Biological Characterization of Naturally Occurring *Citrus tristeza virus* Strains in California Citrus

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ABSTRACT. The *Citrus tristeza virus* (CTV) collection housed at the Central California Tristeza Eradication Agency contains 339 isolates including representatives from the entire state of California, with the majority of the isolates having been collected in the San Joaquin Valley. Isolates have been biologically characterized using the standard host indicators. Individual symptoms are rated on a scale of 0 to 5, 0 being no reaction and 5 being most severe. For each host plant variety, the symptoms are rated individually, then summed. A total rating is then assigned to each isolate tested by summing the ratings for each variety. Isolates can be compared at three levels according to individual symptoms, host indicator variety, or overall severity. This enables researchers to select particular isolates for study and to determine subtle changes in an isolate due to factors such as aphid transmission. Further, this rating system provides a means to identify whether an isolate is exotic to California. The majority of California CTV isolates are mild; however, some have the potential to cause severe symptoms in some host varieties. Quick decline and stem pitting of grapefruit were the symptoms most frequently rated as severe or moderately severe.

Citrus tristeza virus (CTV), a member of the family *Closteroviridae* and genus *Closterovirus*, is generally recognized worldwide as the virus disease that economically impacts commercial citrus the most. It is known to exist as different strains or isolates (terms that can be used interchangeably) that range in their transmissibility, severity, and in their disease reaction within a specific host variety (5, 11, 19). While some citrus varieties can be severely impacted by a few or many strains of the virus, certain other citrus types are tolerant and exhibit no visible symptoms. These tolerant hosts are especially troublesome since they are viable inoculum sources for transmission by both aphid vectors and grafting. Of the numerous disease symptoms, the two most studied maladies are the quick decline reaction of sweet orange varieties grafted onto sour orange rootstock and the stem pitting of trunks and twigs of most varieties. A third symptom category termed seedling yellows is not a disease symptom that one would observe in a citrus grove. Rather, it is a reaction that occurs under greenhouse conditions in the seedling stage of some grapefruit, lemon, and sour orange varieties and typically indicates a potentially severe strain of CTV.

CTV can be detected by a wide variety of methods including biological indicators, serology, and various molecular techniques (4, 7, 10, 12, 13, 15, 20). Which method one uses depends upon the desired outcome or use of the test results. Historically and still today, the particular biological reactivity of an isolate is sorted out by characterizing symptom development on a standard set of host indicator varieties (6, 16, 17, 18, 19). These include Mexican lime, sweet orange (often Madam Vinous or Pineapple Sweet), grapefruit (typically Duncan), lemon (Eureka or Lisbon), sour orange, and sweet orange grafted onto sour orange as the rootstock. Strains can be identified and isolates characterized according to the manifestation and severity of disease symptoms in each individual host type. Whereas this methodology has worked well

for categorizing isolates on a descriptive level, this type of evaluation does not consistently explain the alterations in biological activity that often occur when the virus is vectored by insects. A different approach may be necessary to determine differences in specific experimental situations such as studying the aphid transmission of virus isolates. A discussion of such obstacles is presented here.

The greenhouse facility at the Central California Tristeza Eradication Agency (CCTEA or Agency) in Tulare, California, USA houses an extensive collection of CTV isolates that were collected from field trees in commercial groves at various times since 1993. In many cases, these source trees were scheduled for removal as part of the eradication program and no longer exist in the field. Of the 339 accessions, the majority was collected in the San Joaquin Valley where over 80 per cent of the California citrus industry is located (2). Additionally, isolates from southern California, including Riverside and Ventura counties, are also represented. Several isolates are maintained for specific research projects upon the request of scientists. A total of 225 isolates have been evaluated and a summary of their biological reactivity is reported here.

MATERIALS AND METHODS

Isolate Collection. Although collected from various locations statewide, the majority of isolates was collected in the San Joaquin Valley (the southern portion of the Central Valley) in conjunction with the Central California Tristeza Eradication Agency. Budwood was collected from commercially grown citrus trees diagnosed as positive for CTV by Enzyme Linked Immunosorbent Assay (ELISA) and grafted into Madam Vinous sweet orange for preservation. Prior to tree removal, the field trees were examined for stem pitting symptoms by peeling back the bark on a minimum of eight twigs and a patch on the main trunk. Plants were maintained under greenhouse conditions at the CCTEA in Tulare, CA.

