Serological Detection of Cherry Leafroll Virus in English Walnut Trees

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ABSTRACT

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Direct (D) and indirect (I) enzyme-linked immunosorbent assays (ELISA) were compared for relative reliability in detecting the walnut strain of cherry leafroll virus (CLRV-W), the causal agent of blackline disease in English walnuts. The lowest concentrations of purified CLRV-W8 detected by I-ELISA and D-ELISA with peroxidase-conjugated γ-globulin were 4 ng/ml and 48 ng/ml, respectively, of the virus in phosphate buffer, pH 6.5. Likewise, based on the highest dilution of CLRV-W-infected walnut inner bark tissue and pollen in PBS-Tween buffer at which the virus was detected, I-ELISA was 8 and 16 times more sensitive than D-ELISA, respectively. I-ELISA with peroxidase and alkaline phosphatase was more sensitive than I-ELISA with glucose oxidase for detection of the known concentrations of purified CLRV-W8 or of the virus in infected walnut tissues. I-ELISA with γ-globulin-peroxidase conjugate is efficient and reliable for indexing English walnut seedlings in nurseries for CLRV-W. It also was reliable in detecting CLRV-W in naturally infected orchard trees when a relatively large amount of pollen from each tree (avg 80 g per tree) was collected from which 0.1 g was used for I-ELISA tests. However, the detection of CLRV-W by I-ELISA in orchard trees was not reliable when a relatively small amount of pollen from each tree (avg 5 g per tree) was collected for the assay. The relatively large amount of pollen from each tree was necessary for reliable I-ELISA tests to compensate for the uneven and erratic distribution of CLRV-W in naturally infected walnut orchard trees.

Additional key words: Persian walnuts, virus diseases, indexing for virus.

Walnut blackline (WBL) is an infectious disease of English walnut (Juglans regia) on northern California black (Juglans hindsii) and Paradox (J. hindsii × J. regia) walnut root stocks (12).

WBL of English walnut trees is caused by a graft-, pollen-, and seed-transmissible strain of cherry leafroll virus (CLRV-W) (9–12).

Viruslike leaf symptoms have been observed in some open-pollinated English walnut seedlings naturally infected or artificially inoculated with CLRV-W (8,10,12); however, commercial orchard trees in California are symptomless until the virus reaches the graft union and induces blackline at the graft union (10,12).

Because of a slow movement of the CLRV-W in walnut trees (10), the orchard trees infected through pollination may harbor the virus for a number of years before any visible symptoms of

infection are observed. Thus, infected but symptomless orchard trees can serve as unsuspected sources of infected budwood, graftwood, or seed used for propagation or as sources of infected pollen that contribute to the natural spread of CLRV-W (10–12) unless they were detected by being indexed for the presence of the virus.

Conventional indexing of walnut trees for CLRV-W infection by graft inoculation of English/ *J. hindsii* trees or bioassay of trees by mechanical inoculation of herbaceous hosts (9,12), is time consuming and inefficient. Also, the erratic and uneven distribution of CLRV-W in infected trees, due to the slow movement of the virus, makes it necessary to collect and assay numerous samples from each tree.

Several immunosorbent assay techniques have been employed successfully for the rapid detection of early virus infection in plants (1,3,7,14) where other indexing procedures were inefficient or failed.

In this paper, we report the relative efficiency of direct and indirect enzyme-linked immunosorbent assay (ELISA) for detection of CLRV-W in infected English walnut trees.

MATERIALS AND METHODS

Virus purification and serology. A single-lesion CLRV isolate, CLRV-W8, recovered from walnut trees affected with blackline (10,12) was used for the production of antiserum. The virus was propagated in cucumber (*Cucumis sativus* L. 'National Pickling') seedlings in a greenhouse. The cucumber seedlings were harvested and the virus was purified 10–20 days after mechanical inoculation. A modified procedure for the purification of potato leafroll virus (13) was satisfactory for the purification of CLRV-W8. The modifications are as follows: potassium phosphate buffer (pH 6.5) was used in all steps, *n*-butanol:chloroform (1:1, v/v) was added at the rate of 1.5 ml per 10 ml of extract during the clarification step; and pellets were resuspended in 0.05 M phosphate buffer without urea.

