The Relationship of Cherry Leafroll Virus and Blackline Disease of English Walnut Trees

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ABSTRACT

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Two isolates of cherry leafroll virus (CLRV-W) recovered from walnut trees affected with walnut blackline disease (WBL) induced WBL in inoculated English walnut trees propagated either on rootstocks of *Juglans hindsii* or the natural hybrid Paradox (*J. hindsii* × *J. regia*). A virus isolate (CLRV-W7) from a single local lesion, propagated in cucumber, infected English walnut seedlings following transfer by mechanical inoculation. Thirteen of 17 English/*J. hindsii* and 10 of 10 English/Paradox walnut indicator plants (hereafter called indicators) graft-inoculated with bark patches from English walnut seedlings mechanically inoculated with CLRV-W7 became infected and developed the typical symptom of WBL (a blackline at the graft union) within 2 yr. Control trees similarly grafted with bark patches from healthy English walnut seedlings remained symptomless. Inoculum of a second single-lesion isolate (CLRV-W8) was purified from cucumber, suspended in a mixture of phosphate buffer and glycerin (10:1, v/v), and applied to the cambium under bark flaps in the scion portion of English/*J. hindsii* or English/Paradox walnut indicators. Within 2 yr, six of eight English/*J. hindsii* and five of five English/Paradox inoculated trees became infected and developed the blackline symptom. The blackline at the graft unions of indicators experimentally infected with CLRV-W8 was identical to the blackline symptom in walnut trees naturally infected with CLRV-W. Similar control applications of the phosphate buffer:glycerin mixture without the virus failed to produce the blackline symptom. Virus from the WBL-affected indicators was serologically identical to the original isolates used as the inoculum. Virus was not recovered from the scion of any indicator plant without the blackline at the graft union nor from the rootstock portion of any indicator either with or without a blackline at the graft union. WBL is caused by CLRV-W and the development of blackline at the graft union of English walnuts on rootstocks of *J. hindsii* and Paradox is apparently due to the hypersensitive reaction of the rootstocks to CLRV-W.

Additional key words: black walnut, Persian walnut, virus disease.

Walnut blackline disease (WBL) is a widespread and specific disease that has reached epidemic proportions in several English walnut-producing areas in California (10). English walnut (*Juglans regia*) orchard trees affected with WBL are subject to gradual girdling by a narrow, black, necrotic strip of cambium and phloem tissues (the blackline symptom) at the rootstock-scion union, resulting in decline and death of the English walnut scion (10). The disease occurs in >30 different English walnut cultivars propagated on seedlings of Northern California black walnut (*Juglans hindsii*) and natural hybrid Paradox (*J. hindsii* × *J. regia*), which are standard walnut rootstocks in commercial orchards in California. The disease also has been observed in English walnut trees propagated on seedlings of Chinese wingnut (*Pteriocarya stenoptera*) and several *Juglans* spp. besides *J. regia* (8). WBL has been reported in Oregon (9,16), France (4,6), England (7), and Hungary (13). In California orchards, WBL-affected English walnuts on rootstocks of *J. hindsii* show a narrow strip of necrotic tissue (blackline) at the graft union (Fig. 1A), whereas WBL-affected English walnuts on Paradox rootstock often develop an extensive necrosis of bark in the Paradox rootstock that is clearly delineated by the scion at the graft union (Fig. 1C). Early investigators of the disease attributed the symptoms to various noninfectious causes, of which a spontaneous scion-rootstock incompatibility was most often suggested to be the cause (3,4,6,7,16–18). Recent reports showed that a strain of cherry leafroll virus (CLRV-W) is consistently associated with WBL-affected trees (4,5,10,13). Furthermore, it was demonstrated that the causal agent of WBL spreads naturally in commercial orchards; that CLRV-W is graft-transmissible from diseased to healthy walnut trees; and that the virus is present only in the English scion portion of naturally and experimentally WBL-affected trees on rootstocks of *J. hindsii* or Paradox (10). A probable causal relationship between CLRV-W and WBL was suggested, and it was also suggested that the development of necrosis in cambium and phloem tissues at the graft union of the English scion and rootstocks of either *J. hindsii* or Paradox is due to the hypersensitive reaction of the rootstocks to CLRV-W (10). Recently, however, it has been suggested (4) that WBL may be a noninfectious genetic disorder or that an infectious agent other than CLRV-W may be the cause of the disease.

The purpose of the experiments reported here was to verify the etiology of WBL by inoculating healthy walnut trees with single-lesion isolates of CLRV-W, checking for development of typical symptoms of WBL in experimentally inoculated walnut trees, and then reisolating CLRV-W from experimentally inoculated and WBL-affected walnut trees. A portion of this work has been reported (11).

