A Mild Isolate of Citrus Variegation Virus Found in Florida Citrus[1]

S. M. Garnsey, N. Baksh, M. Davino, and J. P. Agostini

ABSTRACT. Random indexing of symptomless rough lemon in a recently replanted grove site near Dundee, Florida, revealed a mechanically transmissible virus which produced mild leaf mottle symptoms on Etrog citron. Host-range studies, serological tests, and physical properties of purified virus indicated that the virus was an isolate of citrus variegation virus (CVV). This newly discovered CVV isolate produced significantly milder symptoms in diagnostic indicators than the previously described Florida isolate of CVV and was more difficult to detect in graft-inoculated indicators. An antiserum was prepared against the new CVV isolate and used for serological assays. Survey of the discovery site by enzyme-linked immunosorbent assay (ELISA) indexing revealed CVV-infected sprouts only in a limited area.
Index words. Crinkly leaf, citrus leaf rugose virus.

Citrus variegation virus (CVV) has been found in several citrus-growing areas including Florida (4, 6, 10); however, it is usually not widespread or a major disease problem. The isolate of CVV originally described from Florida (6) was considered a typical isolate, and causes strong symptoms in Eureka lemon and Etrog citron. We had assumed that other CVV isolates would be readily detected during routine indexing for citrus exocortis viroid (CEV) on Etrog citron.

A citrus crinkly leaf virus (CCLV) has also been reported in citrus from several locations (4, 5, 10, 12). Symptoms of CCLV on lemon are milder than those described for CVV and consist of chlorotic flecking and mild distortion. A close relationship between CVV and CCLV was suggested when they were first described (4) and later substantiated by further study (2, 10, 12, 13). A crinkly leaf-type virus (CLTV) was described from Florida which produced flecking symptoms similar to CCLV in lemon (8). Subsequent studies showed that CLTV was clearly distinguishable from CVV in biological, physical, and serological properties, and the name citrus leaf rugose virus (CLRV) was proposed (8) and adopted (9).

Recently, we discovered by chance an isolate of CVV in Florida, which produced only mild symptoms in Etrog citron and which, under warm conditions, was difficult to detect by symptoms.

This paper describes the discovery of this mild isolate of CVV, its symptoms in citrus and non-citrus plants, and a survey for its incidence in the discovery location. A companion paper (3) describes purification of the mild isolate, some of its physical properties, and development of a specific antiserum.

METHODS AND MATERIALS

Plant materials and growing conditions. Most indexing work was done in a partially shaded glasshouse cooled by evaporative coolers. Assays on herbaceous plants were made in spring when temperatures normally ranged from 20 to 26°C. Indexing on citrus indicators and observation of symp-

[1]Mention of a trademark, warranty, proprietary product, or vendor does not constitute a guarantee by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

toms were done all year. In summer, glasshouse temperatures frequently reached 30 to 32°C.

Herbaceous plants were frequently grown prior to inoculation under supplemental light provided by Sylvania Gro lux® wide spectrum VHO fluorescent tubes. The combination of natural and supplemental light yielded rapidly-growing, succulent plants suitable for sap inoculation studies. Plants were held under natural light following inoculation.

All plants were grown in a steam-sterilized potting medium and were fertilized periodically to maintain good growth. Plants were grown from seed, except Eureka lemon and Etrog citron which were propagated as rooted cuttings.

Virus isolates. Two isolates of CVV were used in this study. One was a previously described isolate of CVV (6, 7) originally discovered by Grant and Smith (6). A culture of this isolate is now deposited with the American Type Culture Collection as PV 196. We used a single-lesion culture (designated CVV-1) which has been passed repeatedly between citrus and herbaceous hosts and maintained for over 12 years with no apparent change.

The second isolate of CVV was the mild isolate described in the introduction which was coded CVV-2. Unless otherwise noted, a single-lesion isolate from *Crotalaria spectabilis*, which was propagated in citron and coded CVV-2A, was used for all comparative studies.

