Application of the Fluorescence Test to Citrus Affected by Virus and Viruslike Diseases

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A viruslike disease of citrus prevalent in parts of the Araraquara region of the state of São Paulo, Brazil (*8, 10*), has been under investigation the past four years. In 1969, Rossetti *et al.* (*9*) found that extracts of fruit albedo from diseased trees gave chromatographic profiles showing a fluorescent violet-blue spot at R_f 0.08 comparable to the reaction described by Schwarz (*11*) as marker 1-G for both South Africa greening and stubborn disease. Samples from Brazil were sent to A. W. Feldman in Florida, who identified the violet-blue phenolic marker (R_f 0.08) as gentisoyl glucose, which is also found in tissue extracts of greening-affected trees (*1*).

A preliminary test showed that chromatographed extracts of fruit albedo from trees affected by a severe strain of tristeza also exhibited the same phenolic marker (R_f 0.08). In view of these results, additional experiments, including further transmission trials of the Araraquara citrus decline, were carried out, and are described in this paper.

MATERIALS AND METHODS

Citrus decline transmission trials. To maintain Araraquara citrus decline, 45 greenhouse-grown Rangpur lime seedlings were side-grafted with tissue from diseased, six-year-old trees of nucellar Baianinha, Valencia, and Natal sweet oranges from the Araraquara region. For controls, Rangpur lime seedlings were side-grafted with tissue from nucellar trees of the same varieties and age, from the nonaffected Limeira region. In transmission trials of the causal agent, 93 Rangpur lime seedlings were inoculated either by budding or side-grafting with tissue from diseased and healthy trees of the same above-mentioned varieties. Inoculated plants were subsequently top-worked with budwood from healthy nucellar trees of the same varieties, from Limeira. These buddlings, maintained in the greenhouse, served as indicators.

The fluorescence test. From June to September, 1970, experiments were carried out with samples of bark and albedo from declining trees affected by several virus and viruslike diseases, including Araraquara citrus decline; a decline found in the Cajobi region in São Paulo, described in 1967 (*8*); different strains of tristeza from Gerd Müller's collection (Instituto Agronômico, Campinas); exocortis "decaimiento" from Tucumán, Argentina (*3*); "declinamiento" from Misiones, Argentina (*5*); and an undescribed disease observed by O. S. Passos, in Bahia, Brazil.

Water extracts from samples of fruit albedo and bark from two- to three-year-old branches from diseased and healthy trees were prepared and chromatographed following the method described by Schwarz (*11*). Extracts of fruit albedo and bark obtained from a greening-affected tree from South Africa (supplied by Schwarz) were used for a marker comparison. Chromatography profiles, after spraying with a buffered sodium borate solution (pH 8.4), were examined under ultraviolet at 366 mu. Chromatographic profiles of extracts that showed a bright violet-blue fluorescence at R_f 0.08 (comparable to greening) were designated positive, while those having no fluorescence at R_f 0.08 were designated negative.

First experiment. In June, 1970, 16 fruit and 16 branch samples were collected from each of four trees of the following: (1) six-year-old Pera, Baianinha, and Natal sweet orange trees on Rangpur lime rootstocks (decline-affected and apparently healthy), all from the same orchard; (2) 10-year-old trees of the above varieties from the Limeira region, carrying a mild strain of tristeza; (3) six-year-old Pera and Baianinha trees carrying severe Capão Bonito tristeza strain (CBT); and (4) two 10-year-old trees of each of the above varieties of healthy nucellar clones from the nonaffected Bebedouro region, for controls.

After running the chromatograms, we sent extracts of fruit albedo from two trees of Pera orange, representative of each of the three diseases, and one healthy representative from the above to A. W. Feldman for determination of the gentisoyl glucose marker.

Second experiment. In July, 1970, samples from four fruit and four branches were collected from seven-year-old trees of Pera orange on Rangpur lime rootstock from Gerd Müller's tristeza preimmunization experiment at the Instituto Agronômico, Campinas.

