Recent Developments in the Purification and Characterization of Citrus Viruses

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Two very significant developments have occurred since the two previous reviews (*5, 26*) on isolation, purification, and characterization of citrus viruses. The first is the finding that stubborn, greening, and related diseases are not caused by true viruses, but are associated with mycoplasmalike organisms (*10, 15, 18, 29*). For this reason, they are not included in this review. The second is the discovery that citrus exocortis virus (CEV) is a low-molecular weight, free nucleic acid (*34–38*). Because of its special properties, CEV is treated as a separate topic in this review.

Recent findings on other citrus viruses are reviewed under the topics of purification and characterization, the latter including electron microscopy and serological and other related studies. The papers reviewed are essentially those that have appeared since the reviews of 1968 (*26*) and 1969 (*5*), although prior papers containing morphological data are cited in table 1.

Because of their recent discovery or special current interest, certain viruses and virus diseases are also briefly discussed. Some have been transmitted to herbaceous hosts, but have not been purified and characterized. Purification procedures are included.

VIRUS ISOLATION AND PURIFICATION

Citrus leaf rugose virus. Garnsey (*11, 13*) reported purification of leaf rugose virus (originally referred to as the crinkly-leaf-type virus) from citrus leaf tissue. This virus produces a characteristic rugosity of citrus leaves, and is serologically related to citrus infectious variegation virus (*11, 12*). Purification was achieved by extracting infected citrus tissue in an aqueous medium, adsorbing host components with hydrated calcium phosphate, concentrating the virus by differential centrifugation, and finally separating by density-gradient centrifugation. This method yielded highly purified and infectious virus preparations.

Citrus infectious variegation virus. Desjardins and French (*7*) found density-gradient electrophoresis extremely effective for eliminating a partially degraded ribosomal fraction that remained in virus preparations following organic-solvent extraction and differential centrifugation of extracts of citrus leaves. This host ribosomal fraction, which contaminated virus zones in density-gradient tubes, was completely removed by density-gradient electrophoresis. The ribosomal fraction was not a problem when the virus was purified from herbaceous plant tissue. The Florida isolate of citrus infectious variegation virus has been purified by the same procedure used for citrus leaf rugose virus (*12*).

Tristeza virus. Reports from two laboratories have described the preparation of purified or partially purified tristeza virus. The method first reported by Bar-Joseph *et al.* (*1, 2*) and later used by Primo *et al.* (*27*) involves gentle grinding with a mortar and pestle of either freeze-dried host tissue or tissue frozen in liquid nitrogen. Frozen, powdered tissue was suspended in Tris buffer and centrifuged at low speed. This procedure minimized shearing of the flexuous rods (*2*). Following low-speed centrifugation, virus in the supernatant

was precipitated with polyethylene glycol (PEG—mol. wt. 6,000). Differential centrifugation at intermediate speeds and additional PEG precipitations yielded relatively pure preparations of flexuous rods.

Latent Meyer lemon virus and citrus multiple sprouting virus. Majorana and Schwarz (*21*) reported mechanical transmission of these two viruses to herbaceous hosts, and purification of the viruses. They found, however, that the infectivity of their purified preparations was similar to that of crude plant extracts, and they could not find typical virus particles.

Citrus isolate of tobacco necrosis virus. During work on the cristacortis and psorosis concave gum viruses, Yot-Dauthy *et al.* (*45*) isolated a strain of tobacco necrosis virus free of satellite virus. Initial isolation from citrus leaves involved homogenization of the tissue in phosphate buffer, clarification by low-speed centrifugation, precipitation with PEG-6000, and differential centrifugation. This isolate of tobacco necrosis virus was also purified from cowpea by butanol clarification of the homogenate, followed by PEG precipitation and differential and density-gradient centrifugation. The virus was identified as a strain of tobacco necrosis virus by its co-banding with tobacco necrosis virus in CsCl gradients. The virus particles were icosahedral, and similar to those of tobacco necrosis virus. Purified preparations were highly infectious, and produced necrotic local lesions on several species of citrus (*45*).

Majorana (*19*) found that tobacco necrosis virus does not induce concave gum symptoms nor any other systemic symptoms in citrus species.

VIRUS CHARACTERIZATION

Electron microscopy. Morphological features of citrus viruses described to date are summarized in table 1. Characteristics listed for vein enation virus

TABLE 1
MORPHOLOGY AND DIMENSIONS OF CITRUS VIRUSES

Virus	Morphology	Size (nm)	References
Infectious variegation-crinkly leaf*	Icosahedral	30	(4, 7)
		25–30	(44)
		26	(22)
		27–34	(20)‡
Citrus leaf rugose†	Icosahedral	30	(11)
Satsuma dwarf	Icosahedral	26	(16, 40)
		20	(30)
Vein enation	Spherical	24–26	(14)
Citrange stunt	Flexuous rod	650 × 19	(3, 33)
Tristeza	Flexuous rod	2,000 × 10–12	(17, 27)
		2,000 × 10–11	(2)
		2,000 × 11	(25)
		2,000 × 12	(5)
Hassaku dwarf	Flexuous rod	2,000 × 10–12	(39, 41)
Citrus isolate of tobacco necrosis	Icosahedral	30	(45)

* Considered to be strains of the same virus (43).
† Related to infectious variegation (11).
‡ See text for various values for different centrifugal components.

