EARLY DIAGNOSIS OF EXOCORTIS INFECTION IN Poncirus Trifoliata BY A LABORATORY TEST

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INTRODUCTION

A rapid method of detecting exocortis (*6*) or scaly-butt (*2*) virus in *Citrus* species characteristically free of diagnostic symptoms is urgently needed. Heretofore, exocortis virus has been detected in various species of *Citrus* by indexing on rootstock of trifoliate orange, *Poncirus trifoliata* (Linn.) Raf., (*2*). Benton *et al.* (*2*) showed that scaling of the outer layers of bark of *P. trifoliata* rootstocks, characteristic of exocortis infection, usually begins 4 to 8 years after budding.

The Australian and the Florida citrus budwood programs require approved budsource trees to be free of exocortis virus but for somewhat different reasons: In Australia, *Poncirus trifoliata* is a very important rootstock. At present, in Florida, it is not important, but it might assume importance there as a rootstock for exocortis-free scion varieties. However, since exocortis virus is certainly not beneficial, and since a means exists for eliminating it, this is being done as a part of the Florida Citrus Budwood Certification Program.[3] Growers in several citrus-growing regions are especially interested in hybrids of *P. trifoliata* as rootstocks. Some of these hybrids may prove to be desirable rootstocks, but their horticultural worth can be evaluated only through field trials with tops free of exocortis virus. Unless exocortis is eliminated from the budwood source, the commercial use of stocks affected by exocortis is obviously not feasible.

Benton *et al.* (*2*) established the virus nature of exocortis through its transmission to *Poncirus trifoliata*, and reported the range of symptoms expressed. Morton citrange, *Citrus sinensis* (Linn.) Osbeck X *P. trifoliata*, and Rangpur lime, *C. limonia* Osbeck, have been suggested as test plants that react more rapidly than *P. trifoliata* to exocortis virus, but this remains to be proved. For these reasons, *P. trifoliata* seems to be the valid test plant for the presence of exocortis virus, and for the same reasons the Australian and Florida programs require indexing on it. The difficulty with *P. trifoliata* as an index plant is that diagnosis of infection or noninfection is so long delayed.

Virus-infected plants commonly show microscopic tissue changes (*4*, *5*), some of which are so specific for a given virus-host plant combination that they can be used as diagnostic symptoms of infection (*1*, *8*, *9*, *10*). The writers have studied exocortis-infected *Poncirus trifoliata* tissues and have reported some success in detecting infected trees by laboratory methods (*3*).

This paper describes a staining technique which provides a specific color reaction in the phloem ray cells of the bark of exocortis-infected trifoliate orange and presents details of the application of this test in the selection of exocortis-free parent trees of sweet orange, grapefruit, mandarin, and other citrus varieties.

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MATERIALS AND METHODS

Citrus trees on *Poncirus trifoliata* rootstock are scarce in Florida[4] but common in Louisiana.[5] The major portion of the bark specimens examined were collected in the latter area. Bark specimens were also collected from trifoliate rootstocks in the exocortis-indexing plot of the Florida Citrus Budwood Program at Winter Haven, Florida. All specimens were collected across the bud union and initially consisted of wood and attached bark. After it was discovered that exocortis infection could be diagnosed from the bark alone, only bark was collected. For routine examination, bark specimens ¼ inch (6 mm) wide by ¾ inch (20 mm) long, taken across the bud union, were found to be the smallest that could be sectioned conveniently by the method used in these studies. Immediately after excision, specimens were placed in formaldehyde-acetic acid-alcohol solution No. 2 (*7*) for fixing and preserving until examination. The location, top variety, age, condition, etc., of each tree sampled were recorded.

It was thought that such tissue abnormalities as might exist would be microscopic. Accordingly, bark and wood specimens were prepared for microscopic examination by cutting longitudinal (radial) and transverse sections with a block plane (Stanley No. 60½) held in the hand. The blade of this plane is placed at a lower angle of inclination (14°) than most others (*11*) and for that reason is better adapted to sectioning wood and bark without crushing the softer tissues (*3*).

As an aid to differentiating various tissues, Cartwright's stain was used in exploratory examinations of both wood and bark sections. Other stains are discussed in connection with their use.

RESULTS

Development of the Color Test. A number of tissue abnormalities were investigated before the exceptionally constant association of exocortis virus infection in *Poncirus trifoliata* and a reaction of cells in the vascular rays of the bark with Cartwright's stain was discovered. This reaction was more readily distinguished in transverse sections than in radial sections. Phloroglucinol-HCl reagent produced a red color reaction (*3*) that differentiated the same phloem ray cells in exocortis-affected bark sections as Cartwright's stain did, but did so more clearly (fig. 1). Phloroglucinol-HCl is well known as a reagent for detecting aldehydes (wound gum, lignin, etc.).

