

SURVEYS, CITRUS IMPROVEMENT AND OTHER SUBJECTS

Detection of Citrus Viruses by Graft Transmission: A Review

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Plants are still the only "eyes" we have for the detection of most citrus viruses. This review will provide a brief history of the detection of citrus viruses by graft transmission. It will explore some of the factors affecting symptom expression, i.e., the importance of good plant growth and nutrition, temperature, light, inoculation techniques, and use of sensitive indicator plants. Also reviewed will be the importance of a virus bank and some problems of detecting mild virus isolates. An outline will be presented for an "ideal" indexing facility. This review is designed to lend impetus and direction for improving inoculation and detection procedures so that the mildest reacting

isolates, or new and unknown agents, can readily be detected in the shortest possible time.

There are other innovative, rapid, and important means of virus detection, e.g., mechanical transmission to herbaceous hosts, chemical and histological techniques, culturing as with *Spiroplasma citri*, and observation of a virus in the electron microscope. Each method is important. However, there are many citrus diseases for which graft transmission to indicator plants is still the only means of detection. This review focuses on the most effective use of plants for citrus virus detection.

BACKGROUND OF CITRUS VIRUS DETECTION

Fawcett (1933, 1934, 1938) was the first to transmit an identifiable citrus virus experimentally by graft inoculation. It took him 9 years for the first transmission of psorosis virus. Later, Fawcett and Cochran (1942) induced psorosis young-leaf symptoms in field-grown seedlings of sweet orange. They grafted bark patches from infected trees to young seedlings and observed leaf symptoms in less than 6 weeks and bark symptoms in 5 months. However, it was Wallace (1945) who pioneered the concept of seedling indexing as we know it today. He noted that young-leaf symptoms of psorosis were so variable and difficult to detect in

the field that absence of these symptoms, or of bark-lesion symptoms, were no guarantee of virus freedom. He developed the seedling-inoculation technique whereby "bark tissue from a young small twig of a suspect tree was grafted to a corresponding cut in a young indicator seedling." He used electrical splicing tape made of rubber and cut back the inoculated seedlings a few inches above the bark patch. He found that topping stimulated early symptom expression. By this method he induced shock symptoms, leaf flecking, and mottling. Most symptoms were readily detected within 4 weeks. Wallace's technique, modified slightly by

use of buds or chips and different kinds of wrapping tapes, is still the standard method of inoculation for virus detection in citrus. Wallace (1947) also tested the grafting of rectangular leaf pieces inserted into the bark of seedlings and reported transmission of psorosis virus by this means.

The history of the discovery of the West Indian (Mexican or Key) lime as an indicator for tristeza virus is reviewed by Wallace (1959). Through international cooperation and correspondence, information from the Gold Coast of Africa (Hughes and Lister, 1953) that lime trees show specific symptoms for tristeza was quickly disseminated and tested. Wallace (1951) reported successful transmission of tristeza by leaf or bark tissue and confirmed the observations of Hughes and Lister that lime seedlings show stem pitting. Discovery of lime seedlings as indicators for tristeza virus helped solve a worldwide puzzle and united as one disease lime dieback in the Gold Coast, stem pitting of grapefruit, incompatibility of

sweet orange on sour orange rootstock in South Africa and South America, tristeza disease in South America, and quick decline in California. This discovery focused sharply on the importance of indicator seedlings for virus detection and strain separation (Grant, 1959) and indirectly was responsible in part for the formation of the International Organization of Citrus Virologists (Wallace, personal communication). After this beginning, i.e., the use of sweet orange and West Indian lime seedlings for psorosis and tristeza detection, other indicators were discovered; Orlando tangelo for cachexia (Childs, 1951), West Indian lime for vein enation (Wallace and Drake, 1953) and for yellow vein (Weathers, 1957), *Citrus excelsa* for tatter leaf (Wallace and Drake, 1962), sweet orange for stubborn (Calavan and Christiansen, 1965b), citron for exocortis (Salibe, 1961; Calavan *et al.*; 1964; Frolich *et al.*, 1965) and Orlando tangelo for cristacortis (Vogel and Bové, 1968).

