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SURVEY OF STEM PITTING CITRUS TRISTEZA VIRUS IN COMMERCIAL CITRUS GROVES IN FLORIDA

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Abstract. ***Citrus tristeza virus*** **(CTV) has affected how citrus is grown in Florida since the 1950s. The brown citrus aphid, first detected in Florida in 1995, is an efficient vector of CTV and is capable of spreading severe forms of CTV throughout the state. The use of molecular markers for CTV led to the discovery of aphid transmitted stem-pitting forms of CTV (SP-CTV) in Polk County, central Florida. A survey to determine if SP-CTV was present was undertaken for the eleven counties representing 80% of commercial citrus production in Florida. Five sweet orange and two grapefruit sites per county were surveyed using a hierarchical bulk sampling procedure. Immunocapture reverse transcriptase polymerase chain reaction (IC-RT-PCR) with Type II primers was used for initial screening followed by other SP-CTV markers for positive samples. Fifty-five percent (42 of 77) of the sites surveyed tested negative in all samples. The majority of sites testing positive, 61%, had a less than a 5% chance of any single tree in that block testing positive. Only six sites had a greater than a 10% chance of any one tree testing positive for the SP-CTV markers. There were two main pattern profiles of markers present. Not all of these isolates have been evaluated in biological indicators so whether they can cause significant damage and what their threat is to the citrus industry has yet to be determined. Currently, a strong Citrus Budwood Registration Program and increasing the number of budwood source trees under protective screen will prevent the spread of severe forms of CTV throughout the nursery industry.**

Commercial citrus in Florida was thought to be infected mainly with mild and decline-inducing isolates of *Citrus tristeza virus* (CTV) as stem-pitting isolates were not considered to be a problem (Hilf and Garnsey, 2002). Stem-pitting CTV (SP-CTV) that had been detected in Florida had been isolated from Meyer lemon trees and produced mild to moderate stem pitting in grapefruit indicators but not in sweet orange indicators (Lee et al., 1997). Although SP-CTV isolates were not aphid transmitted from these plants (Lee et al., 1997), there has been a continuing awareness of Florida's susceptibility to the possible introduction and dissemination of SP-CTV, particularly with the detection of the brown citrus aphid (*Toxoptera citricida*) in Florida in 1995 (Halbert and Brown, 1996). The severity of stem-pitting isolates has been well documented from California (Calavan et al., 1980; Rocha-Pena et al., 1995), Central and South America (Rocha-Pena et al., 1995), Brazil (Salibe, 1965) and from Florida (Halbert et al., 2004; Tsai et al., 2000).

Evaluation of molecular diagnostic techniques for detection of stem-pitting isolates of CTV determined which marker was suitable for a large-scale survey in Florida (Sieburth et al., 2005). Field testing of these markers was performed on trees removed from the Florida Citrus Budwood Registration Program as the result of MCA-13 positive ELISA tests (Permar et al., 1990). In initial field testing, we found SP-CTV molecular marker positives at four sites in two different Florida counties. This raised concern as to the distribution and extent of stem pitting in Florida and led to this survey. The objectives of this study were: 1) to adapt extraction and PCR procedures to process large numbers of samples quickly; 2) to survey the major citrus growing counties in Florida to determine if stem-pitting markers were present in sweet orange and/or grapefruit groves; 3) to evaluate the level of infection at sites that were positive; and 4) to determine what profiles of stem-pitting markers were present especially in comparison to what was found in Florida previously.

According to the 2002 Florida Commercial Citrus Inventory, there are a total of 648,806 acres of sweet orange production. Eleven counties with more than 20,000 acres of sweet orange production were selected. This represented 84% of commercial sweet orange production.

Materials and Methods

Sampling. Five sweet orange and two grapefruit sites were sampled in each of the following eleven Florida counties: Collier, DeSoto, Hardee, Hendry, Highlands, Hillsborough, Indian River, Manatee, Martin, Polk, and St. Lucie. Blocks of trees of all ages were chosen with the additional criteria of having at least 20 rows of at least 20 trees per each row and few missing trees. Sampling of each block was done by the hierarchical bulk sampling procedure (Hughes and Gottwald, 1999) with 25 composite petiole samples collected at each site. The composite samples consisted of the petioles of two leaves from each of four trees that were cut into small pieces with scissors and placed in pre-labeled collection envelopes while in the field. These were placed in 1 gal freezer bags with 8 mesh Drierite (Hammond Drierite Company, Xenia, Ohio) and were stored on ice during the remainder of sample collection for that day. Samples were dried at room temperature for 2 d, and then stored at -20 °C until they were processed for extraction. To be able to locate sample sites later, GPS readings were taken at Row 1, Tree 1 for each grove. There were a total of 1,925 samples.

