Studies on Strains of Exocortis Virus in Citron and *Gynura aurantiaca*

S. P. Kapur, L. G. Weathers, and E. C. Calavan

Several workers have reported strains of citrus exocortis virus (CEV), distinguished largely by severity of symptoms and incubation period (*4, 5, 6, 7, 10, 11, 13, 14, 20*). These are imperfect criteria for distinguishing strains, because they are dependent on host and environment. While variations in symptoms of infected trees in commercial citrus groves and in rootstock plantings suggest the existence of strains of CEV, differences in symptoms could result from other factors, as reported by Weathers (*18, 19*) and Weathers *et al.* (*23, 24*).

The discovery that gynura (*Gynura aurantiaca*) was a reactive herbaceous host for CEV (*21*) and refinement of extraction procedures (*11*) provided an opportunity for studying strains of CEV in more detail. We report here further studies on the existence of definitive strains of CEV.

Recent findings indicate that the causal agent of exocortis disease is a low-molecular weight, free-RNA molecule about one-tenth the size of other typical viral nucleic acids (*15, 16, 17*). For purposes of convenience, and because most citrus virologists refer to the causal agent as a virus, we shall refer to it as exocortis virus.

MATERIALS AND METHODS

Three isolates of CEV provided by E. C. Calavan served as sources. The three isolates were differentiated as mild, moderate, and severe, based on symptoms in Arizona 861 Etrog citron plants grown under similar environmental conditions. The mild isolate caused slight darkening of the petiole bases and lower side of the principal veins, occasional slight leaf epinasty, and very little or no stunting. The moderate isolate caused moderate epinasty of leaves, marked cracking of midveins, and slight to moderate stunting of plants. The severe isolate caused marked leaf epinasty, small, corky lesions or vertical cracking of stems, and severe stunting. All three isolates remained stable in serial transfers to Etrog citron and were maintained in this host.

Cuttings of gynura and citron plants free of other known citrus viruses were propagated in the greenhouse. They were rooted in silica sand and then transferred into steam-sterilized U. C. soil mix in containers. Receptor plants for host-range studies were grown as seedlings in U.C. soil mix in greenhouses maintained at 75 to 80° F.

Plants were inoculated by modifications of the razor-slash method (*8, 9, 22*). Young gynura and citron leaves with symptoms of CEV were ground in Tris buffer (0.1 M, pH 9.0) with a mortar and pestle. The tissue extract was strained through double layers of cheesecloth before use. Razor blades were dipped in the CEV extract and drawn diagonally six to eight times through the stem of a receptor plant. The cuts were wrapped with self-adhesive tape. Blades were disinfected between use on different plants by dipping in a 1.5 per cent solution of sodium hypochlorite.

Biological activity was determined by the appearance of systemic symptoms within 30 days after inoculation. The total number of "infected-plant days" on five to 10 gynura plants per test comprised the "relative infectivity" value (*17*).

In ultraviolet (UV) irradiation studies, virus extracts were exposed for

0, 2, 5, and 10 minutes to a UVSL-13 Mineralite UV lamp at a distance of 25 cm.

In cross-protection studies, gynura and citron plants were first inoculated with the mild isolate. Ten days later, gynura plants were challenge-inoculated singly with moderate and severe isolates of CEV. Citron plants were challenge-inoculated singly with moderate and severe isolates 30 days after inoculation with the mild isolate.

EXPERIMENTS AND RESULTS

Transmission of CEV isolates. Results of experiments on mechanical transmission of the three isolates of CEV from citron to gynura are presented in table 1. A comparative study of razor-slash inoculations showed better results with phenol and with Tris buffer extracts than with plant-to-plant transfer (table 1). The mild isolate did not induce symptoms in gynura plants by any of the inoculation methods. Symptoms produced in gynura by moderate and by severe isolates varied at the time of infectivity assay (30 days after inoculation) from mild to severe. When gynura plants showing mild or moderate symptoms were cut back, new growth invariably developed only severe symptoms.

Return inoculations from gynura to citron, with Tris buffer extracts, were successful only with moderate and severe isolates, and invariably produced severe symptoms. Return inoculations with the mild isolate were not successful, indicating either that this isolate was not present in gynura plants or that they were infected subliminally.

To further substantiate that the mild isolate could not be detected in gynura, 100 gynura plants were razor-slashed with Tris buffer extracts from citron leaves infected with the mild isolate of CEV. No symptoms developed in any of the gynura plants, and return inoculations to citron again were negative.

Effects of air temperature. The response of these three isolates to different temperatures is shown in table 2. The mild isolate failed to infect gynura plants regardless of the temperature at which the inoculated plants were grown. Temperature did not influence the relative infectivity of the moderate and severe isolates. Symptoms and relative infectivity of both isolates were similar at all temperatures above 21° C. No infectivity was recorded at temperatures below 21° C. Optimum temperature for symptom development was 24 to 30° C.