THE SEARCH FOR NEW AND IMPROVED INDICATOR VARIETIES

It is fortunate that the subfamily Aurantioideae in the family Rutaceae contains 32 citrus-related genera with some 203 species, not counting many manmade and natural hybrids, most of which are graft compatible (Swingle, 1967). This presents the searcher for new indicators with a large selection of graft-compatible plants differing in genetics and chemistry. Of this large bank of materials relatively few have been adequately tested in the search for new indicators. Roistacher (1963, 1964) tested 19 cultivars of mandarin, mandarin hybrids, and sweet orange for sensitivity to concave gum and psorosis. These tests showed the Dweet tangor to be very sen-

sitive and some sweet orange cultivars to be more sensitive than others. Calavan and Christiansen (1965a) tested 73 rootstocks searching for improved cachexia indicators and found sensitive Parsons Special mandarin. Frolich *et al.* (1965) tested four clonal and 16 seedling selections of citron and found USDCS 60-13 to be very sensitive to exocortis virus. Vogel and Bové (1968) tested many species and cultivars and found Orlando Tangelo to be a superior indicator for cristacortis. Miyakawa (1969) tested 18 citrus species and 2 citrus relatives as indicators for Satsuma dwarf virus; all were susceptible and showed symptoms.

FACTORS AFFECTING SYMPTOM EXPRESSION

Plant nutrition. To detect the mild strains of virus in the shortest possible time, it is necessary to use plants of superior growth and vigor. Most symptoms appear in leaves of new growth flushes and may disappear as the flush

hardens. Citrus plants are very subject to micronutrient deficiencies, especially copper, zinc, manganese, and iron deficiencies, so micronutrients must be supplied in adequate amounts or growth flushes will be reduced and mottled, and

cannot reliably be "read" for virus disease symptoms. The growth of citrus in containers for use in virus detection has been reported in detail by Nauer *et al.* (1967), and a recommended soil mix and fertilizer schedule outlined (Nauer *et al.*, 1968). This U. C. soil mix, modified for citrus, has been successfully used at the Rubidoux indexing facility in Riverside for 15 years. It is also used at the University of California Lindcove Field Station greenhouses in central California and at the Central California Tristeza Control Agency greenhouses for growth of large numbers of indicator plants. Recently it has been used at the citrus research glasshouses in Burjasot, Spain. The U. C. system yields plants of good vigor free from micronutrient deficiencies; this system, or a variation of it, is recommended as a prerequisite for any program of citrus virus detection.

Importance of sanitation. A program for proper sanitation essential to the good growth of citrus plants must include elimination or avoidance of all *Phytophthora* spp. pathogenic to citrus. This can be done by testing for the presence of the pathogens by the methods of Klotz and DeWolfe (1958) or Grimm and Alexander (1973), removal of all infected plants, spraying wood benches with copper naphthenate (Roistacher and Baker, 1954), sterilization of all flats and containers, replacement of soil under benches with rock or gravel, minimizing splashing water by using proper hose ends and keeping the host ends off the ground. Personnel working with plants should be made aware of the necessary sanitary procedures for avoiding phytophthora infection. Following these practices, we have avoided phytophthora infection in the Rubidoux glasshouses for 18 years.

Supplemental light. The use of supplemental lighting is recommended for increasing plant growth and for improving symptoms of certain viral infections. Roistacher (1963) found that supplemental artificial light for 5 hours daily during the winter period increased the total number of leaves by 18 per cent for nine cultivars of mandarin and mandarin hybrids. Supplemental light also had a

pronounced effect on symptom development. Under supplemental light, 222 leaves showed concave-gum symptoms compared to 72 leaves without light. Supplemental light in winter also increased growth of sweet orange, sour orange, *C. excelsa*, and West Indian lime and appeared to correct winter yellowing of lime seedlings.

The effect of light intensity on virus symptom expression has not been adequately explored. Kapur and Weathers (1974) indicated that light intensities of 500, 1,000, 1,500, and 2,000 ft-c had no significant effect on development and severity of exocortis symptoms in *Gynura aurantiaca*. They found that increased day length delayed symptom development and decreased relative infectivity.

Temperature. The temperature at which plants are grown has a very significant effect on virus symptom expression. Most citrus viruses can be classified as cool-temperature or warm-temperature pathogens. Our observations indicate that tristeza, psorosis-A, concave gum, infectious variegation, and vein enation are caused by cool-temperature viruses. Desjardins *et al.* (1957) found tristeza symptoms were completely suppressed in West Indian lime plants held in a controlled-temperature cabinet 3 to 4 weeks at 40° C. Roistacher *et al.* (1974) showed that leaf and stem-pitting symptoms were suppressed in West Indian lime inoculated with six tristeza isolates and held in a glasshouse at 28 to 40° C daytime/ 26 to 27° C nighttime temperatures even though the growth of plants at warm temperatures was twice that of plants at cooler temperatures. Roistacher and Calavan (1974) showed that tristeza, psorosis-A, concave gum, infectious variegation, and vein enation viruses were eliminated from the upper portions of infected plants held 3 to 4 months at these temperatures. Studies with tristeza virus by Bar-Joseph and Loebenstein (1974) revealed a lower number of threadlike virus particles in leaf cells at high temperatures, 30 to 36° C, then at 22° C. They found that stem pitting was completely suppressed at 30 to 36° C.