Testing. Extraction of viral RNA for immunocapture RT-PCR was prepared as previously described (Sieburth et al., 2005). Screw-cap microcentrifuge tubes (2 mL, USA Scientific, Ocala, Fla.) with O-rings were used with two 0.25 inch stainless steel balls for grinding in a Mini-BeadBeater-96 (BioSpec Products, Bartlesville, Okla.).

Type II primers were initially used to screen samples for stem pitting since previous testing demonstrated that Type II primers detect a larger percentage of stem pitting isolates than the other primers tested (Sieburth et al., 2005). First strand cDNA synthesis was prepared using immunocaptured virions as previously described (Hilf et al., 1999). For Type II primers, 25 mL reactions were set up with 1X Green GoTaq Buffer with $MgCl_2$ (Promega Corp, Madison Wis.), with an additional 0.5 mM of $MgCl_2$, 0.2 mM dNTPs, 0.625 U of Taq

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polymerase (Applied Biosystems, Foster City, Calif.) and 0.6 mM of each primer. For VT-1 primers, reactions were set up similarly, except 0.2 mM of VT-1 primers was used. The PCR cycling profiles were performed as previously described (Sieburth et al., 2004). All PCR reactions were amplified in 8-strip PCR tubes and subsequently spun in a mini-centrifuge with 8-strip insert. Products were visualized in 2.5% agarose pre-cast gels (Cambrex Bio Science Rockland, Inc., Rockland, Maine). Amplification of the CTV coat protein gene with T36CP primers and subsequent hybridization with oligonucleotide probes (ONP) I, III, IV, V, and VI were performed as previously described (Sieburth et al., 2005).

Results

It took two months to sample all 77 sites, three months for extraction, two months for running Type II primers on all samples, and one month to complete the RT-PCR and perform the hybridization on the positive samples. Fifty-five percent of the sites were negative for stem-pitting markers; the only county that tested negative at all seven sites was Indian River County in central east Florida. The single-tree incidence was calculated for potential transmission by the brown citrus aphid, *Toxoptera citricida* (Hughes and Gottwald, 1999). Twenty-seven percent of the sites had a 1-2% single tree incidence of Type II primers; twelve percent of the sites had a 3-8% single tree incidence, and only five sites (6%) had higher levels, 13.5-41% single tree incidence (Table 1).

Samples that tested positive with Type II primers were further evaluated with VT-1 primers and oligonucleotide probes (ONP). Six different profiles of stem pitting were detected as determined by whether they tested positive or negative for each test (Table 2). The two most prevalent profiles, Profile 1 and 2, both were present in seven counties. Profile 1 isolates were only positive for Type II primers and were present in 34 samples in 17 sites. Profile 2 isolates were positive for Type II and VT-1 primers and ONP III, and were present in 102 samples in 20 sites. The other profiles made up the remaining 13% of the samples. The only two Profile 3 isolates were at a site with a large number of profile 1 isolates and may be a variation of that particular isolate.

Table 1. Single tree incidence of stem pitting CTV molecular markers by county, the type of citrus in which they were found and the profile of stem pitting CTV molecular markers.

County	Positive sites	Type	Single tree incidence	Profiles present
Collier	2	Swt O[z]	1.0%	2
DeSoto	2	Swt O	1.0%	4
Hardee	4	Swt O	1.0-6.4%	1, 2, 10
Hendry	6	Swt O, GF[y]	1.0-15.9%	1, 2, 4, 10
Highlands	4	Swt O, GF	1.0-19.7%	1, 2, 3, 5
Hillsborough	4	Swt O	1.0-5.1%	1, 2, 10
Indian River	0	na[x]	na	na
Manatee	1	Swt O	5.1%	1
Martin	6	Swt O, GF	1.0-28.8%	1, 2, 4
Polk	3	Swt O	2.5-46.9%	2, 4, 10
St. Lucie	3	Swt O	1.0-4.7%	1, 10
Total	35 (77)	na	1.0-46.9%	1,2,3,4,5,10

[z] Sweet orange.
[y] Grapefruit.
[x] Not applicable.

