ABSTRACTS

Molecular Characterization of the Genome of a Grapefruit Stem Pitting Isolate of Citrus Tristeza Virus from Florida

K. L. Manjunath, R. Chandrika, V. J. Febres, R. F. Lee, and C. L. Niblett

ABSTRACT. Citrus tristeza virus (CTV) is widespread and a serious problem in Florida. Previous biological characterization indicates that most of the CTV isolates cause either mild or quick decline symptoms. Stem pitting (SP) isolates have rarely been found in Florida, and these have not spread widely into commercial citrus. The epidemiological situation was altered radically with the arrival of *Toxoptera citricida* in Florida, and SP isolates of CTV may now be spread and threaten commercial citrus. It has become necessary to characterize these isolates at the molecular level so that research can be underway to develop virus resistance in citrus for the SP isolates already present. An isolate from Meyer lemon causing severe SP in grapefruit was selected. Analysis of the consensus sequence of 10 different open reading frames showed closer similarity (about 90% at the nucleotide level) to the Florida quick decline isolate, T36, than to the sequences of other SP isolates. However, the sequence comparison of the coat protein (P25) and the P27 genes of several isolates indicated the relationship of these genes were closer to other SP isolates rather than T36. Interestingly, the base sequence in certain regions of the polyprotein was about 30% different from other known CTV sequences.

Comparison of Biologically Different Citrus Tristeza Virus (CTV) Isolates by Single Strand Conformation Polymorphism (SSCP) Analysis

A. Sambade, S. Gago-Zachert, L. Semorile, O. Grau, N. Costa, M. Peyrou, M. Francis, M. Machado, G. W. Müller, S. M. Garnsey, J. Guerri, and P. Moreno

ABSTRACT. Citrus tristeza virus (CTV) causes variable losses depending on the virus strains predominant in each citrus region. Control of the most severe isolates by quarantine, selective eradication, budwood certification or cross protection procedures requires quick and sensitive methods to detect specific strains responsible for the damage. A major limitation to the use of sensitive techniques such as PCR or hybridization is that CTV pathogenic determinants are presently unknown. In an attempt to identify genomic regions differing in mild and severe strains we compared 20 CTV isolates from different geographic origins and belonging to five different biogroups by single strand conformation polymorphism (SSCP) analysis of genes p13, p18, p20 and p23. Sets of primers based on conserved sequences were used to retrotranscribe and amplify (RT-PCR) the selected genome regions, and the DNA synthesized was denatured and separated in non-denaturing polyacrylamide gel. A variety of SSCP profiles were obtained from different isolates, but none of them could be specifically associated with any of the biogroups. However, two trends were observed: 1) For all genes studied, the mild isolates generally had an SSCP profile with a single sequence variant, whereas the severe isolates usually showed more complex patterns, and 2) In the more severe isolates, the SSCP pattern of genes p20 and p23 showed a major sequence variant, whereas SSCP of genes p13 and p18 showed variants with similar titer. These results suggest that CTV genes have different selective constraints, and that the severe phenotypes are associated with higher genetic heterogeneity.

A Classification of Citrus Tristeza Virus Isolates Based on the Sequence Polymorphism of the 5'—Terminal Region of the Viral RNA

C. López, M. A. Ayllón, J. Navas-Castillo, M. Machado, G. W. Müller, J. Guerri, P. Moreno and R. Flores

ABSTRACT. The wide variability observed in the biological properties of different citrus tristeza virus (CTV) isolates must be the consequence of a rich sequence polymorphism in the viral RNA which presumably propagates as a population of closely related sequences forming a quasi-species. This molecular variability is particularly noticeable in the 5'-terminal region in which less than 70% nucleotide identity was observed between the two reference sequences from T36 and VT isolates. To get a deeper insight on how general this situation is, we prepared and sequenced a series of cDNA clones of this region from several CTV isolates of different pathogenicity and geographical origin. The analysis of the variability observed in the 5' untranslated region (UTR) has led to a classification of the sequences into three groups with intergroup nucleotide identity ranging from 44 to 63%; the intragroup identity was higher than 88%. Some CTV isolates, particularly from Japan, contained sequences belonging to the three groups, whereas in Spanish isolates the predominant sequences belonged to the same group. The sequence polymorphism found in the region of the first ORF adjacent to the 5' UTR is also consistent with this grouping, although as it could be anticipated as a potential coding region, the intra- and intergroup sequence identities of the 5' UTR with two stem loops which is preserved in all cases. The variability is either accommodated in the loops or, when found in the stems, compensatory mutations maintain their general features and stability.

Characterization of Citrus Tristeza Closterovirus Isolates by RFLP of the Coat Protein Gene

V. G. R. Valle, M. A. Machado, G. W. Müller, M. L. P. N. Targon, J. Teófilo Sobrinho, and R. F. Lee

ABSTRACT. Restriction fragment length polymorphism (RFLP) of the coat protein (CP) gene of 32 isolates of citrus tristeza closterovirus (CTV) was used to compare their diversity, and to detect the occurrence of mixtures of isolates in several clones and cultivars of sweet orange, mandarin, lime and grapefruit. *Hinf*I provided better information about diversity and complexity of the isolates than *BamH*I, *BstE*II, *EcoR*I, *Fok*I, *Hae*III, *Mae*I, *Pvu*II and *Sae*3e. The RFLP analysis indicated a complex mixture of different isolates in the plants. At least 17 different combinations of isolates could be detected. Although an association between RFLP patterns and biological activities was not always obvious, many commercial clones of Pera sweet orange showed five to six isolates, whereas severe isolates such as the Capão Bonito complex were detected as mixtures of two or three isolates.

Progress on Citrus Tristeza Virus Strain Differentiation by Serology

O. V. Nikolaeva, A. V. Karasev, S. M. Garnsey, and R. F. Lee

ABSTRACT. Citrus tristeza closterovirus (CTV) has a filamentous, thread-like virion composed of a 25 kDa capsid protein (CP) and a single-stranded positive sense RNA of 19.3 kb. Fusion proteins containing different parts of the CTV CP were constructed and used to characterize the CTV antigenic structure. Thirty monoclonal antibodies (MAbs) to CTV were classified into four groups that react to linear, continuous epitopes, and one group (with 19 Mabs) that react to conformational epitopes. The trapping ability of antibodies reacting to linear epitopes of CTV CP, which is generally poor, was greatly increased by the addition of nominal amounts of Mabs reacting to conformational epitopes. CTV isolates from different countries having different biological activities

were analyzed by indirect DAS-ELISA using different combinations of polyclonal antibodies and Mabs as trapping and intermediate antibodies. All isolates tested could be detected by various trapping/detecting antibody combinations selected for universal detection. Two antibody combinations tested reacted selectively to isolates which produce some degree of stem pitting in Madam Vinous sweet orange seedlings. A combination of a polyclonal antibody raised against bacterially expressed MAb 3E10 as the intermediate detecting antibody and a combination utilizing two different polyclonal antibodies raised against bacterially expressed CP produced similar results. The selectivity of reaction was associated with the interaction between the antigen and specific trapping antibodies. These results demonstrate the potential for constructing antibody systems for selective detection of CTV isolates based at least in part on properties of the trapping antibody.

Diagnosis and Strain Typing of Isolates of Citrus Tristeza Virus by Immunocapture RT- PCR Coupled to a Fluorogenic Exonuclease Assay

G. Nolasco, Z. Sequeira, B. Cevik, R. F. Lee, V. Febres, and C. L. Niblett

ABSTRACT. We have developed a PCR-based test that may be used in large scale screenings and also provides information about the type of citrus tristeza virus (CTV) strains present in the isolates assayed. The test is based on Immunocapture/RT-PCR amplification of part of the coat protein (CP) coupled to a fluorogenic exonuclease assay. This kind of assay is very dependent on sequence specificity. To cover a broad spectrum of isolates, the designs of the fluorogenic probes were based on the sequences of the CP gene of more than 100 isolates from diverse origins. Two different probes labeled with the same fluorochrome were designed and combined in the same reaction, ensuring the detection of a broad spectrum of isolates. The test was validated with isolates from diverse origins and with different biological properties. Under standard conditions it was possible to detect one part of infected extract diluted in 999 parts of healthy extract, enabling the use of larger composite samples than with ELISA. As the biological properties of a large number of strains of CTV have been recently related to certain nucleotide substitutions of the CP gene, we also used this information to design additional fluorogenic probes that would be able to discriminate among the CTV strains. Four probes were designed to detect the different biological groups: severe stem pitting, severe quick decline, Mediterranean, and mild strains. These probes were labeled with different fluorochromes, and are intended to be used in a re-amplification step after the initial IC/RT-PCR. Following the re-amplification, the fluorescent spectrum is measured and the extent of hybridization of each probe computed, giving an insight into the strain composition of the isolate. This approach is being used routinely in the analysis of every new CTV infectious focus found in Portugal, as well as in cross protection studies.

