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The abstracts are arranged in the order they were presented at the conference. The conference program can be found at: https://escholarship.org/uc/item/334952v5.

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MANAGING BIOSECURITY RISKS TO AUSTRALIAN CITRUS

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Citrus is one of the largest and most important horticultural export commodities in Australia producing around 600 thousand tonnes annually, although on the world scale this represents less than 1% of production. The introduction of new pathogens poses a major risk to Australian citrus. Strict post entry quarantine protocols in Australia require that all imported citrus varieties are shoot tip grafted *in vitro* and tested for exotic and endemic pathogens by the Department of Agriculture, Fisheries and Forestry (DAFF) before their release. Today the majority of new varieties are imported by private individuals or variety managers rather than by government or industry organisations.

Auscitrus is the national 'not for profit' industry organisation responsible for the supply of high health status and true to type citrus seed and budwood (private and public varieties) to the Australian citrus industry. It maintains Australia's citrus foundation trees in 2 secure repositories funded by the citrus industry levy and private variety owners. Auscitrus also provides a pathogen testing and pathogen elimination service through a partnership agreement with NSW DPI for locally selected private varieties.

High health status and pre-immunised foundation trees are routinely tested by NSW DPI to provide material for the establishment of budwood supply trees maintained in the field. The rise in importance of private varieties (both imported and local) has put increased pressure on traditional budwood production, requiring a shift for high demand varieties towards rapid nursery multiplication by Auscitrus. Generally nurseries who purchase Auscitrus buds do not multiply their own budwood. Budwood multiplication and seed supply are commercially funded, with Auscitrus budwood and seed operations receiving no monetary support from industry or government.

Although some of the world's worst citrus pathogens like Huanglongbing (HLB) are not known to occur in Australia, Auscitrus has recognised there is a need to move towards a protected (screened) budwood supply system. HLB and its insect vectors pose a significant threat to Australia, given our close proximity to the Indonesian Archipelago and New Guinea where the disease is known to occur and the high amount of visitor traffic from countries where the disease is present. Movement of the pathogen and its vectors eastward from Asia to Australia through cyclonic winds or illegal movement of infected plant material is highly likely. In the event of a HLB incursion it would be difficult to track 'at risk' commodities like citrus and orange jasmine because currently Australia does not have mandatory nursery registration.

Graft-transmissible diseases like *Citrus psorosis virus*, *Citrus tatterleaf virus* and *Citrus exocortis viroid* are rarely seen in Australian orchards due to the success of the Auscitrus budwood scheme. However the use of Auscitrus budwood is voluntary. The Australian citrus industry in partnership with Auscitrus must move towards a mandatory certification scheme to ensure the future of our industry in the face of increasing biosecurity risks.

PROGRESS IN CONTROL OF MAIN GRAFT-TRANSMISSIBLE CITRUS DISEASES IN THE P. R. CHINA

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In the P.R. China, over fifty graft-transmissible fruit tree pathogens have been identified, of which the main disease causing agents of citrus include *Candidatus* Liberibacter asiaticus, *Citrus tristeza virus* (CTV), Citrus tatter-leaf virus and *Citrus exocortis viroid*.

In 1989 and 2000, although the National Governor set up Regulations for Plant Seeds and a Law for Plant Seeds, the requirements for clean fruit nursery trees and seeds were poorly described. Thus, the graft-transmissible disease problem increased in severity due to the use of infected propagation material in the fast growing fruit industry. Fortunately, this law will be modified next year. The only successful case of control via a virus-free scheme in P.R. China is that of the Citrus Industry. Since 2001 ca 100 modern virus-free citrus nurseries have been established in 13 provinces, which have the capacity for producing ca 114 million nursery trees per year. Through the supply of virus-free material, the loss caused by graft-transmissible citrus diseases in the inland area has dramatically decreased. A sub-association for nurserymen was set up in 2008 under the Chinese Society of Citriculture, making propagation of nursery trees more organized than ever before. Guangxi province has been a good example of the successful control of HLB through this scheme, where the percentage of HLB has dropped to less than 1% from over 10%. Other coastal areas have not followed suit due to urbanization and the lower importance of agriculture. Based on the progress in citrus, a state-level cooperative project on controlling virus diseases of fruit trees has been executed since 2012. Similar schemes are being applied to other fruits such as apple, plum, grapevine, peach and banana etc. Mild strain cross protection is another control option for those pathogens spread both by grafting and insects, such as CTV. A few mild isolates with potential protective capability, screened from thousands of field CTV isolates are being applied in field experiments.

POSTER 1

THE CITRUS VARIETY IMPROVEMENT PROGRAM OF INTA IN ARGENTINA

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The Citrus Variety Improvement Program of INTA, in Argentina was established 29 years ago as an industry insurance policy. Since 1984, the Concordia Experiment Station has maintained citrus mother trees tested for freedom from disease and trueness to type. The objective is to secure propagating material of commercial varieties for industry needs. Varieties are selected from research projects and/or introductions and are subject to quarantine controls and a sanitation process. The selected trees are shoot-tip grafted and indexed for tristeza, psorosis, exocortis, cachexia, citrus variegated chlorosis, canker and huanglongbing. Two hundred and eighteen scion and rootstock varieties are maintained and evaluated annually for their agronomic characteristics and sanitary status by visual observation and biological, immunochemical and/or molecular diagnostics. Data obtained on origin, botanical characteristics, agronomic performance, sanitary status and availability of propagation material is recorded. The information is available at INTA's website (www.inta.gov.ar/concordia). Basic material is exported to citrus producing countries and sent to germplasm banks on request. The isolation, annual evaluations, pest monitoring and disease diagnosis guarantee the quality of the material offered by the program to the Argentine citrus industry.

POSTER 2

RAPID SCREENING OF CITRUS PLANTS FROM THE SANITATION PROCESS OF THE IRAN CITRUS RESEARCH INSTITUTE

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In the research program of the Iran Citrus Research Institute (ICRI), indexing of citrus varieties after the sanitation process is done for important virus and virus-like diseases. Rapid screening is necessary for selection of the candidates among the high number of plants produced by shoot-tip grafting (STG) and thermotherapy. Multiplex RT-PCR is performed with a mix of four citrus viroid primers, those for Citrus exocortis viroid (CEVd), Hop stunt viroid (HSVd), Citrus bent leaf viroid (CBLVd) and Citrus dwarfing viroid (CDVd). Indexing of the plants is also carried out for Citrus Tristeza Virus (CTV) with a Direct Tissue Blot Immmuonoassy (DTBIA) using commercial polyclonal antibodies and for *Candidatus* Liberibacter asiaticus by conventional PCR using A2/J5 and OI1/OI2c primers. The selected plants enter a complementary assessment process comprising different methods including biological indexing.

POSTER 3

THE SOUTH AFRICAN POST ENTRY QUARANTINE AND CITRUS IMPROVEMENT SCHEME.

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In 1973, a consultative visit by Dr E.C. Calavan of University of California, Riverside (UCR) led to the official launch of the South African Citrus Improvement Programme (SACIP). Interestingly, similar programmes around the world were launched pre-1973 i.e. Argentina, Brazil and USA, while programmes in Australia and Spain were launched post-1973. Initially, nursery selected sources trees from the field were used. After two years of evaluation for yield, quality and longevity, candidate trees were used to produce multiplication blocks at nurseries. When a shift occurred from rough lemon rootstocks to exocortis sensitive trifoliate and hybrids, biological indexing started. Shockingly, 25% of the initial candidate trees were found to be viroid infected and subsequently removed from the programme. A major break-through was when Murashige and co-workers developed a technique for grafting the small apical shoot of citrus, shoot tip grafting (STG), to the top of a decapitated seedling grown *in vitro*. However, in 1975 the technique was improved and grafted plants were reported free of citrus viruses by Navarro and co-workers. In 1977, Chester Roistacher visited and taught the technique at ARC-ITSC (previously known as Citrus and Subtropical Fruit Research Institute). This led to the development of a nucleus block in insect proof tunnels which houses STG lines. In addition, an evaluation block and the Citrus Foundation Block (to multiply STG budwood) were established at Addo research station and Uitenhage, respectively. Introduction of advanced molecular methods and constant re-indexing revealed that there had been two escape incidents (Viroids II and IV) which were not picked up by visual indexing. A manual on protocols and citrus diseases has been set up and is constantly updated.

POSTER 4

PERFORMANCE OF LEMON LINES RECOVERED THROUGH SHOOT TIP GRAFTING

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Lemon cultivars recovered by shoot-tip grafting (STG) were compared with the original 30-40 year old nucellar clones located in a germplasm block in Tucuman, Argentina. Cultivars evaluated were Frost Eureka, Limoneira 8A Lisbon, Feminello Santa Teresa, and Genoa EEAT. These were grafted on *Poncirus trifoliata* Flying Dragon, except for Frost Eureka which was grafted on 79 AC Cleopatra mandarin x Swingle citrumelo [*Citrus reshni x (C. paradisi x P. trifoliata)* due to its incompatibility with trifoliate rootstock. The selected clones were naturally infected with CTV, an endemic disease, and Frost Eureka was also infected with one viroid (not exocortis). Trees propagated with STG budwood were indexed and confirmed to be free of CTV, psorosis, and citrus viroids before planting. A field trial was planted in November 2007, with four randomized blocks per treatment and four replicate trees per block. Tree height, canopy volume, trunk circumference, fruit yield and quality were evaluated. CTV infection was monitored annually in spring of 2009, 2010 and 2011, and thereafter in spring and fall of 2012 and 2013. Direct immunoprinting ELISA with 3DF1+3CA5 monoclonal antibodies (Plant Print Diagnostics, Valencia) was used to detect CTV in field samples. In 2009, Limoneira 8A had the highest incidence of CTV infected plants (25%) followed by Genoa EEAT (1.6%) while Feminello Santa Teresa remained CTV free. In 2013, 100% of STG lemon lines were infected with CTV. Cumulative yield after four harvests of Limoneira 8A, Feminello Santa Teresa, and Genoa EEAT was no different from trees budded from their respective mother trees. In contrast, the STG line of Frost Eureka yielded 80% more fruit than the source tree with the viroid. In conclusion, CTV did not substantially affect the growth and yield of the lemon cultivars tested, while the viroid in Frost Eureka considerably reduced tree productivity.

CITRUS SHOOT-TIP GRAFTING BEYOND PROFICIENCY

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Shoot-Tip Grafting (STGing) is an important part of a comprehensive certification program. Reduction in the time for certification to an average of two years (to less than one year in some cases) is possible. Components of a highly successful STGing program include good scheduling, dedicated STGing personnel, a comprehensive testing program, a reliable source of seed, and a clean area for working including specialized tools and equipment. Proficiency in both the budstick technique and the leaf-stripping technique will give flexibility in scheduling and allow faster completion times. The use of *Poncirus trifoliata* as a rootstock in culture gave us greater success with fewer root sprouts. Laboratory tests on young STGs should be used for a early indication of failure; follow up testing will determine if elimination of pathogens was successful. Precision trimming starts with the use of Gelrite (or Phytagel) for a solid base of STG set up, allowing the STGs to be evaluated in a horizontal position with a dissection microscope. Detailed notes are taken weekly and micro-trimming can prevent the selection of root sprouts by accident. Most STGs begin sprouting at two to three weeks. Small STGs grafted into rootstocks will grow faster in the greenhouse and be ahead of STGs grown for a longer period in culture tubes in the laboratory. Increased sucrose can speed the development of some selections, but the increased number of root sprouts and associated trimming can be disadvantageous. The transfer to the greenhouse may be a problem area; it is best if personnel involved with STGing do the grafting and follow-up. Kinkoji (*Citrus obovoidea*) is used for grafting of STGs as it is easy to work with year around and has increased our success rate. Differences in personal success rate may be because of the amount of regular practice, critical evaluation to determine the cause of success or failure, sharp tools, and attention to the most minute of details.

DOES PHYTOSANITARY PURITY DIMINISH HORTICULTURAL EXPRESSION AND THEREBY ECONOMIC VALUE?

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The practice of shoot-tip grafting to eliminate viroids and deleterious pathogens has been very beneficial to the citrus industry world-wide over recent decades. This not only allowed for the safe movement of new varieties around the world but also the use of quality-inducing rootstocks like citranges. It has also extended the productive life of citrus orchards and in some cases improved the horticultural performance of cultivars and thereby economic value to farmers. However, the single minded goal of removing all possible pathogens from the plant material seems to have occasional deleterious effects on the horticultural performance of some cultivars. In practice, new citrus varieties are usually discovered via chance mutation in commercial orchards. Such varieties result from old clone material, usually containing one or more viruses/viroids, necessitating clean-up, as specified by relevant Plant Improvement Schemes prior to commercial release. However, the ever-improving science of virus detection and elimination makes the achievement of totally sterile plant material a reality. It is suggested that this influences the horticultural expression of such pure varieties. The authors will present information aimed at demonstrating that complete virus/viroid elimination may satisfy the regulatory hurdles for commercial release but also in some cases result in the loss of economic value. Financial return to the orchard is dependent on a few very basic aspects: early production of good yields of edible (desired) fruit with high pack-outs (size, colour, etc.). Altering one or more of these characteristics may mean the difference between a winner and a loser. Beneficial and/or relatively non-harmful aspects of specific viroids/pathogens to horticultural value in fruit trees/horticulture will be outlined. An argument will be put forward for the characterization of certain citrus viroids relative to their role in horticultural expression and the potential re-introduction of purified strains after the clean-up process to ensure maintenance of such economic value. This presentation aims at stimulating debate and is not intended to criticize or lay blame.

NEXT-GENERATION SEQUENCING: A POWERFUL TOOL TO IDENTIFY KNOWN AND NEW VIRUSES IN CITRUS

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There is a large diversity of viruses and viroids that infect citrus trees, some of them well characterized and known, belonging to the genera *Closterovirus*, *Ilarvirus*, *Capillovirus*, *Sadwavirus*, *Mandarivirus*, *Marafivirus*, *Spirovirus* (*Ophiovirus*), *Badnavirus* and viroids belonging to the genera *Apscaviroid*, *Cocadviroid*, *Hostuviroid* and *Pospiviroid*. In addition, there are a large number of well-known graft-transmissible diseases of unknown etiology, which suggests that there exist unknown viral agents infecting citrus. Although the diagnosis of known agents is straightforward by serological or molecular methods, the detection of viruses without prior knowledge remains a challenge. Currently, only biological indexing is able to address the detection of the majority of graft-transmissible diseases. However, the development of next generation sequencing (NGS) technologies allows exceptional sequence data generation at a fraction of the cost of biological indexing, dramatically modifying the diagnostic landscape. Virus-derived small interfering (si) RNAs (21-24 nucleotides) from citrus trees showing different symptomatologies were analyzed by Illumina sequencing. *De novo* contigs generated by different bioinformatic software were analyzed against the Genbank database, using Blastn, Blastx and Tblastx. Those nucleotide sequences, which had some homology to viral sequences, were used along with all siRNAs to reconstruct the genomes of various viral agents through iterative mapping against contigs. Along with known viruses and viroids, new citrus *Bunyavirus*, *Badnavirus* and *Luteoviridae* species were successfully identified. NGS is a powerful technology that could greatly simplify the screening, routine diagnosis, detection and characterization of citrus pathogens, providing knowledge to generate new diagnostic tools and having the potential to rapidly replace biological indexing.

MECHANICAL INOCULATION OF CITRUS AND PERIWINKLE WITH *Spiroplasma citri*

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Citrus stubborn disease (CSD), caused by the bacterial pathogen *Spiroplasma citri*, is of major concern for citrus production around the world. The development of a reliable virulence assay for *S. citri* is urgently needed, but has been proven to be extremely challenging. With the current project we investigated the mechanical inoculation of plants using an *S. citri* axenic culture. A secondary culture of *S. citri* isolate C189 was injected with a vaccination gun or slashed into the stems of citrus and periwinkle seedlings. The progression of the infection was monitored using a newly developed TaqMan quantitative polymerase chain reaction (qPCR) assay targeting the *S. citri* spiralin gene, as well as an antibody ScCCPP1 detecting a secreted protein of *S. citri* (U.S. patent application no. 61/615760). Our results indicated that *S. citri* entered the phloem tissues and established infection in both citrus and periwinkle. Bacterial cells could be detected by qPCR in plant tissues from newly emerged tissues away from the original inoculation site 1-7 weeks post inoculation. The ScCCPP1 antibody verified the qPCR results by producing positive reactions for the same time frame. *S. citri* detection past 5-7 weeks post mechanical inoculation became erratic by qPCR while the ScCCPP1 antibody continued to produce positive results. These data indicate that *S. citri* may have failed to maintain a long term infection in these plants while *S. citri* secreted proteins were still present in the inoculated plants. This is the first record of successful or at least partially successful mechanical transmission of *S. citri* from axenic culture directly into host plants. Further improvement on the inoculation procedures to achieve long term infection and overcome the possible loss of *S. citri* pathogenicity due to *in vitro* subcultures is underway.