Inactivation by UV light. An experiment was conducted to study the sensi-

TABLE 1

TRANSMISSION OF THREE ISOLATES OF CITRUS EXOCORTIS VIRUS FROM CITRON TO GYNURA AURANTIACA BY THREE VARIATIONS OF RAZOR-SLASH INOCULATION

Method of extracting virus	Infected/inoculated plants with:			Total
	Mild isolate	Moderate isolate	Severe isolate	
Direct*	0/14	6/14	7/12	13/40
Phenol	0/15	9/15	18/20	27/50
Tris buffer	0/15	13/15	9/15	22/45

* Plant-to-plant transfer by razor slash.

TABLE 2
EFFECT OF TEMPERATURE ON RELATIVE INFECTIVITY OF ISOLATES OF CITRUS EXOCORTIS VIRUS IN GYNURA AURANTIACA
(Light intensity, 750 ft-c; R.H., 50 per cent)

Virus isolate	Infected/inoculated plants at:				
	18°C	21°C	24°C	27°C	30°C
Mild	0/10	0/10	0/10	0/10	0/10
Moderate	0/5	5/10	4/5	3/5	4/5
Severe	0/5	6/10	3/5	4/5	4/5

tivity of the three isolates of CEV to UV irradiation. No relative infectivity was obtained in gynura plants inoculated with irradiated extracts containing the mild isolate of CEV. Moderate and severe isolates were neither inactivated nor modified by 10-minute exposure to UV irradiation.

Cross-protection investigations. These studies were initiated to determine if the mild isolate induced a subliminal infection in gynura that afforded protection against more severe isolates. Gynura plants inoculated with the mild isolate were singly challenge-inoculated with moderate and severe isolates of CEV. Both induced severe symptoms of exocortis in the "protected" plants. The percentage of transmission was about equal to that in unprotected plants, suggesting that the mild isolate did not afford protection against either the moderate or severe isolate.

Gynura plants that did not show symptoms after challenge inoculations were tested for the presence of CEV by inoculating citron cuttings with extracts from these plants. No symptoms of exocortis developed in the citron plants, indicating that the symptomless gynura plants were not harboring CEV.

Another experiment on cross-protection was conducted with citron plants. Since Etrog citron, in contrast to gynura, becomes infected and shows mild symptoms with the mild isolate of CEV, it was thought that cross-protection against more severe isolates might be demonstrated with this host. No such protection was afforded, however, by the mild isolate in citron against infection with moderate and severe isolates. Challenge-inoculated plants showed definitive symptoms of CEV corresponding to the challenge inoculum, and transmission was not reduced.

DISCUSSION AND CONCLUSIONS

CEV is apparently transmitted from citron to gynura more readily with phenol or Tris buffer extracts than by direct transfer by razor slashing.

Results of transmission, temperature, and ultraviolet irradiation experiments indicate that the mild isolate of CEV used apparently cannot infect gynura. If it can, such infection must be of a subliminal nature that could not be detected in these experiments. This finding is of great practical importance. Gynura, which is a good herbaceous host for donor and assay purposes, may have limited value as a CEV indicator in certification work because of its failure to react to a mild isolate. Use of gynura may be limited to moderate and severe isolates of CEV.

Strains of a virus have often been shown to differ in their resistance to various inactivating treatments, such as

temperature and ultraviolet irradiation. The ease with which biological activity is altered presumably reflects differences in physical structure of the strains. Studies conducted on the effects of temperature and UV light on isolates of CEV revealed no differences in sensitivity to inactivation by these treatments in our tests.

It is generally accepted that protection of a plant by one strain of a virus against infection with a second strain stems from the first strain's occupation of all "infective sites" in the protected tissue. If infective sites in certain tissue are not occupied by the protecting strain, they should be susceptible to infection with other strains.

The failure of a mild isolate of CEV to afford protection in gynura against infection with moderate and severe isolates can easily be explained by the failure of the mild forms of CEV to infect gynura plants. Results with citron, however, cannot be explained this way because the mild isolate of CEV does infect and induce symptoms in citron plants.

Cross-protection is good evidence for virus strain relationships, but failure to achieve it is not always evidence of unrelatedness (*1, 12*). Cross-protection is rarely absolute even under the most favorable conditions of mechanical inoculation. Moreover, in our cross-protection tests there was uncertainty regarding the occupancy, by the mild isolate, of all the infective sites in the citron plants prior to challenge-inoculation. The mild isolate may not be completely systemic in citron, because symptoms are sporadic.

The reults are in agreement with those of Salibe and Moreira (*14*), who reported that mild forms of exocortis virus used in interference tests did not protect trees against infection with more severe forms of CEV. Similar results of non- or incomplete protection between strains of sugar beet curly top virus, cucumber viruses 3 and 4, and tomato mosaic virus, and strains of tomato spotted wilt virus have been reported (*2, 3, 25*).

From the results reported here and by others, it is clearly evident that variants of CEV do exist. It is also evident that these variants, while very stable in citron, are not so stable in gynura. Whether or not the levels of symptoms noted by us and reported by others are the result of strain differences was not confirmed from studies in gynura. However, because the behavior of the mild isolate in single infections is conspicuously different from that of the other isolates, we believe that it is an identifiable strain of exocortis virus.