Separation and Characterization of Strains of Citrus Tristeza Virus Useful in Mild Strain Cross Protection in South Africa

L. J. Marais, K. L. Manjunath, I. M. Rosales, G. A. Barthe, K. S. Derrick, C. L. Niblett, and R. F. Lee

ABSTRACT. Citrus tristeza virus (CTV) and its efficient aphid vector, *Toxoptera citricida*, are both endemic in South Africa. Severe strains of CTV significantly reduce citrus production. Marketable size grapefruit can be grown only with mild strain protection. In recent years, the mild sources of the Nartia isolate of CTV, widely used for cross protection, have been found to be contaminated with severe strains. Single aphid transmissions were made from Nartia and other potentially useful strains using *T. citricida*. After the establishment of these aphid transmitted sub-isolates in receptor plants, they were tested using probes capable of differentiating groups of strains based on their capsid protein (CP) gene sequence. Amplification and sequencing of the CPs of the original isolates and the aphid transmitted sub-isolates revealed that: 1) *T. citricida* is capable of separating strain mixtures in CTV isolates; 2) the CP gene sequences of some CTV strains from South Africa display a wide diversity; and 3) the CP gene sequences of CTV strains considered mild and useful for cross protection in South Africa differ from those used in other regions of the world.

Occurrence of Severe Stem Pitting Strains of Citrus Tristeza Virus on Madeira Island

G. Nolasco, B. Bonacalza, A. Fernandes, and Z. Sequeira

ABSTRACT. Madeira is a mountainous island lying in the North Atlantic Ocean 400 miles west of the Moroccan coast. Citrus is grown all around the island in small orchards near the farm houses, mostly for home consumption. Diverse rootstocks, including sour orange and various trifoliate hybrids are used. In 1994, the brown citrus aphid *Toxoptera citricida* was found in one location. Despite eradication efforts, it spread all around Madeira. The first focus of citrus tristeza virus was found in 1995. Subsequent annual surveys by ELISA have shown that the virus had an irregular pattern of spread and was still not being detected in most sampling sites. By the beginning of 1998, most of the trees in the initial focus had died. Biological characterization of the isolates on standard indicators in the greenhouse showed typical symptoms of severe strains; stem pitting on Madam Vinous sweet orange, vein corking on Mexican lime and quick decline of sweet on sour orange. Stem pitting symptoms could also be seen on tolerant combinations in the field in 1998. Sequencing of the coat protein gene of some isolates have shown the presence of components with a very close relationship with severe stem pitting strains from South America, such as B249, mixed with mild strains. This was confirmed using fluorogenic strain discriminating probes. The presence of such strains and the brown citrus aphid, the difficulty in achieving eradication and the large numbers of tourists visiting Madeira constitute a serious threat to Mediterranean citriculture.

RFLP and SSCP of Coat Protein Gene of Mild and Severe Citrus Tristeza Virus Isolates of Sweet Orange

A. A. Souza, G. W. Müller, M. L. P. N. Targon, P. Moreno, and M. A. Machado

ABSTRACT. Citrus tristeza virus (CTV) may occur as a large number of distinct strains, some of which show symptoms in the field according to the scion and rootstock variety combination. In Brazil, the Capão Bonito strains are considered most severe, affecting all varieties of sweet orange grafted on Rangpur lime. We have used restriction fragment length polymorphism (RFLP) and single-strand conformation polymorphism (SSCP) analysis of the coat protein (CP) gene to compare the occurrence of mild and severe CTV isolates in Pera sweet orange. The mild and severe isolates were collected from trees in several orchards in São Paulo State. The CP gene of the isolates was reverse transcribed and amplified by RT-PCR. All isolates produced an amplified product of approximately 670 bp, the known size of the CP gene. In both techniques, differences were observed in the CP gene between the mild and severe isolates. The number of strains of isolates ranged from two to five. However, no correlation between strain number and disease severity was observed. Several Capão Bonito isolates presented different patterns. The enzyme *RsaI* generated the highest polymorphism of the CP gene. The pattern of severe isolates differed from other severe isolates, but some RFLP and SSCP fragments were common among them. This suggest the possibility that some strains are the same in different isolates.

Towards Map-Based Cloning of the *Ctv* Resistance Gene from *Poncirus trifoliata*

T. E. Mirkov, Z. N. Yang, D. Fang, F. Moonan, and M. L. Roose

ABSTRACT. *Poncirus trifoliata*, a close relative of citrus, carries a single dominant allele, *Ctv,* that confers resistance to citrus tristeza virus (CTV). From interspecific crosses between *Citrus* spp. and *P. trifoliata,* 21 dominant RAPD markers linked to *Ctv* were identified. Ten of these markers were cloned and converted into co-dominant RFLP markers. The closest single copy RFLP marker co-segregated with *Ctv* in a population of 554 progeny, and is flanked by two single copy RFLP markers at distances of 0.5 and 0.8 cM, respectively. As these markers were present as

single copies in the *Poncirus* genome, they were used directly to screen a bacterial artificial chromosome (BAC) library and initiate a chromosome walk to *Ctv.* The library was constructed in pBeloBAc11 from an individual homozygous for *Ctv,* and over 100 000 clones were obtained with an average insert size of 110 kb. Forty five thousand clones have been stored in triplicate in 384 well microtiter plates, and 18 000 unique clones per filter been spotted in duplicate onto hybridization filters using advanced robotics. Screening two filters (36 000 clones) represents 7× coverage of the *Poncirus* genome and gives greater than a 99.5% probability of completing a 1 megabase walk. A series of BAC clones have now been identified by hybridization that span the co-segregating RFLP marker and the RFLP marker 0.8 cM from *Ctv.* We are continuing the chromosome walk toward the remaining flanking marker to obtain a contiguous sequence of BAC clones for this region. A cDNA library has also been constructed from the same individual. As BACs in the walk are fingerprinted and assembled into the contig, they are being used to screen this cDNA library.

Evaluation of Citrus Tristeza Virus in Grapefruit in Brazil Using Monoclonal Antibodies and SSCP

M. J. Corazza-Nunes, G. W. Müller, D. R. Stach-Machado, and M. A. Machado

ABSTRACT. Increasing grapefruit production in Brazil and its susceptibility to citrus tristeza virus has stimulated research on the use of mild strain protection. A 14-yr field experiment has shown the occurrence of several mild and severe isolates based on field symptoms, but not distinguishable using Galego lime biological indicators. Using monoclonal antibodies (MABs), some correlation was found between titer and symptom severity with CB30 and CB37 MABs, but not with MCA13 nor CB37 in DAS-ELISA. The absorbancy reaction (RAb405) was calculated between the universal MABs and the specific ones; low values of RAb405 as a consequence of high Ab405 values for specific MABs were found for the majority of plants with severe symptoms. Using specific MABs, it was shown that severe strains recognized by MCA13 and CB 39 are present in low titers in the complexes. High titers do not always correlate with symptom severity. SSCP analysis of the coat protein gene showed the occurrence of more than two bands in gel profiles, demonstrating the presence of a complex mixture. Correlation of band patterns and symptomology suggests that some isolates may be useful in mild strain protection programs for grapefruit.