MATERIALS AND METHODS

Indicators for WBL and the cherry leafroll virus isolates. Two-year-old healthy English walnut (*Juglans regia* L. 'Ashley,' 'Chico,' 'Trinta,' 'Sunland,' 'Franquette,' and 'Payne') on rootstocks of Northern California black walnut [(*J. hindsii* Jeps.) Jeps.] and hybrid Paradox (*J. hindsii* × *J. regia*), propagated and grown in the field as described previously (10), served as indicators. Of the 11 single-lesion isolates from WBL-affected orchard walnut trees (10), two designated as CLRV-W7 and CLRV-W8 were used in this study and served as the inocula to experimentally inoculate and induce WBL in the walnut tree indicators. CLRV-W7 was isolated from leaves of Payne and CLRV-W8 was isolated from cambium and inner bark tissues of Eureka walnut orchard trees affected by WBL as described previously (10). CLRV-W7 was used to mechanically inoculate English walnut seedlings, and then bark patches from the experimentally infected seedlings were grafted to

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healthy walnut tree indicators, whereas a purified preparation of CLRV-W8 was used to directly inoculate the walnut tree indicators.

Mechanical inoculation of seedlings of English walnut. In one experiment, CLRV-W7 isolated from a single local lesion in cucumber (*Cucumis sativus* L. 'National Pickling') was propagated in cucumber plants. Systemically infected cucumber leaves were triturated in a freshly prepared, chilled mixture of 5% aqueous solution of nicotine and 1 M phosphate buffer (pH 7.2) (1.5:1.0, v/v) at a ratio of one part cucumber leaves to five parts (v/v) phosphate buffer:nicotine mixture. The homogenate was rubbed on 22-μm (600-mesh) Carborundum-dusted, fully expanded leaves of fifteen 6-mo-old, virus-free, open-pollinated, cultivar Eureka English walnut seedlings grown in 3.7-L containers in the greenhouse as described previously (10). The controls were similarly inoculated with a homogenate of healthy cucumber leaves. The experimentally inoculated Eureka walnut seedlings were grown for 18 mo in the greenhouse and lathhouse before we used bark patches from them to graft-inoculate the WBL indicator trees.

Transmission of CLRV-W from a mechanically inoculated English walnut seedling to English walnut trees and the induction of WBL. In this portion of the experiment, English walnut cultivars Ashley, Chico, and Trinta propagated on rootstocks of *J. hindsii* or Paradox served as indicators. In May 1980, eight to ten 2-yr-old English walnut indicator trees of each cultivar on both rootstocks were graft-inoculated with bark patches ($\cong 1 \times 2.5$ cm) from the mechanically inoculated Eureka English walnut seedlings as described previously (10).

Three bark patches were grafted to the scion portion of each indicator ~5–20 cm above the graft union of the scion and rootstock. Controls consisted of indicators that were similarly graft-inoculated with bark patches from healthy, virus-free Eureka English walnut seedlings; indicators that were graft-inoculated