Mechanically transmitted, pure cultures of CEV, CLRV, citrus ringspot virus (CRSV), tatter leaf-citrange stunt virus (TL-CSV), citrus tristeza virus (CTV) were used for the comparative tests. A psorosis isolate free of other known viruses was also used.

Indexing. Citrus plants were graft-inoculated with leaf pieces or bark chips, and the plants topped to force new growth.

Herbaceous plants were inoculated mechanically as previously described (6). Young leaf tissue was triturated in 10 parts 0.05 M Tris (Tris-hydroxy amino methane) pH 7.8 or 0.05 M potassium phosphate pH 7.0, with or without 0.5% 2-mercaptoethanol. Citrus plants were inoculated mechanically using the same procedure on both surfaces of young leaves.

Infection in symptomless hosts was confirmed by assays to cowpea or by serological tests.

Serology. Serological tests were done by agar gel double diffusion methods as described previously (7, 8) or by double sandwich enzyme-linked immunosorbent assay (ELISA). The ELISA procedures used were essentially those used for citrus tristeza virus (1), except that test samples were normally prepared in 0.05 M Tris buffer. Production of the antiserum, γ-globulin purification and enzyme conjugation are described elsewhere (3).

RESULTS

Initial discovery. The discovery site was a replanted 16-ha block of young, nucellar navel orange trees grafted on Carrizo citrange rootstock. The original planting had been grapefruit on rough lemon rootstock. While indexing the navel trees for an exocortis experiment, we collected several rough lemon root sprouts remaining from the previous planting. A mild, inconspicuous mottle and epinasty were observed in the first flush of growth in Etrog citron following graft inoculation with tissue from one rough lemon sprout. The symptoms faded as the flush matured. Mechanical inoculations from this original source produced necrotic local lesions on *Crotalaria spectabilis*, but no definite symptoms on *Chenopodium quinoa*, Black Local cowpea, and Red Kidney bean. Because of the lack of clear symptoms

in citron, cowpea, and Red Kidney bean, we did not immediately recognize this new virus as CVV.

Symptoms in citrus. The new isolate of CVV was tested on various citrus indicator plants in several experiments to determine its identity, and subsequently its similarity in symptomatology to CVV-1. The CVV-2 isolate produced a mild mottle and some leaf epinasty (a mild shock effect) in the first symptomatic flush of growth following inoculation in Etrog citron. Transitory, mild mottle symptoms were seen in subsequent flushes. The leaf distortion associated with CVV-1 was not detected (fig. 1), but an occasional puckered area which has been described for CLRV (9) was noted. In contrast to CVV-1, chronic infection in citron was difficult to detect (fig. 1D). In Eureka lemon, CVV-2 produced a pinpoint, chlorotic flecking (fig. 1E) very similar to that caused by CLRV (9). We did not observe the leaf distortion or shock reactions expected for CVV-1 (fig. 1B). The leaf flecking in Eureka lemon was the most consistent, persistent symptom of CVV-2 infection observed in citrus.

A chlorotic mottle, sometimes associated with a psorosis-like vein flecking and/or vein banding, was observed in some leaves of sour orange, sweet orange, Duncan grapefruit, Mexican lime and alemow inoculated with CVV-2. A very mild mottle was also observed in *Citrus hystrix* and rough lemon, but no definite symptoms were observed in Rangpur lime. The leaf symptoms described all faded as leaves matured. In comparison, CVV-1 produced stronger flecking and mottle symptoms, often with some evidence of shock in the first symptomatic flush. Leaf distortion was also produced by CVV-1 in all of the above hosts except Mexican lime and sweet orange. No definite symptoms were seen in Rusk citrange.

Although some leaf symptoms of CVV-2 could easily be confused with those produced by psorosis, they were clearly different from the strong shock reactions and persistent, ringlike leaf patterns caused by CRSV. No symptoms of CTV or CEV were observed.