Sequence of the Coat Protein Gene of the Severe Capão BonitoCitrus Tristeza Virus Complex

M. L. P. N. Targon, M. A. Machado, G. W. Müller, K. L. Manjunath, and R. F. Lee

ABSTRACT. The Capão Bonito region in the south of São Paulo State in Brazil is considered a high risk area for citriculture due to the presence of severe stem pitting isolates of citrus tristeza virus (CTV) affecting sweet orange grafted on several rootstocks, including Rangpur lime. A study was conducted to characterize this severe complex by sequencing the coat protein gene (CPG). DsRNA was purified from severe stem-pitted Pera sweet orange and cDNA was synthesized. The CPG was amplified by PCR using specific primers containing *Eco* R1 and *Bgl* II sites, producing a 669 bp fragment in all isolates used. Digestion with *Eco* R1 generated fragments of 669, 500 and 169 bp, indicating the presence of at least two different CTV strains, one with an *Eco* R1 site generating the 500 and 169 bp fragments (CTV-CB3-104), and the other without an *Eco* R1 site (CTV-CB3-22). The analysis of the nucleotide sequence showed 58 different base sequences (= 15 amino acids in the CP) between them, corresponding to a 91% homology. The CTV-CB3-22 strain is very similar to known mild isolates such as T30, whereas the CTV-CB3-104 strain is quite similar to severe stem pitting isolates. The CPG sequence analysis showed that the Capão Bonito isolate is a complex.

Genome Sequencing of the Pera-IAC Citrus Tristeza Virus Cross Protecting Complex

M. L. P. N. Targon, M. A. Machado, G. W. Müller, K. L. Manjunath, R. F. Lee, and C. L. Niblett

ABSTRACT. The genome size and complexity and the occurrence of strain mixtures are challenges to the characterization of the citrus tristeza virus (CTV) genome. Cross protection with mild isolates in some susceptible sweet orange varieties became a powerful tool to control severe isolates causing stem-pitting and stunting in both scions and rootstocks in Brazil. Sequencing of the p18, p25 and p27 genes has pointed out the high complexity and genetic diversity of CTV in Brazil. The protective Pera-IAC complex for sweet orange has been used for over 30 yr with suitable biological stability. This complex has at least two different strains, as evaluated by RFLP and SSCP of the coat protein gene. Complete genome sequencing of the mixture of strains should take into consideration the complexity. In order to overcome such complexity, sequencing has been carried out using both a cDNA library, and long PCR fragments. The cDNA library was constructed using primers derived from the known sequences of T-36, SY568 and VT. To amplify larger fragments, eLONGase (Gibco-BRL) was used. The larger fragments and the overlaps should decrease the possibility of sequencing mosaics. To date, the full CTV genome has been amplified in fragments ranging from 2.0 to 5.6 Kb, and the sequencing is now in progress.

Nucleotide Sequences of p18 and p27 genes from Different Citrus Tristeza Virus Isolates

M. L. P. N. Targon, M. A. Machado, K. L. Manjunath, and R. F. Lee

ABSTRACT. Citrus tristeza virus (CTV) exists as a number of biologically different isolates. Methods used to differentiate these isolates are based mainly on the nucleotide sequence of the coat protein gene, since a close association between biological activity and nucleotide sequence has been observed. We were interested to see if other genes, specifically p18 and p27, displayed a similar association. Double-stranded RNA from 12 different Brazilian isolates was isolated and used in RT-PCR with specific primers to amplify fragments of 500 bp and 700 bp. After cloning into the pGEM-T vector (Promega) and selection by PCR, DNA from positive clones was extracted and sequenced. Large sequence variations between isolates were found, but in a dendrogram generated from the multiple alignment of amino acid sequences of both p18 and p27 of biologically distinct isolates, there was no grouping of isolates of similar biological activity.

Monoclonal Antibodies to Recombinant Coat Proteins of Severe Brazilian Isolates of Citrus Tristeza Virus

D. R. Stach-Machado, M. L. P. N. Targon, G. Arruda, R. A. Barbosa, M. J. Barreto, G. A. Wagner, and M. A. Machado

ABSTRACT. Screening of severe isolates of citrus tristeza virus (CTV) in Brazil has been conducted using RT-PCR, RFLP, SSCP of the coat protein gene, and sequencing part of the genome. However, such methodologies are time consuming and expensive for large-scale use. Severe isolates causing stem pitting and stunting on sweet orange and Rangpur lime are considered the more severe, and are known as the Capão Bonito complex, in which at least two strains (CB22 and CB104) can be detected. To improve the diagnostic capacity of such CTV severe isolates, monoclonal antibodies using recombinant coat proteins of CB22 and CB104 were developed. The proteins were expressed in *Escherichia coli* using the pET22 vector, and used as antigens in Balb/c mice. Although the proteins show a homology of 93% in amino acid sequence, it was possible to obtain three groups of specific monoclonal antibodies, demonstrating the existence of distinct epitopes. The monoclonal antibodies have high specificity for both the cloned coat protein and the virus in tissue, and does not cross react with plant proteins. The three groups include monoclonal antibodies for CB104, CB104 + CB22, and CB22. The monoclonal antibody for CB104 seems to recognize the more severe stem pitting isolate of the complex.

Improved Conditions for Rapid Detection or Typing of Citrus Tristeza Virus and Citrus Psorosis Virus by One-Step PCR

A. Sambade, A. Olmos, M. L. García, G. Legarreta, O. Grau, M. Cambra, J. Guerri, and P. Moreno

ABSTRACT. One-step reverse transcription polymerase chain reaction (RT-PCR) is being increasingly used for detection or typing of RNA viruses. Commercial metal block thermal cyclers (MBTC) are considered to provide higher yield of DNA, whereas air thermal cyclers (ATC) allow amplification in a much shorter time. We have optimized a rapid RT-PCR protocol for detection of citrus tristeza virus (CTV) and citrus psorosis virus (CpsV) with the ATC A20 (Idaho Technologies, ID, USA), and have compared its sensitivity with that obtained using the gene Amp 9600 and 2400 MBTC (Perkin-Elmer, CA,USA). The protocol for ATC A20 included 30 min RT at 46 C and 2 s denaturation (94 C), 2 s annealing (56 C for CTV and 45 C for CpsV) and 20 s DNA synthesis (72 C). The protocol for MBTC had identical RT conditions and 15, 30 and 60 s (for CTV) or 40 s (for CpsV), at the same temperatures, for PCR. RT-PCR done on one or in two steps (cDNA and then PCR) gave similar detection sensitivity, but the first procedure was faster than the second. Using a standard protocol of 40 cycles, the MBTC provided at least 100 times the sensitivity of the ATC. However, reduced denaturation time in the ATC (2 s) delayed inactivation of the Taq polymerase and allowed the use of 60 cycles with increasing yields of a clean PCR product. Under these conditions, CTV and CpsV could be detected in the ATC in 1 hr with a sensitivity similar to that obtained with the MBTC in 3 hr. The use of over 40 cycles in the MBTC resulted in the appearance of a smear of multimeric products. Our results show that ATC can be used for fast experiments in molecular studies with an efficiency similar to that of MBTC.

Evaluation of Natural Transmission and Spatial Distribution of Bahia Bark Scaling in Grapefruit Exposed to Natural Field Infection

C. J. Barbosa, H. P. Santos Filho, P. R. H. Valverde, N. F. Sanches, and O. Nickel

ABSTRACT. The etiology of Bahia bark scaling (BBS) of citrus remains unknown. This disease affects sweet orange and grapefruit scions, and due to its occurrence in nucellar clones shoot tip grafted plants, it appears to be vector-borne. This study was done to determine the incidence and spatial distribution of BBS in the field. A trial was established in 1994 using 360 Marsh grapefruit plants exposed to natural infection and 30 control plants protected in insect-proof cages. The trial was conducted near to and downwind of 30 infected grapefruit trees. Inspections for symptoms were done every 3 mo. In 1998, 24.5% of the exposed plants had developed symptoms, while none of the protected plants had any symptoms. Most of the symptomatic plants were in rows closest to the inoculum source. The results indicate that an aerial vector is involved in the transmission of BBS.