Four trees each of four strains of tristeza virus were used: strain 1, very mild and showing almost no pitting; strain 2, mild with pitting on the branches; strain 3, with severe pitting on the branches, and stunting; and strain 4, very severe, with very severe stunting and pitting, having been previously inoculated with a common strain and then challenge-inoculated with the very severe CBT strain.

Extracts of bark and albedo from each of the above trees were chromatographed along with extracts from greening-affected trees. Extracts from the latter served as reference standards for the presence of gentisoyl glucose. Three positive samples from the albedo extract from trees no. 53 (very mild strain), 193 (mild strain), and 79 (very severe strain) were sent to A. W. Feldman for determination of gentisoyl glucose.

Third experiment. Four fruit and four branch samples from each of three declining eight-year-old trees of Valencia orange on Rangpur lime were obtained from an orchard affected by a viruslike disease of undetermined cause in the Cajobi region (*8*). Similar samples were also collected from two apparently healthy trees in the same orchard as well as from a declining exocortis-affected tree.

In Bahia, three to six fruit samples were also collected from two seven-year-old nucellar Pera orange trees and from three old-clone, 10-year-old trees of the same variety showing severe decline, stunting, and die-back. Samples were extracted and chromatographed as described above.

Various diseases from Argentina. L. Foguet, from Tucumán, Argentina, collected and sent samples from declining and apparently healthy trees, to be tested. These included: six fruit and six branch samples from affected five-year-old trees of Ruby Blood grapefruit on Cleopatra tangerine rootstock; four samples of both fruit and branches from two declining eight-year-old trees of Jaffa orange on Rangpur lime rootstock; fruit and branch samples from two healthy trees of the same variety and age from the El Timbo orchard, Tucumán; and fruit and branch samples from two healthy 30-year-old trees of Ruby Blood on Cleopatra tangerine from the Estación Experimental Agricola de Tucumán.

The following fruit samples from several orchards of the Misiones region were collected and tested: four from each of five trees of 18-year-old Calderón sweet orange on *Poncirus trifoliata* rootstock and from two apparently healthy five-year-old trees of the same variety from a nearby orchard; four from each of five declining trees of Valencia sweet orange on Rough lemon and from two apparently healthy trees of the same variety in the same orchard.

TABLE 1

RESULTS OF FLUORESCENCE TESTS FOR THE PRESENCE OF GENTISOYL GLUCOSE IN EXTRACTS OF BARK AND ALBEDO FROM SEVERAL CITRUS VARIETIES

Grove and variety	Disease condition	No. trees*	No. samples†	
			Albedo	Bark
Bebedouro:				
Pera	healthy	0/2	0/8	0/8
Baianinha	healthy	0/2	0/8	0/8
Natal	healthy	0/2	0/8	0/8
Araraquara:				
Pera	decline	2/4	8/16	5/16
Baianinha	decline	4/4	10/16	1/16
Natal	decline	3/4	7/16	8/16
Pera	healthy	0/2	0/8	0/8
Baianinha	healthy	0/2	0/8	0/8
Natal	healthy	0/2	0/8	0/8
Limeira:				
Pera	tristeza	4/4	14/16	0/16
Baianinha	tristeza	4/4	9/16	1/16
Natal	tristeza	3/4	5/16	3/16
Capão Bonito:				
Pera	severe tristeza	4/4	15/16	1/16
Baianinha	severe tristeza	4/4	13/16	3/16
Campinas:				
Pera	tristeza vm‡	2/4	3/16	0/16
Pera	tristeza m	4/4	14/16	6/16
Pera	tristeza s	4/4	10/16	12/16
Pera	tristeza vs	4/4	11/16	10/16
Cajobi:				
Pera	blight	0/3	0/12	0/12
Pera	healthy	0/2	0/8	0/8
Bebedouro:				
Pera	exocortis	0/1	0/4	0/4
Bahia State:				
Pera nc‡	decline	2/2	9/10	.....
Pera oc	decline	3/3	10/15	.....
Tucumán:				
Ruby blood	healthy	1/1	1/2	0/2
Ruby blood	decline	1/3	5/6	0/6
Jaffa	healthy	0/1	0/2	0/2
Jaffa	decline	1/1	1/2	0/2
Misiones:				
Calderón	healthy	1/2	3/8	.....
Calderón	decline	2/5	4/20	.....
Valencia	healthy	1/2	3/8	.....
Valencia	decline	3/5	8/20	.....