Symptoms in herbaceous plants. Careful comparative tests between CVV-1 and CVV-2 were made on diagnostic indicators using similar sources and dilutions of inocula. The general symptoms of CVV-2 were similar to those of CVV-1 in direct comparison but often differed in degree. The local lesions in cow-

TABLE 1
SYMPTOMS PRODUCED BY A MILD FLORIDA ISOLATE OF CITRUS VARIEGATION VIRUS (CVV-2) IN HERBACEOUS HOSTS

Host*	Symptoms
Vigna unguiculata	Diffuse chlorotic local lesions, systemic necrosis or chlorotic mottle
Phaseolus vulgaris	Mild systemic chlorotic mottle or vein banding
Crotalaria spectabilis	Necrotic, ring-like local lesions, systemic necrosis
Capsicum annuum	Systemic chlorotic mottle
Chenopodium quinoa	Diffuse chlorotic areas on inoculated leaves, mild systemic mottle
Petunia hybrida	Mild systemic mottle
Cucumis sativus	Diffuse, chlorotic local lesions, mild systemic mottle
Momordica balsamina	Systemic mottle
Nicotiana glutinosa	None observed

*See text for specific cultivar.

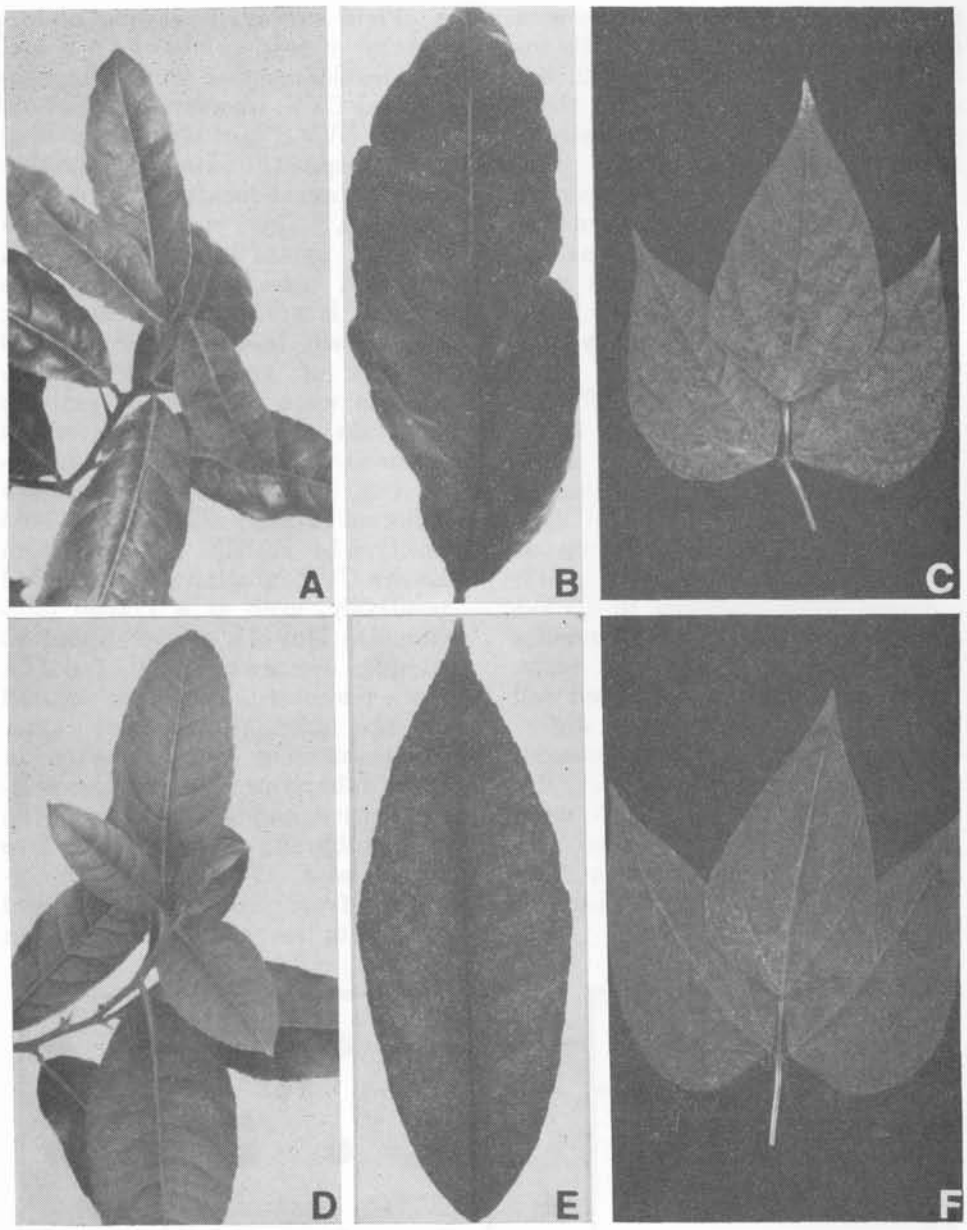

Fig. 1. Comparison of symptoms produced by Florida isolates of citrus variegation virus isolates CVV-1 (top) and CVV-2 (bottom) in A&D) Etrog citron, B&E) Eureka lemon, and C&F) Red Kidney bean.

pea were consistently less distinct, and the chlorotic, systemic symptoms in Red Kidney bean (fig. 1F) and Burpee blue petunia were consistently milder than for CVV-1. Symptoms in *C. spectabilis*, California Sweet pepper, and *C. quinoa* were at least as strong for CVV-2 as for CVV-1. We had less success infecting California Early Wonder pepper plants than California Sweet peppers.

The amount of necrosis in local lesions and the amount of systemic necrosis in *C. spectabilis* and cowpea varied with light and temperature conditions as previously described for CVV (6). National

