Pathogenicity on Walnut and Serological Comparisons of Cherry Leafroll Virus Strains

Adib Rowhani and S. M. Mircetich

Postgraduate research plant pathologist and research plant pathologist, respectively, ARS, USDA, Department of Plant Pathology, University of California, Davis, CA 95616.

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ABSTRACT

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Cherry leafroll virus isolate W8 (CLRV-W8) from English walnut (*Juglans regia*) with blackline symptoms in California was closely related serologically to isolate CLRV-Wsp from symptomless walnut seedlings originally from Spain. These two isolates were very similar to, but distinguishable from, a third isolate from Italy (CLRV-WRS) that induced ringspots on leaves of English walnut. Comparison by immunodiffusion with CLRV isolates from cherry, golden elderberry, dogwood, and rhubarb revealed that among these isolates, the golden elderberry isolate was the most closely related to the three walnut isolates. In pathogenicity tests, all three walnut isolates (CLRV-W8, -Wsp, and -WRS) infected Hartley English walnut cultivar and caused blackline symptoms at the English walnut/*Juglans hindsii* graft union. Uninoculated control trees and trees inoculated with CLRV cherry and golden elderberry strains remained healthy and virus-free.

Additional keywords: Blackline disease of walnut, host specificity.

In California, more than 30 different English walnut cultivars are susceptible to walnut isolates of cherry leafroll virus (CLRV-W), but infected scions develop no recognizable leaf symptoms. CLRV-W causes walnut blackline (WBL) disease resulting in decline and death of English walnut scions (*Juglans regia* L.) propagated on Northern California black (*J. hindsii* Jeps.) or hybrid paradox (*J. hindsii* × *J. regia*) rootstocks (4,8,9). Natural virus spread occurs during cross-pollination, and infected limbs and scaffolds are symptomless until the virus invades the graft union and induces tissue necrosis at the scion-rootstock junction (8). In the United States, other CLRV strains occur naturally in dogwood (CLRV-Dw), golden elderberry (CLRV-Ge), and elm (5,6,7,14). These hosts develop characteristic leaf symptoms upon infection (5,6,14).

In Italy, CLRV strains of two serotypes infect walnut trees and cause yellow mosaic (CLRV-WYM) or ringspot leaf symptoms (CLRV-WRS) (10). An additional serotype of CLRV was isolated from symptomless olive trees in Italy (12). In the United Kingdom, CLRV has been recovered from walnut, cherry, and birch trees (1,2,3).

We investigated the serological properties of three CLRV-W isolates of different origins: United States, Italy, and Spain. These were compared with CLRV isolates from cherry (-Ch), dogwood (-Dw), golden elderberry (-Ge), and rhubarb (-Rh). In addition, we investigated the pathogenicity to English walnut trees of the isolates from walnuts, cherry, and golden elderberry.

MATERIALS AND METHODS

Virus source and propagation. CLRV-W8 isolate was recovered from a blackline-affected English walnut orchard tree (*J. regia*) in California (8,9); CLRV-Wsp was isolated from 4-mon-old symptomless walnut (*J. regia*) seedling grown from seed imported from Spain. CLRV-WRS, recovered from English walnut seedlings in Italy showing chlorotic rings on leaves, was supplied by J. I. Cooper. CLRV strains of cherry, dogwood, golden elderberry, and rhubarb were supplied by R. Stace-Smith. Virus cultures were maintained and propapated in cucumber (*Cucumis sativus* L. 'National Pickling') and/or *Chenopodium quinoa* Willd. Carborundum-dusted leaves were inoculated with a homogenate from infected cucumber or *C. quinoa* leaves in 0.05 M phosphate buffer, pH 6.5.

Purification and antiserum production. The walnut isolates of CLRV were purified as described by Rowhani et al (11) and then used for antiserum production and mechanical inoculation of trees. For different serological tests, the virus isolates were partially purified as described by Rowhani et al (11), except that the sucrose gradient step was omitted.

Antisera against CLRV-Wsp and -WRS were prepared by injecting white New Zealand rabbits with three intramuscular injections at weekly intervals. Virus preparations of 67 and 210 μg for CLRV-Wsp and -WRS, respectively, were emulsified with an equal volume of complete adjuvant (1 ml each) for each injection. Blood was collected 10 and 20 days after the last injection. Antisera to CLRV-W8 and other strains of CLRV (-Ch, -Dw, -Ge, and -Rh) were either prepared in an earlier study (11) or supplied by R. Stace-Smith. The antisera had homologous titers of: CLRV-W8, 1:512; -Wsp, 1:256; -WRS, 1:128; -Ge, 1:1,024; -Ch, 1:2,048; -Dw, 1:512; -Rh, 1:256 in gel immunodiffusion tests; nonspecific titers with sap from healthy plants were not more than 1:2–1:4.