The presence of CLRV-W8 particles in the purified preparation collected from two distinct bands from sucrose density gradients was determined by electron microscopy and the infectivity of the preparation was determined by mechanical inoculation of *Nicotiana megalosiphon* Heurch and Mueller and cucumber seedlings. CLRV-W8 concentration was estimated by assuming that a purified preparation containing 1 mg of virus per milliliter has an $OD_{260\ nm} = 10$. This assumption was based on the average amount of nucleic acid (40%) present in the components of the golden elderberry isolate of CLRV (6) which has a relatively close serological relationship to CLRV-W (4). A mixture of both virus bands from sucrose density gradients was used to obtain antiserum by immunizing New Zealand rabbits. Chicken antibodies were obtained by immunizing White Leghorn, Line 58, hens (supplied by H. Abplanalp, University of California, Davis).

The rabbits were given three intramuscular injections at weekly intervals. Each injection was prepared by emulsifying 1 ml of virus preparation with an equal volume of Freund's complete adjuvant. The amounts of virus used for the first, second, and third injections were 225, 450, and 800 μg, respectively. The rabbit was bled 1 wk after the last injection and thereafter at weekly intervals. A booster injection of 750 μg of purified virus was given to the rabbit after the second bleeding. The antiserum titer was determined by agar gel double diffusion tests.

To obtain chicken antibodies against CLRV-W8, two egg-laying hens were immunized with the virus. Each hen was given two intravenous injections at 4-day intervals and two intramuscular booster injections at 2 wk and 2 mo after the second intravenous injection. For booster injections, the virus preparations were emulsified with an equal amount of Freund's complete adjuvant. The amount of virus used for each injection was 30, 130, 120, and 400 μg, respectively. Ten days after the second injection, the eggs were collected and tested to determine the titer of antibody in each yolk. Antibodies were extracted from the egg yolk by adding 20 ml of PBS (0.02 M phosphate buffer containing 0.15 M NaCl at pH 7.4), then shaking thoroughly, and centrifuging at 10,000 rpm for 10 min in a SS-34 rotor. The supernatants, which contained CLRV-W8-specific antibodies, were mixed with an equal volume of glycerol and stored at −20 C until used. The titer of yolk antibodies collected individually from a number of eggs and saved in the freezer was assessed by ELISA. The titer of all yolk antibodies from different eggs were tested with similar crude extracts from CLRV-W8-infected cucumber seedlings. To obtain the titer of yolk antibodies from different egg yolks, a constant dilution of yolk antibodies was used with variable dilutions (twofold dilutions) of the extracts from cucumber seedlings as an antigen. The antibody titers of individual yolks were then determined on the basis of the highest dilution of antigen in the crude sap detectable by that specific yolk antibody.

Antivirus γ-globulin was prepared from the rabbit antiserum as described by Clark and Adams (3), except the column was prepared with DE52 instead of DE22 cellulose. The DE52 cellulose was mixed with PBS (pH 7.4) and then packed in a 3.5–5.0 cm column. The column was washed with PBS for 4–6 hr. When the pH of the eluted buffer dropped to 7.4, the column was loaded with γ-globulin.

The enzyme most commonly used for preparing γ-globulin-enzyme conjugates employed in ELISA tests for plant viruses is alkaline phosphatase (2). In these studies, however, we compared the relative usefulness of alkaline phosphatase, peroxidase, and glucose oxidase conjugates for the detection of CLRV-W by ELISA.

Gamma-globulin was conjugated with alkaline phosphatase (Sigma type VII-S, from bovine intestine; Sigma Chemical Co., St. Louis, MO) by the previously described method (3) while the method of Johnson et al (5) was followed to conjugate γ-globulin with peroxidase (Sigma type VI, from horseradish) and glucose oxidase (Sigma type VII, from *Aspergillus niger*) with minor modifications as follows: after activation of the enzymes, 175 μl of activated peroxidase or 280 μl of activated glucose oxidase was mixed with 1 ml of 1.5 mg or 2.5 mg of γ-globulin per milliliter in 0.01 M carbonate buffer (pH 9.5), respectively. After the mixture was dialyzed against saline, 5–10 mg of bovine serum albumin (BSA, Sigma fraction V) was added per milliliter and the conjugate was stored at 4 C until used.

