CHAPTER 7

Mechanical Transmission, Isolation, and Purification of Citrus Viruses

A Review of Research on Mechanical Transmission, Purification, and Morphology of Citrus Viruses

W. C. PRICE

A MAJOR STIMULUS was given in 1960 to investigations of the nature of citrus viruses when Grant and Corbett (5) reported the mechanical transmission of citrus variegation virus. Previously, the only known means of transmitting citrus viruses were by grafting and aphids. Since then, there have been reports of mechanical transmission of crinkly-leaf virus (2, 15), tatter-leaf virus (4, 17, 22), stubborn and psorosis-A viruses (19), and Satsuma dwarf virus (20).

Actually, the first virus to be transmitted from citrus by mechanical means was potato mottle virus (PVX). Holmes (9) transmitted PVX to healthy seedlings of Mediterranean sweet orange [*Citrus sinensis* (L.) Osb.], Orlando tangelo (*C. reticulata* Blanco x *C. paradisi* Macf.), and Meyer lemon [*C. limon* (L.) Burm. f. hyb.] by rubbing inoculum into their leaves in the presence of finely powdered Carborundum. Only one chlorotic lesion was produced in a plant of each of these species. Holmes recovered the virus from each lesion by subinoculation to plants of Turkish tobacco and *Nicotiana glutinosa* L. The ease with which the virus was recovered led him to the conclusion that the difficulty previously encountered in many attempts to transmit citrus viruses by mechanical means was not due to inhibitory substances in the citrus foliage, but rather to use of insufficiently susceptible test plants.

However, Holmes was wrong, at least in part; inhibitors are present in citrus foliage, and means have been found to remove them, or to neutralize them, so that the viruses can be transmitted mechanically (3, 5). Other factors have also been important, not the least of which seems to be the low concentration of virus obtainable from infected citrus plants. Holmes may have been partly right in blaming the use of insufficiently susceptible test plants for failure to transmit; young tender shoots of citrus seedlings have proved to be more susceptible to infection with citrus variegation virus than are mature leaves.

Grant and Corbett (5, 6, 8) used 1 ml of 20 per cent sucrose and 0.05 g of activated charcoal per gram of leaf material, the sucrose to prevent disruption of mitochondria and consequent release of enzymes, and the charcoal to absorb inhibitors normally present in the plant juice. They transmitted citrus variegation virus to many different kinds of citrus and non-citrus plants. Inoculations were usually successful when the test plants were maintained in a cool greenhouse in winter or in an air-conditioned (20-22°C) greenhouse in summer. Several other investigators subsequently verified the mechanical transmission of citrus variegation virus to citrus and non-citrus plants.

The discovery that citrus variegation virus can be transmitted mechanically made it possible for Grant and Corbett (7, 8) to determine some of the biological properties of the virus. Knowledge of its biological properties made purification of the virus easier.

Tanaka and coworkers (10, 20, 21) transmitted a virus to sesame (*Sesamum indicum* L.) and to a number of leguminous plants from citrus that had been infected with Satsuma dwarf virus. To prepare the inoculum, juice was taken from young citrus shoots infected with Satsuma dwarf virus and buffered with 0.05-0.1 M di-potassium phosphate solution. The virus was readily transmitted from herbaceous plants to healthy plants of the same species by mechanical inoculation. They believed the virus to be that of Satsuma dwarf, but could not be certain because at the time of their reports they had not transmitted it back to citrus.

Storm and Streets (19) reported transmission of stubborn and psorosis-A viruses from citrus to Early White Spine cucumber (*Cucumis sativus* L.), using methods based on those of Grant and Corbett. So far as I know, their report has not been verified by other workers. Majorana (15) reported the mechanical transmission of crinkly-leaf virus from Eureka lemon [*C. limon* (L.) Burm. f.] to sour orange (*C. aurantium* L.), Rough lemon (*C. jambhiri* Lush.), and cowpea (*Vigna sinensis*

Savi), and of a California strain of citrus variegation virus to Rough lemon and cowpea. Since I have seen only the abstract of the original paper, I do not know what method of transmission he used.

Yarwood (22) transmitted a latent virus, subsequently shown to be tatter-leaf virus (4) from Meyer lemon to cowpea, bean, cucumber, tobacco, sunflower, *Chenopodium amaranticolor,* and *Nicotiana clevelandii.* Infectivity of the inoculum was increased by use of phosphate, sucrose, and N-6 benzyl adenine, by placing plants in the dark before or after inoculation, and by quick drying of the inoculated leaves. Inoculum from cowpea was about 300 times as infectious as that from lemon; whether the difference was due to presence of inhibitors or to a low concentration of virus in lemon was not established. Fulton (4) found that mechanical transmission of tatter-leaf virus was facilitated by grinding the leaf tissue inoculum in 0.03 M, pH 8.0 phosphate buffer containing 0.01 M sodium diethyldithiocarbamate and 0.02 M 2-mercaptoethanol.