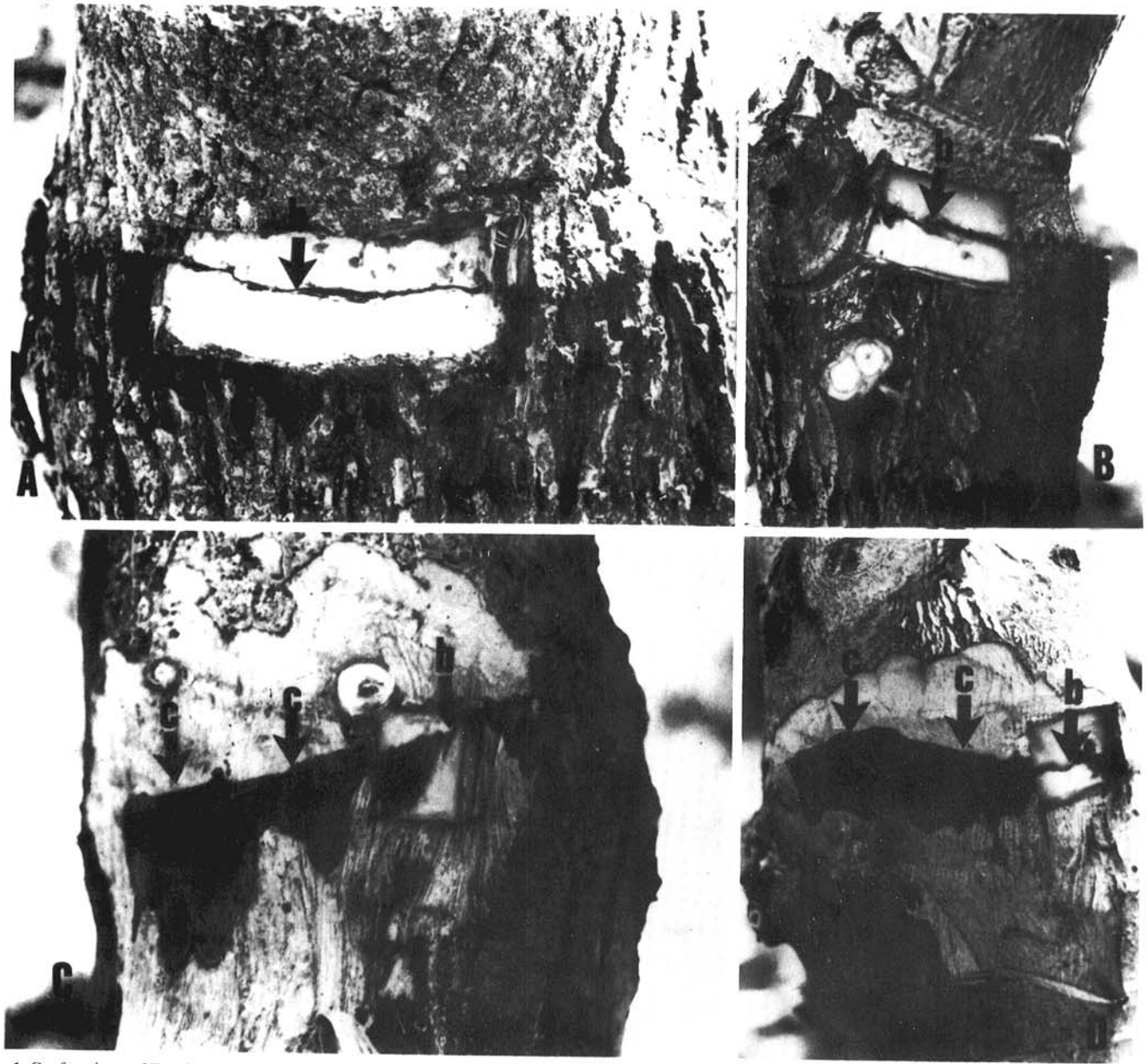

Fig. 1. Graft unions of English walnut trees infected with CLRV-W showing symptoms of the walnut blackline disease. **A and B,** English walnuts propagated on *Juglans hindsii* rootstock. **A,** Naturally CLRV-W-infected orchard tree. **B,** Tree artificially infected with purified CLRV-W. Bark was removed to show the narrow strip of necrotic cambium and phloem tissues—the blackline symptom (b arrows)—at the junction of the scions and rootstocks. **C and D,** English walnuts propagated on Paradox rootstock. **C,** Naturally CLRV-W infected tree. **D,** Tree artificially infected with purified CLRV-W. Outer bark was shaved off to show bark canker in Paradox rootstock (c arrows) developing from the scions downward; note also the blackline (b arrows) at the junction of the scion and rootstock.

with bark patches from walnut orchard trees naturally infected with CLRV-W and affected by WBL; and indicators that received no inoculum. All indicators were observed over a 2-yr period for the development of cambial-phloem necrosis, or blackline, at the graft union and were assayed periodically by enzyme-linked immunosorbent assay (ELISA) for the presence of CLRV-W.

Inoculation of English walnut/*J. hindsii* or Paradox indicators with a purified preparation of CLRV-W. In a second experiment, CLRV-W8 was partially purified from systemically infected cucumber seedlings by procedures described for the purification of potato leafroll virus (15) except that: 0.5 M phosphate buffer (pH 6.5) was used in all steps; a mixture of *n*-butanol and chloroform (1:1, v/v) was added at the rate of 1.5 ml per 10 ml of extract; and the centrifugation pellets were resuspended in 0.05 M phosphate buffer, pH 6.5, without urea. The inoculum was a partially purified virus preparation collected from two well-defined bands in sucrose density gradients that consisted largely of isometric virus particles ~25–26 nm in diameter. The purified virus was suspended in a mixture of 0.05 M potassium-phosphate buffer, pH 7.2, and glycerin (10:1, v/v), at a ratio of 100 μg of virus per milliliter of buffer:glycerin mixture. The virus suspension was tested for infectivity on *Nicotiana megalosiphon* Heurch and Mueller and then used for direct inoculation of walnut tree indicators.

