SEROLOGICAL DETECTION OF A CITRUS VIRUS IN LEAF EXTRACTS FROM FIELD TREES

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ABSTRACT

Serological tests were used to index 239 citrus trees in three groves for a crinkly-leaf-type virus of citrus (CLTV). CLTV was identified by serological tests, in all 12 trees previously shown to be infected by indexing with indicator plants, and in nine previously untested trees. CLTV was readily detected in young leaf tissue collected during the spring flush, but leaf tissue collected during the summer yielded negative or inconsistent results. Leaf tissue was stored dry successfully for several months prior to testing. The agar-gel serological test yielded results in 12 to 24 hours. The CLTV antiserum, prepared by immunizing rabbits with purified virus, did not react with other citrus viruses or extracts from healthy plants.

INTRODUCTION

Citrus viruses have been identified heretofore almost wholly from symptoms produced in indicator plants (2). This procedure requires numerous indicator plants of the proper age, favorable test conditions, experienced personnel, and a test period usually ranging from several months to several years. Symptoms in indicator plants are often difficult to evaluate, because different viruses may cause similar symptoms, strains of the same virus may produce different symptoms, and mixed infections are common.

Serological tests are often used to identify many plant viruses. These tests are rapid, easily performed with simple equipment, and provide a highly specific, direct measurement of the virus (9). Serological tests are based on the specific reaction between a virus and its antiserum. Antisera to plant viruses are usually obtained by injecting a preparation of partially purified virus into a rabbit, causing production of antibodies. These antibodies are small proteins in the serum fraction of blood and are formed specifically to the virus or other antigenic substances injected. Serum containing antibodies to a virus is called an antiserum to that virus.

Application of serological techniques to the practical identification of citrus viruses has been attempted (7,8) but has not met with general acceptance. Lack of sufficient quantities of purified virus has hindered production of specific antisera to citrus viruses, and, until recently, the antisera produced have been of low quality (3,6).

Recently, studies made on a citrus crinkly-leaf-type virus (CLTV) (5) indicated that it could be purified in reasonable quantities. A specific antiserum was produced to purified CLTV, and this antiserum reacted strongly with sap extracts from greenhouse-grown citrus plants infected with CLTV. Initial tests of field trees were also promising and prompted us to conduct some large-scale field tests of serological indexing during the spring and summer of 1969. The results of these tests on the serological indexing for CLTV from citrus leaf tissue are presented in this paper.

METHODS AND MATERIALS

An isolate of CLTV, following serial mechanical transmission through several citrus and herbaceous hosts, was increased in young plants of *Citrus excelsa* Wester. This isolate was free of other citrus viruses and produced typical CLTV symptoms in 'Mexican' lime (*C. aurantifolia* [Christm.] Swing.), 'Eureka' lemon (*C. limon* [L.] Burm. f.) and 'Duncan' grapefruit (*C paradisi* Macf.) (5).

CLTV was purified from young leaves of *C. excelsa* by using a hydrated calcium phosphate gel clarification procedure (4), followed by density gradient centrifugation in sucrose columns. The virus was recovered from the density gradient columns and concentrated by centrifugation. Six to 10 mg. of purified virus from these concentrated and high infectious preparations was injected into a domestic rabbit over a 6-week

period, by intramuscular and intravenous routes.

Virus antibody concentration was maximum approximately 2 weeks after the final injection, and the antiserum used in this study was collected at that time. Antiserum was mixed 1:1 with glycerol and stored at 4° C, or freeze-dried and stored at −25° C. Further details on the purification of CLTV and production of its antiserum will be published elsewhere.

Leaf tissue was collected March 26, 1969 from 239 field trees in three adjacent groves by sampling four to five shoots from the periphery of each tree. Preliminary tests showed that the serological activity of CLTV was not affected by drying, so a composite 1 g sample of young leaf tissue from each tree was air-dried and stored under vacuum at room temperature over anhydrous Ca SO_4 until testing was convenient. During the summer, some of the trees were resampled and the serology tests repeated. These tests were run with fresh tissue, omitting the drying process. Fifty-five of the 239 trees sampled had been indexed on indicator plants the previous year, and 12 tested positively for CLTV. The remaining trees, which had not been tested previously, were selected so that 4 to 5 trees were sampled in all directions from trees known to carry CLTV (see Fig. 1).

All samples were coded so that the identity of the 12 trees known to carry CLTV was concealed while making the serological tests.

Agar-gel plates (1) were prepared by adding 12 ml of a medium containing 0.75% Ionagar No. 2[1] (Consolidated Laboratories) and 0.02% sodium azide to 100 x 15 mm plastic petri dishes. Wells for the reactants were cut by using an Auto-Gel Punch[1] (Grafar Corp., Detroit, Mich.) with cutters 7 mm in diameter spaced at 7mm.