In unfixed, unstained sections of *Poncirus trifoliata* bark infected with exocortis virus, reactive cells in the phloem rays could be detected by their more opaque appearance by transmitted light before phloroglucinol-HCl reagent was applied. Histochemical tests (*3*) of unfixed, unstained sections of bark gave further evidence that aldehydes were characteristically present in ray cells of trifoliate bark infected with exocortis virus and were not found in those of trifoliate bark free of the virus.

Evaluation of the Phloroglucinol-HCl Color Reaction. Bark specimens collected in Florida and Louisiana were subjected to the phloroglucinol-HCl color test in two series (*3*). In the first series, comprising 174 trees of 3 to approximately 40 years of age, diagnosis of exocortis infection was based on the presence or absence of bark scaling on the trifoliate rootstocks. Tops were predominantly sweet orange, *Citrus sinensis*, but a number of mandarin orange, *C. reticulata* Blanco; grapefruit, *C. paradisi* Macf.; and one calamondin, *C. mitis* Blanco, were included. Ninety trees

[4] Through the active cooperation of the Florida State Plant Board, particularly the section concerned with counting commercial citrus trees, about 60 trees on *P. trifoliata* roots were located in Florida.

[5] Ralph Brown, Superintendent of the Plaquemines Parish Experiment Station, and M. W. McEachern, Agricultural Agent, both gave invaluable assistance in the collection of specimens in Louisiana. Wood and bark specimens from trees in Mr. Brown's experiments were especially important in the success of the work reported in this paper.

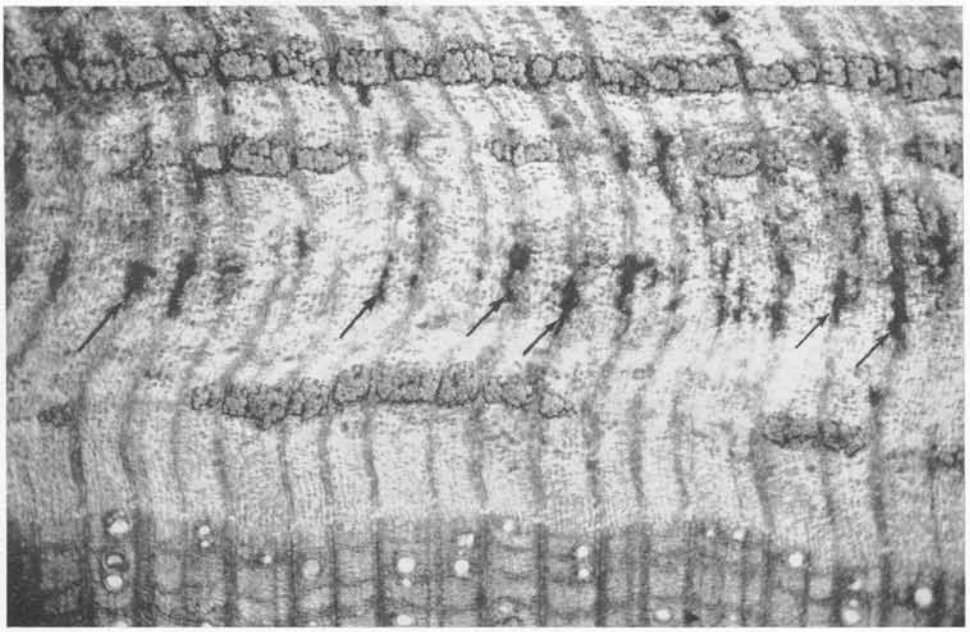

Fig. 1. A cross section of exocortis-infected Poncirus trifoliata bark, treated with phloroglucinol reagent, showing the xylem (at lower edge of illustration) and clusters of fiber cells, in transverse rows, which both stain pink. Normally the medullary rays of the bark do not stain, but in exocortis-infected bark some ray cells stain red-purple (note arrows). Photographed on Panatomic-X film with X-2 (green) filter, 96X.

were diagnosed as infected (as described), and in 88 of them the phloem ray cells developed a color reaction with phloroglucinol-HCl. Eighty-four trees were diagnosed as not infected (on the basis described), and 83 of these gave no color reaction. This amounts to an error of 3 determinations in 174, or 1.7 per cent. In these tests a single bark specimen was taken across the bud union of each tree, and five to eight sections were examined from each specimen. No attempt was made to correct errors by repeated examinations, the purpose of the test being to determine the accuracy of the method.

The second series comprised two lots of Washington Navel orange trees on trifoliate rootstock, 2 and 4 years old, respectively, from time of budding. None of these trifoliate stocks exhibited bark scaling. Of the 2-year-old trees, 8 were propagated with buds from parent trees known to be infected with exocortis virus and 8 from parents known to be free of the virus. There were also 25 trees, 4 years old, all propagated from exocortis-free parents. Of the 8 infected trees, 7 were tested and all gave a color test with phloroglucinol. (One bark specimen damaged in collecting was not sectioned.) The 8 trees 2 years old and the 25 trees 4 years old from exocortis-free parents all gave negative color reactions with phloroglucinol. The error in the second series of determinations was zero per cent.