Serology. Serological relationships among walnut and nonwalnut isolates of CLRV were examined in gel immunodiffusion tests in a 0.4-mm layer of 0.9% noble agar in 0.85% sodium chloride and 0.1% sodium azide (saline). Antisera against different isolates were diluted 1:10 in saline used in central wells. Antigens consisted of partially purified virus preparations or sap extracted from healthy plant tissue and were diluted 1:10 in 0.05 M phosphate buffer, pH 6.5. The intragel cross-absorption tests were performed in 0.9% noble agar-saline as described by Van Regenmortel (13).

Pathogenicity to walnut. In 1984, four to five trees of Eng. ,h walnut cv. Serr on Northern California black (*J. hindsii*) roots per virus isolate were inoculated with purified preparations of CLRV-W8, -Wsp, -Ch, and -WRS. In 1985, CLRV-Ge was included with these isolates in a similar experiment using the Hartley cultivar. Inocula contained purified virus (about 100 μg/ml) in 0.05 M phosphate buffer, pH 6.5, containing 50% glycerol (V/V). Inoculation was made under the bark approximately 3–5 cm above

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the graft union, as previously described (8). Inoculum infectivity was verified on the herbaceous hosts, *C. quinoa* and *Nicotiana megalosiphon* Heurch and Mueller.

Pathogenicity of the CLRV strains to walnuts was assessed after a 3-mon incubation, when inner bark and cambium tissues were collected (1 cm below the inoculation site) and assayed by enzyme-linked immunosorbent assay (ELISA) (11) for the presence of CLRV. The capability of the CLRV strains to cause WBL disease was based on the presence of blackline symptoms at the rootstock-scion junction.

RESULTS

Experimental plant reactions. Because *Chenopodium quinoa* was found to be a universal host for all CLRV strains used in this study, it was used for virus propagation. Among 43 different herbaceous plant species from six different families (*Chenopodiaceae*, *Compositae*, *Cruciferae*, *Cucurbitaceae*, *Leguminosae*, and *Solanaceae*) artificially inoculated with CLRV-W8 and -Wsp, only cucumber and melon (*Cucumis melo* L. 'Honeydew') were useful in differentiating these two strains.