Plants. Seeds were extracted from fruit harvested at the University of California Lindcove Research and Extension Center located in Exeter, CA and treated with 8-hydroxyquinoline and dried (18). Seeds were germinated in Ropak® multipots (Stuewe & Sons, Inc., Corvallis, OR, USA) and later transplanted into 15.24 cm (6-inch), 3.79 l (one-gallon) plastic pots. Three seedlings were planted in each pot and each seedling was given a label with a unique bar code number for tracking and ease of data management. Greenhouse temperature conditions were controlled at 26-32°C (80-90°F) in the winter and 26-38°C (80-100°F) in the summer. Due to the dense Central Valley fog, supplemental lighting was necessary from October to March to provide an additional 6 h of light (16 h/day). Conversely, shade cloth was used from April to September to insulate the greenhouse from the heat and intense sunlight.

Once inoculated, seedlings were moved to a cooler greenhouse where the winter temperature ranges from 20-24°C (68-78°F) and the summer temperature ranges from 20-32°C (68-88°F). Supplemental lighting was also used in this greenhouse from October to March, as was shade cloth from April to September. Lowering the temperature in both greenhouses was accomplished by an evaporative cooling system. All seedlings were trained to a single stem.

Biological Characterization. Individual strains of CTV can be determined through biological characterization on standard citrus indicator hosts under controlled greenhouse conditions. Isolates were characterized using: Mexican lime, Madam Vinous sweet orange, sour orange, Duncan grapefruit, Eureka or Lisbon lemon grafted onto rough lemon, and Madam

Vinous sweet orange grafted onto sour orange rootstock (5, 6). These plants were grown from seed and transplanted, three trees per 1 gallon pot. When the trunks had a pencil-sized diameter, Madam Vinous was grafted onto sour orange and Eureka lemon was grafted onto rough lemon. These plants were ready for inoculation at about 1-year old. Seedlings of similar age were graft-inoculated by "chip budding" and wrapped with clear tape. Three buds were used per seedling. After 2 weeks, the buds were unwrapped and examined to check if the bud was still green and alive. At least two chip buds must have been alive otherwise the seedling was re-inoculated. Seedlings were pruned to 30 cm from the soil and maintained as a single stem. Controls for each experiment included a non-inoculated (healthy) seedling and seedlings inoculated with a seedling yellows strain (Riverside SY 575), a mild strain (Riverside 519), and a severe local isolate (107).

Visual symptoms on all indicators were assessed 3 and 6 mo after inoculation. Mexican lime seedlings were examined for vein flecking or clearing, leaf cupping, vein corking, stunting, and chlorosis. Madam Vinous seedlings were assessed for vein clearing or flecking, stunting, and chlorosis. Sour orange, grapefruit, and lemon hosts were examined for a seedling yellows reaction, and sweet on sour for stunting. The Mexican lime plants were destroyed after the 6-mo reading. Madam Vinous sweet, grapefruit, lemon, and sweet on sour were evaluated again at 9 mo and all varieties except the sweet on sour were destroyed. Sweet on sour seedlings were then transferred to an outdoor location and maintained under heat and water stress conditions for an additional several months to induce quick decline symptoms. As part of each reading, the growth of each seedling was measured from the soil line. Any seedling with a growth greater than 60 cm was cut back to 60 cm. In addition, every seedling was assayed for the virus by ELISA. At the final reading of each host indicator, the final growth measurement was taken and the bark was peeled back to examine for stem pitting symptoms. For each host pant, all growth measurements were summed. Growth was evaluated based on the proportion as compared to healthy controls.

All symptoms were rated for severity according to a scale of 0-5, zero being no reaction and five being a severe reaction. The individual symptom reaction scores for each host plant (n = 3) were averaged. A total rating for each host indicator variety was calculated by summing all reaction scores for that variety. A total rating for each isolate was calculated by a cumulative sum of all reactions of all hosts. If a particular isolate was characterized more than once, the symptom scores from each experiment were averaged for each host plant. In cases where none of the plants from a member of the host range were infected (tested positive by ELISA), these plants were excluded from the average.

ELISA Protocol. Plants were tested for the presence of CTV by indirect-double antibody sandwich, Enzyme Linked Immuno-Sorbent Assay (DAS-I-ELISA). Assays were conducted at the CCTEA following the protocol as outlined in the CCTEA Laboratory Procedures and Quality Assurance Manual (3). Microtiter plates (Immunlon 3 and Nunc Polysorb) were first coated with goat anti-CTV antibody (University of California, Riverside) to trap the virus. Plant sap was extracted from approximately 2 g of leaf petioles by placing in PEP extraction buffer, and homogenizing for 10 seconds in a KLECO Tissue Pulverizer (Kinetic Laboratory Equipment Company, Visalia, CA). The samples (plant sap) were loaded onto microtiter plates and incubated at 4°C overnight. After washing, the plates were loaded with rabbit anti-

CTV polyclonal antibody produced by Nikolaeva et al. (13) and incubated at 34°C for 2 h. Plates were again washed. Anti-rabbit alkaline phosphatase conjugate (MP Biomedicals, Aurora, OH) was added followed by a 2-h incubation at 34°C. Following the addition of alkaline phosphatase substrate, the optical density of the plate wells was read at a wavelength 405 nm using a Molecular Devices reader and Softmax Software. A positive reaction was apparent within 1.5 to 2 h after the addition of the substrate. Samples were considered positive when the optical density value was at least two times greater than the average of the negative controls.