CITRUS VIROIDS: AN 80 YEARS JOURNEY 1934-2013

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Viroids are small, non-coding, non-encapsidated, single-stranded, covalently closed RNAs that replicate autonomously when inoculated in their plant hosts in which they may elicit diseases. Presently, more than thirty viroids have been described and classified into two families, *Pospiviroidae* and *Avsunviroidae*. In the case of citrus, the viroid journey began in 1934 with the report on the xyloporosis disorder of Palestine sweet lime (*Citrus limettioides*) followed by the reports on the cachexia disease of Orlando tangelo (*C. paradisi* × *C. reticulata*) and the exocortis disorder of trifoliate orange (*Poncirus trifoliata*) rootstock in 1948. The etiological agents of these diseases, that were known to be graft-transmissible, were considered to be viruses until viroids were described as a new class of plant pathogens with the discovery of the *Potato spindle tuber viroid* (PSTVd) and the *Citrus exocortis viroid* (CEVd) in the early 1970s.
CEVd was characterized after its transmission to gynura (*Gynura aurantiaca*). However, since nucleic acid technologies were limited in the 1970s, ongoing studies relied on the use of experimental herbaceous hosts displaying viroid symptoms and yielding high viroid titres. This approach prevented the identification of other citrus viroids with narrower host ranges until the following decade. Following the adoption of 'Etrog' citron (*C. medica*) as an indicator for biological indexing of exocortis, in the late 1970s, the range of mild to moderate and severe symptoms observed after graft-inoculation with field isolates were erroneously considered as evidences for the existence of CEVd strains. In the mid-1980s, and after the use of a double gel electrophoresis system (i.e. sequential polyacrylamide gel electrophoresis, sPAGE) and silver staining, four additional viroid-like RNAs with distinct electrophoretic mobilities were identified and assigned the Latin numerals I through IV. Citrus viroid-I, -II, -III, and -IV were further characterized and shown to have distinct biological and molecular properties, thus considered individual viroid species separate from CEVd.
During the following two decades there was much discussion regarding the names adopted for citrus viroids. Presently, after lengthy field characterization experiments and according to the criteria of the International Committee of Virus Taxonomy, the following has been accepted for the five citrus viroids characterized in the mid-1980s. All identified citrus viroids belong to genera of the *Pospiviroidae* family and share some structural (e.g. central conserved region) and replication (e.g. asymmetric-nucleus) properties. CEVd is the causal agent of the exocortis disease and its name refers to the exocortis disease as originally described for the trifoliate rootstock. CEVd belongs to the *Pospiviroid* genus (with PSTVd as its type member). The available data shows that differences in virulence are host dependent and associated with certain nucleotide changes located in a specific region of the viroid RNA. CEVd has a wide host range and has been identified naturally infecting hosts other than citrus. CEVd is the largest among citrus viroids and has the unique property of spontaneously increasing in length its RNA genome by terminal repeats after

prolonged infections on specific solanaceous hosts. *Hop stunt viroid* (HSVd) is the causal agent of the cachexia and xyloporosis disease and its name refers to the stunting effect induced in hops. HSVd belongs to the *Hostuviroid* genus and it has a wide host range. The HSVd variants identified in citrus have a size ranging from 296 to 301 nucleotides and only variants containing specific RNA sequences, also known as cachexia expression motif, cause cachexia disease on sensitive citrus such as mandarins and some of their hybrids. *Citrus bark cracking viroid* (CBCVd) belongs to the *Cocadviroid* genus (*Coconut cadang cadang viroid* as its type member) and its name refers to the symptoms induced on trifoliate rootstock. CBCVd is the smallest of the known citrus viroids and is closely related (both molecularly and biologically) to CEVd. *Citrus bent leaf viroid* (CBLVd) and *Citrus dwarfing viroid* (CDVd) belong to the *Apscaviroid* genus (*Apple scar skin viroid* as its type member). The CBLVd name refers to the symptom induced in 'Etrog' citron whereas CDVd refers to the size reduction of citrus trees propagated on trifoliate rootstock. Two additional citrus viroids, also belonging to the *Apscaviroid* genus, have been identified since the 1980s. *Citrus viroid V* (CVd-V) induces mild reactions in 'Etrog citron' however, synergism with other *Apscaviroids* results in enhanced citron symptoms. CVd-VI (syn. CVd-OS) also induces mild reactions in 'Etrog citron' and has chimeric features related to CDVd, CEVd, and CBCVd. The effects of CVd-V and -VI on citrus under field conditions are still unknown.

In the late 1990s, the term 'transmissible small nuclear ribonucleic acid' (TsnRNA) was introduced to describe well-characterised citrus viroid RNA species that do not induce distinct disease syndromes in most citrus hosts but rather act as regulatory genetic elements modifying tree performance to the benefit of the grower. Since then, the TsnRNA-Ia, -IIa, and -IIIb have been studied in lengthy replicated field trials providing interesting results for reduced tree height and canopy volume, enhanced fruit size and increased yield per canopy volume as well as achievement of high-density plantings in the absence of any adverse effects in fruit quality or tree longevity. It is important to note however, that such results have been achieved only with specific scion/rootstock/TsnRNA combinations (e.g. sweet orange (*C. sinensis*)/trifoliate/TsnRNA-IIIb and clementine (*C. clementina*)/Carrizo citrange (*C. sinensis* x *P. trifoliata*)/TsnRNA-Ia+IIa+IIIb). TsnRNAs had no effect on various other scion rootstock combinations (e.g. lemon (*C. limon*)/*C. macrophylla* and Oroblanco (*C. grandis* x *C. paradisi*)/Citremon (*P. trifoliata* x *C. limon*)) or even on rootstocks such as Carrizo citrange when used as seedlings.

In the following decades, the advent of molecular biology provided a variety of new tools for viroid research and detection. Methods such as imprint and blot hybridization, reverse transcription and polymerase chain reaction (PCR) followed by cloning and sequencing or single-strand conformation polymorphism and transient or transgenic expression of viroid RNA *in planta*, in combination with *in vitro* transcription and plant inoculation or protoplasts transfection and more recently deep sequencing and a real time quantitative PCR protocol for the universal detection of citrus viroids, have transformed our diagnostic capacity. Even though, 'Etrog' citron and sPAGE remain the golden standard for citrus viroid detection, since it can detect all viroid-like molecules regardless of available RNA sequence information, the need for the development of robust, quick, reliable and economical viroid detection methods is always current. Nowadays, the open trade agreements, the global movement of citrus germplasm and competition of citrus producers, in combination with the ever-changing quarantine regulations and the constant need for pathogen-

tested citrus propagative materials, make the use of modern molecular technologies for citrus viroid detection a necessity.
With continuing research much information has been generated regarding viroid replication (e.g. symmetric, asymmetric, and RNA polymerase II), host processing (e.g. cleavage, ligation, and elongated viroid genomes), evolution and population structure (e.g. complex populations of closely related sequence variants), cell to cell (e.g. plasmosdemata) and long distance movement (e.g. phloem companion cells), biologically active RNA secondary structures (e.g. hairpins and Loop E), and mechanisms involved in pathogenesis (e.g. gene silencing) and symptom expression (e.g. cell wall modifications). However, a series of interesting and challenging practical and basic science questions remain open. Are viroids associated with the gummy bark disease of sweet orange? Are viroids associated with “Wood pitting-Gum pocket- Gummy pitting” observed on trifoliate rootstock? Are viroids associated with the Kassala disease of grapefruit? Do modern molecular viroid detection methods need to replace bio-indexing? In the absence of true dwarfing citrus species and rootstocks and in the face of serious citrus production cost and disease challenges, is the use of TsnRNAs for dwarfing and high-density plantings feasible in commercial scale and ethical? In the absence of any viroid encoded proteins, are viroids using a novel process for the suppression of the gene silencing plant antiviral mechanism? Are the viroid-like molecules the evolutionary link between the RNA and DNA world?

We hope that the current and the future generations of citrus scientists will carry on the 80 years old journey of viroid research and that they will provide exciting answers, new discoveries, and even more questions for scientific advancement.

VIROID REAL-TIME PCR FOR RELATIVELY LARGE NUMBERS OF CITRUS SAMPLES

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Real-time RT-PCR has been developed to replace conventional PCR. We have been able to increase our testing from 900 tests in 2005 to a range of 8,000 to 17,000 tests per year depending on need. The Homex-6 has been used to process larger amounts of plant tissue for greater accuracy in certification testing. A liquid handling robot has been incorporated to reduce repetitive hand injury, decrease the probability of pipetting error and increase efficiency. A cherry-picking robot is used for reformatting plates for running consecutive tests on a subset of samples. Published primers were tested and where necessary new primers were developed. Although the goal was to develop two sets of multiplex q-PCR each with three sets of primers, we run all viroids separately. CVd I, CVd II, CVd IV, CVd V and CEVd are run as Taqman assays, with the variable CVd III run as a SYBR green assay.

A SYBR GREEN RT-QPCR METHOD FOR UNIVERSAL DETECTION OF CITRUS VIROIDS

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Seven distinct viroid species representing four genera of the *Pospiviroidae* family have been identified in citrus. *Citrus exocortis viroid* (CEVd, genus *Pospiviroid*)*, Hop stunt viroid* (HSVd, genus *Hostuviroid*)*, Citrus bark cracking viroid* (CBCVd, genus *Cocadviroid*) and *Citrus bent leaf viroid* (CBLVd), *Citrus dwarfing viroid* (CDVd), *Citrus viroid V* (CVd-V) and CVd-VI of the genus *Apscaviroid,* cause various citrus diseases and abnormalities. Reverse transcription (RT) followed by quantitative real time polymerase chain reaction (qPCR) is one of the latest technologies in the field of RNA pathogen detection. We developed two sets of degenerate primers and their respective SYBR Green RT-qPCR protocols capable of detecting all known citrus viroids and their variants in two one-step RT-qPCR reactions (U.S. patent application no. 61/556634). The first reaction detects CBLVd (variants CVd-Ia, -Ib, and I-LSS), CDVd (variants CVd-IIIa and -IIIb), CVd-V, and CVd-VI. The second reaction detects CEVd, HSVd (citrus variants CVd-IIa, -IIb, -IIc, and Ca 909) and CBCVd. 'Etrog' citron Arizona S-1-861 biological indexing was used to validate the newly developed method. Furthermore, a newly developed semi-automated nucleic acid extraction and purification protocol for citrus tissue was integrated into this method for high throughput capacity. In September of 2012, the method was approved by the California Department of Food and Agriculture for use in the "Citrus Nursery Stock Pest Cleanliness Program" (Permit No. QC 1354). So far we have tested successfully over 5,000 nursery samples from various citrus types (i.e. orange, lemon, lime, grapefruit, mandarin, kumquat, etc.) reducing cost (RT-qPCR supplies estimated at US$1.90 per sample) and time (results acquired in 24 hours or less) compared to biological indexing. The described method can benefit citrus germplasm, research, and regulatory programs as well as diagnostic labs around the world. It is universal, accurate, quick, and paired with a high throughput nucleic acid extraction protocol can substantially reduce viroid testing cost and time.

CYTOPATHOLOGY OF A NOVEL VIRUS-LIKE DISEASE OF CITRUS

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Pummelo (*Citrus maxima*) trees with sectorial dieback and foliar symptoms that included severe chlorosis, corkiness, and necrosis of leaf veins, were observed in Honolulu, USA. Symptomatic leaves tested negative using molecular and/or serological assays for all viruses, viroids, and fastidious prokaryotes that cause or are associated with similar symptoms in citrus. No discernible double-stranded RNAs, a hallmark of RNA virus infection, were isolated from symptomatic tissues. Light microscopy of midribs coming from symptomatic leaves revealed the presence of inclusion bodies in cells surrounding the vascular bundle that were absent in similar cells from asymptomatic leaves. Transmission electron microscopy of thin sections also revealed numerous circular, electron-dense inclusion bodies up to 2.5 microns in diameter in symptomatic tissue. Isometric particles approximately 35 nm in diameter were often associated with the inclusion bodies. These particles and inclusion bodies were often, but not always, bound by a double membrane in the vacuole of affected cells.

POSTER 5

SIMULTANEOUS DETECTION OF CTV, CEVd AND CCaVd BY THE USE OF ARIZONA 861 S CITRON AND RT-PCR IN TWO STEPS

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Citrus tristeza virus (CTV), *citrus exocortis viroid* (CEVd) and *citrus cachexia viroid* (CCaVd) are pathogens that affect citrus trees in Chile. The combined use of a rapid diagnostic method with effective cleaning of infected budwood, represents a powerful preventive tool for the control of viral diseases. The objective of this study was the simultaneous detection of CTV, CEVd, and CCaVd through the use of bio-amplification in Arizona 861 S1 citron plants combined with two incubation temperatures and multiple RT-PCR in two steps. Citron indicator plants were inoculated with the three pathogens individually and in combination, with un-inoculated plants as a control. After 4 and 8 months incubation at the two different temperatures both the multiple and individually inoculated plants showed symptoms associated with these pathogens, which were subsequently corroborated through the use of multiplex RT-PCR in two steps. This method could be employed as an alternative efficient and cost-effective diagnostic method to those that are currently in use for detecting CTV, CEVd, and CCaVd. Nevertheless its effectiveness should also be evaluated with tests using different isolates of these pathogens.

POSTER 6

FIRST REPORT OF *Citrus viroid* V INFECTING CITRUS PLANTS IN TEXAS

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Citrus viroid V (CVd-V) is a recently characterized citrus viroid which has a GC rich genome of 292-295 nucleotides belonging to genus *Apscaviroid* within *Pospiviroidae* family. Presence of CVd-V in previously known viroid-infected 'Rio Red' grapefruit plants on sour orange rootstock was determined using reverse transcription-polymerase chain reaction (RT-PCR), sequential PAGE, and single-strand conformation polymorphism (SSCP). RT-PCR with primer pair CVV-P3/P4 consistently detected CVd-V from the samples tested. Highly purified CVd-IIa, CVd-IIb, CVd-IIc, and CVd-V ds RNAs were extracted and sPAGE analysis on these extracts revealed an estimated molecular size of approximately 295 bp for CVd-V by comparison of its migration relative to that of CVd-II. SSCP analysis on direct PCR products of CVd-IIa, CVd-IIb, and CVd-V showed different electrophoretic profile for them indicating sequence variations although they are similar size DNA fragments.

POSTER 7

A GENOMIC SURVEY OF *Citrus exocortis viroid* (CEVd) INFECTION UNDER FIELD CONDITIONS

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Citrus exocortis viroid (CEVd), is a member of the genus *Pospiviroid* within the *Pospiviroidae* family. CEVd is the causal agent of exocortis disease characterised by bark scaling and dwarfing symptoms in sensitive citrus hosts commonly used as rootstocks and limiting crop production of the grafted commercial species. Since symptom expression takes 3-5 years to show on inoculated trifoliate orange (*Poncirus trifoliata* (L.) Raf.), the biological characterization of CEVd usually relies on the use of indicator plants. Etrog citron (*Citrus medica* L.) has been widely used for indexing purposes, bio-amplification of the viroid RNA and strain characterization.
Following infection with CEVd, Etrog citron displays a characteristic syndrome that includes severe stunting, leaf epinasty and midvein necrosis in 3-6 months. Furthermore, the effect of a severe isolate of CEVd on the gene expression of Etrog citron has been examined, using a citrus cDNA microarray approach that revealed that infection triggered important changes in chloroplast, cell wall, peroxidase and symporter activities. However the changes in gene expression found in CEVd infected Etrog citron may not be necessarily responsible for the bark scaling symptoms that are the most characteristic exocortis syndrome in trifoliate orange.
Four trifoliate orange seedlings that were graft inoculated with a severe strain of CEVd and were planted in a field plot in June 1993, developed the characteristic bark scaling symptoms, yellowing of the twigs and remained stunted as compared with the non-inoculated controls. In 2010 plant material was collected in order to accomplish a gene expression analysis using the same genome-wide 20-K cDNA microarray (previously used in Etrog citron) developed under the Citrus Functional Genomic Project (CFGP; http://www.ibmcp.upv.es/ genomics/cfgpDB/). For that, the gene-expression profiles of the CEVd infected trifoliate oranges and uninfected control ones were compared. Subsequently, Significance Analysis of Microarrays (SAM; www-stat.stanford.edu/~tibs/SAM) was performed to identify those genes differentially expressed in both conditions and lastly, they were classified with gene ontology (GO) analysis by using the Blast2GO tool (www.blast2go.org). As expected, results showed similarities and differences with those obtained for Etrog citron that could account for some of the differences found in the symptomatology.

