees were infected by CTV, eleven with HSVd, five with CEVd and nine with CVD-III. Five samples were simultaneously infected by the four pathogens.

Juvenility and Genetic Fidelity in Citrus Sanitized Plants Through Stigma/Style Somatic Embryogenesis

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Somatic embryogenesis proved to be an excellent method for the total recovery of most infected Citrus spp, except for Clementine. Genetic fidelity of sanitized plants could often be limited because of the morphological reversion to the juvenile state. Investigations on the morphological traits of fruits are too long and become rather costly when applied on new mutant somaclones, on regenerants and on micropropagated plants. The degree of juvenility occurring in plants sanitized from *in vitro* stigma/style culture of lemon, mandarin, sour orange and sweet orange was investigated. Comparisons were made between the somatic embryo-derived scion and the mature-phase scion, both grafted onto sour orange rootstock. Growth under screenhouse and in field conditions was also compared. Plants were examined during the first 3 yr of growth after grafting, for differences in stem and leaf growth and for the presence or absence of reproductive structures and thorns. Plants regenerated from stigma/style culture initially showed many features characteristic of seedlings. However, under screenhouse conditions, juvenile characters were lost during the second year after the culture initiation, even after their grafting onto rootstocks. After 3 yr, regenerated plants began fruiting on some branches with different grade according to the species: 50, 20 and 10 % with mandarin, sour orange and lemon, respectively. Flowering usually occurred 1-2 yr later in plants growing in the field. Flow cytometric analysis and two different DNA-based techniques (ISSR and RAPD) were used to detect the genetic fidelity in regenerated plants. Somaclonal variations in regenerants were never observed.

Improved Biological Indexing of the Main Citrus Viruses and Viroids

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Biological indexing is still a compulsory assay in the detection and characterization of the main citrus graft-transmissible pathogens. Space, time and skills for the production of indicator seedlings in the traditional biological indexing are limiting success factors. Rooted cuttings of Etrog citron, Volkameriana lemon, Mexican lime and Madame Vinous sweet orange were used as indicators in the biological indexing of citrus tristeza, psorosis, infectious variegation, exocortis and for viroid replication. After inoculation by chip budding with the specific pathogen source, cuttings were dipped in IBA for rooting and kept in Jiffy pots under plastic bags at cool (virus) and warm temperatures (viroids). The same pathogens were tested using the traditional biological indexing. Based on results on rooting capacity, Mexican lime and Madame Vinous sweet orange were used as grafted cuttings using Volkameriana lemon as rootstock. About 15-20 days after inoculation, clear-cut symptoms of the tested pathogens were observed on the new emerging leaves of the specific indicators using cuttings, whereas the same results were delayed where seedlings were used. Viroid replication was successfully achieved using Etrog citron cuttings. Results of biological indexing by cuttings were confirmed by using serological and molecular assays.