In May 1980, four to five indicators of Sunland/Paradox and Franquette or Payne/*J. hindsii* were inoculated with a purified preparation of CLRV-W8 as follows: the suspension of CLRV-W8 particles in phosphate buffer plus glycerin mixture was applied with a glass rod directly to the cambial tissue under a bark flap (1.5 × 3.0 cm) cut in the English walnut scion portion of the indicators. After the virus suspension was smeared on the cambium, the bark flap was returned into the original cut and the inoculation site was firmly wrapped with an adhesive grafting tape. Each indicator tree was inoculated at three different points in the scion ~5–20 cm above the graft union. Controls consisted of comparable indicator trees similarly inoculated with phosphate buffer plus glycerin mixture without the virus; indicators that were graft-inoculated with bark patches from naturally WBL-affected and CLRV-W-infected walnut orchard trees (10); and indicators that received no inoculum. The walnut indicator trees were examined periodically during a 2-yr period for the development of the blackline symptom at the scion-rootstock graft union and assayed for CLRV-W by ELISA. In addition, all indicators were indexed for the presence of CLRV-W. The virus isolates recovered from the walnut indicator trees were compared serologically in agar gel double-diffusion tests (14), both with each other and with the original CLRV-W8 isolate that had been used as inoculum.

Tests for the ability of CLRV-W to systemically infect rootstocks of English walnut. In our surveys of commercial walnut orchards affected by WBL, we observed that English walnut trees with multiple grafts (scions) on rootstocks of hybrid Paradox or *J. hindsii* often developed blackline at the graft union of a single scion and rootstock. The graft unions of the other English scions on the same trees, separated from the WBL-affected scion by rootstock tissue-interstock, remained unaffected by blackline for several years even though the graft unions of different scions were separated from each other by only a few centimeters of interstock tissue. It appeared from this observation that the WBL causal agent might be incapable of invading the rootstocks of Paradox or *J. hindsii* beyond the blackline at the graft union. Furthermore, the subsequent long-term spread of the WBL into the other scions on the same trees might be by means other than through the rootstocks. We conducted the following experiment to determine whether CLRV-W is capable of invading and spreading through the rootstock. One-year-old Paradox seedlings growing in the field (10) were patch-budded with buds from a healthy Trinta English walnut tree free of CLRV-W. Each rootstock seedling received two buds; the upper bud was placed 10 cm directly above the lower bud. Both buds on each rootstock seedling were allowed to grow so that each indicator tree had two scions. After 1 yr, the upper and lower scions were separated from each other by ~5 cm of Paradox interstock, and one of the two scions was graft-inoculated with three bark patches from the Eureka English walnut seedlings that had been mechanically inoculated with a single-lesion isolate of CLRV-W7 as described earlier. The bark patches were placed in the Trinta scion ~5–10 cm above the graft union. Five of the indicators received the inoculum in the upper Trinta scion and five of the indicators were inoculated in the lower scion. Controls consisted of comparable Trinta/Paradox trees inoculated similarly with bark patches from Eureka English walnut seedlings free of CLRV-W. The graft unions of both scions were observed for the development of the blackline symptom for 30 mo after inoculation. The two Trinta scions, the Paradox interstock between the Trinta scions, and the Paradox rootstocks were also periodically tested by ELISA for the presence of CLRV-W.

Reisolation and confirmation of infection of walnut tree indicators by CLRV-W. Reisolations were done by mechanical transmission of CLRV-W from both scions and rootstocks of indicator trees and from Eureka walnut seedlings. Inner bark and cambium tissues from English walnut cultivars and leaves and inner bark and cambium tissues from Eureka seedling indicators were triturated in a phosphate buffer plus nicotine mixture as described (10) and then rubbed on Carborundum-dusted leaves of *N. megalosiphon* growing in the greenhouse. A successful reisolation of CLRV-W from the walnut indicators was based on the development of virus-induced symptoms in mechanically inoculated *N. megalosiphon*. Both CLRV-W7 and CLRV-W8 isolates induced in *N. megalosiphon* identical symptoms that consisted of necrotic lesions on inoculated leaves followed by the development of systemic mottling and chlorotic spots, rings, and line patterns in uninoculated leaves. All virus isolates reisolated on *N. megalosiphon* were further propagated in cucumber plants and then compared serologically with each other and with the original CLRV-W7 and CLRV-W8 isolates in agar gel double-diffusion tests (14) using sap expressed from cucumber plants as the source of antigens. The antiserum used in these tests was produced by immunizing New Zealand rabbits with a purified preparation of CLRV-W8. The antiserum had a specific CLRV-W titer of 1:2,045 and a nonspecific titer of 1:4. The CLRV-W isolates reisolated from walnut indicator trees were considered identical to each other when the precipitin lines of antigen reactants were confluent in agar gel double-diffusion tests employing CLRV-W8 antiserum.