Reaction of Exocortis-free *Poncirus trifoliata* Seedlings. Discovery of a color reaction between exocortis-infected ray cells and phloroglucinol-HCl and certain other reagents and stains raised a question concerning the reaction of unbudded and presumably exocortis-free seedlings of *P. trifoliata*. Bark samples from seedlings estimated to be 3 to 30 years of age were collected in three different areas. Fifty-three specimens were examined, but none yielded a color reaction of phloem ray cells with phloroglucinol-HCl.

The Color Test Applied to Trees in the Florida Citrus Budwood Program. The primary object of developing a color test for exocortis infection was to hasten the indexing of trees in the Florida Citrus Budwood Program. Each candidate tree in the program was budded on two trifoliate seedlings. Because of inexperience in growing these plants, some had died or the buds had failed and the plants had been rebudded. Thus 370 test trees representing 204 candidate parent trees were available for examination. The oldest trees were 27 months from budding and the youngest, 13 months. One bark specimen from each test tree was examined.

Criteria of Exocortis-Virus Infection. Some bark specimens were very small because of the small size of some test trees. In very young trees the color reaction is usually exhibited by only a few rays. Thus there is some chance that a single bark specimen may not include reactive ray cells. For these reasons certain arbitrary criteria were set up for diagnostic purposes.

From 5 to 8 sections, not in serial order, were mounted and stained for examination. If at least one clearly stained ray cell could be found in each of at least 3 sections, the bark specimen and, by implication, the parent tree were considered infected. If only one or two clearly stained ray cells could be found, especially if cortex and pericyclic parenchyma cells were stained, the bark specimen was recorded as probably infected. If no stained ray cells and no stained cortex or pericyclic parenchyma cells were found, the specimen was recorded as not infected. If stained cells and stained intercellular material were found only in the cortex and pericyclic parenchyma areas, the specimen was listed as probably negative. Doubtful diagnoses were recorded because it is planned to re-examine these trees in 6 or 8 months, and such information may shed light on the earliness of the color reaction.

Exocortis Virus Infection in Index Trees. Results of the examination of index trees of *Poncirus trifoliata* are presented in table 1. A detailed discussion of the results is unnecessary here, but it should be pointed out that 82 of the parent trees (40 per cent) were clearly infected with exocortis virus, as indicated by the color test (+), and 26 others (13 per cent) were probably positive (+?). Thirty-eight of the parent trees (19 per cent) were clearly negative, as indicated by the color test (−), and 58 (28 per cent) were probably negative (−?). Among the 204 trees tested, there were twice as many clearly infected trees as clearly uninfected.

Of the 98 sweet orange parent trees represented, 24 (24 per cent) were clearly positive by the color test and 20 (20 per cent) were clearly negative. Of the 85 grapefruit parent trees examined, 54 (64 per cent) were clearly positive by the color test and 7 (8 per cent) were clearly negative. The high percentage of exocortis-free Dancy tangerine trees in this test is misleading. Actually the old clones of this variety are so thoroughly infected with virus, principally psorosis, that only seedling trees have been entered in the Florida budwood program, and the Dancy trees in this test had been propagated from seedlings which were expected to be free of exocortis virus.

Other Viruses and Their Relation to the Color Test for Exocortis. The 204 parent trees had been indexed previously on appropriate test plants, and the virus complement of each was known with considerable certainty. It was found that 58 trees were free of psorosis, xyloporosis, and tristeza. By the phloroglucinol-HCl color test, 15 of the 58 are free of exocortis virus.

The possibility that xyloporosis, psorosis, or tristeza infection might modify the diagnosis of exocortis was considered. However, no evidence could be found that these three viruses, singly or in combination, affected the phloem ray cells of *P. trifoliata* in such a manner as to confuse the color test for exocortis virus.

More Permanent Evidence Needed of Exocortis Infection. The phloroglucinol-HCl color reaction is transitory, fading after 5 or 6 hours and disappearing entirely in 24 hours. A color reaction of a more permanent nature was desired for purposes of

Table 1. DETERMINATION BY THE PHLOROGLUCINOL COLOR TEST OF EXOCORTIS IN TRIFOLIATE ORANGE (PONCIRUS TRIFOLIATA) ROOTSTOCKS OF INDEX TREES IN THE FLORIDA CITRUS BUDWOOD CERTIFICATION PROGRAM

