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*Proc. Fla. State Hort. Soc*. 110:77-79. 1997.

# INDEXING OF CITRUS TRISTEZA VIRUS USING SEROLOGICAL AND BIOLOGICAL TESTS

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*Additional index words.* Elisa, citrus diseases, quick decline, sadness disease, virus indicator plants.

*Abstract.* **Samples of citrus Leaves were collected from five counties in the Florida Panhandle to detect citrus tristeza virus (CTV). The samples were subjected to ELISA test using two antibodies 11 B1 + 3 E10 and MCA 13 (monoclonal antibodies). Seven of the samples collected were positive as identified by 11B1+ 3E10 test. However, only four of these samples were severely infected with the virus as determined by the monoclonal antibody. MCA13 is a sensitive and useful test for detecting severe cases of CTV in citrus. The use of indicator plants can be useful in detecting some CTV strains which could not be detected by ELISA test.**

Tristeza, also known as quick decline, or sadness disease, is caused by citrus tristeza virus (CTV), which is one of the most destructive diseases of citrus worldwide (6). It has been a major problem in many parts of the world such as Brazil and Spain. The virus is readily transmitted by budding and grafting and in a semipersistant manner by several aphid species, yet not by seed (1, 2). The disease was first recognized by the decline of citrus scions on sour orange rootstock, and was first reported in South Africa about 1910 (8) and subsequently on sour orange in Java in 1928 (15), in Argentina about 1931 (18) and Brazil where it was first called "Tristeza" (11). In the U.S., quick decline of citrus on sour orange rootstock was first noted in California in 1939 (4). Similar diseases were reported in New Zealand (9). The cause of the decline of citrus scion on sour orange was unknown for many years, and was thought to be a graft incompatibility between the scion and sour orange rootstock or a nutritional problem (3).

CTV exhibits three symptoms; quick decline of sweet orange on sour orange rootstock, stem pitting and mild strains. Lee et al. (5) reported that mild strains of CTV cause only slight stem pitting, a little vein clearing and flecking on Mexican lime (*C. aurantifolia*) which is the most commonly used indicator plant. Symptoms of quick decline (QD) of sweet orange, grapefruit and tangerine scions budded on sour orange rootstock in the field can be manifested within three to six weeks. Inoculated indicator seedlings of sweet orange usually display stem pitting when the bark is peeled from the wood, growth reduction, stunting and often chlorosis. The symptoms in the field are often accompanied by reduction in tree and fruit size. A given CTV isolate causing stem pitting on sweet orange may or may not cause stem pitting on grapefruit and vice versa. From most CTV isolates, pitting can be observed within six months after the inoculation (13), however, a study conducted in Florida by McGovern et al. (10) showed that the biological assay method required 10 to 12 rather than 6 months for stem pitting symptoms to be observed.

Indexing trees for graft-transmissible pathogens (GTP's) may be taken as a measure of the tristeza virus reservoir existing in citrus trees. Such indexing can be used to limit and prevent the spread of the disease. The use of biological and serological means to test for the existence or absence of CTV have been useful and effective tools with which to identify the disease and to eliminate its spread. The purpose of this study was to test for the presence of mild and severe strains of tristeza virus by the use of a serological test (Enzyme-linked immunosorbent assay) and to evaluate the biological properties of CTV, vein clearing, seedling yellows and stem pitting in indicator plants.

## Materials and Methods

Citrus plant materials with suspected citrus tristeza virus (CTV) were obtained from five counties in the Florida Panhandle (Franklin, Gulf, Calhoun, Bay, Walton) where citrus is grown on a relatively small scale. Nineteen samples of tender, fully expanded leaves and small stems from nine suspected varieties were collected: grapefruit (*C. paradisi*), lemon (*C. limon*), satsuma (*C. reticulata*), sweet orange (*C. sinesis*), navel orange (*C. sinesis*), Meyer lemon (*C. limon* 'hybrid'), kumquat (*C. fortunella*), and Ponkon (*C. reticulata*). The samples were obtained from four sides of each suspected tree (one to two leaves from each side) using clean clippers and knives which were sterilized by dipping in 0.57% sodium hypochloride solution to avoid contamination. A total of four to five envelops containing the samples inserted into plastic zip-lock bags were transferred the same day to the laboratory in an ice chest. Indicator plants, Key lime (*C. aurntifolia*), Madam vinous sweet orange (*C. sinesis*) and Duncan grapefruit (*C. paradisi*) with single stems 5-10 mm in diameter and 10-15 cm in height were planted in plastic container in a complete randomized design under conducive greenhouse conditions. Key lime and Duncan grapefruits were grafted into Volkamer rootstock. Madam Vinous sweet orange plants were propagated from seeds. Healthy virus free plants were also kept in the greenhouse and used as controls.