The sucrose-charcoal method of Grant and Corbett was used by Dauthy and Bové (3) to transmit crinkly-leaf virus, as well as citrus variegation virus. Charcoal was not particularly effective in enhancing infectivity. There is likelihood, however, that the source of the charcoal influenced the results; charcoal from different sources is apt to have different adsorptive properties and it will sometimes remove a virus from solution.

It is perhaps ironic that the first citrus virus to be electron micrographed was not one of those that has been transmitted mechanically, but was tristeza virus, which is transmitted by aphids. Electron micrographs of thread-like particles 10-12 by 2000 mμ were shown at the Third Conference of the International Organization of Citrus Virologists in September, 1963. It was announced at this meeting by Kitajima *et al.* (11, 12) and by Silva *et al.* (18) that such thread-like particles were associated with tristeza disease and that the association was so constant that they considered the particles to be tristeza virus. The particles were found in both leaf-dip preparations and partially purified preparations from infected seedlings in a greenhouse and from naturally infected field-grown plants.

Although the particles have not been demonstrated to be infectious, there is considerable circumstantial evidence that they are tristeza virus particles. If they are indeed tristeza virus particles, this is the first time that a citrus virus has been obtained in a sufficiently pure form to be characterized with respect to size and shape. This statement can be made despite the report by Klotz (13) that small spherical particles, 13 mμ in

diameter, were isolated from psorosis-infected plants, because there is no evidence that the small particles represent psorosis virus. They might be particles of phytoferritin, or ribosomes; such particles are present in citrus and other species. They are essentially spherical and about 13 mμ in diameter, and they are often difficult to remove from virus preparations during the purification process (1).

Thread-like particles, which appear to be the same as those reported from Brazil, have been seen in electron micrographs of ultrathin sections of West Indian lime leaves infected with the T_3 (Florida) strain of tristeza virus (16). The particles seem to be confined to phloem elements; they were not found in cells of the epidermis, palisade, or spongy parenchyma. Their restriction to phloem cells suggests that it will be difficult, if not impossible, to transmit tristeza virus mechanically because of the necessity of introducing it directly into phloem tissue, a process in which aphids are particularly adept.

In their work on purification of tristeza virus, Silva *et al.* (18) used 0.001 M neutral phosphate buffer containing 0.01 M sodium sulfite when grinding leaves to obtain juice; they treated the juice with ether-carbon tetrachloride or with n-butanol before centrifuging at 30,000 rpm. I was unable to obtain tristeza virus particles when using ether-carbon tetrachloride or n-butanol to purify the T_3 strain of tristeza virus. Partial purification was achieved, however, by freezing the source leaves with liquid nitrogen, grinding to a fine powder, and extracting with 0.2 M tris (hydroxymethyl) aminomethane-HCl buffer at pH 7.4 (16). Even then, the partially purified preparations contained fewer thread-like particles than did those described by Silva *et al.*, probably because the starting material contained fewer particles.

Spherical particles 13-14 mμ in diameter were found by Dauthy and Bové (2) in partially purified preparations of crinkly-leaf virus from citrus, and of citrus variegation virus from cowpea plants. Because they did not find such particles in healthy plants, Dauthy and Bové concluded that the two viruses are strains of the same virus. This conclusion should be re-examined in view of the fact that Corbett and Grant (1) found phytoferritin particles, 10-12 mμ in diameter, in partially purified preparations of citrus variegation virus, but also found another particle about 30 mμ in diameter, which they believe to be the virus itself. Corbett and Grant point out that the phytoferritin particles in both healthy and diseased plants are similar to the 13 mμ particles observed by Dauthy and Bové.

Partially purified preparations of a virus were obtained by Semancik

and Weathers (17) from cowpea plants that had been mechanically inoculated with a virus from citrus plants with symptoms of tatter leaf. Fulton (4) subsequently identified the virus as that of tatter leaf by transmitting it from cowpea to citrus, where it induced symptoms of tatter leaf. The purification procedure of Semancik and Weathers involved the use of 0.01 M neutral phosphate buffer, containing 0.001 M magnesium sulfate and a 1 per cent aqueous suspension of bentonite. According to the authors, bentonite stabilized the virus in juice of herbaceous plants. Bentonite may serve to remove ribosomes from suspension, but it also removes or neutralizes ribonuclease. Care must be exercised in using bentonite, however, because some viruses will become adsorbed to it. In the case of tatter-leaf virus, use of bentonite decreased the number of cycles of centrifugation required to remove extraneous materials from the juice, and yielded preparations 10 to 20 times as infectious as those obtained by using chloroform-butanol or by precipitation at pH 5.0.

