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MECHANICAL TRANSMISSION, PURIFICATION, SEROLOGY, and ELECTRON MICROSCOPY

Mechanical Transmission of Citrus Viruses

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This paper is part of the report of the IOCV Committee on Mechanical Transmission and Purification of Citrus Viruses. It reviews the progress that has been made in mechanical transmission of citrus viruses and updates the reviews made by Price (*35*) and Weathers (*44*). The report will deal largely with the practical aspects of mechanical transmission of citrus viruses, and list some methods and procedures that have been employed successfully by the writers and other workers.

Prior to 1960 there was little information about the viruses that cause diseases of citrus, because we lacked a suitable method for mechanical transmission of these viruses. Little can be learned of the nature and properties of a virus until it can be studied apart from the host plant and then reintroduced and established in the host. Citrus plants do not provide appropriate material for experimental study of citrus viruses. Succulent growth and subsequent development of symptoms occur only periodically. The mature leaves of citrus are tough and dry, and the presence of inhibitors makes them difficult sources for virus extracts.

The first opportunity to study citrus virus came when Grant and Corbett (*19, 20*) successfully transmitted the viruses of citrus infectious variegation by sap inoculations between both citrus and herbaceous plants. This important work stimulated transmission tests with other citrus viruses, and to date sap transmissions of at least eight have been reported (table 1).

For more detailed information on mechanical transmission of viruses see the review articles by Fulton (*12*) and Yarwood (*51*).

RECEPTOR PLANTS

Susceptibility of the receptor plant is of prime importance for mechanical transmission of any plant virus. Many of the obstacles encountered when viruses were confined to citrus tissue were overcome by discovery of herbaceous hosts. These were found to be more susceptible when inoculated by rubbing, and some viruses reached a higher titer than in the citrus host. Transmission between succulent plants could usually be easily made. Some herbaceous plants reacted to infection by forming local lesions, which made quantitative assays of the virus possible.

In the reports of mechanical transmission of viruses from citrus plants, all were found to go to one or more species of herbaceous plants (table 1). The virus of Satsuma dwarf is reported to be mechanically transmissible from citrus to herbaceous plants and back to citrus, but not from citrus to citrus (*28, 29, 39, 40*). The viruses of crinkly leaf, infec-

TABLE 1

VIRUSES REPORTEDLY TRANSMITTED FROM CITRUS TO CITRUS AND TO HERBACEOUS PLANTS BY MECHANICAL INOCULATIONS

Virus	Donor species	Primary receptor species	Selected references
Exocortis	Citrus medica, Petunia hybrida	Citrus medica, Petunia hybrida, Solanum **spp.**, Gynura aurantiaca, Scopolia **spp.**	(13, 16, 18, 37, 42, 43, 48, 49, 50)
Crinkly leaf	Citrus limon	Citrus aurantifolia, C. aurantium, C. limon, C. sinensis, Crotalaria spectabilis, Cucumis sativus	(4, 30)
Infectious variegation	Citrus limon C. paradisi	Citrus aurantium, C. limon, C. sinensis, C. spectabilis, Vigna sinensis, Cucumis sativus, Phaseolus vulgaris, Petunia **spp.**, Antirrhinum majus	(1, 3, 6, 7, 9, 14, 19, 20, 32, 34)
Psorosis	Citrus limon	Citrus aurantifolia, Vinca rosea	(44, 45)
Leaf rugose	Citrus sinensis	Citrus aurantifolia, C. limon, C. paradisi, Crotalaria spectabilis, Cucumis sativus, Gomphrena globosa, Nicotiana tabacum, Vigna sinensis, Phaseolus vulgaris, Petunia hybrida	(15)
Satsuma dwarf	Citrus unshiu	Arachis hypogaea, Astragalus sinicus, Canavalia gladiata, Crotalaria spectabilis, Kummerovia striata, Phaseolus vulgaris, Sesamum indicum, Vigna sesquipedalis	(28, 29, 39, 40)
Citrange stunt	Citrus limon C. excelsa	Chenopodium amaranticolor, C. quinoa, Cucumis sativus, Helianthus annuus, Nicotiana clevelandii, N. tabacum, Phaseolus vulgaris, Vigna sinensis, Poncirus trifoliata × Citrus sinensis	(2, 11, 36, 41, 47, 52)
Tatter leaf	Citrus limon	Citrus medica, Nicotiana clevelandii	**(Garnsey, unpublished)**
Potato virus X	Citrus **spp.**	Nicotiana tabacum	(25)
Tobacco mosaic	Citrus **spp.**	Nicotiana tabacum, N. glutinosa	(44)
Tobacco necrosis	Citrus **spp.**	Phaseolus vulgaris	(33, 53)

