Indexing of Greening and Exocortis Through Fluorescent Marker Substances

R. E. SCHWARZ

THE PRESENCE of specific fluorescent materials can be associated with virus infection of various plants, and Schwarz (2) presented evidence that a specific fluorescent substance is found in the reproductive and vegetative tissues of sweet orange [*Citrus sinensis* (L.) Osb.] infected with the greening virus.

An analysis of the fluorescent materials by thin layer chromatography (TLC) was made for the purpose of developing an index test for greening virus in the fruit and bark of greening-affected citrus and for exocortis virus in the bark of trifoliate orange. The results of this work are reported here.

Materials and Methods

ALBEDO FLUORESCENCE TEST.—Both normal and greened fruits were collected from Valencia, Midseason, and Washington navel varieties of sweet orange in the main greening areas of the Transvaal (White River, Tzaneen, and Rustenburg). Normal fruit was collected from trees in areas free of greening disease. In addition, some stubborn and normal fruits of sweet orange from California were also examined.

Fruits were halved lengthwise and the albedo surface examined under an ultraviolet lamp (Portable ultraviolet lamp, type II, Hanovia, Slough, England), having 95 per cent of the radiation at 365 mμ.

ANALYSIS OF ALBEDO EXTRACTS BY THIN LAYER CHROMATOGRAPHY.—The material for TLC analysis was prepared either from the albedo of the fruit or the bark of the branches. The albedo or bark (0.5 g) was cut into small pieces and transferred to a plastic sampling tube to which 2 ml of water was added. The mixture was shaken for 2 hours, the eluate decanted into evaporation dishes, and the water evaporated at 50°C under reduced pressure. The residue was taken up with 0.5 ml water and spotted in 4 μl portions, on 20 x 20 cm chromoplates coated with 600 μ layer of Mercks' silica gel, according to Stahl's (4) method, and examined under ultraviolet light. The violet fluorescence of the Rf 0.08 marker substance is intensified when the chromoplate, after development and drying, is sprayed with a sodium borate buffer solution of pH 8.7.

Thin layer chromatography analysis was made of the albedo of normal, greened, and stubborn fruits. Samples of bark for analysis were obtained

from sweet orange seedlings experimentally infected with greening by psylla insects, and from naturally infected greened trees in orchards of the main greening areas (White River, Tzaneen, and Rustenburg). In addition, analyses were made of bark from normal trees growing in greening-free areas, and from trees suffering from disorders other than greening.

Analyses were also made of bark from trifoliate orange [*Poncirus trifoliata* (L.) Raf.] rootstocks of 32-year-old Washington navel trees showing severe exocortis symptoms, from trifoliata stocks of healthy trees of the same age, from trifoliata stocks of 6-year-old sweet orange trees previously inoculated with exocortis at the age of 2 years, and from trifoliata stock of normal sweet oranges of the same age.

A few tests were also made of the fruit and bark of other species, including mandarin (*C. reticulata* Blanco), tangelo (*C. reticulata* Blanco x *C. paradisi* Macf.), lemon [*C. limon* (L.) Burm.], grapefruit (*C. paradisi* Macf.), and citron (*C. medica* L.).

Results

ALBEDO FLUORESCENCE TEST: GREENING.—Fruits from greening-affected trees growing in various areas were classified into the following three groups: (a) fruits with typical external symptoms of greening, including lopsidedness and off-color, (b) doubtful fruits, and (c) normal-looking fruits. All fruits of group (a) showed bright violet fluorescence of the albedo; a variable percentage of the fruits in group (b) exhibited the specific fluorescence. Normal fruits show a yellow-brown fluorescence. A statistical study of the correlation between external symptoms and albedo fluorescence is in progress.

The violet-fluorescent substance is usually unevenly distributed in the albedo. The highest concentration occurs in the albedo near the stem-end and in the columella. The concentration on one side, usually the better developed side of the fruit, is often higher. Intensity of fluorescence decreases from Valencias to Midseasons to navels. The fluorescent substance first appears when the fruits are about 20-30 mm long and occasionally even earlier. It is never present in the albedo of fruits of trees free of greening virus, nor is it present in fruits of trees infected only with exocortis, xyloporosis, tristeza, or psorosis virus. Fruits of trees affected by dry root rot or by a deficiency of zinc, boron, manganese, or copper do not show this fluorescence either. Fruits from trees affected by the latter disorders sometimes show a type of fluorescence different from that of normal fruits, but never the bright violet type of fluores-

cence associated with greening. Localized fluorescence similar to, but distinguishable from the one caused by greening, was found under blackspot (*Guignardia citricarpa* Kiely) lesions in decayed fruits and in association with lesions from fruit fly stings and the holes made by the larvae of the false coddling moth.