Corbett and Grant (1) obtained partially purified preparations of citrus variegation virus, from which they characterized the virus as an icosahedron-shaped nucleoprotein about 30 mμ in diameter. The source of most of their preparations was systemically infected cowpea leaves; it had been shown in earlier studies that such leaves had the highest concentration of virus, judged by infectivity. As a check, however, systemically infected citrus leaves were used as starting material in several instances. Several successive steps were used to obtain virus preparations reasonably free of extraneous materials, all the steps being carried out at 0-4°C. Leaf tissue was homogenized with chloroform and 0.5 M sodium citrate containing 0.1 per cent thioglycolic acid, at pH 6.5. The emulsion was broken by low-speed centrifugation. The aqueous phase was filtered through glass wool and dialyzed for 24 hours against 0.005 M borate buffer at pH 9.0. Additional steps included angle centrifugation at alternately high and low speeds, followed by rate zonal density gradient centrifugation. In the density gradients, the infectious zone occurred 2.3-2.8 cm below the meniscus. A non-infectious zone, which was shown to contain phytoferritin particles 10-12 mμ in diameter, occurred 1.7-2.0 cm below the meniscus. The non-infectious zone was present in preparations from healthy plants, but the infectious zone was not. The non-infectious zone appeared as a faint yellowish band when the gradient tube was back-lighted. The infectious zone was subsequently resolved into two distinct zones, both infectious. The component in the zone with the greatest infectivity would seem to have a sedimentation coefficient

of 110 S; this figure is probably low because of small amounts of impurities in the preparation.

The mere fact that a particle is found in a plant showing symptoms of a virus infection is no proof that the symptoms are due to the particle. The particle may be there by accident; it may be a latent virus; it may be a specific by-product of the infection. When attempting to establish the identity between the particle and the disease agent, it would be highly desirable to invoke Koch's postulates. There is presently no way, however, to grow a virus in pure culture. To get around the difficulty, Lauffer (14) some years ago proposed a substitute. I think that a brief review of Lauffer's method of associating form and function is pertinent at this point. The method depends upon demonstrating that a property possessed by the *form* is also possessed by the *function*. Lauffer refers to such a demonstration as a coincidence. An example of a coincidence in properties would be that the particle and the "infectivity" sediment at the same rate under identical conditions in a centrifuge. Another would be that the particle and the "infectivity" have the same isoelectric point. The more such coincidences, the better the chance for identity.

The coincidence that "infectivity" and particles sedimented at the same rate in density gradients was used by Semancik and Weathers (17) as a means of identifying a virus as a flexuous rod 19 mμ by 650 mμ. Both "infectivity" and particles were found in a zone that was not visible in the gradient. Fulton (4) subsequently provided infectivity data to show that this virus is that of tatter leaf.

The same type of coincidence was used by Corbett and Grant (1) to identify citrus variegation virus as an icosahedron 30 mμ in diameter.

Kitajima *et al.* (12) were forced to use another approach. Tristeza virus has not been transmitted mechanically; hence, it is presently not possible to demonstrate a coincidence between a property of the "infectivity" and that of the thread-like particle. The approach used was to show that the particles are associated with diseased plants and not with healthy ones. The evidence for identity would be better if a method such as that proposed by Lauffer were to show that the thread-like particles and the "infectivity" behave in an identical manner, perhaps by allowing the aphid vector to feed on purified preparations of the virus and then on test plants.

In dealing with particles that are essentially spherical, it is even more important to identify the form (that is, the particle) with the function (the infectivity) because it is now known that various kinds of essentially spherical particles are constituents of normal plant cells.

In conclusion, citrus virologists have come a long way since 1960 in understanding the nature of citrus viruses. The ability to transmit some of these viruses by mechanical means has made it possible to study their properties and to identify them by electron microscopy, sedimentation, and other methods. With improved techniques, we may be able to work out methods, such as serological ones, for rapid diagnosis of the viruses in the field. The next six years promise to be exciting ones for citrus virologists.

This paper is the Florida Agricultural Experiment Stations' Journal Series No. 2600.