POSTER 8

A SEMI-AUTOMATED NUCLEIC ACID EXTRACTION AND PURIFICATION PROTOCOL FOR CITRUS TISSUE

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We developed a semi-automated, high throughput, RNA extraction and purification procedure optimized for citrus tissue. The system utilizes the SPEX SamplePrep's Cryo-station and Geno Grinder 2010, and the Applied Biosystems' MagMAX™ Express-96 along with a modified 5x MME-96 Viral RNA Isolation Kit. The detection of viral RNA with reverse transcription quantitative real time polymerase chain reaction (RT-qPCR) requires high quality RNA as defined by concentration, purity, and integrity. RNA concentration and purity were assessed by spectrophotometry at 260 nm, and 260/280 and 260/230 ratios, respectively. The RNA concentration of 304 citrus samples ranged from 14.4 to 886.0 ng/µl with an average of 180.082 (±135.784, n= 304). The majority of the samples (96.1%) had concentrations ≥50ng/µl while 89.1% of the samples had concentrations of 50-400 ng/µl. The RNA purity ratios were higher than the desirable 1.8 (low protein contaminants) for all samples tested with a mean value of 2.404 (±0.272, n= 304) while 98% of the samples had 260/280 ratio ≥2.0. The 260/230 ratios had a broader variation in comparison to the 260/280. The 260/230 ratio mean value was 1.883 (±0.657, n= 304). The majority of the samples (62%) had the desirable 260/230 ratio of 1.8-2.0 (low polyphenols or polysaccharides contaminants). The remaining 27% of the samples had 260/230 ratio of 1.0-1.7 and the remaining 11% had 260/230 ratio <1.0. Subsequent experimentation with adjusted grinding and washing buffers significantly improved and standardized the 260/230 ratios to the desirable values of 1.8-2.0.The RNA integrity of 23 samples was evaluated by 118 RT-qPCR reactions targeting the mRNA of the NADH dehydrogenase citrus gene. The mean Ct value of the RT-qPCR was 21.948 (±3.064, n= 118) with maximum and minimum values 28.5 and 16.29, respectively. The cost of supplies for the presented RNA extraction and purification procedure was estimated at US$4.03 per sample.

POSTER 9

DEVELOPMENT OF A REVERSE TRANSCRIPTION LOOP-MEDIATED ISOTHERMAL AMPLIFICATION FOR RAPID DETECTION OF *Citrus tatter-leaf virus*

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Citrus tatter-leaf virus (CTLV) causes damage to citrus on trifoliate orange or citrange rootstocks. In this study, a reverse transcription loop-mediated isothermal amplification (RT-LAMP) was set up for rapid diagnosis of CTLV, under isothermal conditions. The whole assay procedure was completed in one tube. Six primers spanning eight distinct sequences of the target gene were designed for RT-LAMP. The gene amplification products were visualized by both agarose gel electrophoresis and the naked eye. Gene amplification products were also quantified with a standard curve generated from a scale of gene copy number plotted against time for a positive reaction with the help of a real-time turbidimeter. Specificity was validated by the absence of cross-reactions with other citrus pathogens. The sensitivity was 100-fold higher than that of conventional one-step RT-PCR, and was similar to that of quantitative RT-PCR. Therefore, in a portable device, RT-LAMP has the potential of detecting CTLV in the field.

MOLECULAR DETECTION AND CHARACTERIZATION OF *Citrus vein enation virus* IN PLANT AND APHID TISSUES

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Citrus vein enation disease is characterized by woody galls on the trunk and vein enations on the leaves of susceptible citrus species. This disease is graft transmissible and naturally spread by aphid species in a persistent manner. With the aim of identifying the putative virus associated with vein enation, small interfering (si) RNAs (21-24 nucleotides) from three different field citrus trees that tested positive to citrus vein enation by indexing and that are located in Valencia, Tenerife and Gran Canaria, Spain, were analyzed by next generation sequencing Illumina technology. Bioinformatic analysis of individual samples allowed the identification of a new *Luteoviridae* present in the three samples. Bioinformatic analysis using the CLC Genomic Workbench, Velvet, Geneious and Bowtie software allowed the reconstruction of a sequence of 5077 nucleotides, corresponding to a new viral species for which the name *Citrus vein enation virus* is proposed. Open reading frames (ORF) were identified coding for an hypothetical protein, the polymerase, the aphid transmission protein and the coat protein. Protein homology analyses for these ORFs showed similarities with *Luteoviridae* members: 44% (*Pea enation mosaic virus-1*; Acc. Num. NP_619736), 66% (*Pea enation mosaic virus-1*; Acc. Num. AAA72297), 62% (*Chickpea chlorotic stunt virus*; Acc. Num. AAY90043) and 70% (*Pea enation mosaic virus-1*; Acc. Num. AAA72298), respectively. Specific primers and a TaqMan probe based on the new sequence were designed for real-time RT-PCR detection of the agent. The method allowed the successful detection of this virus in plant material and in various aphid species, even using direct systems of sample preparation. This novel diagnostic could greatly simplify and reduce the cost of routine detection of this highly prevalent disease in certification and sanitary programs.

GENERAL RESISTANCE AGAINST SPECIALIZED FRUIT PATHOGENS THROUGH D-LIMONENE TRANSGENIC DOWN REGULATION IN SWEET ORANGE PLANTS

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It is widely assumed that fleshy fruits are involved in mediating the attraction of seed dispersal organisms and the avoidance of consumption by seed predators. It is thought that the primary function of secondary metabolites present in immature fruits is to defend them against all types of potential consumers. Changes in size, texture, taste, aroma and color occur during ripening. Frugivores include not only legitimate dispersers such as vertebrates and birds but also less appreciated but more abundant consumers of fleshy fruits, microbes. Plant volatile organic compounds (VOCs) comprise a wide diversity of low-molecular-weight secondary metabolites, including terpenoids. In general, flowers and fruits release the widest variety of VOCs, with emission rates peaking before pollination and at ripening. Sweet orange fruits accumulate mainly terpenoids in mature peel (flavedo) oil glands, and D-limonene accounts for about 97% of their content. In nature, D-limonene content is usually low in orange fruits during the 2 to 3 months post-anthesis; it then drastically increases when the fruit is still green but contains seeds and remains at a high level until the fruit becomes fully mature. To investigate the role of VOCs in mature fruit interactions with specialized pathogenic microorganisms, we have generated transgenic orange plants carrying a D-limonene synthase gene in antisense (AS) configuration. Transgenic expression caused a dramatic decrease in the accumulation of D-limonene in fruit peels, being about 80-100 times lower in AS samples than in empty vector (EV) transgenic ones. A global gene expression analysis of these fruits linked the decrease of D-limonene to the upregulation of genes involved in innate immunity. Additionally, this caused the activation of J jasmonic acid signalling and metabolism upon challenge with different economically important fungal and bacterial pathogens, which led to strong general resistance against *Xanthomonas citri* subsp. *citri*, *Penicillium digitatum* and *Phylloctista citricarpa* in AS orange peels, indicating that D-limonene and related terpene accumulation not only attract legitimate seed dispersers but also facilitate infection by specialized microorganisms.

THE FRONTIERS OF THE IOCV: THE QUESTIONS ARE CHANGING

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I will attempt to define some of the frontiers of CTV and other graft transmissible diseases of citrus. Considerable progress has been made in the last couple of decades. However, the result may be more the defining of new questions than an understanding of the disease processes. What are the relevant questions that are evolving? What are the coming opportunities?

SPREAD OF *Citrus tristeza virus* IN A TRIAL PLANTED IN CONCORDIA, ARGENTINA, USING TRANSGENIC ROOTSTOCKS POTENTIALLY RESISTANT TO TRISTEZA

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Citrus tristeza virus (CTV) causes one of the most devastating diseases of citrus worldwide inducing the death of sweet orange, mandarin, lime and grapefruit trees budded on sour orange. The availability of a CTV-resistant rootstock with the sour orange attributes of productivity, fruit quality and tolerance to abiotic stresses would be a major benefit to the citrus industry worldwide. The objective of the field trial was to evaluate the response to CTV of 10 sour orange (*Citrus aurantium* L.) transgenic lines carrying CTV-derived sequences. They were obtained in the laboratories of IVIA, Spain and planted at the INTA Experiment Station in Concordia, Argentina where CTV is endemic and efficiently transmitted by the brown citrus aphid (*Toxoptera citricida* Kirkaldy). Rooted cuttings of transgenic sour orange lines were budded with non-transgenic and virus-free Valencia Late sweet orange (*C. sinensis* Osb.). Valencia trees budded on tolerant rootstocks as well as on non-transgenic sour orange were planted as controls. Trees were planted in a complete randomized design with two trees per plot and 5 replications. Every six months imprints were taken to determine the progress of CTV infection in each tree. Based on direct-immunoprinting-ELISA, differences in disease progress were observed till June 2012 on the different transgenic rootstocks. By December 2012 the percentage of diseased trees was over 80%. The sudden increase in disease progress in the last semester could be due to post-freeze effects. Four years after planting, almost 100 % of the trees are CTV infected, showing stunted growth and yellowing of foliage. Trees from each transgenic line were grouped according to symptom severity in the field. The better looking trees were those of two of the ten transgenic lines carrying CTV-derived sequences.

MOLECULAR IDENTIFICATION OF CTV GENOTYPES FROM NORTH WESTERN ARGENTINA

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- IOCV sponsored student presentation

Citrus tristeza disease was reported in Northeast Argentina in 1930 and in the Northwest in 1947. Later, millions of citrus trees on sour orange died from quick decline in both citrus regions. The most efficient vector, *Toxoptera citricida* and other aphids are present and consequently, the disease is endemic. Nowadays, citrus varieties are only grafted on tolerant rootstocks. Independent of rootstock, grapefruit is affected by stem pitting, and disease expression is severe in some selections. Biological characterization of *Citrus tristeza virus* (CTV) isolates from Northwest Argentina has been carried out since 2008 in the Centro de Saneamiento de Citrus of the EEAOC although molecular identification of isolates has not been performed thus far. In order to identify isolates, a reverse-transcription polymerase chain reaction (RT-PCR) was performed. Five sets of genotype-specific CTV primers within the open reading frame (ORF)-1a of well recognized genotypes (T30, T36; B165; T3 and VT) were used for characterization. CTV isolates were collected from C*itrus limon,* C. *sinensis,* C. *paradisi,* C. *reticulata* C. *reshni,* C. *latifolia,* C. *macrophylla, Poncirus trifoliata and Troyer citrange (C. sinensis x P. trifoliata)* according to the following criteria: species or cultivars of the source tree, visual symptoms on the source tree, and symptom expression in greenhouse tests with Mexican lime (*Citrus aurantifolia*), Pineapple sweet orange (*C. sinensis*), sour orange (*C. aurantium*) and Duncan grapefruit (*C. paradisi*) indicator plants. Most of the source trees showed no remarkable symptomatology in the field tree. Of the five CTV genotypes analyzed, severe genotypes were widely distributed, whereas mild isolates were detected at a very low incidence. The genotypes T3 and VT were predominant in mixed infections, independent of host species and variety. Data obtained are relevant because they complement existing information for CTV biological diversity in Northwest Argentina. This is the first characterization and classification of northwestern CTV isolates.

MOLECULAR CHARACTERIZATION OF *Citrus tristeza virus* ISOLATES FROM HUNAN PROVINCE IN CHINA

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Twelve *Citrus tristeza virus* (CTV) isolates, collected from 5 different orchards in Hunan, China, were simultaneously characterized by RT-PCR of the p23 gene, capillary electrophoresis of single strand conformation polymorphisms (CE-SSCP) of the p23, p25 and p27 genes as well as multiple molecular marker analysis (MMM) with four standard isolates, namely T3, T30, T36, and VT. Results from RT-PCR of the p23 gene indicated that all the isolates were virulent. CE-SSCP and MMM characterization revealed high levels of genetic diversity among the isolates ranging from single genotype infections to highly mixed infections. Out of 12 isolates, 11 contained the T3 genotype, three being single T3 genotypes, two contained T3+VT and six had T3+VT+T36+T30. The remaining isolate was the VT+T36+T30 genotype. T3 and VT, reported to be virulent genotypes, are widely distributed in Hunan Province. As the isolates were from trees showing stem pitting, the single T3 profile of CE-SSCP was likely to be related to stem pitting. Further confirmation is to be performed with more samples showing obvious stem pitting symptoms. In young orchards, single genotype isolates are usually detected, and mixed ones are found in old orchards. Diversity was observed among the isolates within one orchard and between different orchards. The characterization by the three different molecular methods resulted in consistent results with some inconsistency among different methods. In the latter case, sequencing should be conducted for further characterization.

GENOTYPING CTV ISOLATES BASED ON QUADRUPLEX RT-PCR AND MICROARRAY HYBRIDIZATION BY USING THE *InCheck* PLATFORM

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Despite millions of trees being indexed by ELISA, Citrus tristeza disease continues to spread worldwide, confirming that quarantine restrictions, eradication, and tristeza-free propagation material are not enough to combat the virus once it becomes established in an area. Reports from all over the world show that several destructive isolates of CTV, not dependent on sensitive rootstocks, may suddenly appear as a result of rearrangements or mutations of the genome. Also, it appears that bio-indexing on indicator plants or other official equivalent methods cannot help to limit the introduction of exotic strains, given that biological indexing is time consuming and molecular methods have a limited range of discrimination. With this in view, and thanks to the recent progress in CTV genome sequencing, we developed a fast diagnostic assay in which multiplex (4 Plex) RT-PCR combined with a sequential hybridization step on the *InCheck* Platform allows the genotyping of CTV isolates. The 44 probes were designed on the complete genome of 38 CTV representative isolates of six phylogenetic clades (VT, T36, NZ-RB, B165, T3, T30). Tests carried out with isolates from different countries were used to validate the diagnostic procedure: (i) single or mixed MMM genotypes (VT+T3 and T3) inducing seedling yellows (SY) and stem pitting (SP) on sour orange and grapefruit; (ii) VT+T3 and VT genotypes inducing SY/SP on sweet orange; and (iii) mild isolates, with T30+T3 or T30 genotypes. Quadruplex primers Qua1 and Qua2 targeting eight genes, and, coupled with the panel of specific probes, after the hybridization step, resulted in signals of VT-like group probes for SY isolates and RB probes for isolates inducing SY and SP. T30-like isolates from asymptomatic as well as combined infections were also detected. According to the results the detection and hybridization process is easy, rapid and accurate and can also be run by someone with no background in biology. With such potential it could dramatically increase the capability of diagnostic laboratories and contribute to minimizing the impact of new emerging CTV strains.

POSTER 10

ALL PHLOEM CELLS ARE NOT CREATED EQUAL: TISSUE SELECTIVITY DURING *Citrus tristeza virus* INFECTION

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To establish a systemic infection, *Citrus tristeza virus* (CTV) particles move from the site of inoculation to sink tissues in the roots and young flush, then into older tissues. For most strains, this process is inhibited in resistant species such as *Poncirus trifoliata*. We examined the determinants of host selectivity by infecting a range of species, from susceptible *C. macrophylla* to selective *C. reticulata* and *C. sinensis x P. trifoliata*, with the CTV strains T36 and T68, as well as seven T36-T68 hybrids covering the 3' ORFs and the L1/L2 protease domains. The roots, stem, and young flush tissue of inoculated plants were tested by ELISA at three month intervals, which showed that while all isolates tested could readily infect *C. macrophylla*, the selective hosts restricted infection to the roots for T36 and 6 of 7 T36-T68 hybrids. In Swingle citrumelo, isolate T68 and hybrid 1390 were present in the stem tissues, while T68 alone was able to move out of the roots of Carrizo citrumelo. This suggests that systemic infection requires specific variants located in the 3' end of the genome and raises an important question: why are the roots of selective species susceptible to infection by any isolate?