Psorosis-A and concave gum symptoms

TABLE 1
EFFECT OF TEMPERATURE ON SYMPTOMS OF PSOROSIS-A
AND CONCAVE GUM VIRUSES

Virus	No. of leaves with symptoms*/ total no. of leaves			
	Cool room†		Warm room†	
	Sweet orange	Dweet tangor	Sweet orange	Dweet tangor
Concave gum (CGV-301)	22/49	31/50	0/54	0/73
Psorosis-A (PV-200)	0/40	30/42	0/39	9/44
Psorosis-A (PV-209)	Severe shock		0/56	20/46‡

*In two plants for each cultivar 5 weeks after inoculation (first flush).

†Mean maximum temperatures were 38 in the warm room and 29°C in the cool room; mean minimums were 24 and 20°C, respectively.

‡Pinpoint flecking only.



Fig. 1. The effect of temperature on symptom expression by psorosis-A virus: A) shock symptom induced in a sweet orange seedling held at cool temperatures (left) compared to no shock symptom on plant held at warmer temperatures (right); B) symptoms in a Dweet tangor leaf at cool temperatures (left) compared to symptoms on a leaf held at warm temperatures (right).

are expressed best at cool temperatures. Table 1 shows the numbers of leaves with symptoms for the first flush of growth on Dweet tangor and Pineapple sweet orange seedlings inoculated with two isolates of psorosis-A and one of concave gum virus and held at 38/24°C or at 29/20°C. Shock and leaf symptoms for psorosis-A-infected plants were suppressed at warm temperatures (fig. 1A) and symptoms of concave gum were absent. One isolate of psorosis-A virus caused pinpoint leaf spotting only in Dweet tangor at warm temperatures and appeared much different from symptoms produced at cooler temperatures (fig. 1B).

Satsuma dwarf definitely appears to be caused by a cool temperature virus.

Tanaka *et al.* (1969) showed that varying the day/night temperatures markedly affected the type and location of symptoms on Satsuma mandarin leaves infected with Satsuma-dwarf virus. They found that moderate temperatures 28/23°C masked symptoms whereas excellent symptoms were induced at lower temperatures (18/13° or 23/18°C).

Another cool temperature pathogen is responsible for South African greening disease. Bové *et al.* (1974) have shown that symptoms of South African greening were completely suppressed at temperatures of 27 to 32°C, whereas at this temperature regime California stubborn reacted severely. In the same experiment, citrus decline from India and leaf mot-

ting from the Philippines showed moderate to severe symptoms at these temperatures. Schwarz and Green (1972) showed that the greening pathogen was inactivated in budwood held in saturated hot air at 47°C for 240 minutes, 49°C for 120 minutes, or 51°C for 60 minutes. Fiberglass enclosures around entire field trees caused a marked reduction in greening symptoms.

Stubborn disease, exocortis, and cachexia can be considered as warm-temperature diseases because symptoms are best expressed in indicator plants at warm temperatures and the pathogens are difficult to eliminate by thermotherapy (Calavan *et al.* 1972b). Vogel and Bové (1974) reported that symptoms of stubborn were suppressed in field trees at the cool temperatures of Corsica where the total time above 28°C in July was only 27 hours. However, when budwood was collected from symptomless trees and indexed at warmer temperatures, stubborn symptoms developed. Glasshouse temperatures of 28 to 40°C maximum day and 26 to 27°C night are best for rapid development of stubborn symptoms.

Exocortis symptoms develop best at temperatures also favorable to stubborn. Weathers *et al.* (1962) showed that bark scaling in trifoliolate orange inoculated with exocortis developed within 14 months at 30 and 35°C but no bark scaling developed at cooler temperatures. Roistacher

and Calavan (unpublished) inoculated mild isolates of exocortis viroid into citron and observed symptoms at four locations in California. The mild symptoms were considerably suppressed and in some cases absent in the shaded and cool glasshouse at Ivanhoe as compared to the warm glasshouses at Riverside and Indio. Best symptoms were observed in the warmest temperatures, at Indio. Kapur and Weathers (1974) found that 24 to 30°C was optimum for development of exocortis symptoms in gynura.

