Disease Detection and Losses

Serological Detection of Cherry Leafroll Virus in English Walnut Trees

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ABSTRACT


Direct (D) and indirect (I) enzyme-linked immunosorbent assays (ELISA) were compared for relative reliability in detecting the walnut strain of cherry leafroll virus (CLR-V-W), the causal agent of blackline disease in English walnuts. The lowest concentrations of purified CLR-V-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 CLR-V-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 CLR-V-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 CLR-V-W. It also was reliable in detecting CLR-V-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 CLR-V-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 CLR-V-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).

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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 CLR-V-W (10–12) unless they were detected by being indexed for the presence of the virus.

Conventional indexing of walnut trees for CLR-V-W infection by graft inoculation of English J. hindsii trees or bioboy of trees by mechanical inoculation of herbaceous hosts (9, 12), is time consuming and inefficient. Also, the erratic and uneven distribution of CLR-V-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 CLR-V-W in infected English walnut trees.

MATERIALS AND METHODS

Virus purification and serology. A single-lesion CLR-V isolate, CLR-V-W, recovered from walnut trees affected with blackline (10, 12) was used for the production of antisera. The virus was propagated in cucumber (Cucumis sativus L. 'National Pickling') seedlings in a greenhouse. The cucumber seedlings were harvested and the virus purified 10–20 days after mechanical inoculation. A modified procedure for the purification of potato leafroll virus (13) was satisfactory for the purification of CLR-V-W. 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 ml of extract during the clarification step, and pellets were resuspended in 0.05 M phosphate buffer without ura.

The presence of CLR-V-W 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 megalestaphon Heuch and Mueller and cucumber seedlings. CLR-V-W concentration was estimated by assuming that a purified preparation containing 1 mg of virus per milliliter has an OD300 = 10. This assumption was based on the average amount of nucleic acid (40%) present in the components of the golden elderberry isolate of CLR-V (6) which has a relatively close serological relationship to CLR-V-W (4). A mixture of both virus bands from sucrose density gradients was used to obtain antisera by immunizing New Zealand rabbits. Chicken antibodies were obtained by immubizing White Leghorn Line 58, hens (supplied by H. Aplanalp, 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 antisera titer was determined by agar gel double diffusion tests.

To obtain chicken antibodies against CLR-V-W, 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 CLR-V-W-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 CLR-V-W-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 antisera as described by Clark and Adams (3), except that the column was prepared with DE52 instead of DE22 cellulose. The DE52 cellulose was washed 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 CLR-V-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-iso-5-ethylben-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 y-globulin used for coating the plates and for conjugation 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 microplates (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 was recorded 30–45 min after the addition of the substrate.

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

Antiserum production. The maximum homologous titer of CLRV-W8 antisera 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 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 and lowest nonspecific background of healthy plants) were obtained when 1.5 and 2.5 ng 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 ng 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 ng 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 A405 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 y-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 using y-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 A405 nm = 0.022, A414 nm = 0.012, and A405 nm = 0.025, respectively, for wells with buffer when y-globulin-alkaline phosphatase, -glucose oxidase, and -peroxidase conjugates were used.

When samples of CLRV-W8-infected cucumber and walnuts were used to compare sensitivity of D-ELISA with γ-globulin-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-W8 was detected, I-ELISA with y-globulin-peroxidase conjugate was two to 16 times more sensitive than D-ELISA with the same γ-globulin conjugate in detecting CLRV-W8 depending on the plant species and type of tissue assayed (Table 1). I-ELISA with γ-globulin peroxidase would also be more sensitive than D-ELISA with γ-globulin-peroxidase conjugate.
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 α-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 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 tested 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 symptomless. 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).

![Graph](https://example.com/graph.png)

**Fig. 1.** Direct (D)- and indirect (I)-enzyme-linked immunosorbent assay (ELISA) absorbance values obtained by using purified preparation of CLRV-W 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 A₄₅₀ nm of 0.025 for the buffer.
and moves very slowly (~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 1-ELISA with γ-globulin-peroxidase conjugate is very sensitive and reliable and superior to D-ELISA for detecting CLR-V-W in walnuts. In our ongoing research concerned with epidemiology, role of pollen in natural spread of CLR-V-W and the relative resistance of English walnut cultivars to CLR-V-W, 1-ELISA is a very useful and reliable research tool. Likewise, 1-ELISA is efficient, reliable and more economical than other conventional indexing procedures for detecting CLR-V-W in walnut nursery trees and seedlings or indexing for CLR-V-W of seed and graftwood for propagation. However, the cost-benefit ratio for general survey of commercial orchards for CLR-V-W-infected trees may be questionable because the reliable detection of CLR-V-W in orchard trees by 1-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 expense-consuming. Nevertheless, in walnut-growing areas with a low incidence of CLR-V-W-infected trees, 1-ELISA is an efficient and reliable method for locating symptomless infected trees to be rogued to suppress a further natural spread of CLR-V-W.

LITERATURE CITED