Chromogenic substrates for the different enzymes were: *p*-nitrophenyl phosphate (Sigma 104) 0.7 mg/ml in 10% diethanolamine (pH 9.8) for alkaline phosphatase; *o*-phenylenediamine, 0.7 mg/ml in phosphate-citrate buffer (pH 5.0) containing 0.012% hydrogen peroxide for peroxidase; and for glucose oxidase the substrate was 2,2'-azino-di-3-ethylbenz-thiazoline sulfonic acid (ABTS), 0.2 mg/ml in a buffer composed of 25 vol of 0.1 M potassium phosphate buffer (pH 6.0), 4 vol of 18% aqueous D-glucose which was prepared many hours before use to have the β-D-isomer, and 1 vol of 200 μg of peroxidase per milliliter. The glucose oxidase substrate mixture was vigorously shaken to saturate it with oxygen before it was used.

Immunosorbent assays and sensitivity tests. Throughout this work we use the terms "direct ELISA" (D-ELISA) when the γ-globulin used for coating the plates and for conjugating to the enzyme is from the same animal species and prepared against the same antigen. In the case of "indirect ELISA" (I-ELISA) the reactions were done on plates precoated with antiviral rabbit antibodies ("first antibody"). The immobilized virus particles were then reacted with antiviral chicken antibodies ("second antibody"). The level of binding of second antibody was then determined with enzyme-labelled rabbit anti-chicken antibodies. The terminology used is adapted from that of Koenig (7).

All ELISA tests were performed in flat-bottom microelisa plates (Dynatech, M129A or Immulon I from Dynatech Laboratories Inc., Alexandria, VA) and sample reactions were determined by scanning plates in a Titertek Multiskan colorimeter (Flow Laboratories Inc., McLean, VA). Absorbance was measured at 405, 414, or 450 nm for *p*-nitrophenyl phosphate, ABTS, or *o*-phenylenediamine substrates, respectively. The reaction of enzymes with their respective substrates were recorded 30–45 min after the addition of the substrate.

For D-ELISA the previously described method (3) was used,

except that the plates were incubated for 1.0–1.5 hr at 37 C following each step. For I-ELISA, the procedure was similar to that described for D-ELISA except that after incubation with the test samples, the egg yolk-antivirus antibodies diluted 1:1,000 in PBS-Tween containing 2% PVP was added. After washing, rabbit anti-chicken γ-globulin (Miles-Yeda Ltd, IgG Fraction [Miles Laboratory, Inc., Elkhart, IN] purified further by the same procedure described for rabbit antivirus antiserum) conjugated with the enzymes was added at 1.5–2.0 μg/ml diluted in extraction buffer (PBS + 0.05% Tween-20 + 2% PVP + 0.2% BSA).

Sources of antigen in these investigations were: a purified preparation of CLRV-W8, systemically infected leaves of cucumber artificially inoculated with CLRV-W8, and various tissues of English walnut (*J. regia* L.) artificially inoculated with CLRV-W8 or naturally infected trees. Cucumber leaves and walnut inner bark and cambium, leaf and catkin samples were prepared in extraction buffer at an approximate ratio of 1:10 (w/v). The tissue samples were triturated with a mortar and pestle or homogenized with a Polytron homogenizer (Brinkmann Instruments, Westbury, NY). Pollen samples from English walnut trees were sonicated with Braun-Sonic 1510 apparatus (VWR Scientific Inc., San Francisco, CA) for 20 sec at 100 W. Purified CLRV-W8 preparations used in these tests were diluted in 0.05 M potassium phosphate buffer (pH 6.5).