Roistacher *et al.* (1973) induced cachexia gumming symptoms in 30/30 Parsons Special mandarin scions within 1 year of budding in a warm glasshouse whereas only 10/30 plants in a cooler screenhouse showed symptoms.

In summary, the temperatures at which plants are held for observation of symptoms are very important for diagnosis. Tristeza, psorosis-A, concave gum, infectious variegation, Satsuma dwarf, vein enation, and greening can be considered to be caused by cool-temperature pathogens, while stubborn, Asian greening, exocortis, and cachexia have warm-temperature pathogens. An indexing facility should be divided into at least two or three compartments for proper diagnosis of different pathogens. The use of a single glasshouse room to detect a number of citrus pathogens is not recommended.

EFFECT OF INOCULUM TISSUE ON SYMPTOM EXPRESSION

The tissue used and the method of grafting may affect the intensity and time required for development of symptoms. Various tissues, including stem pieces, bark, leaves, buds, chips, roots, and fruit columella, have been used as grafts for indexing. By using bark tissue containing psorosis lesions, Fawcett (1942) and Wallace (1945) induced severe leaf symptoms in inoculated sweet orange plants within 5 weeks. Grafting of buds, shields, or chips is a very convenient means of virus transmission and has been the standard practice by many workers. However, other tissues have been successfully used. Leaf tips (Schwarz, 1968), tri-

angular leaf inserts (Cohen, 1972), and leaf-punch inserts (Blue *et al.*, this volume) are examples of leaf-to-leaf inoculation. These techniques were all successful for transmission of tristeza virus to West Indian lime. However, Blue *et al.* showed that leaf-to-leaf indexing was erratic for detection of psorosis, concave gum, and exocortis pathogens. Leaf pieces grafted to seedling stems have been reported successful for psorosis-virus transmission (Wallace, 1947), stubborn transmission (Calavan *et al.*, 1972a), and for transmission of six different viruses by Garnsey and Whidden (1970).

Calavan *et al.* (1968) found that side

grafting 3- to 5-cm long stems into suitable indicator seedlings was the most effective means for detecting stubborn. A side graft plus a bud from a mature shoot of a field tree were superior to buds, expanding-leaf patches, internodal-bark shields, bark patches from a scion trunk, side grafts from the root section, or fruit columella tissue. Preliminary investigations (Roistacher *et al.*, unpublished) comparing trunk-bark tissue with bud tissue as inoculum for detection of mild isolates of exocortis showed that bark tissue gave a consistently stronger reaction in citron. Bark pieces were found to transmit exocortis from calamondin (Igwegbe, 1967) or from an old-line lemon (Roistacher *et al.*, unpublished) whereas no transmission could be obtained from twig inoculum from the same trees. Blue *et al.* (this volume) have

DETECTION OF MILD ISOLATES

A mild reaction on an indicator plant need not be interpreted as always related to a mild reaction in field trees. McLean (1974) reported that tristeza virus isolate no. 706 caused only a mild reaction in West Indian lime but induced the most severe stem pitting encountered in South Africa. Another extremely mild-reacting isolate of tristeza virus (T-511) was observed by E. C. Calavan and others to induce decline of grapefruit on sour orange rootstock at Bryn Mawr, California. This strain was symptomless in some West Indian lime seedlings, and in one test showed no leaf symptoms or stem-pitting 15 months after inoculation. A few isolates of exocortis viroid escaped detection on eight Arizona 861 citron plants grown under warm conditions but induced moderate bark cracking on trifoliolate rootstocks in the field. A very mild-reacting isolate of exocortis viroid (E805), which induces petiole wrinkle, petiole browning,

ESTABLISHMENT OF A VIRUS BANK

An indexing program should include a virus bank as an integral part of any operation. Each index test for a given virus should be accompanied by inoculation with a severe and a very mild-reacting

shown that leaf-to-leaf indexing for tristeza virus gave a high percentage of successful grafts, was faster and easier than bud inoculation, and induced good leaf-vein clearing and stem-pitting symptoms for very mild-reacting isolates. Their method had an additional advantage in that very small seedlings could be used thus saving up to 6 months of growing time for seedlings. Also, when large seedlings are used, they accept leaf inocula from a number of source trees without undue stem injury.

Each pathogen may be found to have its best tissue for transmission. The various methods and tissues mentioned above should be tested for each pathogen. A specific method which works well for one pathogen will not necessarily be useful for others.

and petiole twist on Arizona 861 citron, is known to induce moderate stunting of Bearss lime on Troyer citrange, trifoliolate orange, and Rangpur lime in the Coachella Valley of California.