RESULTS AND DISCUSSION

Although the majority of isolates tested would not be considered severe by international standards, this data provides sufficient evidence that given the diversity of California citrus plantings, strains of CTV exist in California that have the potential to cause severe disease symptoms. Further, because a particular isolate can produce mild symptoms in one citrus variety but cause a severe reaction in another, a citrus industry can be significantly impacted where plantings are mixed, such as in California with many specialty varieties. Currently, no characterization method, whether molecular, serological, or biological, is a "silver bullet"; as each can give inconsistent results.

California Isolate Collection. The biological reactions of the 225 isolates characterized are summarized in Table 1. A rating of 3 or higher was considered to be severe or moderate-severe. Forty-seven isolates caused a quick decline reaction in sweet orange grafted onto sour orange rootstock, of which 17 were severe. Stem pitting symptoms were observed with 14 isolates in lemon, but only one was severe; with 53 in grapefruit, where 14 were severe; and with 25 in sweet orange, where seven were severe. A seedling yellows reaction was caused by 55 isolates in grapefruit, with seven rated severe; there was 56 in lemon, with three severe; and 18 in sour orange, where seven were severe. Lemons and sweet on sour were most stunted by CTV infection. Isolates were also tested against the monoclonal antibody MCA-13, which is used in Florida to detect strains of CTV that are considered severe because of their capability to cause decline symptoms. No correlation was found between California isolates causing quick decline symptoms (or not) and their reaction with MCA-13. Com-

TABLE 1
SUMMARY OF BIOLOGICAL REACTIONS OF 225 CALIFORNIA CTV ISOLATES CHARACTERIZED USING STANDARD HOST INDICATORS

Biological Reaction	No. showing some symptoms	No. with severe symptoms[1]
Quick Decline	47	17
Stem Pitting		
Grapefruit	53	14
Lemon	14	1
Sour orange	—	—
Sweet orange	25	7
Seedling Yellows		
Grapefruit	55	7
Lemon	56	3
Sour orange	18	7

[1]A severe reaction is considered one with a rating of 3 or higher based on a rating scale of 0 to 5, with 0 being no symptoms observed.

plete reaction information of a particular isolate can be found at the CCTEA website (www.cctea.org).

Exotic Isolates. Biological characterization data is valuable information that can be used to determine the introduction of exotic strains to a particular location, providing there exists a knowledge-base of endemic isolates. In the spring of 1999 during the systematic subsampling survey conducted by the eradication program, a grove was estimated to have a CTV incidence of 2.42% using the hierarchical subsampling method (9). Each tree in this grove was sampled during the following spring and the incidence had increased to 41%. This rapid rate of increase could not be due to natural vector transmission (8). Material was collected from the infected field just prior to tree removal and inoculated into the standard host indicators. In this case not only was the use of the overall severity rating important, but the individual symptom ratings also were important. Typical California isolates exhibit symptoms in Mexican lime including vein flecking where small segments of leaf veins are clear or transparent when backlit. The cupping of leaves and some corking of the leaf veins also occur. Seedlings inoculated with the tissue in question had leaves with the entire vein system almost completely transparent and this could be observed by merely walking along the greenhouse bench. Seedling yellows-like symptoms occurred in several lime seedlings, and plants were severely stunted. Leaves measured 15 to 30 mm as opposed to a similar infected leaf of 40 to 60 mm or a healthy leaf of 80 to 100 mm. In Madam Vinous sweet orange hosts, California CTV isolates rarely cause vein-clearing. However, almost one-fourth of the seedlings inoculated displayed severe vein clearing in this host variety. Severe corking was observed in both Mexican lime and Madam Vinous hosts. Overall, isolates collected from this grove produced symptoms more severe than even the severe California controls (Fig. 1). Armed with this and other molecular evidence, officials concluded that exotic material was illegally brought into the state and levied fines on the grower and other associated entities.