Pickling cucumber and *Mormordica balsamina* were not consistently infected with CVV-1 or CVV-2, but symptoms were similar in those plants infected. Lack of necrotic local lesions in Red Kidney bean and lack of chlorotic to necrotic local lesions in *C. quinoa* were further evidence that CVV-2 was not related to, or contaminated with CLRV, CRSV, or TL-CSV.

Serology. In a preliminary test, extracts of CVV-2 reacted positively in agar gel immunodiffusion tests to an antiserum to CVV-1 (7). The reaction to CVV-2 was indistinguishable from the homologous reaction with no evidence of spur formation at the intersection of precipitin lines. Subsequently, new antisera were prepared to CVV-1 and CVV-2 (3) and similar results were obtained in reciprocal tests. Both isolates also cross reacted well in ELISA tests with little difference between antigen sources (S. M. Garnsey, unpublished). Reactions to CLRV in ELISA were usually negative, but weak reactions were obtained with high concentrations of purified CLRV in some tests.

Field survey. Following identification of this isolate as CVV and the development of an ELISA system for CVV, we surveyed the field where CVV-2 had been discovered to determine the extent of infection and the exact location of the first isolation. The location of the original symptomless RL sprout had not been recorded, but the general location within the block was known. In our first survey, 50 samples of young rough lemon sprouts were collected at periodic intervals over a 5-ha area, which included the initial discovery locality (fig. 2). None of the samples collected March 31, 1982 tested positive by ELISA. Extracts from known CVV-infected plants reacted positively, even at a 1/2000 dilution. On May 11, an additional 63 samples were collected from a more restricted, 1-ha area focused on the original discovery area. These samples were collected in three subgroups. Two samples tested positive, and both were from the same subgroup of 26 samples. Two weeks later, 47 samples were collected from individually tagged sprouts in the subgroup area which