LITERATURE CITED

1. Bennett, C. W.
1953. Interactions between viruses and virus strains. Advan. in Virus Res. 1: 39–67.
2. Bennett, C. W.
1955. Recovery of water pimpernel from curly top and the reaction of recovered plants to reinoculation with different virus strains. Phytopathology 45: 531–36.
3. Best, R. J.
1954. Cross protection by strains of tomato spotted wilt virus and a new theory to explain it. Austral. Jour. Biol. Sci. 7: 415–24.
4. Broadbent, P., L. R. Fraser, and J. K. Long
1971. Exocortis in dwarfed citrus trees. Plant Dis. Reptr. 55: 998–99.
5. Calavan, E. C., and L. G. Weathers
1961. Evidence for strain differences and stunting with exocortis virus. *In:* Proc. 2nd Conf. Intern. Organ. Citrus Virol. (W. C. Price, ed.) Gainesville: Univ. Florida Press, pp. 26–33.
6. Fraser, L. R.
1958. Virus diseases of citrus in Australia. Proc. Linn. Soc. N. S. Wales 73: 9–19.

7. FRASER, L. R., AND E. C. LEVITT
1959. Recent advances in the study of exocortis (scaly butt) in Australia. *In:* Citrus virus diseases. (J. M. Wallace, ed.) Berkeley: University of California Division of Agricultural Sciences, pp. 129–33.
8. GARNSEY, S. M., AND J. W. JONES
1967. Mechanical transmission of exocortis virus with contaminated budding tools. Plant Dis. Reptr. **51:** 410–13.
9. GARNSEY, S. M., AND L. G. WEATHERS
1972. Factors affecting mechanical spread of exocortis virus. *In:* Proc. 5th Conf. Intern. Organ. Citrus Virol. (W. C. Price, ed.) Gainesville: Univ. Florida Press, pp. 105–11.
10. GIACOMETTI, D. C.
1957. Doencas de virus e cavalos para citros. Ceres (Vicosa) **10:** 127–36.
11. KAPUR, S. P.
1971. Ecology of citrus exocortis virus strains in citrus and herbaceous plants and their characterization. Ph.D. Thesis, Univ. Calif., Riverside.
12. KASSANIS, B.
1963. Interactions of viruses in plants. Advan. Virus Res. **10:** 219–55.
13. MOREIRA, S.
1959. Rangpur lime disease and its relationship to exocortis. *In:* Citrus virus diseases. (J. M. Wallace, ed.) Berkeley: University of California Division of Agricultural Sciences, pp. 135–40.
14. SALIBE, A., AND S. MOREIRA
1965. Strains of exocortis virus. *In:* Proc. 3rd Conf. Intern. Organ. Citrus Virol. (W. C. Price, ed.) Gainesville: Univ. Florida Press, pp. 108–12.
15. SEMANCIK, J. S., AND L. G. WEATHERS
1968. Exocortis virus of citrus: association of infectivity with nucleic acid preparations. Virology **36:** 326–28.
16. SEMANCIK, J. S., AND L. G. WEATHERS
1970. Properties of the infectious forms of exocortis virus of citrus. Phytopathology **60:** 732–36.
17. SEMANCIK, J. S., AND L. G. WEATHERS
1972. Exocortis virus: An infectious free-nucleic acid plant virus with unusual properties. Virology **47:** 456–66.
18. WEATHERS, L. G.
1960. The effect of host nutrition on the development of exocortis in *Poncirus trifoliata*. Phytopathology **50:** 87.
19. WEATHERS, L. G.
1964. Nitrogen as a factor in the development of exocortis of citrus. Phytopathology **54:** 968–69.
20. WEATHERS, L. G., AND E. C. CALAVAN
1961. Additional indicator plants for exocortis and evidence for strain differences in the virus. Phytopathology **51:** 262–64.
21. WEATHERS, L. G., AND F. C. GREER, JR.
1968. Additional herbaceous hosts of the exocortis virus of citrus. Phytopathology **58:** 1071.
22. WEATHERS, L. G., F. C. GREER, JR., AND M. K. HARJUNG
1967. Transmission of exocortis virus of citrus to herbaceous plants. Plant Dis. Reptr. **51:** 868–71.
23. WEATHERS, L. G., M. K. HARJUNG, AND R. G. PLATT
1965. Some effects of host nutrition on symptoms of exocortis. *In:* Proc. 3rd Conf. Intern. Organ. Citrus Virol. (W. C. Price, ed.) Gainesville: Univ. Florida Press, pp. 102–07.
24. WEATHERS, L. G., A. O. PAULUS, AND M. K. HARJUNG
1962. Effect of soil temperature on the development of exocortis in *Poncirus trifoliata*. Phytopathology **52:** 32.
25. WU, J. H., AND S. G. WILDMAN
1963. Interference experiments relating to whether tobacco mosaic virus and cucumber virus 3 and 4 are strains of the same virus. Nature **199:** 1015–16.