Reaction of Soybean Cultivars to Inoculation with Citrus Bahia Bark Scaling

C. J. Barbosa, P. R. H. Valverde, H. P. Santos Filho, A. D. Vilarinhos, and O. Nickel

ABSTRACT. The etiology of Bahia bark scaling is unknown, and no indicator plants have thus far been found. Therefore, the objective of this study was to attempt to identify biological indicators for this disease. Purified leaf extracts from affected plants were mechanically inoculated to *Datura metel*, *Chenopodium quinoa*, *C. amaranticolor*, *Phaseolus* cvs. 'IAPAR-57', 'Mateiguinha' and 'Rio', *Crotolaria spectabilis*, *C. ochloleura*, *C. anagirtoides*, *Glycine max* (soybean) cv 'Hardee', 'Braag' and 'FTT', *Nicotiana benthamiana*, *N. tabacum* cv 'TNN', *Passiflora edulis* and *Nicandra*

physaloides. Control plants were inoculated with buffer. Aphid transmission to soybean was also attempted. Aphids (*Toxoptera citricida*) were collected off infected citrus and immediately transferred to plants of soybean cvs 'Hardee' and 'Braag' and left there for 48 h. For controls aphids from healthy citrus were used. Interveinal chlorosis was evident in these two soybean cvs in both the mechanical and aphid transmission experiments. In the former they appeared 45 d after inoculation, while with the latter they took only 15 d to appear. The leaves later became necrotic and dropped off. Symptomatic leaves were tested for citrus tristeza virus by ELISA, but were negative. None of the other test plants developed any symptoms.

Incidence and Severity of Bahia Bark Scaling in a Commercial Orchard of Sweet Orange

C. J. Barbosa, P. R. H. Valverde, H. P. Santos Filho, R. S. Almeida, and O. Nickel

ABSTRACT. Although the etiology of Bahia bark scaling, which affects sweet orange and grapefruit, remains unknown, there is evidence that it is spread by aerial vectors. The present study was conducted to evaluate the incidence and severity of the disease in a grove of 4421 18-yr old sweet orange trees of various varieties (1039 Pera, 854 Natal, 300 Valencia and 2228 Bahianinha). The incidence was evaluated on the basis of visual appearance of symptoms on an increasing scale of 1 to 3. All varieties except Valencia had an incidence of over 80%, with Bahianinha having the highest severity rating (19.75% of the trees scored 2-3).

Detection of Viruses Associated with Citrus Psorosis by RT-PCR

G. A. Barthe, T. L. Ceccardi, and K. S. Derrick

ABSTRACT. The viruses found in citrus associated with citrus psorosis are serologically and biologically diverse. In addition their uneven distribution and often low concentration in citrus makes their detection difficult. We have had little success in detecting them in grove trees using serology, but assays based on RT-PCR were found to detect most Florida isolates. Currently we are using primers designed from the sequence of the coat protein gene of a Florida ringspot isolate (CRSV-4) to amplify a 600 bp product. In an effort to build some confidence in the PCR assays, grove samples are biologically indexed by side grafting into sweet orange or grapefruit seedlings to confirm the PCR results. We are building a database of coat protein sequences of various isolates that will be used to design consensus primers to expand the range of detection. The isolates that have been sequenced vary from 71 to 96% (nucleic acid) and 97 to 99% (amino acid) in comparison to CRSV-4.

Citrus Varieties Affected by Leprosis Virus Disease

A. A. Salibe

ABSTRACT. Leprosis is a destructive disease of citrus caused by a virus tentatively assigned to the Rhabdoviridae, and which is transmitted by the mite *Brevipalpus phoenicis*. It affects leaves, fruits and branches, causing extensive losses to citrus growers. Protective miticide applications in São Paulo State, Brazil are estimated to amount to US $5-10 million annually. Field and laboratory studies have shown the sweet oranges are most susceptible, while most mandarins, grapefruits, shaddocks and citrons are tolerant. While these latter types do not exhibit symptoms, they can act as virus repositories for mite contamination. Within the sweet oranges, the more affected varieties are Hamlin, Salustiana, Pera, Barão, Piralima, Cipó and Seleta de Itaborai. Less susceptible varieties are Coroa do Rei, Moro, Mangaratiba, Jaboticaba and Cacau. The degree of susceptibility varies considerably within varieties, as shown by the number of lesions on leaves, fruit and branches. Inheritance studies in crosses of oranges and mandarins showed that among 12 tangors, only three were susceptible (Reticulata, São João del Rei and Tenagerona) while nine were tolerant (Murcott, Sangue de Boi, Brinco, São Oedro, Céu, Sabará, Maracujá, Umatilla and Temple).

Symptoms of leprosis were also detected in leaves of Troyer citrange and some lemon hybrids (Brazilian rough, Camargo, Gigante and Rio Clara). Rarely, some leprosis-like symptoms were seen in leaves of Ponkan and Cravo mandarins. Severe leprosis was however found outside São Paulo in Cravo mandarin and many sweet orange varieties in the warmer northeast citrus area of Brazil.

Partial Characterization of an Isolate of Citrus Leprosis Virus and Occurrence of a Mite Vector Predator in Rio de Janeiro State

P. S. T. Brioso, J. O. Cunha Júnior, M. de Bonis, H. G. Montano, J. P. Pimental, and J. I. H. Faccini

ABSTRACT. Leprosis is currently one of the major citrus virus diseases in Rio de Janeiro State. The virus is transmitted by the mite *Brevipalpus phoenicis*. The Universidade Federal Rural do Rio de Janeiro has developed the Leprosis Etiology, Epidemiology and Control Program to characterize citrus leprosis virus (CLiV) isolates, observe vector population dynamics and identify vector predators. Buffered extracts of symptomatic Seleta sweet orange leaves were inoculated onto *Chenopodium amaranticolor* plants. Electron microscopy of ultrathin sections of leaf lesions revealed the presence of rhabdovirus-like virus particles in the cytoplasm. Mite populations were monitored from April 1997 to April 1998; higher populations were recorded during drier periods. For the first time, a predator mite, *Iphiseiodes zuluagai* was recorded.

The Nucleotide Sequence of the Coat Protein Genes of Satsuma Dwarf Virus and Related Viruses

T. Iwanami and Y. Kondo

ABSTRACT. The 3'-terminal regions of RNA2 of satsuma dwarf virus (SDV), citrus mosaic virus (CiMV), natsudaidai dwarf virus (NDV), navel infectious mottling virus (NIMV) and two unidentified viruses Az-1 and LB-1 were sequenced. All sequences contained part of a long open reading frame which encodes larger and smaller coat proteins (Cps) followed by a 3' non-coding region and a poly (A) tail. Computer-assisted phylogenetic analysis of the deduced amino acid sequences of the CPs and nucleotide sequences of the 3' non-coding region indicated that these viruses are clustered into three distinctive groups: SDV, CiMV (CiMV, NDV, Az-1, LB-1), and NIMV. The viruses in the CiMV group consisted of two sub-clusters, CiMV (CiMV and LB-1) and NDV (NDV and Az-1). This classification is in accordance with serological relationships. Amino acid sequences identities were 81-84% among viruses of different groups and 90-98% among viruses in the CiMV group. The results suggest that SDV, CiMV and NIMV are distinct viruses, while NDV, Az-1 and LB-1 are strains of CiMV.

A Physical and Genomic Map of *Xylella fastidiosa*

J. Machado, P. Carle, L. Zreik, A. J. G. Simpson, M. Garnier, J. M. Bové, and F. Laigret

ABSTRACT. Citrus variegated chlorosis is caused by a strain of the xylem-limited bacterium, *Xylella fastidiosa*. Koch's postulates were fulfilled using isolate 8.1.b, and this isolate has been cloned. The derived 9a5c clone was chosen for the FAPESP-sponsored *Xylella fastidiosa* genome sequencing project. Genomic DNA was released from agarose embedded cells and digested with the following rarely cutting restriction enzymes: *Asc*I (5'-GGCGCGCC-3'), *Not*I (5'-GCGGCCGC-3'), *Sfi*I (5'-GGCCNNNNNGGCC-3'), *Smi*I (5'-ATTTAAAT-3'). These enzymes yielded 10 to 15 restriction fragments, with sizes ranging from 10 to 400 kbp, and they were separated by pulsed field gel electrophoresis. These fragments were transferred onto nylon membranes and hybridized with DNA probes in order to determine overlaps between fragments. These probes were made of highly conserved sequences such as rDNA coding for ribosomal RNAs or from randomly cloned *X. fastidiosa* genomic DNA, some of which were identified after sequencing. For example, one insert

was found to code for a protein highly similar to Rp1F, a protein involved in *Xanthomonas campestris* pathogenicity, and another was found to code for MP synthase. Because *X. fastidiosa* harbors plasmids, it was necessary to perform hybridizations with plasmid derived probes in order to distinguish between genomic and plasmid DNA fragments. From the physical and genomic map established in this way, several restriction fragments are selected to prepare ordered genomic libraries for the *X. fastidiosa* genome sequencing project.