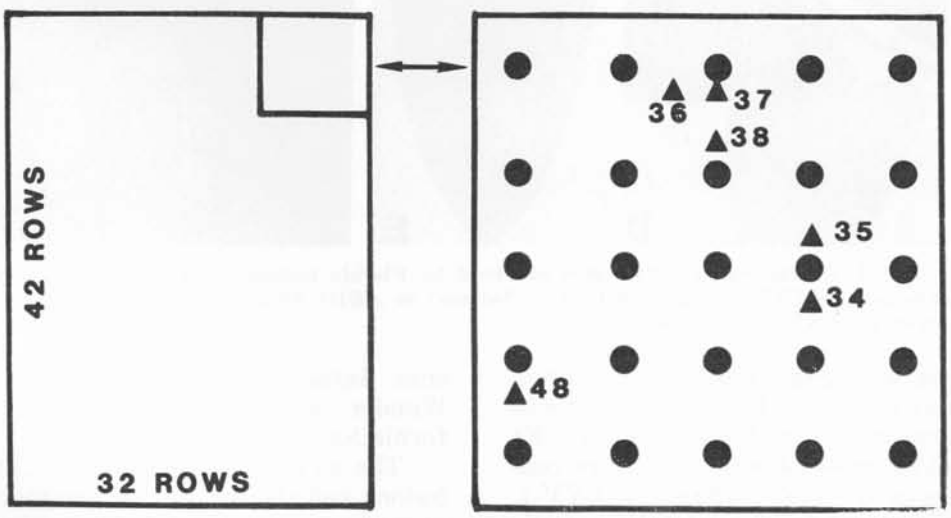

Fig. 2. Location of CVV-infected rough lemon rootsprouts (▲) in replanted block of young navel oranges on Carrizo citrange (●). Samples collected from throughout 32 x 42 tree area at left. All positives were found upper right area shown at larger scale on the far right.

had yielded the two positives. Five of these samples gave a strong positive test. All of these ELISA-positive sprouts were symptomless; however, one symptomatic sprout was subsequently discovered in the same area and confirmed by ELISA. The location of the six positive sprouts (fig. 2), suggested that they arose from three different original trees.

The CVV isolates present in the infected rough lemon sprouts have been transmitted to citron by grafting and subsequently subtransmited mechanically to citrus and herbaceous plants. Some differences in symptoms have been observed in citrus and herbaceous hosts. Several of the CVV-infected sprouts were co-infected with CEV.

DISCUSSION

The virus isolate discovered in a symptomless rough lemon rootsprout at Dundee, Florida is CVV. Although some symptoms differ from those normally described for CVV, its biological properties are generally similar and its identity was confirmed by serology. While leaf-flecking symptoms of CVV-2 in Eureka lemons are similar to those produced by CLRV, other host responses and serological tests do not indicate any closer relationship between CVV-2 and CLRV than that between CVV-1 and CLRV described earlier (7, 8). Psorosis-like symptoms are produced by CVV-1 and CVV-2 in some hosts and are the reason CVV was originally included in the psorosis group (4, 11). However, other symptoms, mechanical transmissibility, and association with a readily purified particle differentiate both CVV isolates from psorosis.

It is unlikely that the symptoms reported here for CVV-2 result from a mixed virus infection, because they were produced by a single lesion isolate transferred serially through different citrus and herbaceous hosts and, also, from purified sources of CVV-2 (3).

Symptoms of the CVV-2 isolate closely resemble the descriptions of citrus crinkly leaf virus (now considered a form of CVV), the main difference being the lack of distortion in lemon leaves infected with CVV-2. Observations on reactions of CCLV in Etrog citron were not found, so direct comparison could not be made in that host; however, Fraser (5) reported mild symptoms in two other citrons. Majorana and Martelli (10) did not observe systemic symptoms of CCLV in bean, but the mild symptoms of CVV-2 are easily missed if conditions are not favorable.

The mild transitory symptoms of CVV-2 in most citrus indicators increase the possibility that it could be overlooked in routine indexing procedures. Eureka lemon may be the best indicator tested. The mild symptoms in cowpea and bean also do not favor easy detection, and development of symptoms in other hosts can be erratic, depending on conditions and procedures. Because detection of CVV-2 can be done rapidly and with great sensitivity by ELISA, this is the indexing method of choice.