POSTER 11

Citrus tristeza virus POPULATION STRUCTURE DOES NOT ALTER SYMPTOM ONSET AND SEVERITY

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Unlike most citrus infecting viruses, *Citrus tristeza virus* (CTV) possesses a number of distinct strains that produce a range of disease syndromes on different host species. In addition, these strains are frequently found in mixed populations within a single host. While we have some knowledge of the symptoms produced by a single strain, there is little understanding of how combinations of strains affect symptom expression and disease severity. To test the effect of population structure on symptom expression, we inoculated *Citrus paradisi* and *C. aurantii* with a range of single strains and mixed field populations of CTV. Seedling yellows symptom development was assessed six months post-inoculation. Real-time qRT-PCR was used to assess relative population titre, and these data were compared with symptom severity. We found that there was no obvious correlation between the dominance or presence of specific isolates in the population and the incidence or severity of seedling yellows; there was also no correlation between total viral load within infected tissue and symptom severity. These data further suggest that symptom severity is not due to quantitative differences between strains in a population, but indicates that qualitative differences within and between each population influence symptom development.

POSTER 12

GENERATION OF TRANSGENIC *Citrus macrophylla* TOLERANT AND OR RESISTANT TO *Citrus tristeza virus*

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Citrus tristeza virus (CTV) is the cause of an important and devastating citrus disease with great economic loss to some citrus species in certain regions of the world. In Chile, the disease has spread throughout the entire national citrus zone. Tthe major damage has been caused in the northern region where isolates capable of causing severe stem pitting have been detected. The CTV genome is close to 20.000 bp and encodes three suppressors that block the natural defence strategy of the plant, the “silencing mechanism”. This research work proposes a strategy to generate transgenic rootstocks resistant or tolerant to the disease through post-transcriptional gene silencing (PTGS) in which the silencing signals will move from rootstock to scion preventing future infections. We have developed two hybrid genetic-constructs CTV-p25-p20 (p25-3´region - p20-5´region) and CTV-p20-p23 (p20-3´region - p23-5´region) based in the T36-strain. These transgenes were synthesized and integrated in hairpin mode (sense-intron antisense) in the pHellgate12 plasmid. These constructs were used in transformation of *Citrus macrophylla* in order to obtain rootstocks resistant or tolerant to CTV. The methodology employed was the transformation of epicotyl tissue; the transformation was mediated by *Agrobacterium tumefaciens,* strain EHA105. The transformed explants were incubated at 25ºC in co-cultivation medium in the dark for 72 hrs and then the explants were regenerated in a medium with kanamycin selection. We have obtained transgenic shoots that show transgene integration into the plant-DNA. In the near future these shoots will be grafted with a CTV infected Lime (*Citrus aurantifolia*) scion to challenge the transgenic rootstocks and evaluate the silencing process in the scion.

POSTER 13

MOLECULAR CHARACTERIZATION OF C*itrus tristeza virus* ISOLATES FROM THE PHILIPPINES

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Twenty four samples from non-symptomatic Pummelo trees as well as trees expressing virus-like symptoms such as inverted leaf cupping and/or stem pitting were collected randomly across citrus-growing regions in the Philippines between 2012 and 2013. Samples were propagated on calamandarin (*Citrofortunella microcarpa* x *Citrus reticulata*) rootstock and maintained under standard greenhouse conditions at the Bureau of Plant Industry in Davao City, Philippines. Pummelo budwood obtained from the greenhouse propagations was shipped to the University of California, Riverside under permit. Total RNA was extracted and analyzed by reverse transcription polymerase chain reaction (RT-PCR) using primers targeting the major coat protein (CP) gene of the *Citrus tristeza virus* (CTV). All samples from Luzon Island tested negative for CTV while eleven samples from Mindanao and Visayas Island were CTV positive. The CTV genotype of the positive samples was determined using multiple molecular markers targeting different CTV genome regions. The predominant CTV genotype identified was VT in single or mixed infections with T30. A single sample contained the T36 CTV genotype. Preliminary phylogenetic analysis based on the full length sequence of the CP gene indicated that ten Philippines CTV isolates with VT and VT+T30 genotypes clustered in one clade closely related to VT isolates from China (AT-1 and CT14A) and Japan (NUagA). The single Philippines isolate with a T36 genotype clustered with CTV isolates from India (Kpg 3), Hawaii (HA18-9 and HA16-5), and Taiwan (Pum/SP/T1).

SOUTH AFRICAN *Citrus tristeza virus* CROSS-PROTECTION AND SOURCE CHARACTERISATION.

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To minimize losses in the local citrus industry, the South African Citrus Improvement Scheme (CIS) implemented cross-protection using mild CTV sources to reduce the effect of challenges by endemic severe CTV strains. This management strategy also referred to as “pre-immunisation” or “mild strain protection” was implemented by the CIS at its initiation. The use of cross-protection in South Africa has been mostly successful, but cases of cross-protection breakdown have been experienced and a change in the pre-immunising source for grapefruit cross-protection was made to address this.
A number of countries apply cross-protection and report diminished expression of disease and improved production including Australia, Japan, Brazil, Argentina, Peru and South Africa. All pre-immunisation sources used for cross-protection, except the South African sources, are single variant sources.
CTV is a complex of strains. This insight and the subsequent development of diagnostics for genotyping enabled the analysis of mixed populations. We have expanded on a published CTV genotype testing system and have tested various maintenance sources of the GFMS12 and GFMS35 pre-immunisation sources at 3 different institutions including grapefruit mother trees maintained at the Citrus Foundation Block. Also segregation of genotypes is noted in different multiplications of the two sources and this is in all probability a contributing factor to cross-protection breakdown in the field.

THE COMPLETE GENOME OF TWO ITALIAN ISOLATES OF *Citrus tristeza virus* OBTAINED THROUGH DEEP SEQUENCING OF SMALL RNAs

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Bio-indexing and characterization by CE-SSCP, MMM and phylogenetic analysis of representative samples collected in a highly infected *Citrus tristeza virus* area of Sicily reveals that two main groups are present: one includes isolates inducing severe seedling yellows and a second is asymptomatic on sour orange. Severe isolates cluster in a single subclade within the group of seedling yellows and stem pitting isolates (VT-like); mild ones are similar to T30-like isolates. In order to investigate the phylogenetic relationship of the CTV population with the isolates of respective clades we undertook the complete genome analysis of two of them, namely SG29 and Bau282.
SG29 is a severe isolate, from Sanguinello sweet orange, inducing seedling yellows on sour orange and, rarely, stem pitting on Duncan grapefruit, but not on sweet orange. Bau282 is from sweet orange TDV and host susceptibility showed it is asymptomatic on sour orange and Duncan grapefruit. The genomes were obtained after sequencing of the small RNAs and assembly of overlapping sequences by reference alignment from libraries sequenced by Next-generation platforms. The viral small interfering RNAs found were in the predominant 22 and 21 nucleotide-size classes. The complete SG29 and Bau282 genome in length are 19,259 bp and 19,250 bp, respectively, with 12 open reading frames (ORFs), structurally identical to the other known CTV isolates. Phylogenetic analysis based on 31 full CTV genomes showed that SG29 clustered with the “Asian” VT-lineage in which T318A (Spain), AT-1 (China), Nuaga (Japan) and CT11A (China) isolates segregate and has the highest homology identity with T318A (98.4 %) and AT-1 (97.4%). Bau282 clustered within the mild isolates T30 (Florida) and T385 (Spain) and BLAST analysis showed a very high identity equal to 99%.

MOLECULAR CHARACTERIZATION OF CTV ISOLATES IN URUGUAY: A PICTURE FROM THE PAST COMPARED TO PRESENT

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- IOCV sponsored student presentation

Since its appearance two centuries ago, Tristeza has been classified as the most devastating viral disease which affects citrus worldwide. Its causative agent, *Citrus tristeza virus* (CTV), is transmitted by infected grafts or by insect vectors such as the aphid *Toxoptera citricida*. In all affected citrus growing areas, the existence of genetic variants of the virus with different degrees of severity has been reported. Characterization of CTV isolates can provide epidemiological information and can be useful for disease control. The presence of CTV and its efficient vector has been known in Uruguay since the 1940s. However, there is no data based on molecular biology reflecting genetic variants circulating in the country. In the present study, using RT-PCR amplification of three regions of the CTV genome (p20, p23 and p25), we established phylogenetic relationships of the strains in the country at the present time and 20 years ago. The samples used were collected in 1990 and maintained *in planta* in vector free greenhouses, and also were compared with current field samples. This valuable historical collection provides a sample of past CTV occurrence in Uruguay. The results show that circulating strains in the country are severe and unsurprisingly resemble strains reported in Argentina. However, some of the strains under study are similar to reference strains from Israel or Hawaii. This may reflect the introduction of infected buds or trees in the past.

DEEP SEQUENCING ANALYSIS OF TOTAL RNA FROM CSD DISEASE CITRUS-TREES: THE CTV AND CSDaV PICTURE.

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Citrus Sudden Death (CSD) remains a challenge for citrus production in non-irrigated areas in the North and Northwest regions of Sao Paulo State, Brazil. Its incidence has increasingly affecting the drought-tolerant but CSD-susceptible 'Rangpur lime' rootstock. So far, since the first report of the disease in the 2000s its etiology remains uncertain, but vectored-viruses (Tymovirus and/or a CTV haplotype) have been considered as the main hypothesis (Macheroni *et al*., – J. Virol., 2005). Here we checked the occurrence of these viruses in a deep sequencing dataset of total RNA by Illumina platform run for transcriptomic studies of sweet orange. Pools of reads of Valencia graphed on Rangpur lime and Sunki mandarin, both CSD-symptomatic (-S) and also from its non-symptomatic pairwise trees (-NS) growing side-by-side were analyzed. Around 74 million reads for each experimental condition were assembled using the CLCbio platform *de novo* assembly algorithm using a CTV reference genome (GenBank AY 170468). For the NS-CSD dataset only 0.005% of reads matched with CTV virus sequences, whereas for the S-CSD dataset 0.014% of reads come from the CTV virus. However, almost three times more reads of CSD-disease trees matched with CTV. A careful comparison of CTV reads from S-CSD with NS-CSD allowed us to identify some sets of reads more frequently occurring in diseased trees, as also some CTV reads specifically occurring in the CSD diseased plants. Those genetic differences which make the CTV-reads specific for the CSD symptomatic plants were mapped through the whole CTV genome reference. Primers were designed for these specific reads and are under testing. On the other hand, using the same assembly strategy we observed only few reads matching the Tymovirus genome (GenBank NC006950) in the analyzed dataset, independent of whether they come from diseased or non-diseased trees. Despite being poorly represented, the Tymovirus's reads were shown to be genetically diverse, which was confirmed by Sanger sequencing of amplicons from five different Tymovirus' genomic regions amplified by specific primers.

MULTIPLEX RT-PCR SPANNING THE *Citrus tristeza virus* GENOME FOR ISOLATE CHARACTERIZATION BY A LAB-ON-CHIP, THE VERECHIP™.

A. Lombardo, G. Scuderi, G. Licciardello and A. Catara.

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Citrus tristeza virus (CTV) is the causal agent of the most important citrus disease and exists as numerous strains which may cause different symptoms. Due to the wide biological diversity of the virus, identification of the actual genotypes present in an area is useful in adopting adequate control strategies.
In recent years a plethora of methods for molecular characterization of the virus have been developed. Among others RT-PCR, Real-Time PCR and CE-SSCP have been adapted for discrimination of severe and mild CTV strains.
In this work, based on the fully sequenced genomes of the virus available on GenBank, we have developed two quadruplex primer sets, one for simultaneously obtaining amplicons from the 5'UTR, ORF1a, RdRp and p27 regions (the latter acts as a positive control), the other for p20, p23 and p33, and the Citrus elongation factor EF 1 alpha (as a quality control of the RNA extracted).
One Verechip™, an electrically active system that integrates a PCR module, consisting of two independent PCR reactors, and a hybridization chamber, is used for both quadruplex reactions. RNA and RT-PCR master mix are combined and loaded to the chip, which is placed in a thermal reactor for rapid end point PCR. Hybridisation mix is then added and the RT-PCR products, with sizes between 73 and 239 bp, flow into the microarray portion of the chip, which is returned to the thermal reactor. Hybridisation complete, the array is then read. The array was represented by 44 probes designed for the phylogenetic groups with type strains T36, T3, T30, VT, NZ-RB, B165 and other emerging clusters such as HA16-5. The distribution of the target genes throughout the genome and the specific probes designed on the nucleotide sequences, enables the identification of any known strain even if recombinant or in mixed infections, and is likely to distinguish new groups.

MAPPING DECLINE DETERMINANTS OF C*itrus tristeza virus*

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Historically, decline (or tristeza) has been the most devastating disease caused by *Citrus tristeza virus* (CTV), although stem pitting greatly limits production in many citrus industries around the world. Decline has been the major problem caused by CTV in Florida because fortunately severe stem-pitting isolates have been kept out so far. Decline is a man-made disease based on propagation of sweet orange, grapefruit, and mandarins on the sour orange rootstock. Although this disease can be controlled by using alternative rootstocks, there are soils in which all other rootstock choices are less desirable in terms of fruit quality and yield. One of our major goals has been to develop a way to allow growers to use the sour orange rootstock in the presence of CTV. Florida has two predominant strains of CTV, a decline (T36) strain and a non-decline strain (T30). A first goal has been to map the viral determinant that induces decline. This was done by creating hybrids by substituting T30 sequences into T36. A parallel goal was to use a non-decline T36 hybrid to cross protect trees on the sour orange rootstock from decline. This project was delayed considerably because we were not able to definitively assay decline in the greenhouse because we were unable to distinguish decline symptoms from seedling yellows symptoms with these hybrids. In order to examine decline in field trees, we had to obtain permission to do a field test of recombinant DNA produced virus hybrids from the USDA Biotechnology Regulatory Service. Valencia sweet orange on sour orange rootstocks were inoculated with T30/T36 hybrids in the greenhouse and transferred to the field in 2010. The test will end August 2013. Results of this field test will be presented showing that the p23 and 3' non-translated sequences of T36 contain the determinant of decline.

DECLINE OF CITRUS TREES IN IRAN

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There are two major citrus growing areas in Iran, the Caspian Sea belt with a Mediterranean climate and sour orange (*Citrus aurantium*) as the major rootstock and the southern region with tropical and subtropical climates. Mexican lime (*C. aurantifolia*) and Bakraee (*Citrus* sp.) are the common rootstocks of the latter region. From the beginning of 2010 a decline inducing disease appeared in fruiting trees on Bakraee rootstock in Jiroft (Southern region). In September and December 2011, 6 plots of approximately 100 trees each, were analyzed for the presence of *Candidatus* Liberibacter asiaticus, the agent of Huanglongbing (HLB) by conventional PCR using A2/J5 and OI1/OI2c primers. The survey found a 2 to 9 percent infection of HLB in symptomatic trees of all plots. In 2012, quick and slow decline symptoms were observed in sweet orange trees budded on sour orange rootstock in Sari (Caspian Sea belt). Preliminary serological analysis of the samples from symptomatic trees for *Citrus Tristeza Virus* (CTV) with Direct Tissue Blot Immmuonoassy (DTBIA) using a commercial polyclonal antibody (Bioreba), showed infection in 41 of the 46 samples.

DETECTION OF *Citrus tristeza virus* IN FOUR CITRUS SPECIES IN SOUTH-WESTERN NIGERIA

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The occurrence of *Citrus tristeza virus* (CTV) in Nigeria has been previously reported, but in order to ascertain its presence and distribution in tangelo (*Citrus reticulate* × *C. paradisii*), pomelo (*C. maxima*), sour orange (*C. aurantium*) and calamondin (*C. microcarpa*) trees, field surveys were conducted in eight orchards from three states of south-western Nigeria. A total of 98 trees were examined for stem pitting, vein clearing and leaf curling. Leaf samples were collected and RT-PCR was used to detect CTV presence using CTV-specific primers PIN1 and PIN2 directed to the conserved 3' untranslated region. Results show the presence of CTV in the three states and in all the species while symptoms differed with location. Disease incidence was highest in Ogun state (68.9%) followed by Osun state (57.1%) and Oyo state (48.8%). This confirms the widespread presence of CTV as a threat to citrus production.