Materials and sampling procedures for virus detection in naturally infected walnut trees. To assess the reliability of ELISA for indexing nursery trees for CLRV-W infection we conducted the following experiment. Nuts were collected from naturally CLRV-W-infected English walnut trees that also showed blackline at the graft union of the scion and *J. hindsii* (Jeps.) Jeps. rootstock and then planted in nursery rows in December 1979. In the spring of 1980, when the resulting seedlings were ~20-cm tall, they were bioassayed on cucumber and *N. megalosiphon* and also tested by I-ELISA. From these seedlings, 15 CLRV-W-infected and 20 CLRV-W-free seedlings were selected and grown for an additional 3 yr in the nursery and were tested at least four times per year by I-ELISA for the presence of virus in leaf, inner bark, and cambial tissues.

In experiments designed to evaluate ELISA for detecting CLRV-W in orchard walnut trees, first we attempted to determine possible advantages, if any, of using inner bark and cambium, leaves, catkins, or pollen as assay tissue. In these experiments, we used blackline-affected English walnut (*J. regia*) trees propagated on northern California black walnut (*J. hindsii*) rootstock. From each tree assayed by I-ELISA, inner bark and cambial tissues of the English walnut scion were collected ~10 cm above the blackline at the graft union, leaves and catkins were collected and combined from each of 10 randomly selected terminal shoots, and pollen was collected from 10 catkins. Thus, four different samples from each of 148 trees were tested for CLRV-W by ELISA.

The results from the above tests suggested that pollen may be more useful than either inner bark or leaf tissue samples for detection of CLRV-W in orchard walnut trees by I-ELISA. In November 1980, a walnut orchard with 25-yr-old trees of Ashley and Serr cultivars on rootstock of *J. hindsii* was visually surveyed for the incidence of blackline-affected trees. The survey revealed that 14 of 89 trees had blackline at the graft union of the scion and rootstock whereas 75 trees were symptomless. In March 1981, catkins were collected from each of the 89 trees by shaking the entire tree with a shaker used for mechanically harvesting walnuts. All catkins that fell to the ground under each individual tree were hand picked and placed in an enclosed building on a wire screen and allowed to shed pollen. The amount of pollen collected from each tree ranged from 5 to 170 g (mean, 82 g per tree). Approximately 0.1 g of pollen from each tree was processed as described above and assayed by I-ELISA for the presence of CLRV-W. In March 1983, we retested in the same manner 21 of the 89 trees assayed in 1981. In March 1982, in another commercial orchard, we evaluated the reliability of I-ELISA in detecting CLRV-W in walnut trees by collecting ~100 catkins from each of 60, 25-yr-old orchard trees of cultivar Ashley on rootstock of *J. hindsii*. Nine of 60 trees had blackline at the graft union and the amount of pollen collected from each tree ranged from 1.8 to 9.8 g (mean, 5.3 g per tree).

RESULTS AND DISCUSSION

Antiserum production. The maximum homologous titer of CLRV-W8 antiserum was 1:256 before the booster injection and 1:4,096 after the final injection compared with a titer of 1:4 for healthy sap determined by agar gel double-diffusion tests. The maximum titer of yolk antibodies was 1:5,120 (dilution of crude sap from CLRV-W8-infected cucumber seedlings) in an I-ELISA test. The titer of the yolk antibodies began to increase 10 days after the first booster injection, leveled off for about 1 wk, and then declined. One week after the second booster injection, the titer began to increase again, 4 days later it reached the highest peak and leveled off for 5 days, then declined. All yolk antibodies used in our experiments were collected after the second booster injection.

Comparative sensitivity of different immunoassays. In preliminary experiments, we compared the relative sensitivity of I-ELISA with glucose oxidase, peroxidase, and alkaline phosphatase conjugated to antibodies to detect CLRV-W8 in artificially inoculated cucumber leaves. The concentrations of γ-globulin conjugated with enzymes and the molar ratio of enzyme and γ-globulin affected the level of nonspecific background in healthy cucumbers and the sensitivity of I-ELISA in detecting CLRV-W8 in artificially inoculated cucumber plants. In our system, the best results (based on the positive detection of CLRV-W8 at the highest dilution of infected cucumber sap with lowest nonspecific background of healthy plants) were obtained when 1.5 and 2.5 mg of γ-globulin per milliliter was conjugated with peroxidase and glucose oxidase, respectively, at a molar ratio of enzyme:γ-globulin of 1:1. Likewise, the best results with alkaline phosphatase were obtained when 1.5–2.0 mg of γ-globulin per milliliter was conjugated with the enzyme at the enzyme:γ-globulin ratio of 4:1. Furthermore, in these tests, we obtained the highest sensitivity of I-ELISA when the microtiter plates were coated with 1.5 μg of γ-globulin per milliliter of coating buffer and crude yolk antibody dilution of 1:1,000. The optimum incubation time for each of the steps was 1.0–1.5 hr at 37 C.