The stem-end part of the albedo of several affected mandarins and tangelos also exhibited violet fluorescence, but it was much less conspicuous than in sweet orange fruit. A violet fluorescence was also found in the local lesions in the albedo of lemons severely affected by greening, and a much more pronounced violet fluorescence occurred in the albedo of greening-affected fruit of the Corsican citron. However, the relation of the violet fluorescence to greening virus in these latter instances remains to be confirmed. No fluorescence was observed in the albedo of grapefruit, even in fruits with pronounced external symptoms of the disease such as lopsidedness and aborted seeds.

ALBEDO FLUORESCENCE TESTS: STUBBORN.—The albedo of stubborn-affected Olinda Valencias from California showed fluorescence identical in color and intensity to that of greened fruits. However, the violet fluorescence in the stem-end albedo and in the columella of stubborn-affected fruits of the sweet orange varieties Campbell Valencia, Frost Valencia, Madam Vinous, and Ruvel navel was relatively weak. None of the healthy fruits from any variety from California showed the violet fluorescence.

CHROMATOGRAPHIC ANALYSIS OF THE ALBEDO EXTRACTS.—The TLC profiles prepared from the albedo extracts of greened and normal oranges are compared in Figure 1. The former show a violet marker substance 1g (g indicates marker substance specific for greening), with an Rf of 0.08, and in addition, there often is a second violet marker substance 2g, with an Rf of 0.4. Both are absent in the chromatographic profiles of normal oranges. Marker substance 1g is responsible for the violet fluorescence of the albedo of affected fruits, and is of high diagnostic value. Investigations on the chemical nature of the substance, probably a coumarin glycoside, are in progress. Other fluorescent materials were found in extracts of healthy fruits, but were absent in those of greened fruits. Furthermore, their number varied in different samples. Thus, their absence in a certain sample is not regarded as significant.

Albedo extracts from the 5 fresh, stubborn fruits, and from 3 of the 5 dried samples of albedo from stubborn fruits, contained marker substance 1s (s indicates a marker substance specific for stubborn). Marker substance 1s and 1g are identical. The absence of the marker substance

in 2 of the dried samples of stubborn-affected fruits might be caused by the drying process. None of the 5 fresh or 7 dried samples of healthy fruits from California contained the marker substance. A second marker substance, 2s, was found in 2 of the 5 fresh fruit samples of stubborn fruits.

Albedo extracts of greened and healthy fruits of mandarins, lemons, Seminole tangelo, citron, and grapefruit were also tested with the TLC method. Although the results indicate that greened fruits of mandarins and tangelos contain the violet Rf 0.1, this marker is not found as regu-

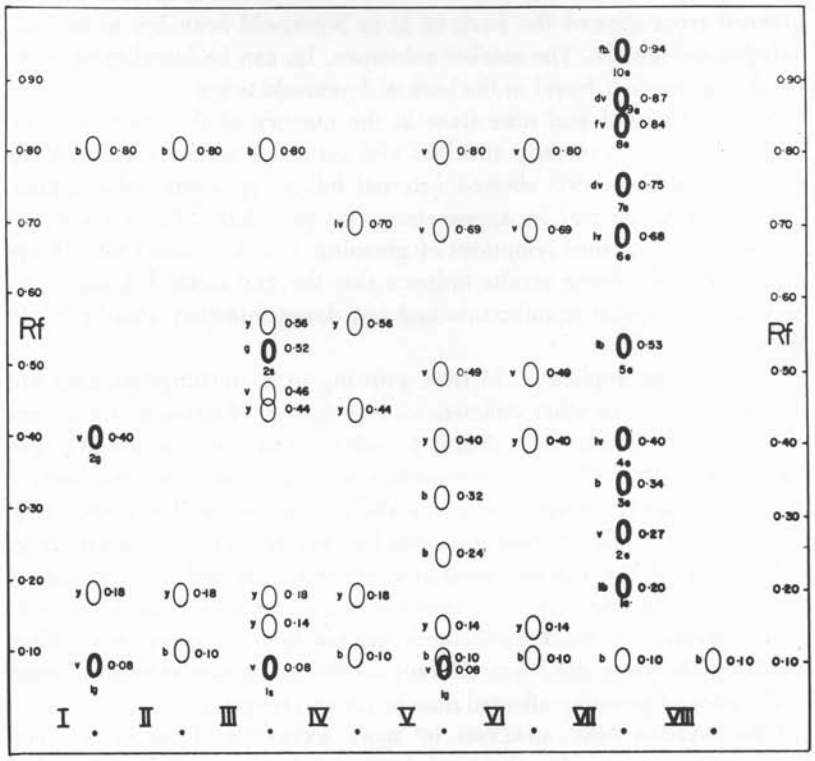

FIGURE 1. *Thin layer chromatographic profiles (under ultraviolet light) of albedo extracts. I. Greening-affected sweet orange fruits. II. Healthy fruits. III. Stubborn-affected sweet orange fruits* (U.S.A.). *IV. Healthy fruits* (U.S.A.) *and also the profiles of bark extracts of V-VIII. V. Greening-affected sweet orange. VI. Healthy sweet orange. VII. Exocortis-affected trifoliate orange. VIII. Exocortis-affected trifoliate orange. The figures next to the spots indicate the Rf values. v = violet, b = blue, y = yellow, l = light, d = dark.*

larly and is not present in such high concentration as it is in sweet orange.