POSTER 14

NATURAL SPREAD OF *Citrus tristeza virus* IN SICILY

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In citrus areas affected by citrus tristeza disease the knowledge of transmission rates as well as the characteristics of CTV isolates is of interest in order to predict the epidemiology of the disease and to prevent the spread of the virus. Such information is more relevant on consideration that, under field conditions, citrus species are differently susceptible to infection. The CTV population in Sicily is mainly composed by three CTV sub-populations consisting of: (i) severe and widely diffused (up to 72%), (ii) mild and homogeneous (about 17%) and (iii) mixed severe and mild CTV isolates.

We have examined the natural spread of CTV isolates in experimental plots, including commercial groves and nursery blocks in a highly CTV infected zone of Eastern Sicily, by DTBIA and ELISA followed by CE-SSCP. In a 344 tree plot of Tarocco Tapi grafted on Troyer citrange, aged 13 years and spaced 6x4m, infected tree increased from 17% to 97% in five years. At the initial stage the mild isolate was the most represented, but after five years the severe isolate predominated in the trees.

As far as nursery blocks were concerned the rootstock most susceptible to infection was *Citrus macrophylla* with up to 25% of seedlings infected, showing vein clearing and different degrees of stem pitting. Volkameriana lemon was infected up to 20% but showed vein clearing and stem pitting only when infected by severe isolates. Only a very low percentage (< 1%) of sour orange was infected, and Troyer and Carrizo citranges were not affected at all. Representative samples analysed by CE-SSCP revealed the prevalence of severe isolates inducing SY two months after inoculation on sour orange seedlings. One, despite being genotypically close, was still asymptomatic one year later and is being evaluated in greenhouse tests to investigate its ability to cross protect against severe CTV strains.

The study confirms (i) the susceptibility of *C. macrophylla* to natural transmission as already reported in other countries, (ii) the field efficiency of *Aphis gossypi* in vectoring CTV strains, shown also on greenhouse tests, (iii) the fast colonization of the host plant by severe strains.

POSTER 15

EVALUATION OF VIRUS-DERIVED dsRNA TO PROMOTE THE RESISTANCE OF CITRUS TO *Citrus tristeza virus*

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Citrus tristeza virus (CTV) is an economically important virus disease in many citrus producing areas of the world. In antiviral research, double stranded RNA (dsRNA) homologous to genes of the virus is transferred into organisms to repress virus replication, preventing infection. Here, the possibility of an RNAi construction expressing dsRNA of the CTV p23 gene to make citrus resistant to CTV was evaluated. Via the agro-infiltration transient expression system, the RNAi could be quickly transferred into a leaf and yielded siRNA in the plant. After leaves were inoculated with CTV, the results of RT-qPCR and ELISA analyses indicated that there was an inhibitory effect mediated by the dsRNA on CTV infection. This indicates the feasibility of using this construct for CTV resistance in transgenic plants. Compared to the control, the agro-infiltrated plants had normal growth without being affected by the dsRNA construct, and did not show any CTV-like symptoms. Resistance assay of the clones is currently in progress and the virus inhibition will be evaluated.

POSTER 16

QUANTITATION BY REAL-TIME RT-PCR OF CTV TARGETS IN *Toxoptera citricida* AFTER DIFFERENT ACQUISITION PERIODS

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Citrus tristeza disease caused by *Citrus tristeza virus* (CTV) is of major economic importance and spread by grafting and several aphid species. *Toxoptera citricida* (Kirkaldy) is the most efficient vector and the mode of CTV transmission by the aphid is semi-persistent. Real-time quantitative RT-PCR is a simple and sensitive method for the detection of CTV in aphids. Primer set HD-F/R was designed within a highly conservative region of *CP25*, and a SYBR Green 1 real-time RT-PCR detection system was established with optimized reaction conditions. The RT-qPCR was used to detect four CTV isolates in 15 aphids after a period of 0.5-48 h. Results showed that the mean copies of acquired CTV-targets were 10^6, the lowest 10^5 and the highest 10^7 for CT11A, CT16-2 and CT Lijian. For CT14A they were 10^5, 10^4 and 10^6, respectively. The amount of CTV in aphids reached a peak at 4 h with CT Lijian, and at 6 h with CT11A or CT16-2. However, for CT14A, there was a small peak around 6-12 h before a second peak at 24 h. The accumulation of CTV in aphids was at its highest at the end of monitoring.

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POSTER 17

GENE SILENCING OF VIRAL *24K*-GENE INDUCES UNCONTROLLED INFECTION OF *Citrus psorosis virus* (CPsV) BUT NOT OF THE UNRELATED *Citrus tristeza virus* (CTV)

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The ophoivirus *Citrus psorosis virus* (CPsV) is the causal agent of a serious disease affecting citrus trees. Post-transcriptional gene silencing (PTGS), can be induced by transgenic expression of pathogen-derived sequences encoding hairpin RNAs. Using this strategy is possible to knock down gene expression in order to study gene functions. *Citrus sinensis* plants were transformed with a hairpin construct derived from the *24k* gene (ihp24K) from the Argentine CPsV 90-1-1 isolate. Several independent ihp24k transgenic lines were generated and after regeneration, two of these lines (6117 and 6119) were propagated in the greenhouse and challenged by graft-inoculation with CPsV 90-1-1. Symptom observation and molecular analyses (RT-PCR and TAS-ELISA) were performed. Both challenged lines ihp24K were highly susceptible to the virus when assayed under greenhouse conditions showing more severe symptoms and higher viral titers than the non-transgenic control. Symptoms were manifested in successive flushes reflecting an uncontrolled and longer lasting infection in the ihp24K silenced plants.

To gain insight into the specificity of the hyper susceptibility triggered in the ihp24K silenced plants, a second assay was performed challenging 12 propagations of both ihp24K lines and non-transgenic control plants either with the unrelated closterovirus *Citrus tristeza virus* (CTV) or with CPsV 90-1-1. Symptom observation and ELISA evaluation reflected the expected severe symptoms in the CPsV inoculated transgenic plants compared to the non-transgenic control but first evaluation of the CTV inoculated transgenic plants did not show the same clear hyper-susceptibility behavior. Further evaluations along time should be conducted but these results suggest a specific mechanism triggered in the ihp24K silenced plants leading to an unregulated infection.

Studies focused on the understanding of the molecular mechanisms behind uncontrolled infection should also be run in order to unravel this putative *24k* regulation function of the viral cycle.

UNBIASED, NEXT-GENERATION SEQUENCING FOR THE CHARACTERIZATION OF *Citrus tristeza virus* POPULATIONS

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A high-throughput sequencing pipeline to characterize *Citrus tristeza virus* isolates was developed. Three alternative viral templates (total RNA, double-stranded RNA and virus particles) combined with random RT-PCR amplification were first tested on a single, previously characterized GFMS12 sub-isolate for their enrichment qualities and subjected to Illumina paired-end sequencing. Comparisons between the sequencing data obtained and gene-specific phylogenies were also made in order to test their validity. Double-stranded RNA was found to be most enriching and was utilized for further characterization of additional glasshouse-kept isolates. A novel T68-1-like South African genotype, named CT-ZA3 was assembled *de novo* and shown to be the dominant component in all GFMS12 sub-isolates tested. This study underlined the effectiveness of next-generation sequencing for genotype discovery as well as whole-genome characterization of CTV isolates to a level of detail not previously attainable with classical methods such as single stranded conformational polymorphism (SSCP) and Sanger sequencing of multiple clones, which have been shown previously to yield incongruences in genotype identification.

THE USE OF NEXT-GENERATION SEQUENCING TO STUDY VIRUS DISEASE ETIOLOGY

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Next-generation sequencing (NGS) has been established as a reliable approach to study metagenomic samples. In plant pathology, the application of NGS has not been limited to sequencing for pathogen discovery, but has also been used for applications such as the study of plant-pathogen interactions. In this study, we used metagenomic NGS to establish the virome (viral profile) of citrus samples. Double-stranded RNA was extracted from the cambium tissue of virus diseased citrus trees with a cellulose extraction protocol and sequenced in an unbiased manner using Illumina sequencing-by-synthesis technology on a HiScanSQ. Two samples were analyzed; one displayed atypical “psorosis” symptoms and the other was a source plant containing *Apple stem grooving virus* (ASGV) and *Citrus tristeza virus* (CTV). Small datasets were generated (1-3 million reads) for each sample and used in bioinformatic analysis through *de novo* assemblies and read-mapping. Assembled contigs were identified and classified according to the sequence they aligned to with the highest bit score in BLAST searches against the NCBI non-redundant DNA and protein databases. The Capillovirus, ASGV was identified in the known sample together with several CTV genotypes. However, the atypical “psorosis” sample had a more complex virome that include three viroids (*Citrus exocortis viroid*, *Hop stunt viroid*, and *Citrus dwarfing viroid*), as well as several CTV genotypes. The presence of multiple CTV genotypes was confirmed for both samples by read-mapping to full-length reference genomes. The results of this proof of principle experiment indicate that the metagenomic sequencing approach of dsRNA can be successfully implemented to establish the virome of citrus trees with an unknown virus etiology.

HLB RESEARCH IN BRAZIL – FROM ETIOLOGY TO DISEASE MANAGEMENT

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Huanglongbing (HLB) was first reported in the center of São Paulo State (SPS) in Brazil in early 2004. It was soon demonstrated that the disease was associated with the putative liberibacter species *Candidatus* Liberibacter asiaticus (Las) associated with HLB in Asia (6) and with a new putative species, '*Ca.* L. americanus' (Lam) (17). Although inducing similar symptoms in citrus, Lam and Las have shown contrasting behavior. In 2004 Lam was by far the most prevalent species, being present in 98% of all positive samples analyzed at the Fundecitrus lab (10). This indicated that Lam may have been introduced to Brazil earlier than Las. Shortly after the discovery, Lam incidence declined. Within three years Lam and Las occurred in similar proportions, and by 2012 Lam was present in less than 0.5% of the samples, in contrast to over 99.5% for Las (Wulff, unpublished data). Also, Lam has spread less rapidly than Las and remained confined almost exclusively within SPS, while over the years Las has been progressively found in all regions of SPS as well as in the States of Paraná and Minas Gerais, and in neighboring Argentina and Paraguay. However, in SPS, where regular surveys have been undertaken (fundecitrus.com.br), HLB (in this case associated mainly with Las) has progressed irregularly to and within the distinct citrus growing regions. From the center of the State, where the disease is assumed to have been first introduced to Brazil and where a higher incidence has been recorded, HLB has moved to the other regions with different speeds and intensity despite the conditions, namely proximity of citrus orchards and presence of the insect vector, *Diaphorina citri*, favoring disease spread uniformly in all directions (11). In the north and northwest HLB was first reported in 2007 and 2008, and, in these regions, as well as in the south, the incidence of affected trees has been lower than in the eastern or central regions of the State (3). The northern region is characterized by hot summers and mild winters and the south by relatively mild summers and cold winters.

Although factors influencing incidence and spread of the disease and the associated pathogens are not completely understood, research on host-pathogen-vector interactions since 2004 has generated revealing information, important not only for an understanding of the epidemiological aspects of the disease but also for improving management practices.

Initial research focused on Lam and sought to determine if pruning could be used to restore the health of a tree, thus circumventing the need to remove diseased trees (8). The work involved 592 trees and indicated that Lam would colonize a citrus tree and the rootstock rapidly, before manifestation of leaf symptoms. Eight months after pruning, symptoms were again present in 62.5% of the 376 trees in which the entire scion was removed 15–20 cm above the grafting line, and in 58.3% of the 216 trees in which only the symptomatic branch was removed, regardless of the variety of sweet orange used as scion ('Valencia', 'Hamlin' or 'Pêra'), age of the tree (3, 5, 9 or 16 years), or symptom status of the tree before pruning (symptoms only at the end of the branch or on the entire branch). The study also revealed latent infections in 7.8% of 79 caged trees which were not showing any symptoms when they were pruned and subsequently expressed HLB symptoms on new flushes. This work served as a basis for the implementation of the laws to enforce elimination of diseased trees, and

a three-pronged-system (1), crucial for success in minimizing the impact of the disease. Recent work involving only Las has provided more detailed information about citrus tree colonization by the pathogen (14). Las was detected in the roots of potted seedlings of Swingle citrumelo, Cravo Rangpur lime and Sunki mandarin, the main rootstocks used in SPS, some 50 cm below the inoculation site, 45 days after inoculation, and in the scion and roots of all 4 and 10-year-old orange trees grown on 13 distinct rootstocks.

Research subsequently focused on other aspects of the disease epidemiology: impact of ambient temperatures and graft transmission and multiplication of the pathogens in citrus and orange jasmine (*Murraya exotica* L., not *Murraya paniculata* (L.) Jack as incorrectly referred to in other publications).

The research on the impact of ambient temperatures focused on exposing Lam+ve and Las+ve trees to distinct daily temperature regimes (11). The research was motivated by the already known contrasting responses to ambient temperatures of plants affected by Las or *Ca*. L. africanus (5,16) and by the complete lack of information on this subject for Lam. After a series of growth chamber experiments it was demonstrated that Lam is more heat sensitive than Las. Fully symptomatic orange trees affected by Lam exposed to daily regimes of 27 to 32°C, 24 to 32°, or 35 to 38ºC for 60 days were totally cleared of symptoms and of the pathogen, while fully symptomatic trees affected by Las were only partially cleared of symptoms and the pathogen (Las titer decreased from average 107 to 103 cells per gram of tissue) only when exposed to 24 to 38ºC for the same duration. More recently it was shown that this same temperature regime leads to a decline in Las titers in new flushes on symptomatic branches, an impact which would lead to a significant reduction in pathogen acquisition rates by the insect vectors feeding on them (15). Although field work will add important information on this aspect of the HLB pathosystem, data so far accumulated indicate that high summer temperatures may restrict rates of spread of the disease and help to explain the irregular dissemination patterns of HLB in SPS. Field and greenhouse experiments involving even higher temperatures (40 to 60°C) for different durations were also conducted, with the aim of curing Las+ve trees, but with limited success (13). The reasons for the limited success were apparently related to the sensitivity of the citrus tree to high temperatures and to the ability of the pathogen to survive in roots. The temperature-time combinations necessary to kill the bacterium were apparently close to those that would kill a citrus tree, and in the roots, the bacterium remains protected from heat.

Studies on graft transmission of Las and Lam were conducted with the objective of comparing graft transmission efficiencies and the ability of both bacteria to multiply, individually or simultaneously, in potted Valencia, Hamlin, Pera, and Natal (the main sweet orange varieties grown in SPS), under conditions favorable for disease development (9,10). Lam was less efficiently transmitted and less able to multiply in citrus leaves of all sweet orange varieties (10). The percentage of plants that became infected varied from 10.0 to 23.3% for Lam and 66.7 to 73.3% for Las, and the cycle threshold values (Cts) varied from 24.14 to 24.97 for Lam and 19.42 to 20.92 for Las. These Cts corresponded to average 106 and 107 cells per gram of tissue for Lam and Las, respectively. Similar values were obtained also when field samples, collected from three distinct regions of SPS, were analyzed (unpublished data). No apparent effect of one species over the other was observed in plants inoculated simultaneously with both pathogens. Lower titers of Lam appear to be the main factor explaining its conspicuous decline over the years in SPS. Lower titer would reduce the chances of pathogen acquisition by the insect vector and its consequent

transmission to healthy trees, in a pattern similar to the one observed for Las in new flushes exposed to heat (see above). This work also showed that, contrary to Lam+ve plants, Las+ve plants harbored the bacterium at titers (6.75 log Las cells/g tissue) close to maximal values, three months before symptom expression, an indication that asymptomatic trees may be serving as a source of inoculum, contributing to dissemination of HLB in the field.