(*14*) were based only on electron microscope studies of sectioned infected tissue. Hassaku dwarf (*41*), tristeza (*25*), and Satsuma dwarf (*30*) viruses have been studied in tissue sections and in purified preparations. The difference between the diameter reported for Satsuma virus in the isolated state (26nm) and that observed in the cell (20nm) (table 1) may have resulted from the method of measurement. While the shape of viruses can be determined in sections, there are problems with this approach. Tubular structures that are host components (*32*) can be mistaken for rod-shaped viruses. The plane of sectioning also affects the particle size and shape recorded.

In studies of the centrifugal components of infectious variegation and crinkly leaf viruses, Majorana (*20*) found differences in particle diameter between the two viruses and between the two major components of both viruses. For unfixed top and bottom components of infectious variegation, he found 32.73 and 33.89 nm, respectively, and for crinkly leaf virus, 27.27 and 33.56 nm, respectively. Particles fixed with osmium tetroxide were found to have slightly smaller diameters in all cases.

Serology. Antisera have been prepared to citrus leaf rugose virus (*11, 13*) and to the related Florida isolate of infectious variegation virus (*12*). The antiserum to citrus leaf rugose virus has been used for detection of virus in field trees (*13*).

Oliveira (*23*) and Primo *et al.* (*27*) prepared antisera to the flexuous rods associated with tristeza virus disease with antibody titers of 1/1024. Such sera should be of practical value in diagnosing diseased trees in the field, provided care is taken in preparing the test antigens.

Yot-Dauthy *et al.* (*45*) failed to obtain a positive cross-reaction of the citrus isolate of tobacco necrosis virus with antiserum prepared to a type strain of the virus. The citrus isolate may be a different serotype of this virus.

Sedimentation studies. Bar-Joseph *et al.* (*2*) reported a calculated S-value for tristeza virus at zero depth of 140 ± 10. S values determined in sucrose density gradients varied from 105 to 131, and varied with depth in the gradient.

An S value of 115 and a buoyant density in CsCl of 1.373 at 20°C was reported for the citrus isolate of tobacco necrosis virus (*45*).

The two major centrifugal components present in leaf rugose virus (*11*) were similar to those reported for citrus infectious variegation virus.

Majorana (*20*) reported S values of 95 and 120 for the two components of infectious variegation virus, and 100 and 125 for the two components of crinkly leaf virus.

Electrophoretic heterogeneity. Electrophoresis of infectious variegation virus in polyacrylamide gels (*8*) revealed fast and slow electrophoretic forms. Both forms were found in virus purified from citrus and cowpea tissue. Relative amounts of the two forms varied with the length of infection in cowpea. The slow form tended to decrease in older infections.

CHARACTERIZATION OF EXOCORTIS VIRUS

Despite the lack of a local-lesion assay host or characteristic particles that could be monitored during purification (*34*), great progress was made in the purification and characterization of the citrus exocortis virus (CEV).

The preliminary work of Semancik and Weathers (*34*) indicated that CEV was not readily sedimented at 100,000 *g* for 2 hours, and that viruslike particles were not present in infectious fractions. The presence of infectivity in the upper one-third of 10 to 40 per cent sucrose gradients after 2.5 hours of centrifugation was also atypical for plant viruses. These workers reported that

CEV could be precipitated by ethanol and that phenol extracts were highly infectious. These findings, which suggested that CEV might be a free nucleic acid, were supported by the observed sensitivity of infectious fractions to pancreatic ribonuclease (*34*).

Later research by these authors (*35*) revealed two components with different sedimentation characteristics: a major component with a 10 to 15 S value and a minor component with an S value of 25+. Behavior of CEV on methylated, albumin-coated Kieselguhr columns and on cellulose columns suggested a double-stranded RNA species. This was contradicted, however, by melting studies and by lack of resistance to RNase inactivation in high ionic-strength media.

Subsequently (*36*), the major component was found to be soluble in 2M LiCl, and resistant to heat and to concentrations of diethylpyrocarbonate that inactivated single-stranded viral RNA. Infectivity distribution was heterogeneous after Cs_2SO_4 equilibrium sedimentation, and had a lower buoyant density than did single- and double-stranded RNA markers. This suggests CEV is either a low-molecular-weight RNA similar to transfer RNA, or possibly a RNA:DNA hybrid.

Later, Semancik and Weathers (*37*) showed that infectivity is associated with a low-molecular weight (10S—125,000 daltons) RNA species, and identified the infectious RNA on polyacrylamide gel columns after electrophoresis.