The ELISA procedure, based on the double antibody sandwich indirect technique (DAS-I), was used as described by Roistacher (14). A solution of twenty ul rabbit antiserum (908 - $\frac{1}{8}$) diluted in 20 ml of coating buffer was prepared to coat each ELISA plate, and 200 ul of the solution was pipetted in each well in each plate. The plates were incubated for 4 hr at 37°C then washed three times with phosphate buffer saline Tween 20C (PBS-T). A 0.25g portion of leaf petiole from each sample was ground using mortar and pestle and then diluted with 5.0 ml PBS-T to be used for extraction. 200 ul of the sample extraction solution per well were added to each plate. Since only nineteen samples were available, it was decided to use four replicates for each sample. Sixteen other wells representing four controls (buffer solution), four healthy, four T-30 (a mild isolate of tristeza), and four T-36 (a severe strain of tristeza) samples were also prepared and added to each plate. The plates were incubated overnight at 4°C, and then washed three times with PBS-T. Taiwan mixture and monoclonal antibodies (11B1 + 3E10) to detect most tristeza strains, and monoclonal antibody 13 (MCA 13) to identify severe strains of tristeza were added to different plates. The plates were incubated for 2 hr at 37°C and washed three times with PBS-T. Following washing with the phosphate buffer, 200 ul of the goat anti-mouse phosphatase was added to each well. The plates were incubated for 2 hr at 37°C followed by washing

with PBS-T three times. A buffer solution consisting of 4P-Nitrophenyl phosphate PNP (Sigma Chemical Corp.) dissolved in 20 ml substrate buffer was added to each plate. Wells were read by the ELISA plate reader using a wavelength of 405 nm.

The indicator plants were inoculated with buds and tissue pieces from samples obtained from the Florida Panhandle and were confirmed to be CTV positive by the ELISA test, following the technique used by Wisler et al. (17). Two or four buds were grafted onto each of the indicator plants. The plants were monitored and the development of morphological changes such as leaf chlorosis, vein clearing etc. were recorded.

## Results and Discussion

The reaction of citrus isolates collected from different locations in the Florida Panhandle to Elisa test using the Taiwan monoclonal antibody (11 B1 + 3E 10) is shown in Table 1. From a total of 19 samples obtained, seven were positive to CTV. When the latter samples were tested using monoclonal antibody MCA 13, three of these samples were found to be infected with severe isolates of CTV (Table 1). According to McGovern et al. (10), brown citrus aphid (*Toxopters citricida*), which is the most efficient vector in spreading CTV, is not found in the Florida Panhandle region. In spite of this, and the fact that citrus plantings are limited in this area, almost one third of citrus trees in the area are infected with CTV. Proposed causes of tristeza infection included the presence of different strains of aphids such as *Aphis gossypii*, which is also considered to be an efficient vector for transmitting the CTV disease (12), *Aphis spiraecola* and *Toxopters aurantii*. It is estimated that approximately 20% of citrus trees in Florida are budded on sour orange rootstock, one of the most susceptible rootstock to CTV (7). Brown citrus aphid, another efficient vector for CTV, is presumably absent from the Florida Panhandle (10). However, the area is exposed annually to a number of hurricanes, which may have contributed to the introduction of brown citrus aphid (BCA) in the area. Extensive efforts have been made to develop rootstocks resistant to CTV disease. Sour orange rootstock, however, is still the dominant rootstock in all citrus plantings in the Panhandle. The trees may be carrying the disease, yet they are still in production. This could be due to the high yield, fruit quality, and the resistance of sour orange rootstocks to water stress and phytophthora.

Leaf chlorosis: Seedlings of Mexican lime growing under optimum conditions in the greenhouse developed distinct dark greenish color which showed vein clearing when inoculated with MCA 13 reactive isolates, designated as severe samples. Chlorotic patterns on young leaves appeared three weeks after inoculation. These patterns were accompanied by mild cases of vein clearing which intensified four weeks later.

Vein-clearing: In Mexican lime seedlings inoculated with samples obtained from MCA 13 reactive isolates, the leaves developed areas of distinct dark greenish color which showed a strong vein-clearing. On the other hand, seedlings infected with MCA 13 non-reactive isolates, showed relatively mild cases of vein clearing with occasional mild flecking which were obvious on young leaves. However, as the leaves became mature, the symptoms became more difficult to be detected.

Seedling yellow: When Duncan grapefruit seedlings were infected with samples obtained MCA 13 reactive samples they developed small, pointed-tip leaves with chlorotic patterns resembling zinc deficiency symptoms two weeks after inoculation (Table 2). These symptoms progressed rapidly, and the leaves became completely yellow within a two-week period (seedling yellow). Shoot sizes were significantly reduced, thus giving the plant a stunted appearance. There was no morphological differences in leaf color or plant size between MCA 13 non-reactive samples and the healthy control plants. These finding were similar to those obtained earlier by Roistacher (14).