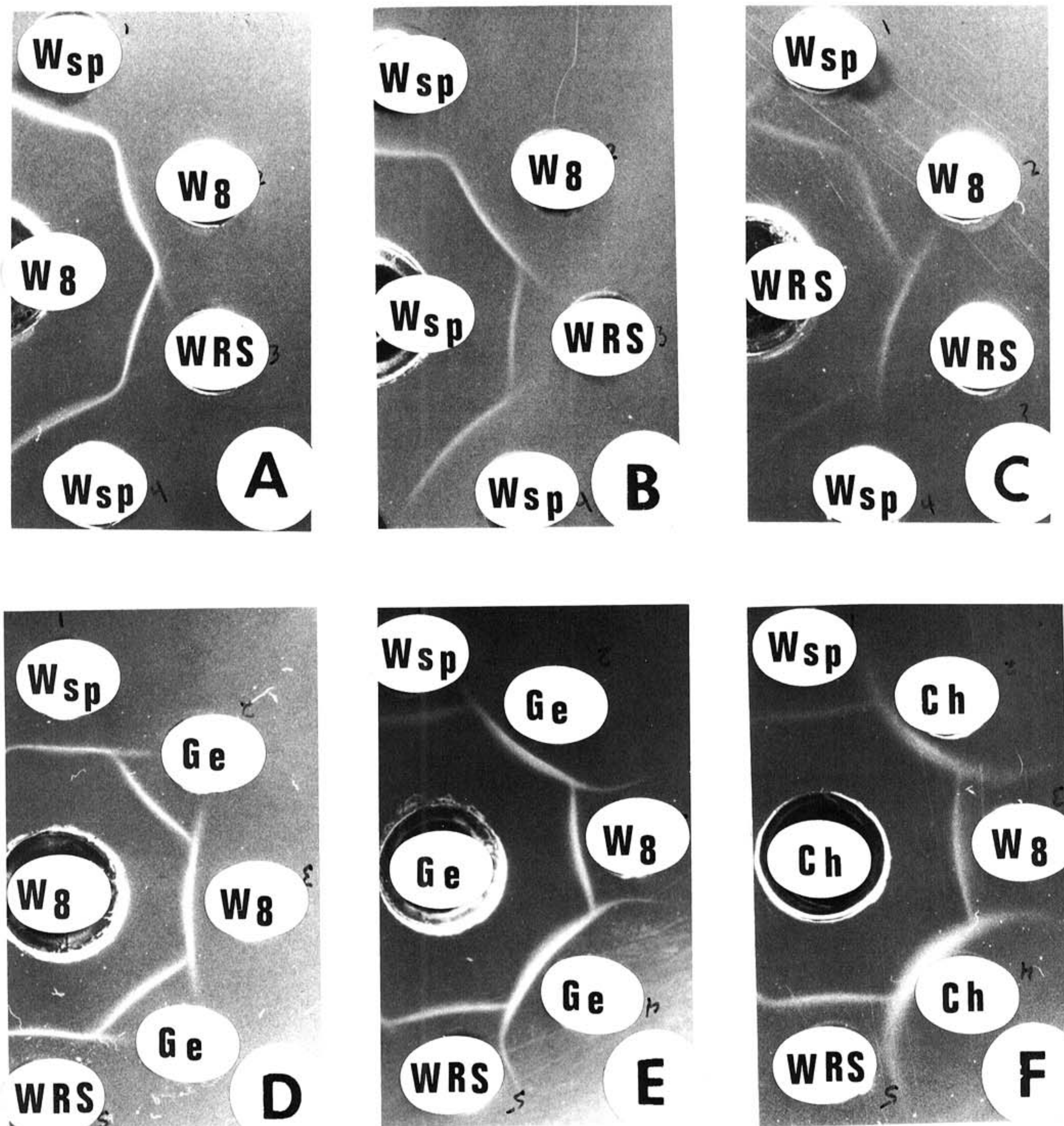

Fig. 1. Comparison of the strains of cherry leafroll virus (CLRV) in gel immunodiffusion tests using antisera produced against walnut isolates of CLRV from California (W8), Spain (Wsp), Italy (WRS), and nonwalnut isolates from golden elderberry (Ge) and cherry (Ch) (central wells). In the peripheral wells, the respective antigens (partially purified material) were used. Note spur formation when CLRV-W8 or -Wsp were used (**A** and **B**). This was also the case when antiserum against CLRV-WRS was used (**C**), but in all tests no spur formation occurred between CLRV-W8 and -Wsp (**A–C**). Spur formation occurred between walnut isolates and a nonwalnut isolate (Ge) when antiserum against CLRV-W8 was used (**D**) and also when antiserum against nonwalnut isolates were used (**E** and **F**). Sap from healthy plants did not show any reaction in these experiments.

Cherry leafroll virus-W8 induced chlorotic lesions on inoculated cotyledonary leaves and later caused systemic chlorotic spots and ringspot patterns on uninoculated leaves, whereas CLRV-Wsp induced chlorotic lesions on inoculated leaves that later became necrotic and in severe instances, often caused death of the inoculated leaves. No systemic symptoms were observed in either cucumber or melon plants.

Serology. Serological relationships among CLRV isolates, examined by agar gel immunodiffusion tests, showed that CLRV-W8 and -Wsp are very closely related, if not identical (Fig. 1), and antigenically distinct from CLRV-WRS, -Ge, -Ch, -Dw, and -Rh isolates (Table 1). CLRV-W8 and -Wsp formed a confluent precipitin line regardless of the source of CLRV antiserum (Table 1). However, this isolate formed a spur when tested against -WRS antigen with CLRV-W8 or -Wsp antisera, indicating that CLRV-W8 and -Wsp were more distantly related to -WRS than to each other. In intragel cross-absorption tests, CLRV-W8 and -Wsp eliminated all reactions. Homologous reactions remained when CLRV-WRS atigen was used to absorb CLRV-W8 or -Wsp antisera. This result is in agreement with the data presented above, indicating that CLRV-WRS is not identical to either -W8 or -Wsp. In other comparisons, precipitin reactions between walnut and nonwalnut isolates showed lines of partial fusion (Table 1). Even so, test results indicated that CLRV-Ge has more determinants in common with the walnut isolates, especially CLRV-W8, than with the nonwalnut isolates (Table 1).

