Natural Spread, Graft-transmission, and Possible Etiology of Walnut Blackline Disease

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ABSTRACT

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Walnut blackline (WBL), a disease characterized by necrosis at the rootstock-scion junction, is widely distributed and commonly associated with declining English walnuts (*Juglans regia)* propagated on Northern California Black walnut *(J. hindsii)* or Paradox *(J. hindsii* × *J. regia)* seedling rootstocks in California. Natural spread of WBL from infected to healthy trees was demonstrated by the results of annual surveys of commercial orchards. Typically, the disease spreads from infected to adjacent healthy orchard trees. A virus, identified as a walnut isolate of cherry leafroll virus (CLRV-W) on the basis of its serological reaction with antisera to several strains of CLRV, was consistently isolated from English walnut scions but never from *J. hindsii* and Paradox rootstocks of naturally infected orchard trees. The causal agent of WBL was readily graft-transmitted by bark patches from English walnut scions of naturally infected trees to healthy English walnuts on *J. hindsii* or Paradox rootstocks only when the inoculum was applied to the English walnut scion; indicator trees developed characteristic blackline at the union within 1 yr. No transmission of CLRV-W or blackline occurred when bark patches from *J. hindsii* or Paradox rootstock of WBL-affected trees were applied to English walnut scion or *J. hindsii* or Paradox rootstock of the indicator trees. The WBL agent induced chlorotic spots, rings, and line patterns in leaves of graft-inoculated open-pollinated seedlings of English walnut cultivar Ashley, but English walnut cultivar Trinta remained symtomless. Leaf symptoms similar to those in graft-inoculated Ashley seedlings occurred in Eureka English walnut seedlings that had been mechanically inoculated with CLRV-W, but no infection occurred in *J. hindsii* and Paradox walnut rootstock seedlings. Apparently, CLRV-W is present only in the English walnut scions of naturally WBL-affected trees. The development of blackline at the union of English walnut on *J. hindsii* or Paradox rootstocks appears to be due to the hypersensitive reaction of the rootstocks to the WBL agent.

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

In the last 10 yr an increasing incidence of declining English walnut trees has been observed in numerous commercial orchards in some of the most important walnut-producing areas in California. Extensive surveys of California walnut-growing districts during the last 4 yr revealed a widespread and high incidence of the walnut blackline (WBL) disorder associated with the decline of English walnut trees propagated on *Juglans hindsii* or Paradox seedling rootstocks. This disorder also has been observed in Oregon (12,20), France (7), and England (8).

WBL was observed for the first time in Oregon in 1924, and was attributed to noninfectious cause(s) (20). In California, the same disease was noted in a few walnut trees near San Francisco in 1929 (6,22). It has commonlv been assumed that WBL is confined to commercial orchards of the central coastal counties in the vicinity of San Francisco Bay and restricted to older, mature trees more than 20 yr old (6,12,20–22). However, our recent survey of commerical walnut orchards revealed a high prevalence of WBL in several of California's most important walnut-growing regions in the central San Joaquin and Sacramento valleys. Because of its wide geographical distribution, destructiveness, its high incidence in the orchards, and because a number of walnut cultivars on the standard *J. hindsii* and Paradox rootstocks are affected, WBL is now considered the most important factor limiting walnut production in some regions and it is a serious threat to the walnut industry in California.

Since the disease was noted in Oregon in 1924, investigators suggested a wide variety of noninfectious causes of WBL (6–8,12,20,21). Because blackline was not observed in English walnuts propagated on English walnut seedlings rootstock (11,21,22), and because no specific virus-like symptoms have been observed in the leaves of WBL-affected English walnut cultivars, a spontaneous scion-rootstock incompatibility was most often suggested as the cause of WBL (6–8,12,20). This led to the conclusion that WBL is not caused by a graft-transmissible virus (21). However, there was no convincing experimental etiological evidence in the literature to support a noninfectious cause of WBL or to exclude an infectious agent causing this disease. Therefore, we investigated the etiology, natural spread, and graft transmission of WBL. Moreover, we investigated the presence and involvement of viruses in the disease. A portion of this work has been reported in abstract form (14).

MATERIALS AND METHODS

Orchard surveys for geographical distribution, incidence, and natural spread. Numerous California commercial orchards with declining walnut trees were surveyed for the presence and incidence of WBL. To determine possible relation between particular walnut rootstocks and cultivars and occurrence of diseased trees, we surveyed orchards having trees of the same age and the same rootstocks, but located in different walnut-growing areas known to differ in the incidence of WBL. To assess the rate and pattern of WBL spread, we surveyed two commercial orchards annually for 4 yr. Positive diagnosis of the disease in walnut trees was based on the presence of the narrow dark strip of cambial and phloem tissue; ie, the blackline symptom at the union of the English scion and the rootstock.