In addition to bioassay, we also used ELISA (1) to ascertain the presence or absence of CLRV-W in the walnut indicator trees. Antigen sources for ELISA were: leaf tissue from Eureka seedlings and inner bark and cambium tissues from Eureka seedlings and rootstock and scion portions of walnut cultivar indicators. One gram of test sample was triturated in 10 ml of phosphate-buffered saline (PBS) amended with 0.05% Tween 20, 2.0% polyvinyl pyrrolidone, and 0.2% ovalbumin (1). All ELISAs were conducted according to previously described procedure (1) in flat-bottom microtiter plates (M129A; Dynatech Laboratories, Alexandria, VA 23314). ELISA absorbance values were measured at $A_{405\ nm}$ with a Titertek Multiskan colorimeter (Flow Laboratories, Inglewood, CA 90302) and recorded within 30–40 min after the addition of the substrate. A series of tests were conducted to assess the relative efficiency and reliability of the ELISA procedure before employing the test in these studies. The results of these tests revealed that ELISA absorbance values were 20- and 3-fold greater for a purified preparation of virus at the concentration of 2,500 and 15 μg CLRV-W per milliliter of PBS-Tween, respectively, than the absorbance values (0.02) for PBS-Tween alone. ELISA absorbance values of healthy cucumber and walnut tissues (plant tissue plus PBS-Tween, 1:10, w/v) ranged from 0.02 to 0.06, depending on the experiment. On the other hand, ELISA absorbance values of CLRV-W-infected cucumber or walnut tissues in the same experiments ranged from 0.70 to 1.50. Tissues from indicators infected with CLRV-W were assayed at dilutions up to 1:160 (w/v, tissue:PBS-Tween) and still had ELISA absorbance values at least threefold greater than the absorbance values of tissues of virus-free indicators at the same dilution. Thus, we felt confident in the use of ELISA for confirming CLRV-W infection in walnut indicators throughout this investigation. Indicators were considered to be infected with CLRV-W when the ELISA absorbance values of the samples were at least three times higher than the absorbance values

of samples from uninfected walnut indicators tested at the same time and in the same microtiter plate.

RESULTS

Return of CLRV-W to English walnut seedlings. Eleven of 15 Eureka English walnut seedlings became infected with CLRV-W when mechanically inoculated with a homogenate of cucumber leaves that were infected with a single-lesion isolate of CLRV-W. The infection of the seedlings of Eureka English walnut was confirmed by ELISA and bioassay. The ELISA absorbance values for leaf and cambium samples from individually tested CLRV-W-infected seedlings ranged from 0.80 to 1.20, whereas the absorbance values for noninfected seedlings ranged from 0.04 to 0.06. Five of the 11 CLRV-W7 infected Eureka seedlings developed leaf symptoms consisting of mottling, chlorotic spots or rings, and necrotic spots. These seedlings also showed pronounced stunting or dieback of terminal shoots compared to the uninoculated control Eureka walnut seedlings. Six of 11 CLRV-W7-infected Eureka walnut seedlings remained symptomless and there was no

TABLE 1. Efficiency of bark patch inoculum taken from Eureka English seedlings mechanically inoculated with a single-lesion isolate of cherry leafroll virus (CLRV-W7) in inducing blackline in graft-inoculated English Walnut cultivars on Paradox or *J. hindsii* rootstock

Source of inoculum	Indicator (cultivar scion/rootstock)	Fraction[a] of indicators: With blackline at the graft union	Infected with CLRV-W7[b]: Scion	Infected with CLRV-W7[b]: Rootstock
CLRV-W infected[c]:				
Eureka English walnut seedlings mechanically inoculated with a single lesion isolate of CLRV-W7	Trinta/Paradox	10/10	10/10	0/10
	Ashley/*J. hindsii*	8/10	8/10	0/10
	Chico/*J. hindsii*	5/7	5/7	0/7
Eureka/*J. hindsii* orchard tree naturally blackline affected and CRLV-W infected	Ashley/*J. hindsii*	5/5	5/5	0/5
	Chico/*J. hindsii*	3/5	3/5	0/5
CLRV-W free[d]:				
Eureka English walnut seedlings	Trinta/Paradox	0/5	0/5	0/5
	Ashley/*J. hindsii*	0/5	0/5	0/5
	Chico/*J. hindsii*	0/5	0/5	0/5
Eureka/*J. hindsii* healthy orchard tree	Trinta/Paradox	0/5	0/5	0/5
	Ashley/*J. hindsii*	0/4	0/4	0/4
	Chico/*J. hindsii*	0/4	0/4	0/4
Uninoculated controls	Trinta/Paradox	0/5	0/5	0/5
	Ashley/*J. hindsii*	0/12	0/12	0/12
	Chico/*J. hindsii*	0/9	0/9	0/9

[a] Number of indicators with blackline or infected with CLRV-W per number of plants inoculated.
[b] Infections determined by assay with enzyme-linked immunosorbent assay (ELISA). The ELISA absorbance values of cambium and inner bark tissues from individual indicators designated as CLRV-W7-infected ranged 0.70–1.50; the absorbance values for indicators designated CLRV-W7-free ranged 0.02–0.06.
[c] Experimentally or naturally CLRV-W infected trees. Three bark patches ~2×3 cm from the scion of the donor tree were grafted to the scion portion of each indicator.
[d] Naturally CLRV-W-free, healthy trees. Three bark patches from the scion of the donor tree were grafted to the scion portion of each indicator.