tious variegation, leaf rugose, citrange stunt, tatter leaf, and exocortis were mechanically transmitted between both herbaceous and citrus plants. Weathers (*44, 45*) obtained infections of lime seedlings and *Vinca rosea* plants inoculated with sap from a tree showing leaf symptoms which appeared to be those of psorosis A. This virus has been transmitted from *V. rosea* to *V. rosea* by sap and from *V. rosea* back to lime by dodder. Storm and Streets (*38*) reported transmission of psorosis A virus to cucumber, but not from cucumber to cucumber or back to citrus. Surprisingly, the first virus to be transmitted from citrus by mechanical means was potato virus X (PVX) (*25*). Subsequently, two other noncitrus viruses were mechanically transmitted from citrus (*33, 44, 53*).

No clear pattern has emerged to indicate what herbaceous plants will be susceptible to citrus viruses. Fulton (*12*) claims that "probability of successful transmission of an unknown virus is certainly better with hosts of proven susceptibility to many viruses than with untried hosts." This conclusion is borne out with citrus viruses. Cucumber, cowpea, and *Crotalaria spectabilis* are reported more commonly as herbaceous hosts of citrus viruses than are any other plants. Their use in experiments with citrus viruses probably reflects their accessibility as well as their reputation for susceptibility to other woody plant viruses (*12*).

Some herbaceous species are susceptible to a large number of viruses, including ones normally found in woody plants (table 2). These plants either have been used most frequently by workers in the past or are known to offer some particular advantage. It is advisable, therefore, to include them in any host-range studies.

TABLE 2
PLANT SPECIES SUGGESTED FOR USE IN STUDIES ON HOST RANGE AND SYMPTOMATOLOGY OF CITRUS VIRUSES

Species	Variety or selection
Chenopodium quinoa	Purdue source
Crotalaria spectabilis	Any selection
Cucumis sativus	National Pickling
Gomphrena globosa	Any selection
Lycopersicon esculentum	Bonny Best
Nicotiana tabacum	Turkish, Samsun, Burley
N. glutinosa	Any selection
Phaseolus vulgaris	Red Kidney, Bountiful, Pinto, Pencil Pod Wax
Petunia hybrida	Burpee Blue
Vigna sinensis	Early Ramshorn, Black

More desirable herbaceous plants than those reported probably exist. Many of the citrus viruses probably have wide host ranges. The virus of exocortis has been transmitted by grafting from petunia to several species ot solanaceous plants (*50*). Citrange stunt and infectious variegation viruses have been transmitted mechanically to many species of plants in several families (table 1). The search for additional and more susceptible host species for a given virus is not very appealing once good systemic and local-lesion hosts are found.

All of the viruses listed in table 1 are reported to have been transmitted mechanically from citrus to herbaceous plants; not all have been transmitted mechanically between citrus plants. Satsuma dwarf, so far as the writers know, has not been mechanically transmitted from citrus plants to citrus plants. With some of the citrus viruses, as with many woody-plant viruses, the citrus host is difficult to infect by mechanical inoculation. Citrus plants can vary in their susceptibility to viruses that are transmitted mechanically. For instance, the exocortis virus is transmitted readily when citron serves as the receptor host, but with difficulty when sweet orange and grapefruit plants serve as receptors (*13, 18*).