Isolation of viruses from walnut trees. All mechanical virus transmission attempts from scion and rootstock of walnut trees were made by triturating freshly harvested inner bark and cambium tissues or leaf tissue, approximately one part of plant issues in five parts (v/v) of cold (4 C) freshly prepared phosphate:nicotine buffer mixture consisting of 1.5 volumes of 5% aqueous solution of nicotine and 1.0 volume of 0.1 M phosphate buffer, pH 7.2 (16). The homogenates were rubbed onto Carborundum-dusted (22-μm [600-mesh]) cotyledons or leaves of cucumber (*Cucumis sativus* L. 'National Pickling'), cowpea (*Vigna unguiculata* [L.] Walp. 'Ramshorn'), bean (*Phaseolus vulgaris* L. 'Bountiful'), and

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tobacco (*Nicotiana tabacum* L. 'Havana 425') plants. The inoculated herbaceous plants were placed in the greenhouse and observed for symptom development. Virus isolates recovered from walnut trees were compared serologically with each other and with known strains of tomato ring spot virus (TomRSV) (13), tobacco ring spot virus (TobRSV), and cherry leafroll virus (CLRV) in agar-gel double-diffusion tests (15) and by the enzyme-linked immunosorbent assay (ELISA) as described by Clark and Adams (2). Antigen sources were expressed sap from *Chenopodium quinoa* Willd. plants. The symptomatology and herbaceous host range of virus isolates recovered from WBL-affected trees were studied in greenhouse experiments. The herbaceous plant species were mechanically inoculated in the usual manner with heavily infected tobacco or cucumber leaves homogenized in 0.03 M phosphate buffer, pH 7.2. The infection of herbaceous hosts was based on the presence of symptoms and on back-inoculation of homogenates to cucumber, cowpea, bean, and tobacco plants.

Field and greenhouse graft-transmission experiments. Orchard trees with an advanced stage of blackline at the union, and from which virus was recovered, served as inoculum sources. The inocula were bark patches from both English walnut *(Juglans regia* L.) scion and from Northern California black walnut (*J. hindsii* Jeps.) or Paradox (*J. hindsii* × *J. regia*) rootstock. The indicator trees were English walnut scions propagated on *J. hindsii* and Paradox seedling rootstocks or *J. hindsii* scions propagated on seedling rootstocks of English walnut cultivars Eureka and Ashley.

In several attempts to determine whether virus was present in both the rootstocks and the English scions of WBL-affected trees and to determine a possible causal relation between a virus and WBL disease in orchard trees, we used four combinations of inocula and grafted walnut indicator trees. Basically, the graft-transmission experiments consisted of: English walnut scion inoculum applied to the English walnut portions or to the *J. hindsii* or Paradox rootstock portions of the indicator trees; and *J. hindsii* or Paradox rootstock inoculum applied to the English walnut portions or to the *J. hindsii* or Paradox rootstock portions of the indicator trees. The inocula from both scion and rootstock of the same donor walnut tree were used to inoculate indicator trees in all four combinations (Table 1). Controls were comparable indicator trees inoculated in the same manner with inocula from symptomless orchard walnut trees and those that received no inoculum.

On orchard trees the graft procedure was as follows: a gasket cutter (diameter 3.8 cm) was used to cut circular bark patches in indicator trees about 20–30 cm distant from the union of scion and rootstock. The bark patches were removed from the scion and rootstock. Similarly, bark patches were cut and removed from scion or rootstock of diseased walnut trees and placed in the circular cuts in the trunks of indicator trees. The inocula were fastened in place with four small nails and sealed with grafting wax. Two bark patches from either rootstock or scion inoculum were applied to the scion or to the rootstock of each indicator tree.

In two different experiments we inoculated a total of 156 9-yr-old healthy English walnut trees located in a walnut cultivar orchard at the University of California, Davis. Twenty trees were inoculated in May 1976 and 136 trees were inoculated in June 1977. These experiments included 35 English walnut cultivars, each represented by four or more trees of which half of the scions were propagated on *J. hindsii* and the other half were propagated on Paradox rootstock. The final results of these experiments were collected in November 1979.

Because of the contentions of others (6,21,22) the WBL affects

TABLE 1. Efficiency of bark patches from English walnut scion and two different rootstocks of naturally infected orchard trees in transmitting the causal agent of walnut blackline disease to 2-yr-old healthy walnut trees

Source of inoculum		Indicator cultivars (scion/rootstocks)	Part of indicator inoculated (scion/rootstock)[a]	Fraction of indicators with blackline at the union[b]	Results of back indexing of indicators[c]	
Scion cultivar/ rootstock seedling	Inoculum-bark patches from				Scion	Rootstock
Orchard trees with blackline at the union[d]						
Eureka/*J. hindsii*	Scion[f]	Trinta/*J. hindsii*	Scion	11/15	+	–
	Scion[f]	Trinta/*J. hindsii*	Rootstock	0/15	–	–
	Rootstock[g]	Trinta/*J. hindsii*	Scion	0/15	–	–
	Rootstock[g]	Trinta/*J. hindsii*	Rootstock	0/15	–	–
Ashley/Paradox	Scion[f]	Trinta/*J. hindsii*	Scion	6/10	+	–
	Scion[f]	Trinta/*J. hindsii*	Rootstock	0/10	–	–
	Rootstock[h]	Trinta/*J. hindsii*	Scion	0/10	–	–
	Rootstock[h]	Trinta/*J. hindsii*	Rootstock	0/10	–	–
Hartley/*J. hindsii*	Scion[f]	*J. hindsii*/Eureka	Scion	0/10	–	–
	Scion[f]	*J. hindsii*/Eureka	Rootstock	9/10	–	+
	Rootstock[g]	*J. hindsii*/Eureka	Scion	0/12	–	–
	Rootstock[g]	*J. hindsii*/Eureka	Rootstock	0/13	–	–
Orchard trees without blackline at the union[e]						
Eureka/*J. hindsii*	Scion[f]	Trinta/*J. hindsii*	Scion	0/10	–	–
	Scion[f]	Trinta/*J. hindsii*	Rootstock	0/10	–	–
	Rootstock[g]	Trinta/*J. hindsii*	Scion	0/10	–	–
	Rootstock[g]	Trinta/*J. hindsii*	Rootstock	0/10	–	–
Hartley/*J. hindsii*	Scion[f]	*J. hindsii*/Eureka	Scion	0/10	–	–
	Scion[f]	*J. hindsii*/Eureka	Rootstock	0/10	–	–
	Rootstock[g]	*J. hindsii*/Eureka	Scion	0/10	–	–
	Rootstock[g]	*J. hindsii*/Eureka	Rootstock	0/10	–	–
Uninoculated controls	None	Trinta/*J. hindsii*	None	0/20	–	–
		J. hindsii/Eureka		0/20	–	–