Literature Cited

1. CORBETT, M. K., and GRANT, T. J. 1967. Purification of citrus variegation virus. Phytopathology 57: 137-143.
2. DAUTHY, D., and BOVÉ, J. M. 1964. Isolement du virus de la frisolee des Citrus (crinkly leaf virus.) 1. Note preliminaire. Fruits (Paris) 19: 187-193.
3. DAUTHY, D., and BOVÉ, J. M. 1965. Experiments on mechanical transmission of citrus viruses, p. 250-253. *In* W. C. Price [ed.], Proc. 3d Conf. Intern. Organization Citrus Virol. Univ. Florida Press, Gainesville.
4. FULTON, R. W. 1966. Mechanical transmission of tatter leaf virus from cowpea to citrus. Phytopathology 56: 575.
5. GRANT, T. J., and CORBETT, M. K. 1960. Mechanical transmission of the infectious variegation virus of citrus. Nature 188: 519-520.
6. GRANT, T. J., and CORBETT, M. K. 1961. Mechanical transmission of infectious variegation virus in citrus and non-citrus hosts, p. 197-204. *In* W. C. Price [ed.], Proc. 2nd Conf. Intern. Organization Citrus Virol. Univ. Florida Press, Gainesville.
7. GRANT, T. J., and CORBETT, M. K. 1964. Properties of citrus variegation virus. Phytopathology 54: 946-948.
8. GRANT, T. J., and CORBETT, M. K. 1965. Progress in citrus virology: mechanical transmission, p. 244-249. *In* W. C. Price [ed.], Proc. 3d Conf. Intern. Organization Citrus Virol. Univ. Florida Press, Gainesville.
9. HOLMES, F. O. 1959. Transmission of potato mottle virus to, and from, citrus plants by mechanical inoculation. Phytopathology 49: 729-731.
10. KISHI, K., and TANAKA, S. 1964. Studies on the indicator plants for citrus viruses. II. Mechanical transmission of the virus causing Satsuma dwarf to sesame (*Sesamum indicum* L.). Ann. Phytopath. Soc. Japan 29: 142-148. (In Japanese; English summary).
11. KITAJIMA, E. W., SILVA, D. M., OLIVEIRA, A. R., MÜLLER, G. W., and COSTA, A. S. 1964. Thread-like particles associated with tristeza disease of citrus. Nature 201: 1011-1012.
12. KITAJIMA, E. W., SILVA, D. M., OLIVEIRA, A. R., MÜLLER, G. W., and COSTA, A. S. 1965. Electron microscopical investigations on tristeza, p. 1-9. *In* W. C. Price [ed.], Proc. 3d Conf. Intern. Organization Citrus Virol. Univ. Florida Press, Gainesville.
13. KLOTZ, L. J. 1950. Virus diseases of citrus. Proc. 7th Intern. Botan. Congr. 712-715.
14. LAUFFER, M. A. 1952. Form and function: a problem in biophysics. Sci. Monthly 75: 79-83.

15. MAJORANA, G. 1963. Richerche sulla 'psorosia folgia bollosa' en sulla 'variegatura infettiva' degli Agrumi. Riv. Pat. Veg. Pavia, Ser. 3, 3: 251-270. (Rev. Appl. Mycol. 43: 478- 1964; original not seen).
16. PRICE, W. C. 1966. Flexuous rods in phloem cells of lime plants infected with citrus tristeza virus. Virology 29: 285-294.
17. SEMANCIK, J. S., and WEATHERS, L. G. 1965. Partial purification of a mechanically transmissible virus associated with tatter leaf of citrus. Phytopathology 55: 1354-1358.
18. SILVA, D. M., OLIVEIRA, A. R., and KITAJIMA, E. W. 1965. Partial purification of tristeza virus, p. 10-13. *In* W. C. Price [ed.], Proc. 3d Conf. Intern. Organization Cirus Virol. Univ. Florida Press, Gainesville.
19. STORM, L. W., and STREETS, R. B. 1963. Transmission of the stubborn and psorosis "A" viruses of citrus to an herbaceous host. Phytopathology 53: 1142.
20. TANAKA, S., and KISHI, K. 1963. Studies on indicator plants for citrus viruses. 1. Mechanical inoculation on leguminous plants with sap from Satsuma dwarf tree. Ann. Phytopath. Soc. Japan 28: 262-269. (Japanese; English summary).
21. TANAKA, S., KISHI, K., and YAMADA, S. 1965. Researches on the indicator plants of Satsuma dwarf and Hassaku dwarf viruses, p. 260-267. *In* W. C. Price [ed.], Proc. 3d Conf. Intern. Organization Citrus Virol. Univ. Florida Press, Gainesville.
22. YARWOOD, C. E. 1963. Mechanical transmission of a latent lemon virus. Phytopathology 53: 1145.