The research on orange jasmine aimed at (i) determination of the distribution, based on sampling at 76 urban locations over two time intervals, of orange jasmine trees infected by Lam or Las, and (ii) determination of levels of genetic and pathogenic similarities among the orange jasmine and citrus liberibacters, based on sequences of the rplJ gene and on cross inoculation experiments (12). The work was motivated by the detection of Lam in a single mature orange jasmine tree growing in front of the manager's house in the citrus farm most affected by Lam in 2004, by the detection of Las in 2005 in orange jasmine trees growing in urban areas and, more importantly, by suspicion that infected *M. exotica* trees may play an important role in the HLB epidemics. In the years 2005/2006 Lam was detected in 56 (11.7%) and Las in 2 (0.4%) of the 477 orange jasmine trees from 10 locations and, in 2009, Lam was detected in an additional 5 (1.6%) and Las in 28 (9.1%) of the 309 orange jasmine trees from seven locations. Lam titers were higher in Lam+ve (4.3±0.68 log cells/g tissue) than in Las+ve trees (3.0±0.92 log cells). As happens with infected citrus under favorable conditions for disease development, symptom severity was stronger on the orange jasmine trees infected by Lam than on those infected by Las. The higher symptom severity in *M. exotica* may not be related only to the higher bacterium titers in this host since in citrus, Lam reaches lower titers than Las. In Las-infected orange jasmine the infection seemed to be transient. This was observed in naturally infected field trees and in graft-inoculated plants. This work also showed that the infected orange jasmine trees were in locations relatively close to each other and, coincidently, in the area of highest incidence of HLB in citrus at that time, a clear indication of pathogen transmission from host to host by *D. citri*. Similarity among citrus and orange jasmine liberibacters, in terms of pathogenicity, could not be fully determined due to the strong tissue incompatibility observed between citrus and orange jasmine during the cross inoculation experiments. Most budwood used as inoculum died in heterologous combinations. On those plants in which the budwood survived, only Lam was successfully transmitted and the plants remained infected. Comparative analysis of the rplJ gene from the liberibacters found in orange jasmine with those found in citrus showed that Lam or Las from both hosts were identical. The importance of orange jasmine and citrus as source of Lam to citrus in SPS was investigated in further work involving the insect vector for bacterium inoculation (7). Higher Lam transmission rates occurred from orange jasmine than from citrus. As orange jasmine trees infected with liberibacter are not systematically eliminated in urban areas, and vector populations not suppressed, orange jasmine may represent a constant risk to neighboring citrus orchards. Also, since nursery production and sale of orange jasmine are not regulated (as they are for citrus), asymptomatic orange jasmine trees may be important for distributing liberibacters to distant citrus areas still free from the disease.

An overview of the HLB epidemics in Brazil, particularly in SPS, and the main research findings on the HLB pathosystem were briefly presented here. Other field work and studies (2,4), and the daily experience of the citrus growers with the disease, have confirmed the necessity of eliminating symptomatic trees and controlling the insect vector on an area-wide basis in order to optimize opportunities

for successfully minimizing the spread and impact of HLB. Although many research questions still require answers, research has provided a better understanding of the distinct patterns of spatio-temporal progress of the disease, and knowledge required for official responses and establishment of management practices. Among research outcomes, impacts of high temperatures on Las multiplication in new flushes may have some potential for the development of new, less costly and less insecticide-dependent strategies to manage HLB.

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HUANGLONGBING IN TEXAS

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Following the confirmation of Huanglongbing in Florida in 2005, surveys for the disease have been on-going in Texas, with citrus leaf samples with suspicious foliar symptoms and *Diaphorina citri* samples being subjected to qPCR analysis. By the end of 2011, 17,000 leaf samples and 38,000 psyllid samples had been tested without any detection of HLB-associated *Ca.* Liberibacter spp. In January 2012, a leaf sample collected from a 5-year old Valencia sweet orange tree tested positive by qPCR; this was confirmed by USDA-APHIS by qPCR, and conventional PCR and DNA sequencing to be *Ca.* L. asiaticus (CLas). The disease was also transmitted by grafting to healthy citrus seedlings. Subsequent delimiting surveys confirmed CLas in 56 trees in the Valencia orchard, 19 trees in a neighboring grapefruit orchard, and 3 trees in nearby residential properties. During 2012, 31 *D. citri* samples from several locations throughout the citrus production area of south Texas gave positive qPCR results, but none have been confirmed by conventional PCR and sequencing**.** Attempts to mitigate the spread include voluntary area wide psyllid control in autumn and winter, removal of infected trees, and spraying locations where qPCR positive psyllids have been found. In April 2013, one psyllid sample produced a positive qPCR result for *Ca.* L. americanus (CLam) which was confirmed by the USDA, with 98% homology to CLam from Brazil.

RECENT RESEARCH IN MANAGEMENT OF CITRUS HUANGLONGBING IN GUANGDONG, CHINA

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Citrus is an economically important crop worldwide. Citrus Huanglongbing (HLB, yellow shoot disease) is highly destructive in southern China, particularly in Guangdong and the neighboring provinces. According to literature, HLB was observed in Chaoshan area of Guangdong in the late 1890s, and the disease has remained endemic since then. The infectious nature of HLB was recognized in the 1940-1950s. The association of *Candidatus* Liberibacter asiaticus with HLB was confirmed in 1996. Based on the infectious pathogen theory, control strategies for HLB control were developed. These include clean nursery stocks, insect vector control and removal of infected trees. While elimination of the HLB pathogen is still a priority for HLB management, there is also high interest in how to improve productivity of HLB-affected trees. Current research has focused on heat and soil treatments. Heat treatment was developed in the 1950s, mainly for elimination of HLB pathogen in scions. We recently studied the effects of high summer temperature on the reduction of *Ca*. L. asiaticus titers in HLB-affected citrus trees in field. Heat treatment was delivered via covering a tree with a temporary enclosed tent of plastic sheeting, allowing natural sunlight to raise ambient temperature. After treatment three times in summer, significant reductions of *Ca*. L. asiaticus titers in the treated trees were observed in October, November, and December. In soil treatment, a soil conditioner rich in P, N, K, Mn, and organic matter was added to soil surrounding HLB-affected mandarin citrus trees. We observed that HLB trees showed more vigorous growth and lower titer of *Ca*. L. asiaticus two months post treatment. Titer reduction was most obvious 7 months post treatment. However, the titer of *Ca*. L. asiaticus resurged ten months after treatment. We also observed an increase in fruit yield and quality with the soil conditioner treatment.

HUANGLONGBING PREVENTION IN THE CANARY ISLANDS AND MAINLAND SPAIN: SURVEYS OF *Candidatus* Liberibacter SPECIES AND *Trioza erytreae*

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The economically and socially important Spanish citrus industry (about 310,000 ha) is totally based on pathogen-free certified cultivars locally produced. In spite of this, Huanglongbing (HLB) is considered a serious threat, preventive methods being essential to maintain the HLB-free status. With this purpose extensive surveys and routine analyses were conducted in 2009, 2012 and 2013 in the Canary Islands (Spain) where *Trioza erytreae* was reported in 2002 (close to 9,300 trees inspected, 535 analyzed and 783 *T. erytreae* tested). In addition, surveys were also performed in the main citrus growing areas in mainland Spain. In Comunidad Valenciana (180,000 ha) annual surveys include 100 sampling points in selected orchards and in surrounding areas of 3 ports, 2 airports and 20 packinghouses. In all these localized points arthropods caught on yellow sticky traps are identified monthly and citrus trees are visually inspected for HLB symptoms (62,500 trees inspected, 385 analyzed). Suspicious HLB-symptomatic leaves were directly printed on paper membranes during the field inspection and in the Canary Islands *T. erytreae* specimens were directly squashed. Samples were analyzed by real-time PCR using a commercial kit (HLB/100, Plant Print Diagnostics, www.plantprint.net). Neither the analyzed citrus samples nor psyllid species tested positive for '*Ca*. Liberibacter' HLB-species. *T. erytreae* was not found in mainland Spain. Implementation of surveys in all Mediterranean countries and legislation for eradication should be a priority for HLB prevention.

INSIGHTS INTO HUANGLONGBING-*Phytophthora* INTERACTIONS

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Huanglongbing (HLB), caused by *Candidatus* Liberibacter asiaticus (*Las*), is well known to be a destructive disease of citrus that results in losses of fruit quality and quantity, canopy dieback, and tree decline. Recently, early *Las* infection of roots and associated root loss has been identified. The role of root infection in disease development has focused attention on the potential for interactions of HLB with soil-borne pathogens and pests. Concurrent with the spread of HLB through Florida citrus groves an unexpected rise in populations of *Phytophthora* spp., especially *P. nicotianae* (*Pn*), was observed in a statewide survey during unfavourable environmental conditions (i.e., periods of exceptional drought). A greenhouse study demonstrated that HLB induces a rapid increase in *Pn* propagule counts in the soil, but that the interaction is observed only until HLB drastically reduces root mass, confirming most recent field population results. The presence of *Pn* at the time of inoculation caused a significant shift in *Las* colonization to the root system associated with a delay in foliar symptom development. Hence, damage to the root system caused by *Las*-*Pn* interactions may reduce stress tolerance of infected trees before appearance of HLB symptoms. Fourteen months after inoculation, the HLB-*Pn* interaction reduced total leaf area which could limit the total photosynthetic capacity of the tree, exacerbating the disruption of carbohydrate supply. Although both HLB and *Pn* reduced fibrous root mass, no interaction was observed. Based on preliminary results, *Las*-*Pn* interactions are likely to increase the rate of decline of HLB-affected trees.

POSTER 18

THREAT OF HUANGLONGBING IN ARGENTINA

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Huanglongbing (HLB), probably the most destructive disease of citrus, is a threat to the citrus industry worldwide. Detection of *Candidatus* Liberibacter species associated with HLB is essential for the control and management of the disease. In 2009, the Ministry of Agriculture, Livestock and Fisheries of Argentina implemented a National Program for Prevention of HLB in order to protect the citrus industry from the disease. The actions carried out by the program are the control of borders and domestic roads to prevent the spread of the HLB vector (*Diaphorina citri* Kuwayama) to psyllid-free regions and a survey of citrus orchards, nurseries and trees in public spaces. Plant material with suspicious HLB symptoms and psyllids (adults, 4th and 5th instar nymphs) collected in the surveys is analyzed by real time PCR (qPCR) with Taqman probes for the presence of *Ca.* Liberibacter asiaticus (LAS) and *Ca.* Liberibacter americanus (LAM) in laboratories located in the citrus regions and in Buenos Aires city. Under the HLB Prevention Program to date, all psyllid samples have been analyzed with negative results for the presence of LAS and LAM while 17 plant samples from the Province of Misiones were positive for LAS.

POSTER 19

EVALUATING SWEET ORANGE CLONES FOR GREENING RESISTANCE

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Citrus greening disease remains a threat in the cooler citrus production areas of southern 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 on diseased fruit. The embryos were obtained from wide, asymptomatic sections of symptomatic fruit and cultured on Murashige and Tucker medium. Once the clones had developed sufficiently, they were challenged with *Candidatus* Liberibacter africanus by means of the Triozid insect vector in the laboratory. Two clones remained negative after challenges and a third had a low percentage infection. The ultimate evaluation will be in the field where they are exposed to repeated natural infection by the insect vector. Trees of the three clones were planted in November 2007 amongst old trees with greening symptoms. This report is on their current status regarding greening infection as well as growth, production and fruit quality.

POSTER 20

CHEMICAL OR TRANSGENIC ENHANCEMENT OF SYSTEMIC ACQUIRED RESISTANCE (SAR) DOES NOT REDUCE HLB DISEASE DEVELOPMENT

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The objective was to evaluate soil-applied SAR inducers or transgenic enhancement of SAR expression for effect on HLB disease progress in citrus trees challenged by graft-inoculation or psyllid-mediated infection. One yr-old Hamlin sweet orange trees were planted in May 2009 and treated as follows: 1) non-treated check (UTC), 2) foliar insecticide to control psyllids, 3) soil-applied imidacloprid/thiamethoxam (IMID/THIA) to induce SAR, 4) soil-applied IMID/THIA plus foliar insecticides, 5) graft-inoculated UTC, 6) graft-inoculated with IMID/THIA. A randomized block design was used with 5 repetitions of 10 tree plots. In 2010, the SAR inducer acibenzolar-S-methyl (ASM, Actigard 50WP) which does not control psyllids was substituted in treatments 3, 4 and 6. At 24 months after treatments were established in a field site, 105 trees were PCR+ (35%) in the trial. Higher numbers of PCR+ trees occurred in the UTC, the UTC with graft inoculation, and the IMID/THIA/ASM with graft-inoculation. A lower number of PCR+ trees occurred in the treatments with SAR inducers, foliar insecticides, and foliar insecticide plus SAR inducers, but no effect of SAR treatment on rate of HLB disease progress was detected. Transgenics of Duncan grapefruit and Hamlin trees were constructed that expressed the NPR1 gene from Arabidopsis (AtNPR1), a key positive regulator of SAR. Over-expression of AtNPR1 in transgenic lines of these susceptible hosts reduced citrus canker lesions. Resistance to *Xanthomonas citri* subsp. *citri* infection was related to expression levels of AtNPR1. Two lines each of ‘Duncan’ grapefruit and Hamlin with the highest expression of AtNPR1 were screened in a greenhouse containing HLB-infected plants and psyllids. Rate of infection progress over 12 months was similar for transgenic lines and non-transgenic controls. Again, an effect of SAR on HLB disease progress was not-detected which confirmed the ineffectiveness of enhanced SAR for HLB disease control.

POSTER 21

POPULATION DYNAMICS AND SEASONAL FLUCTUATION IN THE PERCENTAGE INFECTION OF *Trioza erytreae* WITH *Candidatus* Liberibacter africanus, IN AN ORCHARD FULLY INFECTED WITH AFRICAN GREENING

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Trioza erytreae (Del G.) (Hemiptera: Triozidae) is in itself a minor pest of citrus, but its significance is attributed to its ability to vector the citrus African Greening disease pathogen, *Candidatus* Liberibacter africanus. The population fluctuation of the citrus triozid is correlated to the flushing rhythm of the citrus host, but seasonal fluctuation in infection of populations carrying the Greening pathogen have been difficult to monitor in the past due to limited detection methodologies. This study explored this fluctuation in infectivity in an orchard entirely infected with African greening by using PCR to test individual triozids caught on sticky traps placed weekly in the orchard. Two season's data are presented from a small sour orange orchard in the Nelspruit district in South Africa. The triozid population fluctuations correlated to previous findings of population peaks following the citrus flush cycles and responses to specific climatic influences. Fluctuations in the percentage infectivity of the *T. erytreae* populations were observed, with infectivity peaking at or just after the citrus flush seasons, but with peaks in infectivity differing to population peaks.

TEMPORAL PROGRESSION OF *Candidatus* Liberibacter INFECTION OF CITRUS AND TRANSMISSION BY ADULTS OF *Diaphorina citri*

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Over the last decade the plant disease Huanglongbing (HLB) has emerged as a primary threat to citrus production worldwide. HLB is associated with infection by one of a group of phloem-limited bacteria (*Candidatus* Liberibacter spp.) that are readily transmitted by the Asian citrus psyllid, *Diaphorina citri*. However, the temporal progression of infection, time to symptom expression (i.e. latent period), and how they relate to transmissibility remains unresolved. We graft inoculated sweet orange trees with *C.* Liberibacter asiaticus, then at different times after inoculation, we inspected plants for HLB symptoms, measured bacterial infection levels (i.e. titre or concentration) in plants, and measured acquisition by psyllid adults that were confined on the trees. Plant infection levels increased rapidly over time, saturating at uniformly high levels (approximately 10^8 CN per g of plant tissue) near 200 days after inoculation – the same time at which all infected trees first showed disease symptoms. Pathogen acquisition by vectors was positively associated with plant infection level and time since inoculation, with acquisition occurring as early as the first introduction 60 days after inoculation. These results suggest that there is ample potential for psyllids to acquire the pathogen from trees during the asymptomatic phase of infection. If so, this could limit the effectiveness of tree rouging as a disease management tool and would likely explain the rapid spread observed for this disease in the field.