[a] Three bark patches ~2 × 3 cm from either scion or rootstock of donor trees were grafted to the scion or rootstock of each indicator plant.
[b] Number of plants with blackline per number of plants inoculated.
[c] Back-indexed on cucumber, bean, cowpea, and tobacco plants: cherry leaf roll virus (walnut strain) (CLRV-W) + = recovered; – = not recovered.
[d] CLRV-W recovered from English walnut scion only.
[e] No virus was recovered from either English walnut scion or rootstock.
[f] One- to 3-yr-old terminal shoots from English walnut scion.
[g] One- to 3-yr-old sucker shoots growing from rootstock.
[h] Roots ranging 2–5 cm in diameter.

English walnuts only when they commence full bearing or trees that are 20 yr old or more, we conducted a graft-transmission experiment by using 2-yr-old walnut trees as indicators. In this experiment, *J. hindsii* and Eureka English walnut seedlings were grown in the greenhouse and then transplanted (1 × 4 m apart) to the field in the fall of 1974. In May 1975, actively growing *J. hindsii* and Eureka English walnut seedlings were cleft grafted, respectively, with healthy scions of cultivar Trinta English and *J. hindsii* walnut seedlings. In the following year (May–June 1976), bark patches from 1 to 3-yr-old wood of English scion and from sucker shoots or roots of *J. hindsii* or Paradox rootstock of diseased trees were used to inoculate 120 indicators. Each indicator received three inoculum bark patches (2 × 3 cm) from scions or rootstocks (Table 1). The indicator trees were observed for 3 yr.

In a greenhouse experiment 40 trees of 2-yr-old Ashley English scions on *J. hindsii* rootstock growing in 11.5-L cans were inoculated in the same manner as the 2-yr-old field trees, and observed for WBL union symptoms. All indicator trees were indexed for the presence of virus by mechanical inoculation of two-to-five plants of each herbaceous host (cucumber, tobacco, bean, and cowpea).

To determine whether the virus associated with WBL-affected English walnut cultivars is capable of inducing leaf symptoms in walnut seedlings and the blackline symptom at the union of grafted walnuts, we conducted the following experiments in the field: 1-yr-old Ashley English walnut seedlings growing in the field were inoculated with diseased bark patches from a Eureka English walnut tree from which virus had been recovered, but which showed no leaf symptoms. The Ashley seedlings were inoculated in September 1976 and concomitantly 10 of the inoculated Ashley seedlings were grafted with healthy buds of Trinta English walnut or *J. hindsii* trees. The healthy buds were placed about 50 cm above the highest point of inoculum insertion on the trunk of the Ashley seedlings. These buds developed into vigorous scion shoots the following season. Experimental controls were Trinta English walnut or *J. hindsii* on Ashley seedling rootstocks that received bark patches from healthy, virus-free Eureka English walnut. All trees were observed for symptoms in developing leaves of the Ashley seedling rootstocks and in scion leaves of cultivar Trinta or *J. hindsii*. The scion-rootstock unions also were examined periodically over a 3-yr period. All indicator trees were bioassayed for virus by test inoculations of cucumber, tobacco, cowpea, and bean plants.

A single-lesion virus isolate recovered from symptomatic leaves of the Ashley seedling rootstock was used when attempts were made to return virus to Eureka English walnut seedlings. Infected cucumber leaves were triturated in the nicotine:phosphate buffer mixture and the homogenate was rubbed on Carborundum-dusted, fully expanded leaves of 6-mo-old Eureka walnut seedlings growing in the greenhouse.

RESULTS

Field symptoms, distribution, and natural spread. General aboveground symptoms of WBL-affected English walnut trees are similar to those caused by soilborne pathogens, improper cultural practices, nutrient deficiencies, and incompatibility between scion and rootstock. WBL-affected English walnut trees show poor terminal growth, yellowing, and drooping of leaves and premature defoliation, particularly on the top (Figs. 1A,B). Later, affected trees show small-sized, yellowed, and drooping leaves, dieback of terminal shoots, general tree decline, and profuse suckering of the rootstock (Fig. 1C). Subsequently, the entire English scion dies (Fig. 1D). The presence of excessive suckering is a good indication that walnut trees may be affected with WBL, although sprouting alone is not an infallible diagnostic indicator of the disease. However, positive diagnosis requires a careful examination of the union. Trees with WBL show small holes and cracks in the bark at the scion-rootstock junction, and upon removal of bark a narrow strip of darkened cambium and phloem tissue (blackline) is found (Figs. 1A–C). In early stages of disease development, however, the blackline symptom is not continuous around the union (Fig. 1B). Thus, to ascertain an incipient infection, it may be necessary to examine the union at several points. Eventually, the blackline completely encircles and girdles the tree at the scion/rootstock junction resulting in death of the scion within 2–6 yr. (Fig. 1D). The necrosis of cambium and phloem at the graft union of English scion and *J. hindsii* rootstock appears as a narrow strip at the union (Figs. 1B,C). However, in trees of English/Paradox combination an extensive necrosis of bark tissue also may develop after onset of the narrow cambial necrosis at the union (Fig. 1E). The necrosis and bark canker in the Paradox rootstock of WBL-affected English walnut trees often may extend to the ground level. Diseased trees on Paradox rootstocks showed a quicker decline and death than did those on *J. hindsii* rootstocks. *J. hindsii* and Paradox are the standard walnut rootstocks in California; however, WBL also has been observed in English walnut trees propagated on wingnut *(Pterocarya stenoptera)* and on several *Juglans* spp. other than English Walnut *(J. regia)* seedlings (11).