Mechanical transmission frequently can be improved by employing methods that introduce the virus more efficiently into the susceptible tissue of the host. Young, tender shoots of citrus seedlings have proved more susceptible to infection with citrus infectious variegation virus than have mature leaves (*19, 20*). Majorana (*31*) easily transmitted infectious variegation virus by injecting partially purified virus preparations under the cortex of orange and lemon seedlings. Exocortis virus frequently was more readily transmitted from citrus to citrus (*16*) and from petunia to petunia (*43, 50*) by inoculation methods that deposited the virus extract into stem tissue than by the usual sap-inoculation methods.

DONOR PLANTS AND SOURCE OF INOCULUM

Not only is citrus tissue apparently difficult to infect by mechanical inoculation, but also, with some viruses, it is a relatively poor source of inoculum. Some hosts may provide more highly infectious inoculum than others that appear equally affected, but selection of a more favorable donor plant has been largely neglected in studies of mechanically transmitted citrus viruses. Once herbaceous hosts are located, there is little incentive to search for more suitable citrus donor plants.

With the possible exception of exocortis virus, most citrus viruses are most readily transmitted from extracts prepared from very succulent young leaves. Transmissibility is apparently correlated with either high virus content or lower content of inhibitory materials in rapidly developing leaves. Exocortis virus can be readily transmitted by knives, needles, and so forth contaminated by first inserting them into infected young stem tissue and then into stem tissue of healthy test plants, but not so readily with extracts of leaves applied by conventional methods (*16, 50*). Kapur (*27*) found that Tris buffer extracts of young, succulent leaves were equal in infectivity to partially purified extracts of the virus and more infectious than extracts derived by razor slashing of infected stems. Infectious variegation virus has been transmitted to several species of plants with extracts prepared from petals and albedo (*23*).

Mechanical transmission may be made possible by first transmitting the virus to a more suitable herbaceous host by some other means and then transmitting it mechanically from that host to other plants. Exocortis virus was readily transmitted mechanically after it was established in petunia by dodder and then transmitted from petunia to petunia by sap inoculation and by rubbing healthy stems with pieces of infected stems (*42, 43, 46, 50*).

Establishing infection can depend on what substances other than the virus are present in the inoculum. Since Duggar and Armstrong (*10*) reported that ordinary pokeweed markedly inhibited the infectivity of tobacco mosaic virus, many plant extracts have been shown to inhibit the infectivity of plant viruses.

Grant and Corbett (*21, 22*) reported that citrus foliage, like that of many plants, contains substances inhibitory to the infectivity of citrus variegation virus. When they diluted cowpea inoculum vol./vol. with leaf extracts from sweet orange, grapefruit, and Eureka lemon, the number of local lesions produced on cowpea test plants was greatly reduced. Heating the sap containing the inhibitor for 10 minutes at 60 or 72°C failed to decrease its inhibitory effect.

Weathers (*44*) diluted extracts of cucumber mosaic virus with leaf extracts from leaves of several healthy *Citrus* spp. and found that infectivity to cowpea was reduced. Inhibitory activity was obtained immediately on mixing, but infectivity could be restored by filtration through activated charcoal.

Grasso *et al.* (*24*) observed that extracts of healthy leaves of many *Citrus* spp. interfere with the transmission of citrus variegation virus, citrange stunt virus, and tobacco mosaic virus to healthy indicator plants. They confirmed that the activity of the inhibitor was instantaneous and that no further reduction occurred on standing. Preliminary evidence indicated that the inhibitor acts on the host rather than on the virus, and that the inhibitor in citrus is probably protein in nature. Selective removal of protein from fractions with high inhibitor activity fully restored the infectivity of virus extracts.

PREPARATION OF INOCULUM

Inoculum for mechanical transmission is most commonly prepared by grinding infected tissue in a mortar in the presence of buffers or other diluents and pressing the juice through cheesecloth.