* Number of trees showing gentisoyl glucose in chromatograms at R_f 0.08/number of trees sampled.
† Number of samples showing gentisoyl glucose in chromatograms at R_f 0.08/number of samples examined.
‡ vm = very mild; m = mild; s = severe, vs = very severe; nc = nucellar clone, oc = old clone.

RESULTS

Citrus decline transmission trials. No plants from the Araraquara region grafted with diseased material used either for perpetuation or for inoculation with citrus decline have shown any symptoms of decline during the last two years.

Fluorescence tests. Results of all tests are summarized in table 1.

First experiment. The marker of gentisoyl glucose was found in 75 per cent of the 12 declined trees from different varieties and was considered positive. Of the 48 albedo and bark samples, 52 and 29 per cent, respectively, were positive. Ninety-one per cent of the 12 trees affected by common Limeira tristeza had albedo and bark samples showing positive in 58.3 and 8.3 per cent of the samples, respectively. Of the eight trees affected by CBT, 32 albedo and bark samples showed a positive response for 87.5 and 12.5 per cent of the samples, respectively. None of the samples from Bebedouro or Araraquara control trees gave a positive fluorescence reaction.

Chemical analysis of the positive samples was made by A. W. Feldman. These samples showed the presence of gentisoyl glucose in the two Pera trees that were declining and in the two Pera trees affected by severe CBT, all from Araraquara. Samples from the two healthy Pera trees (control) from Bebedouro and the two Pera trees from Limeira carrying a mild strain of tristeza which showed no fluorescence were also negative for gentisoyl glucose.