In contrast to the previous assumption that WBL is limited to certain orchards in the central coastal counties (6,21,22), our survey revealed that the WBL disease is commonly present in high incidence in many of California's walnut-producing areas. The incidence of affected trees in surveyed orchards ranged from a few to more than 80%. The disease was observed in English walnut orchard trees ranging in age from 5 to over 70 yr old. The disease is commonly present in high incidence in numerous orchards in Stanislaus, San Joaquin, Merced, and Yolo counties which account for about 40% of the total walnut acreage in California. The disease was observed as far north as Butte County and as far south as Tulare County where one affected tree was observed in a commerical orchard near Visalia. However, the highest incidence of WBL and the most serious damage by this desease were observed in the coastal walnut-growing areas and in the lower Sacramento and upper and central San Joaquin valleys. Apparently WBL is much more widely distributed in California's walnut-producing areas than previously suspected.

Our surveys revealed commonly extensive and high incidence (over 70%) of diseased trees in commercial walnut orchards in Stanislaus County, which were free of WBL disease 10 yr ago. However, in Tulare County orchards trees of the same age and with the same cultivar-rootstock combinations were free of WBL disease. Apparently, active spread of WBL had occurred in Stanislaus, but not in Tulare County. Furthermore, the great difference in disease incidence in Tulare and Stanislaus counties rules out an involvement of noninfectious rootstock incompatibility.

Annual surveys of two commercial walnut orchards in Stanislaus County revealed natural spread of WBL disease. One orchard had 361 10-yr-old trees with an equal number of Trinta and Payne English walnuts on *J. hindsii* rootstock planted 10 m apart. This orchard was surveyed for four consecutive years. In 1975, 58 trees were affected, and these occurred in groups. In 1979, this orchard had 132 affected trees of which 60% involved Trinta scions. The average number of diseased trees increased by 19% per year. Typically, the spread of WBL was from a diseased to an adjacent healthy tree; 90% of newly infected trees neighbored previously diseased trees. The second orchard had 1,044, 15-yr-old Payne English walnut scions on Paradox rootstocks planted 12 m apart. In 1977, 97 trees, again grouped in clusters of several trees, were affected. In 1979, this orchard had 139 WBL-affected trees. The predominant spread of WBL was from a diseased to an adjacent healthy tree. Seventy percent of the newly infected trees were adjacent to known diseased trees.

Recovery of virus from walnut trees. Although walnut trees affected with WBL exhibited no viral-type leaf symptoms, we repeatedly mechanically transmitted a virus to cucumber, tobacco, cowpea, and beans from cambium and inner bark or leaves of English scions of walnut trees that showed blackline symptoms at the union of the scion and rootstock. However, we consistently failed to recover this virus from similar tissue extracts of Paradox or *J. hindsii* rootstocks. During a 3-yr period, we recovered the same virus from only the English scion portion of 202 diseased orchard trees; such trees were located in various California walnut-growing regions. Likewise we failed in numerous attempts to

recover this virus from 109 walnut trees free of WBL symptoms. The highest efficiency, often more than 90%, of virus recovery from cambial and inner bark tissues was from the early spring through the early summer. The virus was extremely difficult to recover from those tissues in the late summer or fall. However, the same virus frequently was recovered from fully expanded leaves of walnut trees in the late spring and summer, but it was difficult to recover it from young and rapidly expanding leaves in the early spring. The leaves of English walnut offered higher recovery of the virus than cambial tissue in the late summer and fall. All virus isolates, from different California walnut regions, induced identical symptoms in the herbaceous indicator plants. In cucumber, primary chlorotic spots appeared on cotyledons and systemic mottling, line patterns, and rings were induced on newly developed leaves (Fig. 2A); in beans, chlorotic and necrotic lesions occurred on inoculated and uninoculated leaves (Fig. 2B), followed by death of apical growth; in tobacco, chlorotic and white rings and line patterns were produced in inoculated and uninoculated leaves (Figs. 2C, D); and in cowpea, chlorotic spots and necrotic rings were induced on primary leaves followed by systemic necrosis of stems, and vein