The following procedure, a slight modification of that described by Garnsey and Weathers (*17*), is satisfactory for most viruses, and should be tried before employing more specialized techniques. Macerate a small amount of young, succulent leaf tissue in cold phosphate buffer (pH 7.0, 0.01 to 0.05 M), using a prechilled, porcelain mortar and pestle. A ratio of 0.1 gm of leaf tissue to 1 ml of buffer is satisfactory. With viruses of succulent plants, this procedure is usually satisfactory for routine transmission. Addition of reducing agents, such as sodium sulfite, sodium thyoglycolate, or cysteine hydrochloride, may be desirable to suppress inhibitors.

Grant and Corbett (*19, 20*) used 1 ml of 20 per cent sucrose and 0.05 gm of activated charcoal per gram of leaf tissue to transmit citrus infectious variegation virus to citrus and noncitrus hosts. The sucrose was used to prevent disruption of mitochondria and subsequent release of enzymes, and the charcoal, to adsorb inhibitors. Majorana (*30*) used this method to transmit crinkly leaf virus of citrus. Dauthy and Bové (*4, 5*) used the sucrose-charcoal method to transmit crinkly leaf virus as well as citrus infectious variegation virus. The sucrose-charcoal method was used by Weathers (*44*) to transmit an isolate of psorosis from citrus to citrus. The use of charcoal in extraction does not appear to be essential for transmission of either crinkly leaf virus or citrus infectious variegation virus (*4, 5, 7, 9*).

In transmitting the Satsuma dwarf virus to sesame and several leguminous plants (*29, 39, 40*), the juice of young infected shoots was extracted in 0.05 to 0.1 M di-potassium phosphate buffer. The virus was readily transmitted to herbaceous hosts by mechanical inoculation.

Desjardins and Wallace (*7, 8*) easily transmitted citrus infectious variegation virus by grinding young infected citrus leaves in liquid nitrogen in a mortar and suspending the powder in 10 per cent sucrose solution without charcoal, This method of preparing inoculum often resulted in 90 to 100 per cent transmission.

The virus of citrange stunt is readily transmitted by macerating young citrus leaves in phosphate buffer in a mortar and inoculating by the conventional methods (*12, 36, 47, 52*). The mechanical transmission of citrange stunt virus is facilitated by the addition of sucrose, N-6-benzyladenine (*52*) and 0.01 M sodium diethyldithiocarbamate, and 0.02 M 2-mercaptoethanol (*12*).

Weathers *et al.* (*50*) mechanically transmitted exocortis virus from petunia to petunia and citrus using various diluents and stabilizing additives. Most diluents and additives provided similar infectious inoculum for mechanical inoculation, but did not markedly increase transmission. The infectivity of the inoculum was increased to 70 per cent by grinding petunia leaf tissue in 0.1 M Na_2SO_3 at pH 8.0. Kapur (*27*), on the other hand, obtained almost 100 per cent transmission of exocortis virus from gynura to gynura when the virus was extracted in Tris buffer (0.1 M, pH 9.0).

METHODS OF APPLYING INOCULUM

The conventional methods of inoculating by applying infective plant extracts to young, developing, fully expanded leaves that have been dusted with carborundum (400- to 600-mesh), with a finger, cheesecloth pad, spatula, pestle, or the like, are commonly used in transmitting viruses of citrus. Rinsing leaves after inoculation is recommended, to remove plant substances that injure leaves of herbaceous plants. Rapid drying of the leaves after rinsing is also recommended, to be followed by incubation in moderate light at 21 to 24°C. Citrus viruses are no different from most others in that infectivity is greater in the presence of agents that stabilize the virus and minimize the effect of inhibitors.

Methods of applying inoculum other than by rubbing extracts on leaves have been described and may have advantages with particular viruses. Garnsey and Jones (*16*) reported that exocortis virus could be mechanically transmitted from citron to citron as a contaminant on grafting tools. Weathers *et al.* (*50*) reported that exocortis virus was transmitted from petunia to petunia by using contaminated needles and razor blades and by rubbing infected stem pieces of petunia on stems of healthy plants. These techniques were usually more effective than conventional leaf-rubbing methods (*18*), probably because they introduced the virus into tissue that would be less accessible by conventional techniques.