To make the serological tests, 50 mg of coarsely powdered leaf tissue from each sample was ground with a mortar and pestle in the presence of 1 to 1.2 ml of buffer solution, pH 7.4, made .02 M in sodium phosphate, .01 M in sodium diethyldithiocarbamate, and .02 M in sodium thioglycollate. This ratio of dry tissue to buffer was equivalent to a fresh tissue: buffer ratio of approximately 1:4. The resultant macerate was placed in wells in agar-gel plates as follows: 1) undiluted; 2) diluted 1:1 with buffer; and 3) mixed 1:1 with a 50 mg/ml macerate of dried *C. excelsa* leaves infected with CLTV. *The last* treatment was a control included to: 1) measure any deleterious sap effects on CLTV and the reaction process; and 2) to confirm the identity of any CLTV precipitation lines from the samples. The antiserum was used at 1/8, 1/32 and 1/128 dilutions in normal saline. The dilution end point for the antiserum used was 1/256 against the CLTV control macerate containing 25 mg dried tissue/ml. The placement of the reactants in the test plate is illustrated in Fig. 2a.

Each sample was also tested against normal rabbit serum in separate plates to detect any nonspecific reactions between the citrus leaf extracts and serum.

Plates were observed periodically on a viewing box designed to provide annular back lighting. Observations were begun 12 to 24 hours after the reactants were added and continued for several weeks. Final results were recorded photographically.

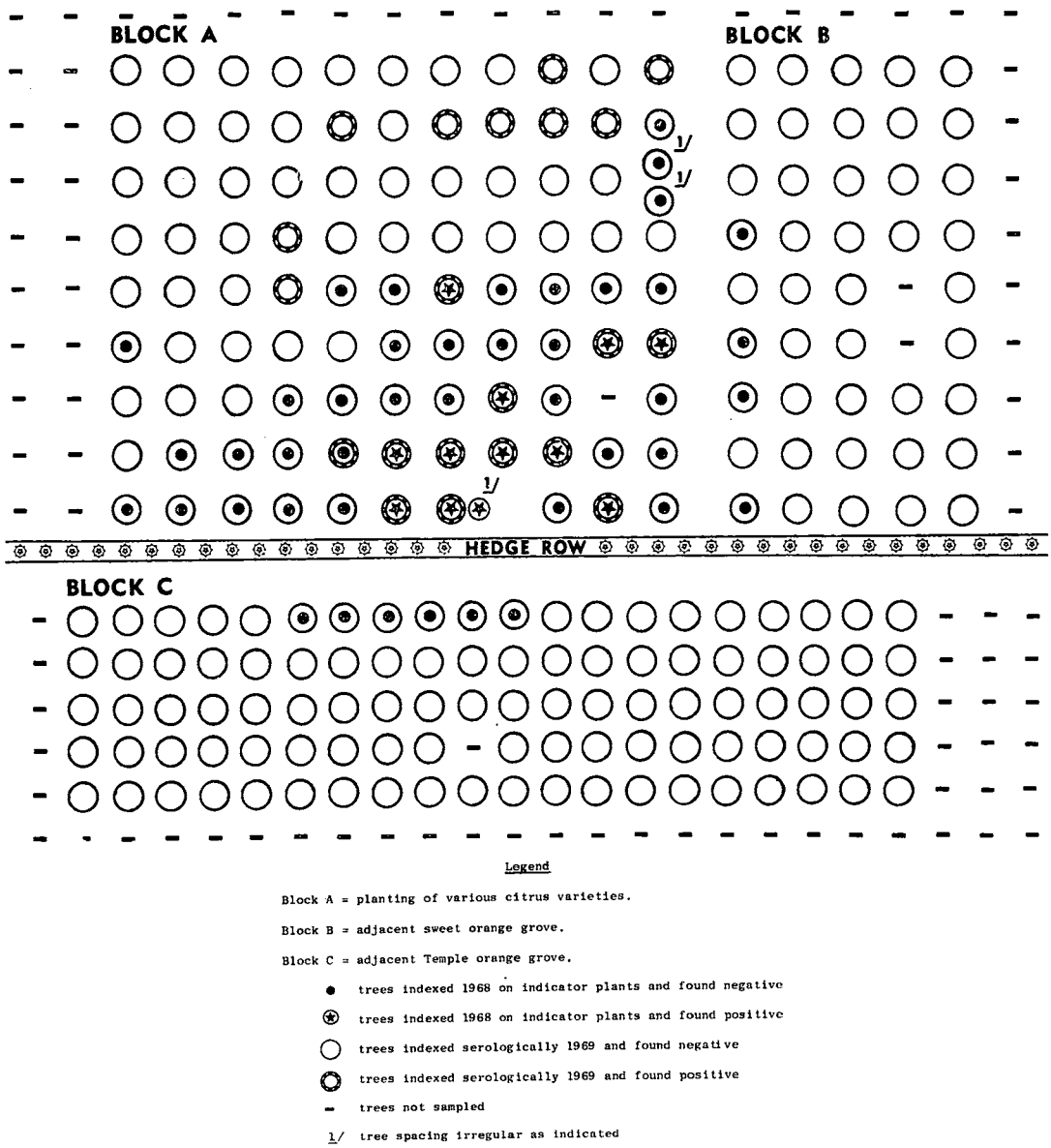
Additional tests were made on selected samples by different personnel, who used the general procedure outline above, with the following modifications: 1) tissue was macerated in 0.02 M potassium phosphate buffer containing 0.5% sodium sulfite and adjusted to pH 8.0; 2) agar-gel plates were prepared with a medium containing 0.85% Noble agar[1], 0.85% sodium chloride and 0.1% sodium azide.