The relative sensitivities of D- and I-ELISA with γ-globulin-peroxidase conjugate based on the lowest limit of detection of known concentration of purified CLRV-W8 are shown in Fig. 1. The absorbance values $A_{450\,nm}$ for CLRV-W8 concentrations at 25 ng and 2.5 ng/ml were 0.05 and 0.03, respectively, for D-ELISA whereas the absorbance values for the same CLRV-W8 concentrations were 0.25 and 0.1 for I-ELISA, as compared to the absorbance value of 0.024 for buffer controls. I-ELISA was >10-fold more sensitive than D-ELISA.

We also used purified preparations of CLRV-W8 to compare the sensitivity of I-ELISA with different γ-globulin enzyme conjugates. The lowest limits of detection were 4 ng and 8 ng of CLRV-W8 per milliliter by I-ELISA by using γ-globulin conjugated with peroxidase and alkaline phosphatase, respectively, while the lowest limit of detection was 24 ng of CLRV-W8 per milliliter by I-ELISA by using γ-globulin conjugated with glucose oxidase. These results are the averages of four replications of each I-ELISA-γ-globulin conjugate. I-ELISA absorbance values for CLRV-W8-loaded wells were at least three times higher than the absorbance $A_{405\,nm} = 0.022$, $A_{414\,nm} = 0.012$, and $A_{450\,nm} = 0.025$, respectively, for wells with buffer when γ-globulin-alkaline phosphatase, -glucose oxidase, and -peroxidase conjugates were used.

When samples of CLRV-W infected cucumber and walnuts were used to compare sensitivity of D-ELISA with peroxidase labeled antibody and I-ELISA with different γ-globulin-enzyme conjugates we obtained similar results (Table 1) to those results from the experiments employing a purified preparation of CLRV-W8. Based on the highest dilution at which CLRV-W was detected, I-ELISA with γ-globulin-peroxidase conjugate was two to 16 times more sensitive than D-ELISA with the same γ-globulin conjugate in detecting CLRV-W depending on the plant species and type of tissue assayed (Table 1). I-ELISA with γ-globulin peroxidase

conjugate was as sensitive or more sensitive than the same ELISA procedure with alkaline phosphatase depending on the type of infected cucumber and walnut tissues assayed (Table 1). I-ELISA with γ-globulin-glucose oxidase conjugate was generally less sensitive than I-ELISA with peroxidase or alkaline phosphatase (Table 1). The nonspecific background of glucose oxidase conjugate was usually very low, as observed by Johnson et al (5), but its reaction after the addition of substrate was slow, and generally its sensitivity for the purified preparation or CLRV-W-infected plant tissues was not as good as that obtained with either alkaline phosphatase or peroxidase conjugates. Although it has been reported (2) that alkaline phosphatase conjugate is more sensitive and has a better reproducibility than peroxidase conjugate, in our tests, we experienced no difficulties with peroxidase conjugate in reproducing results. Likewise, we had no problems of high nonspecific background and light sensitivity of the o-phenylenediamine substrate as experienced by other workers (2). Peroxidase is less expensive than alkaline phosphatase and conjugate of the former enzyme, and in our system, was as sensitive and occasionally more sensitive than alkaline phosphatase conjugate (Table 1). However, the chromogenic substrate, o-phenylenediamine for peroxidase is listed as having mutagenic activity and must be handled with caution.