The detection of the mildest strains of any virus should be one of the objectives of a good indexing program. Unless a broad spectrum of indicator plants is used and combined with utmost consideration for good plant growth under proper temperature conditions, a virus may be missed. A strain of concave gum virus was reported which rarely caused patterns in sweet orange but consistently caused a mild reaction in mandarin and Dweet tangor (Roistacher and Calavan, 1965). The Dweet-mottle virus causes no symptoms in sweet orange, mandarin or West Indian lime, but induces a definite irregular psorosis-like leaf mottle in Dweet tangor (Roistacher and Blue, 1968).

isolate of known performance to serve as positive controls, and some noninoculated plants as negative controls. An index test can be considered complete when the mildest known isolate has given

a positive reaction.

In the California Citrus Variety Improvement Program we have maintained a virus bank for over 15 years and have kept records on the performance of each virus isolate. Each of the known virus-infected plants was tested for other viruses. The severity of symptoms, date of inoculation, and kind of indicator plant are recorded for each index test. Some viruses may weaken or disappear but, over the years, only a few isolates of tristeza have become weaker while most isolates have given a consistent uniform reaction in West Indian lime. Exocortis has been persistent, is rarely lost, and our mild isolates have consistently induced mild reactions in all tests. Moderate-reacting isolates of exocortis vary from mild to moderately severe in citron, depending on temperature. Severe isolates almost always cause severe reactions with bud or bark grafts but not necessarily with leaf grafts. Some concave gum and

psorosis virus isolates have caused uniform symptoms for many years; however, some have disappeared from source plants, perhaps due to a prolonged period of hot weather. Symptoms of stubborn have a tendency to disappear if plants are not kept continually under warm conditions. Three isolates of cachexia virus differ in intensity of symptom reaction, but have been consistently transmitted to Parsons Special mandarin or Orlando tangelo by two bud-graft inoculations. One hundred per cent transmission has been obtained from three source plants over a 10-year period.

Transmission of infectious variegation virus has been variable from sweet orange and mandarin but consistent from infected citron or lemon. Tatter-leaf virus, present in three tristeza-free sources of Meyer lemon, has consistently caused a strong reaction when bud transmitted to *C. excelsa* or Troyer citrange.

AN "IDEAL" INDEX FACILITY AND RECOMMENDED PROCEDURES

(1) A glass or fiberglass house is a necessity. It should be partitioned into at least 3 sections: a cool section with refrigeration or evaporative-cooling, an intermediate-temperature section for growing plants, and a warm section for indexing or preconditioning and thermotherapy (Roistacher & Calavan, 1972, 1974). The area under the benches should be gravel over porous soil. Benches can be preferably of wood or of other materials spaced for drainage and should be sprayed with copper naphthenate preservative or dusted with Bordeaux mixture.

(2) The soil mix used should be well aerated, of high water-holding capacity, balanced for micro- and macronutrients—preferably a U.C.-type mix (Nauer *et al.*, 1968). The soil should be well mixed and preferably steam sterilized. All flats and containers should be sterilized. Fertilization should be supplied regularly, preferably with each watering, using a metering device. The pH of the soil should be maintained between 5.0 and 6.5 through a balanced fertilizer mix (Nauer *et al.*, 1968). Hose ends should be selected for a soft flow to minimize

splash and always hung up, never left on the ground.

(3) A sanitation program should be established to eliminate any *Phytophthora*, and precautions taken to maintain the house free of this and other organisms detrimental to plant growth.

(4) Lights should be provided above index benches and controlled by a timing switch.

(5) A virus bank should be established and maintained. A record should be kept of each virus source test. All virus-source plants should be indexed for presence of other viruses.

(6) Each index test should include the mildest-reacting isolate as a positive control. An index test can be terminated when this mild isolate has induced clearly defined symptoms compared to a negative control.

(7) The temperatures during indexing should be recorded for each experiment and reported if results are published.

(8) When indexing for a new pathogen the widest range of conditions and procedures should be tried. Leaves, buds, stem pieces, and bark tissue should be

indexed to a wide range of species and varieties of indicator plants under varying temperatures. Electron microscopy, chemical tests, and mechanical inoculations to herbaceous hosts should be attempted whenever possible.

The above procedures should lend impetus and direction toward the "ideal" of rapid, efficient detection of new pathogens or the mildest strains of known pathogens, as well as good routine indexing for citrus viruses.

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