Comparison of Citrus Infectious Variegation and Citrus Crinkly-Leaf Virus Isolates from Italy and California

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INFECTIOUS VARIEGATION (CIVV) and crinkly leaf (CCLV) are common virus diseases of citrus in Italy, and their occurrence has been recorded at different times in practically all the citrus-growing areas of the country. Field trees exhibit symptoms similar to those described in California for the same diseases (3), and the two syndromes often coexist on the same tree.

The similarity of the properties and behavior of the causal agents of these disorders (CIVV and CCLV) encouraged some authors (1, 11, 15) to consider them as closely related entities or as strains of the same virus. In spite of the relative ease with which both viruses are mechanically transmitted to herbaceous plants (1, 2, 5, 10), the current literature indicates that little comparative work has been done with isolates of different origin. Except for a report by Servazzi *et al.* (13), there is no well-substantiated evidence that the viruses associated with infectious variegation and crinkly-leaf diseases in Italy are comparable to the virsuses associated with these disorders in Florida (5, 6), California (2, 10), and Corsica (1).

To investigate this subject, comparative studies of Italian and California isolates of CIVV and CCLV were undertaken in 1964. Comparison was based upon: a) symptomatological responses of sour orange (*Citrus*

aurantium L.) seedlings inoculated with chip-buds from naturally in-fected plants; b) reaction of herbaceous hosts following mechanical inoculation; c) cross-protection tests; and d) physical properties of the virus *in vitro*. Some early results were reported (11) briefly. This paper reports also the preliminary results of hot-air treatment for the cure of both diseases.

Methods and Results

VIRUS SOURCES.—The Italian isolates of CIVV and CCLV used in these trials came from lemon plants on sour orange rootstocks in orchards of central and southern Italy. The California isolate of CIVV is the original one of Fawcett and Klotz (3), while one of the two CCLV isolates from California derives from a naturally infected seedling (seed-transmis-sion?) and the other from a mechanically inoculated seedling.

GRAFT TRANSMISSION TO SOUR ORANGE.—All virus isolates were trans-mitted by chip budding of diseased material into apparently healthy sour orange seedlings. Symptoms appeared in from 20 days to 3 months, de-pending on the vegetative condition of the host. Positive transmission was achieved whenever infected material was used, whereas the unin-oculated checks remained apparently healthy. In several attempts to sap-inoculate herbaceous hosts, no success was achieved.

All the CIVV isolates caused characteristic chlorotic patterns of the leaves accompanied by reduction in size and malformation. No differ-ences in symptom expression were observed on the seedlings graft-inoculated with Italian or with California isolates; both produced equally strong reactions. (All CCLV isolates, irrespective of their origin, caused similar puckering and crinkling of the foliage, but without the clear tissue discoloration that is typical of the disease.) These results are in line with those obtained previously by Majorana (9) and Servazzi *et al.* (13) and indicate that the CIVV and CCLV isolates used in our studies cannot be differentiated from one another on sour orange.

MECHANICAL TRANSMISSION TO HERBACEOUS PLANTS.—All the trans-mission tests were performed on potted plants in an air-conditioned greenhouse at an average temperature of 22-24°C. Affected leaves of graft-inoculated sour orange seedlings were macerated in a mortar with an equal amount (w:v) of phosphate buffer 0.1 M, pH 7.2. The juice was then gently rubbed on corundum-dusted leaves of the herbaceous plants. Crude or clarified and concentrated sap of French beans and cowpea was used for inoculation in several instances.

The principal assay hosts were *Vigna sinensis* (L.) Endl., *Phaseolus*

vulgaris L., *Dolichos lablab* L., and *Tithonia speciosa* Hook. On the varieties Black local, Iron clay, and Early ramshorn of *V. sinensis*, all isolates of CIVV and CCLV caused yellow chlorosis of the veins, mosaic mottle, curling of the trifoliate leaves (Fig. 1,A), and stunting of growth. Later, the top foliage became bushy, and occasionally necrosis of the stem developed.