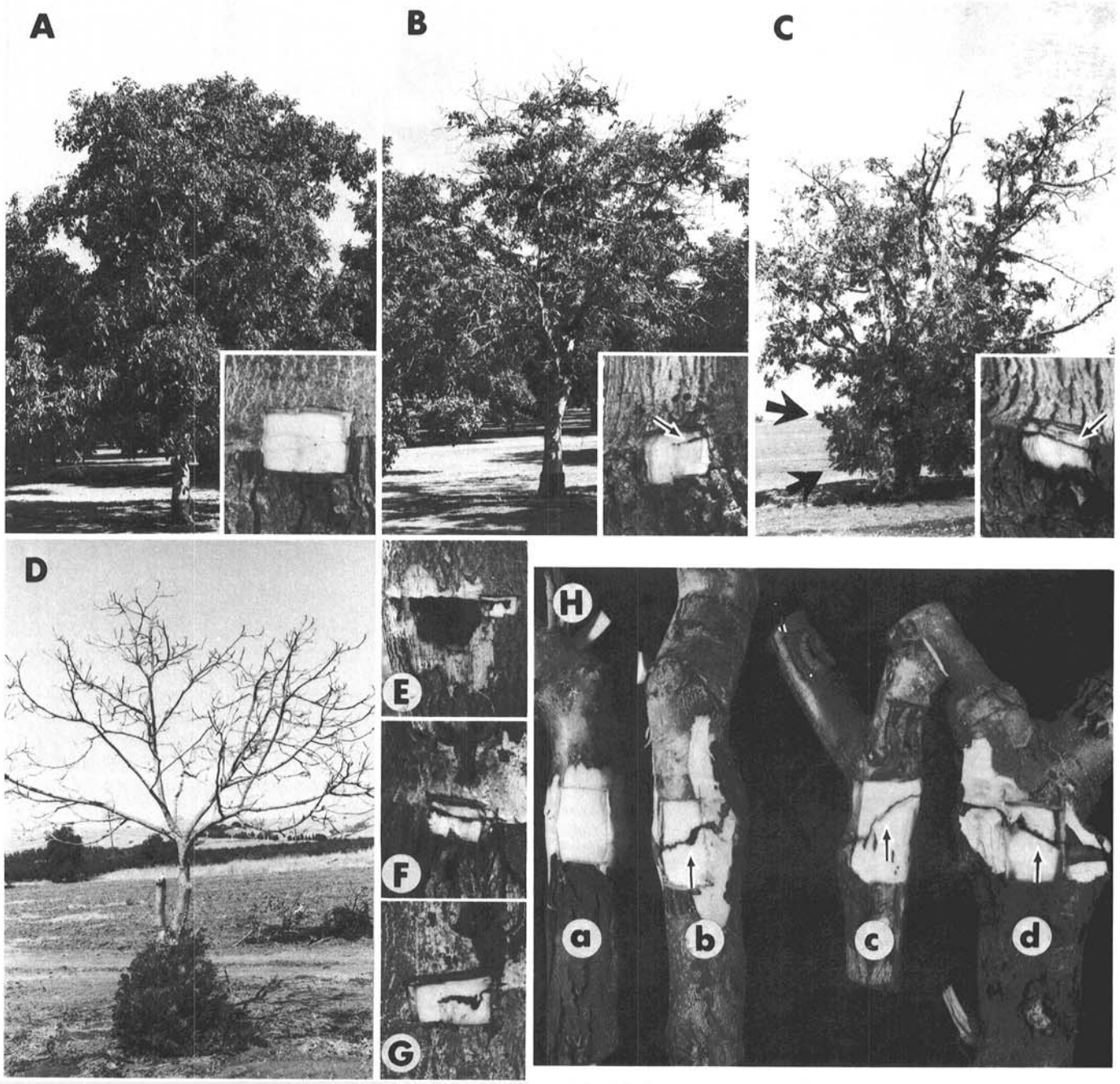

Fig. 1. Symptoms of walnut blackline disease (WBL) in naturally and experimentally infected walnut trees. (A–D) Orchard English walnuts on *Juglans hindsii* rootstock. **A,** Healthy 8-yr-old walnut tree; graft union with bark removed to show the absence of the blackline symptom (insert). **B,** Infected 8-yr-old tree in an early stage of WBL exhibiting poor terminal shoot growth, small, chlorotic, and drooping leaves. Graft union partially girdled by blackline (insert, arrow). **C,** Twenty-five-year-old walnut in advanced stage of WBL with the top of the tree dying and vigorous growth of sprouts from the rootstock (arrows); graft union completely girdled by blackline (insert, arrow). **D,** Diseased 10-yr-old tree with top killed by WBL and profuse sprouting from the rootstock. **E,** Graft union of naturally WBL-infected English walnut on Paradox rootstock showing bark canker developing downward from the scion into the rootstock. **F and G,** Graft unions of 9-yr-old English walnuts on *J. hindsii* and paradox rootstocks, respectively, inoculated with bark patches from diseased English scion of naturally WBL-affected tree. Blackline symptoms are similar to that shown in inserts B and C. **H,** Graft unions of 2-yr-old English walnuts on *J. hindsii* rootstock inoculated with bark patches from English scion of orchard walnuts: a, received inoculum from healthy tree; b, c, and d, each of these indicators received inoculum from different orchard WBL-infected trees; note blackline symptom at the unions (arrows).