Exocortis virus has been reciprocally transmitted between citrus and petunia by approach grafts and other grafting procedures, even though these species are widely different and failed to form a functional graft union (*50*). Whether these tissue connections represent a mechanical transmission occurring between donor and receptor plants is not clear. It is possible that protoplasmic connections between these plants were established.

ENVIRONMENTAL EFFECTS

In all reports of mechanical transmission of citrus viruses in which temperature was studied, all viruses, with the possible exception of exocortis, were favored by low temperatures. Grant and Corbett (*19, 20, 21, 22*) reported that infection of Eureka lemon and *Citrus spectabilis* by citrus infectious variegation virus was favored by temperatures of 20 to 21°C as compared with fluctuating temperatures of 20 to 35°C. Plants of *C. spectabilis* developed necrotic rings at 20 to 35°C. Sour orange and grapefruit plants inoculated with citrus infectious variegation virus and held at 20 to 21°C developed only variegation symptoms. When inoculated and held at fluctuating temperatures of 20 to 35°C, such plants showed a variation of symptoms. Desjardins and Wallace (*7*) found that infection of cucumber by citrus infectious variegation virus was favored by cool temperatures.

Tanaka *et al.* (*40*) found that sesame plants exposed to temperatures above 34°C following inoculation with Satsuma dwarf virus usually developed no symptoms. Sesame plants kept at 25°C for more than eight hours following inoculation with Satsuma dwarf virus showed severe symptoms even if they were later exposed to temperatures above 36°C.

Effects of temperature, light, day length, season, and carbohydrate content are all interrelated, so that a study of one may, in part, be involved with another.

Tanaka *et al.* (*40*) found that in midsummer, sesame plants inoculated in the evening with Satsuma dwarf virus exhibited 100 per cent infection, but showed a very low rate of infection when inoculated in midday. Yarwood (*52*) found that infectivity of citrange stunt virus was favored by placing indicator plants in the dark before or after inoculation and by quick drying of the inoculated leaves. Garnsey (*14*) showed that systemic symptoms of infectious variegation virus *Citrus spectabilis* were uniform and more severe with a light intensity of 1,800 ft-c under greenhouse conditions. Systemic infection was not observed when light intensity was reduced to 700 ft-c.

Kapur *et al.* (*26*) found that temperatures of 24 to 30°C, with low light intensity and an eight-hour day, were optimal for infection and symptom development of exocortis virus in gynura. Symptoms of exocortis were less severe in gynura plants grown at temperatures below 24°C, and at 18°C most of the plants were symptomless carriers of the virus.

RETURN INOCULATIONS FROM HERBACEOUS PLANTS TO CITRUS

Reproduction of the disease in the original citrus host by a virus that has been mechanically transmitted to an herbaceous plant is necessary to prove that the virus transmitted to the herbaceous host was indeed the cause of the disease. Viruses in experimentally-infected plants have too often been associated with diseases without evidence that they caused those diseases.

Absence of symptoms must never be taken as evidence of lack of infection. That is, a symptomless test plant should not be judged a nonsusceptible host unless attempts to retrieve virus from it are consistently unsuccessful. Viruses vary in the rate at which they systemically invade plants. Recovery tests and return inoculations should be made two or more weeks after inoculation.

The viruses of crinkly leaf, exocortis, psorosis, infectious variegation, leaf rugose, Satsuma dwarf, citrange stunt, and tatter leaf have been returned to citrus from herbaceous plants and have caused the definitive disease. This is sufficient proof that herbaceous plants are hosts of these viruses.

The viruses that have been returned to citrus from herbaceous plants have infected citrus plants inoculated mechanically. It is not necessary to inoculate mechanically, however, to establish that a virus isolate is the cause of the disease. Exocortis and psorosis viruses were transmitted with dodder back to citrus from petunia and periwinkle, respectively, to initially establish the identity of the virus in the herbaceous host (*43, 45*).

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