CHROMATOGRAPHIC ANALYSIS OF BARK EXTRACTS: GREENING.—The bark of greening-affected sweet orange trees regularly contained the marker substance 1g. In certain cases, especially in old orchard trees, the presence of a blue substance with a similar Rf rendered detection of the marker substance difficult. In such cases, spraying the chromoplate with sodium buffer solution changed the blue color to yellow, but intensified the violet color of the marker substance, 1g.

Comparison of the TLC profiles of bark extracts from various parts of greened trees showed the bark of 2- to 3-year-old branches to be most suitable for the test. The marker substance, 1g, can be found even in the trunk, but was not found in the bark of 1-year-old twigs.

Eleven hundred and nine trees in the nursery of the CSFRI were indexed by the TLC method and 798 (72 per cent) were positive. Of the 798 positive trees, 663 showed external foliage symptoms of greening, but 135 were normal in appearance. One year later, 78 of these 135 trees showed external symptoms of greening, but the remainder still appeared normal. These results indicate that the TLC method is more discerning than visual examination and can detect infection while it is still latent.

The test was applied to 74 trees growing in a greening-free area but affected by various other deficiencies and diseases. Sixty-nine trees were negative, but 5 trees were found to contain traces of the marker substance, 1g. Three of the 5 trees were suffering from iron deficiency, 1 showed a general decline, and 1 was affected by rootrot. The trees giving the reaction originated from nurseries believed to be free from greening. Production of the marker substance perhaps was induced by factors other than greening virus, but latent infection cannot be ruled out completely because the marker substance was not found in other trees suffering from the same disorders. Marker substance 1g was regularly found in the bark of greening-affected mandarins and tangelos.

CHROMATOGRAPHIC ANALYSIS OF BARK EXTRACTS: EXOCORTIS.—The TLC profiles of samples prepared from trifoliate orange rootstocks of trees infected with exocortis revealed marker substances that were not present in the trifoliata stocks of normal trees (see Figure 1). In stocks of trees 30 years of age, the profiles showed 10 markers, but only 1 in recently infected trees, the 1 being the same as 9e in the older trees. Of 40 recently infected trees, 28 showed the marker 9e, whereas all profiles from 40 healthy trees were negative. Marker 9e, which is common

to both old and newly infected trees, may be the first marker substance to appear following infection with exocortis virus.

Discussion

Specific violet fluorescence was observed in the albedo of all sweet orange fruits showing external symptoms of greening and from trees with recognized leaf and fruit symptoms of greening disease. This indicates that the violet fluorescence shown by greened fruits when exposed to ultraviolet light of 365 mμ is a highly specific indicator of the presence of the greening virus in sweet orange. The method facilitates the indexing of greening infection in areas where the symptoms are masked by climatic conditions or other factors (3) and also makes possible an accurate determination of the annual fluctuation of the percentage of greened fruits on a single tree. With this method the percentage of greening can be determined three to four months before harvesting and should be valuable for yield estimates. The specific fluorescence is not very marked in the albedo of mandarins and tangelos and, consequently, it is not possible to apply the method to the fruits of those species.

A marker substance identical to that found in the albedo is present in the bark of sweet orange, mandarin, and tangelos. Water extraction of the phenolic marker followed by chromatographic analysis makes it possible to use the bark to index greening in these species. Furthermore, chromatography is the first step towards determining the identity of the markers.

A method involving the use of paper chromatography is being used by N. D. Elphinstone of Letaba Estates, a large citrus estate in the Northern Transvaal, to index nursery trees before transplanting. All trees showing external symptoms of greening were positive with the fluorescent bark test. In addition, another 10-20 per cent of nursery trees gave a positive result, although they appeared normal. These results are similar to those obtained at the CSFRI and indicate, on the one hand, that the bark test can be used in commercial-indexing and, on the other hand, that the bark test is more sensitive than indexing based on leaf symptoms. In a few instances, traces of the marker substances were present in the bark of old sweet orange orchard trees supposed to be free from greening. Nevertheless, the bark test is regarded as reliable when sweet orange trees are uniform and grown under optimum conditions. It is advisable to re-investigate the specificity of the method in each country, as disorders, deficiencies, or diseases other than those investigated in the present study may also cause changes in the metabolism of the phenolics.

The fact that a marker substance identical to that in the albedo of greened fruits was also found in the albedo of stubborn fruits suggests that this test may prove useful for diagnosing stubborn.

Indexing methods for exocortis virus, such as the Etrog citron test (1), are so rapid and specific that the use of the fluorescence test for indexing exocortis in trifoliate orange appears not to be practical. However, should specific markers be found in latent hosts of exocortis virus such as sweet orange, indexing exocortis by phenolics might prove to be of considerable value.

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