Recently these workers (*38*) transmitted purified exocortis virus to tomato plants, which subsequently developed bunchy top symptoms similar to those produced by potato spindle tuber virus in tomato. Diener and Raymer (*9*) showed earlier that potato spindle tuber virus is an infectious, free nucleic acid.

FUTURE WORK

Although considerable progress has been made during the past three or four years, obviously much remains to be done on purification and characterization of other citrus viruses. For example, citrus ringspot virus, which has been transmitted to herbaceous hosts and back to citrus by the dodder species *Cuscuta subinclusa* (*6*), remains uncharacterized. Desjardins and Drake (unpublished) found rod-shaped particles of various lengths in extracts from infected hosts, but their relationship to citrus ringspot virus is as yet unproved.

The current interest in the cristacortis virus (*42*) and its common association with psorosis concave gum make it a worthy candidate for intensive study.

The causal agent of impietratura has not been studied, although the disease is destructive and widespread (*24, 31*). Certainly it would be interesting to know whether or not the eruptive gummosis disease recently described by Pujol (*28*) is a form of impietratura.

The causal agent of xyloporosis also remains uncharacterized.

The flexuous rods associated with tristeza virus-infected tissues are generally considered to be virus particles, but to our knowledge, no one has shown these rods to be infectious. Desjardins and French (unpublished) attempted, unsuccessfully, to do this by feeding *Aphis gossypii* through membranes on extracts enriched with the flexuous rod-shaped particles. Similar experiments should be done with the more efficient vector, *Toxoptera citricidus.*

Development of antisera to more citrus viruses and greater application of serological techniques for citrus virus identification are also needed. This work can proceed more readily as more citrus viruses are characterized and purified.

SUGGESTED PURIFICATION PROCEDURES

This section contains some general methods and suggestions which may be useful for the purification of hitherto uncharacterized citrus viruses.

Extraction. The type of increase host and the virus morphology dictate to some extent the extraction procedure. For example, citrus leaf tissue is much more fibrous and difficult to homogenize than is most herbaceous plant leaf tissue. One approach is to freeze tough tissues in liquid nitrogen and grind them to a powder with a mortar and pestle. The frozen powdered tissue can then be extracted in phosphate or Tris buffer (pH 7.0–8.0) in a mechanical blender. When citrus is the increase host, use of very young tissue is desirable. Herbaceous plant tissue can be homogenized in a high-speed blender, but a mortar and pestle or a food grinder is less likely to shear long, flexuous rods. In most cases, extraction should be carried out in a cold, buffered medium. Different ionic strengths and hydrogen ion concentrations should be tested, and inclusion of reducing agents and/or chelating agents, such as sodium sulfite, sodium thioglycolate, 2-mercaptoethanol, and sodium diethyldithio-carbamate, has often proved useful.

Clarification. Low-speed centrifugation removes gross cellular debris, but does not remove other components. Further clarification can be effected by: (1) emulsifying the extract with an immiscible organic solvent (chloroform-butanol); (2) adsorption of host components with hydrated calcium phosphate gel; or (3) differential precipitation of virus or host components with polyethylene glycol (PEG—Carbowax 6,000). If organic solvents are used, possible deleterious effects on the virus should be determined. If hydrated calcium phosphate gel is used, conditions (pH, ionic strength, etc.) necessary for differential adsorption of host components must be determined by clarifying extracts of healthy tissue.

Concentration. Differential centrifugation is commonly used to concentrate viruses. If the virus is difficult to resuspend from compacted, high-speed pellets, PEG precipitation, alcohol precipitation, ultra-filtration or dialysis against concentrated PEG can be tried.

Further purification. Density-gradient centrifugation and density-gradient electrophoresis are effective. Gel filtration (granulated agar or Sephadex), ion-exchange chromatography, and iso-electric precipitation are alternatives. Two-phase aqueous polymer systems might also be considered after preliminary trials to determine into which phase the virus partitions.

If chemical and physical characterization of the virus is contemplated, determination of the purity of the virus preparation is imperative. Specific infectivity of the final purified virus preparation should also be demonstrated by transmission to a diagnostic host, preferably a local-lesion host.

SUMMARY

New procedures have been described for purification of citrus infectious variegation virus. Citrus leaf rugose virus and an isolate of tobacco necrosis from citrus have been purified. Partially purified preparations of tristeza particles have also been obtained.

More information has been obtained on the morphology of citrus viruses by examination of thin sections of infected tissue and preparations of purified virus.

Antisera to tristeza, citrus leaf rugose, and infectious variegation viruses have been prepared. The causal agent of exocortis has been characterized as a low-molecular weight RNA species with high thermal stability. It is soluble in LiCl, resistant to diethylpyrocarbonate,

and susceptible to RNase. A relationship to potato spindle tuber virus has been shown.

A list of general procedures for use in purifying citrus viruses is presented, and problems for future study are outlined.

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