Obstacles to Biocharacterization. In typical bio-characterization experiments, three seedlings of each host variety are used per isolate. For this purpose, three can be sufficient providing the virus becomes established in at least two of the three seedlings inoculated. However, CTV is a complex virus making it difficult to work with and variability in resulting symptoms occurs on several levels and for a number of reasons. Health quality of indicator plants, differential expression of host defense mechanisms in individual seedlings and location of seedlings on the greenhouse bench are initial factors. Further, not all inoculations take and the number of successful inoculations is especially low in sour orange and lemon hosts. Often an experiment must be repeated but symptom severity may differ significantly between individual experiments even when the same plant was used as the inoculum source. The time of year the experiment was initiated appears to be of importance. In California the number of successful inoculations was found to be greatest in the fall season (October-November). Other researchers, as discussed in these proceedings, reported on the difficulty and the length of time in inducing quick decline symptoms in sweet orange grafted onto sour orange rootstock under greenhouse conditions (14).

The complexity of this pathosystem is further realized when investigating the transmission of CTV by insect vectors (1). A detailed analysis is in preparation describing the differences in biological symptoms and molecular patterns of the tristeza virus when transmitted by aphid vectors and graft inoculation (D. Ullman, personal communication). Data

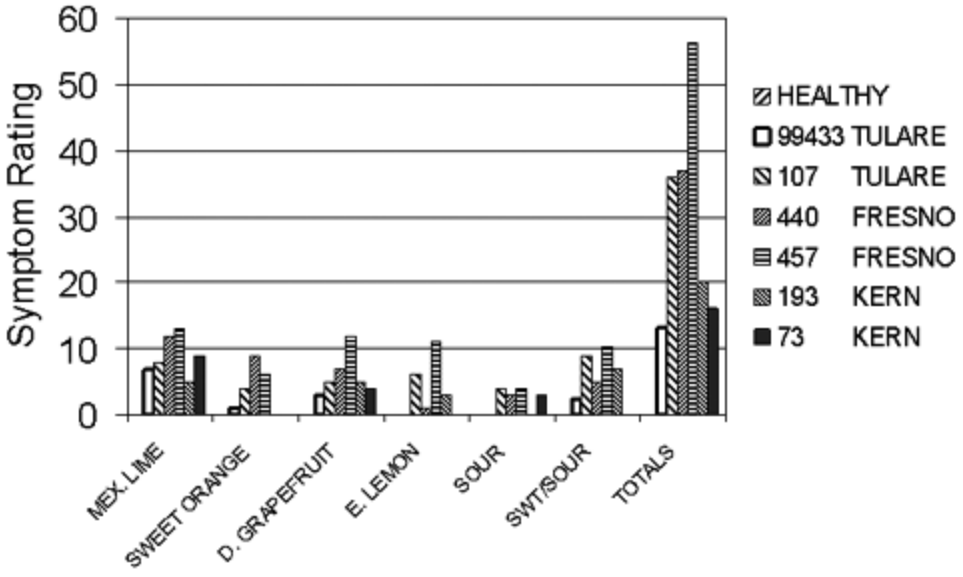

Fig. 1. Symptom ratings summed for each individual host indicator and then summed across all host indicator varieties for a total rating. Isolate 107 is typically used as a severe control in all experiments. Isolates 440 and 457 were collected as part of an investigation regarding the illegal importation of budwood. Isolate 440 was collected from a mature Bonanza navel that was part of the original grove planting whereas isolate 457 was collected from the newly planted mandarin scion.

analysis for this purpose has proven to be difficult on each of the aforementioned three levels (overall, host, and symptom) and raises many interesting questions. While trends may be fairly obvious, specific differences are not easily identified and are often not consistent between replicate experiments. But it is these differences that are necessary to answer questions such as whether all aphids transmit the virus in the same way, whether virions are altered during transmission and how the specific virus population affects the transmission process. One way to get around these obstacles is to use 20 to 30 replications or inoculated seedlings per host indicator and conduct a statistical analysis of the data obtained. This solution is not practical due to space limitations in the greenhouse and leaves one suspicious of diluting severe reactions in some hosts when averaging data.

Inconsistent symptom development can be attributed to the actual source of inoculum. Virion populations systemically move throughout the tree so the placement of aphids for virus acquisition and the collection of a particular branch from which budwood is used for inoculation from field trees introduces variability. Further, virus populations change over time due to super-infections or natural forces such as evolution, mutation, and genetic recombination (21). Thus, the reproducibility of such experiments over time becomes a paradox.

Despite these limitations, valuable information is gained through characterization experiments. When interpretations and conclusions of data are presented, researchers must adopt a level of acceptance and understand that the world of CTV is imperfect.

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