Variety	No. of trees	Exocortis diagnosis*			
		+	+?	−	−?
Sweet orange (*C. sinensis*)					
Hamlin	18	3	1	5	9
Jaffa	2	2	0	0	0
Parson Brown	2	0	0	0	2
Lue Gim Gong	2	1	0	1	0
Pineapple	38	11	4	8	15
Pope Summer	4	0	0	1	3
Seedling, sweet	1	0	0	0	1
Valencia	26	6	6	4	10
Washington Navel	5	1	3	1	0
Total	98	24	14	20	40
Grapefruit (*C. paradisi*)					
Duncan	11	5	1	2	3
Foster	2	2	0	0	0
Marsh	19	10	3	2	4
McCarty	2	0	0	1	1
Ruby	20	15	3	1	1
Thompson	31	22	3	2	4
Total	85	54	10	8	13
Mandarin (*C. reticulata*)					
Dancy	7	0	0	6	1
Mandarin	1	0	0	0	1
Total	8	0	0	6	2
Hybrids					
Tangor (*C. sinensis* × *C. reticulata*)					
Temple orange	5	4	1	0	0
Tangelo (*C. paradisi* × *C. reticulata*)					
Lake	1	0	0	1	0
Orlando	3	0	0	2	1
Seminole	1	0	0	1	0
Minneola	1	0	1	0	0
Calamondin (*C. mitis*)	1	0	0	0	1
Limequat (*C. aurantifolia* × *F. japonica*)	1	0	0	0	1
Total	13	4	2	4	3
Grand total	204	82	26	38	58

* Infection of parent trees (determined by color test): positive (+), probably positive (+?), negative (−), or probably negative (−?).

record. Safranin and light green (3) gave the desired result. When properly handled, this staining procedure appears to be as precise as phloroglucinol-HCl in differentiating exocortis-affected ray cells and is more striking. Because it can be wrongly applied, however, and because it is slower, safranin-light-green stain is not as satisfactory for routine examinations as phloroglucinol-HCl. For record purposes and for confirmation of the phloroglucinol-HCl color test, it has been our practice to stain several sections with safranin-light green after a color reaction has been obtained with phloroglucinol.

DISCUSSION

Esau (5) pointed out that viruses often effect changes in the anatomy of the host that may "prove of diagnostic value especially if viruses are otherwise difficult to distinguish." Atypical cells (more opaque by transmitted light) found in the phloem rays of *Poncirus trifoliata* infected with exocortis virus seem to be such an effect. The contents of such cells react with aldehyde-coupling reagents such as phloroglucinol and others (3) by forming colored products that facilitate their demonstration. As indicated by the color reaction with phloroglucinol, atypical cells are characteristically present in *P. trifoliata* infected with exocortis virus 2 years or longer and characteristically absent in trifoliate trees that are free of exocortis virus. In 41 bark specimens from trifoliate trees and rootstocks 2 and 4 years of age, the phloroglucinol color test substantiated without error the known exocortis infection and the known exocortis-free condition of nonscaling young trees. Likewise, it substantiated the presumed exocortis infection or the presumed exocortis-free condition (based on the presence or absence of bark scaling) in 212 of the specimens from 215 mature trees (98.6 per cent) (3).

The color test with phloroglucinol-HCl was employed in making a tentative diagnosis of exocortis infection in 204 parent trees of miscellaneous varieties (candidates for certification as approved bud sources in the Florida Citrus Budwood Certification Program) budded 13 to 27 months previously on *Poncirus trifoliata* rootstocks. By this test 82 trees (40 per cent) were clearly infected with exocortis virus, 26 (13 per cent) were probably infected, 38 (19 per cent) were clearly free, and 58 (28 per cent) were probably free. With repeated testing, these figures undoubtedly will change.

There was no evidence that the presence of the viruses of psorosis, xyloporosis, and tristeza confused the diagnosis of exocortis in *Poncirus trifoliata*. Until the reliability of the color test has been fully substantiated, final diagnosis must be determined by the symptom of bark scaling. In the meantime, the data obtained by this method are of considerable immediate value because they provide a basis for making an early choice of budwood. Certainly it is unwise to propagate for commercial use from the 82 clearly infected parent trees. Propagation from among the 38 clearly negative trees would be the logical choice. For research purposes there is here a basis for selecting with considerable assurance bud sources infected with or not infected with exocortis.

SUMMARY

1. Atypical cells are characteristically present in the phloem rays of *Poncirus trifoliata* trees, 2 years or older, infected with exocortis virus.

2. The contents of atypical ray cells react with phloroglucinol and other aldehyde-coupling reagents to produce a characteristic color compound.

3. The color reaction between phloem ray cells and phloroglucinol seems to be a reliable indication of exocortis infection in *P. trifoliata.*

4. In 204 citrus and citrus hybrids indexed on *P. trifoliata* rootstocks in the Florida Citrus Budwood Certification Program, 82 (40 per cent) were clearly infected with exocortis as revealed by the color test.