Effectiveness of the Chromatographic Method for Detecting Exocortis Virus Infection in Poncirus trifoliata

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ABSTRACT

Free and bound forms of scopoletin and umbelliferone accumulate in the bark of *Poncirus trifoliata* (at the bud union) within 3 years after budding with a citrus exocortis virus-infected (CEV) scion. These coumarins, detected by thin-layer chromatography (TLC), were used to ascertain CEV infection in the candidate scion. Effectiveness of the TLC method for detecting CEV in candidate trees was compared with the phloroglucinol-HCl color test and with the standard citron (*Citrus medica* L.) indexing. Percentage of correct diagnoses by TLC (using data from citron indexing as a standard) was highest (90%) when extracts were prepared from *P. trifoliata* bark collected in May and June. Diagnoses were correct in 70% of samples collected in April and July, and in 55% of samples collected in October. Scion variety did not appear to modify the specific coumarins that accumulated in the CEV-infected *P. trifoliata* bark or affect their detection by TLC. Infection with xyloporosis, tristeza, or psorosis virus(es) did not cause accumulation of coumarins in *P. trifoliata* bark. Phloroglucinol-HCl color tests correlated closely (ca. 90%) with the citron index standards only when the candidate trees had been budded for less than 8 years. Phytopathology 61: 1338-1341.

Additional key words: citrus virus, phenolics.

Phenolic substances, primarily free and bound forms of scopoletin and umbelliferone, accumlate in the bark of trifoliate orange (*Poncirus trifoliata* Raf.) as well as in the leaf midribs and young bark of Etrog citron (*Citrus medica* L.) plants infected by citrus exocortis virus (CEV) (3, 8, 14). Although the first conspicuous symptom of CEV infection in *P. trifoliata* (bark scaling) may not appear until 4 to 12 years after inoculation, coumarins are usually present within 3 years after inoculation (3, 14). Ethanolic extracts of bark from CEV-infected *P. trifoliata*, when chromatographed by thin-layer chromatography (TLC) and viewed under ultraviolet light, reveal one to three, bright-blue to violet fluorescent bands composed essentially of free and bound coumarins (3, 14). Duration of infection and time of the year at which bark samples are collected appear to influence the number and intensity of these fluorescent bands (3, 14).

Poncirus trifoliata and some of its hybrids are potentially important rootstocks for citrus in Florida, but their use is predicated on the availability of budwood free of CEV. Indexing citrus budwood source trees for CEV currently is done by bud-inoculating CEV-sensitive citron clones. In such clones, symptoms of CEV often appear in 2-6 months; but with some strains of CEV, symptoms may not be evident until 1 or more years after inoculation. A limited amount of indexing for CEV infection in *P. trifoliata* has also been done, using the phloroglucinol-HCl method (2).

In these investigations, we are concerned with (i) the results of the chromatographic method of determining CEV infection in *P. trifoliata* as compared with those obtained by the citron and phloroglucinol-HCl indexing method; and (ii) whether there is an optimum time of the year for collection of the bark samples from *P. trifoliata* rootstocks.

MATERIALS AND METHODS.—*Source trees.*—The 155 trees selected for these tests were located in the Florida Budwood Foundation Grove in central Florida. All were growing on *P. trifoliata* rootstock, and had been budded at various times between 1955 and 1965. Less than 5% of the trees exhibited bark scaling on the stock. Each scion represented a different source of budwood and included cultivars of *C. sinensis* Osbeck 'Hamlin', 'Valencia', 'Queen', 'Pineapple', 'Sweet Seedling', 'Enterprise', 'Homosassa', 'Navel', 'Parson Brown', and 'Blood Orange'; *C. paradisi* Macf. 'Marsh', 'Duncan', 'Thompson', and 'Ruby' grapefruit; *C. paradisi* x *C. reticulata* 'Orlando' tangelo; *C reticulata* Blanco 'Dancy' tangerine; *C. temple* 'Temple'; and *C. reticulata* hybr. Blanco 'Murcott'.

Indexing on citron.—Five different citron clones (from seven citron clones, X-186, OES-2, OES-4, OES-7, OES-9, OES-10, and Arizona-861) were used for indexing each source tree. These citron clones were budded during April and early May of 1969, and maintained in a screenhouse. During this same period, buds from each source tree were also grafted on five citron plants of clone OES-4, which were then maintained in field plots. A designation of CEV-positive was used for the source tree when two citron plants either in the screenhouse or in the field plots exhibited symptoms (2). If no symptoms were noted after 1 year, the source tree was considered CEV-free in accordance with the current standard procedure of the Florida Department of Agriculture.