Second experiment. Positive fluores-

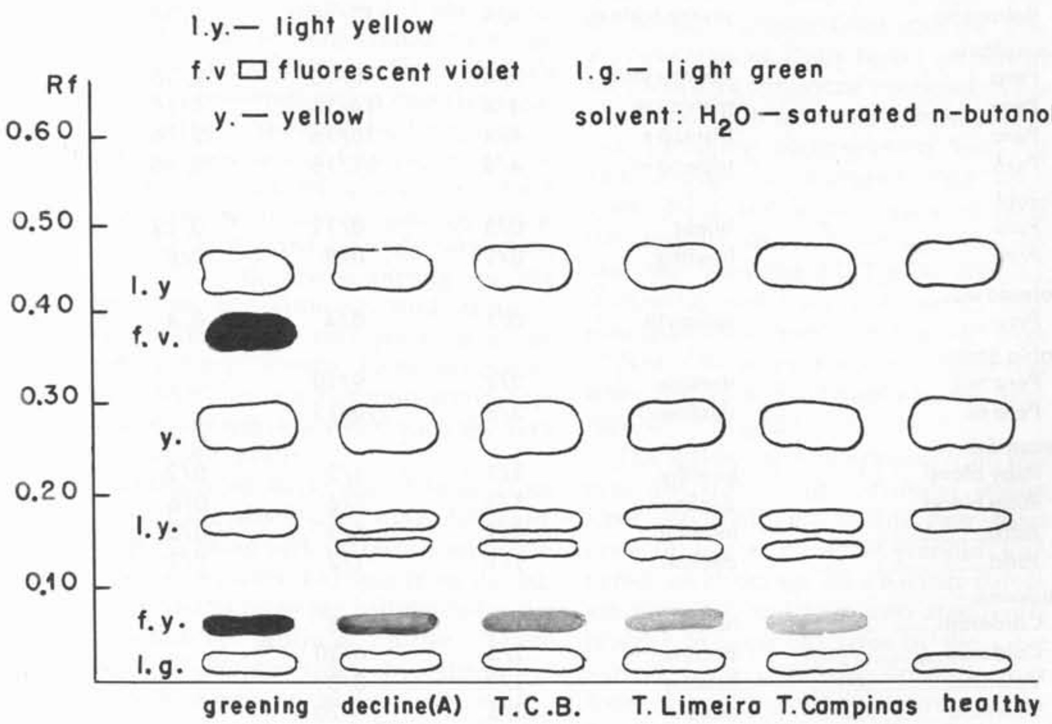

Fig. 1. Results of fluorescence test applied to citrus decline and two strains of tristeza, as compared with greening.

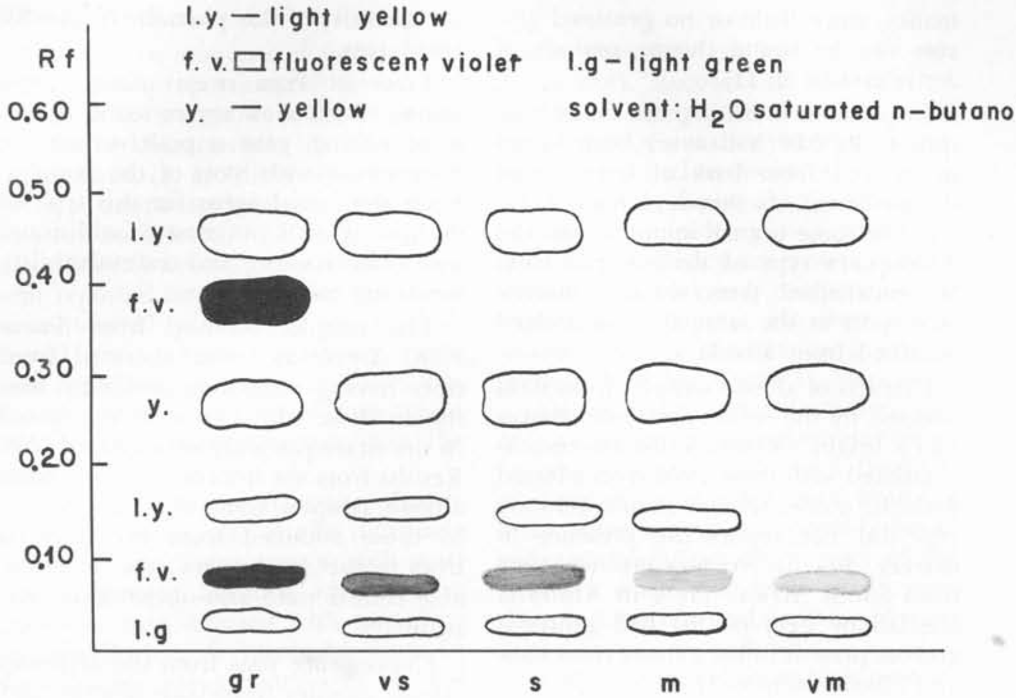

Fig. 2. Results of fluorescence test applied to very severe, severe, mild, and very mild strains of tristeza, as compared with greening.

cence tests fom the four trees representing each strain of tristeza (very mild to very severe) were 50, 100, 100, and 100 per cent, respectively; albedo tests were positive in 25.0, 87.5, 62.5, and 68.5 per cent, respectively, of the 16 samples of each tristeza strain; and bark tests were positive in 0.0, 37.5, 75.0, and 62.5 per cent, respectively, for the 16 samples of each tristeza strain category.

In extracts of this experiment, A. W. Feldman found gentisoyl glucose in samples from trees affected by the very severe strain of tristeza, while samples from trees affected by milder strains were negative for gentisoyl glucose marker.

Marker 2-G (R_f about 0.36), which is normally present in greening material, was never detected in any of the chromatographed tissue samples.

Third experiment. Fluorescence at R_f 0.08 was obtained in extracts of samples from all Pera trees from Bahia. Of 25 tissue samples collected, 76 per cent were positive for marker 1-G. No fluorescence at R_f 0.08 was noted on any of the chromatograms prepared from tissue extracts from any of the other trees tested.