TABLE 2. Inoculation of 2-yr-old healthy English walnut trees with partially purified cherry leafroll virus (CLRV-W) and with bark patches from WBL-affected orchard trees

Source of inoculum	Indicator (cultivar scion/rootstock)	Fraction[a] of indicators: With blackline at the graft union	Infected with CLRV-W[b]
CLRV-W partially purified preparation suspended in PO_4 buffer:glycerin mixture (10:1, v/v)	Sunland/Paradox	5/5	5/5
	Franquette/*J. hindsii*	2/4	2/4
	Payne/*J. hindsii*	4/4	4/4
Controls, PO_4 buffer:glycerin mixture (10:1, v/v)	Sunland/Paradox	0/3	0/3
	Franquette/*J. hindsii*	0/3	0/3
	Payne/*J. hindsii*	0/3	0/3
Bark patches from a blackline-affected Payne/*J. hindsii* orchard tree[c]	Sunland/Paradox	2/2	2/2
	Franquette/*J. hindsii*	2/2	2/2
	Payne/*J. hindsii*	3/3	3/3
Controls, uninoculated	Sunland/Paradox	0/5	0/5
	Franquette/*J. hindsii*	0/5	0/5
	Payne/*J. hindsii*	0/5	0/5

[a] Number of indicators with blackline or CLRV-W per number of indicators inoculated.
[b] Determined by ELISA and bioasssay. ELISA absorbance values of cambium and inner bark tissues from individual indicators designated as CLRV-W ranged 0.60–1.20); the absorbance values for cambium and inner bark tissues for indicators designated noninfected ranged 0.04–0.06. CLRV-W was mechanically transmitted to *N. megalosiphon* only from scions of indicators that were ELISA-positive for CLRV-W; no virus was transmitted to *N. megalosiphon* from any of the controls or from the rootstock portion of any indicator.
[c] Naturally, CLRV-W-infected tree. Three bark patches ~ 2 × 3 cm from scion of the donor tree were grafted to the scion portion of each indicator.

difference in the vigor or growth between the six symptomless CLRV-W7-infected and noninfected, control Eureka walnut seedlings. The viruses reisolated from infected seedlings of cultivar Eureka with and without leaf symptoms were serologically identical to each other and to the CLRV-W7 isolate used as the inoculum in this experiment as determined by agar gel double-diffusion tests. Apparently, we successfully returned CLRV-W to English walnut seedlings. All of the Eureka English walnut seedlings in this experiment were from the nuts of a single walnut tree; however, some seedlings were affected more severely than the others by the same isolate of CLRV-W. Apparently, even though we detected no relationship between the ELISA absorbance values and severity of symptoms in these seedlings, differential susceptibility to CLRV-W exists among seedlings of Eureka English walnut within the same population.

Return of CLRV-W from mechanically inoculated English walnut seedlings to English walnut cultivars and induction of blackline symptoms. Twenty-one of 27 Trinta, Ashley, and Chico/*J. hindsii* or Paradox indicators developed blackline at the graft union following graft-inoculation of their scions with bark patches from Eureka seedlings that were mechanically inoculated with a single-lesion CLRV-W7 isolate (Table 1). The first incipient development of blackline (≅2 cm long) at the graft union was observed directly below the inoculum within 3 mo after inoculation. Subsequently, the blackline spread around the entire circumference of the graft union and caused total girdling within 2 yr. The indicators inoculated with bark patches from either the experimentally CLRV-W7-infected Eureka seedlings or from naturally CLRV-W-infected and WBL-affected trees of Eureka/*J. hindsii* (Table 1) had identical blackline symptoms. Likewise, the blackline symptom at the graft union of indicators artificially inoculated with bark patches from mechanically inoculated Eureka seedlings were identical to the blackline symptom of naturally WBL-affected orchard trees. Controls graft-inoculated with CLRV-free materials remained symptomless during the entire 2-yr experimental period (Table 1). The English walnut scions of all inoculated indicators that developed blackline at the graft union were infected with CLRV-W (Table 1). The virus isolates recovered from the indicators with blackline at the graft union were serologically identical to each other and to the CLRV-W7 isolate that served as the original inoculum in these tests. No virus was recovered from any of the symptomless indicators or from the rootstock portion of any indicator tree.