Pathogenicity to walnut. In the 1984 trials, isolates CLRV-W8 and -Wsp, but not -WRS or -Ch, were successfully transmitted to the Serr walnut cultivar (Table 2). In a similar experiment conducted in 1985, the Hartley walnut cultivar was used, and, in addition to CLRV-W8 and -Wsp, we also succeeded in transmitting -WRS but not -Ch or -Ge isolates. All CLRV-W8, -Wsp, and -WRS infected walnut trees (diagnosed by ELISA) developed the blackline symptom at the graft union within a year after inoculation.

TABLE 1. Serological relationship between walnut isolates and other nonwalnut strains of cherry leafroll virus (CLRV) in agar gel double immunodiffusion tests

	Antiserum[a]						
Antigen pairs	W8	Wsp	WRS	Ch	Ge	Dw	Rh
W8, Ch	W8[b]	W8	W8	Ch	W8	Ch	W8
W8, Ge	W8	W8	W8	···[c]	Ge	···	···
W8, Dw	W8	W8	W8	Dw	W8	Dw	W8
W8, Rh	W8	W8	W8	Rh	W8	Rh	Rh
W8, Wsp	···	···	···	···	···	···	···
W8, WRS	W8	···	WRS	W8	W8	···	W8
Wsp, Ch	Wsp	Wsp	Wsp	Ch	Wsp	Ch	Ch
Wsp, Ge	Wsp	Wsp	Wsp	···	Ge	···	Ge
Wsp, Dw	Wsp	Wsp	Wsp	Dw	Wsp	Dw	Dw
Wsp, Rh	Wsp	Wsp	Wsp	Rh	Wsp	Rh	Rh
Wsp, WRS	···	Wsp	WRS	···	Wsp	···	Wsp
WRS, Ch	WRS	WRS	WRS	Ch	WRS	Ch	Ch
WRS, Ge	WRS	WRS	WRS	Ge	Ge	Ge	Ge
WRS, Dw	WRS	WRS	WRS	Dw	WRS	Dw	Dw
WRS, Rh	WRS	WRS	WRS	Rh	WRS	Rh	Rh

[a] W8 = cherry leafroll virus (CLRV) isolated from infected walnut trees in California showing no leaf symptoms but exhibiting the typical blackline symptoms at the graft union; Wsp = CLRV isolated from symptomless 4-mo-old walnut seedlings grown from seed imported from Spain; WRS = CLRV isolated from naturally infected walnut trees in Italy showing chlorotic rings on leaves; Ch = CLRV isolated from cherry trees; Ge = CLRV isolated from golden elderberry trees; Dw = CLRV isolated from dogwood trees; Rh = CLRV isolated from rhubarb plants.

[b] Antigen whose precipitin line extended to form a spur.

[c] Confluent precipitin line.

DISCUSSION

Serological comparisons distinguished the walnut isolates of CLRV (CLRV-W8, -Wsp, and -WRS) from nonwalnut isolates (Fig. 1 and Table 1). Isolates CLRV-W8 and -Wsp are essentially identical antigenically because in intragel cross-absorption tests, each virus eliminated the antibody activity of the other isolate. These three isolates appear to share some serological relationship with nonwalnut isolates of CLRV, but the extent of relationship differed with the different strains (Fig. 1, D–F, and Table 1).

Although CLRV-W8 does not induce virus-like leaf symptoms in naturally infected English walnut, it causes WBL in English/*J. hindsii* or Paradox orchard trees (8,9). The Spanish isolate, CLRV-Wsp, causes no virus-like leaf symptoms in naturally infected English walnut seedlings; the Italian isolate, CLRV-WRS, induces characteristic chlorotic rings in leaves of naturally infected English walnut seedlings (10). We are not aware of any report of the occurrence of blackline disease in walnut trees in Italy or Spain, but our experiments showed that walnut isolates of CLRV (-Wsp and -WRS) present in Spain and Italy, respectively, are as capable as the California isolate of inducing blackline disease of English walnut trees propagated on *J. hindsii* or paradox rootstook (Table 2). The Serr cultivar (1984 experiment, Table 2) was not infected with CLRV-WRS, whereas the same strain infected the Hartley cultivar (1985 experiment, Table 2), and this response might be caused by differences in environmental conditions or physiological state of walnut trees when the inoculation was performed. In general, the CLRV isolates showed considerable host specificity because CLRV-Ch and CLRV-Ge failed to infect walnuts in two experiments in which CLRV-W8, -Wsp, and -WRS did infect.