Most of the markers were detected in sweet orange, except for three grapefruit sites. There was a single positive sample in grapefruit in Hendry County and a 19.7% single-tree incidence in grapefruit in Highlands County; both were profile 1. The grapefruit site with profile 2 in Martin County had a 28.8% single-tree incidence and was located next to an orange grove with a 26.3% single-tree incidence. This might imply that these were aphid-transmitted to the same degree. One hundred and forty-one of the positive samples contained mild CTV isolates (positive reaction with ONP VI) and 147 contained decline isolates (positive reaction with ONP I).

Discussion

Modifications were made to create an efficient means for testing with the stem pitting molecular markers. The Mini-BeadBeater-96 allowed the processing of 24 samples at a time, but the greater pressures of the Mini-bead beater-96 grinding process resulted in cracked tubes. USA Scientific 2 mL screw-cap microcentrifuge tubes with O-rings were the only tubes tested that did not crack with the two 0.25 inch stainless steel balls necessary for thorough grinding of dried, rehydrated petioles. Of the eight molecular markers tested for detection of SP-CTV (Sieburth et al., 2005), RT-PCR was chosen instead of hybridization with oligonucleotide probes since the hybridization procedure would have been much more time consuming and was not suitable for screening large numbers of samples.

The use of 8-strip PCR tubes and a mini-centrifuge with an 8-strip tube insert reduced the amount of labor and time in individually labeling and spinning tubes. The Green GoTaq buffer used in the PCR reactions contains a loading dye which allows samples to be loaded directly into the gels following PCR. Pre-cast gels reduced the labor in making the gels. As they were shorter gels than our poured gels, they ran for shorter periods of time.

Stem pitting markers are present in commercial Florida sweet orange and grapefruit. However, the majority of the sites tested negative for the markers in all samples, and only five of the 77 sites tested had a greater than 10% single tree incidence of stem-pitting isolates of CTV. Because of the long duration of sampling, some of the collections took place during the hot summer months when the CTV viral titer can be low, so the actual incidence of the markers could be higher. Of the nine different profiles from isolates collected in Florida previously (Sieburth et al., 2005), six of them were detected in this survey. By the profiles of the stem pitting markers detected in both grapefruit and sweet orange, it appears that we possibly have more than one type of stem pitting: Profile 1 and Profile 2. The high CTV incidence rates at the two grapefruit sites are of concern, since most Florida SP-CTV isolates cause severe stem-pitting in grapefruit. These profiles have all been seen in Florida before, but then Profile 5 was more prevalent among isolates collected from previous surveys than either Profile 1 or Profile 2 SP-CTV (Sieburth et al., 2005).

Widespread SP-CTV which was transmissible and caused stem pitting when inoculated into sweet orange and grapefruit seedlings, was reported in Florida (Feldman and Hanks, 1977). The scion variety was thought to influence the severity of the pitting in indicators. However, the start of the mandatory budwood program had an impact in reducing the incidence of decline isolates in citrus nurseries (Powell and Pelosi, 1993). In a study published in 2002, the majority of the isolates recovered from commercial citrus were Florida de-

Table 2. Positive (POS) and negative (Neg) results for stem pitting molecular markers showing the profiles represented, the number of positive composite samples for each profile, and the number of counties represented.

Profile	Type II	VT-1[z]	Probe III[z]	Probe IV[z]	Probe V[z]	No. counties	No. sites	No. pos.
1	POS	Neg	Neg	Neg	Neg	7	17	34
2	POS	POS	POS	Neg	Neg	7	20	102
3	POS	Neg	Neg	Neg	POS	1	1	2
4	POS	Neg	POS	Neg	Neg	4	6	9
5	POS	POS	POS	Neg	POS	1	1	1
10	POS	POS	Neg	Neg	Neg	5	6	9

[z]Results for VT-1 and Oligonucleotides Probes represent positive results of the 158 samples that tested positive with Type II primers.

cline (T-36 genotype), mild (T-30 genotype) and the only VT genotypes recovered from Meyer lemon not commercial citrus (Hilf and Garnsey, 2002). These Meyer lemon isolates caused mild to moderate stem pitting in grapefruit and not in sweet orange, but were not thought to be aphid transmitted (Lee et al., 1997). The Profile 2 stem pitting found in northern Polk County causes mild to moderate stem pitting in sweet orange biological indicators and so far, only mild stem pitting in field trees. We do not yet know if any of the other isolates that react to the stem-pitting markers cause stem pitting in field trees or in biological indicators. Not all of these isolates have been evaluated in biological indicators so whether they can cause significant damage and what their threat is to the citrus industry has yet to be determined. Currently, a strong Citrus Budwood Registration Program and increasing the number of budwood source trees under protective screen will prevent the spread of severe forms of CTV throughout the nursery industry.

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