Various diseases from Argentina. In samples obtained from the Misiones area (table 1), the fluorescence marker was found in three of the five-year-old healthy Calderón trees, in one 18-year-old diseased tree of the same variety, and in two of the diseased Valencia trees. Samples from the remaining trees exhibited a faint fluorescence at R_f 0.08. Three of the four trees sampled in the Tucumán area showed fluorescence at R_f 0.08.

DISCUSSION AND CONCLUSIONS

Tests described herein were carried out with samples collected during dormancy, since little or no gentisoyl glucose can be found during periods of active growth (*1, 11*).

In previous work (*9*), the violet-blue spot at R_f 0.08 had never been found in extracts from bark of branches of diseased trees. As shown in table 1, extracts of some branch samples from the Araraquara type of decline and from tristeza-affected trees showed fluorescent spots at the same R_f as did those obtained from albedo.

Extracts of albedo samples from trees affected by the severe strain of tristeza had a bright violet-blue fluorescence as compared with those from trees affected with the milder tristeza strains. Schwarz (*12*) did not report the presence of marker 1-G in tristeza-affected trees from South Africa. Trees in Australia affected by stem pitting had gentisoyl glucose present in the extract from bark and albedo samples (*1*).

Tristeza-affected trees may, in certain instances, show foliar symptoms similar to greening, such as zinc deficiency symptoms and yellowing of the leaf veins. Other leaf and fruit symptoms characteristic of stubborn or greening are very rarely found in São Paulo. Declining trees from Araraquara always show root-rot. Declining trees from the Capão Bonito area with the severe tristeza strain produce severe pitting in all sweet orange varieties, and in the Rangpur lime, rootstock and young branches break very easily. Such symptoms, however, do not seem to be associated with greening in South Africa.

Feldman (*2*) reported that sinapic acid is found at an elevated level in virus-infected trees. Its increase in diseased trees may account for the hardened conditions of the wood tissue since this phenolic is utilized as a lignin precurson (*2*). Brittle branches, prevalent in trees affected by severe tristeza strains (*4*), in declined trees in the Araraquara region of Brazil, and in Hassaku dwarf-infected trees in Japan (Rossetti, unpublished report), might be related to the quantity of this phenolic in the diseased trees.

Trees of Pera sweet orange from Bahia, which show severe stunting and some pitting, gave a positive test for fluorescence with most of the samples. Since the causal agent for this type of decline is still unknown, additional studies on etiology and transmissibility are being continued.

The samples received from Tucumán, Argentina, were obtained from trees having symptoms strikingly similar to those with citrus decline found in the Araraquara region in Brazil (*10*). Results from the fluorescence tests from albedo samples were also comparable to those obtained from the diseased trees in the Araraquara area. Comparable results were also obtained by Ramallo (*7*).

Fluorescence tests from the Misiones albedo samples from both healthy and diseased trees gave apparently contradictory results, since samples from both healthy and diseased trees showed a faint fluorescent spot at R_f 0.08. Similar results were also reported by Pujol *et al.* (*6*).

Schwarz (*12*) indicated that marker 1-G is not uniformly distributed in greening-affected trees. Feldman (*2*) also noted that tissue age and season seem to influence the quantity of phenolics in plant tissue. Oliveira and Müller (unpublished report on the study of the Araraquara type of citrus decline) noted that certain fluorescent substances may accumulate during different stages of the vegetative development.

Thus, data on the inconsistent accumulations of gentisoyl glucose make it difficult to standardize the sampling techniques for applying the fluorescence tests in Brazil, where climatic variations are frequent. Moreover, the fact that Feldman also found gentisoyl glucose in several of the diseased trees affected either by the Araraquara type of decline or by the severe tristeza strains in

Brazil, leads us to conclude that the fluorescent-marker technique cannot be used to index trees for greening or stubborn in São Paulo, Brazil.

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