Reliability of I-ELISA for indexing naturally CLRV-W-infected walnut trees. I-ELISA with a γ-globulin-peroxidase conjugate repeatedly detected CLRV-W in each of 15 naturally infected English walnut seedlings each time they were indexed during the period from May 1980 through March 1983. The virus was detected consistently in inner bark and cambium and leaf tissues of each of 15 seedlings that were initially CLRV-positive in 1980 regardless of what time of the year they were indexed. No virus was detected in any of the 20 seedlings during the same 3-yr period that were initially CLRV-negative for CLRV-W in May 1980 and which were grown in the same nursery. Apparently, I-ELISA with γ-globulin-peroxidase conjugate is an efficient and reliable method to index for CLRV-W in nursery English walnut seedlings.

Detection of CLRV-W in different tissues from orchard trees of English walnut on *J. hindsii* rootstock by I-ELISA was investigated. CLRV-W was detected by I-ELISA in 148 of 148 trees when inner bark and cambial tissues of the English scion were taken ~10 cm directly above the blackline area regardless whether the trees were completely or partially girdled by the blackline at the graft union. In 92 of 148 trees in this test, the blackline symptom extended <50% of the graft union circumference. CLRV-W was detected in 23 of the 92 trees when English scion samples were taken at 10 cm above the graft union and 10 cm around the circumference of a tree from the end of the blackline. No CLRV-W was detected in

any of the 92 trees when samples were taken >15 cm from the end of the blackline. When composite leaf or catkin samples collected from 10 terminal shoots of the same 148 blackline-affected orchard trees were assayed, CLRV-W was detected in only 27 and 22 trees, respectively. However, 84 of the same 148 trees were indexed as positive for CLRV-W when composite samples of pollen from the same 10 terminal shoots per tree were assayed by I-ELISA. CLRV-W is not uniformly distributed throughout infected walnut orchard trees. Furthermore, pollen from walnut trees is more useful than either leaves or catkins for indexing for CLRV-W by I-ELISA.

In a commercial orchard of 89 walnut trees examined, 14 trees had blackline at the graft union and 75 were symptomatic. Five to 170 g (avg 82 g per tree) of pollen was collected from each surveyed tree and tested for the presence of CLRV-W by I-ELISA. Pollen samples from 13 of 14 trees with blackline symptom were positive, and pollen samples from 45 of the 75 symptomless trees that were tested were positive for CLRV-W. In the repeated I-ELISA tests in the same orchard, eight of 10 trees that were positive for CLRV-W in 1981 tests also showed a positive reaction for the virus in the March 1983 test. Among the 11 trees that were negative for CLRV-W in 1981, two trees tested positive for CLRV-W in March 1983; presumably these two trees became naturally infected during 1981–1982. These tests revealed that in WBL-affected commercial orchards, in addition to those trees exhibiting blackline symptom, a considerable number of other trees also may be infected with CLRV-W that remain symptomless until the virus reaches the graft union and induces the blackline symptom (12). In contrast, in the test conducted in March 1982, 1.8–9.8 g (avg 5.3 g per tree) of pollen was collected from each surveyed tree and tested for the presence of CLRV-W by I-ELISA. Of nine trees with blackline symptom, a pollen sample from only one tree was positive for CLRV-W. Of 51 symptomless trees tested, pollen samples from only five trees were positive. Apparently, the amount of pollen collected from each tree (1.8–9.8 g per tree) in the 1982 test was not sufficient to compensate for the erratic distribution of the virus in orchard walnut trees. Nevertheless, these tests revealed that pollen testing by I-ELISA with γ-globulin-peroxidase conjugate is more reliable and accurate in determining CLRV-W infection in walnut orchard trees than visual surveys based on the presence of blackline at the graft union of orchard walnut trees. The CLRV-W is pollen transmitted (11)

TABLE 1. The relative sensitivity of different immunoassay methods and enzymes labelled γ-globulin in detection of the walnut isolate of cherry leaf roll virus (CLRV-W) in infected plants