Phloroglucinol-HCl color test.—One bark sample

from the *P. trifoliata* stock of each source tree was collected during April and early May of 1969, and processed according to the procedure of Childs (2).

Samples for chromatography.—Bark samples were collected from the *P. trifoliata* stock, just below the bud union, from April through July and in October of 1967 through 1969. Each sample consisted of a single 50- x 30-mm section of bark with cambial tissue. Approximately one-third of these trees were sampled twice during this 3-year period. All samples were frozen at time of collection. For processing, bark was finely chopped and extracted in 50% ethanol (1:5 fresh wt/v) at 4 C for 72 hr. Twenty-five or 50 μliters of the bark extract were charged onto TLC plates (Mallinckrodt-7G), developed in water-saturated *n*-butanol, air-dried, and viewed under ultraviolet. Typical fluorescent zones, indicative of CEV infection, appear as a violet band at R_f 0.55 to 0.63; generally, a second or third band is evident at R_f values of 0.67 to 0.72 and 0.78 to 0.82. The fluorescent band at R_f 0.55 to 0.63 is the principal fluorescence associated with CEV infection in *P. trifoliata* (3, 8).

Because CEV can be mechanically transmitted in some species of *Citrus* (9) and possibly in *Poncirus*, extensive precautions were taken to disinfest the tools used for budding, pruning, and bark sampling (9).

RESULTS.—*Citron indexing.—Screenhouse.*—Fifty-six of the 155 source trees indexed were found to be CEV-infected (Table 1). Citron clones OES-2, OES-4, OES-9, and Arizona-861 were the most sensitive of the seven clones used, and accounted for ca. 75% of the initial detection of CEV infection. Data from these tests were used as a standard for comparing the performance of the other indexing procedures, and hereafter will be referred to as the citron index standards.

Field plots.—These tests showed that 53 of the 155 source trees indexed were CEV-infected (Table 1). All 53 trees coincided with those shown to be infected by the citron index standards. Three trees diagnosed as infected by the citron index standards were negative in this test. These three trees were scions of sweet orange (Hamlin, Pineapple, and Sweet Seedling) that had budded in 1955, 1961, and 1964, respectively.

Phloroglucinol-HCl color tests.—Thirty trees were rated as CEV-infected, 29 of which coincided with ratings obtained by the citron index standards (Table 1). The remaining 27 source trees, indexed CEV-free by the phloroglucinol-HCl method but CEV-infected by the citron index standards, were predominately from trees budded prior to 1961. Of the 125 source trees rated as CEV-free, 97 were similarly rated by the citron index standards (Table 1).

Chromatography and time of sampling.—Chromatographed extracts of the *P. trifoliata* bark from 52 of the source trees exhibited typical fluorescent pattern(s) indicative of CEV infection (Table 1). Of this group, 39 trees were also rated CEV-infected by the citron index standards. Thus, 13 trees rated as CEV-infected by TLC were rated as CEV-free by the citron index standards. TLC of extracts from 11 of these 13 trees revealed only a single fluorescent band of low intensity (low concentration of coumarins) at R_f 0.55 to 0.63.

The 103 trees shown to be CEV-free by TLC included 86 source trees that were also comparably rated by the citron index standards (Table 1).

The above data on indexing by the TLC method are from all sampling times during the 3-year period. The per cent correct diagnoses of both healthy and CEV-infected source trees ascertained by TLC varied according to the time of year when bark samples were collected (Fig. 1). Thus, chromatographed extracts of bark collected in October from CEV-infected trees frequently failed to exhibit the fluorescent patterns typical of CEV infection. These fluorescent patterns were occasionally lacking in some of the extracts of bark collected in April and in July. In the October sampling, a correct diagnosis was obtained for 55% of the healthy and CEV-infected source trees. The April and July samplings gave a correct diagnosis of ca. 70%, whereas the May and June samples yielded a correct diagnosis of ca. 90% (Fig. 1).

Scion variety did not appear to modify the specific coumarins that accumulated in the CEV-infected *P. trifoliata* bark, nor alter their detection by TLC. Also, coumarins did not accumulate in the bark of the *P.*

TABLE 1. Diagnoses of healthy and exocortis-infected citrus cultivars on *Poncirus trifoliata* rootstock as determined by citron indexing, phloroglucinol-HCl color tests, and thin-layer chromatography (TLC)

Method of indexing[a]	Number diagnosed as exocortis-negative	Number diagnosed as exocortis-positive
Citron (screenhouse)[b]	99	56
Citron (field plots)[c]	102 (99)[e]	53 (53)
Phloroglucinol-HCl	125 (97)	30 (29)
TLC[d]	103 (86)	52 (39)

[a] Bud samples for citron indexing and bark samples for phloroglucinol-HCl tests were collected in April and early May of 1969. Bark samples for TLC analyses were collected from April through July, and in October of 1967 through 1969.