Transmission Efficiency of *Xylella fastidiosa* to Citrus by Sharpshooters and Identification of Two New Vector Species

R. Krügner, M. T. V. de C. Lopes, J. S. Santos, M. J. G. Beretta, and J. R. S. Lopes

ABSTRACT. This study evaluated the transmission efficiency of *Xylella fastidiosa,* the causal organism of citrus variegated chlorosis (CVC) to citrus by five species of sharpshooter leafhoppers (Hemiptera: Cicadellidae, Cicadellinae) commonly found in citrus groves, *Acrogonia* sp., *Bucephalogonia xanthopis*, *Dilobopterus costalimai*, *Oncometopia facialis* and *Plesiommata corniculata.* Groups of field-collected adults of each species were allowed a 48-hr acquisition access period (AAP) on infected citrus and transferred (five per plant) to healthy sweet orange seedlings for a 48-hr inoculation access period. At least 150 individuals were tested for each species, divided into 3-4 replications. Approximately 10 test plants were inoculated in each replication. Before the AAP, the field collected sharpshooters were confined for 48 hr on test plants (10 per plant) to determine whether they were already infective. Inoculated plants were rated monthly for CVC symptoms and tested for *X. fastidiosa* infection by DIBA and PCR 6 mo after inoculation. Transmission rates for single insects were 1.3, 2.3, 2.9, 5.5 and 11.7% respectively for *O. facialis*, *Acrogonia* sp., *P. corniculata, D. costalimei* and *B. xanthopis*. Pre-infective individuals were detected in field-collected samples of all species except *B. xanthopis*. The results indicate that overall transmission efficiency to citrus is low and that members of the tribe Cicadellini are more efficient vectors than the Proconiini species (*Acrogonia* sp. and *O. facialis*). This is the first report of *B. xanthopis* and *P. corniculata* as vectors of CVC.

Citrus Variegated Chlorosis: Resistance Evaluation of Natal Sweet Orange Clones

W. Li, L. C. Donadio, and O. R. Sempionato

ABSTRACT. To evaluate the resistance to citrus variegated chlorosis (CVC), 13 clones of Natal sweet orange were grafted onto Rangpur lime and inoculated by grafting CVC-infected Pera sweet orange twigs. The trials was laid out in a randomized block design, with eight plants per clone. Although all inoculated plants began to develop CVC symptoms and gave positive ELISA reactions 3 mo after inoculation, there were some differences in disease severity. 'Felicio Sasso 1969' was the most susceptible clone. 'Milton Teixeira', '7 Lagos', 'Natal PI' and 'Pedro Sanches 1944' showed very severe symptoms. 'Natal Murcha', 'Nair Sasso Hernandes 1944' and 'Ivan Aidar 1954' all had medium symptom severity. The severity on 'Fazenda União 35 anos', 'Natal 2', 'Antonio Sasso 1961' and 'Fazenda Vila São João' was low, whilst 'Natal 1' was the clone with the lowest severity. Although none of the clones was completely resistant to CVC, the fact that some clones developed markedly less severe symptoms indicates that they may be productive in CVC-endemic situations.

Susceptibility of Different Citrus Species and Related Plants to Citrus Variegated Chlorosis in the Field

F. F. Laranjeira, J. Pompeu Junior, R. Harakava, J. O. Figueiredo, S. A. Carvalho, and H. D. Coletta Filho

ABSTRACT. Plants of *Citrus, Poncirus* and *Fortunella* genera were evaluated in the field in five areas of São Paulo State, Brazil, for their susceptibility to *Xylella fastidiosa*, the causal organism of citrus variegated chlorosis (CVC). The plants were examined visually for foliar and fruit

symptoms, tested serologically by DIBA and immunoblotting and by PCR. All sweet orange varieties evaluated under high inoculum pressure showed CVC symptoms. The only other plants with symptoms were some mandarins, tangors, tangelos and one sour orange. Pathogen detection was negative for *Poncirus trifoliata*, *Fortunella margarita*, *Citrus yuzu*, *C. limettoides*, *C. latifolia* and grapefruit. Eureka, Femminello and Monachello lemons were all negative even under high inoculum pressure, but Camargo, Sanguino 2 and Amber lemons were positive. Comprida citron and Periforme pummelo were found to be symptomless hosts of *X. fastidiosa*.

Modeling of Citrus Variegated Chlorosis-Induced Yield Damage in Fifteen Sweet Orange Varieties

F. F. Laranjeira and J. Pompeu Junior

ABSTRACT. Citrus variegated chlorosis (CVC) symptoms and associated yield damage were quantified in plants of 15 sweet orange varieties (Westin, Lue Gim Gong, Pera, Valencia, Bahianinha, Gardner, Sunstar, Barão, Ovale, Folha Murcha, Rubi, Berna, Pineapple and two Cardenera selections) established in a high inoculum pressure area of São Paulo State in Brazil. No correlation between symptoms and yield damage was found. In Rubi, almost no foliar symptoms were found, but yield damage was always over 90%. The least affected in terms of yield were Westin and Lue Gim Gong. Selection of productive selections on the basis of foliar symptoms is therefore not reliable.

The Use of 2DCORR Software for Spatial Distribution Analysis of Citrus Variegated Chlorosis

W. M. C. Nunes, M. A. Machado, F. Laranjeira, and E. L. Furtado

ABSTRACT. The orientation and distance between infected plants can be one of the more important considerations for constructing epidemiological models. The approach of the 2DCORR software is concentrated on the aspect of spatial distribution. The method produces, independently, an estimate for extension of the scale on which the disease is correlated and the probability of deviation of an aleatory space distribution. The incidence of citrus variegated chlorosis, based on disease symptoms, was evaluated in orchards of sweet orange in Bebedoura and Colina, São Paulo State. Six evaluations were conducted and the data submitted to 2DCORR software. There was a better understanding of the epidemiological parameters using the 2DCORR approach than the 2DCLASS.

Genetic Diversity of Brazilian Strains of *Xylella fastidiosa* Infecting Citrus and Coffee

R. P. Leite Jr., A. Mehta, F. M. S. Carvalho, and B. Ueno

ABSTRACT. In Brazil *Xylella fastidiosa* causes citrus variegated chlorosis (CVC) in citrus in southern Brazil and a dieback disease of coffee in all major growing areas. We conducted studies on the genetic diversity of *X. fastidiosa* associated with these two diseases. Fifteen strains from diseased citrus and coffee were used. The ATCC type strain (35879) was also included in the study. The strains were characterized on the basis of genomic fingerprints generated by using the rare-cutting restriction enzymes *Not*I and *Sif*I. The restriction fragments were separated by pulse-field gel electrophoresis. Restriction fragment patterns were determined by direct comparison and the data was used to determine the genetic relationships between the strains based on the proportion of shared DNA fragments. Strains of *X. fastidiosa* associated with CVC exhibited a diverse restriction pattern. The genetic similarity ($F = 2n_{xy}/(n_x + n_y)$) ranged from 0.77 to 1.00 among citrus strains when the *Not*I and *Sif*I values were averaged. They were 069 to 0.84 similar

to the coffee isolates. In relation to the type strain, the citrus strains were 0.31-0.45 and the coffee 0.50 similar respectively. On the basis of this genetic analysis, the citrus and coffee strains appear to be closely related.

Plasmid DNA Profile of Brazilian Strains of *Xylella fastidiosa* Associated with Citrus Variegated Chlorosis

A. Mehta, B. Ueno, and R. P. Leite Jr.

ABSTRACT. The objective of this study was to determine the plasmid profile of *Xylella fastidiosa*, the bacterium which causes citrus variegated chlorosis. Twenty-one strains of *X. fastidiosa* from different areas were grown on BCYE medium and plasmid DNA extracted using Kado and Liu's salt method. Plasmid DNA was analyzed by agarose gel electrophoresis and visualized in UV light after ethidium bromide staining. *Erwinia stewartii* isolate SW2 was included to provide a size marker. The number and size of plasmids was diverse. Plasmids were detected in 18 of the 21 strains analyzed. The number in each strain ranged from one to three. Based on electrophoretic mobility, the sizes ranged from 5.3 to 263.3 kb. Five different plasmid profiles were determined for the *X. fastidiosa* strains, with a maximum of three plasmid classes in two profiles. A plasmid of 38 kb was present in 17 different strains in four different plasmid profiles. The diverse plasmid content illustrates frequent mobility of plasmids between strains.