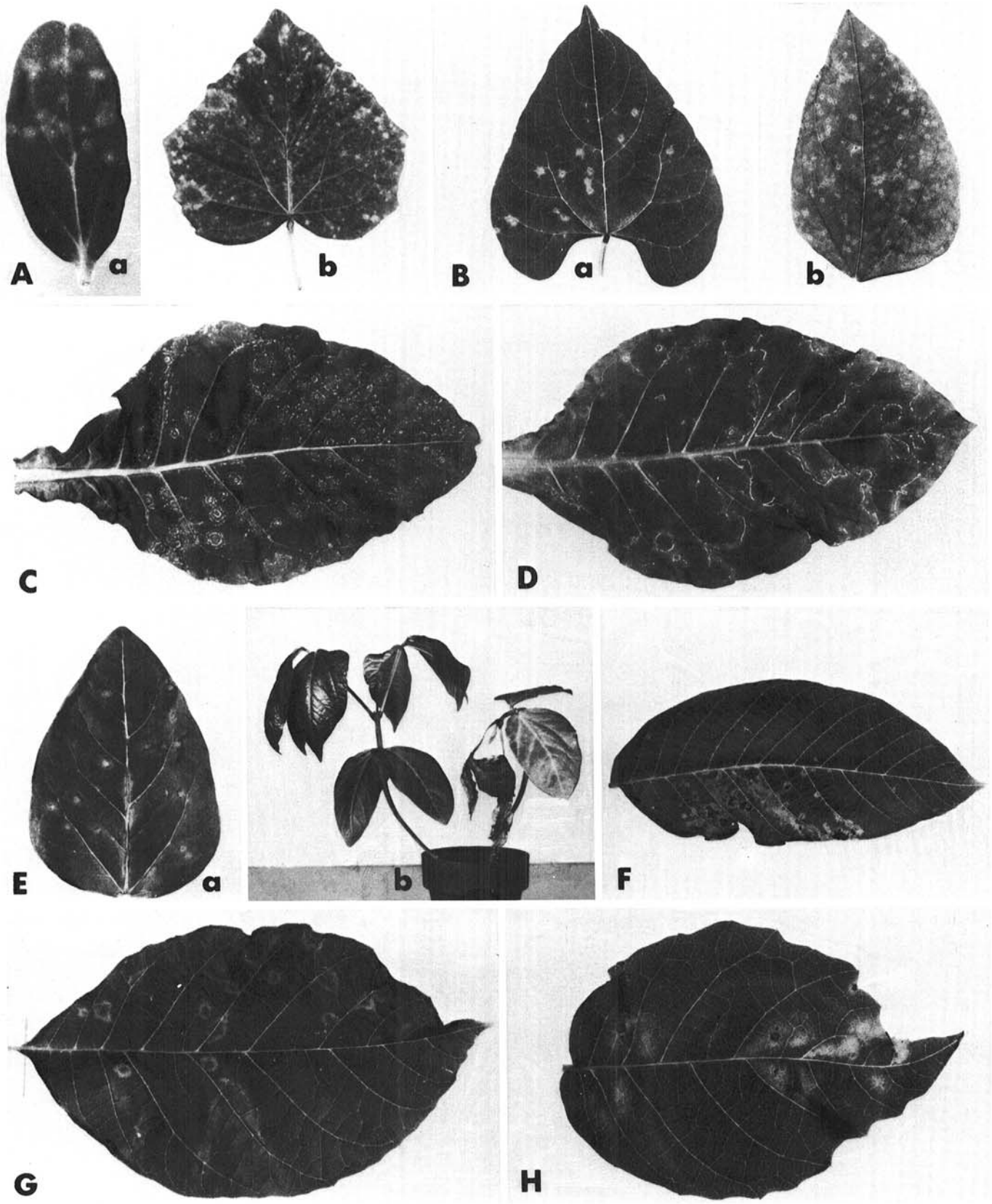

Fig. 2. Symptoms induced by the walnut strain of cherry leafroll virus (CLRV-W) in: **A,** Cucumber cultivar National Pickling; **a,** chlorotic spots on inoculated cotyledon; **b,** chlorotic spots and rings in a systemically infected leaf. **B,** Bean cultivar Bountiful; **a** and **b,** chlorotic and necrotic lesions in inoculated and systemically infected leaves, respectively. **C** and **D,** Tobacco cultivar Havana 425; white rings and line pattern in inoculated and systemically infected leaves, respectively. **E,** Cowpea cultivar Ramshorn; **a,** chlorotic spots and necrotic rings in inoculated leaf; **b,** uninoculated healthy plant (left) and inoculated plant with systemic stem necrosis and general collapse (right), **F** and **G,** Leaves from two different open-pollinated Ashley English walnut seedlings that were graft inoculated with CLRV-W showing chlorotic spots and rings and necrotic lesions. **H,** Systemically infected leaf of an open-pollinated Eureka English walnut seedling showing chlorotic spots and rings and necrotic lesions after it was mechanically inoculated with CLRV-W isolated from the walnut seedling leaf shown in F.

necrosis of new leaves (Fig. 2E). Seven single-lesion isolates were compared and all induced local and systemic symptoms in *Chenopodium amaranticolor* Coste & Reyn., *C. quinoa* Willd., and *Cucurbita pepo* L. These isolates caused latent infections in *Nicotiana glutinosa* L. and *N. rustica* L.; none of the isolates infected *Gomphrena globosa* L., *Petunia hybrida* Vilm., and *Datura stramonium* L. In a comparative host range study, the golden elderberry strain of cherry leafroll virus (CLRV-GE) caused symptoms in *N. rustica* and *N. glutinosa,* but failed to induce symptoms in Ramshorn cowpea, and Bountiful bean.