[b] Five different citron clones, selected from seven citron clones (X-186, OES-2, OES-4, OES-7, OES-9, OES-10, and Arizona-861) were used for indexing each of the 155 source trees. Data from these tests were used as a standard for comparing the performance of the other indexing procedures, and are referred to as the index standards.

[c] Five citron plants of clone OES-4 were used for indexing each source tree.

[d] Performance from all sampling periods (see METHODS).

[e] Numbers in parentheses refer to the total number of source trees that coincide with the results of the citron (screenhouse) index standards.

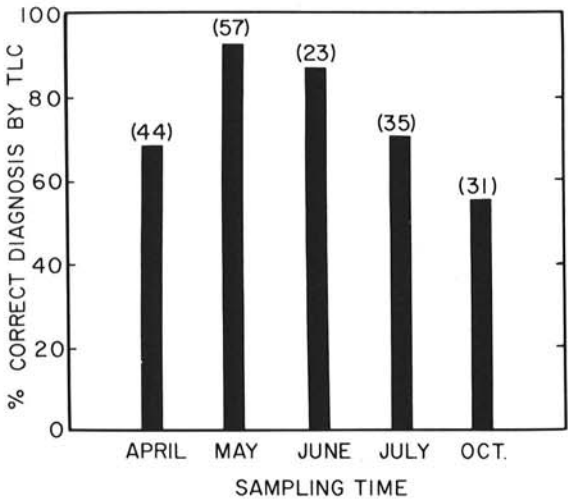

Fig. 1. Per cent correct diagnoses (using data from citron indexing as a standard) of healthy and exocortis-infected citrus cultivars on *Poncirus trifoliata* rootstock as determined by the thin-layer chromatographic (TLC) method. Figures in parentheses indicate the total number of samples analyzed each month from collections made from 1967 through 1969.

trifoliata stock at the bud union when the scion was infected with xyloporosis, tristeza, or psorosis virus(es).

DISCUSSION.—Infection by CEV appears to be tolerated by *P. trifoliata*, with the consequence that symptoms are usually delayed or are very gradual in developing. The accumulation of free and bound forms of scopoletin and umbelliferone in the *P. trifoliata* rootstock (3, 6), however, are evident within 3 years after inoculation, and forms the basis for CEV detection by TLC (3, 14). These coumarins were not found in extracts of bark from CEV-infected Rangpur lime (*C. limonia* Osbeck) or from Troyer and Carrizo citranges *(C. sinensis* x *P. trifoliata) (unpublished data).* Though bound forms of scopoletin and umbelliferone are normal constituents of *Citrus* and *P. trifoliata* (4, 5, 6) as well as of other plants, particularly when subjected to stress (8, 10, 11), they do not seem to be normal constituents of the *P. trifoliata* bark used in these tests.

The accumulation and disappearance of these coumarins in the *P. trifoliata* bark does not appear to be scion-related. These coumarins seem to be influenced by seasonal factors, and may coincide with fluctuations in the virus titer in the host. Recent experiments with CEV-infected citron indicate that a direct relationship appears to exist between the ratio of scopoletin to umbelliferone and the titer of virus in citron tissue (S. M. Garnsey & A. W. Feldman, *unpublished data*).

Extracts of bark collected in May and June from CEV-infected *P. trifoliata* consistently exhibited strong fluorescent bands on TLC, whereas fluorescent bands were either generally weaker or were occasionally absent, indicating a random distribution (8) as well as a lower concentration of these coumarins in bark samples collected in April and July. Fluorescent bands on TLC characteristic of CEV infection were occasionally absent in extracts of bark samples collected in October.

This cyclic pattern of increase and decrease of coumarins may be a reflection of seasonally induced changes in host physiology. Should the CEV titer in the scion be related to the coumarin concentration present in the *P. trifoliata* bark, then budwood for indexing on citron might best be collected during May and June when coumarins are most prominent in the bark of CEV-infected *P. trifoliata.* Samples collected at this time of the year for indexing in citron might yield a higher incidence of correct diagnosis, particularly with the milder strains of CEV.

It is possible that the 13 trees, rated as CEV-infected by TLC but rated as CEV-free by the citron index standards, would have had a different rating by the latter method had budwood for indexing been collected in June. Additional studies will be needed to elucidate this point. The time of year in which budwood is collected for diagnoses of greening and stubborn diseases in *Citrus* has also been shown to be an important variable in indexing for these diseases (1, 15).