Virus isolates of different origins did not cause noticeably different symptoms. However, the CIVV isolates showed a more consistent tendency to induce yellowish local lesions on inoculated primary leaves than did the CCLV isolates.
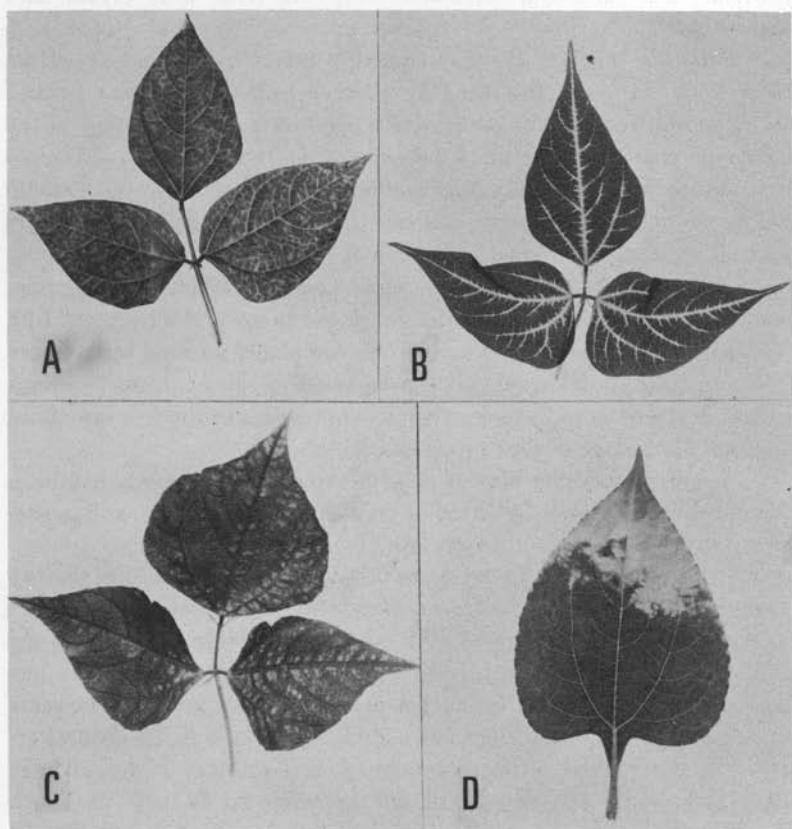

FIGURE 1. *A. Mosaic mottle on trifoliate* V. sinensis *leaf, caused by CCLV. B. Bright yellow chlorosis of veins of trifoliate* P. vulgaris *leaf infected by CIVV. C. Interveinal yellow mottle and veinlet clearing produced by CIVV on* D. lablab. *D. Bright yellow chlorosis of* T. speciosa *leaf systemically invaded by CIVV.*

Irrespective of their origin, the CIVV isolates caused bright-yellow chlorosis of first-order veins of the trifoliate leaves (Fig. 1,B), chrome yellow-mosaic mottle of the interveinal tissues, and top necrosis on the varieties Bountiful, Price, Tender green, Geneva market, and Satisfaction of *P. vulgaris* L. Although systemically invaded by all the CCLV isolates, this host does not react visibly to them.

ON *Dolichos lablab* L.—Bright yellow systemic mottle and yellowing of the veinlets (Fig. 1,C) developed following infection by CIVV. No differences in the severity and type of symptoms were noticed between isolates from several areas in Italy. The CCLV isolates caused a similar syndrome, but successful infections with this virus were erratic and inconsistent.

ON *Tithonia speciosa* HOOK.—Systemic bright yellow mosaic of the leaves (Fig. 1,D) was produced by all CIVV isolates. The CCLV isolates caused similar reactions, but erratically, and on a low percentage of the inoculated plants. Of the other herbaceous hosts tested, only *Chenopodium quinoa* Willd., *Chenopodium amaranticolor* Coste et Reyn., *Petunia hybrida* Vilm., and *Sesamum indicum* L. var. "Morada" were latently invaded by CIVV. Occasional infections of these plants by CCLV were obtained, but the results were inconsistent and contradictory. The symptomatological responses of this host range are in general agreement with those previously reported (1, 5, 10). All the plants infected by CIVV are also hosts of CCLV. However, CIVV virus usually induces strong reactions in these herbaceous indicators, whereas CCLV seldom produces symptoms on a high percentage of the inoculated plants.

P. vulgaris is the only host of those assayed that appears to exhibit a clear-cut difference in its reaction to CIVV and CCLV infection, and to provide a reliable means for distinguishing the two viruses.

CROSS-PROTECTION TESTS.—Due to the differential response to the two viruses obtained with *P. vulgaris*, Bountiful beans were used as test plants in subsequent trials. One or 2 days after unfolding, and when the host's susceptibility seemed highest (11), the primary leaves were mechanically inoculated with California or Italian CCLV isolates. The same primary leaves were challenge inoculated with CIVV 8 to 10 days afterwards. In the control series, corundum-dusted primary leaves of bean plants were gently rubbed with phosphate buffer 0.1 M, pH 7.2, 1 to 2 days after their opening and inoculated as follows: a) with CIVV 8 to 10 days afterwards; b) at time of leaf unfolding with CCLV only; and c) 8 to 10 days after leaf opening with CIVV.

Between the first and second challenge inoculation all plants remained

in the dark and received 3 min of artificial illumination every 2 hr. With such treatment, test plants retained a satisfactory degree of susceptibility as demonstrated by the high percentage of infections (up to 80 per cent) obtained on control c plants that were inoculated on fully expanded primary leaves. Under normal light conditions, plants inoculated at this stage of growth usually contracted few and irregular infections (11).