In gel-diffusion tests all seven virus isolates reacted positively with antisera prepared against the following isolates of cherry leafroll virus (CLRV): the golden elderberry strain (CLRV-GE) (9), the rhubarb isolate (CLRV-R) (18), (antisera supplied by R. Stace-Smith, Agriculture Canada, Research Station, Vancouver, B.C., Canada) and the dogwood isolate (CLRV-D) (23) (antiserum PVAS #142, from the American Type Culture Collection, ATCC). All these walnut isolates were serologically identical, but were not identical with the CLRV-GE isolate. The precipitin line of homologous reactants extended beyond the precipitin line of heterologous reactants. In other gel diffusion or ELISA tests, none of the seven walnut isolates reacted positively with antisera prepared against tobacco ring spot virus isolate TobRSV #438 (supplied by K. Kimble, Dept. of Plant Pathology, University of California, Davis 95616), and two different tomato ring spot virus (13) antisera (PVAS #174 and PVAS #239 obtained from ATCC). The virus associated with WBL-affected walnut is a strain of CLRV (5) hereafter referred to as CLRV-W.

Graft transmission experiments in the field. The WBL causal agent was graft-transmitted to 30 of 50 9-yr-old healthy orchard English walnut trees on *J. hindsii* or Paradox rootstocks when bark patches from English scions of naturally WBL-affected trees were applied to the English scions of the indicator trees. The cambial necrosis-blackline at the graft union of inoculated indicator trees developed within 1 yr and was identical to that in naturally WBL-affected orchard trees (Figs. 1F, G). Development of blackline at the union progressed 5 to 21 cm/yr. In contrast, no disease resulted when similar inocula were applied to *J. hindsii* or Paradox rootstocks of 50 indicator trees or when bark patches from *J. hindsii* of naturally WBL-affected trees were applied to either English scions or *J. hindsii* and Paradox rootstocks of 56 indicator trees. In our graft-transmission tests, the following English walnut cultivars or selections produced blackline symptoms: 53-517, Sinensis, Idaho, PI 18256, PI 159568, Ehrhardt, Placentia, Hodges, 0-20-1073, 0-13-1048, Blackmer, Sharkey, Franquette, Eureka, Westside, Marchetti, Trinta, 56-224, and 56-176. No blackline symptoms developed in any 42 indicator trees that served as controls in this experiment. All indicator trees that developed blackline yielded CLRV-W from the English scion and the virus was serologically identical to the CLRV-W from the naturally WBL-affected walnut trees. No virus was recovered from *J. hindsii* or Paradox rootstock of any indicator tree or from the English scions of the indicators that were free of blackline symptoms.

Graft-transmission experiments of 2-yr-old trees are summarized in Table 1. A year after bark patch inoculation, 17 of 25 Trinta scions on *J. hindsii* indicator trees developed blackline at the union (Fig. 1H) and nine of 10 *J. hindsii* scions on Eureka seedling indicator trees developed blackline at the union when the bark patches from English scions of naturally infected trees were applied to the Trinta scions and Eureka English seedling rootstocks of the indicator trees (Table 1). Extracts of Trinta and Eureka were infectious on herbaceous host plants and serological tests confirmed that the virus was CLRV-W. When a similar bark patch inoculum was applied to the *J. hindsii* portions of the indicator trees, none developed blackline (Table 1). Controls had remained symptomless for 3 yr when the experiment was terminated (Fig. 1H, a).

Graft transmission experiments in the greenhouse. Within 1 yr of inoculation, seven of 10 2-yr-old Ashley English walnut on *J. hindsii* indicators developed blackline, when bark patches from English scions of naturally WBL-affected trees were grafted to the Ashley scions of the indicator trees. When similar inocula were applied to the *J. hindsii* rootstocks, none of 10 showed disease. Likewise, none of 20 indicator trees that received bark patches from the *J. hindsii* rootstocks of the same naturally WBL-infected trees developed blackline. Controls had remained symtomless for 3 yr when this experiment was terminated. CLRV-W was recovered only from Ashley English sions of the indicator trees affected by blackline.

Induction of leaf symptoms by CLRV-W in Ashley English walnut seedlings. One year after graft-inoculation, four of 10 *J. hindsii* on Ashley seedlings and five of 10 Trinta on Ashley seedling indicator trees developed distinct viruslike leaf symptoms in the side and sucker shoots of Ashley seedling rootstock when bark patches from Eureka English walnut scions of WBL-affected orchard trees were applied to the Ashley portions of the indicator trees. Leaf symptoms consisted of chlorotic spots, mottling, necrotic lesions, chlorotic rings, and line pattern (Figs. 2F, G). No viruslike symptoms were observed in Trinta English and *J. hindsii* scions of the same trees. CLRV-W was recovered from symptomatic leaves of Ashley rootstock; virus also was recovered from the symptomless leaves of Trinta English scions, but not from *J. hindsii* scions. Also, on four *J. hindsii*/Ashley combinations in which symptoms showed in the Ashley rootstock, blackline developed at the union between the scion and the rootstock 1 yr after inoculation. Although extracts of Trinta/Ashley combinations contained CLRV-W none of the trees developed blackline at the union. All controls had remained symtomless for 3 yr after inoculation when the experiment was terminated.