Return of purified CLRV-W directly to English walnut/*J. hindsii* or Paradox indicators and induction of WBL. The results from the experiments concerned with the direct inoculation of walnut tree indicators with a partially purified preparation of CLRV-W to reproduce WBL are summarized in Table 2. Within 2 yr, 11 of 13 Sunland/Paradox and Franquette or Payne/*J. hindsii* indicators developed blackline at the graft union when they were inoculated in the scion portion with a partially purified preparation of a single-lesion CLRV-W8 isolate. Initially, a blackline, ~3 cm long appeared at the graft union directly below the inoculation sites on the indicator scions and then advanced around the graft union at the rate of ~7–12 cm per year depending on the cultivar/rootstock combination. Franquette and Payne/*J. hindsii* indicators inoculated with purified CLRV-W8 developed a narrow strip of darkened, dead cambium and phloem tissue at the graft union (ie, blackline) that also is a characteristic symptom of naturally WBL-affected English walnut trees propagated on rootstock of *J. hindsii* (Fig. 1A and B). In Sunland/Paradox indicators that received the same inoculum, the appearance of blackline at the graft union resulted in the additional development of a bark canker in the Paradox rootstock just as in naturally WBL-affected English walnut trees on Paradox rootstock (Fig. 1C and D). Controls remained symptomless during the entire 2-yr experimental period (Table 2). The English walnut cultivar scions of all indicators that developed blackline at the graft union were also infected with CLRV-W. The virus isolates recovered from the indicators with blackline at the graft union were serologically identical to each other and to the CLRV-W8 isolate used as the original inoculum when compared in agar gel double-diffusion tests using antiserum produced against CLRV-W8. No virus was recovered from the scions of any of the symptomless indicator trees. Likewise, no virus was recovered from the rootstock portion of any indicator tree regardless of the presence or absence of a blackline symptom at the graft union. These results showed that CLRV-W is the causal agent of WBL disease of English walnut trees.

Inability of CLRV-W to systemically infect Paradox walnut rootstock. Ten of 10 double-scion, Trinta/Paradox interstock/Trinta/Paradox rootstock, indicators graft-inoculated in one of the two scions with bark patches from Eureka seedlings mechanically inoculated with CLRV-W7 developed blackline only at the graft union between the inoculated Trinta scion and the Paradox interstock or rootstock depending on the position of the inoculated scion on the indicator. For example, if the upper Trinta scion was inoculated, then blackline developed at the graft union of this scion and the Paradox interstock, while the union of the lower, uninoculated Trinta scion and the Paradox interstock and rootstock, separated from the affected union by ~5 cm, remained symptomless during the 2-yr experimental period. Likewise, when the lower Trinta scion was inoculated, blackline developed at its union with the Paradox rootstock interstock only while the graft union of the upper, uninoculated Trinta scion and Paradox interstock remained symptomless (Fig. 2). ELISA tests detected the presence of CLRV-W in all inoculated Trinta scions. ELISA absorbance values for samples from inoculated scions exceeded 0.7, whereas control sample absorbance values were less than 0.06.