Fast Evaluation for Resistance to Citrus Variegated Chlorosis in Citrus Varieties

C. He, E. M. G. Lemos, and W. Li

ABSTRACT. Seedlings of Pera, Natal, Valencia and Caipira sweet orange and grafted plants of these varieties on Rangpur lime were graft-inoculated with *Xylella fastidiosa*-infected Pera sweet orange twigs in the greenhouse. DAS-ELISA and PCR were used to detect the presence of the bacterium in the leaves, stems and roots of the inoculated plants. All the inoculated seedlings of all four varieties gave positive ELISA and PCR results 2 mo after inoculation, a month before symptoms appeared. Detection in the grafted plants was positive 3 mo after inoculation, again before any symptom development. Detection in root tissues took much longer; 1 yr in the seedlings and 1.5 yr in the grafted plants. Caipira and Pera developed more severe leaf symptoms than Natal or Valencia. The use ELISA and PCR on inoculated seedlings gives quicker results for testing varietal resistance.

Citrus Variegated Chlorosis: Field Selection and Evaluation of Sweet Orange Varieties for Tolerance

W. Li, A. Coutinho, L. C. Donadio, and O. R. Sempionato

ABSTRACT. The use of resistant or tolerant varieties may be one way to reduce losses cause by citrus variegated chlorosis (CVC). In 1995 and 1996, we selected 65 sweet orange trees in heavily infected groves in São Paulo and Minas Gerias states, Brazil; these 'escape' trees had no obvious CVC symptoms whereas most of the surrounding trees were diseased. Of the 65 trees, 32 were Pera, 26 Natal, six Valencia and one Hamlin. Buds from them were grafted onto Rangpur lime seedlings in October 1996, and planted out in randomized block design with 10 plants per selection at the Bebedouro experiment station in April 1997. Five trees of each variety were in a vector-transmission evaluation trial where one CVC-infected tree was planted between each pair of test trees as an inoculum source. The other five trees of each selection were planted in a separate trial where four of each were graft-inoculated with a CVC-infected Pera twig, the fifth being an uninoculated control. Some graft-inoculated trees began to develop symptoms 3 mo after inoculation, and by April 1998, 25 of the selections had conspicuous symptoms. The remainder were still symptomless. The controls were also symptomless, as were all the trees exposed to natural vector transmission. The trees will continue to be evaluated for possible tolerance.

Victoria Tangor—A Natural Hybrid Tolerant to Citrus Variegated Chlorosis

W. Li, V. Rossetti, and N. de Oliveira Machado

ABSTRACT. Citrus variegated chlorosis (CVC) affects almost all commercial sweet orange varieties grown in Brazil. Some mandarin varieties have been found to be tolerant in field and greenhouse evaluation. Victoria tangor, a natural hybrid found in an orchard in southern São Paulo state, was grafted onto Rangpur lime some 60 yr ago. It has the same canopy characteristic of sweet orange, its fruit matures early, and is highly productive with fruit and juice of good quality. Its identity as a tangor was confirmed by plant morphology and isozyme analysis. In 1986, five plants were grafted onto Rangpur lime, along with 3000 Pera sweet orange trees. All the Pera have developed severe CVC symptoms, but none of the five tangor plants show any fruit or leaf symptoms. They were also negative for CVC in ELISA and PCR tests. In June 1996, Victoria tangor grafted onto Cleopatra mandarin was graft-inoculated with CVC in the greenhouse. At the same time, the tangor and some sweet orange varieties were top-worked onto CVC-affected Natal trees. In January 1997, 20 tangor seedlings were graft-inoculated. None of the inoculated tangor plants have developed symptoms, but the seedlings were positive in PCR tests. The top-worked tangor did develop symptoms 6 mo after the top-worked sweet orange trees. The conclusion is that Victoria tangor does possess some tolerance to CVC.

Occurrence of *Xylella fastidiosa* in Budwood Source Trees Without Citrus Variegated Chlorosis Symptoms

H. D. Coletta Filho, K. M. Borges, and M. A. Machado

ABSTRACT. Citrus variegated chlorosis (CVC) has spread quickly in São Paulo State, and many production areas are now infected. Within orchards, transmission is by several species of sharpshooter leafhoppers, but dissemination over long distances occurs mainly through the movement of infected budwood and nursery trees. Because of the limited supply of healthy budwood, some growers have collected budwood from apparently healthy trees in commercial orchards. Since CVC symptoms can take some time to develop, these trees can be infected without the grower realizing it. PCR-based diagnosis was carried out to survey the frequency of the causal bacterium, *Xylella fastidiosa*, in symptomless trees used as budwood sources. The samples were collected by the growers and were symptomless. The observed frequencies of CVC positive samples was 17% and 25% in two areas of central São Paulo state. In the south of the state it was 26%.

Transmission of *Xylella fastidiosa* by Spliced Approach Grafting

C. L. Medina, W. M. C. Nunes, M. A. Machado, and E. C. Machado

ABSTRACT. The causal organism of citrus variegated chlorosis (CVC), *Xylella fastidiosa*, was transmitted from infected sweet orange tree to healthy nursery trees by spliced approach grafting. Eighty 16-mo old Pera sweet orange on Rangpur lime nursery trees were transplanted into 100-liter plastic bags in an insect-proof screenhouse. One month after transplanting, they were each splice approach grafted to a CVC-infected Pera sweet orange tree in the screenhouse. The graft was done 10 cm below the main branches, with a contact length of 5 cm. For controls, healthy nursery plants were grafted to healthy trees. Transmission was confirmed by PCR in 52.5% of the trees 5 mo after grafting, which is quicker than by conventional bud inoculation.

Comparative Foci Study of Citrus Variegated Chlorosis Using Symptomology and Serology

W. M. C. Nunes, M. A. Machado, F. F. Laranjeira, G. M. Tonon, and E. L. Furtado

ABSTRACT. A preliminary analysis of citrus variegated chlorosis (CVC) foci in the field was done by evaluating the incidence of the disease by both symptoms and DIBA. The experimental area was an orchard of 300 Pera sweet orange trees, divided into two plots. Four evaluations were done 2 mo apart. Initially, the symptoms foci were lower in number than the DIBA foci, but in the second, third and fourth evaluations the DIBA foci were two, five-and-a-half and five times more numerous, respectively. The DIBA foci, however, were always larger than the symptoms foci. DIBA evaluations were thus found to give a more accurate picture of CVC incidence.

Efficiency of PCR Detection of *Xylella fastidiosa* for Epidemiological Studies on Citrus Variegated Chlorosis

W. M. C. Nunes, M. A. Machado, F. F. Laranjeira, G. M. Tonon, and E. L. Furtado

ABSTRACT. Citrus variegated chlorosis (CVC) evaluations in epidemiological studies is usually done on the basis of symptoms. Because of the sensitivity of PCR, a comparative study of the two was conducted in a sweet orange orchard with low CVC incidence. Seventeen trees with symptoms were marked, and together with surrounding trees. They were sampled six times at intervals of 2 mo. Trees surrounding PCR-positive symptomless trees were included in the subsequent sampling. Thus 153 trees were sampled in the first evaluation, and 731 in the final one. PCR detected 24% more infected trees than were apparent on the basis of symptoms, yet it was not significantly better for forecasting the spread of the disease. Further studies are being conducted.