Table 1. Mean values of Elisa results using monoclonal antibody 13 (MCA 13) and the Taiwan monoclonal antibody (11B1 + 3E10) for citrus samples (extracted leaves) collected from citrus trees in the Florida Panhandle compared to healthy trees.

| Treatments | MCA 13 Mean[1] | 11B1 + 3E10 Mean[1] |
|---|---|---|
| Healthy | 0.040 + 5.393 | 0.040 + 0,010 |
| T-36 | 0.067 + 0.074* | 2.102 + 0.246* |
| S1 | 0.030 + 3.500 | 0.043 + 2.273 |
| S2 | 0.051 + 0.012 | 0.051 + 6.898 |
| S3 | 0.026 + 5.041 | 0.039 + 2.272 |
| S4 | 0.066 + 0.018 | 0.926 + 0.016* |
| S5 | 0.047 + 6.481 | 1.175 + 0.024* |
| S6 | 0.488 + 0.026* | 2.768 + 0.000* |
| S7 | 0.035 + 2.136 | 1.024 + 0.037* |
| S8 | 0.052 + 0.017 | 0.043 + 5.437 |
| S9 | 0.037 + 7.937 | 0.038 + 3.400 |
| S10 | 0.044 + 5.452 | 0.034 + 2.179 |
| S11 | 0.430 + 0.018 | 0.038 + 4.010 |
| S12 | 0.037 + 3.428 | 0.040 + 3.637 |
| S13 | 0.031 + 3.276 | 0.032 + 5.148 |
| S14 | 0.031 + 3.257 | 0.038 + 2.869 |
| S15 | 0.039 + 4.644 | 0.360 + 9.978* |
| S16 | 0.174 + 0.023* | 0.832 + 0.020* |
| S17 | 0.038 + 3.379 | 0.038 + 4.750 |
| S18 | 0.039 + 2.550 | 0.053 + 0.014 |
| S19 | 0.122 + 0.012* | 0.122 + 0.012* |

[1]Means based on 4 observations.
*Means are significantly different from healthy (0.04) at $p < 0.01\%$ using Dunnett's test.
T-30 a mild isolate of tristeza.
S. sample collected from the Florida Panhandle.

Stem-pitting: Infected seedlings of Duncan grapefruit and Madam vinous did not develop stem-pitting symptoms even after six months of inoculation as stated by Wallace (16). McGovern et al. (10), on the other hand, believed that the symptoms would appear on infected trees 10-12 rather than 6 month after inoculation. The results of this study confirm these findings. Thus, it is possible that the plant can be infected with CTV, yet stem pitting symptoms may not appear within 6 months of inoculation with infected butwood.

Table 2. Duncan grapefruit reaction to seedling yellow strains of CTV*.

| Indicator plants | Sample No. | Train type | Seedling yellow |
|---|---|---|---|
| Duncan grapefruit | 4 | Mild | N |
| | 5 | Mild | N |
| | 6 | Severe | Y |
| | 7 | Mild | N |
| | 15 | Mild | N |
| | 16 | Severe | Y |
| | 19 | Severe | Y |
| | control | | N |

*Observations were made on two inoculated seedlings of each sample compared with one non- inoculated control.
N = Negative.
Y = positive.

In comparing the two methods (Elisa vs. Indicator plants, it was concluded that Elisa was faster and more accurate, saving time

and labor. The use of indicator plants can be useful in detecting some CTV strains which could not be detected by Elisa test. However, the method is considered costly and requires specific environmental conditions such as 24-27°C/20-22°C day/night temperature, respectively.


### Acknowledgment:

The authors would like to acknowledge the sincere assistance and contribution of Dr. Larry G Brown, Citrus Virologist, Division of Plant Industry, U.S. Department of Agriculture and Consumer Services, Gainesville, Florida.


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Reprinted from

*Proc. Fla. State Hort. Soc*. 110:79-82. 1997.


# PERENNIAL PEANUT GROUND COVER IN CITRUS ORCHARD ROW MIDDLES AND DISCUSSION OF POTENTIAL ENVIRONMENTAL BENEFITS


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*Additional index words. Arachis glabrata,* cover crop, sustainable agriculture.



*Abstract.* **Rhozomal (perennial) peanut (*Arachis glabrata* Benth.) establishment was monitored following planting in row middles of a one-year-old citrus grove in southwest Florida. Treatments of herbicide and fertilizer were evaluated for effect on perennial peanut plant density. Treatments were Fluazifop-p-butyl (Fusilade 2000 1E) herbicide, K-Mag fertilizer, Fluazifop-p-butyl + K-Mag only, Fluazifop-p-butyl + K-Mag + nitrogen (N), and an untreated check. A randomized complete block design with four replications were arranged between tree rows on the bed top of a typical flatwoods two-row bedded grove. Three years after planting, there were no significant differences in plant density between treatments (96%) and the check (89%). Applications of Fluazifop-p-butyl in years one and two were effective in controlling grassy weeds such as common bermudagrass [*Cynodon dactylon* (*L*.) Pers]. In this experiment, perennial peanut without inputs (herbicide, fertilizer), was able to suppress common bermudagrass and to obtain a high level (89%) ground cover in three years (1991-1994).**


The rhizoma (perennial) peanut is a tropical perennial legume introduced into Florida from Brazil in 1936 (French and Prine, 1991b) Cultivar 'Florigraze' rhizoma peanut, released in 1979, is commercially grown on 14,000 acres in Florida (French et al., 1993) This rhizomatous legume has been primarily grown as a forage crop (Saldivar et al., 1992) for hay and in pasture, though it can be used as an ornamental, conservation cover, and living mulch.


Florida Agricultural Experiment Station Journal Series No. N-01453.