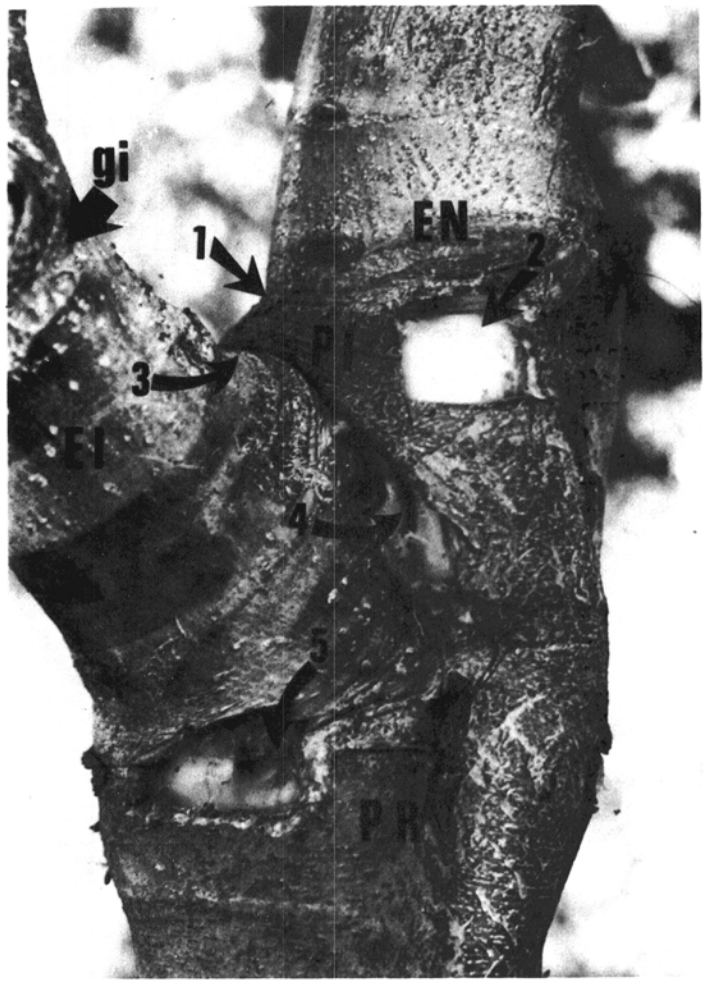

Fig. 2. Graft unions of English scion (EN)/Paradox interstock (PI)/English scion (EI)/Paradox rootstock (PR) walnut indicator tree that received CLRV-W infected graft-inoculum (gi arrow) at the lower English scion (EI). Note the presence of blackline at the graft union of the inoculated scion (EI) and the Paradox interstock (PI) and Paradox rootstock (PR) (3, 4, and 5 arrows), but the absence of blackline at the graft union of the uninoculated scion (EN) and the Paradox interstock (PI) (1 and 2 arrows).

ELISA tests also revealed that CLRV-W was spreading from the point of inoculation in the Trinta scions at the average rate of 40 cm per year. No virus was detected in any of the uninoculated Trinta scions nor in the Paradox interstock or rootstock portion of the Trinta/Paradox interstock/Trinta/Paradox rootstock indicators since ELISA absorbance values for any of these samples were less than 0.04. The controls remained symptomless and virus-free throughout the experimental period. Apparently, Paradox is hypersensitive to CLRV-W, and the causal agent of WBL cannot advance into and systemically infect the rootstock portion beyond the necrotic cambial and phloem tissues or blackline at the graft union of WBL-affected English walnut/Paradox rootstock trees.

DISCUSSION

In these studies, we completed Koch's postulates and unequivocally established the causal relationship of CLRV-W and WBL of English walnuts on *J. hindsii* and Paradox rootstocks. Furthermore, our research showed that CLRV-W systemically infects English walnut (*J. regia*) but it does not infect *J. hindsii* and Paradox walnut rootstocks. Apparently the necrosis of cambium and phloem tissues or blackline at the graft union of an English scion on either rootstock is the result of a hypersensitive reaction of the *J. hindsii* and Paradox (*J. hindsii* × *P. regia*) rootstocks to CLRV-W.

These studies also revealed marked differences in tolerance or resistance among open-pollinated Eureka English walnut seedlings originating from the same tree when mechanically inoculated with a single-lesion CLRV-W7 isolate. Recently, we detected a hypersensitive reaction in open-pollinated seedlings of *J. regia* to CLRV-W. Two of 64 English cultivars/Eureka English walnut seedling trees developed blackline at the graft union similar to that observed in *J. hindsii* and Paradox following graft-inoculation of the English cultivar's scion (S. M. Mircetich, *unpublished*). These observations and results suggest that the selection and breeding of English walnuts for resistance to CLRV-W is feasible.

The natural spread of the causal agent of WBL in California's commercial walnut orchards has been reported (10). However, these studies showed that the causal agent of WBL, CLRV-W, does not systemically infect *J. hindsii* and Paradox, the standard and almost exclusive walnut rootstocks in California; therefore, nematodes, which have been reported to be vectors of certain strains of CLRV (2), cannot play a role in the spread of CLRV-W in California's commercial walnut orchards. A recent report (12) showed that CLRV-W is transmitted through seed from CLRV-W-infected English walnut trees to seedlings and is also transmitted by pollen from naturally CLRV-W infected to healthy orchard English walnut trees. The studies reported here further support the contention (10,12) that pollen is implicated in the natural spread of WBL in California's commercial walnut orchards.

English walnut trees are monoecious with unisexual flowers. English walnut cultivars are self-fertile, but various degrees of self-unfruitfulness exist in different cultivars due to dichogamy. Since a reduction in yield may occur due to dichogamy and insufficient self-pollination in certain English walnut cultivars, ~8,094 hectares (20,000 acres) of California's commercial orchards are artificially pollinated each year by dispersing walnut pollen from aircraft. Because the causal agent of WBL, CLRV-W, is pollenborne and can be transmitted by infected pollen to healthy trees, control measures should include the careful selection of walnut pollen sources to avoid trees infected with CLRV-W. Rogueing of trees infected with CLRV-W immediately after they occur in orchards should be practiced. Since CLRV-W is present only in the scion of English walnut orchard trees on rootstocks of *J. hindsii* or Paradox, the cutting of infected trees below the graft union and regrafting of the rootstock portion with CLRV-W-free graftwood may be practiced. Because the WBL-causal agent is seed- and graft-transmitted, control measures should also include the use of propagation materials from healthy and CLRV-W-free trees. Research on the relative efficiencies of enzyme-linked and radio-immunosorbent assays (ELISA and RISA) for the rapid detection of CLRV-W in orchard and nursery walnut trees and walnut pollen is in progress.

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