Gas Exchange and Water Relations of Drought-Stressed Orange Trees Infected by *Xylella fastidiosa*

C. L. Medina, M. M. A. Gomes, E. C. Machado, A. M. M. A. Lagôa, G. Habermann, and M. A. Machado

ABSTRACT. The effects of drought stress on *Xylella fastidiosa*-infected 1-yr old Pera sweet orange trees in 100 liter pots was studied using CO_2 assimilation (*A*), transpiration (*E*) and stomatal conductance (*g*) rates measured at 9:00h, 12:00h and 15:00h, and water leaf potential (*Ø*) measured at 6:00h and 14:00h. The *Ø* at 6:00h was considered as an evaluation of soil water potential. The treatments were: Irrigated healthy plants (HP), drought stressed healthy plants (DHP), infected plants (IP) and drought stressed infected plants (DIP). Water was withheld and measurements made until the plants presented *A* near zero, when they were rehydrated for recovery. The *Ø* at 6:00h in IP and HP was similar, while at 14:00h it was smaller in IP. During water stress development, the *Ø* at 6:00h for DHP decreased faster than for DIP, showing higher water consumption rate. *A*, *E* and *g* IP rates were generally smaller than HP rates, mainly at 12:00h. During the first 4 days of water deficit development, DHP and DIP showed similar decay for *A*, *E* and *g* and *Ø* during the day. The *Ø* at 14:00h was smaller for infected plants. After 8 days *A*, *E* and *g* for DHP were smaller at 9:00h and 15:00h, but similar at 12:00h, probably due to the rapid loss of soil moisture. After 10 days, *A*, *E* and *g* were almost zero for the drought stressed treatment, and *Ø* at 6:00h was near -2.5 MPa. DHP recovered total *A*, *E* and *g* rates after 10 days, while DIP recovered partially in relation to IP at the same time. The smallest *Ø* at 14:00h and smallest *A*, *E* and *g* rates for IP at 12:00h can be related to the possible blockage of xylem by bacteria in the infected plants. The partial DIP recovery suggests that drought stress is more harmful to infected plants than to healthy.

Viroid Dwarfing of Valencia Sweet Orange in High Density Plantings

R. J. Hutton, P. Broadbent, and K. B. Bevington

ABSTRACT. Australian field trials have shown that inoculation of citrus trees with viroid CVd-IIIb with and without CVd-IIa reduced the size of selected sweet orange scions on Australian trifoliate orange and citrange rootstocks, without causing deleterious effects on tree health. Closely spaced viroid-dwarfed trees have been shown to out yield conventional plantings on a planted area basis and fruit quality was not affected by either presence of viroids or close planting. The time lag observed between effects on canopy size or yield became apparent following viroid inoculation, is one of the principal advantages of using viroids for regulating tree size. No effects on canopy development become apparent until 4 yr after inoculation. This ensures rapid early development of a high canopy bearing volume per hectare to promote high early orchard productivity. Yield efficiency (yield/tree size) of viroid inoculated trees was not adversely affected by the subsequent reduced canopy growth which was due to a reduction in magnitude of vegetative growth occurring during summer and autumn. Inoculation had no effect on spring flush growth or timing and duration of vegetative flushes, flowering intensity, inflorescence type, fruit set or fruit growth rates. Ideal stock/scion combinations for high density planting should exhibit moderate vigor and high early cropping efficiencies. Trees on trifoliate orange are better suited to high density planting than trees on citrange, although in combination with viroid dwarfing, high density plantings on citrange are also highly productive. Matching tree growth rates to spacing is important to optimize productivity per hectare and maximize potential returns from high density plantings. Economic factors favoring high density plantings of viroid dwarfed trees are the early income on investment and the containment of fixed costs by increasing production efficiency.

Citrus Viroids Detected in Citrus Trees in Japan

T. Ito, H. Ieki, and K. Ozaki

ABSTRACT. Cloning and sequencing of viroid RNAs from citrus trees in Japan showed some genomic diversity among variants in five citrus viroids (CEVd, CVd-I, CVd-II, CVd-III and CVd-IV) and the existence of two other viroids. One of the two consists of 331 nt and has 67% sequence similarity with CVd-III, while the other consists of 327 nt and has 84% sequence similarity with CVd-I. Following the scheme of viroid classification that viroids sharing less than 90% sequence homology should be considered different viroid species, these two would be classified as distinct species.

Segregation of Viroid Isolates Used as Dwarfing Agents for Marsh Grapefruit on Trifoliate Orange

E. S. Stuchi, N. Duran-Vila, and L. C. Donadio

ABSTRACT. Four viroid isolates, three with CEVd plus CVd-II and CVd-III and one with CVd-II and CVd-III, were each inoculated onto 10 6-mo old Marsh grapefruit on trifoliate orange trees in the field by grafting one bud onto the scion and one onto the rootstock. After 3 yr, buds from all the inoculated trees and 10 uninoculated controls were collected for indexing on Etrog 861-S1 citron. The isolates with CEVd caused typical symptoms, but the CVd-II + CVd-III isolate did not. Symptoms varied from trees in the same plot, and in one there were no symptoms. Samples from the indicator plants were collected for S-PAGE analysis. For two trees which had both dwarfing and trunk symptoms, only CEVd was detected in the indicators, but two others also with field symptoms, only CVd-II could be detected. Another tree with field symptoms had no detectable viroids in the indicator. It appears that segregation of the viroids had occurred in the trees.

Detection of Citrus Exocortis Viroid and Citrus Viroid Groups II, III and IV in Brazil by PCR

C. J. Barbosa, M. I. S. Rodrigues, H. P. Santos Filho, A. D. Vilarinhos, and P. E. Meissner Filho

ABSTRACT. Citrus species can be infected by five groups of viroids with different biological and molecular properties. Using indicators and S-PAGE, the occurrence of citrus exocortis viroid (CEVd) and citrus cachexia viroid (CCaVd) has been confirmed. To determine if any other viroids are present, RNA was extracted from different isolates in a collection at Embrapa—Mandioca e Fruticultura in Bahia, for PCR analysis. cDNA was produced from the purified RNA using reverse transcriptase (M-MLV) and using specific primers the presence of CEVd, CVd-II (which includes cachexia), CVd-III and CVd-IV in Brazil was confirmed.

Spiroplasma citri: Phytopathogenicity, Fructose Utilization and Functional Complementation

P. Gaurivaud, F. Laigret, J. L. Danet, M. Garnier, and J. M. Bové

ABSTRACT. By random insertion of transposon Tn4001 into the genome of *Spiroplasma citri* strain GII3, we have obtained several mutants of which one, mutant GMT 553, is non-phytopathogenic. In GMT 553, Tn4001 is inserted in the first gene (*scrX*) of the fructose operon where the second is *fruA* (fructose permease) and the third *fruK* (fructose-1-phosphate kinase). The product of *scrX* has homology to regulatory proteins of sugar phospho-transfer systems. In GMT 553, expressions of the fructose operon is abolished and, hence, fructose cannot be utilized by the mutated spiroplasma. To confirm the involvement of the fructose operon genes in phytopathogenicity, the wild type genes of the fructose operon have been isolated, and functional complementation of the mutant with various combinations of these genes have been achieved. With *fruA* + *fruK*, the mutant fully recovers its ability to use fructose and becomes phytopathogenic again. These results indicate that *fruA* + *fruK*, but not *scrX* are the key genes, and that utilization by the spiroplasma of fructose from the sieve tubes is somehow involved in symptom development.

Molecular Characterization of Two Proteins Associated with Citrus Blight

T. L. Ceccardi, G. A. Barthe, and K. S. Derrick

ABSTRACT. Citrus blight is a disease of unknown etiology that affects bearing trees wherever citrus is grown in hot, humid areas. An estimated one million trees in Florida and 12 million trees in Brazil are lost each year to blight. Several distinct proteins are present in affected trees, and serological assays for two of the proteins, p12 and p35, have been used to distinguish blight from other citrus declines. Protein p35 accumulates in the xylem of affected trees and was shown to be a α-1,3-glucanase by amino acid sequencing and serology. The function of p12 is unknown, but it is present in leaves of blight-affected trees, and serological detection of p12 in leaf extracts is a reliable indicator for blight. Cloning and sequencing of the p12 gene revealed a 393 nt ORF which included a sequence predicted by the N-terminal amino acid sequence of p12 and an N-terminal hydrophobic signal peptide. The amino acid sequence based on the p12 ORF was 49% similar and 31% identical to *Arabidopsis thaliana* expansin, which has been attributed with loosening cell walls during longitudinal growth. However, p12 did not show significant expansin activity in Acreep@ assays using cucumber hypocotyl walls. Experiments in progress to determine the function of p12 include production of transgenic tobacco and citrus plants with the sense and antisense ORFs of p12 DNA.