COMPARISON OF GENE EXPRESSION CHANGES IN SUSCEPTIBLE, TOLERANT, AND RESISTANT HOSTS IN RESPONSE TO INFECTION WITH *Citrus tristeza virus* AND HUANGLONGBING

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The diseases Huanglongbing (HLB) and citrus tristeza are both phloem limited and have significant economic impact on citrus production wherever they are found. Studies of host resistance have indicated that *Poncirus trifoliata* has tolerance/resistance to both diseases, suggesting there may be some common factors in the two kinds of resistance. We have conducted studies of host gene expression changes that occur in response to infection to gain further insight. Controlled inoculation by grafting infected budwood was used to infect potted greenhouse plants of Cleopatra mandarin (*Citrus reticulata*), US-897 (*C. reticulata* x *P. trifoliata*), and US-942 (*C. reticulata* x *P. trifoliata*) with CTV and with *Candidatus* Liberibacter asiaticus, the pathogen associated with HLB. Stem and leaf tissue was collected at 10, 20, and 30 weeks after inoculation. DNA-free RNA was subjected to real-time one-step SYBR Green RT-PCR analysis and relative gene expression was determined using the $2^{-\Delta\Delta Ct}$-method. Differences in gene expression between mock-inoculated and *Citrus tristeza virus* (CTV)-inoculated plants were generally not prominent. Differences in gene expression between mock-inoculated and Las-inoculated plants were most pronounced in susceptible Cleopatra plants and at the later stages of infection. Transcripts for a constitutive disease resistance protein (CDR1) were induced in response to Las in susceptible plants, but showed higher expression levels independent of infection in the tolerant genotypes. A gene for a Myb-like transcriptional regulator family protein which is associated with resistance to some phytoplasma, responded strongly to Las, but also responded to CTV in tolerant plants. Transcript levels for other genes, such as a 2-oxoglutarate and Fe(II)-dependant oxygenase and a plastidic glucose transporter (GLT1), were considerably higher in US-897 and US-942 plants compared with Cleopatra plants independent of infection with either pathogen. It is hypothesized that these genes play a role in the resistance or tolerance of trifoliate orange and its hybrids to HLB and CTV.

HLB PHYTOPLASMA CAUSES WITCHES'-BROOM AND VIRESCENCE IN *Crotolaria juncea* (L.): OCCURRENCE, DETECTION, QUANTIFICATION AND MOLECULAR CHARACTERIZATION

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When, in 2004, HLB symptoms were first seen in São Paulo State (SPS), Brazil, the search for HLB-associated liberibacters identified a new species, *Candidatus* Liberibacter americanus, as well as the known species, *Ca*. L. asiaticus. In 2007, trees with characteristic HLB symptoms, but testing negative for liberibacters, were demonstrated to be infected with a group IX phytoplasma. The same phytoplasma was found infecting Sunn Hemp (*Crotalaria juncea* L.) plants displaying symptoms of witches`-broom and virescence. In SPS, Sunn Hemp is a major, widely distributed cover crop. The "HLB phytoplasma" was present in sunn hemp from seven SPS municipalities, mainly in central and northern regions of the State. Transmission electron microscopy (TEM) analysis of *C. juncea* stalks revealed a profusion of phytoplasma cells inside phloem sieve tubes from symptomatic samples. *Scaphytopius marginelineatus*, a common leafhopper in citrus orchards, was shown to efficiently acquire HLB phytoplasma from affected Sunn Hemp plants, although no transmission to citrus has yet been confirmed. The HLB phytoplasma was successfully transmitted by grafting from citrus to citrus, and symptoms developed after 26 months. The HLB phytoplasmas from citrus and crotalaria samples were characterized by amplification and sequencing of the 16SrDNA gene and the 16S/23S intergenic spacer region and by amplification and sequencing of *rps*C*_rplV* genes. We developed a semi nested PCR and a qPCR based on the more divergent sequences from ribosomal proteins to detect the HLB phytoplasma from plant and insect samples. Our molecular characterizations show that the same HLB phytoplasma is found in citrus as well as Sunn Hemp plants, where the main symptoms are witches'-broom and virescence.

PHLOEM ANATOMY OF SWEET ORANGE AND *Poncirus trifoliata* INFECTED BY *Candidatus* Liberibacter asiaticus

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Huanglongbing (HLB) is a highly destructive disease that affects citrus production in several regions of the world and is caused by three species of phloem limited biotrophic bacteria *Candidatus* Liberibacter spp. HLB causes a range of symptoms that result in fruit downgrading and lead to plant death. Previous microscopy studies reported callose deposition in sieve plates as a response to the bacteria that contributes to the development of HLB symptoms. Since distinct responses are observed among different hosts, we performed anatomical analyses in petioles of sweet orange (*Citrus sinensis* (L.) Osb.) cv. Hamlin (susceptible), and *Poncirus trifoliata* (L.) Raf. cv Rubidoux (tolerant) with *Ca.* Liberibacter asiaticus infection and compared them with non-inoculated plants. Petioles were processed according to the usual techniques of plant anatomy. To visualize the callose deposition, petioles fixed in FAA solution were sectioned on a sliding microtome, staining with 1% aniline blue and analyzed using epi-fluorescence microscopy. Cells of phloem parenchyma of sweet orange showed hypertrophy and hyperplasia that promotes internal pressure in this tissue leading to the collapse of the sieve tube elements (STE). *P. trifoliata* did not exhibit any structural alterations. In sweet orange callose deposition occurred in several collapsed STE, in contrast to *P. trifoliata* where the callose was restricted to one single phloem sieve tube (ST). The susceptible host presented hypertrophy and hyperplasia of phloem parenchyma cells and callose deposition in the collapsed STE while the tolerant host presented only callose deposition in a single ST. This study shows that the collapse of STE that occurs in sweet orange plants could considerably impair phloem transport, whereas this occurs to a greatly lesser extent in the phloem of the tolerant *Poncirus* plants.

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NEW *Candidatus* Liberibacter VARIANTS IN FOUR INDIGENOUS RUTACEOUS SPECIES FROM SOUTH AFRICA

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Citrus greening disease in South Africa is associated with the bacterium *Candidatus* Liberibacter africanus (Laf), which is vectored by *Trioza erytreae*. A Liberibacter related to Laf, *Ca.* Liberibacter africanus subsp. capensis (LafC) was found to infect *Calodendrum capense* and is widespread in South Africa in this indigenous host. In addition to *C. capense*, other members of the Rutaceae from this country may be infected with Liberibacters related to Laf. The current study aimed to investigate this possibility. Samples from 234 *Clausena anisata*, 289 *Vepris lanceolata* and 231 *Zanthoxylum capense* were collected where they occurred naturally throughout South Africa. Total DNA was extracted and tested by real-time PCR for the presence of Liberibacters in general. *Candidatus* Liberibacters from positive samples were characterized by amplifying and sequencing the *rplJ, 16S* and *omp* gene regions. To confirm the identity of the tree host species from which Liberibacter sequences were obtained, the *rbcL* gene of tree hosts was sequenced. In total 33 *C. anisata*, 17 *V. lanceolata*, 9 *Z. capense* and 1 *Zanthoxylum davyi*, tested positive for a Liberibacter. Phylogenetic analysis of the *rplJ* and *omp* gene regions revealed unique clusters for Liberibacters associated with each tree species. Phylogenetic analysis of aligned *16S* rDNA sequences indicated that Liberibacters obtained from *V. lanceolata* and *C. anisata* were similar to *16S* sequences for LafC, whereas those from *Zanthoxylum* species grouped separately. Further studies are needed to test whether Liberibacters related to Laf within indigenous rutaceous species can infect and be transmitted to commercial citrus. These Liberibacters present a unique opportunity to investigate the origin of Laf on citrus which has thus far only been identified from Africa and the Mascarene islands.

VARIABILITY OF *Candidatus* Liberibacter asiaticus BY MULTILOCUS MICROSATELLITE ANALYSIS IN A GROWING AREA IN THE NORTHWEST OF PARANÁ-BRAZIL

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Huanglongbing (HLB) is currently considered the most destructive citrus disease in the citrus producing countries. The disease is associated with phloem-limited *Candidatus* Liberibacter asiaticus, *Ca.* L. africanus and *Ca*. L. americanus. These bacteria are transmitted in a persistent manner by citrus psyllid species, *Trioza erytreae*, that naturally transmit *Ca*. L. africanus and *Diaphorina citri* that can transmit *Ca*. L. asiaticus and *Ca*. L. americanus. Recently, the psyllids, *Cacopsylla citrisuga* in China and *D. communis* in Bhutan, have been reported as new vectors of the HLB agents. In Brazil, the *Ca*. L. asiaticus species is predominant and it was detected for the first time in Maringá, PR in the year 2008. Surveys in an experimental plot located in Maringá were carried out in 2009, 2010, 2011 and 2012 to detect and evaluate HLB spread. In 2010 *Ca*. L. asiaticus was detected for the first time in the experimental plot in 7 plants, the number of infections increased in 2011 to 32 and in 2012 to 67 newly infected trees. Genetic analysis of *Ca.* L. asiaticus was performed using multilocus microsatellite analysis. Three different microsatellite markers were used, one located in the adenosine deaminase open reading frame (ORF) (TATTCTG), the second in the phosphohydrolase ORF (CAGT) and the third in the transcriptional regulator ORF (CTTGTGT). Six different alleles were identified for the adenosine deaminases, three alleles for the phosphohydrolases and ten for the transcriptional regulator gene. In addition, variability was observed within the same tree. A UPGMA dendrogram showing the genetic relationships of '*Ca*. L. asiaticus' isolates was performed to identify the major genetic groups. The use of these molecular markers could contribute to solving questions on the origin and dissemination of HLB-associated *Ca*. L. asiaticus in the region.

DETECTION AND CHARACTERIZATION OF MINIATURE INVERTED-REPEAT TRANSPOSABLE ELEMENTS IN *Candidatus* Liberibacter asiaticus

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Miniature inverted-repeat transposable elements (MITEs) are non-autonomous transposons (devoid of the transposase gene, *tps*) that affect gene functions through insertion/deletion events. No transposon has yet been reported to occur in *Candidatus* Liberibacter asiaticus, an alpha-proteobacterium associated with citrus Huanglongbing (HLB, yellow shoot disease). In this study, two MITEs, MCLas-A and MCLas-B, in *Ca*. L. asiaticus were detected with the genome characterized through a pan-genomic analysis using 326 isolates collected in China and Florida. MCLas-A had three variants, ranging from 237 to 325 bp, and was inserted into a TTTAGG site of a prophage region. MCLas-A had a pair of 54-bp terminal inverted repeats (TIRs) which contained three tandem repeats of TGGTAACCAC. Both “filled” (with MITE) and “empty” (without MITE) states were detected, evidence of the MITE mobility. MCLas-A was more active in Florida than in China. The “empty” sites of all bacterial isolates had TIR tandem repeat remnants (TRR). Frequencies of TRR types varied according to geographical origins. MCLas-B had four variants, ranging from 238 to 250 bp, and was inserted into a TA site of another Liberibacter prophage. The MITE, MCLas-B, had a pair of 23 bp TIRs containing no tandem repeats. No evidence of MCLas-B mobility was found. An identical open reading frame was found upstream of MCLas-A (229 bp) and MCLas-B (232 bp) and was predicted to be a putative *tps*, which might belong to the IS3 family. MCLas-A and MCLas-B were predominantly co-present in Florida isolates, whereas, MCLas-A alone or MCLas-B alone was found in Chinese isolates.

CALIFORNIA'S PURSUIT OF EARLY DIAGNOSTICS FOR HUANGLONGBING

Marylou Polek

Citrus Research Board

The Asian citrus psyllid (ACP) is fairly well established in all counties in southern California and is spreading up the coast into Ventura and Santa Barbara Counties, but is not established in the major citrus production area of the Central Valley. The current status of Huanglongbing (HLB) in California remains as only one backyard tree in Los Angeles County. This tree was identified as being infected with *Candidatus* Liberibacter asiaticus (CLas) in March 2012 and removed. Despite continued surveys in that area, no additional trees have been found positive for HLB-associated pathogens. California is committed to find and eradicate infected trees as a first response. As a means of early diagnosis, adult and nymph ACP are collected and tested to determine whether HLB-associated bacteria are present in a localized area. The current approved diagnostic method for HLB in the United States is q-PCR; however, as demonstrated in Florida, this method may detect HLB-associated bacteria too late for the implementation of an effective eradication strategy. Hence, the Citrus Research Board has made the development of early HLB diagnostic methods their highest research priority. Methods being pursued involve the detection of molecules or compounds unique to Liberibacter infection within the host plant including volatile organic compounds (VOC), proteins secreted by the bacteria, small RNAs, and metabolomic compounds. These methods are very promising and are being tested against the approved q-PCR assay in time-course experiments conducted within a containment facility on the University of California, Davis campus. In addition, the Jet Propulsion Laboratories/NASA will monitor the reflectance properties of leaves for changes during the course of an infection. If this method is successful, JPL will further evaluate whether a large scale survey can be conducted by air or satellite. These new methods are also being field tested in Los Angeles County in the neighborhood where the CLas-infected tree was found in 2012 and in Texas in the vicinity of previous detections of HLB-positive trees.

POSTER 22

DEVELOPING A LAB-ON-CHIP (LOC) DEVICE FOR MULTIPLEX DETECTION OF *Candidatus* Liberibacter SPP. IN HOST PLANTS AND VECTORS

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Candidatus Liberibacter is a genus of plant entophyte gram-negative bacteria in the Rhizobiaceae family vectored by psyllids. Most of the members of the genus are associated with damaging plant diseases and are subject to phytosanitary regulation. *Ca*. L. asiaticus, *Ca*. L. africanus and *Ca*. L. americanus are associated with Huanglongbing, one of the most destructive diseases of citrus. The disease is present in different countries of Asia, America and Africa but the three pathogens and their vectors *Diaphorina citri* and *Trioza erytreae* have a more complex distribution. The disease continues to spread rapidly and is a threat to the Mediterranean Basin countries, where the disease has not been reported. Early detection of the citrus Liberibacter in host plants and vector insects is essential to prevent its introduction and spread and for HLB control and management in countries where the disease is present. Diagnostic methods have to cope with the uneven distribution of the bacterium in plant tissues associated with irregular vector transmission, low bacterial titer during early stages of infection in host plants and vector insects and require differentiation of the individual species.
By using the *InCheck* Platform (STMicroelectronics S.r.l), which combines multiplex PCR and microarray hybridisation on a single chip, we are developing a diagnostic assay to identify and discriminate among the species of Liberibacter in plant DNA extracts.
The design of the probes on the microarray module for the identification and discrimination was based on the sequences of the following species: *Ca*. L. asiaticus, *Ca*. L. africanus, *Ca*. L. americanus, *Ca*. L. solanacearum, *Ca*. L. europeus, *Ca.* L. crescens and outgroup species within the Rhizobiaceae family.

POSTER 23

DEVELOPMENT OF A LOOP-MEDIATED ISOTHERMAL AMPLIFICATION ASSAY FOR THE DETECTION OF *Candidatus* Liberibacter asiaticus

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Huanglongbing (HLB) is one of the most destructive diseases of citrus worldwide. In China, 11 out of the 20 citrus producing provinces have suffered damage from HLB. An important step in HLB management is early detection of *Candidatus* Liberibacter asiaticus. Due to highly variable bacterial titers and its uneven distribution *in planta*, quick detection of HLB bacteria is of importance. Based on the outer membrane protein (omp) gene of Ca. L. asiaticus, a loop-mediated isothermal amplification (LAMP) assay for rapid detection of HLB was developed. The results showed that ladder-like products were found from those HLB-positive samples by LAMP. Amplification products can be visualized by turbidity or the form of a color change when SYBR Green I, a fluorescent dsDNA intercalating dye, was used. The detection limits of the LAMP were 39 copies. Its sensitivity is 100 fold higher than that of conventional PCR. The result of field application demonstrated that the HLB detection rate was 54.20% (68/120), compared to 39.2% (47/120) by conventional PCR. Real-time PCR detection was applied to confirm the higher detection rate. The LAMP method described was rapid, simple, and sensitive, which could be useful for large-scale detection in field samples.