TABLE 2. Differential transmission and induction of blackline symptom by different walnut and nonwalnut strains of cherry leafroll virus (CLRV) in artificially inoculated English walnut/*Juglans hindsii* trees

	1985 experiment[b]; fraction of indicators[c]			1984 experiment[b]; fraction of indicators[c]		
	With blackline at	Infected with CLRV[d]		With blackline at	Infected with CLRV[d]	
Inocula[a]	the graft union	Scion	Rootstock	the graft untion	Scion	Rootstock
W8[e]	4/4	4/4	0/4	4/4	4/4	0/4
Wsp	4/5	4/5	0/4	2/4	2/4	0/4
WRS	0/5	0/5	0/5	2/5	2/5	0/5
Ch	0/5	0/5	0/5	0/4	0/4	0/4
Ge	···	···	···	0/4	0/4	0/4
Control	0/5	0/5	0/5	0/5	0/5	0/5

[a] Inocula were 100 μg/ml suspensions of purified preparations of strains of cherry leafroll virus in 0.05 M phosphate buffer, pH 6.5: glycerol mixture (50:50 V/V).

[b] Serr and Harley cultivars of English walnut on *J. hindsii* rootstock were used in 1984 and 1985 experiments, respectively.

[c] Number of indicators with blackline or infected with CLRV per number of plants inoculated.

[d] Infection determined by enzyme-linked immunosorbent assay (ELISA). ELISA absorbance values at least threefold higher than the absorbance values of comparable controls (CLRV-free tissues) on the same microtiter plates were considered as positive for infection (values for control at A_{450nm} ranged from 0.03–0.06).

[e] See Table 1 for information on virus strains. Control = trees inoculated with 0.05 M phosphate buffer, pH 6.5, and glycerol mixture (50:50 V/V).

LITERATURE CITED

1. Cooper, J. I. 1980. The prevalence of cherry leafroll virus in *Juglans regia* in the United Kingdom. Acta Phytopathol. Acad. Sci. Hung. 15:139-145.
2. Cooper, J. I., and Atkinson, M. A. 1975. Cherry leafroll virus causing a disease of *Betula* spp. in the United Kingdom. Forestry 48:193-203.
3. Cropley, R. 1961. Cherry leafroll virus. Ann. Appl. Biol. 49:524-529.
4. De Zoeten, F. A., Lauritis, J. A., and Mircetich, S. M. 1982. Cytopathology and properties of cherry leafroll virus associated with walnut blackline disease. Phytopathology 72:1261-1265.
5. Fulton, J. P., and Fulton, R. W. 1970. A comparison of some properties of elm mosaic and tomato ringspot viruses. Phytopathology 60:114-115.
6. Jones, A. T., and Murant, A. F. 1971. Serological relationship between cherry leafroll, elm mosaic and golden elderberry viruses. Ann. Appl. Biol. 69:11-15.
7. Mayhew, D. G., and Epstein, A. H. 1971. Elm mosaic virus in Iowa. (Abst.) Phytopathology 61:1024.
8. Mircetich, S. M., and Rowhani, A. 1984. The relationship of cherry leafroll virus and blackine disease of English walnut trees. Phytopathology 74:423-428.
9. Mircetich, S. M., Sanborn, R. R., and Ramos, D. E. 1980. Natural spread, graft transmission, and possible etiology of walnut blackline disease. Phytopathology 70:962-968.
10. Quacquareli, A., and Savino, V. 1977. Cherry leafroll virus in walnut. II. Distribution in Apulia and transmission through seed. Phytopathol. Mediterr. 16:154-156.
11. Rowhani, A., Mircetich, S. M., Shepherd, R. J., and Cucuzza, J. D. 1985. Serological detection of cherry leafroll virus in English walnut trees. Phytopathology 75:48-52.
12. Savino, V., and Gallitelli, D. 1981. Cherry leafroll virus in olive. Phytopathol. Mediterr. 20:202-203.
13. Van Regenmortel, M. H. V. 1967. Serological studies on naturally occurring strains and chemically induced mutants of tobacco mosaic virus. Virology 31:467-480.
14. Waterworth, H. E., and Lawson, R. H. 1973. Purification, electron microscopy, and serology of the dogwood ringspot strain of cherry leafroll virus. Phytopathology 63:141-146.