The origin of CVV-2 in the discovery location is unknown. There is no evidence that CVV-2 originated from the present planting of nucellar navels. The exact history of the previous planting is not known. If CVV-2 had been distributed by propagation in the previous planting, wider distribution of the virus would have been expected. The variation in symptoms among the natural CVV infections found plus variation in exocortis infection observed among different rough lemon sprouts tested further indicates that trees in the original planting may not have originated from a common source. At this point, we are still unsure which (if any) of the six infected rough

lemon sprouts located is the source of CVV-2. Since rough lemon sprouts have been continually treated with herbicides and removed from the planting, our survey did not include all the original sources. This general location was also an area used by the Glen St. Mary nursery many years ago, and this may account for the presence of different virus cultures here. Natural spread from a noncitrus host is also possible, although so far we have not recovered CVV from weeds in the field.

Although CVV has not been a major citrus production problem, repeated discovery of the virus in unexpected and unrelated sites indicates that continued surveillance is necessary, especially in certification programs. The mild symptoms produced by CVV-2 in Etrog citron indicate that this indicator is not reliable for all isolates of CVV. Fortunately, the development of sensitive serological procedures described elsewhere (3) facilitates accurate detection of CVV-2.

ACKNOWLEDGMENTS

The authors acknowledge the technical assistance of Sharon Cullen, Carmen Henderson, Randall Smith, and Rodney Whidden and the original suggestion by C. O. Youtsey to test rough lemon sprouts in the discovery area.

LITERATURE CITED

1. BAR-JOSEPH, M., S. M. GARNSEY, D. GONSALVES, and D. E. PURCIFULL
   1980. Detection of citrus tristeza virus. I. Enzyme-linked immunosorbent assay (ELISA) and SDS-immunodiffusion methods, p. 1-8. *In* Proc. 8th Conf. IOCV. IOCV, Riverside, CA.
2. DAUTHY, D. and J. M. BOVÉ
   1965. Experiments on mechanical transmission of citrus viruses, p. 250-53. *In* Proc. 3rd Conf. IOCV, Univ. Florida Press, Gainesville.
3. DAVINO, M. and S. M. GARNSEY
   1985. Purification, characterization and serology of a mild strain of citrus infectious variegation virus from Florida, p. 196-203. *In* Proc. 9th Conf. IOCV. (In press.)
4. FAWCETT, H. S. and A. A. BITANCOURT
   1943. Comparative symptomatology of psorosis varieties on citrus in California. Phytopathology 33: 837-64.
5. FRASER, L. R.
   1961. Lemon crinkly leaf virus, p. 205-10. *In* Proc. 2nd Conf. IOCV, Univ. Florida Press, Gainesville.
6. GARNSEY, S. M.
   1968. Additional non-citrus hosts for the Florida isolate of citrus variegation virus. Phytopathology 58: 1433-34.
7. GARNSEY, S. M.
   1974. Purification and serology of a Florida isolate of citrus variegation virus, p. 169-175. *In* Proc. 6th Conf. IOCV, Univ. California, Div. Agric. Sci., Richmond.
8. GARNSEY, S. M.
   1975. Purification and properties of citrus-leaf-rugose virus. Phytopathology 65: 50-57.
9. GARNSEY, S. M. and D. GONSALVES
   1976. Citrus leaf rugose virus. Descriptions of Plant Viruses No. 164. C.M.I./A.A.B., Kew, Surrey, England.
10. MAJORANA, G., and G. P. MARTELLI
   1968. Comparison of citrus infectious variegation and citrus crinkly-leaf virus isolates from Italy and California, p. 273-80. *In* Proc. 4th Conf. IOCV, Univ. Florida Press, Gainesville.
11. WALLACE, J. M.
   1959. A half century of research on psorosis, p. 5-21. *In* J. M. Wallace (ed.), Citrus Virus Diseases. Univ. California, Div. Agric. Sci., Richmond.

12. WALLACE, J. M.
1968. Recent developments in the citrus psorosis diseases, p. 1-9. *In* Proc. 4th Conf. IOCV, Univ. Florida Press, Gainesville.
13. YOT-DAUTHY, D. and J. M. BOVÉ
1968. Purification of citrus crinkly-leaf virus, p. 255-63. *In* Proc. 4th Conf. IOCV, Univ. Florida Press, Gainesville.