POSTER 24

PERFORMANCE STUDY OF A COMPLETE KIT FOR DIAGNOSIS OF *Candidatus* Liberibacter HLB-AGENTS BASED ON DIRECT REAL-TIME PCR

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Huanglongbing (HLB) disease is seriously threatening the citrus industry worldwide. The accurate detection of any of the three species *Candidatus* Liberibacter asiaticus (Las), *Ca.* L. africanus (Laf) or *Ca.* L. americanus (Lam) associated with HLB is essential for the prevention and containment of the disease. Real-time PCR coupled with direct systems of sample preparation (tissue-print, squash or spot) is the method of choice for rapid and accurate detection. A complete kit that includes all reagents, lyophilized master mix and immobilized controls (Plant Print Diagnòstics, Ref. HLB/100, www.plantprint.net) based on TaqMan chemistry was developed and successfully validated by intra- (3 IVIA laboratories using 3 different thermocyclers) and inter-laboratory (30 labs from 15 countries) performance studies. Ten blind samples spotted/immobilized on paper were used: 6 were positive and 4 negative. The positive samples consisted of three Las, two Laf and one Lam sweet orange plant crude extracts and the negative samples were crude extracts of HLB-healthy citrus. Results were recorded as positive or negative for each sample and Ct values were annotated when amplification occurred. The results of all the 400 analyses were used for the estimation of the diagnostic parameters according to www.antonio-olmos.com/parametrs/online/calculator.html, regardless of the laboratories involved. Sensitivity, specificity and accuracy values were for the intra-laboratory studies 1.0, 0.91 and 0.96, respectively and for inter-laboratory studies 0.97, 0.94 and 0.96. Due to these excellent values, the system is proposed in the OEPP/EPPO standard protocol for HLB diagnosis. The usefulness and robustness of this kit coupled to the direct system of sample preparation will facilitate the analyses of large numbers of samples even in field conditions. The accuracy of the HLB analysis will greatly contribute to improve the preventive control and management of the disease.

AERIAL SPREAD OF *Citrus psorosis virus* (CPsV) BY INSECTS IN ARGENTINA

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The theory of natural spread of psorosis by insects in Argentina was stated by Rodríguez Pujol and Beñatena, researchers from the Concordia Experiment Station (EEA Concordia) of the National Institute of Agricultural Technology (INTA), in 1965. In 1966, Rodríguez Pujol mentioned natural spread of psorosis in seedlings in the citrus region along the Uruguay River in Argentina. Timmer and Beñatena (1977) reported 0.1 - 1% of the rootstock seedlings in the nursery were rogued because of psorosis. Timmer and Garnsey (1980) observed in Texas (USA) natural spread of Citrus ringspot virus (currently CPsV) in grapefruit at a rate less than 2%. Beñatena and Portillo (1984) showed psorosis transmission from diseased plants to healthy citrus seedlings by *Toxoptera citricidus* and *T. aurantii* winged individuals, by wingless adults of *Toxoptera* spp., by colonies of *Toxoptera* spp. and *Aphis citricola,* with a long incubation period and erratic symptom appearance. Since then, there have been advances in the study of the causal agent, diagnostic methods, and possible transgenic lines tolerant and /or resistant to CPsV but little has been mentioned about the natural spread of this virus. This work confirms the aerial spread by insects of CPsV in the field, both in a plot of certified citrus plants free of CPsV and in trials for testing aerial and/or soil spread of the virus in the Uruguay River citrus region in Argentina.

GRAFTING AND ITS BEHAVIOR ON CPsV-RESISTANT TRANSGENIC ORANGES

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- IOCV sponsored student presentation

Citrus psorosis virus (CPsV) is the causal agent of Psorosis, an important disease causing significant economic losses in Argentina and Uruguay. CPsV is the type member of the genus *Ophiovirus*, family *Ophioviridae*. Its genome has three single-stranded RNAs of negative polarity, encapsidated with a coat protein (CP). As there is no natural resistance to Psorosis, transgenic Pineapple sweet orange (SwO) plants were obtained expressing an intron-RNA hairpin from the *cp* gene (ihpCP). This RNA transcript induces the post-transcriptional gene silencing (PTGS) mechanism, degrading all RNA with *cp* gene sequences, thus preventing the progress of the infection.

When ihpCP citrus trees were CPsV-challenged by graft-transmission, at least two SwO lines (6110 and 6115) did not show any symptoms and CPsV was not detected by molecular and serological analysis. The hallmark of the PTGS is the presence of small RNAs (siRNA), which were detected in the transgenic tissue indicating that resistance is PTGS-mediated.

Our interest is to evaluate whether ihpCP is able to transfer this feature of resistance to susceptible grafted-tissue. For that, non-transgenic buds were grafted on the two resistant ihpCP lines used as rootstocks, which had previously been inoculated with CPsV. In another assay, a susceptible CP-line, expressing the complete CP-mRNA, was grafted on the same two ihpCP resistant rootstocks. Both, the non-transgenic and transgenic CP were susceptible. New flushes showed characteristic symptoms (flecking, spot and shock) and TAS-ELISA and qRT-PCR analysis were positive.

These results suggest that the ihpCP lines, with no evidence of infection, allow virus movement to the susceptible tissues (non-transgenic and CP-line). It seems that the virus could reach susceptible tissues before PTGS has been established.

Therefore, we are currently performing a new assay grafting the susceptible non-transgenic and CP-line scions before CPsV infection, giving kinetic advantage to PTGS establishment.

UPDATES ON LEPROSIS RESEARCH: WHAT HAVE WE LEARNED IN THE LAST DECADE?

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Leprosis is a complex pathosystem that involves an atypical virus, *Citrus leprosis virus C* (CiLV-C), an unusual mite vector, *Brevipalpus* sp., and host plants belonging to at least 18 botanical families. CiLV-C causes only localized lesions and does not spread systemically within its hosts, originally thought to be restricted to *Citrus* sp. Most of what was accepted about leprosis until the early 2000's was shown to be inaccurate. Exactly ten years ago the first sequences of *Citrus leprosis virus C* (CiLV-C) were obtained and used for the development of an RT-PCR-based method for the diagnosis of leprosis. Since then, much has been done by our and other research groups and more light has been shed onto the pathosystem. Data obtained during the last decade will be discussed in the conference; mainly on CiLV-C taxonomy (it is now the type member of the genus *Cilevirus*), variability (contrary to the initial data suggesting low variability among CiLV-C isolates, new reports have shown higher variability and even a probable new related species causing leprosis symptoms in sweet oranges), serological detection (using ELISA and *in situ* immunolocalization), and biological and molecular relationships with other viruses, with its mite vector and with its natural and experimental hosts, now expanded to dozens of plant species.

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MITE VECTOR/VÍRUS RELATIONSHIPS IN THE CITRUS LEPROSIS PATHOSYSTEM AND AN EVALUATION OF EXPERIMENTAL HOST RANGE

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Ultrastructural studies made on viruliferous *Brevipalpus phoenicis* (Acari: Tenuipalpidae) detected presumed *Citrus leprosis vírus C* (CiLV-C) particles between membranes at the basal part of the caeca, dorsal podocephalic gland and adjacent cells. Their viral nature was confirmed by *in situ* immunogold labeling using an antibody against the p29 protein of CiLV-C, the putative capsid gene. No evidence of viral replication was obtained and it was concluded that CiLV-C circulates but does not replicate in the mite. A meticulous anatomical study was made parallel to this study, to map the sites where CiLV-C was present within the mite. However, how the vírus enters, circulates and exits the mite body is still an open question. For feeding, it is believed that *B. phoenicis* uses the stylet to perforate the epidermis to reach parenchymal cells below,and injects saliva for a predigestion of the cell content, when CiLV-C must be injected into the host cell. Then the stylet is withdrawn and the cell sap is sucked with the help of cell turgor and the pharyngeal pump, and is delivered through the esophagus to the ventriculus and midgut. This process is being monitored by an adaptation of the EPG (electric penetration graph). The graphs obtained are being interpreted and tentatively associated with different phases of the feeding process. Using common bean as indicator plant, a series of parameters (vírus acquisition feeding period [4h], vírus inoculation feeding period [4h], latency [less than 4 h], vírus retention [at least 12 days] and % of viruliferous mites in populations colonizing infected fruits or field plants [<50%] was obtained. One hundred and fifty different plant species were assayed for CiLV-C susceptibility following mite inoculation, resulting in 42 species producing local lesions, in which the vírus was detected by at least one of the different methods (ELISA, RT-PCR, transmission eléctron microscopy and immunofluorescence).

GENETIC VARIABILITY OF *P29*, THE PUTATIVE COAT PROTEIN GENE OF *Citrus leprosis virus* C

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Citrus leprosis, transmitted by the tenuipalpid mite *Brevipalpus phoenicis,* is caused by *Citrus leprosis virus C* (CiLV-C), the type member of the genus *Cilevirus*. The disease is responsible for losses of ~US$ 80 million every year to the Brazilian citrus industry due to the localized lesions it induces on leaves, stems and fruits, and to severe dieback and fruit drop it can cause. The disease occurs in several countries in South and Central America and is spreading towards the north of the Continent, having reached Mexico. Preliminary, unpublished data on the variability of the putative movement protein and replicase-associated protein genes suggest very low genetic variability among isolates. Additionally, the only three complete genome sequences of CiLV-C available in the GenBank, two from Brazilian isolates and one from a Panamanian isolate of the virus, share sequence identities of 99%. Altogether, these data suggest low divergence amongst isolates. However, since Brazil is likely to be the center or origin of this virus, we decided to determine the variability of the *p29* ORF (putative coat protein gene, which is more likely to exhibit variability) of 22 CiLV-C isolates from nine Brazilian states and one isolate from Argentina. RT-PCR products were cloned, sequenced and compared among them and with the GenBank sequences of the virus. Overall, *p29* sequence identity ranged from 98% to 100%, with the exception of one isolate from São José do Rio Preto, São Paulo State. The sequence identity of this isolate ranged between 85% and 86% when compared to the other 25 sequences available. This shows that, even though CiLV-C variability may be considered low, there are isolates with significantly higher variability infecting citrus in Brazil. It is even possible that other species of cilevirus causing leprosis-like symptoms are present in the country, as recently reported in Colombia.

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HOSTS AND SPATIAL SCALING OF THE GENETIC STRUCTURE OF A VECTOR-BORNE PLANT PATHOGEN *Xylella fastidiosa*

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The ecology of plant pathogens of perennial crops is impacted by the long-lived nature of their immobile hosts. Over time, host plants are subject to changes to the genotype structure of pathogen populations that may impact disease spread and management practices; examples include the local adaptation of more fit genotypes, or the introduction of novel genotypes from geographically distant areas via human movement of infected plant material or insect vectors. We studied the genotype mixture of *Xylella fastidiosa* populations causing disease in sweet orange and coffee crops in Brazil at multiple scales, using fast-evolving molecular markers (simple sequence DNA repeats, SSR). Results show that populations of this bacterial plant pathogen were regionally isolated, as were the hosts. Independently of host and geographic origin the data suggest that the populations evolved locally and were not the result of migration. At a smaller spatial scale (individual citrus trees), results suggest that *X. fastidiosa* isolates within plants originated from a shared common ancestor, indicating that despite the long-term exposure of trees to infection, infection occurred only once, even though the vector visited the plant multiple times. It is possible that systemically infected trees are less susceptible to new invasions by competing *X. fastidiosa* genotypes. In summary, new insights to the ecology of this economically important plant pathogen were obtained by sampling populations at different spatial scales and from two different hosts.

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N-ACETYLCYSTEINE IN AGRICULTURE, A NOVEL USE FOR AN OLD MOLECULE: FOCUS ON CONTROLLING THE PLANT-PATHOGEN *Xylella fastidiosa*

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Xylella fastidiosa is a plant pathogenic bacterium that causes diseases in many different crops. The mechanism of pathogenicity of this bacterium is associated with its capacity to colonize and form a biofilm in the xylem vessels of host plants. There is no method to control this pathogen in field. In this study, we investigated the inhibitory effect of N-Acetylcysteine (NAC), a cysteine analogue used mainly to treat human diseases, on *X. fastidiosa* under different experimental conditions. Concentrations of NAC over 1 mg/mL reduce bacterial adhesion to glass surfaces, biofilm formation and the amount of EPS. The minimal inhibitory concentration of NAC was 6 mg/mL. NAC was supplied to infected plants in hydroponics, by fertigation, and adsorbed to organic fertilizer (NAC-fertilizer). HPLC analysis indicated that plants absorbed NAC at concentrations of 0.48 and 2.4 mg/mL, but not at 6 mg/mL. Sweet orange plants with typical CVC symptoms and treated with NAC (0.48 and 2.4 mg/mL) in hydroponic solutions showed clear symptom remission and reduced the bacterial population to less than 5% compared with untreated plants, as analyzed by quantitative PCR and bacterial isolation. Fertigation and NAC-fertilizer experiments were done to simulate a condition closer to that normally used in the field. For both, significant symptom remission and reduced bacterial replication rate were observed. Using NAC-Fertilizer the lag for resurgence of symptoms on leaves after interruption of the treatment was increased to around eight months. This is the first report on the effect of NAC as an anti-bacterial agent against a phytopathogenic bacterium. The use of NAC in agriculture might be a new and sustainable strategy for controlling plant pathogen bacteria.

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Candidatus Liberibacter asiaticus MULTIPLICATION IN *Diaphorina citri* IS AFFECTED BY TEMPERATURE

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Ca. L. asiaticus (Las) is the most prevalent liberibacter species associated with HLB in Brazil. In previous works we showed that Las titers and Las acquisition by its insect vector *D. citri* are decreased in plants exposed to daily temperature regime of 24-38°C (Plant Dis. 93:257-262; Plant Dis. *in press*). Since Las transmission seems to require Las multiplication also in the body of the vector the potential effect of temperature on this process also was investigated. Third-day-old adult insects were removed from Las-free *D. citri* colonies and caged for 48 h on new flushes of fully symptomatic branches of Las-infected 1-yr-old Valencia plants grown in greenhouse. The insects were removed from the infected plant, caged on healthy potted 6-mo-old orange jasmine (*Murraya exotica*) plants, and immediately transferred to growth chambers where they remained at temperature constant of 14, 20, 26, 32, or 38°C, and daily light:darkness photoperiod of 12:12h. Insects were then collected from the orange jasmine and individually processed for DNA extraction and qPCR analysis after 3, 6, 12, 24 h and, thereafter, every 48h, up to 15 days of exposure. Ct values varied among individual insects, exposure temperatures, and exposure times. Average Ct values remained higher after most exposure times in the groups maintained at 14 (30.5 to 33.5) and 38°C (29.3 to 32.5) than in the groups maintained at the other temperatures. At 26°C average Cts declined from 31.1 to 25.7. The higher Cts indicated that14 and 38°C are not favorable to Las. Conversely, the constant overtime declining Cts indicated that the temperature of 26°C is favorable to Las multiplication in the body of *D. citri*. The experiment was repeated with similar results. Assuming that the lower titers assessed in the temperature-exposed insects lead to a reduction in the effectiveness of Las transmission (which was not determined in this work), HLB progress would be lower in areas or seasons of similar temperatures. Coincidently this is the situation in Sao Paulo state where in regions of hotter summers (northwestern) and cooler winters (southeastern). As already demonstrated by others (Liu & Tsai, 2000) lower temperatures also impair *D. citri* multiplication, which might be contributing to reduce HLB progress in the south.

PROPHAGES IN C*andidatus* Liberibacter asiaticus AND S*piroplasma citri*

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A prophage is bacteriophage DNA integrated into a bacterial chromosome or existing as a plasmid inside the bacterial cell. It provides important biological traits of bacteria that may involve virulence, environmental adaptation, strain specification and genome evolution. Genome sequence analyses indicate that both *Candidatus* Liberibacter asiaticus and *Spiroplasma citri* harbor prophages. *Ca.* L. asiaticus is associated with citrus Huanglongbing (HLB, yellow shoot disease) and *S. citri* causes citrus stubborn disease (CSD). *Ca.* L. asiaticus is not culturable *in vitro*. *S. citri* is culturable but the process is highly challenging. For these reasons, knowledge of the biology of the two bacteria is very limited. To study the prophage diversity of *Ca*. L. asiaticus in China where HLB has been endemic for over 100 years, 12 consecutive open reading frames (ORFs) in a prophage of a Florida *Ca*. L. asiaticus isolate were selected and primers were synthesized. Using these primers with PCR, 150 *Ca*. L. asiaticus samples from southern China were examined. At least three prophage types were detected, indicating a possible rich pool of *Ca*. L. asiaticus prophage in southern China. Since *S. citri* was culturable, pure culture of nine *S. citri* strains were obtained and whole genome sequences were generated. Two prophage/phage genes were selected and their abundance in each of the 9 whole genome sequences was estimated. Copy number of one gene varied from 13 to 154. Copy number of the other gene varied from 11 to 56. These results suggest that in addition to the chromosomal form, prophages of *S. citri* may also exist in extrachromosomal forms. Overall, these studies demonstrate that prophages are important constituents of both *Ca*. L. asiaticus and *S. citri*. More information on prophages are needed for better understanding of the two fastidious prokaryotes important to world citrus industry.