In the cross-protection test, plants previously inoculated with CCLV were noticeably less susceptible (down to 5 per cent) to CIVV inoculation. Also, the number of infections decreased considerably when plants received no treatment other than rubbing with abrasive and phosphate buffer, control a. Infections were always very high on controls b and c. These results suggest that merely rubbing the surface of very young primary leaves of P. vulgaris is sufficient to reduce greatly their susceptibility to CIVV. The decrease seems irreversible, because treatments such as exposure to darkness seldom change this pattern.

This phenomenon may be explained by the existence of specific receptors in the host for each virus to which it is susceptible (8, 14). The ease with which susceptibility to CIVV is lost and restored by exposure to darkness supports the assumption that in the CIVV-P. vulgaris system, the infection sites developed by abrasion are limited in number and period of susceptibility. Most of these sites would be available at the moment of rubbing with buffer, but, owing to their short lifetimes, would disappear without being converted into infective centers (4, 14). Consequently, the chances of infecting P. vulgaris would be reduced in proportion to the time elapsed between abrasion and inoculation. This hypothesis would apply especially to a virus of low-concentration and rather unstable nature, such as CIVV.

In several experiments, the number of double-inoculated (CCLV + CIVV) plants that developed symptoms was lower than that of control a. However, the differences were neither large nor consistent. Consequently, there was no clear indication that the two viruses really interferred with one another.

PHYSICAL PROPERTIES in vitro.—The physical properties of the two viruses were determined with standard procedures (12), starting from infected sap of P. vulgaris and V. sinensis for CIVV and CCLV, respectively. The dilution end points usually lay between 10^{-2} and 10^{-3} for most CIVV and CCLV isolates. However, one Italian isolate of CCLV lost infectivity at dilutions between 10^{-1} and 10^{-2}, and one Italian isolate of CIVV possessed a fairly high concentration, its dilution-end point being between 10^{-3} and 10^{-4}

Both viruses survived for a very short time in untreated infectious sap at room temperature (22 to 24°C). In particular, CCLV isolates usually lost infectivity after 2 hr, whereas CIVV isolates remained infective for 6 to 8 hr. As expected, the virus survival was somewhat extended at lower temperatures. For example, a California isolate of CCLV lost infectivity after 2 to 4 hr at 22 to 24°C, after 8 to 14 hr at 18°C, and after 24 to 48 hr at 6°C.

The low concentration and the great instability of these viruses, as demonstrated by their low tolerance to dilution and aging *in vitro,* had a direct effect on their thermal inactivation points which were erratic and inconsistent in a long series of trials. Generally, CCLV isolates were infectious after 10 min of exposure at 45°C but not at 50°C, whereas CIVV isolates had a thermal inactivation point between 55 and 60°C.

Although differences in the *in vitro* properties of CIVV and CCLV exist, they are too small and too inconstant to differentiate the two viruses satisfactorily. Furthermore, no significant differences in *in vitro* properties were found between isolates of various geographical origins. Thus, our determinations are in close agreement with those of Grant and Corbett (6, 7).

HEAT TREATMENT.—In these tests, sour orange seedlings graft-inoculated with California and Italian isolates of CIVV and CCLV and with foliage showing clear symptoms were kept for 4 weeks in an artifically illuminated heat chamber at a temperature of 38 ± 1°C. Bean and cowpea plants were sap-inoculated and sour orange seedlings were graft inoculated at regular intervals prior to, during, and after, the heat treatment. Before the heat treatment all inoculations resulted in infection, whereas after 6 days of treatment no transmission to herbaceous plants occurred and no symptoms developed on sour orange seedlings graft-inoculated with material heat treated 28 days. In the 10-month-period following heat treatment, no symptoms appeared on new vegetation of the treated seedlings. Attempts to recover mechanically CIVV or CCLV from symptom-showing leaves were unsuccessful. This suggests that both viruses are totally inactivated in the host tissues or that their concentration drops so low that their recovery becomes exceedingly difficult or impossible.

Conclusions

The similarity in behavior of these two viruses, CIVV and CCLV, is striking. Irrespective of their origin, they showed virtually identical properties *in vitro* and were equally susceptible to heat inactivation *in vivo.* A

clear-cut difference occurred only in the response of one herbaceous host (*P. vulgaris*) to manual inoculation, whereas the reaction of the other herbaceous indicators suggested close relationship or identity of the two viruses. Other workers (1, 10, 15) have concluded from similar evidence that CIVV and CCLV are strains of the same virus. Our findings strengthen that hypothesis.

No evidence was obtained that the Italian and California isolates of CIVV and CCLV considered in our studies are sufficiently different that separation of strains is possible.

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