Macrophylla Decline: A Citrus Disorder with Similarities to Citrus Blight

K. Taylor, D. Ellis, L. Paiva, and H. Geitzenauer

ABSTRACT. Macrophylla decline is a disorder of unknown etiology which occurs in Arizona. It is characterized by early redistribution of zinc (Zn) within the tree, with accumulation in trunk vascular tissue and depletion occurring in the leaves. Later manifestations are decreases in xylem conductivity and necrosis of phloem tissue. It thus has a number of similarities with blight. Therefore, the two disorders were compared on a physiological, structural and molecular basis. Both demonstrated Zn reduction from the leaf canopy to the trunk tissue above the bud union. This accumulation appears to be mediated by a cysteine-rich 5 kD protein with amino acid sequence identity to the chitin binding domain of a number of wound inducible proteins. Following Zn accumulation in the phloem, it accumulates in the xylem. We isolated a 22 kD zinc binding protein that appears correlated with this increase. Macrophylla decline trees were also characterized by a marked reduction in water conductivity, similar to blight-affected trees that have undergone plugging of xylem vessels. Trees were assessed for 12 and 23 kD blight-specific protein (BSP). Differences in BSP accumulation appeared to vary among rootstocks tested. Another protein (14 kD) was identified in the leaf extracts of blight and Macrophylla decline trees that was absent in leaves of healthy trees. Structural similarities in the phloem of trees affected by both diseases were observed. It had previously been suggested that Macrophylla decline was an incompatibility between scion and rootstock, but the above results suggest that it resembles citrus blight in many respects.

Current Situation of Citrus Virus and Viruslike Diseases in Chile

X. A. Besoain, M. P. Valenzuela, M. Castro, and J. F. Ballester-Olmos

ABSTRACT. Citrus trees in Chile have not been adversely affected by virus disease in the past. However, in recent decades there has been the introduction of new varieties, possibly symptomlessly infected. To survey the current virus situation, 160 samples from five regions of Chile (I, IV, V, VI and Metropolitan) were tested by inoculating onto a range of biological indicators in the greenhouse. Citrus tristeza virus was found in regions I, IV and VI, but no decline symptoms of trees on sour orange rootstock or stem pitting in sweet orange or grapefruit has been found. Vein clearing in lime in region I was observed. Concave gum symptoms (blind pocket and oak leaf patterns) were observed in regions I and VI. Exocortis was detected in all regions, with characteristic symptoms in trees on trifoliate orange. Cachexia was detected in the important lemon producing regions IV, V, VI and Metropolitan; field symptoms of lemon on *Citrus macrophylla* were found, and indexing on Parson's Special mandarin in a warm (28-32 C) greenhouse confirmed the diagnosis.

Virus and Viroid Characterization of the "Centro de Citricultura-IAC" Citrus Germplasm Collection

S. A. Carvalho, M. A. Machado, and G. W. Müller

ABSTRACT. The citrus germplasm bank of the "Centro de Citricultura Sylvio Moreira-IAC" in Brazil is one of the largest in the world. It is currently being evaluated for its viral health status, beginning with biological indicators for the presence of citrus tristeza virus (CTV), citrus psorosis virus, citrus exocortis viroid and cachexia viroid. The total number of clones tested so far is 372, consisting of oranges, mandarins, lemons, citrons, grapefruit, acid limes, and various hybrids and relatives. CTV was verified in 97% of the clones, about 27% of which induced severe reaction, and 3% very severe, in lime indicators. The only CTV-free trees were one lemon variety, trifoliate orange, its hybrids and some relatives. Psorosis, exocortis and cachexia were found only in old lines at 19%, 16% and 9% respectively. Exocortis was present in oranges, limes, lemons and mandarins, while psorosis and cachexia were found mostly in sweet orange varieties.

Shoot-Tip Grafting and Tristeza Cross Protection of the "Centro do Citricultura-IAC" Citrus Germplasm Collection

S. A. Carvalho, M. A. Machado, M. A. Baptista, and G. W. Müller

ABSTRACT. The germplasm collection of the "Centro do Citricultura Sylvio Moreira-IAC" in Cordeiropólis, SP, Brazil has almost 1800 accessions of different citrus species, hybrids and relatives. Many are infected with viruses and viroids. Shoot-tip grafting for eliminating viruses and viroids has been conducted on 358 clones, with 100% efficiency for tristeza, exocortis and cachexia elimination, and 63% for psorosis. To improve psorosis elimination, thermotherapy was also used. After elimination of these agents, the material is cross protected against severe tristeza using mild isolates specific for each. The "Pera IAC isolate" is used for sweet oranges and mandarins, the "A141 isolate" for citrons and limes, and the "50 isolate" for grapefruit. The trees are horticulturally evaluated in the field for true-to type characteristics.

Results of Indexing for Viruses and Viroids in the Citrus Germplasm of São Paulo, Brazil

A. A. Salibe, A. Tubelis, F. A. A. Mourão Filho, A. P. Jacomino, and A. B. Salibe

ABSTRACT. Several hundred trees representing most citrus varieties available in São Paulo state, Brazil were biologically indexed for viruses and viroids. Trees were both old and nucellar lines, and were selected from commercial orchards or from germplasm banks, and where possible the geographic origin was noted. The results showed that tristeza, psorosis, vein enation/woody gall, exocortis and cachexia were widespread in old line trees. Higher percentages of exocortis were found in sources originating from California, Spain and Italy. Cachexia was restricted to fewer varieties from California and Portugal, while most psorosis was found in varieties from the Mediterranean region. Psorosis was found in some nucellar lines, suggesting possible natural slow transmission. The first tristeza strains were introduced from South Africa, but more severe strains came in later with illegal imports from southeast Asia.

Citrus Budwood Registration and Certified Nursery Tree Program in São Paulo, Brazil

S. A. Carvalho, E. Feichtenberger, H. Tosin, and A. J. Aires

ABSTRACT. In 1969 regulations covering the Mother Tree Program were established. Since then, blight and citrus variegated chlorosis (CVC) have become the cause of major losses to the citrus industry in Brazil, and the Citrus Budwood Scheme has had to be restructured, and the rules for production of certified citrus nursery trees have been revised. The program is coordinated by the "Comissão Técnica de Citricultura" of the "Secretaria de Agricultura e Abastecimento do Estado de São Paulo". The new rules were established in collaboration with scientists at the "Centro de Citricultura Sylvio Moreira-IAC", "Cordenadoria de Assistência Técnica Integral (CATI)", "Instituto Biológico" and "Fundo de defensa da Citricultura (Fundecitrus)", and were approved by the "Câmara Setorial do Citros" and the "Comissão Estadual de Sementes e Mudas-CESM/SP". The main modifications relate to the indexing for CVC and blight, and the demand for the obligatory maintenance of the mother trees under screen to avoid the insect vectors of CVC. It also introduces the registration of increase blocks, produced from screen-protected mother trees, that should be maintained under screen. The number of rootstock and scion varieties has increased. The program also requires that seed has to come from registered trees, all nursery-tree production has to be done under screen, containers have to be above a minimum size, and soil and water must be disease-free.

The Belize Citrus Certification Program (BCCP)-a Solution for the Management of Graft-Transmissible Citrus Diseases

P. S. Reddy and F. Majil

ABSTRACT. Citrus is the second largest foreign exchange earner for Belize, with a per capita value of US$200. The total acreage is approximately 60 000, of which 60% is on sour orange rootstock. Valencia orange and white grapefruit are the major varieties grown, and 98% of the fruit is processed into frozen juice concentrate for export. Citrus tristeza virus is widely distributed in both mild and severe forms, and the arrival of the efficient aphid vector, *Toxoptera citricida*, in 1996 poses a major threat to the industry. Exocortis, which affects some of the tristeza-tolerant rootstocks, psorosis and blight are widespread. A mandatory certification scheme called the Belize Citrus Certification Program (BCCP) is being implemented by the Citrus Research and Education Institute (CREI), with assistance from the Food and Agriculture Organization. Disease-free parent trees maintained at CREI will be periodically indexed. Registered nurseries which comply with strict guidelines will be allowed to establish quick multiplication blocks with buds cut from the approved parent trees.