Immunosorbent assay method[b] and enzyme used for γ-globulin conjugation	The highest dilution at which CLRV-W was detected[a]				
		English walnut trees			
				Pollen[d]	
	Leaves of cucumber seedlings[c]	Inner bark and cambium[c]	Leaves[c]	Ashley cultivar	Payne cultivar
D-ELISA, peroxidase	640	160	320	160	160
I-ELISA, peroxidase	5,120	1,280	640	2,560	2,560
I-ELISA, alkaline phosphatase	2,560	1,280	320	2,560	1,280
I-ELISA, glucose oxidase	640	640	320	1,280	1,280

[a] ELISA absorbance values at least threefold higher than the absorbance values of comparable controls (CLRV-W-free tissues) on the same microtiter plates.
[b] D-ELISA and I-ELISA are direct (D) and indirect (I) enzyme-linked immunosorbent assays.
[c] Artifically inoculated with CLRV-W8.
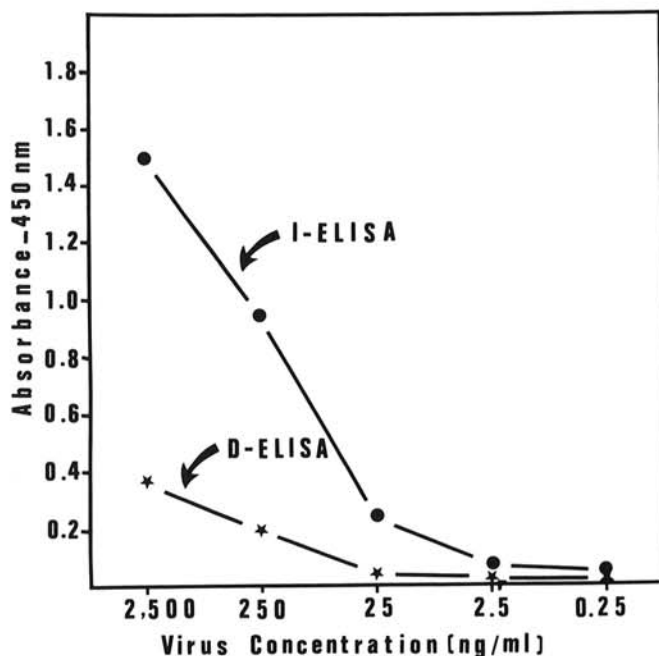[d] Naturally infected with CLRV-W.

Fig. 1. Direct (D)- and indirect (I)-enzyme-linked immunosorbent assay (ELISA) absorbance values obtained by using purified preparation of CLRV-W8 in 0.05 M phosphate buffer (pH 6.5) and peroxidase-conjugated γ-globulin. Each point is the mean of four replications with a maximum $A_{450\ nm}$ of 0.025 for the buffer.

and moves very slowly ($\sim$ 14–32 cm/yr) through infected trees of English walnut cultivars (S. M. Mircetich and A. Rowhani, *unpublished*), thus the infected orchard trees may remain symptomless for a number of years until the virus reaches the graft union of the English scion and *J. hindsii* or Paradox rootstocks and induces the blackline symptom (10,12).

Our research showed that I-ELISA with γ-globulin-peroxidase conjugate is very sensitive and reliable and superior to D-ELISA for detecting CLRV-W in walnuts. In our ongoing research concerned with epidemiology, role of pollen in natural spread of CLRV-W and the relative resistance of English walnut cultivars to CLRV-W, I-ELISA is a very useful and reliable research tool. Likewise, I-ELISA is efficient, reliable and more economical than other conventional indexing procedures for detecting CLRV-W in walnut nursery trees and seedlings or indexing for CLRV-W of seed and graftwood for propagation. However, the cost-benefit ratio for general survey of commercial orchards for CLRV-W-infected trees may be questionable because the reliable detection of CLRV-W in orchard trees by I-ELISA required a relatively large pollen sample (eg, 82 g per tree) to compensate for an uneven and erratic distribution of the virus in naturally infected trees. The collection of such a pollen sample from individual trees is time- and space-consuming. Nevertheless, in walnut-growing areas with a low incidence of CLRV-W-infected trees, I-ELISA is an efficient and reliable method for locating symptomless infected trees to be rogued to suppress a further natural spread of CLRV-W.

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