Results and Discussion

Serological tests of the leaf tissue harvested during the spring flush showed that 21 of the 239 trees tested were infected with CLTV (Fig. 1). When the sample contained CLTV, a precipitation line formed between the well containing CLTV antiserum and the three sample wells (Fig. 2b). When the sample did not contain CLTV, a precipitation line formed only between the serum well and sample well no. 3 containing the CLTV control (Fig. 2c). Results of these serological tests correlated well with indexing results obtained in 1968 which were based on symptoms in indicator plants. The only discrepancy in results from the 55 trees indexed by both methods was that 1 of the 43 trees, which indexed negatively on indicator plants, indexed positively by serological test (Fig. 1). This discrepancy could indicate a failure of the indicator test, or may indicate that the tree became infected during the 12 months between assays.

To see if serological testing results were re-

[1]Mention of a trademark name or a proprietary product does not constitute a guarantee or warranty of the product by the USDA, and does not imply its approval to the exclusion of other products that may also be suitable.



Legend

Block A = planting of various citrus varieties.

Block B = adjacent sweet orange grove.

Block C = adjacent Temple orange grove.

● trees indexed 1968 on indicator plants and found negative

⊛ trees indexed 1968 on indicator plants and found positive

○ trees indexed serologically 1969 and found negative

◎ trees indexed serologically 1969 and found positive

– trees not sampled

1/ tree spacing irregular as indicated

Fig. 1. Diagram of area where leaf samples were collected for serological testing.

producible, 32 samples, composed of 21 positives and 11 negatives, were retested by different personnel who used the modified procedure described earlier. The status of each sample was not divulged until second tests were complete. Results of the second test were the same as the first.

Precipitation lines were usually visible within 12 hours after placing the reactants in the plate. These lines became stronger with time, as precipitate continued to form between the diffusing antibodies and virus, but changes in the precipitation lines were minor after 48 hours.

The strongest and most distinct precipitation zones form when the ratio of antibodies to antigens is optimum. When the ratio of these reactants is out of balance, precipitation lines

may be diffuse or fail to form. Since the antigen concentration in the leaf samples was unknown, six combinations of antiserum and sample concentrations were tested (Fig. 2a). Eighteen of the 21 samples gave the strongest reaction at 50 mg/ml concentration against the serum dilution of 1/32. The remaining three were strongest with serum diluted 1/8. All but one sample gave a visible reaction at the 1/8 serum dilution, while only 12 of 21 gave a visible reaction at the 1/128 dilution.

Mature, spring flush leaves were collected July 8, 1969, from the 21 trees found infected with CLTV in the first test. Macerates of fresh leaf tissue were prepared by using a 1:4 and 1:8 tissue/buffer ratio and the same test procedure used earlier; but none of the samples tested positively for CLTV.

Young, summer flush leaf tissue was collected from 8 of the 21 CLTV-infected trees on July 28, 1969. Very young leaf tissue and tissue from young, but nearly expanded, leaves were tested fresh at 1:4 and 1:8 dilutions against 1/16 and 1/64 dilutions of antiserum. Positive reactions were obtained with 2 of the 8 samples. Macerates of tissue from the young, expanded leaves of both samples gave better results than macerates from the youngest leaves.

The accuracy of our serological tests, therefore, depended on the selection of tissue used to make the test. Failure to detect CLTV in leaf tissue harvested in summer was probably due to a decrease in the amount of virus present. Some CLTV was still present since it could be transmitted by grafting from leaf tissue that gave negative results serologically. Concentrating the virus or development of a more sensitive testing procedure may improve summer test results.

One of the prominent features of serological tests is the speed with which results are obtained for large numbers of samples. The apparent seasonal limitation to serological testing is offset, to some extent, by the fact that leaf tissue can be harvested at the proper time and stored dry for several months until testing is convenient.