The low incidence of confirmation of CEV infection by the phloroglucinol-HCl method was especially noticeable for trees budded prior to 1961, and would indicate that the reliability of this diagnostic method is considerably reduced for trees budded longer than 8 years. It is quite conceivable that the aldehydes, which are responsible for the color formation with phloroglucinol-HCl, may be transitory in a manner similar to the coumarins, and thus were in low concentration or were lacking in bark collected in the spring from many of the older, CEV-infected trees.

There appear to be considerable similarities in the pattern of coumarin accumulation in CEV-infected *P. trifoliata* with that of gentisoyl-glucose accumulation in greening-affected *Citrus* (7, 12, 13, 14). In both diseases, the phenolic compound is manifested in a specific host tissue, often randomly distributed (albedo and bark of 2- to 3-year-old branches for greening); each accumulates at a specific time during the year (fall and winter for greening); and each is evident prior to the appearance of visible symptoms. Like gentisoyl-glucose in greening-affected *Citrus*, the bound and free coumarins that accumulate in CEV-infected *P. trifoliata* can be used as an early and presumptive diagnosis of CEV infection. The TLC method may be especially useful for detecting the presumably milder strains of CEV. As these coumarins are randomly distributed in the *P. trifoliata* bark and are in higher concentrations during certain times of the year, it is desirable to collect several bark samples from each source tree, particularly during May and/or June.

LITERATURE CITED

1. CALAVAN, E. C., C. N. ROISTACHER, & D. W. CHRISTIANSEN. 1968. Distribution of stubborn disease virus in trees of Citrus sinensis and C. paradisi at different seasons, p. 145-152. *In* J. F. L. Childs [ed.]

4th Conf. Int. Organ. Citrus Virol. Proc. Univ. Fla. Press, Gainesville.

2. CHILDS, J. F. L. (Chm.). 1968. Indexing procedures for 15 virus diseases of citrus trees. U.S. Dep. Agr. Handbook No. 333. Washington, D.C. 96 p.
3. FELDMAN, A. W. 1969. Chemical changes induced in citrus plants by viruses, p. 1495-1503. *In* H. D. Chapman [ed.] 1st Int. Citrus Symp. Proc., Vol. III. Univ. Calif., Riverside.
4. FELDMAN, A. W., & R. W. HANKS. 1965. Phenolic compounds in roots and leaves of four citrus cultivars. Nature 207: 985-986.
5. FELDMAN, A. W., & R. W. HANKS. 1968. Phenolic content in the roots and leaves of tolerant and susceptible citrus cultivars attacked by Radopholus similis. Phytochemistry 7:5-12.
6. FELDMAN, A. W., & R. W. HANKS. 1968. Identification and quantification of phenolics in the leaves and roots of healthy and exocortis infected citrus, p. 292-299. *In* J. F. L. Childs [ed.] 4th Conf. Int. Organ. Citrus Virol. Proc. Univ. Fla. Press, Gainesville.
7. FELDMAN, A. W., & R. W. HANKS. 1969. The occurrence of a gentisic glucoside in the bark and albedo of virus-infected citrus trees. Phytopathology 59:603-606.
8. FELDMAN, A. W., R. W. HANKS, S. M. GARNSEY. 1969. Localization and detection of coumarins in exocortis virus-infected citron. *In* W. C. Price [ed.] 5th Conf. Int. Organ. Citrus Virol. Proc., Univ. Fla. Press, Gainesville (in press).
9. GARNSEY, S. M. 1967. Exocortis virus of citrus can be spread by contaminated tools. Fla. State Hort. Soc. Proc. 80:68-73.
10. HUGHES, J. C., & T. SWAIN. 1960. Scopolin production in potato tubers infected with Phytophthora infestans. Phytopathology 50:398-400.
11. MINAMIKAWA, T., T. AKAZAWA, & I. URITANI. 1963. Analytical study of umbelliferone and scopoletin synthesis in sweet potato roots infected by Ceratocystis fimbriata. Plant Physiol. 38:493-497.
12. SCHWARZ, R. E. 1965. A fluorescent substance present in tissues of greening-affected sweet orange. South Africa J. Agr. Sci. 8:1177-1180.
13. SCHWARZ, R. E. 1968. Thin layer chromatographic studies on phenolic markers of the greening virus in various citrus species. South Africa J. Agr. Sci. 11:797-802.
14. SCHWARZ, R. E. 1968. Indexing of greening and exocortis by means of fluorescent marker substances, p. 118-124. *In* J. F. L. Childs [ed.] 4th Conf. Int. Organ. Citrus Virol. Proc. Univ. Fla. Press, Gainesville.
15. SCHWARZ, R. E. 1968. Indexing and epidemiology of greening and some other citrus virus diseases in South Africa. D.Sc. Thesis, Univ. Pretoria, South Africa. 87 p.