Sap inoculation of English walnut seedlings with CLRV-W. In the greenhouse, four of seven Eureka English walnut seedlings developed systemic leaf symptoms 4 mo after they were mechanically inoculated with a single-lesion isolate of CLRV-W obtained from the Ashley seedlings with leaf symptoms. Leaf symptoms consisted of chlorotic spots and rings that often became necrotic (Fig. 2H). None of seven seedlings each of *J. hindsii* or Paradox that were similarly mechanically inoculated developed leaf symptoms during 8 mo of incubation. CLRV-W was recovered only from Eureka seedlings that showed leaf symptoms. Eureka English walnut, *J. hindsii,* and Paradox seedling controls remained symptomless throughout the experimental period; no virus was transmitted from these trees.

DISCUSSION

Our studies clearly showed that WBL spreads naturally in orchards and is caused by a graft-transmissible agent. The present study is the first to implicate an infectious agent in the WBL disorder and our results disagree with previous contentions that WBL is a disorder caused by noninfectious factors (20,21). This is also the first report of a virus disease of walnut trees in the United States

The host range and symptom expression of the CLRV-W isolates were similar, but not identical with those of several other strains of CLRV (4,5,9,17–19,23). The symptoms induced by CLRV-W in tobacco, *C. amaranticolor, C. quinoa,* cucumber, and bean plants were similar to those reported for the cherry isolate of CLRV (4). However, the walnut isolates, in contrast to the cherry isolate of CLRV, infected but induced no symptoms in, *N. glutinosa* and *N. rustica.* Several strains of CLRV, notably the Italian walnut ring spot (19) and the dogwood (D), (17) and rhubarb (R) strains (18) of CLRV, failed to infect cowpea, whereas CLRV-W caused primary and secondary symptoms (Fig. 2E).

In gel-diffusion tests, seven CLRV-W isolates reacted positively with three sources of cherry leafroll virus antisera (ie, CLRV-R, CLRV-D, and CLRV-GE antisera). Furthermore, when CLRV-GE antiserum was tested against CLRV-W and CLRV-GE virus isolates, homologous precipitin lines spurred over heterologous lines; no spurs developed among CLRV-W isolates. Thus, the CLRV-GE and CLRV-W are related, but not identical.

CLRV-W was repeatedly isolated from English walnut scions, but never from the *J. hindsii* or Paradox rootstocks of WBL-affected trees. We also easily graft-transmitted CLRV-W and blackline disease by applying bark patches from diseased English scions to the English walnut portions of healthy indicator trees. On

the other hand, no graft-transmission of the virus nor blackline symptom were observed when bark patches from infected English walnut scions were applied to the *J. hindsii* or Paradox portions or when bark patches from *J. hindsii* or Paradox from WBL-affected trees were used to inoculate either English walnut scions or *J. hindsii* or Paradox rootstocks of indicator trees. Apparently CLRV-W is present only in the English walnut scions of WBL-affected orchard trees; both *J. hindsii* and Paradox rootstocks are resistant to CLRV-W, and the necrosis of cambium and phloem tissue; ie, blackline at the union of the English scion and the rootstocks is the result of a hypersensitive reaction of the *J. hindsii* and Paradox rootstocks to the CLRV-W. The constant association of CLRV-W and WBL disease strongly suggests a causal relationship.

We observed no difference in the incidence of WBL in naturally or experimentally infected trees propagated on *J. hindsii* and Paradox rootstock. However, generally a quicker decline resulted on Paradox than on *J. hindsii* rootstock, which was attributed to a more rapid development of bark canker in Paradox rootstock (Fig. 1E).

The natural spread of WBL and CLRV-W in commerical orchards was observed in this study, but the mode of spread or the vectors involved were not determined. The absence of virus in walnut rootstocks and apparent immunity of the *J. hindsii* and Paradox rootstocks to CLRV-W suggests that nematodes, even though capable of transmitting certain strains of CLRV (5), are unlikely vectors of WBL and CLRV-W in California's walnut orchards. Some of the CLRV strains are seed-borne and pollen transmitted (1,3,5,9,10,16). Although we have no experimental evidence that WBL agent or CLRV-W is seed- or pollen-transmitted in walnut, we have observed that natural spread does not occur in young orchards until the walnut trees bear flowers. Incidence and spread of WBL was observed to be proportional to the precocity of the English walnut cultivars; ie, more precocious cultivars show greater number of diseased trees. Furthermore, we observed a natural spread of WBL in commercial orchards from infected Payne to healthy Payne or Trinta cultivars and no spread from infected Payne to Franquette cultivars. This may be explained by the fact that pollen shedding of Payne and peak of pistillate bloom of Payne and Trinta cultivars is separated by approximately 3–5 days; whereas the pollen shedding of Payne and the peak of pistillate bloom of Franquette is separated by approximately 27 days. Similarly a high rate of natural spread from diseased Ashley to healthy Ashley English walnuts was observed, with no spread to neighboring Hartley walnut trees. The periods of pollen shedding and pistillate bloom for Ashley and Hartley cultivars are separated by 3 and 15 days, respectively. Since we graft-transmitted WBL and CLRV-W with equal ease to cultivars Trinta, Ashley, Hartley, and Franquette, these field observations suggest that pollen may be involved in transmission of the WBL agent.

Because the WBL causal agent is graft-transmitted, control measures should include careful selection of propagating material to avoid infected trees. Since WBL-affected trees do not recover, and since natural spread was confirmed in this study, immediate roguing of infected trees in orchards or nurseries should be practiced. Research on the relative resistance of English walnut cultivars and rootstocks to WBL and on the role of pollen in natural spread of the disease is in progress.

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