The deleterious effects of citrus leaf components on the serological tests, as measured by including a known source of CLTV (Fig. 2a, c, Well 3), were minor. Actually, citrus leaf extracts apparently disrupted CLTV into smaller components, which migrated more rapidly through the gel than intact virus particles, therefore speeding test results. None of the sample extracts formed nonspecific precipitates which could be confused with the virus-antibody precipitation line.

The CLTV antiserum did not react with leaf extracts from citrus plants infected singly with tristeza, psorosis, exocortis, tatter leaf, or infectious variegation viruses. The first three viruses were present in some of the field trees infected with CLTV, but did not interfere with its serological detection. These results confirm the specificity expected of this serological test (9).

The 21 trees which indexed positively for CLTV are located in the same planting (Fig. 1) but include 11 different varieties of different ages and origin. Healthy and infected trees of six varieties coexist in the planting. No CLTV-in-

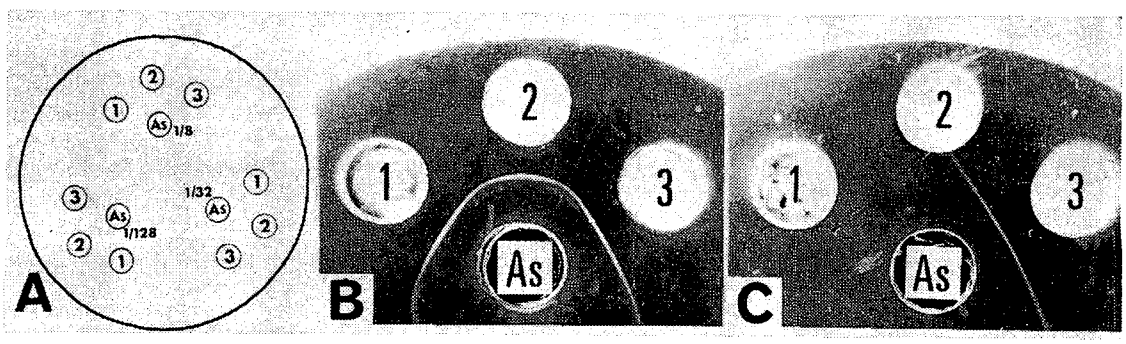


Fig. 2. Placement of reactants in serology plates and formation of precipitation zones between CLTV and its antiserum. The placement of reactants is shown in Fig. A. Wells marked A were filled with CLTV antiserum diluted as indicated. Wells marked No. 1 were filled with buffered macerate of dried leaf tissue (50 mg/ml); wells marked No. 2 were filled with macerate used in No. 1, but diluted 1:1 with buffer and wells marked No. 2 were filled with a 1:1 mixture of macerate used in No. 1, plus macerate from dried lea ves of Citrus excelsa infected with CLTV (50 mg/ml). When the leaf sample macerate contained CLTV, a precipitation line formed between the A well and all three antigen wells (Fig. B). When the leaf sample macerate did not contain CLTV, a precipitation line formed only between the A well and well No. 3 containing the known source of CLTV used as a control (Fig. C).

fected trees were found among the 142 trees tested in the two adjacent plantings. No varietal effects on test results were observed, but no tests were conducted to measure this factor specifically.

Serological diagnosis of citrus viruses has considerable potential; however, further work is needed, even with CLTV, to develop a test procedure with the best combination of speed, sensitivity, simplicity, and reliability. Serological detection of other citrus viruses will not be possible until the necessary antisera to those viruses are developed, and this will be a difficult task for citrus viruses that have not been transmitted mechanically, or which occur in relatively low concentrations in their hosts.

Serological indexing will not replace indexing procedures based on the use of indicator plants, but where applicable, it will provide an attractive alternative. A highly specific test that can be completed in as little as 12 hours, requires only several drops of leaf tissue extract, several drops of antiserum, and a minimum of equipment or facilities, offers many advantages.

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FROST CONDITIONS AND DAMAGE TO CITRUS DURING TWO CONSECUTIVE RADIATION FREEZES

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ABSTRACT

Damage to citrus was different during two consecutive radiation freezes. The more damaging freeze to citrus was characterized by a more favorable condition for frost formation. The beginning of heavy frost conditions was accompanied by a change in wind direction to the S, greater than 90% relative humidity, and frost points equal to temperature of leaves exposed to sky radiation.

INTRODUCTION

It is difficult to determine whether a radiation freeze will or will not damage citrus when active growth is not visible on the trees (2). In this situation, a major problem is whether to heat or not to heat citrus groves. To heat when it is not necessary, or not to heat when it is necessary, is a costly error in judgment. Most of this judgment is based on the extent of minimum air temperatures.

An example of whether to heat or not to heat citrus groves during a radiation freeze occurred during two consecutive freezes this past December in Florida. The trees, in general, were relatively dormant, as a result of previous cool